Functional analyses of porcine T-cell responses upon viral infections

–Lessons learned from pigs as natural hosts and biomedical models–

I n a u g u r a l d i s s e r t a t i o n

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Universität Greifswald

vorgelegt von

Alexander Schäfer

Greifswald, Dezember 2020

Dekan: Prof. Dr. Gerald Kerth

1. Gutachter: Prof. Dr. Dr. h.c. Thomas C. Mettenleiter

2. Gutachter: Prof. Dr. Artur Summerfield

3. Gutachter: Univ.-Prof. Dr. Armin Saalmüller

Tag der Promotion: 19.03.2021

Meinen Eltern.

"There are these two young fish swimming along and they happen to meet an older fish swimming the other way, who nods at them and says 'Morning, boys. How's the water?' And the two young fish swim on for a bit, and then eventually one of them looks over at the other and goes 'What the hell is water?'

[…]

It is about the real value of a real education, which has almost nothing to do with knowledge, and everything to do with simple awareness; awareness of what is so real and essential, so hidden in plain sight all around us, all the time,
that we have to keep reminding ourselves over and over: This is water.

This is water."

David Foster Wallace

In: This Is Water — Some Thoughts, Delivered on a Significant Occasion, about Living a Compassionate Life

Contents

LIST OF ABBREVIATIONS

αGC	α-Galactosyl-C16-Ceramide
ASF	African Swine Fever
ASFV	African Swine Fever Virus
Bcl-6	B-cell lymphoma 6
CD	Cluster of Differentiation
cTC	Conventional T Cells
CTLA-4	Cytotoxic T-Lymphocyte-associated Protein 4
DAMP	Danger-associated Molecular Pattern
DC	Dendritic cell
DN	$CD4^-/CD8\alpha^-$ double negative
DP	$CD4^+/CD8\alpha^+$ double positive
dpi	days *post infectionem*
FasL	Fas Ligand
FoxP3	Forkhead Box P3
GALT	Gut-associated Lymphoid Tissue
H1N1pdm09	H1N1 Influenza A Strain of the 2009 Pandemic
HA	Hemagglutinin
huIAV	Human Influenza A Virus
IAV	Influenza A Virus
ICOS	Inducible T-cell Co-Stimulator
IFNγ	Interferon-γ
IL	Interleukin
iNKT cell	Invariant Natural Killer T Cell
mAb	Monoclonal Antibody
MAIT cell	Mucosal-associated invariant T Cell
MALT	Mucosa-associated Lymphoid Tissue
MDSC	Myeloid-derived Suppressor Cells
MHC I/II	Major Histocompatibility Complex Class I/II
MICA/B	MHC Class I Chain-related Protein A/B
NA	Neuraminidase
NK	Natural Killer Cell
NKG2D	Natural Killer Group 2 Member D
PBMC	Peripheral-Blood Mononuclear Cells
PD-1	Programed Death-1
PLZF	Promyelocytic Leukemia Zinc Finger Protein
PRR	Pattern Recognition Receptor
RORγt	Retinoic Acid Receptor-related Orphan Receptor-γt
SCID	Severe-Combined Immunodeficiency
SLA I/II	Swine Leukocyte Antigen I/II

SPF	Specific-Pathogen-free
swIAV	Swine Influenza A Virus
T-bet	T-box Transcription Factor TBX21
TCR	T-Cell Receptor
T_{FH}	Follicular Helper T Cell
TGFβ	Transforming Growth Factor-β
Th	T Helper
TNFα	Tumor Necrosis Factor-α
Treg	Regulatory T Cell

1 INTRODUCTION

1.1 From Farm Animal to Biomedical Model — Immunological Research in Pigs

1.2 Porcine Viruses investigated in this Thesis

1.3 Porcine Immunity in the Context of Viral Infections

Domestic pigs have lived with humans for ages. Initially, they were used as a source of food. In recent years, pigs were used not only for nutrition but also as companion animals, as hunters of precious truffles, as donors for xenotransplantation, and as a new biomedical model species. However, living in close proximity within a population or to other species harbors a noteworthy risk: infectious diseases. Pigs are hosts to various infectious agents that can also infect humans. For both, the main reason they do not succumb to every infection they encounter, is the individual's immune system. It distinguishes between self and foreign and thus, protects its host. Yet, infectious agents evolved a plethora of ways to overcome host barriers and immune responses — all in order to ensure their own survival. Understanding of the mechanisms underlying infectious diseases in their hosts but also of the host response is pivotal for survival.

1.1 From Farm Animal to Biomedical Model — Immunological Research in Pigs

After domestication of wild boar (*Sus scrofa scrofa*), domestic pigs (*Sus scrofa domesticus*) were used for different purposes by humans for ages. In science, after being intensively studied as a donor species for xenotransplantation [1], domestic pigs have received more attention as a potential new biomedical model species for other immunological research areas as well. This comes from the fact that pigs are considerably more similar to humans than other model species, e.g., mice (*Mus musculus*). Pigs have a body size and weight comparable to humans. Moreover, their whole anatomy and physiology resembles those of humans [2]. On a genomic level, more than 80% of the analyzed parameters involved in immune responses showed a considerable homology between pigs and humans, compared to less than 10% between humans and mice [3]. Several lymphatic tissues, i.e., thymus, spleen, mucosa-associated lymphoid tissue (MALT), and gut-associated lymphoid tissue (GALT), exhibit a high degree of similarity between pigs and humans [4]. The same is true for receptors of the innate immune system, i.e., Toll-like receptors and NOD-like receptors or the complement system (extensively reviewed by Saalmüller and Gerner [5]). In recent years, the use of "dirty" animals with a largely intact and considerably more diverse microbiome reflecting their wild counterparts increased and revealed dramatic disparities to gnotobiotic (germ-free) and SPF (specific-pathogen-free) animals [6]. Moreover, to mirror the genetic background in a genetically diverse population like humans, outbred animals like pigs are especially useful [2].

However, research in pigs is not as advanced as it is in other, still more often used model species. To differentiate cells by cell-surface and functional markers, immunologists use a standardized nomenclature, termed clusters of differentiation (CD). Currently, 371 CD markers have been described for humans [7]. Although porcine homologues have been found for most human CD markers [8], the lack of available monoclonal antibodies (mAb) reacting with those markers is

a major obstacle in porcine immunology. Moreover, information about the expression of CD markers on the different leukocyte subsets is still much less detailed as in humans or mice [5]. Nevertheless, porcine immunology is advancing, cross-reacting or even anti-porcine-specific mAb are developed, and multiple T-cell subsets have been identified and correlated with human and/or murine counterparts [9].

The described characteristics of pigs make them an exceptionally suitable model species for immunological research, especially for infections with viruses of the respiratory and digestive tract [2, 10].

1.2 Viruses investigated in this Thesis

All living beings, from bacteria to complex animals, are exposed to a constant storm of infectious microorganisms – viruses. Viruses come in many different shapes and with highly variable characteristics. Some are cleared right away, some replicate but are not a cause for disease, others infect and stay for the rest of the host's life, and some cause severe disease and lethality in many, if not all, infected individuals. In this thesis, two different viruses were investigated: One causes mostly minor disease in pigs but has zoonotic potential, the other represents a major infectious threat for the porcine population with severe disease and high lethality.

1.2.1 Influenza A Virus

Influenza viruses contain a multi-segmented, negative sense, single-stranded viral RNA genome. The virus family, *Orthomyxoviridae*, encompasses seven genera, four of which contain Influenza viruses. Influenza A virus (IAV) belongs to the genus *Alphainfluenzavirus* [11]. The IAV genome consists of eight segments, which encode at least 10 proteins: polymerase basic protein 2 (PB2, segment 1), polymerase basic protein 1 (PB1, segment 2), polymerase acidic protein (PA, segment 3), hemagglutinin (HA, segment 4), nucleoprotein (NP, segment 5), neuraminidase (NA, segment 6), matrix proteins (M1 and M2, segment 7), and non-structural proteins (NS1 and NEP/NS2, segment 8) [12]. The number of expressed proteins is larger than the number of genome segments because IAV uses a variety of molecular mechanisms, e.g., leaky scanning or alternative splicing [11, 12]. IAV, like other RNA viruses, have a high genetic diversity [13], which in IAV is increased by two mechanisms. The first is antigenic drift, where point mutations are inserted through the lack of proof-reading by the viral polymerase [11, 12]. Moreover, their genome structure enables IAV to share genome segments of different origin

within one infected host cell (but only within the same genus), in a process called reassortment or, concerning HA or NA with a subsequent phenotypic change, antigenic shift [11, 12, 14].

Based on the two viral surface glycoproteins, HA and NA, IAV are subtyped into 16 HA- and 9 NA-subtypes (two additional HA subtypes, H17N10 and H18N11, have only been found in bats [15]) [14]. All IAV subtypes were isolated from avian species, with the exception of bat-origin IAV [16]. IAV, as well as other Influenza viruses, are often associated with mammalian and avian hosts [17]. However, Influenza-like viruses infecting amphibians and fish have recently been described, indicating that their host range might be broader than thought [18, 19].

IAV is transmitted mostly via aerosols from infected individuals [20], but infection can also occur after contact to fomites [21]. Although viral shedding typically correlates with signs of disease and is restricted to the first few days after infection, non-classical courses and unspecific symptoms limit the ability to isolate infected individuals and thus, might promote viral transmission [22]. IAV particles enter their host cells by HA-mediated binding to sialic acid residues on the cell surface [23]. Virus-specific HA variants bind to differently linked sialic acids, which is the key factor determining host range [14, 23]. Sialic acids are found in two variants, α-2,3-linked and α-2,6-linked. The former is predominantly found in bird's intestines and cells from the lower respiratory tract in mammals, while the latter is present in tissue from the mammalian upper respiratory tract [24, 25]. Accordingly, avian IAV preferentially bind α-2,3-linked sialic acids, while mammalian IAV prefer α-2,6-linked sialic acids [24, 25]. Of note, the distribution of sialic acids in the respiratory tract is highly comparable between humans and pigs [26].

Antigenic drift and reassortment have resulted in annual seasonal epidemics and five historically documented pandemics with more than 50 million fatalities in the 1918-1919 pandemic alone [27]. Variants of the 1918 pandemic IAV strain are responsible for the majority of human IAV infections since [28], which illustrates the long-lasting consequences of viral pandemics beyond immediate morbidity and mortality [29]. The most recent IAV pandemic in 2009 was caused by an H1N1 subtype (H1N1pdm09). H1N1pdm09 had an exceptional genome constellation: the PB2 and PA segments were of avian origin, the PB1 segment came from a human H3N2 IAV, NA and M segments were derived from an avian-like swine IAV, while HA, NP, and NS segments came from another classical swine H1N1 IAV [14, 30]. Interestingly, the H1N1pdm09 variant circulated in pigs for several years before it was transmitted to humans and even then, it took months before the outbreak was recognized [31]. This emphasizes a central role of pigs for IAV evolution.

Pigs have also become increasingly important for IAV spread for other reasons. Traditionally, pigs were held in relatively small farms (~100 animals) and were not shipped in large quantities. This has changed dramatically over the last decades, with a shift to large-scale farms (~1,000

animals) and increased worldwide live animal shipments [32]. Moreover, pigs "learned to fly" and are now shipped by plane between the biggest pig producers, Southeast Asia, Europe, and North America in large numbers [32]. Since IAV infections in pigs are multifaceted, ranging from clinically inapparent to highly acute [33], and shipped pigs are usually not tested for IAV, international transport contributes to the global spread of IAV [34]. Additionally, there are molecular reasons that brought pigs into the focus of IAV research. As introduced before, IAV have different host ranges, based on the differently linked sialic acid on the host cell surface. In contrast to humans, pigs express both sialic acid variants in their upper respiratory tract, enabling infection with and reassortment of both avian and mammalian IAV [25, 35]. Therefore, the term "mixing vessel" has been coined [16, 36]. This notion has been challenged by data showing limited expression of α-2,3-linked sialic acids on porcine respiratory cells [37] and might indicate that the high number of animals in close proximity is the main determinant for inter-species transmissions [38]. However, emergence of avian and mammalian IAV reassortants has been shown in contact animals of experimentally infected pigs but not in humans [39, 40].

There have been numerous outbreaks , both global pandemics [30] and regional epidemics [39, 41], caused by zoonotic transmission of IAV from pigs to humans. However, there have also been infections of porcine populations by transmission from humans [42]. The IAV strain in this thesis, H1N1pdm09, has been studied as a cause of a clinically mild disease in pigs with zoonotic potential. However, there are also viral infections in pigs that cause severe disease and lethality.

1.2.2 African Swine Fever Virus

The cause for the devastating viral hemorrhagic fever-like illness African swine fever (ASF) is the ASF virus (ASFV). It is a complex, large, double-stranded DNA virus and the only member in the genus *Asfivirus* of the *Asfarviridae* family [43, 44]. ASFV strains have a genome size of 170-190 kbp [45], encoding more than 150 viral proteins [46]. Based on differences in the major ASFV capsid protein, p72, 22 genotypes have been defined [45, 47]. ASFV virions are usually enveloped after budding from the host cell [48]. However, their outer envelope is lost in later stages of infection, when infected host cells are lysed, without negative effects on infectivity [49].

ASFV infects all members of the *Suidae* family, with considerable differences depending on the respective subspecies. In its native geographical origin, sub-Saharan Africa, ASFV circulate in a sylvatic cycle with soft ticks of the genus *Ornithodoros* in bushpigs (*Potamochoerus larvatus*) and warthogs (*Phacochoerus africanus*) [50]. ASFV infection is usually clinically inapparent in both reservoir species [51]. Outside of Africa, ASFV infects domestic pigs and wild boar,

causing major disease with high lethality [52], and making it responsible for millions of fatalities and major economic challenges of global extent [53]. ASFV affects only suids and has no zoonotic potential [54].

Until the 1950s and 1960s, there were only minor ASF outbreaks in European countries, but ASFV became endemic in Spain and Portugal and spread to other European and American countries. These outbreaks were eradicated in the mid-1990s, except for Sardinia [55]. After ASFV was introduced into Georgia in 2007, it spread to many other European and Asian countries [55-59]. In September 2020, a wild boar found dead in Brandenburg was tested positive for ASFV, representing the first case of ASF in Germany [60]. Since then, ASF has spread regionally and dozens of further cases have been detected. Wild boar seem to play a major role for ASFV maintenance and spread outside of Africa, considering that ASFV-transmitting *Ornithodoros* ticks (or ticks from other genera) are found only in Southern but not Northern European or Asian countries [61]. This is especially true if close contact between domestic and wild pigs is not prevented [62, 63]. The initial introduction into wild or domestic pig populations, however, is usually caused by human negligence [64]. Infectious ASFV contaminations can be found in multiple places, from pig secretions, meat from infected animals, to farmer's clothing (although infection by fomites has not yet been shown) [64]. ASFV introduction into Georgia was likely caused by contaminated pork from Madagascar [65].

ASFV has a distinct cell tropism and infects and replicates in cells of the myeloid lineage [66]. These includes professional antigen-presenting cells (APC), such as monocytes [67], macrophages [68], and dendritic cells (DC) [69], but also neutrophil granulocytes [70]. CD163, a specific marker for macrophages [71], has been discussed as a cellular receptor needed for viral entry [72]. However, later studies found no correlation between susceptibility and CD163 expression [73] and CD163 knockout pigs were still susceptible to ASFV infection [74]. Thus far, the only cellular marker correlating with viral susceptibility was CD45 [73], a pan-leukocyte marker [75]. Interestingly, the extracellular ASFV protein CD2v, which is indispensable for viral replication in its arthropod vectors [76], shares high homology with porcine CD2, an adhesion receptor found on porcine T cells [77]. CD2v is pivotal for hemadsorption of erythrocytes to infected cells [77]. Since attenuated strains often have truncated versions of CD2v [78], it is probably also important for pathogenesis [77].

Two different ASFV strains of the Georgia lineage were used in this thesis. The first, "Armenia2008" is a highly virulent strain with high lethality in both wild boar and domestic pigs [79]. The second, "Estonia2014", is a moderately virulent strain that induces acute disease without fatalities in domestic pigs but severe disease and high lethality in experimental infections of wild boar [80]. Serological evidence found in hunted animals in Northern Estonia suggested that at least some wild boar survive ASFV field infections [81].

1.3 Porcine Immunity in the Context of Viral Infections

RNA viruses, like IAV, have a high diversity and fast evolution. DNA viruses, like ASFV, on the other hand, have a vast repertoire of viral proteins to evade the host's unspecific innate immune responses. The adaptive arm of the immune system provides more specific responses and is a crucial player for immunity at mucosal boundaries [82]. Moreover, as obligate intracellular parasites, viruses are often not completely neutralized by antibody responses mounted by the host [83]. Therefore, a more specialized part of the immune system has to get involved: T cells.

1.3.1 *E pluribus unum* – The diverse T-cell Compartment

T cells, named for their characteristic development in the thymus, respond to foreign antigen as a part of the adaptive immune system. Their (at least in theory) virtually unlimited ability to respond to antigens is based on the molecular structure of a key T-cell molecule, the T-cell receptor (TCR). The TCR is expressed on the cell surface in a complex with the T-cell lineage marker, CD3 [84]. It is comprised of two eponymous chains, α and β, or γ and δ, in the two subsets αβ and γδ T cells, respectively. The chains consist of up to four domains: constant (C), variable (V), and joining (J) regions in α and γ chains, and additionally diversity (D) segments in β and δ chains. V(D)J recombination, the process of somatic recombination of the three TCR domains, results in an estimated 10^{15} potential individual αβ T-cell clonotypes (the total of T cells with an identical TCR) [85]. γδ T cells possess an even greater potential diversity, with an estimated upper limit of 10^{18} clonotypes [86]. However, only about 10^{6}-10^{8} T-cell clonotypes are expressed in any given individual [87, 88], and there is a considerable bias in favor of some clonotypes [89]. Still, this illustrates the vast potential of possible antigen-specific T-cell responses. Both T-cell subsets will be introduced below.

1.3.1.1 The T-cell Blueprint — αβ T cells

αβ T cells are the subset of $CD3^{+}$ lymphocytes typically addressed when T cells are mentioned. They represent the majority of T cells in humans and mice, but also in pigs.

Pigs, like humans and mice, have both conventional αβ T-cell subsets, $CD4^{+}/CD8\alpha^{-}$ ($CD4^{+}$) and $CD4^{-}/CD8\alpha^{+}$ ($CD8\alpha^{+}$). Interestingly, whereas humans and mice usually have twice as many CD4 T cells as CD8α T cells, this ratio is reversed in pigs [90]. The $CD4^{+}$ subset is currently thought to mirror human and murine T helper (Th) cells [5]. Among $CD4^{+}$ αβ T cells, pigs have a population of $CD25^{+}$ cells expressing the transcription factor FoxP3 and exerting regulatory effector functions, thereby meeting the classical definition of regulatory T cells (Tregs) [91].

CD8α$^+$ can be subdivided in two subsets, CD8αα$^+$ and CD8αβ$^+$, depending on the expression of CD8 as a homo- or heterodimer, respectively. Porcine CD8αβ$^+$ αβ T cells exert activities of cytotoxic T cells [92], while the functions of CD8αα$^+$ αβ T cells are not fully understood [90]. At least some evidence suggests that human CD8αα$^+$ αβ T cells are terminally differentiated memory cells that downregulated the CD8 β-chain to decrease antigen reactivity [93-96].

Porcine CD4$^+$ αβ T cells are able to upregulate the expression of CD8α on their surface resulting in a considerable population of CD4$^+$/CD8α$^+$ (DP) T cells [97]. This upregulation is dependent on antigen contact [5] and is also induced during viral infections or immunizations, including swine Influenza viruses [98] and vaccines against H1N1pdm09 [99]. A considerable DP T-cell population in the human and murine periphery is only found as prematurely released precursor T cells from the thymus [100], during autoimmune diseases, or at the site of inflammation [101].

T-cell activation generally requires several signals. Mammalian αβ TCR bind the antigen presentation molecules major histocompatibility complex (MHC) class I and II on the surface of APC, where CD8α$^+$ and CD4$^+$ T cells are restricted to MHC I and MHC II, respectively [102, 103]. In pigs, MHC molecules are referred to as swine leukocyte antigen (SLA) I and II. The interaction of TCR and MHC/SLA is termed signal 1. The second signal is acquired by interaction of either pro- or anti-inflammatory molecules on the surface of APC, like CD80/86 or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152), respectively, with CD28 on the T-cell surface. The third signal is given by secreted cytokines from the APC, to fine-tune the T-cell response [84, 102].

αβ T cells utilize a plethora of cellular effector mechanisms to fight pathogens or other threats. Based on extra- and intracellular markers and the cell-specific use of these effector functions, multiple T-cell subsets have been defined. Among them, cytotoxic T cells play a major role during viral infections. Cytotoxic responses kill infected or otherwise compromised cells, which can then be cleared by phagocytes. This limits pathogen spread, especially for intracellular pathogens such as viruses, but also prevents proliferation of mutated cells that pose a risk for cancer development [104]. Cytotoxicity is exerted by a few key proteins, perforin and granzymes or interaction of Fas (CD95) and Fas ligand (CD95L/CD178).

Cytotoxic cells, identified by expression of perforin or other cytotoxic molecules, are usually found in the CD8αβ$^+$ population [92]. In pigs, however, perforin$^+$ cells have also been identified among DP αβ T cells during infection with ASFV and pseudorabies virus [105-107]. These data are still under discussion as other groups have been unable to reproduce these results [9]. Moreover, it is unknown whether the DP cytotoxic T cells originate from CD4$^+$ or CD8α$^+$ T cells [92, 107].

$CD4^+$ Th cells act in a more indirect way, predominantly by secretion of cytokines that orchestrate further responses. Th responses are classified based on the expression of specific surface markers, transcription factors, and cytokines [84, 102]. The key cytokines produced by Th1 cells are interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), mediating responses against intracellular pathogens like viruses [108]. Differentiation of Th1 cells is associated with expression of the T-box transcription factor TBX21 (T-bet) [109]. Th2 cells, characterized by secretion of interleukin (IL)-4, IL-5, and IL-13 are defined by expression of GATA-3 and have recently also been described in the pig [110]. They are induced mainly during infections with extracellular pathogens, demonstrated in pigs during infection with a swine-specific parasite, *Trichuris suis* [110]. Th17 cells, defined by expression of the transcription factor retinoic acid receptor-related orphan receptor gamma t (RORγt) and secretion of IL-17, take part in responses against extracellular pathogens, like fungi and bacteria, predominantly at mucosal surfaces [111, 112]. Follicular helper T (T_{FH}) cells in humans and mice are pivotal orchestrators of humoral immune responses but are scarcely investigated in pigs. In humans, they are characterized by surface expression of CXC chemokine receptor 5 (CXCR5), inducible T-cell co-stimulator (ICOS, CD278), programed death 1 (PD-1) and intracellular expression of B-cell lymphoma 6 (Bcl-6) and IL-21 [113]. Porcine T_{FH} cells have recently been described during comparative immunization studies in mice and pigs [114]. Mitigation of pro-inflammatory responses and induction of tolerance to certain antigens is executed Tregs. Tregs, characterized by expression of the transcription factor forkhead box P3 (FoxP3), express the anti-inflammatory cytokines IL-10 and Transforming growth factor-β (TGFβ) and can suppress T-cell responses by binding of CTLA-4 to CD28 on activated T cells [115]. Moreover, they can directly kill antigen-specific T cells granzyme-dependently similar to cytotoxic T cells [116].

Pigs belong to a group of animals with another peculiarity. Unlike humans and mice, the porcine T-cell compartment has another major player: γδ T cells. In early studies of porcine peripheral blood, γδ T cells were incorrectly identified as $CD4^-/CD8\alpha^-$ (DN) αβ T cells [97, 117]. By using antibodies against the porcine γδ TCR, it was later shown that most of the DN T cells belong to the γδ T-cell population [118]. Although these cells undergo thymic development like αβ T cells, they are a distinct population with specific properties.

1.3.1.2 A Blend of both Systems — γδ T cells

Depending on age, γδ T cells represent up to 85% of all peripheral porcine T cells [5, 119, 120]. This is in stark contrast to humans and mice, where γδ T cells only account for less than 10% of all T cells. Still, even in the low numbers found in humans and mice, γδ T-cell responses are indispensable as they bridge innate and adaptive responses [121]. However, our understanding of γδ T cells is far from complete.

In pigs, γδ T cells are divided into three major subpopulations, based on the expression of CD2 and CD8α. $CD2^-/CD8\alpha^-$ γδ T cells are mostly naïve, $CD2^+/CD8\alpha^-$ γδ T cells are composed of activated or effector cells, while the $CD2^+/CD8\alpha^+$ subset consists of terminally differentiated effector cells [119, 122]. Porcine $CD2^+$ γδ T cells are also discussed as a separate lineage with distinct development in the thymus [123]. However, this is contrary to other results showing CD2 upregulation *in vitro* and, therefore, remains under discussion [124]. Multiple γδ T-cell subsets are described in humans or mice. These are based on the expressed γ and δ chains of the γδ TCR. Similar to pigs, the majority of human γδ T cells does not express classical T-cell surface markers like CD4 and CD8, although a subset might express low levels of CD8α [125-127]. However, they do express molecules typically associated with the innate lymphocyte population of Natural Killer (NK) cells, such as CD16 or the C-type lectin-like receptor NK group 2 member D (NKG2D) [128]. Since porcine NK cells are only rudimentarily described, it is difficult to compare surface markers on porcine γδ T cells with their counterparts on porcine NK cells. Still, the major marker for porcine NK cells, CD8α, is known to be upregulated in cytotoxic γδ T cells [105]. The increased expression of CD8α on effector γδ T cells indicates similar innate-like or even cytotoxic responses.

Activation of γδ T cells is thought to be independent of conventional antigen presentation, although γδ TCR ligands might interact with MHC I and II or MHC-related proteins [129]. Instead, antigen detection by γδ TCRs is more similar to antigen binding by immunoglobulins or pattern recognition receptors (PRR) [130-132]. Multiple host-derived proteins are able to induce γδ T-cell responses [133]. Among them are the MHC-related proteins T10 and T22 in mice [134, 135] and the MHC class I chain–related proteins A and B (MICA, MICB) or CD1c in humans [136, 137]. Moreover, human herpesvirus 1 transmembrane glycoprotein gI [138] and fibroblasts infected with human herpesvirus 5 [139] are recognized by γδ T cells, indicating that pathogen-derived antigens interact with γδ TCRs as well. Recently, γδ T cells have been shown to bind to the non-classical MHC I-like molecule MR1 [140]. MR1 is typically associated with presentation of riboflavin metabolites derived from bacteria to another unconventional T-cell population, mucosal-associated invariant T (MAIT) cells. Most γδ-TCR ligands described so far are typically upregulated during infection or other cellular dysregulations, regardless of the chemical nature of the specific γδ TCR ligands [129]. This resembles recognition of danger-associated molecular patterns (DAMP) by the innate immune system and thus, emphasizes the role of γδ T cells during early immune responses [129]. However, this could also mirror experimental biases, because the usual experimental approach includes stimulation with tumor cells or host cells [130]. γδ T cells can also be activated independent of TCR interaction in an innate-like way by cytokines, especially those produced early during an evolving immune response, i.e., IL-1 and IL-23 [141]. The broad range of activation mechanisms further underlines the importance of γδ T cells.

Depending on the mode of activation, γδ T-cell reactions differ significantly. The responses are fine-tuned by the activating ligands: NKG2D induces cytotoxic responses [142, 143], PRR-dependent activation (usually concomitant with cytokines) triggers secretion of inflammatory cytokines [144, 145], while activation via their γδ TCR results in both cytotoxic and inflammatory responses but also proliferation and other immunoregulatory functions [129]. In response to innate activation by cytokines, human and mouse γδ T cells primarily secrete IFNγ and IL-17 [141, 146]. Responses of activated porcine γδ T cells are largely comparable to human γδ T cells. Cytotoxic capabilities [147-149] and secretion of pro-inflammatory cytokines, i.e., IFNγ, TNFα, and IL-17, have been demonstrated [124, 150]. Moreover, porcine γδ T cells can also be activated by innate cytokines such as IL-12 and IL-18 [124]. Presentation of antigens similar to professional APCs has been shown for activated human γδ T cells. Of note, even though human αβ T cells also expressed antigen presentation molecules like MHC II and CD80/86, γδ T cells displayed higher expression levels and were the only T-cell subset capable of inducing proliferation to the same extent as DCs [151]. Comparable antigen presentation capabilities have also been implicated for porcine γδ T cells [152].

Besides γδ T cells, another innate-like T-cell population exists with major implications for immunity: invariant Natural Killer T (iNKT) cells.

1.3.2 Same Same but Different – invariant Natural Killer T cells

Porcine iNKT cells are largely similar to conventional T cells (cTC): they develop in the thymus, express CD3 and a TCR on their surface, and are able to mount antigen-specific responses [84]. However, they also have some very peculiar differences to cTC. Their eponymous characteristic is a TCR with strikingly reduced diversity. Most iNKT cells express the same TCR, and thus, react to the same antigenic stimuli [153]. Moreover, they exit the thymus with an already activated phenotype and even some functions usually attributed to memory cells [154-156]. This pre-activated phenotype enables iNKT cells to react within hours or few days to further activation, emphasizing their innate-like characteristics [157]. Although they are considered a rare population, accounting for often far less than 1% of all peripheral T cells, they still outnumber any αβ or γδ T-cell clonotype.

Development, differentiation, and many effector mechanisms of iNKT cells are primarily controlled by a single transcription factor, promyelocytic leukemia zinc finger (PLZF) [158, 159], which is actively suppressed in cTC [160]. Based on the expression of other lineage-defining transcription factors, i.e., T-bet, RORγt, and GATA-3, murine iNKT cells are divided into multiple subsets [161]. $PLZF^{+}$/$T\text{-}bet^{+}$ iNKT1 cells produce IFNγ and TNFα, exert cytotoxic properties, and thus, resemble Th1 cells. $PLZF^{hi}$/$GATA\text{-}3^{+}$ iNKT2 cells are comparable to Th2

cells by secretion of IL-4 and IL-13. PLZF^{lo}/RORγt^{+} iNKT17 cells express IL-17A, similar to Th17 cells [162-167]. Functional subsets of human iNKT cells are not as well-defined [161]. Human DN and CD8α^{+} iNKT cells display similarities to murine iNKT1 cells, as they express IFNγ and exert cytotoxic functions upon activation [168, 169]. Moreover, IL-17-producing human iNKT cells have been shown [170]. However, data on more detailed subsets or tissue distribution are essentially missing. There is a significant difference in the expression of classical T-cell surface markers between human and murine iNKT cells. While human iNKT cells are predominantly CD8α^{+} or DN with a minor CD4^{+} population, murine ones display a major CD4^{+} and a minor DN subset but do not express CD8α at all [171].

In contrast to cTC, which are activated by interaction with MHC I and II molecules, the ligand for the iNKT-cell TCR is CD1d [172]. CD1d presents glycolipids, although the cognate antigens are still not entirely understood [173]. In research scenarios, the synthetic CD1d ligand α-galactosyl-C16-ceramide (αGC) is used to activate iNKT cells *in vivo* and *in vitro* [174, 175]. PBS57, a glycolipid related to αGC [176], is also used to detect iNKT cells by flow cytometry applying CD1d tetramers developed earlier [177, 178]. CD1d is highly conserved among the species investigated thus far, underlining the pivotal importance of iNKT cells for immunity even in an evolutionary context [179]. Interestingly, the extended CD1 family in humans and other mammals (CD1a to CD1e) is reduced in mice, where CD1d is the only member known to be expressed [173]. Expression of CD1d is also indispensable for iNKT-cell development in the thymus [180]. Human γδ T cells can also be detected by αGC-loaded CD1d tetramers, indicating intersecting responses of iNKT cells and γδ T cells. [181].

Current understanding suggests three strategies for iNKT-cell activation, as discussed by Kumar *et al.* [182]: The first is presentation of strong agonists, usually pathogen-derived antigens, such as αGC, in the context of CD1d. These signals do not require further stimulation to induce iNKT-cell responses (TCR-driven activation). However, not all pathogen-derived antigens are potent agonists like αGC. Moreover, CD1d is also able to present host-derived glycolipids, which are also less potent triggers. Therefore, these antigens require a second signal in the form of cytokines for broad iNKT-cell activation (TCR + cytokine-driven activation). This second signal comes from APCs that detected antigens with their PRRs during early pathogen encounter [173, 183, 184]. In this context, iNKT-cell activating cytokines are IL-12 or type I interferons [185, 186]. The last mode of activation is entirely dependent on concomitant secretion of pro-inflammatory cytokines (cytokine-driven activation). Known inducers of iNKT-cell responses are IL-12 and IL-18 [187-189]. Cytokine-driven signals are discussed as the predominant mode of activation during infections *in vivo* [190]. Of note, these modes of iNKT-cell activation resemble those of γδ T cells.

Investigation of iNKT cells in pigs has mostly focused on the potential use of αGC as a vaccine adjuvant against infectious diseases like IAV [191-196]. Beyond that, little has been known about porcine iNKT cells in general. Similar to humans, pigs express all members of the CD1 family [197]. Analogous to humans and mice, pigs artificially disrupted of CD1d expression do not develop iNKT cells [198]. The porcine iNKT-cell TCR consists of Vα and Jα segments homologous to the Vα24-Jα18 and Vα14-Jα18 structures found in humans and mice, respectively [199]. The main antigen binding domains (complementarity-determining regions 1α and 3α) are conserved between humans and pigs [179]. The frequency of porcine iNKT cells has been found to be lower than 1% of $CD3^+$ cells in the blood [191, 200, 201], which is in the typical range of human peripheral iNKT cells [202]. In swine, iNKT cells are usually $CD8\alpha^+$ and lack a significant $CD4^+$ population [191, 192, 201]. Previous studies also showed high PLZF expression levels in porcine iNKT cells [203]. Moreover, some evidence indicated that αGC stimulation of porcine PBMCs resulted in iNKT-cell proliferation [192, 203]. Although these previous studies indicated a comparable role and function of porcine iNKT cells, many characteristics were left unexplored.

T-cell populations take part in antiviral immune responses. Still, many aspects have not been thoroughly investigated so far. The following chapter shall introduce the previously existing knowledge about porcine antiviral T-cell responses.

1.3.3 Molecular Biological Warfare – Antiviral T-Cell Immunity in Pigs

The late Eckhard Podack coined the phrase of "Immunology [as] the science of biological warfare" [204]. The analogy of combat between intruding pathogens and the host's defense system is quite fitting: For the host, it can be a fight-or-die situation; for the infecting agent, it is more like live-or-die. Both sides need to be victorious in order to ensure survival. In this thesis, the fight between quite different pathogens in the same host, the pig, was investigated. One, IAV, typically causes clinically inapparent disease and the infection is eventually cleared. The other, ASFV, usually causes severe disease with high lethality. Because of the delicate structure of the lung, inflammatory responses need to be carefully balanced and cytotoxic T cells in particular require strict regulation [205]. The pivotal importance of T-cell responses for immunity in the lung becomes obvious even in the cell numbers: there are about 10^{10} T cells in the healthy human lung, comparable to the size of the T-cell compartment in the peripheral blood [205]. The T-cell response of the porcine host against IAV and ASFV infections shall be introduced in this chapter.

The general analysis of immune responses against IAV infections is hampered by the fact that different virus strains are used to study porcine immunity. In some studies, IAV strains from

pigs (swIAV) were used, while human strains (huIAV) were used in others. This is reflected by differences in the antiviral responses, which makes it challenging to deduce universally applicable conclusions. In order to reduce the complexity of previous results from the literature, this chapter will focus on responses against H1N1 swIAV and huIAV and studies with detailed immune phenotyping.

The central role of T-cell immunity during respiratory infections has also been demonstrated for IAV infections in numerous studies before. Chemical depletion of alveolar macrophages in pigs before infection with H1N1 huIAV resulted in increased clinical signs and mortality, which correlated with reduced levels of pro-inflammatory cytokines in lungs and lower frequencies of IFNγ-producing CD8α$^+$ lymphocytes in BAL fluids [206]. T cells were able to reduce disease severity, viral load, and mortality from lethal challenge infection after heterosubtypic priming in the absence of antibodies in mice [207], ferrets [208], and non-human primates [209]. In a similar approach, the intranasal administration of H1N1 T-cell epitopes reduced the viral load after heterologous challenge infection in pigs [210]. These studies underlined not only the significance of antiviral T-cell responses, but also demonstrated cross protection by pre-existing T-cell immunity to infection with heterologous viruses. However, only few studies analyzed the porcine T-cell response against IAV infection in detail.

In an attempt to use pigs as a model for human IAV infections, Khatri *et al.* infected pigs with H1N1 swIAV and provided the first phenotyping of responding porcine immune cells [211]. They found an influx of γδ T cells as well as cytotoxic CD8α$^+$ αβ T cells in the bronchoalveolar lavage (BAL) 6 dpi. Moreover, they showed activated, CD25$^+$ T cells in lungs and BAL of infected pigs 6 dpi, concurrent with an increase of DP αβ T cells in BAL, tonsils, and regional lymph nodes, and increased Treg frequencies in the lung. This mirrored human IAV infections and implicated pigs as a suitable model species for IAV infections [211]. Heinen *et al.* infected pigs with H1N1 swIAV and collected BAL fluid every other day in the first week and less frequently in the following weeks after infection [212]. They found an influx of lymphocytes 4-8 dpi, which primarily consisted of cytotoxic CD8α$^+$ αβ T cells and CD4$^+$ αβ Th cells but also NK cells. Frequencies of cytotoxic CD8α$^+$ αβ T cells showed a second, prolonged increase, beginning 15 dpi and lasting at least until the challenge infection 42 dpi. They also showed a subtype-specific proliferative response in lymphocytes from infected animals [212]. Pomorska-Mól *et al.* found antigen-specific T-cell responses after *in vitro* re-stimulations beginning 7 dpi and decreased lymphocyte counts in pigs after infection with H1N2 swIAV, but performed no further differentiation of the lymphocyte response [213]. The same authors found a similar proliferative T-cell response after *in vitro* re-stimulation beginning 7 dpi and pro-inflammatory cytokines in the lung of infected pigs in a later study with H1N1 swIAV [214]. A more detailed immune phenotyping was done by Sinkora *et al.* in BAL fluids of pigs infected with H1N1

swIAV 28 dpi [215]. They found increased frequencies of αβ T cells in general, and significantly elevated levels of $CD4^+$ Th cells and DP effector/memory cells. Although γδ T-cell frequencies decreased, the remaining cells differentiated into $CD2^+$/$CD8\alpha^+$ effector cells. In regional lymph nodes, they described elevated percentages of DP αβ T cells with concurrent decreased levels of $CD4^+$ Th cells. These local responses were not mirrored on a systemic level, as there was only a small increase of DP αβ T cells but no other changes [215]. Tungatt *et al.* used MHC I tetramers loaded with IAV peptides in Babraham inbred pigs immunized twice with H1N1 swIAV. They showed frequencies of up to 40% of $tetramer^+$ $CD8\beta^+$ αβ T cells in BAL fluids 28 dpi using just three peptides [216]. In a subsequent experiment, the group infected Babraham pigs with a swine isolate of H1N1pdm09 and found the first antigen-reactive, $tetramer^+$ $CD8\beta^+$ αβ T cells 5 dpi and a strong response 14 dpi in BAL fluid [216]. Although the group sizes were small (only three pigs were immunized and infected, respectively), this was the first successful attempt to describe the kinetics of antigen-reactive $CD8\beta^+$ αβ T cells in immunized and infected pigs.

The most recent and also most detailed analyses of porcine T-cell responses during H1N2 swIAV infection were done by Talker *et al.* in two consecutive papers. In the first study, pigs were infected twice with H1N2 swIAV at an interval of four weeks [217]. Typical clinical signs of respiratory disease were found in all infected individuals after primary infection but were reduced after the second infection. A cytotoxic and proliferative response in peripheral $CD8\beta^+$ T cells was detected early after the first infection but did not occur after the second infection. A similar but less distinct course was observed for CD4 Th cells, which proliferated and upregulated CD8α, primarily after the first infection. Interestingly, the amount of PBMCs that produced IFNγ after *in vitro* re-stimulation increased over the course of the study and a boost effect of the second infection was detectable in most individuals. This was also true for the numbers of proliferating cells producing more than one of the investigated cytokines, IFNγ, TNFα, and IL-2, and demonstrating signs of cytotoxic activity, measured by increased surface expression of the degranulation marker CD107a [217]. In the second study, the groups used the same H1N2 swIAV again, but infected only once [218]. They found proliferating $CD4^+$ Th cells with an activated phenotype ($CD8\alpha^+$/$CD27^+$) in lung lymph nodes between 4 and 12-15 dpi, and proliferating, activated $CD8\alpha^+$ cytotoxic T cells ($CD27^+$) in the lungs at the same time. IFNγ-producing cells were found in blood, lung, and lung lymph nodes 6-15 dpi, except for the lung, where IFNγ-producing cells were still found 44 dpi. In line with this finding, they showed that most IFNγ+ cells were found in the lung, while TNFα was predominantly produced in lung lymph nodes. Interestingly, they also showed cytokine production after re-stimulation with heterosubtypic swIAV, which was generally higher in lymphocytes isolated from the lung [218].

Studies investigating the porcine T-cell response after infection with huIAV in detail are also scarce. A first study with H1N1pdm09 showed mild clinical signs and increased frequencies of

cytotoxic CD8α$^+$ T cells and CD4$^+$ Th cells in the blood of infected pigs [219]. Mair *et al.* described lymphocytes with an innate, NKT-like phenotype (CD3$^+$NKp46$^+$) that expanded in the lung of pigs infected with H1N1pdm09. *In vitro*, these cells showed cytotoxic potential and secretion of IFNγ upon stimulation, indicating that they play a role in the antiviral response [220]. Rajajo *et al.* underlined the importance of T-cell responses against H1N1pdm09 with a different approach. They investigated pigs with a genetic background of severe-combined immuno-deficiency (SCID), characterized by an almost complete lack of lymphocytes [221]. H1N1pdm09-infected SCID pigs showed higher viral loads in the lung, prolonged shedding, and delayed viral clearance compared to non-SCID heterozygous carrier pigs. Interestingly, SCID pigs also showed fewer lung lesions and a lower lung pathology score [221]. Detailed immune phenotyping for infections of pigs with H1N1pdm09 has not yet been published.

Although CD1d presents bacteria-derived glycolipids only, various groups showed iNKT-cell activation and participation in anti-IAV responses in humans and mice [188, 222, 223]. During IAV infections in humans and mice, myeloid-derived suppressor cells (MDSC) suppress IAV-specific antiviral responses. Adoptive transfer of iNKT cells eliminated the immunosuppressive activities of MDSC and promoted antiviral immunity and viral clearance [222]. Activation of murine iNKT cells during IAV infection reduced viral titers by early enhancement of innate immune responses [224]. Moreover, iNKT-cell-derived IFNγ promotes potent antiviral effector functions in CD8α$^+$ T cells and NK cells in mice [225]. In pigs, there is only little data available regarding iNKT-cell responses during viral infections. Studies in pigs involving iNKT cells and IAV typically focused on immunizations rather than infections [191, 195]. Artiaga *et al.* investigated the effects of iNKT-cell activation by addition of αGC as an adjuvant to UV-inactivated H1N1pdm09 prior to challenge with live H1N1pdm09 in comparison to pigs primed with UV-inactivated virus alone [192]. αGC-adjuvanted priming significantly reduced viral loads in nasal swabs and resulted in undetectable levels of challenge virus in the lower respiratory tract. Moreover, levels of IFNγ-producing cells and virus-specific antibody titers were elevated in pigs primed with αGC-adjuvanted, UV-inactivated virus compared to pigs primed with UV-inactivated virus alone [192]. In a subsequent study, the group challenged unprimed pigs with H1N1pdm09 and used simultaneous systemic and local iNKT-cell activation by intramuscular and intranasal αGC administration, respectively, as a potential treatment [193]. Intranasal administration was more potent in regard to reduction of clinical signs, lung pathology, and viral load in lungs and nasal swabs. Interestingly, activation and expansion of iNKT cells 9 or 2 days prior to IAV infection by intramuscular or intranasal αGC treatment produced no beneficial outcome [226].

Literature regarding the second viral pathogen investigated in this thesis, ASFV, is scarce. Still, the pivotal importance of cellular immune responses against ASFV infections has been

demonstrated unambiguously in previous studies [227]. Oura *et al.* infected naïve pigs with the avirulent ASFV strain OUR/T88/3. Afterwards, CD8α$^+$ lymphocytes were depleted by anti-CD8α antibodies (clones 76-2-11 and 11/295/33) and the pigs were challenged with the homologous virulent strain OUR/T88/1. CD8α-depleted animals succumbed to challenge, whereas undepleted controls survived with mild disease [227]. However, since CD8α is expressed on a wide range of lymphocytes, from NK cells to various T-cell subsets [149, 228], it remained unknown, which cell type(s) conferred protection. Therefore, the authors tried to specifically deplete CD8β$^+$ cytotoxic T cells with an anti-CD8β mAb (clone PPT22). However, a significant depletion of CD8β$^+$ T cells was only achieved in one of seven pigs. Remarkably, this CD8β-depleted individual was the only one succumbing to the challenge infection [227]. This is the most conclusive evidence for the importance of CD8α$^+$ T cells in anti-ASFV responses so far, although the groups were relatively small. Still, this *in vivo* data is supported by earlier *in vitro* studies, where PBMCs from pigs after recovery from ASFV infection were tested in a killing assay. *In vitro* re-stimulation led to specific killing of ASFV-infected macrophages, but not uninfected controls or cells infected with classical swine fever virus [229]. Similar cytotoxic responses without further investigation of CD8α dependency have been shown by other groups as well [230, 231].

As mentioned, CD8α is also found on DP T cells, which account for a considerable population in pigs and might also exert cytotoxic responses. DP T cells from ASFV-recovered pigs also have been shown to proliferate and express perforin and IFNγ upon *in vitro* re-stimulation with ASFV-derived antigens [107, 232]. Moreover, the aforementioned depletion experiments [227] suggest that DP T cells, like CD8α$^+$ cells, might play a role in antiviral immunity during ASFV infection.

The predominant focus of previous studies investigating cellular immunity against ASFV infection was put on CD8α$^+$ lymphocytes, while CD4$^+$ T-cell responses are less well investigated. Canals *et al.* demonstrated a first insight into ASFV-specific reactions of CD4$^+$ T cells. They cultivated PBMCs from ASFV-immune pigs in the presence of live (i.e., intracellular antigen) or UV-inactivated (i.e., extracellular antigen) virus. Proliferation of CD4$^+$ T cells in the presence of UV-inactivated virus was entirely abrogated by treatment with mAbs against CD4, while the proliferative response was only partially impaired but not abolished by anti-CD8α, anti-SLA I, and anti-SLA II mAbs [233]. CD4$^+$ T cells also provide T-cell help to B cells in order to induce antibody production and maturation. Casal *et al.* showed that depletion of CD4$^+$ T cells by mAb treatment or rosetting resulted in significantly reduced production of ASFV-specific antibodies, indicating a T cell-dependent class switch [234].

Regulatory T-cell responses during ASFV infections are often not investigated. This is primarily due to the short period of time between infection and death of the infected individual, providing

not enough time for Tregs to fully develop and unfold their effector functions. Recently, Treg responses were investigated in an immunization study with two attenuated ASFV strains, the naturally attenuated OURT88/3 and the deletion mutant BeninΔMGF. Treg frequencies were elevated 130 days after immunization and seemingly inhibited protective responses after challenge infection because IL-10 serum levels increased considerably [235].

Porcine γδ T cells might take part in protective responses during ASFV infection, because a positive correlation between circulating γδ T-cell frequencies and survival was found [236]. γδ T cells might also play another role during ASFV infection. They have been shown to be able to induce T-cell proliferation in an SLA II-dependent manner in the absence of professional APC, indicating that they present viral antigen to other T-cell populations [120]. This might even compensate for antigen presentation deficiencies in ASFV-infected APCs [66]. However, the evidence is weak and has never been explored further.

Since porcine iNKT cells have only been investigated during immunizations or in healthy individuals, there is no knowledge about possible contributions of this cell type to the antiviral response against infections. Previously, PBMCs from ASFV-recovered pigs with an innate phenotype ($CD3^+/CD6^-/CD8\alpha^+/CD16^+$) similar to NKT cells demonstrated cytotoxic activity, but a further characterization of these cells has not been done [92]. Still, this indicates that unconventional T cells might play a role in the ASFV-specific immune response.

1.4 The Objectives

Infectious diseases harbor a risk for both pigs and humans but most research so far was focused exclusively on humans, which left the porcine immune system's composition as well as characteristics of specific immune-cell populations during infections largely unexplored.

Little general data is available regarding porcine iNKT cells, let alone about their activation or effector capacities during viral infections. Human and murine studies have shown that iNKT cells are pivotal to immunity. However, these studies have also proven considerable differences between murine and human iNKT cells. In the first part of this thesis, it was one objective to investigate porcine iNKT cells in greater detail and expand the understanding about them in healthy individuals as well as during viral infections. This serves two major purposes: First, this knowledge allows further research into viral diseases and antiviral vaccine candidates for pigs, ultimately improving animal health and wellbeing. Second, it provides the necessary prerequisites for the establishment of a new model species closer to humans, improving current animal models especially for infections concerning both pigs and humans.

In a second part, the immune responses of pigs against two different viruses were investigated. For the first virus, IAV, pigs are not only susceptible hosts and of epidemiological importance but also a supposedly more suitable model species for human infections. Additionally, pigs are susceptible to mammalian influenza viruses but can also be infected by avian strains, enabling recombination events and the emergence of new influenza virus strains. This has been the cause for a multitude of major outbreaks and pandemics in the last century. Moreover, pigs are increasingly utilized as a model species in different research settings because their physiological and immunological properties resemble the human ones more closely than those of mice do. However, most studies focused on responses of either peripheral cells or isolated time points only. In contrast, immune responses in this thesis were investigated both locally along the route of viral entry and systemically over a prolonged study period. The results from these studies in the porcine model were compared to human responses against IAV infections.

For the second virus, ASFV, the pig is the only natural host. Although enormous losses caused by ASF already occurred globally, antiviral responses are insufficiently investigated. However, an improved understanding of the porcine T-cell immunity is needed to develop more precise countermeasures against the current near-global spread of ASFV. Moreover, although wild counterparts of domestic pigs, wild boar, play a crucial role in ASFV dissemination, there is virtually no data about their immune response. Therefore, antiviral responses against both moderately virulent as well as highly virulent ASFV isolates were studied in domestic pigs and wild boar.

2 PUBLICATIONS

2.1 Publication I

Porcine Invariant Natural Killer T Cells:
Functional Profiling and Dynamics in Steady State and Viral Infections

2.2 Publication II

Experimental H1N1pdm09 infection in pigs mimics human seasonal influenza infections

2.3 Publication III

Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain

2.4 Publication IV

T-cell responses in domestic pigs and wild boar upon infection with the moderately virulent African swine fever virus strain "Estonia2014"

2.5 Publication V

Comparative Pathology of Domestic Pigs and Wild Boar Infected with the Moderately Virulent African Swine Fever Virus Strain "Estonia 2014"

2.1 Publication I

Porcine Invariant Natural Killer T Cells: Functional Profiling and Dynamics in Steady State and Viral Infections

Alexander Schäfer, Jane Hühr, Theresa Schwaiger, Anca Dorhoi, Thomas C. Mettenleiter, Sandra Blome, Charlotte Schröder, Ulrike Blohm

Frontiers in Immunology

Research Topic "Porcine Anti-Viral Immunity"

doi: 10.3389/fimmu.2019.01380

June 2019

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 June 2019
doi: 10.3389/fimmu.2019.01380

Porcine Invariant Natural Killer T Cells: Functional Profiling and Dynamics in Steady State and Viral Infections

*Alexander Schäfer[1], Jane Hühr[1], Theresa Schwaiger[2], Anca Dorhoi[1], Thomas C. Mettenleiter[3], Sandra Blome[4], Charlotte Schröder[2] and Ulrike Blohm[1]**

[1] Institute of Immunology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, [2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, [3] Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, [4] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

OPEN ACCESS

Edited by:
John E. Butler,
University of Iowa, United States

Reviewed by:
Nicolas Bertho,
INRA Biologie, Épidémiologie et Analyse de Risque en santé animale (BIOEPAR), France
Sankar Renu,
The Ohio State University, United States

***Correspondence:**
Ulrike Blohm
ulrike.blohm@fli.de

Specialty section:
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

Received: 29 October 2018
Accepted: 31 May 2019
Published: 18 June 2019

Citation:
Schäfer A, Hühr J, Schwaiger T, Dorhoi A, Mettenleiter TC, Blome S, Schröder C and Blohm U (2019) Porcine Invariant Natural Killer T Cells: Functional Profiling and Dynamics in Steady State and Viral Infections. Front. Immunol. 10:1380. doi: 10.3389/fimmu.2019.01380

Pigs are important livestock and comprehensive understanding of their immune responses in infections is critical to improve vaccines and therapies. Moreover, similarities between human and swine physiology suggest that pigs are a superior animal model for immunological studies. However, paucity of experimental tools for a systematic analysis of the immune responses in pigs represent a major disadvantage. To evaluate the pig as a biomedical model and additionally expand the knowledge of rare immune cell populations in swine, we established a multicolor flow cytometry analysis platform of surface marker expression and cellular responses for porcine invariant Natural Killer T cells (iNKT). In humans, iNKT cells are among the first line defenders in various tissues, respond to CD1d-restricted antigens and become rapidly activated. Naïve porcine iNKT cells were $CD3^+/CD4^-/CD8^+$ or $CD3^+/CD4^-/CD8^-$ and displayed an effector- or memory-like phenotype ($CD25^+/ICOS^+/CD5^{hi}/CD45RA^-/CCR7^{\pm}/CD27^+$). Based on their expression of the transcription factors T bet and the iNKT cell-specific promyelocytic leukemia zinc finger protein (PLZF), porcine iNKT cells were differentiated into functional subsets. Analogous to human iNKT cells, *in vitro* stimulation of porcine leukocytes with the CD1d ligand α-galactosylceramide resulted in rapid iNKT cell proliferation, evidenced by an increase in frequency and Ki-67 expression. Moreover, this approach revealed CD25, CD5, ICOS, and the major histocompatibility complex class II (MHC II) as activation markers on porcine iNKT cells. Activated iNKT cells also expressed interferon-γ, upregulated perforin expression, and displayed degranulation. In steady state, iNKT cell frequency was highest in newborn piglets and decreased with age. Upon infection with two viruses of high relevance to swine and humans, iNKT cells expanded. Animals infected with African swine fever virus displayed an increase of iNKT cell frequency in peripheral blood, regional lymph nodes, and lungs. During Influenza A virus infection, iNKT cell percentage increased in blood, lung lymph nodes, and broncho-alveolar lavage.

Our in-depth characterization of porcine iNKT cells contributes to a better understanding of porcine immune responses, thereby facilitating the design of innovative interventions against infectious diseases. Moreover, we provide new evidence that endorses the suitability of the pig as a biomedical model for iNKT cell research.

Keywords: iNKT cells, T cells, pig, biomedical model, influenza A virus, African swine fever virus

INTRODUCTION

Biomedical research is in need of large animal models that reflect human infectious diseases better than current rodent models (1, 2). Because of the striking resemblances between porcine and human immune system and physiology, pigs could be a superior model species (3–10). In order to establish pigs as a new biomedical model species, a more detailed understanding of porcine immune responses and leukocyte subsets is strongly needed.

Leukocytes at systemic and peripheral sites are eminently important for control of microbial colonization and defense against infections by induction of protective immunity (11). One of those leukocyte subsets are invariant Natural Killer T (iNKT) cells. These cells bridge and orchestrate both untargeted innate and specific adaptive responses, which are crucial for pathogen clearance and survival. In contrast to the vast heterogeneity of T cell receptors (TCR) among conventional $CD3^+$ T cells (cTC), iNKT cells possess a semi-invariant TCR. This TCR is restricted to the non-classical major histocompatibility complex (MHC) class I-related CD1d, presenting lipid or glycolipid antigens. iNKT cells can be activated antigen-dependently with glycolipids derived from microbes or the host by TCR-CD1d interactions or antigen-independently via cytokines, mainly interleukin-(IL-)12 and IL-18 or type I interferons (IFN). The induction of cytokine expression in iNKT cells does not require classical co-stimulation, whereas iNKT cell proliferation depends on co-stimulatory signals by B7/CD28 or CD40/CD40L (12). Additionally, effector cytokines are present as immediately available preformed mRNA transcripts in iNKT cells (13). Therefore, iNKT cells rapidly proliferate and secrete effector molecules like IFNγ, IL-17 or granulocyte-macrophage colony-stimulating factor after activation (14). Moreover, they are able to lyse infected cells by perforin and Fas/FasL interaction (15–18). iNKT cells also augment B cell responses, class switching and affinity maturation independently of classical helper cells (19, 20). Several iNKT cell subsets have been identified by their expression of different transcription factors, like promyelocytic leukemia zinc finger protein (PLZF) and T-bet, most notably in mice, where they are primarily differentiated into iNKT1 ($T\text{-bet}^+/PLZF^+$), iNKT2 ($T\text{-bet}^-/PLZF^{hi}$), and iNKT17 ($T\text{-bet}^-/PLZF^{lo}$) (21–26). Differentiated subsets of human iNKT cells are not as well-defined as in mice (27).

At present, most studies focusing on porcine iNKT cells investigated the potential use of the cognate CD1d ligand α-galactosyl-C16-ceramide (αGC) as an adjuvant for vaccines against Influenza A virus (IAV) and other infectious diseases (28–32). Knowledge about phenotype, response kinetics, and functional aspects of iNKT cell responses in pigs is scarce. The antigen presentation molecules of the CD1 family are of particular importance for iNKT cells. In addition to CD1d, there are four other members in the CD1 family, CD1a, CD1b, CD1c, and CD1e. All of these molecules are expressed in pigs as well as in humans, while mice lack proteins other than CD1d (33). It has been shown that expression of CD1d on thymocytes is required for iNKT cell development, in mice (34) as well as in pigs (35). Murine CD1d tetramers loaded with the αGC analog PBS57 have been shown to detect porcine iNKT cells (7, 36). Their frequency is typically between 0.01 and 1% among $CD3^+$ T cells, thereby resembling human iNKT cell frequencies (7, 28). Porcine iNKT cells are $CD8\alpha^+$ or $CD8\alpha^-$ but lack expression of CD4 in most tissues (28, 29, 37). In contrast to human and murine iNKT cells, naïve porcine iNKT cells express high levels of CD44, as most lymphocytes in swine (38, 39). Moreover, the iNKT cell-specific transcription factor PLZF is highly expressed in porcine iNKT cells (36). Some evidence indicates that iNKT cells expand upon stimulation of porcine PBMC with αGC (29, 36). Using next generation sequencing, it has recently been shown that porcine iNKT cells predominantly use Vα and Jα segments homologous to the Vα24-Jα18 and Vα14-Jα18 rearrangements used in humans and mice, respectively (40). Moreover, molecular investigations demonstrated that the antigen-binding domain of the invariant α-chain, CDR1α, is conserved between pigs and humans (40). This indicates that responses of porcine iNKT cells mimic responses of human iNKT cells, thereby further underlining the suitability of the pig as a biomedical model species for human iNKT cells.

In order to comprehensively understand porcine iNKT cells and advance research, we investigated their phenotype, dynamics and functional responses in-depth in steady state and during IAV and African swine fever virus (ASFV) infection.

MATERIALS AND METHODS

Pigs and Biological Samples

In total 13 German landrace pigs for IAV and 12 for ASFV experiments were obtained from a commercial breeding unit (BHZP-Basiszuchtbetrieb Garlitz-Langenheide, Germany) with high biosecurity standards and hygiene (free of IAV and Porcine reproductive and respiratory syndrome virus among others). Samples for the investigation of age-dependent changes in iNKT cell frequency were obtained during routine veterinary check-ups from the same commercial breeding unit (BHZP-Basiszuchtbetrieb Garlitz-Langenheide, Germany).

Swine (Danish landrace/Danish Large White/Danish Duroc hybrid) used for *in vitro* experiments involving PBMC, were kept at the Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems under conventional conditions.

Viruses and Infection Experiments

Influenza virus A/Bayern/74/2009 was propagated on Madin-Darby canine kidney cells (MDCKII) cells in MEM supplemented with 0.56% bovine serum albumin, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 2 µg/ml L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, USA). For viral titration by $TCID_{50}$ assay, serial 10-fold dilutions of virus suspensions were prepared, added to MDCKII cells in 96-well plates, and incubated for 3 days at 37°C and 5% CO_2. Cytopathic effect was microscopically evaluated. Titers were calculated according to Spearman-Kärber (41, 42). Four-week-old piglets were obtained from a commercial breeding facility directly after weaning. Absence of acute IAV infection of pigs used for the IAV study was confirmed by real-time PCR (AgPath.IDTM One-Step RT-PCR Kit, Applied Biosystems, USA) of nasal swabs prior to transport to the FLI (modified from Spackman et al. (43)). IAV infection was performed 3 weeks after transport to our facility by intranasal administration of 2 ml virus suspension (10^6 $TCID_{50}$/ml) using mucosal atomization devices (Wolfe Tory Medical, USA).

ASFV Armenia08 was propagated and titrated using mature porcine PBMC-derived macrophages as previously described (44). For back titration, virus hemadsorption test was performed by endpoint titration of the diluted inoculation virus. In brief, 100 µl virus dilution were incubated for 24 h on PBMC-derived macrophages in 96-well plates. Thereafter, 20 µl of a 1% homolog erythrocyte suspension were added and hemadsorption read after 24 and 48 h. All samples were tested in quadruplicates. Hemadsorbing units (HAU) were used for read-out. For infection, 2 ml macrophage culture supernatant containing $10^{6.25}$ HAU ASFV Armenia08 were inoculated oro-nasally. All work involving ASFV was done in the high containment facility ($L3^+$) at the Friedrich-Loeffler-Institut.

Cell Isolation and Culture

For isolation of peripheral blood mononuclear cells (PBMC), whole blood was separated by density gradient centrifugation using Pancoll (PAN-Biotech, Germany). PBMC were collected and washed with PBS-EDTA (1 mM; used for all analyses). Cell count was determined using Neubauer improved haemocytometer. Single cell suspensions from spleen and lymph nodes were prepared by mechanically disrupting tissue with a sieve. Lymphocytes from liver were isolated following a modified protocol previously described (45). In brief, liver samples were perfused with ice-cold PBS-EDTA. Perfused regions were minced with sterile scissors, resuspended in PBS-EDTA supplemented with 100 µM $CaCl_2$, and digested with Collagenase D (1 mg/ml; Sigma-Aldrich) for 40 min at 37°C. Remaining tissue was removed by short centrifugation. Cell pellet was resuspended in PBS-EDTA and used for flow cytometry. Lymphocytes from lung tissue were isolated by mincing non-perfused lung tissue, followed by enzymatic digestion as described for liver samples. Lung tissue was additionally mashed through a cell strainer with the plunger of a syringe after digestion. Unless otherwise stated, cells were cultured in Ham's F12/IMDM (1:1), supplemented with 10% fetal calf serum (FCS), 2-mercaptoethanol (50 µM), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Cell Stimulation

For iNKT cell stimulation, freshly isolated PBMC were seed into round-bottom 96-well plates at a density of 10^7 PBMC/ml. αGC (Toronto Research Chemicals, Canada) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) or DMSO as vehicle control diluted in cell culture media were added. αGC was used at 0.1 µg/ml (low-dose) and 1 µg/ml (high-dose). Cells were incubated at 38.5°C, 5% CO_2 for the indicated time. After incubation, cells were harvested, washed with PBS-EDTA, and analyzed by flow cytometry.

For detection of IFNγ and perforin, cells were stimulated as previously described (46). After 4 days, fresh medium with αGC in the corresponding concentrations was added to the cells. After another 2 h incubation, Brefeldin A (10 µg/ml, Biolegend, USA) was added to enable intracellular accumulation of target proteins. Cells were incubated for 4 h and then stained and analyzed by flow cytometry.

For analysis of CD107a surface expression as a marker of degranulation, cells were treated as previously described (47). Briefly, freshly isolated PBMC were seed into 96-well plates at a density of 10^7 PBMC/ml and rested overnight. For antigenic stimulation, αGC was added for a final concentration of 0.1 µg/ml or 1 µg/ml. As unspecific inducers, Phorbol-12-myristat-13-acetat (PMA; Sigma-Aldrich, USA) and ionomycin (Sigma-Aldrich, USA) were added for final concentrations of 50 ng/ml and 1 µg/ml, respectively. The cells were stimulated in the presence of anti-CD107a antibodies (clone 4E9/11, Bio-Rad, USA; 4 µg/ml). After 1 h incubation, Brefeldin A (10 µg/ml), and Monensin (4 µM, Biolegend, USA) were added and the cells were incubated for another 5 h and then stained and analyzed by flow cytometry. Specific degranulation was calculated as the difference in surface expression of stimulated and control cells and is given as ΔCD107a.

For *in vitro* activation assays, porcine $CD172a^+$ cells were purified using monoclonal antibodies (cone 74-22-15) and magnetic anti-mouse IgG1 beads (BD Bioscience, USA). $CD172a^+$ cells were infected with IAV (MOI 1) or ASFV (MOI 0.1) for 48 h. Then, supernatants were collected, cleared of debris by centrifugation, and stored at −80°C until further use. Freshly isolated porcine PBMC were stimulated with the respective supernatants or 1 µg/ml αGC as a positive control for 4 days and then stained and analyzed by flow cytometry.

Cell Proliferation Assay

Freshly isolated PBMC were stained using Tag-it Violet Proliferation and Cell Tracking Dye (Biolegend, USA) according to the manufacturer's instructions. Briefly, total PBMC were

adjusted to 1×10^7 cells/ml in PBS and incubated with the Tag-it Violet Proliferation and Cell Tracking Dye at a concentration of 5 μM. Cells were incubated for 20 min at 37°C in the dark. Quenching was done by addition of cell culture media supplemented with 10% FCS. After washing, stained cells were used for visualization of iNKT cell proliferation after stimulation with αGC.

Flow Cytometry

Stainings for flow cytometry were performed using either single cell suspensions or whole blood. All incubation steps were carried out for 15 min at 4°C in the dark, unless otherwise stated. Antibodies used for flow cytometry are shown in **Supplementary Table 1**. For iNKT cell staining, first tetramers were added at the predetermined concentration (1:500) and incubated at room temperature in the dark for 30 min. Antibodies for staining of additional surface markers were added without washing and incubated with the tetramers for another 15 min. After washing, antibody staining was proceeded. Unconjugated antibodies were detected by isotype-specific fluorochrome-conjugated secondary antibodies. When whole blood or tissue samples containing erythrocytes were stained, erythrocytes were lysed after surface staining prior to fixation by lysis buffer (1.55 M NH_4Cl, 100 mM $KHCO_3$, 12.7 mM Na_4EDTA, pH 7.4, in A.dest.). For intracellular staining, cells were fixed after surface staining using the True-Nuclear Transcription Factor Buffer Set (Biolegend, USA) according to the manufacturer's instructions. Murine CD1d tetramers, empty or loaded with PBS57, were obtained from the NIH Tetramer Core Facility.

Doublets were excluded by consecutive gating FSC-W/FSC-H and SSC-W/SSC-H. Living lymphocytes were gated based on their forward-scatter (FSC) and side-scatter (SSC) properties. Live, single lymphocytes were further separated in conventional T cells (cTC; $CD3^+$/CD1d-Tet^-) and iNKT cells ($CD3^+$/CD1d-Tet^+) for subsequent analysis.

Per sample, at least 1×10^5 single cTC were recorded. BD FACS Canto II or BD LSRFortessa with FACS DIVA Software (all BD Bioscience, USA) and FlowJo V10 (Treestar, USA) were used for all analyses.

Statistical Analysis

GraphPad Prism 7 (Graphpad Software Inc., USA) was used for statistical analysis and graph creation. Normality was tested with the Shapiro-Wilk normality test. Subsequent analysis was performed with either parametric tests for normally distributed data sets or non-parametric tests for non-normally distributed data sets. For analysis of data sets with three or more groups, Repeated-measures one-way ANOVA was used to investigate statistically significant differences between the groups. Multiple comparisons were performed for differences between iNKT cells and cTC subsets, respectively. No statistical analyses were performed for differences between iNKT cells and cTC. Holm-Sidak's *post-hoc* test was used for correction of multiple comparisons. For analysis of data from the IAV and ASFV trial, ordinary one-way ANOVA with Holm-Sidak's *post-hoc* test for correction of multiple comparisons was used. For analysis of data sets of two groups, paired *t*-tests were used. Unless indicated otherwise, data is shown as mean (SD). Statistical significance was defined as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Naïve Peripheral Porcine iNKT Cells Are Mostly $CD8\alpha^+$ and Display an Effector- and Memory-Like Phenotype

To investigate porcine iNKT cells in peripheral blood by flow cytometry, we used murine CD1d tetramers loaded with the αGC analog PBS57 (PBS57 Tet) as previously described (7, 36). After exclusion of doublet cells, live cTC were gated as $CD3^+$/PBS57 Tet^- cells and iNKT cells were defined as $CD3^+$/PBS57 Tet^+ among the lymphocyte gate [**Figure 1A** (48)]. In healthy animals, the average iNKT cell frequency was 0.5%, comparable to frequencies in blood published earlier (36, 37). Most naïve iNKT cells expressed CD8α, while only a small amount was $CD4^+$, although a considerable proportion of iNKT cells expressed low levels of CD4 (**Figures 1B,C**). Thereby, we confirmed previous findings showing similar results (36, 37). iNKT cells expressed neither CD8β nor γδTCR (**Figure 1B**). For differentiation of iNKT cell subsets, expression of the classical T cell markers CD8α and CD4 was analyzed. The subsets were gated according to the respective expression of these markers on cTC. The major subset of peripheral iNKT cells in swine was $CD8\alpha^+/CD4^-$ (73.7 ± 11.9%; **Figure 1C**). There was no distinct $CD4^+$ population; however, a minor fraction of iNKT cells was $CD8\alpha^+/CD4^{lo}$ (DP; 3.6 ± 1.9%). Moreover, there was a considerable $CD8^-/CD4^-$ (DN) population (21.5 ± 10.8%).

For further differentiation, we investigated steady state expression of surface markers that are frequently associated with an effector phenotype in mice and humans, i.e., CD5 (49), CD25 [IL-2Rα (50)], CD278 [Inducible T-cell co-stimulator, ICOS (51)], and the major histocompatibility complex class II [MHC II (52)] in naïve iNKT cells and cTC. All iNKT cells were positive for CD5 whereas only around 60% of cTC showed CD5 expression (**Figure 2A**). Around half of all iNKT cells expressed CD25 at low levels on their surface, while cTC displayed only a minor $CD25^{hi}$ fraction (**Figure 2A**). ICOS was expressed on virtually all iNKT cells, while only on a minor fraction of cTC (**Figure 2A**). MHC II was expressed on most iNKT cells at medium or high levels (**Figure 2A**). In contrast, cTC were mostly MHC II^- but the MHC II^+ fraction expressed it at comparable levels (**Figure 2A**). Because CD25 and MHC II were not expressed by all iNKT cells, we investigated which iNKT cell subset expressed the proteins (**Figure 2B**). Differential staining with CD8α revealed that both proteins were predominantly expressed on $CD8\alpha^+$ iNKT cells. DN iNKT cells showed no or low expression of CD25 and MHC II.

To investigate the functional differentiation of porcine iNKT cells, we analyzed the expression of markers regularly associated with antigen experience and memory status, CD45RA, C-C

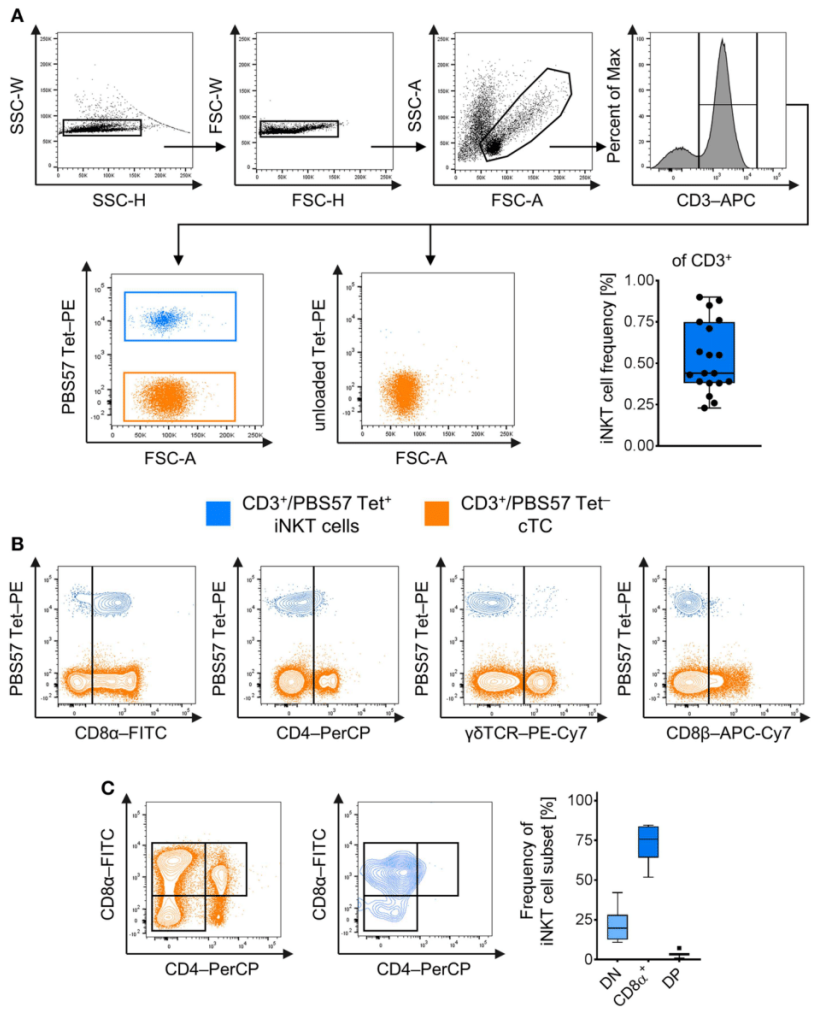


FIGURE 1 | Identification and phenotype of naïve peripheral porcine iNKT cells**.** Expression of surface markers was analyzed by flow cytometry. **(A)** Doublets were excluded by SSC-W vs. SSC-H gates, followed by FSC-W vs. FSC-H gates. Live lymphocytes were identified according to their FSC/SSC characteristics. All T cells were identified using antibodies against CD3. $CD3^+$ T cells stained with the PBS57-loaded CD1d tetramer (left plot) were defined as invariant Natural Killer T cells (iNKT; blue), tetramer-negative cells were defined as conventional T cells (cTC; orange). Unloaded tetramers served as control (right plot). Frequency of iNKT cells in peripheral blood among $CD3^+$ lymphocytes shown as Tukey box plot ($n = 19$). **(B)** Evaluation of expression of CD8α, CD4, γδTCR, and CD8β on iNKT cells and cTC. Representative plots of at least four experiments are shown. **(C)** Representative plots of CD8α and CD4 co-expression by cTC and iNKT cells. $CD8\alpha^-/CD4^-$ (DN; light blue), $CD8\alpha^+/CD4^-$ ($CD8\alpha^+$, blue), and $CD8\alpha^+/CD4^+$ (DP; dark blue) subsets among iNKT cells were identified according to their expression pattern in cTC. Frequency of iNKT cell subsets shown as Tukey box plots ($n = 7$).

chemokine receptor type 7 (CCR7), and CD27 (53–56). cTC displayed a minor fraction of naïve $CD45RA^+$ cells, while the larger fraction consisted of antigen-experienced $CD45RA^-$ cells (**Figure 2C**). $CD45RA^-$ cTC were further divided into $CD27^+/CCR7^+$ central memory cells, $CD27^+/CCR7^-$ transitional memory cells and $CD27^-/CCR7^-$ effector memory cells. There was only a minor population of $CD27^-/CCR7^+$ activated effector memory cells (**Figure 2C**). All iNKT cells were $CD45RA^-$ and thus displayed an antigen-experienced phenotype. Most iNKT cells were $CD27^+/CCR7^+$, resembling central memory cells. A second major fraction expressed CD27 but was $CCR7^-$, similar to transitional memory cells. About a third of iNKT cells was $CD27^-$ and could further be divided into equal fractions of $CCR7^-$ and $CCR7^+$, thereby differentiating subsets comparable to resting and activated effector memory cells, respectively (**Figure 2C**). Expression of CD27 and CCR7 was also investigated for co-expression with $CD8\alpha^+$. Most $CD8\alpha^+$ iNKT cells expressed CCR7 as well as CD27 (**Figure 2D**).

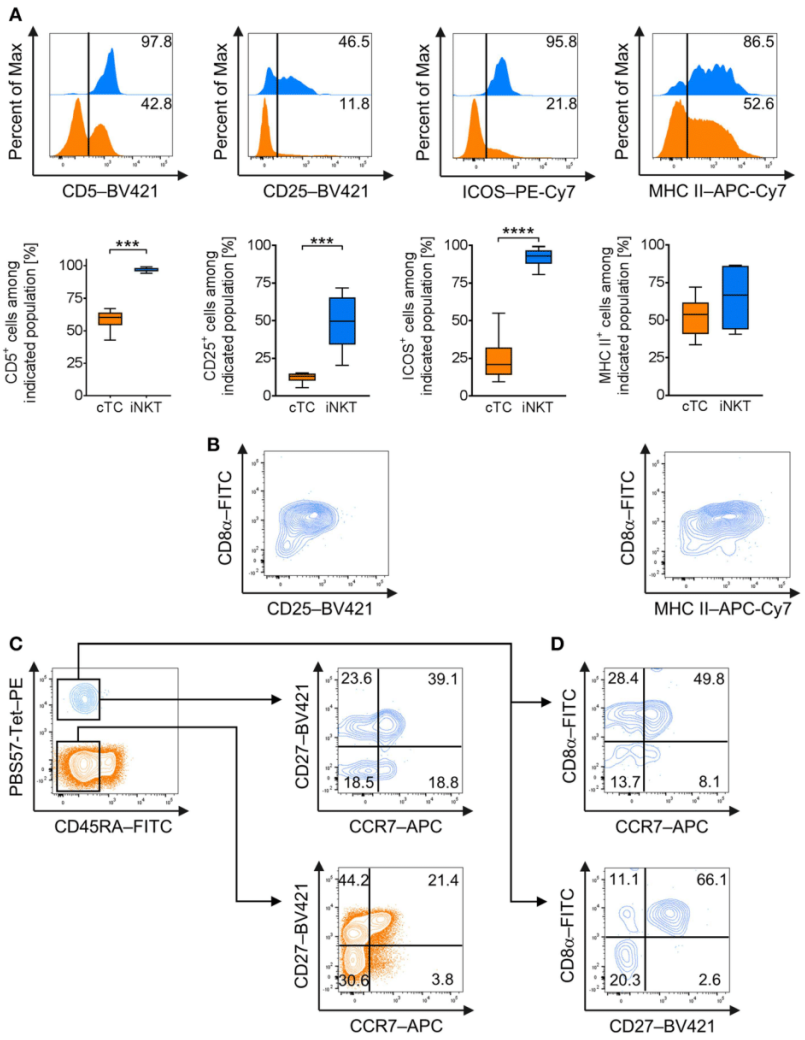


FIGURE 2 | Expression of effector and memory cell-associated markers on naïve porcine iNKT cells. Expression of surface markers associated with effector functions and memory status on naïve peripheral iNKT cells was analyzed by flow cytometry. **(A)** Representative flow cytometric plots of CD5, CD25, MHC II, and ICOS expression by iNKT cells (blue) and cTC (orange). Summarized data show frequencies of iNKT cells or cTC expressing CD5, CD25, MHC II, and ICOS (mean (SD), $n = 6$). **(B)** Representative flow cytometric plots showing differential expression of CD8a and CD25 or MHC II on iNKT cells. **(C)** Expression of CD45RA (left) on iNKT cells and cTC. CD45RA$^-$ cTC (middle) and iNKT cells (right) were further investigated for expression of CD27 and CCR7 and divided into CD27$^+$/CCR7$^+$ central memory cells, CD27$^+$/CCR7$^-$ transitional memory cells, CD27$^-$/CCR7$^-$ effector memory cells, and CD27$^-$/CCR7$^+$ activated effector memory cells. **(D)** Differential expression of CD8α and CCR7 or CD27. Representative plots and histograms of at least three experiments are shown. $^{***}p < 0.001$, $^{****}p < 0.0001$, paired *t*-test.

DN iNKT cells were negative for CD27 while a fraction expressed CCR7 (**Figure 2D**).

In mice, intracellular staining of transcription factors, including T-bet and PLZF, is used to define functional iNKT cell subsets iNKT1 (T-bet$^+$/PLZF$^+$), iNKT2 (T-bet$^-$/PLZF^{hi}), and iNKT17 (T-bet$^-$/PLZF^{lo}) resembling the T-helper cell populations Th1, Th2, and Th17, respectively (21–26). Currently, there is no such differentiation available for porcine iNKT cells. Therefore, we investigated the expression of T-bet and PLZF in porcine iNKT cells. We confirmed that porcine iNKT cells express higher levels of PLZF than cTC (**Figure 3A**). Moreover, we detected expression of T-bet in a subset of porcine iNKT cells (**Figure 3A**). Co-expression of T-bet and PLZF was used to define iNKT cell subsets corresponding to the ones described in other species (**Figures 3B–E**). In naïve swine, the largest iNKT cell subset had a T-bet$^+$/PLZF$^+$ phenotype and was therefore defined as iNKT1 (49.8 ± 14.8%). The second major group was T-bet$^-$/PLZF^{hi}, thereby resembling

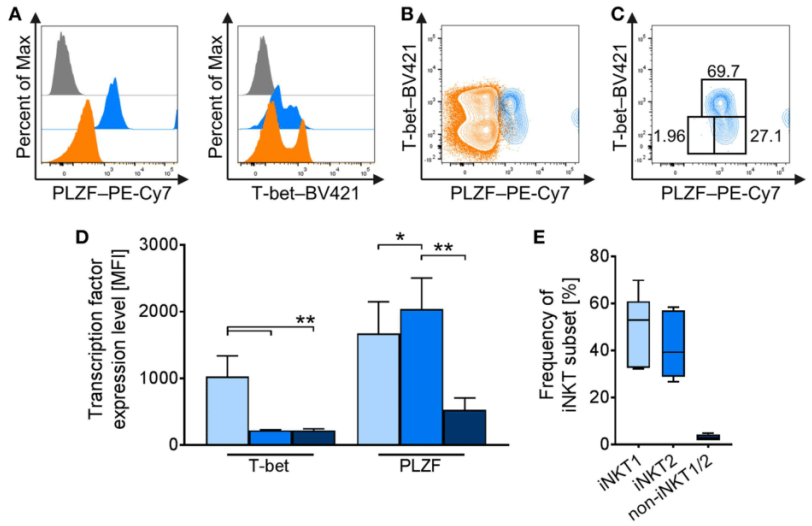


FIGURE 3 | Differential profiling of naïve porcine iNKT cells. Peripheral iNKT cells were differentiated by flow cytometry according to their expression of the transcription factors PLZF and T-bet. **(A)** Representative flow cytometric histograms of PLZF (left) and T-bet (right) expression in iNKT cells (blue) and cTC (orange). Control stainings are shown in gray. **(B)** Representative flow cytometric plot showing co-expression of T-bet and PLZF in iNKT cells and cTC. **(C)** Differential gating of iNKT cells according to their T-bet and PLZF expression. iNKT1 were defined as T-bet^{+}/PLZF^{+} (light blue), iNKT2 as T-bet^{-}/PLZF^{hi} (blue), and non-iNKT1/2 as T-bet^{-}/PLZF^{lo} (dark blue). **(D)** Expression profiles of T-bet and PLZF in iNKT1 (light blue), iNKT2 (blue), and non-iNKT1/2 (dark blue) ($n = 6$). **(E)** Frequency of iNKT subsets in naïve swine shown as Tukey box plots ($n = 6$). *$p < 0.05$, **$p < 0.01$, repeated-measures one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

iNKT2 (41.7 ± 13.3%). A small fraction was T-bet^{-}/PLZF^{lo} (3.1 ± 1.2%) and was defined as non-iNKT1/non-iNKT2 (non-iNKT1/2) because additional differentiating markers were not available. Appropriate differentiation was confirmed by analysis of T-bet and PLZF expression levels in the iNKT cell subsets. T-bet expression in iNKT1 was significantly higher than in iNKT2 and non-iNKT1/2 (**Figure 3D**). PLZF expression level was the highest in iNKT2 and the lowest in non-iNKT1/2 (**Figure 3D**).

Taken together, we confirmed the phenotype of naïve iNKT cells from peripheral blood of healthy pigs to be predominantly CD3^{+}/CD4^{-}/CD8^{+} or CD3^{+}/DN. All iNKT cells expressed the αβTCR but not the CD8 β-chain. Moreover, we showed that naïve porcine iNKT cells display an effector-like (CD5^{hi}/CD25^{+}/MHC II^{+}/ICOS^{+}) and memory-like phenotype (CD45RA^{-}/CCR7^{+}/CD27^{+}). The CD8α subset expressed higher levels of CD25 and MHC II, as well as higher levels of CCR7 and CD27. Additionally, analogous to classifications in rodents, we were able to divide peripheral iNKT cells into three subsets, iNKT1, iNKT2, and non-iNKT1/2, according to their respective T-bet and PLZF expression.

Antigenic Activation Induces Strong Proliferation in Porcine iNKT Cells

A major feature of iNKT cells is their ability to proliferate rapidly upon activation. For porcine iNKT cells, data regarding responses after antigenic activation is limited. Therefore, we investigated the proliferative ability by quantifying iNKT frequency among CD3^{+} lymphocytes and expression of the proliferation marker Ki-67 after stimulation of porcine PBMC with low-dose (0.1 μg/ml) and high-dose (1 μg/ml) αGC or DMSO as vehicle control for 4 days. We also visualized cell divisions by staining with a fluorescent cell tracking dye. Upon stimulation with αGC, iNKT cell frequency among CD3^{+} lymphocytes strikingly increased. After 4 days of stimulation with low-dose αGC, iNKT cells accounted for about 9.7 ± 7.9% of CD3^{+} T cells, a 20-fold increase. High-dose αGC stimulation resulted in a 30-fold increase, leading to an iNKT cell frequency of 16.9 ± 9% among CD3^{+} lymphocytes (**Figure 4A**). To verify that this increase was due to proliferation, we investigated the expression of Ki-67, a widely used marker specific for proliferating cells (57). Most iNKT cells were Ki-67^{+} after stimulation (85.3 ± 10.6% and 91.7 ± 8.7% after low- and high-dose αGC stimulation, respectively), thereby demonstrating that the majority of iNKT cells were proliferating (**Figure 4B**). Expression of Ki-67 in cTC remained on a low level of background activation, proving the specific activation of iNKT cells by αGC (**Figure 4B**). Staining of PBMC with a fluorescent cell tracking dye (Tag-it Violet) before stimulation allows for detection of single proliferation steps upon activation. Again, nearly all iNKT cells were in a highly proliferative state and up to seven proliferation steps were detectable (**Figure 4C**). There was no detectable difference between both αGC doses. cTC demonstrated no proliferation over background level upon αGC stimulation.

Taken together, we demonstrated the rapid proliferative abilities of peripheral porcine iNKT cells in response to antigenic stimulation.

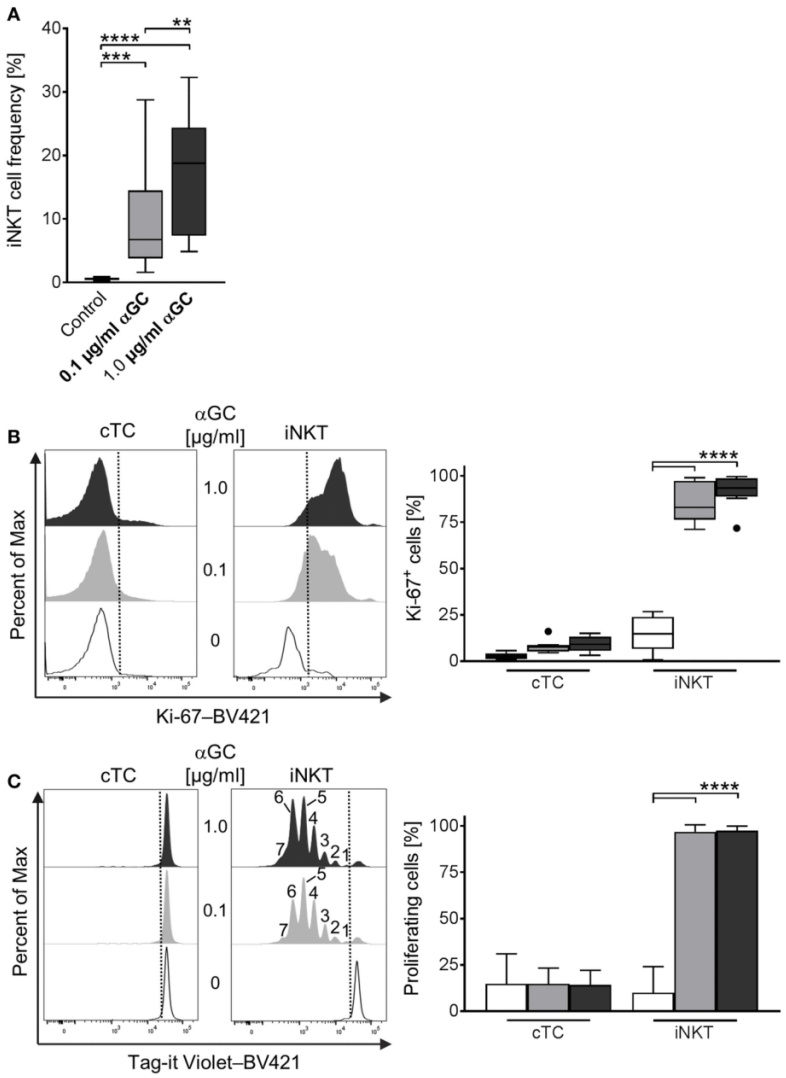


FIGURE 4 | Proliferative activity of porcine iNKT cells upon antigenic activation. **(A)** PBMC were cultivated in the presence of 0.1 μg/ml (gray) or 1 μg/ml (black) αGC or DMSO as vehicle control (white) for 4 days. Proliferation measured as iNKT cell frequency among $CD3^+$ lymphocytes shown as Tukey box plots ($n = 17$). **(B)** Cell proliferation was investigated by measuring Ki-67 expression in cTC and iNKT cells. Representative histograms showing expression of Ki-67 in cTC (left) and iNKT cells (right). Dotted lines indicate the threshold according to the control staining. Frequency of proliferating, Ki-67^+ iNKT cells and cTC ($n = 6$). **(C)** Porcine PBMC were stained with Tag-it Violet and cultivated in the presence of 0.1 μg/ml (gray) or 1 μg/ml (black) αGC or DMSO as vehicle control (white) for 4 days. Proliferating cells were defined as Tag-it $Violet^{lo}$. Representative histograms showing proliferating, Tag-it $Violet^{lo}$ cells in cTC and iNKT cells. Dotted lines indicate the threshold according to the control staining. Numbers indicate individual proliferation steps. Frequency of proliferating, Tag-it $Violet^{lo}$ cTC and iNKT cells (mean (SD), $n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, repeated-measures one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

Porcine iNKT Cells Upregulate Expression of CD25, MHC II, ICOS, and CD5 and Differentiate Into iNKT1 Upon Antigenic Stimulation

Whereas several iNKT cell activation markers are known in mice and humans, for porcine iNKT cells, no markers have been described to investigate their immune response or differentiation status. We therefore analyzed low- and high-dose αGC-stimulated porcine PBMC for activation-dependent changes in expression of multiple surface and intracellular markers. Among the established activation markers used for human and murine T cells are CD25 (50), MHC II (52), ICOS (51), and CD5 (49). Multicolor flow cytometry revealed that the

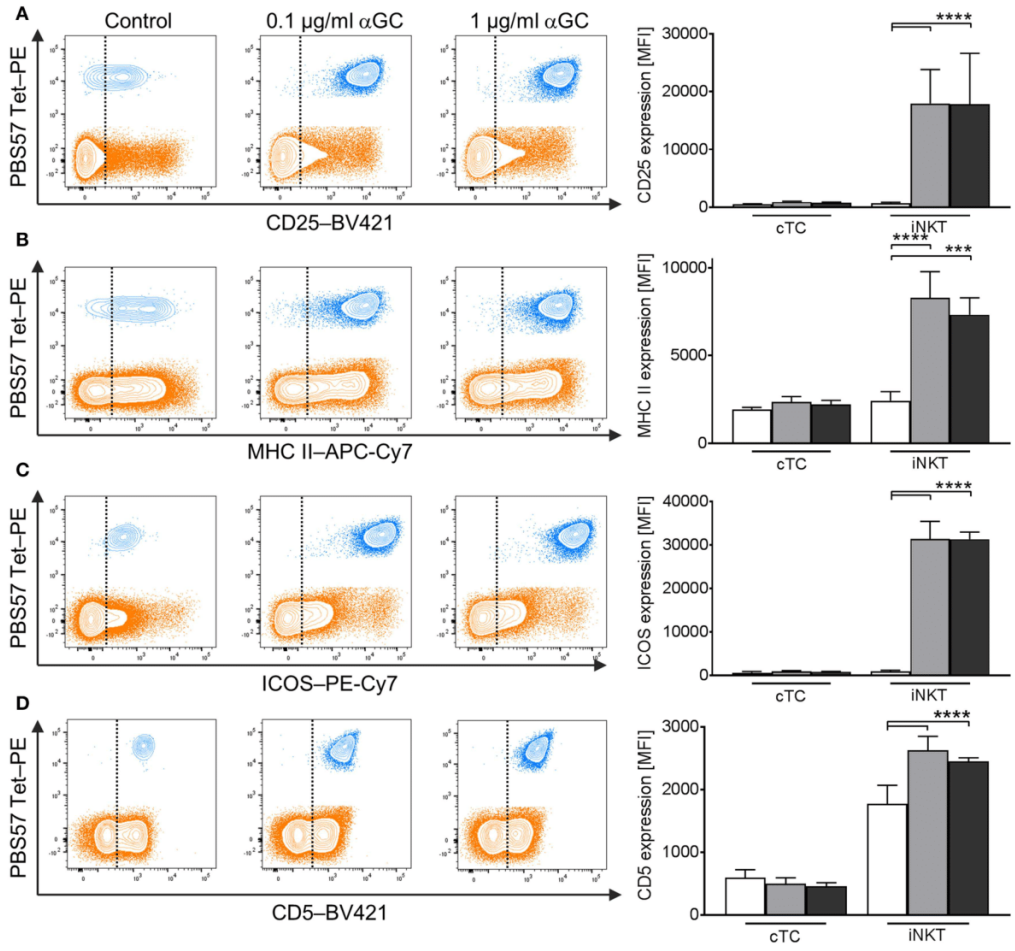


FIGURE 5 | Changes in surface marker expression on porcine iNKT cells upon antigenic activation. Porcine PBMC were incubated in the presence of 0.1 µg/ml or 1 µg/ml αGC or DMSO as vehicle control for 4 days. Representative flow cytometric plots showing expression of **(A)** CD25, **(B)** MHC II, **(C)** ICOS, and **(D)** CD5 on cTC (orange) and iNKT cells (blue). Expression level of control cells (white) and cells stimulated with 0.1 µg/ml (gray) and 1 µg/ml αGC (black) are shown (n = 3-4). Dotted lines show threshold according to marker expression by cTC. Data shown as mean (SD), ***$p < 0.001$, ****$p < 0.0001$, repeated-measures one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

expression of these markers on the surface of cTC did not change significantly irrespective of stimulus or dose. In contrast, upon activation, iNKT cells significantly upregulated the expression of all investigated surface markers. Since most naïve iNKT cells were already positive for MHC II, ICOS, and CD5, the percentage of cells positive for the respective markers did not change. Only the frequency of $CD25^+$ iNKT cells increased significantly after stimulation. However, the expression level of all markers on the surface of iNKT cells increased significantly upon αGC stimulation, as evidenced by heightened MFI. CD25 expression (**Figure 5A**) increased from controls (MFI: 591 ± 106) to low-dose and high-dose αGC-stimulated iNKT cells (MFI: 15,427 ± 3,717; 13,593 ± 2,568, respectively). MHC II (**Figure 5B**) was markedly upregulated between controls (MFI: 2,341 ± 1,248) to low-dose (MFI: 9,653 ± 1,527) and high-dose αGC-stimulated iNKT cells (MFI: 8,159 ± 1,215). ICOS expression (**Figure 5C**) rose from controls (MFI: 849 ± 678) to low-dose (MFI: 35,309 ± 2,548) and high-dose αGC-stimulated iNKT cells (MFI: 31,366 ± 4,013). Expression levels of CD5 (**Figure 5D**) increased from controls (MFI: 1,776 ± 294) to low-dose (MFI: 2,626 ± 170) and high-dose αGC-stimulated iNKT cells (MFI: 2,448 ± 59). There were no significant differences in the expression levels of CD25, MHC II, ICOS, and CD5 on cTC after stimulation with low or high concentrations of αGC.

In mice, activated iNKT cells have been shown to regulate the expression of T-bet and PLZF, depending on the type of activating stimulus (21–26). Comparable data for swine is missing. To investigate the differentiation status of αGC-activated porcine

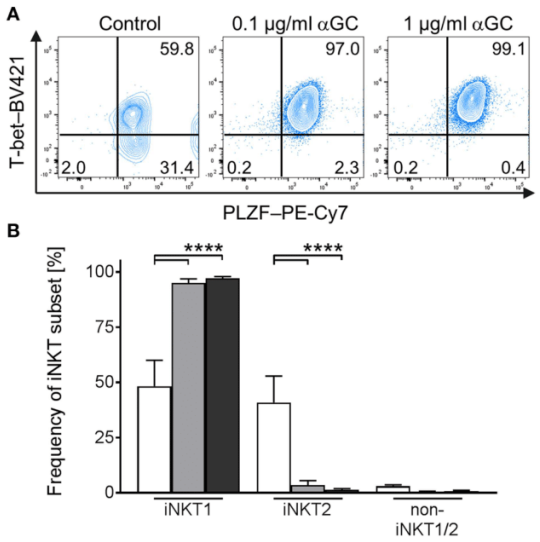


FIGURE 6 | Differentiation of activated porcine iNKT cells. Porcine PBMC were cultivated in the presence of αGC or DMSO as vehicle control. Intracellular expression of T-bet and PLZF was analyzed by flow cytometry after stimulation for 4 days. **(A)** Representative plots of T-bet/PLZF co-expression in untreated control iNKT cells (left) and iNKT cells stimulated with 0.1 µg/ml (middle) or 1 µg/ml (right) αGC. **(B)** Frequency of iNKT1, iNKT2, and non-iNKT1/2 upon stimulation with 0.1 µg/ml (gray) or 1 µg/ml (black) αGC or DMSO as vehicle control (white) ($n = 6$). Data shown as mean (SD), $^{****}p < 0.0001$, repeated-measures one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

iNKT cells, we used the differential staining of T-bet and PLZF established in this study. While PLZF expression was not regulated in iNKT cells (**Figure 6A**), T-bet expression increased in iNKT cells after stimulation with low- and high-dose αGC (**Figure 6A**). Co-expression analysis revealed that all αGC-activated iNKT cells were T-bet$^+$/PLZF$^+$ iNKT1 (**Figure 6B**). There was no difference between low- and high-dose stimulated iNKT cells.

In short, we established a stimulation protocol for porcine iNKT cells. Activation by αGC results in strong upregulation of CD5, CD25, ICOS, and MHC II on iNKT cells and differentiation into T-bet$^+$/PLZF$^+$ iNKT1.

Porcine iNKT Cells Upregulate CD8 and CD4 Expression Upon Antigenic Stimulation

Naïve porcine iNKT cells were mostly CD8α$^+$ or DN and did not display a distinct CD4$^+$ population. However, after stimulation with αGC, frequencies of iNKT cell subsets changed significantly: DN iNKT decreased from 21.5 ± 10.8% in controls to 4.1 ± 3.6% in low-dose and 4.0 ± 2.8% in high-dose αGC-stimulated samples (**Figures 7A,B**). At the same time, the frequency of CD8α$^+$ iNKT cells increased from 73.7 ± 11.9% in controls to 84.1 ± 6.5% in low-dose and 79.6 ± 6.2% in high-dose stimulated samples (**Figures 7A,B**). Stimulation of porcine iNKT cells with αGC resulted in a significant increase of DP iNKT cells. In controls, only 3.6 ± 1.9% of all iNKT cells were DP. Stimulated iNKT cells upregulated CD4 expression, resulting in 11.2 ± 4.4% DP iNKT cells in low-dose and 15.5 ± 5.2% DP iNKT cells in high-dose αGC-stimulated samples (**Figures 7A,B**). Whether these subsets have functional implications was analyzed by investigation of CD25, ICOS, and MHC II expression on DN, CD8α$^+$ and DP iNKT cells. All three proteins tended to be expressed in higher levels on CD8α$^+$ and DP iNKT cells, while expression levels on DN iNKT cells were always the lowest (**Figure 7C**). However, this was statistically significant only for ICOS expression.

Porcine iNKT Cells Express IFNγ, Upregulate Perforin, and Display Fast Degranulation Upon Antigenic Activation

Among the effector mechanisms of iNKT cells are the secretion of effector cytokines and cytotoxicity. Porcine iNKT cells have been shown to secrete IFNγ upon unspecific stimulation with phorbol myristate acetate (PMA) and ionomycin (36, 37). Knowledge about antigen-specific induction of effector molecule production in porcine iNKT cells is missing. Therefore, we investigated expression of IFNγ and perforin and the cytolytic capacities of cTC and iNKT cells after stimulation of porcine PBMC with αGC.

iNKT cells showed a dose-dependent increase of IFNγ expression. Treatment of PBMC with low-dose αGC resulted in 8.4% IFNγ$^+$ iNKT cells, while high-dose αGC resulted in 15.9% IFNγ$^+$ iNKT cells (**Figure 8A**). Perforin expression (**Figure 8B**) increased significantly in iNKT cells upon treatment with both low- and high-dose αGC (MFI: 822.3 ± 280.3 vs. 1,095 ± 175, respectively) in contrast to naïve cells (MFI: 294 ± 33). In contrast, cTC did not express IFNγ (**Figure 8A**) after stimulation with low- and high-dose αGC. There was, however, an increased frequency of perforin$^+$ cTC (**Figure 8B**) after stimulation with αGC. Porcine iNKT cells also displayed cytolytic capacities in response to αGC stimulation. While low-dose αGC did not result in significant changes in CD107a expression on iNKT cells or cTC, high-dose αGC resulted in a significantly higher frequency of CD107a$^+$ iNKT cells (**Figure 8C**). Unspecific stimulation with PMA/ionomycin resulted in an even higher frequency of CD107a$^+$ iNKT cells. However, cTC did not show significant changes of CD107a expression. Thus, we demonstrated antigen-specific induction of the effector molecules IFNγ and perforin and degranulation of porcine iNKT cells.

iNKT Cell Frequency in Swine Decreases With Age

Previous studies in humans indicated an important role for iNKT cells in early stages of life, evidenced by a higher percentage of iNKT cells in young individuals. To investigate whether the iNKT cell frequency in swine is also age-dependent, we analyzed the percentage of CD3$^+$ cells among lymphocytes (**Figure 9A**) and iNKT cells among CD3$^+$ lymphocytes (**Figure 9B**) in blood of healthy pigs two-weeks, four-weeks, 10-12-weeks, 16-weeks, and 25-weeks of age. There was no difference in the frequency of

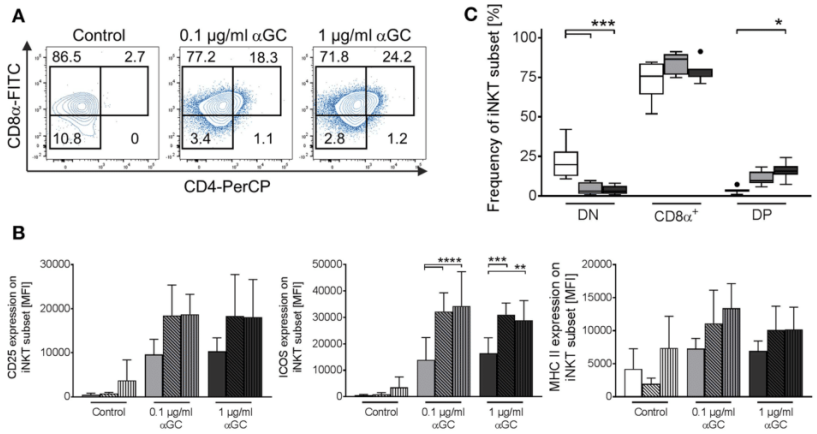


FIGURE 7 | Changes in iNKT cell subset frequency upon antigenic activation. Porcine PBMC were incubated in the presence of of 0.1 µg/ml or 1 µg/ml αGC or DMSO as vehicle control for 4 days. **(A)** Expression of CD8α and CD4 on iNKT cells was investigated. Representative flow cytometric plots of controls (left plot), low-dose (middle plot), and high-dose αGC (right plot) stimulated iNKT cells are shown. $CD8\alpha^-/CD4^-$ (DN), $CD8\alpha^+/CD4^-$ ($CD8\alpha^+$), and $CD8\alpha^+/CD4^+$ (DP) subsets in iNKT cells were identified as previously shown. Numbers in the plot show the frequencies of the respective gate. **(B)** Frequency of iNKT cell subsets in controls (white) and after stimulation with 0.1 µg/ml (gray) or 1 µg/ml αGC (black) shown as Tukey box plots ($n = 7$). **(C)** Expression level of CD25 (left graph), ICOS (middle graph), and MHC II (right graph) on DN (empty bars), $CD8\alpha^+$ (diagonal black hatching), and DP (vertical black stripes) iNKT cells was investigated in control cells (white) and cells stimulated with 0.1 µg/ml (gray) or 1 µg/ml (black) αGC (mean (SD), $n = 4$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, repeated-measures one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

$CD3^+$ cells among the different groups. In contrast, we found the frequency of iNKT cells in 2-week-old piglets (1.1% ± 0.19) to be significantly higher than in all other groups. iNKT cell frequency declined rapidly with increasing age. Four-week-old piglets still displayed significantly higher proportions of iNKT cells (0.48% ± 0.25) than older animals. The frequency of iNKT cells was still elevated, but not statistically significant, in 10–12 week-old pigs (0.24% ± 0.1) compared to older animals. In comparison to 2-week-old piglets, iNKT cell frequency was markedly reduced. In 16-week-old and 25-week-old pigs, iNKT cell frequency remained on a comparable low level (0.09% ± 0.06, 0.08% ± 0.04, respectively).

Virus-Infected Swine Display Increasing Frequencies of iNKT Cells in Disease-Related Tissues

iNKT cells are among the first responders after microbial infection. In pigs, iNKT cell dynamics upon infection have not been investigated so far. Hence, we measured iNKT cell frequency in viral infections of either high zoonotic potential, i.e., IAV (H1N1; **Figure 10A**), or of high veterinary and economic importance, i.e., ASFV strain Armenia08 (**Figure 10B**). Over the course of the study, IAV-infected animals showed no clinical signs of disease. However, during subclinical IAV infection (**Figure 10A**), we found a significant increase in iNKT cell frequency in lung lymph nodes (*Nodus lymphaticus tracheobronchales inferiores*) at 4 days post infection (dpi), which decreased until 7 dpi to levels still higher than in control animals. In line with this finding, iNKT cell frequencies tended to increase non-significantly in peripheral blood, broncho-alveolar lavage (BAL), and lung at 4 dpi, which returned to control levels at 7 dpi in all tissues. In spleen, iNKT cell frequency peaked at 7 dpi. There were no changes in iNKT cell frequency in the gut. In contrast to IAV, ASFV-infected swine showed typical clinical signs of severe disease. ASFV infection was fatal in all animals in this study. During ASFV infection, we detected a significant increase in iNKT cell frequency in blood and lung at 5 dpi. In the lung, frequency dropped to control levels at 7 dpi, while they remained elevated, although not significantly, in blood. iNKT cell frequency was also increased in liver and one of the liver lymph nodes (*Nodi lymphatici hepatici*) 5 dpi but was on control levels again at 7 dpi. We therefore described the first iNKT cell dynamics in virus-infected swine.

To further evaluate the role of porcine iNKT cells in the aforementioned viral infections and to test our findings with the synthetic CD1d ligand αGC, we stimulated porcine PBMC with supernatant of $CD172a^+$ cells infected with IAV or ASFV. We found a small but significant increase of $CD25^+$, $ICOS^+$, and Ki-67^+ iNKT cells after stimulation with IAV-conditioned supernatant (**Figure 11A**). For ASFV, there was no detectable activation of iNKT cells (**Figure 11B**).

DISCUSSION

iNKT cells are a subset of innate lymphocytes located at potential pathogen entry sites at mucosal surfaces and lymphoid tissues. Even though they are a rare population in the vast pool of lymphocytes, they are pivotal orchestrators of innate and adaptive responses (58–60). Because of this central role in immunity, iNKT cells and their cellular responses have

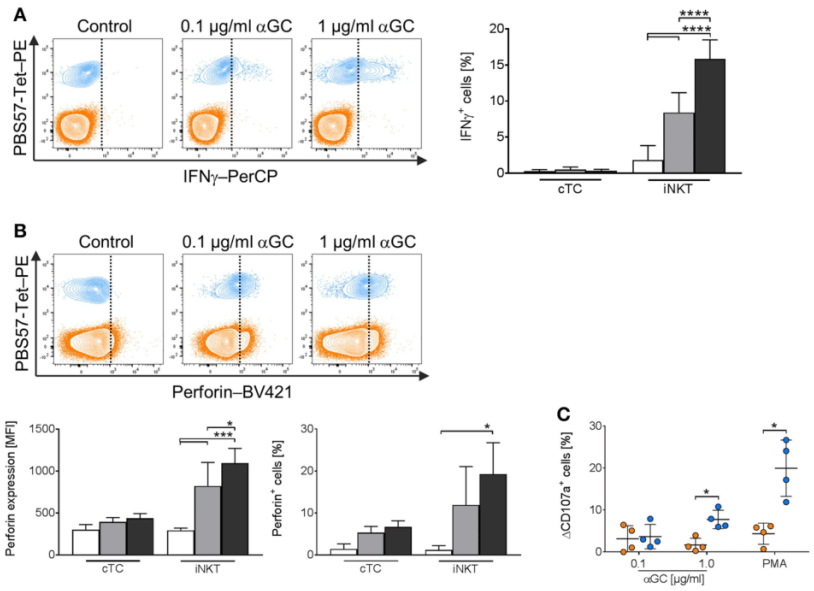


FIGURE 8 | Expression of IFNγ and perforin and degranulation of activated porcine iNKT cells upon antigenic activation. Porcine PBMC were cultivated in the presence of 0.1 µg/ml (gray) or 1 µg/ml αGC (black) or DMSO (white) as a control for 4 days. At day 4, the cells were restimulated with medium containing the respective treatment. 2 h later, Brefeldin A was added and the cells were incubated for another 4 h. Intracellular expression of IFNg and perforin in iNKT cells (blue) and cTC (orange) was analyzed by flow cytometry. **(A)** Representative flow cytometric plots showing IFNγ expression in iNKT cells and cTC (vertical line indicates threshold based on expression level in controls). Frequency of IFNγ-expressing iNKT cells and cTC in controls and after stimulation with 0.1 µg/ml or 1 µg/ml αGC (n = 5-7). **(B)** Representative flow cytometric plots showing perforin expression (vertical line indicates threshold based on expression level in controls). Expression level of perforin in iNKT cells and cTC in controls and after antigenic stimulation. Perforin$^+$ iNKT cells and cTC after antigenic stimulation (n = 4). **(C)** CD107a surface expression on iNKT cells and cTC after stimulation with 0.1 µg/ml or 1 µg/ml αGC and PMA/ionomycin. Specific degranulation was calculated as the difference in surface expression of stimulated and control cells and is given as ΔCD107a (n = 4). Data shown as mean (SD), $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$, repeated-measures one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

been investigated extensively in mice and humans. However, a large animal model for immunological research in general and iNKT cell research in specific is still needed. Because of their physiological and immunological similarities with humans, pigs exhibit high potential as a biomedical model for infectious diseases (3–10). Moreover, because pigs are of high veterinary and economic importance, understanding of their immune system has an invaluable relevance exceeding mere scientific modeling.

We comprehensively characterized peripheral porcine iNKT cells and provided evidence for similarities with their human and murine counterparts. Naïve peripheral iNKT cells in swine were predominantly CD8α$^+$ and did not display a distinct CD4$^+$ population, which confirms earlier data (36, 37). These characteristics are comparable to human peripheral iNKT cells, which are predominantly CD8α$^+$ or DN, and in contrast to murine iNKT cells, which are mostly CD4$^+$ and lack CD8 surface expression (61). CD8α$^+$ iNKT cells in swine and humans are principally CD8αα$^+$ but do not express the CD8 β-chain (61). Differences in the study design in various species may hamper accurate comparative analysis of iNKT. Human and murine iNKT are often expanded *in vitro* before experimental investigations, resulting in significant changes of the subset distribution (61, 62). Therefore, some authors question the reliability of functional classifications of CD4/CD8 subsets in iNKT. We investigated freshly isolated iNKT. Hence, the described characteristics of iNKT subsets in this study are largely unaffected by *ex vivo* sample processing.

Porcine iNKT cells displayed an antigen-experienced phenotype, indicated by lack of CD45RA expression on the cell surface. Applying the characterization of human memory T cells to swine, porcine CCR7$^+$/CD27$^+$ iNKT cells represent a subset functionally similar to central memory cells, while CCR$^-$/CD27$^+$ and CCR$^-$/CD27$^-$ iNKT cells resemble transitional memory cells and effector memory cells, respectively (56). Upon activation, central memory cells produce IL-2 and rapidly proliferate. After differentiation into effector memory cells, they express cytokines, including IFNγ (55). An elevated fraction of CD27$^-$/CCR7$^+$ iNKT cells, probably representing activated effector cells with *de novo* expression of CCR7 (63), further strengthens our hypothesis that iNKT cells exhibit a preactivated phenotype in naïve animals. A comparable phenotype has been shown in human and murine iNKT cells as well (13, 64–66). This preactivation is discussed as the result of a lower activation threshold of iNKT cells in comparison to cTC (67) or exposition to endogenous ligands (68). Either

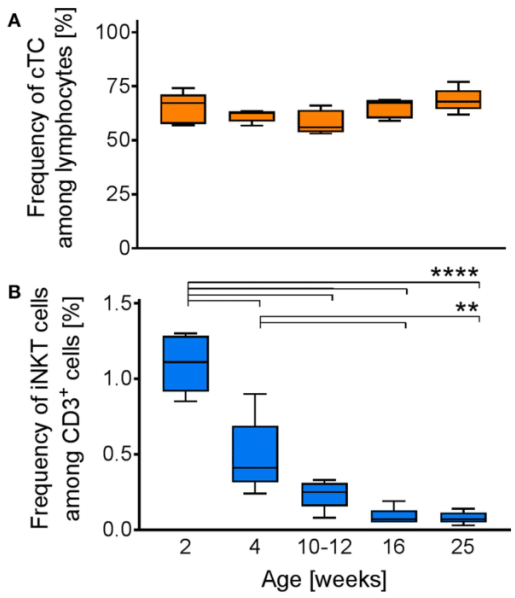


FIGURE 9 | Age-dependent changes in iNKT cell frequency. Whole blood from swine of the indicated age ($n = 5$) was investigated for the frequencies of iNKT cells and cTC among $CD3^+$ lymphocytes. Box plots showing the frequency of **(A)** $CD3^+$ cells among lymphocytes and **(B)** iNKT cells among $CD3^+$ cells. $^{**}p < 0.01$, $^{****}p < 0.0001$, ordinary one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

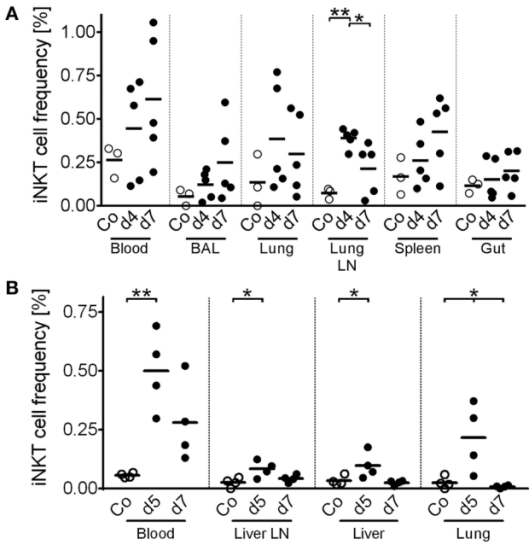


FIGURE 10 | *In vivo* dynamics of porcine iNKT cells during viral infections. Pigs were experimentally infected with **(A)** IAV ($n = 3$-5) or **(B)** ASFV ($n = 4$). At the indicated time after infection, animals were euthanized and lymphocytes of the indicated tissues were isolated. Frequency of iNKT among $CD3^+$ lymphocytes was then investigated using flow cytometry. Open circles show uninfected control animals (Co). Closed circles show infected animals at the indicated time post infection. Each symbol represents an individual animal with a line indicating mean. BAL, Broncho alveolar lavage. Lung LN, lung lymph node (*Nodi lymphatici tracheobronchales inferiores*). Liver LN, liver lymph node (*Nodi lymphatici hepatici*). $^{*}p < 0.05$, $^{**}p < 0.01$, ordinary one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

way, preactivation of iNKT cells *in vivo*, in line with preformed cytokine mRNA (13), additionally emphasizes the ability of iNKT cells to respond immediately to stimuli (66). About half of all porcine iNKT cells expressed CCR7, which is critical for T cell extravasation and migration into T cell areas of secondary lymphoid tissues (69). CCR7 expression on porcine peripheral iNKT cells could therefore also explain the significant *in vivo* increases of iNKT cell frequencies in regional lymph nodes during infection with IAV (H1N1) as well as with ASFV strain Armenia08. Functional CCR7 expression, i.e., chemotactic migration to CCR7 ligands, has been shown for peripheral murine and human iNKT cells (70, 71). Moreover, CCR7 seems to be pivotal for the differentiation into effector subsets in the periphery (71). This indicates that porcine $CCR7^+$ iNKT cells are licensed to early migration from peripheral blood to secondary lymphoid tissues as well. Additionally, we found high levels of CD27 on porcine $CD8\alpha^+$ iNKT cells but not on DN iNKT cells. CD27 is essential for survival of $CD8^+$ effector cells, especially after multiple rounds of cell division (72, 73). Ligand-binding of CD27 on $CD8^+$ cells induces proliferation even in the absence of *bona fide* stimuli such as IL-2 (74). Cumulatively, our results emphasize that porcine iNKT cells display an effector-memory phenotype and that activation of porcine $CD8\alpha^+$ iNKT cells may occur in the absence of co-stimulation by other cells.

Our phenotypic characterization further indicated the presence of two main iNKT subsets, iNKT1 and iNKT2, and a minor subset, non-iNKT1/2. According to studies in mice, these subsets differ not only phenotypically but also functionally. iNKT1 exhibit properties associated with Th1 cells, like production of IFNγ, while iNKT2 produce the Th2 cytokine IL-4. The non-iNKT1/2 fraction may contain several other iNKT cell subsets, which at present cannot be further investigated in swine due to lack of detection systems. Among the possible iNKT subsets are iNKT17, producing IL-17, and regulatory and follicular helper iNKT cells. Different regulatory iNKT cells have been identified by the expression of FoxP3 or E4BP4 in mice (75, 76), follicular helper iNKT have been described as $Bcl\text{-}6^+$ (19). A recent study has provided evidence indicating that porcine iNKT cells also provide non-cognate B cell help (32), indicating that follicular helper iNKT cells exist in pigs as well. In humans, $CD4^+$ iNKT cells are the most efficient B cell helpers (77). Upregulation of CD4 and MHC II in activated porcine iNKT cells suggests that $CD4^+$ iNKT cells in swine have similar functions. MHC II expression by human cTC and iNKT cells is also upregulated upon activation during viral infections (78, 79). Both cell types may act as effective Antigen-presenting cells (APC) (52). Expression of MHC II on the surface of T lymphocytes has also been shown for a variety of other species, including rats, canine, bovine, and equine

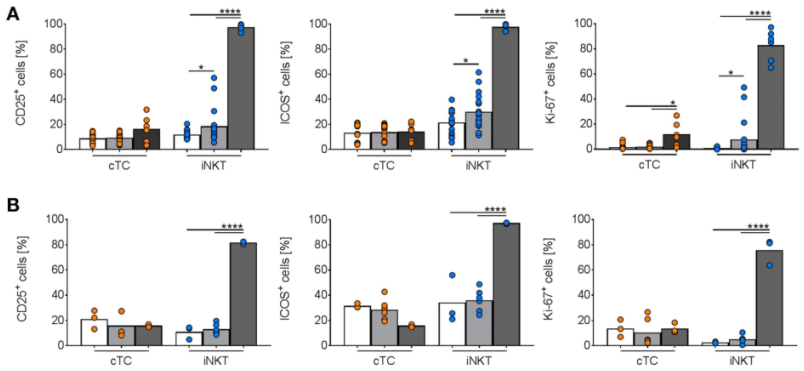


FIGURE 11 | *In vitro* activation of porcine iNKT cells during viral infections. Freshly isolated porcine PBMC were incubated without stimulation (white bars) or in the presence of supernatant of purified CD172a$^+$ cells infected with **(A)** IAV (MOI 1; gray) or **(B)** ASFV (MOI 0.1; gray), or 1 μg/ml αGC (black) for 4 days. Frequencies of iNKT cells (blue circles) and cTC (orange circles) positive for CD25, ICOS, and Ki-67 are shown. Pooled data of two experiments with six individual pigs for IAV and three individual pigs for ASFV shown as mean (SD), $^*p < 0.05$, $^{****}p < 0.0001$, ordinary one-way ANOVA with Holm-Sidak's *post-hoc* test for multiple comparisons.

(52). Murine T cells, unlike cells from other mammals, do not express MHC II on their own but rather acquire it from other cells (52). We found that activation of porcine iNKT cells with αGC leads to abundant cell surface expression of MHC II. Thus, porcine iNKT cells reflect human iNKT cells better than murine iNKT cells. However, the role of MHC II$^+$ iNKT cells is not entirely understood. Earlier studies indicated that interaction of MHC II and CD4 on CD4$^+$ iNKT cells could boost previous TCR-dependent activation by *trans*-interaction of MHC II$^+$ and CD4$^+$ iNKT cells, respectively (80). Upregulation of CD4 as well as MHC II by activated porcine iNKT cells indicates that such a process may occur in swine as well.

Human as well as murine iNKT cells primarily display an effector phenotype (61). We showed that porcine iNKT cells have similar characteristics. Naïve porcine iNKT cells abundantly expressed CD5 and were positive for CD25, MHC II, and ICOS. These markers are associated with effector cells (49–52). Expression of CD25 on naïve iNKT cells has also been shown in humans and mice (81, 82), ensuring prompt iNKT cell responsiveness (83). Expression of CD5 is upregulated by human cTC upon activation (49). We observed a corresponding increase of CD5 on the surface of activated porcine iNKT cells, thereby demonstrating highly activated cells (49). However, the role of CD5 on cTC and iNKT cells is not entirely understood. Molecular studies with human CD5 indicate that homophilic interactions with CD5 on other T cells or APC in *trans* or on the same cell in *cis* are needed for the regulation of T cell immunity (84). CD5 could also be used for regulation of iNKT cell responses, because expression of CD5 has been shown to inhibit TCR-dependent cell activation (49). Therefore, the high levels of CD5 on activated iNKT cells could limit further activation and possible immunopathology. Responses of iNKT cells are further regulated by ICOS. Interaction of ICOS with ICOSL, expressed exclusively on APC (85), is essential for homeostasis, activation, and survival of iNKT cells (51, 86, 87). Expression of ICOS, along with the receptors for IL-12 and IL-18, is induced by PLFZ, thereby shaping the ability of iNKT cells to respond to stimuli (88). This underlines the importance of ICOS for iNKT cell functionality. Some studies indicate that ICOS expression is associated with a Th1-based response, because ICOS expression on iNKT cells correlates with a pro-inflammatory phenotype and expression of IFNγ (86, 87). Other studies suggest that ICOS is not linked to a certain Th-subset differentiation but rather identifies cells in an effector state (89). Thus, ICOS expression on porcine iNKT cells does not necessarily indicate differentiation of iNKT cells into iNKT1. However, in context with the expression of other markers, such as CD25 or CD5, their shift to a T-bet$^+$ iNKT1 phenotype, and their expression of IFNγ and perforin, it seems accurate that αGC-stimulated porcine iNKT cells differentiate primarily into Th1/iNKT1.

In this study, we observed a higher expression of activation and effector markers, like CD25 and MHC II, on CD8α$^+$ iNKT cells than on CD8α$^-$ iNKT cells. Moreover, CD8α$^+$ iNKT cells were the primary subset we detected during Th1-biased iNKT cell responses. Comparable results have been described in earlier studies, where CD8α$^+$ iNKT cells produced higher amounts of IFNγ during unspecific activation with PMA/ionomycin (37). CD8α has also been described as an activation marker on another population of unconventional porcine lymphocytes, γδ T cells (90). This indicates that CD8α also acts as a marker of maturation and effector functions on porcine iNKT cells. Additionally, since activation resulted in a loss of DN iNKT cells and an increase of DP and CD8α$^+$ iNKT cells, both with a significantly higher ICOS expression than DN iNKT cells, CD8α$^+$ and DP iNKT cells might represent highly differentiated effector subsets. The high expression of effector markers even in steady state indicates that porcine iNKT cells are also licensed for rapid responses. Overall, porcine iNKT cells phenotypically mimic human iNKT cells significantly better than murine iNKT cells.

Porcine iNKT cells upregulated perforin expression and displayed degranulation as shown by increased CD107a surface expression upon antigenic stimulation. This feature is shared with iNKT cells in mice and humans (17, 91–93). Cytotoxicity, including perforin production, is induced independent of TCR stimulation by pro-inflammatory cytokines like IL-12 or IL-18 secreted by APC (91, 94). How iNKT cells precisely mediate cytotoxicity is still under discussion. Perforin-mediated lysis was described in viral infections and cancer models (15, 16, 91), while other studies suggested rather Fas/FasL-dependent lysis of target cells by iNKT cells (18, 94). Both pathways are CD1d-dependent, although CD1d-independent lysis mediated by NKG2D has also been described (95). Which of these pathways prevails in a specific context likely depends on the activation mode and availability of relevant molecules on the surface of target cells (14). Deciphering activation and mode of porcine iNKT cell cytotoxicity require in-depth analysis. Our phenotypic and functional characterization paves ways for such studies. The increase of perforin-expressing cTC we saw in our studies is likely due to direct and indirect effects on cTC during iNKT cell activation. IFNγ, highly expressed by activated iNKT cells, is known to enhance the cytotoxic activity of T cells by autocrine and paracrine stimulation (96). Moreover, IFNγ has been shown to induce the expression of the high-affinity IL-12 receptor (97). This enables cTC to react to IL-12, secreted by APC during αGC stimulation. IL-12 in turn is known to enhance perforin responses (98). This bystander activation provides further protection during ongoing immune responses and demonstrates the linkage of both early iNKT cell and subsequent cTC responses.

Interestingly, we only saw significant differences between the effects of the two concentrations of αGC in three cases. High-dose αGC resulted in heightened proliferation, increased perforin expression, and increased frequencies of IFNγ-producing iNKT cells. As iNKT cells do not require co-stimulation for cytokine-production (12, 13), dose-dependent effects are explained by interaction loops between APC and iNKT cells. Activated iNKT cells readily produce cytokines, like IFNγ, and induce maturation of dendritic cells, which in turn increase production of iNKT cell-stimulating cytokines, like IL-12 (99). Notably, αGC is a highly potent antigen with a high affinity to the iNKT cell TCR and a long half-life (100). Therefore, αGC likely induces stronger responses in iNKT cells than activation with natural ligands (27). The distinctive phenotype of *in vitro*-stimulated porcine iNKT cells requires *in vitro* verification with other CD1d ligands or bacterial/viral antigens and, most importantly, *in vivo* validation in infection studies.

We found a rapid age-dependent decrease in iNKT cell percentage during the first 12 weeks of life. In animals older than 12 weeks, iNKT cells maintained the same abundance. Since the frequency of $CD3^+$ lymphocytes did not change, the drop in iNKT cell frequency cannot be explained by changes in the overall T cell frequency. The high iNKT cell frequencies in young individuals indicate that iNKT cells play a critical role for immunological responses in the first weeks of life. Similar age-dependent decreases are known in humans as well (101, 102). The frequency of iNKT cells in swine investigated for age-related changes was lower than in swine investigated in other experiments. This might be explained by different housing conditions of the pigs used for different experiments. The samples for investigation of age-dependent iNKT cell frequency were obtained from a pig farm with strict biosafety standards, limiting contact with pathogenic microorganisms. In contrast, animals for other experiments were kept at our institute under conventional conditions, enabling microbial colonization. In mice, iNKT cell frequencies decrease if animals are kept under germ-free conditions (103, 104). This indicates that iNKT cell homeostasis in swine is also at least partially dependent on the microbiome of the respective animal.

We described the first iNKT cell kinetics in pigs in two viral infections, IAV and ASFV. We observed increases in iNKT cell frequency in blood and mucosal tissues at early time points during both infections, in line with the well-documented relevance of this lymphocyte population at the onset of infection (58, 105). However, iNKT cell dynamics changed at later stages of infection and their frequencies decreased over time. Moreover, we detected small but significant activation of iNKT cells in *in vitro* assays using IAV-conditioned supernatant. A host-beneficial expansion of iNKT cells with antiviral properties during IAV infection has been described in other species as well [reviewed in Crosby and Kronenberg (27)]. Mechanistically, iNKT cells act in various ways during IAV infection. Via secreted IFNγ, iNKT cells activate multiple bystander cells and induce protective adaptive responses. Moreover, iNKT cells facilitate conventional antiviral $CD8^+$ T cell responses (106), inhibit immunosuppression by myeloid-derived suppressor cells (107), and produce cytokines critical for mucosal immunity and integrity, like IL-22 (108). The iNKT cell influx during IAV infection in swine peaked significantly in the lung lymph nodes and non-significantly in lung and broncho-alveolar lavage in our study. Comparable expansions of iNKT populations in these tissues were shown during IAV infection in humans (27). This, in connection with our *in vitro* data, indicates that iNKT cells in swine may play a similar role during respiratory infections as in humans. Future studies will clarify precisely how iNKT cells are involved in antiviral immunity against IAV in swine. In response to ASFV infections, $CD8^+$ T cells seem to play a major role (109). Moreover, higher IFNγ production correlated with higher protection from ASFV challenge (110). A role for iNKT cell-like cells in the immunity against ASFV infection has been discussed earlier, as cells with a NKT-like phenotype ($CD3^+/CD4^-/CD8^+/CD5^{\pm}/CD6^-/CD11b^+/CD16^+$) expanded after co-culture of porcine PBMC with ASFV *in vitro* (110). However, as others and we showed, the phenotype of these cells resembled real iNKT cells only rudimentarily. It is therefore questionable whether the described expansion and effects are attributable to iNKT cells. Contrary to the changes *in vivo*, we did not see any ASFV-induced iNKT cell activation *in vitro*. This might be explained by immune evasion mechanisms used by pathogenic ASFV strains, that were recently been shown to block type I IFN responses in infected cells (111). Since type I IFN are potent inductors of iNKT cell activation (14), blockage of type I IFN expression would impair iNKT cell activation. However, these evasion mechanisms might at least partially be counteracted

in vivo, which cannot be simulated *in vitro*. Our *in vivo* data still indicates that iNKT cells are involved in the immune response against ASFV because the iNKT cell frequency increased locally in affected tissues and systemically in peripheral blood. However, an immunopathological role of iNKT cells during ASFV infection cannot be excluded. Overactivation of iNKT cells may result in a cytokine storm, which further weakens the animals and contributes to morbidity (112, 113). Furthermore, excessive local responses result in immunopathology, like liver damage (114, 115), as typically seen in animals succumbed to ASFV infection. The role of porcine iNKT cells in ASFV infections has to be evaluated in future studies.

iNKT cells are increasingly coming to the fore as target cells for novel vaccine adjuvants. New and more effective vaccines against infectious diseases are needed for swine as well as for humans. This is especially true for zoonotic diseases, like IAV, for which humans and swine are susceptible. αGC or αGC-analogs have been shown to induce high IgG and IgA titers against co-administered proteins in mice (116) as well as in swine (28, 29, 31). Expression of IFNγ by iNKT cells is crucial for B cell help and induction of class switch (20). We observed IFNγ expression by porcine iNKT cells upon antigenic stimulation, which indicates that pathways similar to those in mice are used by porcine iNKT cells to help B cells. αGC-adjuvanted vaccines also prime and potentiate cytotoxic CD8 T cell responses in mice (116, 117) and non-human primates (118). Targeting iNKT cells during vaccination represents a promising new way to increase vaccine efficacy. However, cellular and molecular interactions of porcine iNKT cells with effector cells of the adaptive immune system need further evaluation.

Taken together, we established a multicolor flow cytometry platform for analysis of porcine iNKT cells. Our study pioneered detailed phenotyping and differentiation of porcine iNKT cells and their effector molecules. Moreover, we provided first insights into the relevance of iNKT cells in viral diseases in pigs. We demonstrated that porcine iNKT cells display striking phenotypic and functional similarities to human but less to murine iNKT cells. Therefore, pigs were shown to be a valuable large animal model for immunological studies, especially for, but not limited to, iNKT cell research.

ETHICS STATEMENT

All animal experiments were approved by the ethics committee of the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) with reference numbers 7221.3-1-035/17 for IAV and 7221.3-1.1-064/17 for ASFV. All applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration.

AUTHOR CONTRIBUTIONS

AS and UB: conceived and designed *in vitro* experiments. TS, TM, SB, CS, and UB: conceived and designed animal experiments. AS, JH, TS, SB, and CS: acquired animal samples. AS: performed *in vitro* experiments. AS, AD, TM, and UB: data analysis and interpretation. AS and UB: manuscript preparation. All authors reviewed and approved the final version of the manuscript.

FUNDING

This study was funded by Federal Excellence Initiative of Mecklenburg Western Pomerania and European Social Fund (ESF) Grant KoInfekt (ESF_14-BM-A55-00xx_16).

ACKNOWLEDGMENTS

The authors thank Stefanie Knöfel and Silke Rehbein for outstanding technical assistance. For excellent care of animals, we thank the veterinarians and animal keepers of the FLI. The CD1d tetramer was thankfully provided by the NIH Tetramer Core Facility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01380/full#supplementary-material

Supplementary Table 1 | Antibodies used in this study.

REFERENCES

1. Gutierrez K, Dicks N, Glanzner WG, Agellon LB, Bordignon V. Efficacy of the porcine species in biomedical research. *Front Genet*. (2015) 6:293. doi: 10.3389/fgene.2015.00293
2. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA*. (2013) 110:3507–12. doi: 10.1073/pnas.1222878110
3. Fairbairn L, Kapetanovic R, Sester DP, Hume DA. The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. *J Leukoc Biol*. (2011) 89:855–71. doi: 10.1189/jlb.1110607
4. Giraud S, Favreau F, Chatauret N, Thuillier R, Maiga S, Hauet T. Contribution of large pig for renal ischemia-reperfusion and transplantation studies: the preclinical model. *J Biomed Biotechnol*. (2011) 2011:532127. doi: 10.1155/2011/532127
5. Mair KH, Sedlak C, Kaser T, Pasternak A, Levast B, Gerner W, et al. The porcine innate immune system: an update. *Dev Comp Immunol*. (2014) 45:321–43. doi: 10.1016/j.dci.2014.03.022
6. Swindle MM, Makin A, Herron AJ, Clubb FJ Jr., Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol*. (2012) 49:344–56. doi: 10.1177/0300985811402846
7. Renukaradhya GJ, Manickam C, Khatri M, Rauf A, Li X, Tsuji M, et al. Functional invariant NKT cells in pig lungs regulate the airway hyperreactivity: a potential animal model. *J Clin Immunol*. (2011) 31:228–39. doi: 10.1007/s10875-010-9476-4
8. Ugolini M, Gerhard J, Burkert S, Jensen KJ, Georg P, Ebner F, et al. Recognition of microbial viability via TLR8 drives TFH cell differentiation and vaccine responses. *Nat Immunol*. (2018) 19:386–96. doi: 10.1038/s41590-018-0068-4

9. Maisonnasse P, Bouguyon E, Piton G, Ezquerra A, Urien C, Deloizy C, et al. The respiratory DC/macrophage network at steady-state and upon influenza infection in the swine biomedical model. *Mucosal Immunol.* (2016) 9:835–49. doi: 10.1038/mi.2015.105
10. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. *Trends Microbiol.* (2012) 20:50–7. doi: 10.1016/j.tim.2011.11.002
11. Iwasaki A, Foxman EF, Molony RD. Early local immune defences in the respiratory tract. *Nat Rev Immunol.* (2017) 17:7–20. doi: 10.1038/nri.2016.117
12. Uldrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM, et al. NKT cell stimulation with glycolipid antigen *in vivo*: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J Immunol.* (2005) 175:3092–101. doi: 10.4049/jimmunol.175.5.3092
13. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med.* (2003) 198:1069–76. doi: 10.1084/jem.20030630
14. Kohlgruber AC, Donado CA, LaMarche NM, Brenner MB, Brennan PJ. Activation strategies for invariant natural killer T cells. *Immunogenetics.* (2016) 68:649–63. doi: 10.1007/s00251-016-0944-8
15. Bassiri H, Das R, Guan P, Barrett DM, Brennan PJ, Banerjee PP, et al. iNKT cell cytotoxic responses control T-lymphoma growth *in vitro* and *in vivo*. *Cancer Immunol Res.* (2014) 2:59–69. doi: 10.1158/2326-6066.CIR-13-0104
16. Bassiri H, Das R, Nichols KE. Invariant NKT cells: killers and conspirators against cancer. *Oncoimmunology.* (2013) 2:e27440. doi: 10.4161/onci.27440
17. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med.* (2002) 195:625–36. doi: 10.1084/jem.20011786
18. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells *in vivo* is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol.* (2010) 185:2721–9. doi: 10.4049/jimmunol.1001018
19. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol.* (2011) 13:35–43. doi: 10.1038/ni.2166
20. Doherty DG, Melo AM, Moreno-Olivera A, Solomos AC. Activation and regulation of B cell responses by invariant natural killer T cells. *Front Immunol.* (2018) 9:1360. doi: 10.3389/fimmu.2018.01360
21. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. *Science.* (2002) 296:553–5. doi: 10.1126/science.1069017
22. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol.* (2013) 25:161–7. doi: 10.1016/j.coi.2013.01.003
23. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol.* (2013) 14:1146–54. doi: 10.1038/ni.2731
24. McNab FW, Berzins SP, Pellicci DG, Kyparissoudis K, Field K, Smyth MJ, et al. The influence of CD1d in postselection NKT cell maturation and homeostasis. *J Immunol.* (2005) 175:3762–8. doi: 10.4049/jimmunol.175.6.3762
25. Strong BS, Newkold TJ, Lee AE, Turner LE, Alhajjat AM, Heusel JW, et al. Extrinsic allospecific signals of hematopoietic origin dictate iNKT cell lineage-fate decisions during development. *Sci Rep.* (2016) 6:28837. doi: 10.1038/srep28837
26. Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, et al. Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol.* (2012) 10:e1001255. doi: 10.1371/journal.pbio.1001255
27. Crosby CM, Kronenberg M. Tissue-specific functions of invariant natural killer T cells. *Nat Rev Immunol.* (2018) 18:559–74. doi: 10.1038/s41577-018-0034-2
28. Artiaga BL, Whitener RL, Staples CR, Driver JP. Adjuvant effects of therapeutic glycolipids administered to a cohort of NKT cell-diverse pigs. *Vet Immunol Immunopathol.* (2014) 162:1–13. doi: 10.1016/j.vetimm.2014.09.006
29. Artiaga BL, Yang G, Hackmann TJ, Liu Q, Richt JA, Salek-Ardakani S, et al. α-Galactosylceramide protects swine against influenza infection when administered as a vaccine adjuvant. *Sci Rep.* (2016) 6:23593. doi: 10.1038/srep23593
30. Artiaga BL, Yang G, Hutchinson TE, Loeb JC, Richt JA, Lednicky JA, et al. Rapid control of pandemic H1N1 influenza by targeting NKT-cells. *Sci Rep.* (2016) 6:37999. doi: 10.1038/srep37999
31. Dwivedi V, Manickam C, Dhakal S, Binjawadagi B, Ouyang K, Hiremath J, et al. Adjuvant effects of invariant NKT cell ligand potentiates the innate and adaptive immunity to an inactivated H1N1 swine influenza virus vaccine in pigs. *Vet Microbiol.* (2016) 186:157–63. doi: 10.1016/j.vetmic.2016.02.028
32. Renu S, Dhakal S, Kim E, Goodman J, Lakshmanappa YS, Wannemuehler MJ, et al. Intranasal delivery of influenza antigen by nanoparticles, but not NKT-cell adjuvant differentially induces the expression of B-cell activation factors in mice and swine. *Cell Immunol.* (2018) 329:27–30. doi: 10.1016/j.cellimm.2018.04.005
33. Eguchi-Ogawa T, Morozumi T, Tanaka M, Shinkai H, Okumura N, Suzuki K, et al. Analysis of the genomic structure of the porcine CD1 gene cluster. *Genomics.* (2007) 89:248–61. doi: 10.1016/j.ygeno.2006.10.003
34. Bendelac A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med.* (1995) 182:2091–6. doi: 10.1084/jem.182.6.2091
35. Yang G, Artiaga BL, Hackmann TJ, Samuel MS, Walters EM, Salek-Ardakani S, et al. Targeted disruption of CD1d prevents NKT cell development in pigs. *Mamm Genome.* (2015) 26:264–70. doi: 10.1007/s00335-015-9564-0
36. Thierry A, Robin A, Giraud S, Minouflet S, Barra A, Bridoux F, et al. Identification of invariant natural killer T cells in porcine peripheral blood. *Vet Immunol Immunopathol.* (2012) 149:272–9. doi: 10.1016/j.vetimm.2012.06.023
37. Yang G, Artiaga BL, Lewis ST, Driver JP. Characterizing porcine invariant natural killer T cells: a comparative study with NK cells and T cells. *Dev Comp Immunol.* (2017) 76:343–51. doi: 10.1016/j.dci.2017.07.006
38. Yang G, Richt JA, Driver JP. Harnessing invariant NKT cells to improve influenza vaccines: a pig perspective. *Int J Mol Sci.* (2017) 19:68. doi: 10.3390/ijms19010068
39. Yang H, Binns RM. Expression and regulation of the porcine CD44 molecule. *Cell Immunol.* (1993) 149:117–29. doi: 10.1006/cimm.1993.1141
40. Yang G, Artiaga BL, Lomelino CL, Jayaprakash AD, Sachidanandam R, McKenna R, et al. Next generation sequencing of the pig alphabeta TCR repertoire identifies the porcine invariant NKT cell receptor. *J Immunol.* (2019) 202:1981–91. doi: 10.4049/jimmunol.1801171
41. Spearman C. The method of "right and wrong cases" (constant stimuli) without Gauss's formula. *Br J Psychol.* (1908) 2:227–42. doi: 10.1111/j.2044-8295.1908.tb00176.x
42. Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol.* (1931) 162:480–3. doi: 10.1007/BF01863914
43. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol.* (2002) 40:3256–60. doi: 10.1128/JCM.40.9.3256-3260.2002
44. Pietschmann J, Guinat C, Beer M, Pronin V, Tauscher K, Petrov A, et al. Course and transmission characteristics of oral low-dose infection of domestic pigs and European wild boar with a Caucasian African swine fever virus isolate. *Arch Virol.* (2015) 160:1657–67. doi: 10.1007/s00705-015-2430-2
45. Kitani H, Yoshioka M, Takenouchi T, Sato M, Yamanaka N. Characterization of the liver-macrophages isolated from a mixed primary culture of neonatal swine hepatocytes. *Results Immunol.* (2014) 4:1–7. doi: 10.1016/j.rinim.2014.01.001
46. Fernandez CS, Cameron G, Godfrey DI, Kent SJ. *Ex-vivo* α-galactosylceramide activation of NKT cells in humans and macaques. *J Immunol Methods.* (2012) 382:150–9. doi: 10.1016/j.jim.2012.05.019
47. Singh D, Ghate M, Godbole S, Kulkarni S, Thakar M. Functional invariant natural killer T cells secreting cytokines are associated with non-progressive human immunodeficiency virus-1 infection but not with suppressive anti-retroviral treatment. *Front Immunol.* (2018) 9:1152. doi: 10.3389/fimmu.2018.01152

48. Zekavat G, Mozaffari R, Arias VJ, Rostami SY, Badkerhanian A, Tenner AJ, et al. A novel CD93 polymorphism in non-obese diabetic. (NOD) and NZB/W F1 mice is linked to a CD4+ iNKT cell deficient state. *Immunogenetics*. (2010) 62:397–407. doi: 10.1007/s00251-010-0442-3
49. Domingues RG, Lago-Baldaia I, Pereira-Castro I, Fachini JM, Oliveira L, Drpic D, et al. CD5 expression is regulated during human T-cell activation by alternative polyadenylation, PTBP1, and miR-204. *Eur J Immunol*. (2016) 46:1490–503. doi: 10.1002/eji.201545663
50. Waldmann TA. The multi-subunit interleukin-2 receptor. *Annu Rev Biochem*. (1989) 58:875–911. doi: 10.1146/annurev.biochem.58.1.875
51. Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature*. (2001) 409:97–101. doi: 10.1038/35051100
52. Holling TM, Schooten E, van Den Elsen PJ. Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men. *Hum Immunol*. (2004) 65:282–90. doi: 10.1016/j.humimm.2004.01.005
53. Larbi A, Fulop T. From "truly naive" to "exhausted senescent" T cells: when markers predict functionality. *Cytometry A*. (2014) 85:25–35. doi: 10.1002/cyto.a.22351
54. Okada R, Kondo T, Matsuki F, Takata H, Takiguchi M. Phenotypic classification of human CD4+ T cell subsets and their differentiation. *Int Immunol*. (2008) 20:1189–99. doi: 10.1093/intimm/dxn075
55. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol*. (2004) 22:745–63. doi: 10.1146/annurev.immunol.22.012703.104702
56. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol*. (2013) 43:2797–809. doi: 10.1002/eji.201343751
57. Soares A, Govender L, Hughes J, Mavakla W, de Kock M, Barnard C, et al. Novel application of Ki67 to quantify antigen-specific *in vitro* lymphoproliferation. *J Immunol Methods*. (2010) 362:43–50. doi: 10.1016/j.jim.2010.08.007
58. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol*. (2003) 4:1230–7. doi: 10.1038/ni1002
59. Liew PX, Kubes P. Intravital imaging - dynamic insights into natural killer T cell biology. *Front Immunol*. (2015) 6:240. doi: 10.3389/fimmu.2015.00240
60. Metelitsa LS. Anti-tumor potential of type-I NKT cells against CD1d-positive and CD1d-negative tumors in humans. *Clin Immunol*. (2011) 140:119–29. doi: 10.1016/j.clim.2010.10.005
61. Garner LC, Klenerman P, Provine NM. Insights into mucosal-associated invariant T cell biology from studies of invariant natural killer T cells. *Front Immunol*. (2018) 9:1478. doi: 10.3389/fimmu.2018.01478
62. Chan AC, Leeansyah E, Cochrane A, d'Udekem d'Acoz Y, Mittag D, Harrison LC, et al. *Ex-vivo* analysis of human natural killer T cells demonstrates heterogeneity between tissues and within established CD4(+) and CD4(-) subsets. *Clin Exp Immunol*. (2013) 172:129–37. doi: 10.1111/cei.12045
63. Sallusto F, Kremmer E, Palermo B, Hoy A, Ponath P, Qin S, et al. Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur J Immunol*. (1999) 29:2037–45. doi: 10.1002/(SICI)1521-4141(199906)29:06<2037::AID-IMMU2037>3.0.CO;2-V
64. D'Andrea A, Goux D, De Lalla C, Koezuka Y, Montagna D, Moretta A, et al. Neonatal invariant Vα24+ NKT lymphocytes are activated memory cells. *Eur J Immunol*. (2000) 30:1544–50. doi: 10.1002/1521-4141(200006)30:6<1544::AID-IMMU1544>3.0.CO;2-I
65. Park SH, Benlagha K, Lee D, Balish E, Bendelac A. Unaltered phenotype, tissue distribution and function of Vα14(+) NKT cells in germ-free mice. *Eur J Immunol*. (2000) 30:620–5. doi: 10.1002/1521-4141(200002)30:2<620::AID-IMMU620>3.0.CO;2-4
66. Cole SL, Benam KH, McMichael AJ, Ho LP. Involvement of the 4–1BB/4–1BBL pathway in control of monocyte numbers by invariant NKT cells. *J Immunol*. (2014) 192:3898–907. doi: 10.4049/jimmunol.1302385
67. van den Heuvel MJ, Garg N, Van Kaer L, Haeryfar SM. NKT cell costimulation: experimental progress and therapeutic promise. *Trends Mol Med*. (2011) 17:65–77. doi: 10.1016/j.molmed.2010.10.007
68. Van Rhijn I, Kasmar A, de Jong A, Gras S, Bhati M, Doorenspleet ME, et al. A conserved human T cell population targets mycobacterial antigens presented by CD1b. *Nat Immunol*. (2013) 14:706–13. doi: 10.1038/ni.2630
69. Kim CH, Johnston B, Butcher EC. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among Vα24+Vβ11+ NKT cell subsets with distinct cytokine-producing capacity. *Blood*. (2002) 100:11–6. doi: 10.1182/blood-2001-12-0196
70. Johnston B, Kim CH, Soler D, Emoto M, Butcher EC. Differential chemokine responses and homing patterns of murine TCRαβ NKT cell subsets. *J Immunol*. (2003) 171:2960–9. doi: 10.4049/jimmunol.171.6.2960
71. Wang H, Hogquist KA. CCR7 defines a precursor for murine iNKT cells in thymus and periphery. *Elife*. (2018) 7:e34793. doi: 10.7554/eLife.34793
72. Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol*. (2000) 1:433–40. doi: 10.1038/80877
73. Hendriks J, Xiao Y, Borst J. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J Exp Med*. (2003) 198:1369–80. doi: 10.1084/jem.20030916
74. Carr JM, Carrasco MJ, Thaventhiran JE, Bambrough PJ, Kraman M, Edwards AD, et al. CD27 mediates interleukin-2-independent clonal expansion of the CD8+ T cell without effector differentiation. *Proc Natl Acad Sci USA*. (2006) 103:19454–9. doi: 10.1073/pnas.0609706104
75. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nat Immunol*. (2015) 16:85–95. doi: 10.1038/ni.3047
76. Monteiro M, Almeida CF, Caridade M, Ribot JC, Duarte J, Agua-Doce A, et al. Identification of regulatory Foxp3+ invariant NKT cells induced by TGF-β. *J Immunol*. (2010) 185:2157–63. doi: 10.4049/jimmunol.1000359
77. Zeng SG, Ghnewa YG, O'Reilly VP, Lyons VG, Atzberger A, Hogan AE, et al. Human invariant NKT cell subsets differentially promote differentiation, antibody production, and T cell stimulation by B cells *in vitro*. *J Immunol*. (2013) 191:1666–76. doi: 10.4049/jimmunol.1202223
78. Ibarrondo FJ, Wilson SB, Hultin LE, Shih R, Hausner MA, Hultin PM, et al. Preferential depletion of gut CD4-expressing iNKT cells contributes to systemic immune activation in HIV-1 infection. *Mucosal Immunol*. (2013) 6:591–600. doi: 10.1038/mi.2012.101
79. Montoya CJ, Catano JC, Ramirez Z, Rugeles MT, Wilson SB, Landay AL. Invariant NKT cells from HIV-1 or Mycobacterium tuberculosis-infected patients express an activated phenotype. *Clin Immunol*. (2008) 127:1–6. doi: 10.1016/j.clim.2007.12.006
80. Thedrez A, de Lalla C, Allain S, Zaccagnino L, Sidobre S, Garavaglia C, et al. CD4 engagement by CD1d potentiates activation of CD4+ invariant NKT cells. *Blood*. (2007) 110:251–8. doi: 10.1182/blood-2007-01-066217
81. Jukes JP, Wood KJ, Jones ND. Bystander activation of iNKT cells occurs during conventional T-cell alloresponses. *Am J Transplant*. (2012) 12:590–9. doi: 10.1111/j.1600-6143.2011.03847.x
82. Schneiders FL, Prodohl J, Ruben JM, O'Toole T, Scheper RJ, Bonneville M, et al. CD1d-restricted antigen presentation by Vgamma9Vdelta2-T cells requires trogocytosis. *Cancer Immunol Res*. (2014) 2:732–40. doi: 10.1158/2326-6066.CIR-13-0167
83. Ladd M, Sharma A, Huang Q, Wang AY, Xu L, Genowati I, et al. Natural killer T cells constitutively expressing the interleukin-2 receptor alpha chain early in life are primed to respond to lower antigenic stimulation. *Immunology*. (2010) 131:289–99. doi: 10.1111/j.1365-2567.2010.03304.x
84. Brown MH, Lacey E. A ligand for CD5 is CD5. *J Immunol*. (2010) 185:6068–74. doi: 10.4049/jimmunol.0903823
85. Wikenheiser DJ, Stumhofer JS. ICOS co-stimulation: friend or foe? *Front Immunol*. (2016) 7:304. doi: 10.3389/fimmu.2016.00304
86. Akbari O, Stock P, Meyer EH, Freeman GJ, Sharpe AH, Umetsu DT, et al. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *J Immunol*. (2008) 180:5448–56. doi: 10.4049/jimmunol.180.8.5448
87. Kaneda H, Takeda K, Ota T, Kaduka Y, Akiba H, Ikarashi Y, et al. ICOS costimulates invariant NKT cell activation. *Biochem Biophys Res Commun*. (2005) 327:201–7. doi: 10.1016/j.bbrc.2004.12.004

88. Gleimer M, von Boehmer H, Kreslavsky T. PLZF controls the expression of a limited number of genes essential for NKT cell function. *Front Immunol.* (2012) 3:374. doi: 10.3389/fimmu.2012.00374
89. Burmeister Y, Lischke T, Dahler AC, Mages HW, Lam KP, Coyle AJ, et al. ICOS controls the pool size of effector-memory and regulatory T cells. *J Immunol.* (2008) 180:774–82. doi: 10.4049/jimmunol.180.2.774
90. Stepanova K, Sinkora M. The expression of CD25, CD11b, SWC1, SWC7, MHC-II, and family of CD45 molecules can be used to characterize different stages of gammadelta T lymphocytes in pigs. *Dev Comp Immunol.* (2012) 36:728–40. doi: 10.1016/j.dci.2011.11.003
91. Dao T, Mehal WZ, Crispe IN. IL-18 augments perforin-dependent cytotoxicity of liver NK-T cells. *J Immunol.* (1998) 161:2217–22.
92. Ichikawa T, Negishi Y, Shimizu M, Takeshita T, Takahashi H. α-Galactosylceramide-activated murine NK1.1(+) invariant-NKT cells in the myometrium induce miscarriages in mice. *Eur J Immunol.* (2016) 46:1867–77. doi: 10.1002/eji.201545923
93. Van Der Vliet HJ, Nishi N, Koezuka Y, Peyrat MA, Von Blomberg BM, Van Den Eertwegh AJ, et al. Effects of alpha-galactosylceramide. (KRN7000), interleukin-12 and interleukin-7 on phenotype and cytokine profile of human Vα24+ Vβ11+ T cells. *Immunology.* (1999) 98:557–63. doi: 10.1046/j.1365-2567.1999.00920.x
94. Leite-De-Moraes MC, Hameg A, Arnould A, Machavoine F, Koezuka Y, Schneider E, et al. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J Immunol.* (1999) 163:5871–6.
95. Kuylenstierna C, Bjorkstrom NK, Andersson SK, Sahlstrom P, Bosnjak L, Paquin-Proulx D, et al. NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. *Eur J Immunol.* (2011) 41:1913–23. doi: 10.1002/eji.200940278
96. Bhat P, Leggatt G, Waterhouse N, Frazer IH. Interferon-gamma derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death Dis.* (2017) 8:e2836. doi: 10.1038/cddis.2017.67
97. Gollob JA, Kawasaki H, Ritz J. Interferon-gamma and interleukin-4 regulate T cell interleukin-12 responsiveness through the differential modulation of high-affinity interleukin-12 receptor expression. *Eur J Immunol.* (1997) 27:647–52. doi: 10.1002/eji.1830270311
98. Ebert EC. Interleukin-12 up-regulates perforin- and Fas-mediated lymphokine-activated killer activity by intestinal intraepithelial lymphocytes. *Clin Exp Immunol.* (2004) 138:259–65. doi: 10.1111/j.1365-2249.2004.02614.x
99. Gottschalk C, Mettke E, Kurts C. The role of invariant natural killer T cells in dendritic cell licensing, cross-priming, and memory CD8(+) T cell generation. *Front Immunol.* (2015) 6:379. doi: 10.3389/fimmu.2015.00379
100. Cerundolo V, Silk JD, Masri SH, Salio M. Harnessing invariant NKT cells in vaccination strategies. *Nat Rev Immunol.* (2009) 9:28. doi: 10.1038/nri2451
101. Jing Y, Gravenstein S, Chaganty NR, Chen N, Lyerly KH, Joyce S, et al. Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood. *Exp Gerontol.* (2007) 42:719–32. doi: 10.1016/j.exger.2007.01.009
102. Patin E, Hasan M, Bergstedt J, Rouilly V, Libri V, Urrutia A, et al. Natural variation in the parameters of innate immune cells is preferentially driven by genetic factors. *Nat Immunol.* (2018) 19:302–14. doi: 10.1038/s41590-018-0049-7
103. Wei B, Wingender G, Fujiwara D, Chen DY, McPherson M, Brewer S, et al. Commensal microbiota and CD8+ T cells shape the formation of invariant NKT cells. *J Immunol.* (2010) 184:1218–26. doi: 10.4049/jimmunol.0902620
104. Wingender G, Stepniak D, Krebs P, Lin L, McBride S, Wei B, et al. Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice. *Gastroenterology.* (2012) 143:418–28. doi: 10.1053/j.gastro.2012.04.017
105. Crosby CM, Kronenberg M. Invariant natural killer T cells: front line fighters in the war against pathogenic microbes. *Immunogenetics.* (2016) 68:639–48. doi: 10.1007/s00251-016-0933-y
106. Paget C, Ivanov S, Fontaine J, Blanc F, Pichavant M, Renneson J, et al. Potential role of invariant NKT cells in the control of pulmonary inflammation and CD8+ T cell response during acute influenza A virus H3N2 pneumonia. *J Immunol.* (2011) 186:5590–602. doi: 10.4049/jimmunol.1002348
107. De Santo C, Salio M, Masri SH, Lee LY, Dong T, Speak AO, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest.* (2008) 118:4036–48. doi: 10.1172/JCI36264
108. Paget C, Ivanov S, Fontaine J, Renneson J, Blanc F, Pichavant M, et al. Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages. *J Biol Chem.* (2012) 287:8816–29. doi: 10.1074/jbc.M111.304758
109. Oura CA, Denyer MS, Takamatsu H, Parkhouse RM. *In vivo* depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J Gen Virol.* (2005) 86:2445–50. doi: 10.1099/vir.0.81038-0
110. Takamatsu HH, Denyer MS, Lacasta A, Stirling CM, Argilaguet JM, Netherton CL, et al. Cellular immunity in ASFV responses. *Virus Res.* (2013) 173:110–21. doi: 10.1016/j.virusres.2012.11.009
111. Garcia-Belmonte R, Perez-Nunez D, Pittau M, Richt JA, Revilla Y. African swine fever virus Armenia/07 virulent strain controls IFN-beta production through cGAS-STING pathway. *J Virol.* (2019). doi: 10.1128/JVI.02298-18
112. Scheuplein F, Thariath A, Macdonald S, Truneh A, Mashal R, Schaub R. A humanized monoclonal antibody specific for invariant Natural Killer T. (iNKT) cells for *in vivo* depletion. *PLoS ONE.* (2013) 8:e76692. doi: 10.1371/journal.pone.0076692
113. Van Kaer L, Parekh VV, Wu L. The response of CD1d-restricted invariant NKT cells to microbial pathogens and their products. *Front Immunol.* (2015) 6:226. doi: 10.3389/fimmu.2015.00226
114. Osman Y, Kawamura T, Naito T, Takeda K, Van Kaer L, Okumura K, et al. Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide. *Eur J Immunol.* (2000) 30:1919–28. doi: 10.1002/1521-4141(200007)30:7<1919::AID-IMMU1919>3.0.CO;2-3
115. Takeda K, Hayakawa Y, Van Kaer L, Matsuda H, Yagita H, Okumura K. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci USA.* (2000) 97:5498–503. doi: 10.1073/pnas.040566697
116. Lee YS, Lee KA, Lee JY, Kang MH, Song YC, Baek DJ, et al. An alpha-GalCer analogue with branched acyl chain enhances protective immune responses in a nasal influenza vaccine. *Vaccine.* (2011) 29:417–25. doi: 10.1016/j.vaccine.2010.11.005
117. Venkataswamy MM, Baena A, Goldberg MF, Bricard G, Im JS, Chan J, et al. Incorporation of NKT cell-activating glycolipids enhances immunogenicity and vaccine efficacy of *Mycobacterium bovis bacillus* Calmette-Guerin. *J Immunol.* (2009) 183:1644–56. doi: 10.4049/jimmunol.0900858
118. Padte NN, Boente-Carrera M, Andrews CD, McManus J, Grasperge BF, Gettie A, et al. A glycolipid adjuvant, 7DW8-5, enhances CD8+ T cell responses induced by an adenovirus-vectored malaria vaccine in non-human primates. *PLoS ONE.* (2013) 8:e78407. doi: 10.1371/journal.pone.0078407

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.2 Publication II

Experimental H1N1pdm09 infection in pigs
mimics human seasonal influenza infections

Theresa Schwaiger, Julia Sehl, Claudia Karte, **Alexander Schäfer**, Jane Hühr,
Thomas C. Mettenleiter, Charlotte Schröder, Bernd Köllner, Reiner Ulrich, Ulrike Blohm

PLoS ONE

doi: 10.1371/journal.pone.0222943

September 2019

PLOS ONE

RESEARCH ARTICLE

Experimental H1N1pdm09 infection in pigs mimics human seasonal influenza infections

Theresa Schwaiger[1], Julia Sehl[1,2], Claudia Karte[3], Alexander Schäfer[4], Jane Hühr[4], Thomas C. Mettenleiter[2], Charlotte Schröder[1], Bernd Köllner[4], Reiner Ulrich[1,5], Ulrike Blohm[4]*

1 Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, **2** Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, **3** Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, **4** Institute of Immunology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, **5** Institute of Veterinary Pathology, Faculty of Veterinary Medicine, University of Leipzig, Leipzig, Germany

* Ulrike.Blohm@fli.de

OPEN ACCESS

Citation: Schwaiger T, Sehl J, Karte C, Schäfer A, Hühr J, Mettenleiter TC, et al. (2019) Experimental H1N1pdm09 infection in pigs mimics human seasonal influenza infections. PLoS ONE 14(9): e0222943. https://doi.org/10.1371/journal.pone.0222943

Editor: Balaji Manicassamy, University of Iowa, UNITED STATES

Received: May 28, 2019

Accepted: September 10, 2019

Published: September 20, 2019

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pone.0222943

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Abstract

Pigs are anatomically, genetically and physiologically comparable to humans and represent a natural host for influenza A virus (IAV) infections. Thus, pigs may represent a relevant biomedical model for human IAV infections. We set out to investigate the systemic as well as the local immune response in pigs upon two subsequent intranasal infections with IAV H1N1pdm09. We detected decreasing numbers of peripheral blood lymphocytes after the first infection. The simultaneous increase in the frequencies of proliferating cells correlated with an increase in infiltrating leukocytes in the lung. Enhanced perforin expression in αβ and γδ T cells in the respiratory tract indicated a cytotoxic T cell response restricted to the route of virus entry such as the nose, the lung and the bronchoalveolar lavage. Simultaneously, increasing frequencies of CD8αα expressing αβ T cells were observed rapidly after the first infection, which may have inhibited uncontrolled inflammation in the respiratory tract. Taking together, the results of this study demonstrate that experimental IAV infection in pigs mimics major characteristics of human seasonal IAV infections.

Introduction

Influenza A virus (IAV) infections cause low mortality but high morbidity in humans worldwide annually, while pandemics that occur at irregular intervals may have a disastrous impact on global human health [1–3]. Because newly emerging IAV were often of swine origin or arose from reassortments in the pig as mixing vessel [4–7], pigs have increasingly been evaluated as a biomedical model for human influenza [8]. Pigs are anatomically, physiologically and genetically similar to humans indicating a closer mimic of the situation in humans than rodent models. Studies on the immune response in pigs have long been biased by the lack of specific reagents. Especially the variety of antibodies available for the pig is still far lower to that of mice. As a result, the knowledge of the porcine immune response in general is much smaller compared to that of mice. Despite these disadvantages, pigs have decisive advantages as a

Funding: This study was funded by Federal Excellence Initiative of Mecklenburg Western Pomerania and European Social Fund (ESF) Grant KoInfekt (ESF_14-BM-A55-00xx_16) to TCM.

Competing interests: The authors have declared that no competing interests exist.

model for human IAV infection: they are themselves a natural host for influenza viruses and source of new IAV pathogenic for humans. Thus, understanding the immune response of pigs upon IAV infection and protection by tailored vaccines would assist in the burden on human health by minimizing the emergence of new viruses in swine and zoonotic transmission [4, 5, 9]. Initially, these approaches primarily aimed at identification of antigen preparations that elicit a protective antibody response against surface glycoproteins [10–13], which are effective in controlling IAV infection. However, constant antigenic drift requires an incessant update of the vaccines to match circulating strains. Thus and by expanding the number of reagents for porcine immunological analyses, in the past two decades research increasingly focused on analysis of the immune response against conserved antigenic regions unlikely to change extensively, including cellular immunity. These studies provided evidence for the importance of the cellular immune response in eliminating IAV, also in pigs. These studies have in common that they either only investigated blood-derived cells [14–16] or analyzed the systemic and local immune response only at a very early [17] or late time points of infection [15]. The situation is further complicated by the use of different IAV strains. Recent work recorded for the first time the kinetics of T cell responses and their phenotyping, which supported the previous assumption of the induction of IAV-specific $CD4^+$ and $CD8^+$ T cells [18, 19]. This is in line with several publications reporting IAV-specific $CD8^+$ T cell responses in humans associated with cytolytic [20, 21] as well as memory characteristics [22]. Further, IAV-specific memory T cells were reported to reside in human lungs [23]. Altogether, these porcine studies provided further evidence for similarities of the IAV immune response in pigs and humans [22, 24, 25]. However, it is important to note that the prominent porcine populations of $CD4^+/CD8^+$ double positive T cells [26] as well as the high number of peripheral γδ T [27] cells are virtually absent in humans [28, 29], representing a major difference. Besides the numerical difference, the functionality of the two cell populations is comparable in both human and swine. $CD4^+/CD8^+$ double positive T cells are mature effector cells with memory characteristics that rapidly mount antigen-specific responses upon antigenic challenge [18, 30]. Besides acting as innate immune cells via pattern recognition receptors and direct killing of infected cells, γδ T cells do play a major role in antigen processing and presentation in human and swine [31, 32]. The two most detailed characterizations of T cell dynamics in influenza-infected pigs were performed by Gerner's group [18, 19]. They reported increased capacity of $CD4^+/CD8\alpha^+$ T cells to co-produce IFN-γ, TNF-α and IL-2 as well as the appearance of blood-derived IAV-specific $CD8\beta^+$ T cells [18]. Further investigations revealed increased frequencies of IFN-γ and/or TNF-α producing, influenza-specific $CD4^+$ and $CD8^+$ T cells in the lungs of infected pigs, resulting in an accumulation of memory cells in the lungs at 6 weeks post infection [19]. Although these porcine IAV studies report a pronounced involvement of the cellular immune response, a global analysis and comparison of immune cells and their functions along the route of entry (nose, lung and lung lymph node) as well as the systemic responses (blood) after infection with H1N1pdm09 were still missing. To ascertain the value of the pig as a reliable model mimicking human IAV infection, we compared the immune response in pigs after a natural IAV infection without inducing major clinical signs, as usually occurs in seasonal human influenza.

Material and methods

Ethical statement and study design

All animal experiments were approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) with reference numbers 7221.3-1-035/17.

Table 1. Summary of sampling days and animals during study.

	d0 1st infection	d2pi	d4pi	d7pi	d14pi	d21pi 2nd infection	d22pi	d25pi	d31pi
Blood samples	6	6	6	6	6	6	6	6	6
Organ samples			5	5		5		5	3

Blood samples were taken from the same six animals, randomly chosen on day 0. Three control animals underwent necropsy on day 30 post infection.

https://doi.org/10.1371/journal.pone.0222943.t001

In total 29 four-week old German landrace pigs were obtained from a commercial high health status herd (BHZP-Basiszuchtbetrieb Garlitz-Langenheide, Germany). This farm is free from the following diseases or pathogens: Pseudorabies, classical swine fever virus (CSFV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), *Actinobacillus pleuropneumoniae*, *Mycoplasma hypopneumoniae*, ascaris, mange, *Brachyspira hyodysenteriae* and salmonella category I. Vaccination program does not include vaccination against influenza viruses but the following vaccines were administered: Sows were vaccinated against porcine circovirus type 2 (once), *Erysipelothrix rhusiopathiae*/Porcine parvovirus (twice), salmonella (twice) and *Haemophilus parasuis* (twice). Piglets were vaccinated against PCV2 once and *Mycoplasma hypopneumoniae* twice. Piglets for this study were kept under BSL2 conditions in the animal facilities of the Friedrich-Loeffler-Institut, Greifswald-Riems. IAV infections were performed twice during study period (Table 1), whereby first infection occurred three weeks after transport to our facility by intranasal administration of 2 ml virus suspension (10^6 $TCID_{50}$/ml) using mucosal atomization devices (MAD) (Wolfe Tory Medical, USA) with 26 animals. A second infection was performed on day 21 after the first infection. Three animals were mock infected with medium only and served as controls. Blood samples from the same six randomly selected animals were taken on day 0, 2, 4, 7, 14, 21 (prior to second infection), 22, 25 and 31 after first infection for kinetics of blood cells. After euthanasia with Release® (IDT, Germany), necropsy was performed on five animals on day 4, 7, 21 and 25 post first infection. Control animals were euthanized on day 30 after first mock-infection.

Virus

Influenza virus A/Bayern/74/2009 (hereafter referred to as H1N1pdm09) was propagated on Madin-Darby canine kidney cells (MDCKII) in MEM supplemented with 0.56% bovine serum albumin, 100 U/ml Penicillin, 100 μg/ml Streptomycin and 2 μg/ml L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, USA). For viral titration by $TCID_{50}$ assay, serial ten-fold dilutions of virus suspensions were prepared, added to MDCKII cells in 96-well plates, and incubated for three days at 37˚C and 5% CO_2. Cytopathic effect was examined microscopically. Titers were calculated according to Spearman-Kärber [33, 34]. An acute IAV infection of pigs acquired prior to the study was excluded by real-time PCR (AgPath.ID™ One-Step RT-PCR Kit, Applied Biosystems, USA) of nasal swabs immediately before transport to the experimental facility (modified from [35]).

Sample preparation

Necropsy and pathological gross examination was performed according to standard guidelines under BSL3** conditions. For extracorporeal bronchoalveolar lavage (BAL), the left main bronchus was cut with sterile scissors and 200 ml of sterile PBS solution was injected with a syringe through the main bronchus into the left lung lobe, which was then kneaded softly. BAL fluid was recovered by syringe.

For flow cytometric analyses organ samples from the following organs were taken during necropsy and stored in ice-cold PBS until further use: mucosa of nasal cavity, lung tissue (*lobus dexter medius*) and lung lymph node (*nodi lymphatici tracheobronchiales inferiores*).

Specimen from the following organs were collected for histopathology and fixed in 4% neutral buffered formaldehyde: nasal cavity with conchae, trachea, lungs (*lobus sinister cranialis pars cranialis and caudalis*, *lobus sinister caudalis*, *lobus dexter cranialis*, *lobus dexter medius*, *lobus dexter accessorius*, *lobus dexter caudalis*), lymph node from the lymphocentrum bronchiale.

Lungs were scored for macroscopically detectable atelectasis (reddish-tan, consolidated, lobular to lobar parenchyma) and scored as follows: (shown as percentage of the total parenchyma analyzed for each pulmonary lobe): 0 = no atelectasis, 1 = mild atelectasis (0–30%), 2 = moderate atelectasis (30–60%), 3 = severe atelectasis (60–100%). The max atelectasis score of all seven scored pulmonary lobes was taken to set up one gross lesion score for each individual pig. For histopathological examination, formaldehyde-fixed tissue samples were embedded in paraffin and cut at 3μm. The sections were mounted on Super-Frost-Plus-Slides (Carl Roth GmbH, Karlsruhe, Germany) and stained with hematoxylin-eosin for light microscopical examination using a Zeiss Axio Scope.A1 microscope equipped with 5x, 10x, 20x, and 40x N-ACHROPLAN objectives (Carl Zeiss Microscopy GmbH, Jena, Germany). All tissue sections were scored blind and investigated for signs and severity levels of inflammation (rhinitis, tracheitis, bronchointerstial pneumonia) as follows: 0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, 3 = severe inflammation. The final score for each organ of an individual animal was raised on the basis of the max value of the respective scores.

Immunohistochemistry was performed to detect Influenza A virus antigen in paraffin embedded tissue sections using a mouse monoclonal antibody against the matrixprotein of influenza A virus (M21C64R3, ATCC, Manassas, VA) as previously described [36]. Briefly, tissue sections were dewaxed and rehydrated, and endogenous peroxidase was blocked with 3% H_2O_2 (Merck, Darmstadt, Germany) for 10 min. After demasking of antigens with 10mM Na-citrate buffer (pH = 6, 700 W) for 20 min in a microwave oven, sections were incubated with undiluted normal goat serum for 30 min at room temperature to block nonspecific binding sites. Thereafter, section were incubated with anti-Influenza A matrix protein antibodies, diluted 1: 200 in Tris-buffered saline (TBS) at 4˚C overnight. This incubation was followed by a biotinylated goat anti-mouse IgG (1:200, LINARIS biologische Produkte, Dossenheim, Germany) and the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min each at room temperature. Positive antigen-antibody reactions were visualized by incubation with AEC-substrate (Dako, Hamburg, Germany) for 10 min. Sections were washed with deionized water and counterstained with Mayer‘s hemalaun for 30 s. Sections that stained positive for IAV matrix protein were investigated for cell-specific viral antigen localization and scored for viral antigen distribution in each inspected organ: 0 = negative, 1 = focal to oligofocal, 2 = multifocal, 3 = diffuse as previously described [37]. For each organ the max values of all scores were taken into analysis.

Cell preparation and antibody staining for flow cytometric analysis

Whole blood was diluted with PBS 1:10 and overall number of white blood cells and different leukocyte subpopulations were determined by blood counting device. Flow cytometric analysis of single cell suspensions from mucosa of nasal cavity, BAL, lung tissue and lung lymph node were performed. Cell from BAL fluid were enriched by centrifugation and discarding the supernatant. Leukocytes from mucosa of nasal cavity, lung lymph node and spleen were prepared by mechanical disruption on metal sieves with plungers and washed with PBS. For

isolation of leukocytes from the lung, tissue was minced with scissors, resuspended in PBS-EDTA supplemented with 100 μM $CaCl_2$, and digested with Collagenase D (1 mg/ml; Sigma-Aldrich) for 40 min at 37˚C. After pressing the digested tissue gently through a cell strainer with a plunger, remaining tissue was removed by short centrifugation.

Cell pellets were suspended in FACS buffer for flow cytometric antibody staining. Antibodies used in this study are listed in S1 Table. Unless otherwise stated, all incubation steps were carried out at 4 ˚C in the dark for 15 min in case of extracellular staining and for 30 min for intracellular staining. Staining of whole blood required erythrocyte lysis after surface staining by conventional lysis buffer (1.55 M NH_4Cl, 100 mM $KHCO_3$, 12.7 mM Na_4EDTA, pH 7.4, in A. dest.). For intracellular staining, the True-Nuclear™ Transcription Factor Buffer Set was used according to manufacturer's instructions (BioLegend, Germany).

Software and statistics

Flow cytometric analyses were run on BD FACS CantoII with BD FACS DIVA Software and analyzed using FlowJo Software. GraphPad Prism was used to visualize data and perform statistical analyses. All animal groups examined on days 4, 7, 21, 25 and 30 (control) were analyzed by the nonparametric Kruskal-Wallis test followed by pairwise Dunn's *post hoc* tests compared to control. For blood analyses same tests were used but *post hoc* test was compared to day 0. Statistical significance was designated as $p \leq 0.05$ indicated by an asterisk (*) in the graphs.

Results

Intranasal infection of pigs with H1N1pdm09 induced macroscopic and microscopic lesions in the lungs

After intranasal primary IAV infection, multifocal, reddish-tan consolidated areas (pulmonary atelectasis) of different sizes were macroscopically observed in inoculated animals after 4, 7 and 21 days (Fig 1A), mainly in the *lobus dexter medius* and in the *lobus sinister cranialis pars caudalis* (Fig 1B). 4 dpi, the animals reached the highest atelectasis score compared to pigs which were analyzed after 25 dpi (Fig 1C). One control pig showed a minimal, focal atelectasis in the *lobus dexter cranialis*.

Inflammatory changes were detected in the nasal mucosa, trachea and lung. Results from histopathological investigations of nasal mucosa and lungs are summarized in Fig 2. Starting at 4 dpi pigs showed mild, focal, necrotizing rhinitis with loss of epithelial cells (Fig 2 left panel) and IAV matrix protein-positive respiratory epithelial cells within the lesions. Mild, focal, subacute, lymphohistiocytic rhinitis have been observed 7, 21 and 25 dpi. Until 25 dpi inflammation decreased constantly whereas control pigs were free of rhinitis. One infected pig showed mild, necrotizing tracheitis at 4 dpi compared to all other infected and control pigs, which lacked similar lesions. Lung lesions were mainly localized in bronchi, bronchioles and bronchioloalveolar transition zone leading to mild bronchiolointerstitial pneumonia as shown in Fig 2 (right panel). 4 dpi, mild necrosis and loss of bronchial and bronchiolar epithelium was evident in H1N1pdm09 inoculated pigs followed by the infiltration of lymphocytes, macrophages and few neutrophils into the affected tissue (Fig 2C, right panel). At 7 dpi, mild alveolar edema was present whereas necrosis extended to the bronchi-alveolar transition zone (Fig 2E, right panel). At that time, lymphocytes and macrophages increasingly infiltrated the pulmonary interstitium (Fig 2E, right panel), but Influenza A matrix protein was not detectable at any time point later than 4 dpi (Fig 2B, 2D, 2F, 2H and 2J, right panel). 21 dpi, inflammatory cells were still evident (Fig 2G, right panel). Still negative for viral antigen (Fig 2J, right panel), the amount of infiltrating inflammatory cells slightly decreased at 25 dpi (Fig 2I, right panel).

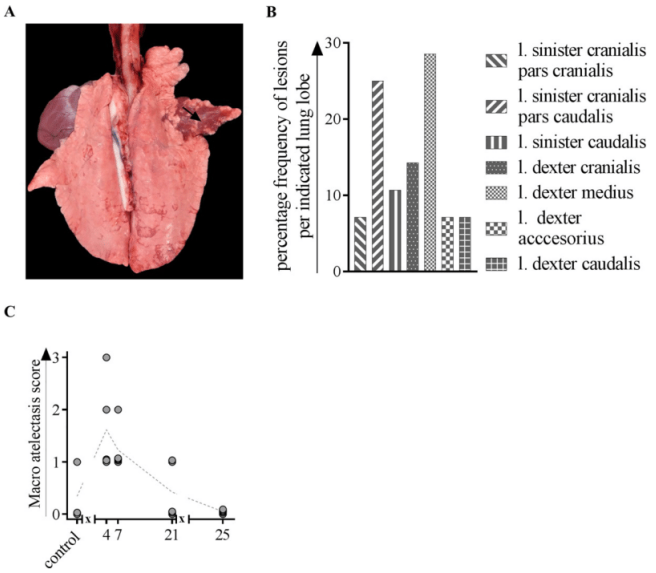


Fig 1. Gross pathologic changes after macroscopic investigation of lungs from H1N1pdm09-infected pigs. At indicated time points, three to five animals were subjected to necropsy for macroscopic investigation of artelectasis. (A) Lung from a pig inoculated with H1N1, 4 dpi. Acute, diffuse atelectasis of the *lobus dexter medius* (arrow). (B) Frequency distribution of macroscopic lesions in different lung lobes. (C) Atelectasis scores after 4, 7, 21 and 25 days of H1N1-inoculated and mock-infected animals. l. = lobus; *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g001

Data from histopathological scoring are summarized in Fig 3. As indicated, IAV matrixprotein was only detectable 4 dpi in the nose, trachea and lung (Fig 3A). At 7 dpi, infected animals showed the highest inflammation score in the nose and lung which then slightly decreased and remained constant until the end of the experiment (Fig 3B). Of note, a moderate significant positive correlation (Spearmann r = 0.464; $p<0.0001$) was found between macroscopic (atelectasis) and microscopic lesions in the lung ($p < 0.0001$) (Fig 4).

IAV H1N1pdm09 infection led to a shift in distribution of blood immune cell subsets but not to leucopenia

Counts of total and different subpopulations of white blood cells did not change significantly during the course of infection (Fig 5). A slight decrease in total cell numbers was observed two days after the first infection but they recovered until day four and stayed at a comparable number until day 14 post first infection. On day 21, prior to second infection, cell number was slightly elevated, stayed at a comparable level the following day and returned to cell numbers comparable to those on day 0 and remained stable until the end of the study (Fig 5A).

Of the myeloid cells in blood, dendritic cell and neutrophil count decreased initially on day two post infection but their numbers as well as monocyte count increased on day four post infection (Fig 5B). On average, monocyte and neutrophil counts remained stable from day 14 post infection until the end of the study, regardless of a second infection, whereas dendritic cell numbers decreased to a hardly detectable level after the second infection and did not recover until the end of the study (Fig 5B). Although neutrophil counts decreased initially after first infection, the frequency of CD14 expressing cells among them increased after first as well as after second infection (Fig 5C).

Regarding cell number of lymphocytes, we observed a decrease for αβ and γδ T cells, as well as for B cells initially after first infection that lasted until day seven and for the latter until the end of the study (Fig 5D). On day 14 post infection cell numbers of γδ T cells and B cells recovered, but αβ T cells numbers were elevated compared to day 0. After second infection αβ T cells decreased to basal level and remained unchanged for the rest of the study, whereas second infection led to another increase in γδ T cells on day 22. Three days later (day 25) and on day 31 numbers of these cells reflected basal levels (Fig 5D).

Proliferation of $CD8^+$ αβ T cells in the blood was increased upon infection with H1N1pdm09

In line with absolute cell numbers obtained from blood counting device (Fig 5), we also observed a decreased frequency of αβ T cells from lymphocytes both after first and second infection with H1N1pdm09 in blood of pigs (Fig 6A). 14 days after first infection, they returned to normal levels. After the second infection recovery time was faster and original frequencies were reconstituted on day 25 (four days after second infection).

To determine subpopulations, frequencies of T cells expressing CD4 and/or CD8 were distinguished into naïve Th cells ($CD4^+/CD8^-$), extrathymic $CD4^+/CD8^+$ cells (including memory as well as cytotoxic cells) and cytolytic T cells ($CD4^-/CD8^+$) (Fig 6B). Overall frequencies of different subpopulations remained stable until day 14 after first infection. Afterwards frequency of cytolytic T cells in bloodstream increased at the expense of naïve cell frequencies. From day 25 (4 days after second infection) increasing frequencies of double-positive Th cells were detected with further decreasing naïve cells. Frequency of proliferating $CD8^+$ cells–characterized by Ki-67 expression–increased both after first and to a lesser extent after second infection with H1N1pdm09. $CD8^-$ αβ T cells did not show signs of proliferation (Fig 6C).

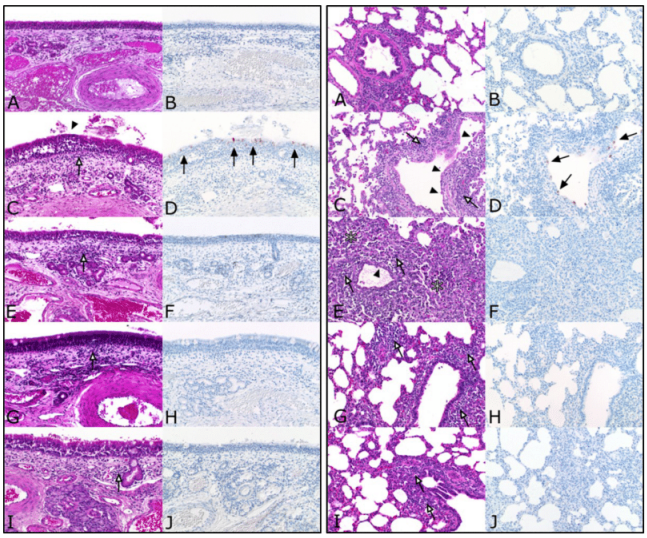


Fig 2. Histopathology from nose (left panel) and lung (right panel) of H1N1-infected pigs. At indicated time points, three to five animals were subjected to necropsy. Lungs, trachea and conchae were fixed in 4% formaldehyde, embedded in paraffin and cut at 3μm. Hematoxylin-Eosin (A, C, E, G, I) and anti-Influenza matrixprotein immunohistochemistry (B, D, F, H, J) were performed on nose and lung tissue. A-B) mock-control. (C-D) 4 dpi. (E-F) 7 dpi. (G-H) 21 dpi. (I-J) 25 dpi. White arrows: infiltration of inflammatory cells; black arrows: Influenza A matrix protein-positive cells; arrowheads: flattening and loss of epithelial cells; asterisks: necrotic lung tissue.

https://doi.org/10.1371/journal.pone.0222943.g002

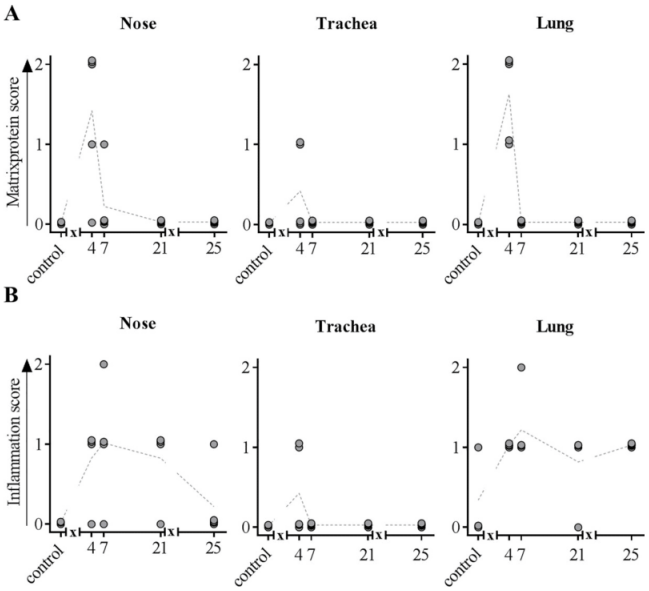


Fig 3. IAV matrixprotein score (A) and inflammation score (B) obtained from the nose, trachea and lung. At indicated time points, three to five animals were subjected to necropsy. Lungs, trachea and conchae were fixed in 4% formaldehyde, embedded in paraffin and cut at 3μm. Hematoxylin-eosin staining was used to determine inflammatory score, immunohistochemistry allowed the detection of IAV matrixprotein. *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g003

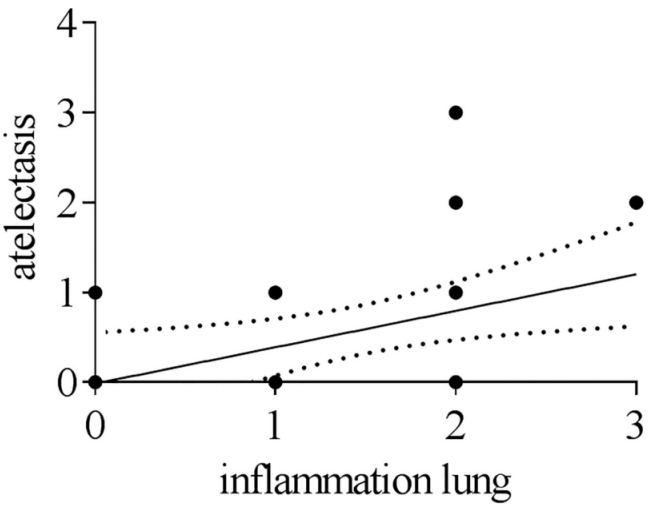


Fig 4. Correlation between macroscopic and microscopic lesions. Correlation between scores of atelectasis and simultaneous inflammatory processes in the lung obtained by histopathological investigation. Spearman r = 0,4642 ($p < 0,0001$).

https://doi.org/10.1371/journal.pone.0222943.g004

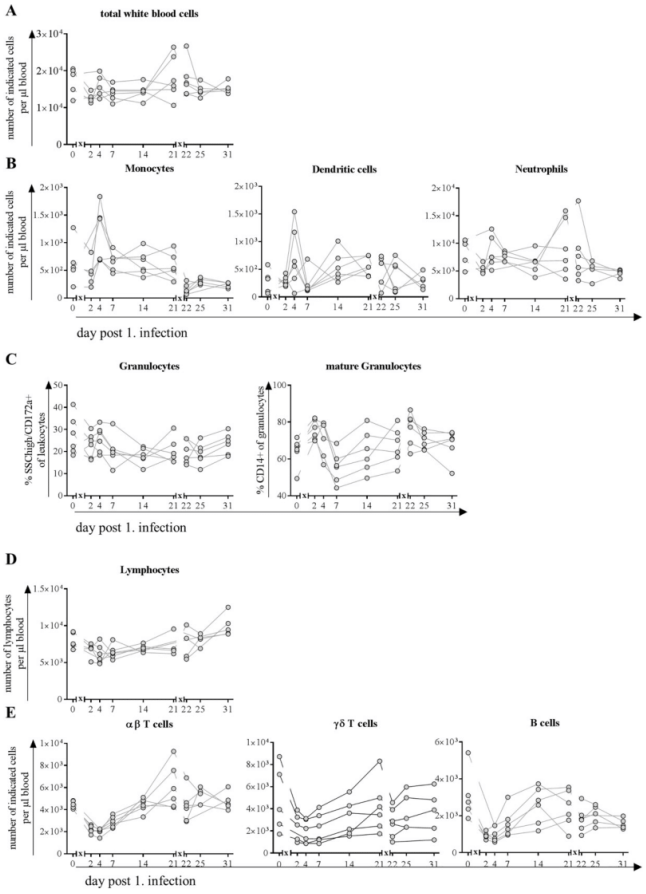


Fig 5. Counts of peripheral blood leukocytes after infection with H1N1pdm09. At indicated time points, blood was collected from the same six pigs, randomly chosen on day 0. Blood counting device revealed total count of A) white blood cells and B) myeloid cells (monocytes, dendritic cells and neutrophils). C) Within the granulocytic population frequency of mature granulocytes is indicated by expression of CD14. D) Total count of lymphocytes and E) main subpopulations: αβ T cells, γδ T cells and B cells. *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g005

γδ T cells proliferated and increased CD8 expression in the blood after infection with H1N1pdm09

Because γδ T cells do play a major role in pigs during infections, we analyzed these cells and the distribution of different subtypes in the course of IAV infection. Frequencies remained stable during the first infection but were increased immediately after the second infection, which was congruent with decreased frequencies of αβ T cells (Fig 7A).

CD2 and subsequent CD8 expression on the cell surface of γδ T cells resembles activation and maturation steps, the former being observed 14 days after first and one day after second infection with H1N1pdm09 (Fig 7B). Differentiated effector γδ T cells (characterized by simultaneous expression of CD2 and CD8) increased after second infection only. In addition, Ki-67 expressing cells increased among activated and differentiated effector memory T cells after the

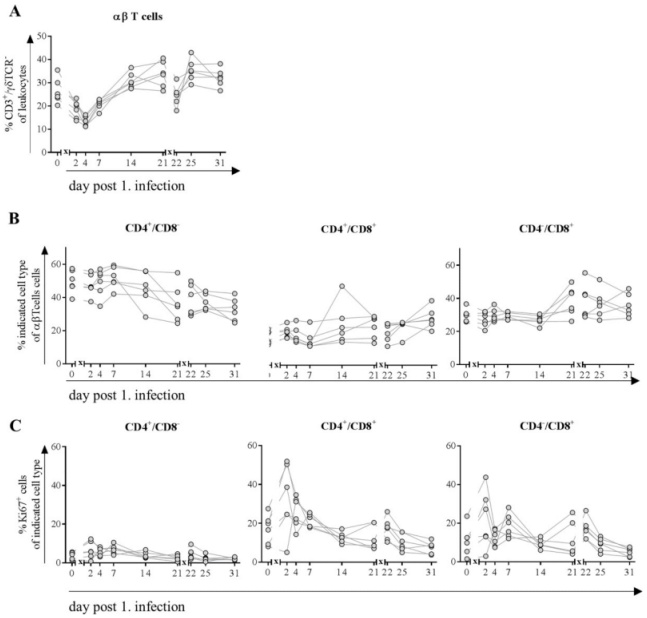


Fig 6. Frequencies of αβ T cells (A), their subpopulations (B) expressing Ki67 (C). At indicated time points, blood was collected from the same six pigs, randomly chosen on day 0. Flow cytometric analyses were performed to determine frequency of $CD3^+$/ $\gamma\delta TCR^-$ T cells (A). Further classification (B) was made based on the expression of $CD4^+$ single positive cells (naive Th cells), $CD4^+/CD8^+$ (memory as well as cytotoxic T cells) and $CD4^-/CD8^+$ cytolytic T cells. Expression of Ki67 (C) indicated proliferative capacity of cells. *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g006

first infection (Fig 7C). After the second infection, only the latter showed higher frequencies of proliferating cells. Naïve γδ T cells did not show proliferative activity.

Frequency of αβ T cells along the respiratory tract increased after the first infection only and expressed mainly CD8αα homodimers

To investigate the immune response along the proposed route of infection, frequencies and functional properties of αβ T cells from lymphocytes in mucosa from nasal cavity, BAL, lung and lung lymph node were analyzed. Increasing frequencies for these cells could be observed four days after first but not after second infection in mucosa of nasal cavity, BAL, lung tissue and lung lymph node and until day seven after first infection in the former two (Fig 8A). Frequencies were still elevated on day 21 compared to control animals and returned to normal levels on day 25, four days after second infection in all organ samples.

Differentiation in subtypes expressing either CD8αα homo- or CD8αβ heterodimers revealed that under physiological conditions (control) $CD8^+$ αβ T cells from tissues of the respiratory tract–nose, BAL and lung–are mainly composed of cells expressing the CD8αα homodimers (Fig 8B). In contrast, in the lymph node CD8αβ expressing T cells were the predominant population. After an initial slight drop in nose and BAL four days after first infection, CD8αα expressing cells increased until day seven in all organs, whereby the ratio in the lymph node was completely reversed towards the homodimer expressing $CD8^+$ T cells. On day 21, prior to second infection, ratio of CD8αα to CD8αβ expressing cells was the same as for control animals. Four days after second infection, the previously observed ratio shift was

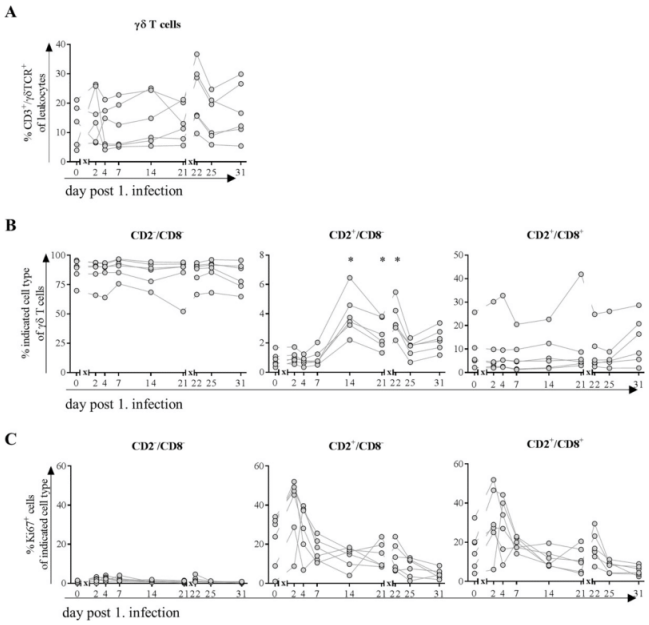


Fig 7. Frequencies of γδ T cells (A), their subpopulations (B) expressing Ki67 (C). At indicated time points, blood was collected from the same six pigs, randomly chosen on day 0. Flow cytometric analyses were performed to determine frequency of $CD3^+$/ $\gamma\delta TCR^+$ T cells. Further classification (B) was made based on the expression of CD2 and CD8: $CD2^-/CD8^-$ = naïve γδ T cells, $CD2^+/CD8^-$ = activated γδ T cells and $CD2^+/CD8^+$ = differentiated effector γδ T cells. Expression of Ki67 (C) indicated proliferative capacity of cells. * = $p \leq 0.05$ Kruskal-Wallis test followed by Dunn's *post hoc* test compared to day 0. *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g007

repeated and resembled the ratio on day seven except for cells in the lung lymph node, for which the ratio was the same as on day four (Fig 8B).

In both αβ T cell subsets expression of perforin increased after the first infection

To investigate the functionality of αβ T cells, expression levels of perforin were investigated along the infection route. In all organs, expression of perforin in $CD8\alpha\alpha^+$ cells increased on the fourth day after first infection but returned to levels as observed in control animals already three days later and did not change during the rest of the study (Fig 8C). $CD8\alpha\beta^+$ T cells showed a similar pattern, although the level of expression was higher, both under basal (control) as well as in infection conditions (day 4) (Fig 8D). For $CD8\alpha\beta^+$ T cells in lung and BAL expression of perforin returned to basal levels on day 7 and remained unchanged. In lung and to a greater extent in the lymph node, expression of perforin was lowest on day 7, returned to basal levels on day 21 but decreased once again after the second infection (Fig 8D).

Frequency of γδ T cells in the nose and CD8 expressing subpopulations were increased after first infection

Because frequencies of γδ T cells remained stable in the blood, we investigated whether there would be changes in organs from the respiratory tract. In the nose, frequency of cells increased slightly over the fourth to seventh day, stayed elevated until day 21 and returned to levels

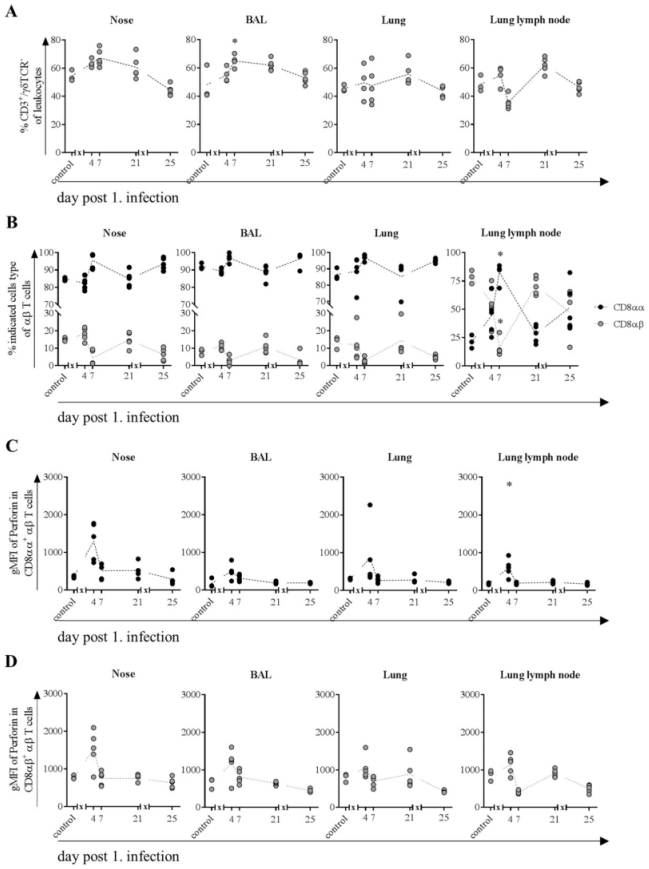


Fig 8. Frequencies of αβ T cells (A) and CD8αα$^+$ and CD8αβ$^+$ subpopulations (B) expressing perforin (C and D). At indicated time points, three to five animals were subjected to necropsy. After preparation of single cell suspensions from nasal mucosa, BAL, lung tissue and lung lymph node, flow cytometric analyses were performed to determine frequency of CD3$^+$/ γδTCR$^-$ T cells (A). Further classification (B) was made based on the expression of CD8αα homo- or CD8αβ heterodimers. MFI (mean fluorescence intensity) indicated cytolytic activity of CD8αα (C) and CD8αβ (D) T cells. * = p≤0.05 Kruskal-Wallis test followed by Dunn's *post hoc* test compared to control. *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g008

comparable to those in control animals on day 25 (four days after second infection) (Fig 9A). In BAL, frequencies continuously decreased over study period. This also applies to the lymph node, albeit to a lesser extent. In the lung, frequency of γδ T cells halved after first and decreased by 1/3 after second infection (Fig 9A).

Investigations on the activation status revealed that in the nose around 40% and in BAL, around 60% of γδ T cells already express the CD8α molecule under physiological conditions (Fig 9B). In contrast to cells in BAL, where frequencies decreased by 10%, activated γδ T cells increased to 60% in the nose four days after infection. In lung and lymph node, frequencies increased from 20% to more than 40% on day four after first infection. For nose, lung and the lymph node, frequency of activated γδ T cells returned to basal levels on day seven and stayed there for every other time point (Fig 9B).

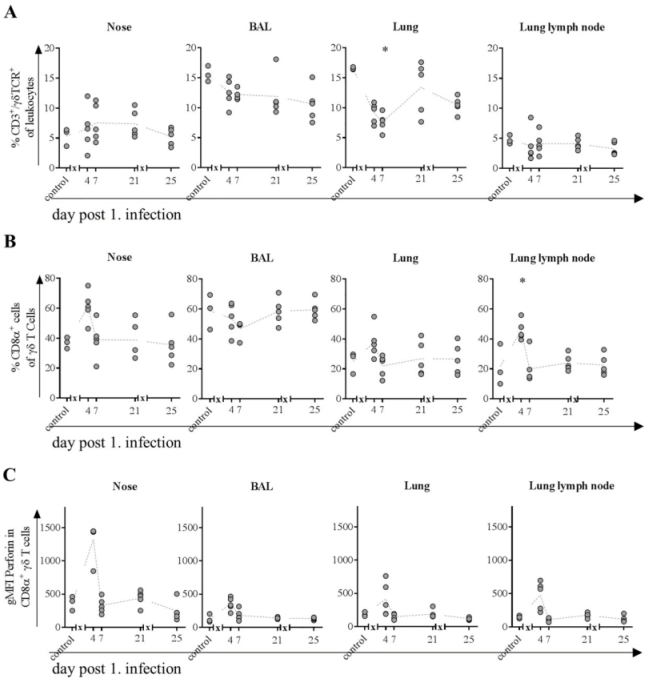


Fig 9. Frequencies of γδ T cells (A) and CD8α$^+$ subpopulation (B) expressing perforin (C). At indicated time points, three to five animals were subjected to necropsy. After preparation of single cell suspensions from nasal mucosa, BAL, lung tissue and lung lymph node, flow cytometric analyses were performed to determine frequency of CD3$^+$/ γδTCR$^+$ T cells (A). The CD8α expressing subpopulation (B) was further tested for intracellular perforin expression (C). * = p≤0.05 Kruskal-Wallis test followed by Dunn's *post hoc* test compared to control. *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g009

Nasal and BAL γδ T cells show increased expression of perforin and/or transcription factors associated with activation

Related to effector functions of γδ T cells in nose perforin expression was increased only at day four after first infection and did not change at any other day of study period (Fig 9C). For γδ T cells in BAL, lung and lung lymph node, expression of effector molecule perforin was slightly increased on the same day.

Because activation of γδ T cells does not necessarily lead to upregulation of CD8 or perforin, the transcription factors T-bet and EOMES of these cells were also investigated in mucosa from the nasal cavity as well as the BAL. Frequency of γδ T cells expressing T-bet only increased in the nose four days after infection, whereas EOMES single-positive cells increased four days after second infection only (Fig 10A). Frequency of cells expressing both transcription factors did not change during study period. In BAL T-bet expressing cells increased both after first and second infection, whereas EOMES as well as double positive cells remained unchanged (Fig 10B).

Discussion

Using two different approaches, continuous peripheral blood cell analysis from the same animals as well as analysis of organ samples from the respiratory tract at specific time points after

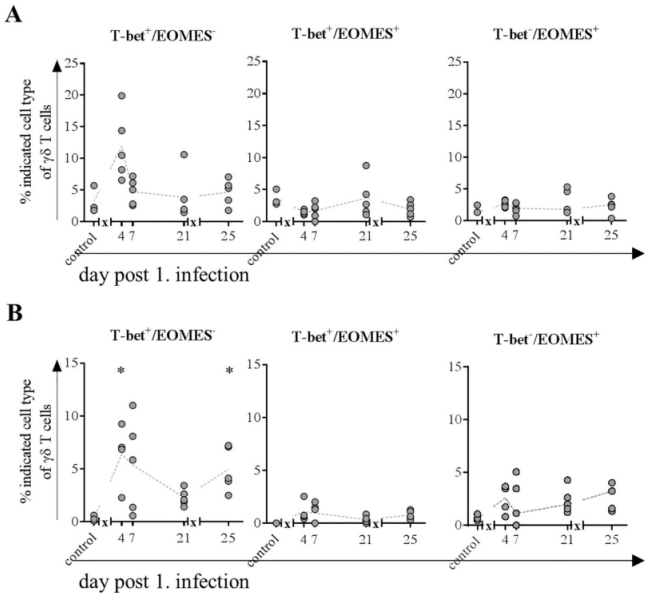


Fig 10. Frequencies of γδ T cells expressing transcription factors EOMES and/or T-bet. At indicated time points, three to five animals were subjected to necropsy. After preparation of single cell suspensions from nasal mucosa and BAL, flow cytometric analyses were performed to determine frequency of T-bet$^+$/EOMES$^-$, T-bet$^+$/EOMES$^+$ and T-bet$^-$/EOMES$^+$ γδ T cells in nose (A) and BAL (B). * = $p \leq 0.05$ Kruskal-Wallis test followed by Dunn's *post hoc* test compared to control. *x* in graph axis indicates infection.

https://doi.org/10.1371/journal.pone.0222943.g010

infection, we characterized in detail the kinetics of the porcine immune response after intranasal infection with H1N1pdm09. In contrast to previous studies reporting fever as well as clinical sings after intranasal infection of pigs with IAV [10, 14, 38, 39], pigs in our study did not develop either. Since different virus strains as well as doses were used, this finding was not unexpected. While a study by Pomorska-Mól *et al.* reported mild clinical signs without fever in intranasally H1N1-infected pigs they neither observed significant changes in hematological parameters nor in numbers of subsets of T and B cells [40]. However, the decreasing lymphocyte count caused by decreasing numbers of T cells and B cells within the first week after infection, correlated well with the other studies [17, 41]. Khatri *et al.* detected also increasing frequencies of CD8$^+$ αβ as well as γδ T cells in lungs and lung LN of H1N1-infected pigs [17], which is in line with our findings. We could further show that this also holds true at the site of first contact, the nose. Increased numbers correlated with an increased amount of perforin pointing toward enhanced cytotoxic function. An increase in CD4$^+$ T cells in the lungs, which was reported by two groups that observed high fever and strong clinical signs [14, 17], was not observed in our study.

The overall leukocyte count was only slightly decreased on day two after the first infection, which was mainly due to a decrease of the neutrophil population and to a lesser extent to decreasing numbers of αβ T cells and B cells. Two days later, absolute numbers of WBC were restored to normal levels because of elevated numbers of myeloid cells–monocytes, dendritic cells and neutrophils–whereas numbers of lymphocytes decreased further until day seven. These observations parallel the mild lymphopenia with monocytosis in human patients infected with IAV [42–44], which was proposed as a screening tool for influenza infection [45,

46]. An increase in neutrophils of IAV patients within the first days of infection described by some groups [42, 47, 48] could not be detected in our study. However, we observed an increase in frequency of CD14 expressing cells after both infections. CD14 upregulation is associated with activation in granulocytes [49, 50] and serves as a coreceptor for TLR7 mediated recognition of ssRNA [51]. We detected a higher number of dendritic cells in the blood in infected pigs four days after the first infection with simultaneously increased number of monocytes, which was not reported for human patients infected with H1N1pdm09. This might be because in most of the studies characterizing the immune response in H1N1 patients definite time points are hard to determine e.g. time of the infection. In contrast, the decrease in αβ T cells and B cells in pigs covers a period of four to seven days with recovery until day 14 and is comparable with decreasing frequencies in patients infected with H1N1pdm09 although the period of mild lymphopenia varies between studies [43, 46, 52]. However, after the second infection, recovery was faster compared to the first infection and thus comparable to vaccinated humans [53, 54]. Further, we detected an increased proliferative activity in $CD4^+/CD8^+$ T cells (constituting of cytotoxic and memory cells) and cytotoxic ($CD8^+$) subtypes of αβ T cells, which was pronounced after first infection with a lesser increase after second infection resembling a memory response. These observations are in line with the findings in an experimental human influenza experiment that additionally reported those $CD4^+$ T cells to have cytolytic and thus direct antiviral characteristics like perforin expression [55, 56]. Description of prominent $CD4^+$ T cell responses being strong in numbers, by contrast, are primarily associated with severe influenza cases [57].

Given that the H1N1pdm09 infection of the pigs in our setup with MAD leads to an infection that is almost exclusively localized to the respiratory tract, the increase in αβ T cells frequencies in nose and BAL were more prominent starting as early as day 4 post infection and peaking at day 7. Interestingly, in 14 weeks old healthy pigs the ratio of CD8αα to CD8αβ expressing αβ T cells isolated from nasal mucosa, the BAL or lung tissue is shifted towards CD8αα cells with frequencies of 85%, 93% and 85% respectively. This is the first description of the distribution of these two subtypes in respiratory organs of pigs and is in line with finding in humans and mice investigating the role of CD8αα expressing cells among the intraepithelial leukocyte population [58–60]. Because CD8αα is known to be a corepressor of TCR avidity and diminishes activation [61, 62], it is tempting to speculate that infection with H1N1pdm09 leads to activation whilst upregulation of CD8αα inhibits at the same time an excessive immune response. In line with this, due to homeostasis, frequencies of cytolytic CD8αβ T cells decreased further enhancing an anti-inflammatory environment. Several studies in mice and humans do further attribute CD8αα expressing T cells to have memory function [63–65]. Our findings point in the same direction, as CD8αα expression of αβ T cells in the respiratory tract of pigs increased more rapidly after second infection. In control (healthy) pigs, the expression level of perforin per cell within the CD8αα subpopulation in respiratory tract samples is only a third of the expression level in CD8αβ expressing T cells supporting in addition that they do not primarily have cytolytic activity. CD8αα expressing T cells arise mainly from CD8αβ T cells by downregulation of the β-chain and thus diminishing activation [64, 65]. Therefore, it is conceivable that the absence of perforin expression after the second infection is due to inhibition by increased frequencies of CD8αα T cells.

Because lymphocytes in the blood of pigs comprise up to 50% of γδ T cells (~3000 cells/μl blood), which strongly contrasts the maximum of 5% (<500 cells/μl blood) in healthy humans [66], it is challenging to draw conclusions with regard to comparability. Nevertheless, absolute numbers of these cells remained relatively constant throughout the study decreasing only slightly after the first infection but increasing after the second infection at the expense of αβ T cells in the blood. The latter observation might be explained by the different time points of

blood sampling: after the first infection, blood was taken at day two whereas after the second infection it was taken on the first day p.i., to gather a potential memory response. The hypothesis is supported by a pronounced increase in proliferative activity in both activated subtypes of γδ T cells after first infection and a subsequent increase of activated γδ T cells in the blood, supporting that this cell population serves as first line defense. The rapid decrease in frequency of proliferative cells might be explained by the recruitment to the site of inflammation (nose and lung), where their frequencies increased as early as day four after infection with a pronounced perforin expression. Frequencies of γδ T cells in lungs of pigs are comparable to those of humans [67] and they are known not only to maintain pulmonary homeostasis [68] but also to efficiently kill IAV infected epithelial cells and macrophages [69, 70]. In line with these findings from human *in vitro* experiments [70, 71], we observed a notable increase in perforin expression of γδ T cells after first infection in mucosa of nasal cavity constituting an entrance for the virus. Furthermore, concurred increase in frequency of CD8 expressing γδ T cells was observed along the route of entry from nose to lung and lung lymph node, in the latter most probably constituting antigen-presenting cells. Because perforin expression did not increase after the second infection and frequencies of γδ T cells in blood where increased only at day 14 p.i., it is likely that these cells play a major role in recovery from influenza infections as described earlier [29]. Finally, also T-bet expression in γδ T cells increased after both infections in nose and BAL, which is associated with improved recovery from influenza infections [72].

Given that in a natural H1N1 infection in humans the day of infection is not exactly clear, it seems obvious that a day-to-day comparison of patient data with the predefined time points in this study is challenging. Further, for obvious reasons, analyses of lung or respiratory epithelial tissue from patients with mild influenza virus infections are limited. However, transiently decreased numbers of lymphocytes along with increased monocyte counts in the blood seems to be a common feature in mild human and porcine subclinical H1N1pdm09 infections. Further, we could show the presence of and increase in CD8αα expressing αβ as well as CD8α$^+$ γδ T cells in mucosa from respiratory tract after infection in pigs, indicating that these cells have the same dual role as in humans. They do rapidly respond with perforin expression to H1N1pdm09 but simultaneously increase expression of the inhibitory CD8αα molecule to prevent excessive harmful immune responses. Therefore, even though pigs in our study did not show overt clinical signs, underlying immune and pathogenic mechanisms seem to be similar. The results from this study further expand the knowledge of the porcine immune response to pandemic IAV infection and thus, support the use of the pig as a large animal model for human seasonal influenza infections. This offers not only a model for testing the efficacy of new influenza vaccines regarding cellular immune responses but also to expand the model for further investigations of influenza induced pneumonia especially in bacto-viral coinfection scenarios.

Supporting information

S1 Table. Antibodies used in flow cytometric analyses.
(TIF)

Acknowledgments

The authors thank Regine Kasper, Stefanie Knöfel, Silke Rehbein and Silvia Schuparis for outstanding technical assistance. For excellent care of animals and support during trial, we thank the animal keepers Kerstin Kerstel, Thomas Möritz and Lukas Steinke. Further, we thank Christian Loth and Ralf Redmer for necropsy assistance in singular quality.

Author Contributions

Conceptualization: Theresa Schwaiger, Reiner Ulrich, Ulrike Blohm.

Data curation: Theresa Schwaiger, Ulrike Blohm.

Formal analysis: Theresa Schwaiger, Julia Sehl, Alexander Schäfer, Ulrike Blohm.

Funding acquisition: Thomas C. Mettenleiter, Bernd Köllner, Ulrike Blohm.

Investigation: Theresa Schwaiger, Julia Sehl, Reiner Ulrich, Ulrike Blohm.

Project administration: Thomas C. Mettenleiter.

Resources: Theresa Schwaiger, Julia Sehl, Claudia Karte, Alexander Schäfer, Jane Hühr, Charlotte Schröder, Bernd Köllner, Reiner Ulrich.

Supervision: Reiner Ulrich, Ulrike Blohm.

Validation: Theresa Schwaiger, Julia Sehl, Ulrike Blohm.

Visualization: Theresa Schwaiger, Ulrike Blohm.

Writing – original draft: Theresa Schwaiger, Julia Sehl, Ulrike Blohm.

Writing – review & editing: Theresa Schwaiger, Claudia Karte, Alexander Schäfer, Jane Hühr, Thomas C. Mettenleiter, Reiner Ulrich, Ulrike Blohm.

References

1. Glezen WP. Emerging Infections: Pandemic Influenza. Epidemiologic Reviews. 1996; 18(1):64–76. https://doi.org/10.1093/oxfordjournals.epirev.a017917 PMID: 8877331
2. Jester B, Uyeki T, Jernigan D. Readiness for Responding to a Severe Pandemic 100 Years After 1918. American Journal of Epidemiology. 2018; 187(12):2596–602. https://doi.org/10.1093/aje/kwy165 PMID: 30102376
3. Kilbourne ED. Influenza pandemics of the 20th century. Emerging infectious diseases. 2006; 12(1):9–14. https://doi.org/10.3201/eid1201.051254 PMID: 16494710.
4. Nelson MI, Gramer MR, Vincent AL, Holmes EC. Global transmission of influenza viruses from humans to swine. The Journal of general virology. 2012; 93(Pt 10):2195–203. https://doi.org/10.1099/vir.0.044974-0 PMID: 22791604; PubMed Central PMCID: PMC3541789.
5. Nelson MI, Vincent AL. Reverse zoonosis of influenza to swine: new perspectives on the human–animal interface. Trends in Microbiology. 2015; 23(3):142–53. https://doi.org/10.1016/j.tim.2014.12.002 PMID: 25564096
6. Nelson MI, Wentworth DE, Culhane MR, Vincent AL, Viboud C, LaPointe MP, et al. Introductions and evolution of human-origin seasonal influenza a viruses in multinational swine populations. Journal of virology. 2014; 88(17):10110–9. https://doi.org/10.1128/JVI.01080-14 PMID: 24965467; PubMed Central PMCID: PMC4136342.
7. Imai M, Kawaoka Y. The role of receptor binding specificity in interspecies transmission of influenza viruses. Current opinion in virology. 2012; 2(2):160–7. https://doi.org/10.1016/j.coviro.2012.03.003 PMID: 22445963.
8. Rajao DS, Vincent AL. Swine as a Model for Influenza A Virus Infection and Immunity. ILAR Journal. 2015; 56(1):44–52. https://doi.org/10.1093/ilar/ilv002 PMID: 25991697
9. Terebuh P, Olsen CW, Wright J, Klimov A, Karasin A, Todd K, et al. Transmission of influenza A viruses between pigs and people, Iowa, 2002–2004. Influenza and other respiratory viruses. 2010; 4(6):387–96. https://doi.org/10.1111/j.1750-2659.2010.00175.x PMID: 20958933; PubMed Central PMCID: PMC4634614.
10. Larsen DL, Karasin A, Zuckermann F, Olsen CW. Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. Veterinary Microbiology. 2000; 74(1):117–31. https://doi.org/10.1016/S0378-1135(00)00172-3.
11. Heinen PP, van Nieuwstadt AP, de Boer-Luijtze EA, Bianchi ATJ. Analysis of the quality of protection induced by a porcine influenza A vaccine to challenge with an H3N2 virus. Veterinary Immunology and

Immunopathology. 2001; 82(1–2):39–56. https://doi.org/10.1016/s0165-2427(01)00342-7 PMID: 11557293

12. Weaver EA, Rubrum AM, Webby RJ, Barry MA. Protection against Divergent Influenza H1N1 Virus by a Centralized Influenza Hemagglutinin. PLoS ONE. 2011; 6(3):e18314. https://doi.org/10.1371/journal.pone.0018314 PubMed PMID: PMC3065472. PMID: 21464940

13. Kim W-I, Wu W-H, Janke B, Yoon K-J. Characterization of the humoral immune response of experimentally infected and vaccinated pigs to swine influenza viral proteins. Archives of Virology. 2006; 151 (1):23–36. https://doi.org/10.1007/s00705-005-0615-9 PMID: 16132180

14. Heinen PP, de Boer-Luijtze EA, Bianchi ATJ. Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. Journal of General Virology. 2001; 82 (11):2697–707. https://doi.org/10.1099/0022-1317-82-11-2697 PMID: 11602782

15. Kappes MA, Sandbulte MR, Platt R, Wang C, Lager KM, Henningson JN, et al. Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. Vaccine. 2012; 30(2):280–8. https://doi.org/10.1016/j.vaccine.2011.10.098 PMID: 22067263

16. Loving CL, Vincent AL, Pena L, Perez DR. Heightened adaptive immune responses following vaccination with a temperature-sensitive, live-attenuated influenza virus compared to adjuvanted, whole-inactivated virus in pigs. Vaccine. 2012; 30(40):5830–8. https://doi.org/10.1016/j.vaccine.2012.07.033 PMID: 22835742

17. Khatri M, Dwivedi V, Krakowka S, Manickam C, Ali A, Wang L, et al. Swine Influenza H1N1 Virus Induces Acute Inflammatory Immune Responses in Pig Lungs: a Potential Animal Model for Human H1N1 Influenza Virus. Journal of virology. 2010; 84(21):11210–8. https://doi.org/10.1128/JVI.01211-10 PMID: 20719941

18. Talker SC, Koinig HC, Stadler M, Graage R, Klingler E, Ladinig A, et al. Magnitude and kinetics of multifunctional CD4+ and CD8β+ T cells in pigs infected with swine influenza A virus. Veterinary research. 2015; 46(1):52–. https://doi.org/10.1186/s13567-015-0182-3 PMID: 25971313.

19. Talker SC, Stadler M, Koinig HC, Mair KH, Rodríguez-Gómez IM, Graage R, et al. Influenza A Virus Infection in Pigs Attracts Multifunctional and Cross-Reactive T Cells to the Lung. Journal of virology. 2016; 90(20):9364–82. https://doi.org/10.1128/JVI.01211-16 PubMed PMID: PMC5044846. PMID: 27512056

20. Quinones-Parra S, Grant E, Loh L, Nguyen TH, Campbell KA, Tong SY, et al. Preexisting CD8+ T-cell immunity to the H7N9 influenza A virus varies across ethnicities. Proc Natl Acad Sci U S A. 2014; 111 (3):1049–54. Epub 2014/01/08. https://doi.org/10.1073/pnas.1322229111 PMID: 24395804; PubMed Central PMCID: PMCPMC3903243.

21. van de Sandt CE, Kreijtz JH, de Mutsert G, Geelhoed-Mieras MM, Hillaire ML, Vogelzang-van Trierum SE, et al. Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus. Journal of virology. 2014; 88(3):1684–93. Epub 2013/11/22. https://doi.org/10.1128/JVI.02843-13 PMID: 24257602; PubMed Central PMCID: PMCPMC3911609.

22. Wang Z, Wan Y, Qiu C, Quinones-Parra S, Zhu Z, Loh L, et al. Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8(+) T cells. Nat Commun. 2015; 6:6833. Epub 2015/05/15. https://doi.org/10.1038/ncomms7833 PMID: 25967273; PubMed Central PMCID: PMCPMC4479016.

23. Pizzolla A, Nguyen THO, Sant S, Jaffar J, Loudovaris T, Mannering SI, et al. Influenza-specific lung-resident memory T cells are proliferative and polyfunctional and maintain diverse TCR profiles. The Journal of Clinical Investigation. 2018; 128(2):721–33. https://doi.org/10.1172/JCI96957 PMID: 29309047

24. Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, et al. Cellular immune correlates of protection against symptomatic pandemic influenza. Nat Med. 2013; 19(10):1305–12. https://doi.org/10.1038/nm.3350 http://www.nature.com/nm/journal/v19/n10/abs/nm.3350.html#supplementary-information. PMID: 24056771

25. Piet B, de Bree GJ, Smids-Dierdorp BS, van der Loos CM, Remmerswaal EBM, von der T, et al. CD8+ T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung. The Journal of Clinical Investigation. 2011; 121(6):2254–63. https://doi.org/10.1172/JCI44675 PMID: 21537083

26. Pescovitz MD, Lunney JK, Sachs DH. Murine anti-swine T4 and T8 monoclonal antibodies: Distribution and effects on proliferative and cytotoxic T cells. Journal of Immunology. 1985; 134(1):37–44.

27. Šinkora M, Butler JE. The ontogeny of the porcine immune system. Developmental & Comparative Immunology. 2009; 33(3):273–83. https://doi.org/10.1016/j.dci.2008.07.011.

28. Ortolani C, Forti E, Radin E, Cibin R, Cossarizza A. Cytofluorometric Identification of Two Populations of Double Positive (CD4+,CD8+) T Lymphocytes in Human Peripheral Blood. Biochemical and

Biophysical Research Communications. 1993; 191(2):601–9. https://doi.org/10.1006/bbrc.1993.1260 PMID: 8461016

29. Carding SR, Egan PJ. γδ T cells: functional plasticity and heterogeneity. Nature Reviews Immunology. 2002; 2:336. https://doi.org/10.1038/nri797 PMID: 12033739

30. Nascimbeni M, Shin E-C, Chiriboga L, Kleiner DE, Rehermann B. Peripheral CD4+CD8+ T cells are differentiated effector memory cells with antiviral functions. 2004; 104(2):478–86. https://doi.org/10.1182/blood-2003-12-4395 %J Blood

31. Brandes M, Willimann K, Moser B. Professional Antigen-Presentation Function by Human γδ T Cells. 2005; 309(5732):264–8. https://doi.org/10.1126/science.1110267 %J Science

32. Takamatsu HH, Denyer MS, Wileman TE. A sub-population of circulating porcine γδ T cells can act as professional antigen presenting cells. Veterinary Immunology and Immunopathology. 2002; 87(3):223–4. https://doi.org/10.1016/S0165-2427(02)00083-1.

33. Spearman C. The Method of "Right and Wrong Cases" (Constant Stimuli) without Gauss's Formula. British Journal of Psychology, 1904–1920. 1908; 2(3):227–42. https://doi.org/10.1111/j.2044-8295.1908.tb00176.x

34. Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Naunyn-Schmiedebergs Archiv für experimentelle Pathologie und Pharmakologie. 1931; 162(4):480–3. https://doi.org/10.1007/bf01863914

35. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 2002; 40(9):3256–60. Epub 2002/08/31. https://doi.org/10.1128/JCM.40.9.3256-3260.2002 PMID: 12202562; PubMed Central PMCID: PMCPMC130722.

36. Graaf A, Ulrich R, Maksimov P, Scheibner D, Koethe S, Abdelwhab EM, et al. A viral race for primacy: co-infection of a natural pair of low and highly pathogenic H7N7 avian influenza viruses in chickens and embryonated chicken eggs. Emerging Microbes & Infections. 2018; 7(1):1–12. https://doi.org/10.1038/s41426-018-0204-0 PMID: 30514922

37. Mamerow S, Scheffter R, Röhrs S, Stech O, Blohm U, Schwaiger T, et al. Double-attenuated influenza virus elicits broad protection against challenge viruses with different serotypes in swine. Veterinary Microbiology. 2019; 231:160–8. https://doi.org/10.1016/j.vetmic.2019.03.013 PMID: 30955804

38. Ferrari M, Candotti P, Lombardi G, Amadori M, Dotti S, Guana S, et al. A comparison of the humoral and cell-mediated response of pigs experimentally infected with either influenza or PRRS viruses. 2008; 32(1):199–201. https://doi.org/10.1007/s11259-008-9161-8 PMID: 18726247

39. Khatri M, Dwivedi V, Krakowka S, Manickam C, Ali A, Wang L, et al. Swine Influenza H1N1 Virus Induces Acute Inflammatory Immune Responses in Pig Lungs: a Potential Animal Model for Human H1N1 Influenza Virus. 2010; 84(21):11210–8. https://doi.org/10.1128/JVI.01211-10 PMID: 20719941

40. Pomorska-Mol M, Markowska-Daniel I, Kwit K, Czyzewska E, Dors A, Rachubik J, et al. Immune and inflammatory response in pigs during acute influenza caused by H1N1 swine influenza virus. Arch Virol. 2014; 159(10):2605–14. https://doi.org/10.1007/s00705-014-2116-1 PMID: 24846450; PubMed Central PMCID: PMC4173111.

41. Forberg H, Hauge AG, Valheim M, Garcon F, Nunez A, Gerner W, et al. Early Responses of Natural Killer Cells in Pigs Experimentally Infected with 2009 Pandemic H1N1 Influenza A Virus. PLOS ONE. 2014; 9(6):e100619. https://doi.org/10.1371/journal.pone.0100619 PMID: 24955764

42. Chen WW, Xie YX, Zhang YH, Feng YQ, Li BA, Li B, et al. [Changes and analysis of peripheral white blood cells and lymphocyte subsets for patients with pandemic influenza A virus (H1N1) infection]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi. 2010; 24(5):331–3. PMID: 21280315.

43. Cunha BA, Pherez FM, Schoch P. Diagnostic Importance of Relative Lymphopenia as a Marker of Swine Influenza (H1N1) in Adults. Clinical Infectious Diseases. 2009; 49(9):1454–6. https://doi.org/10.1086/644496 PMID: 19824851

44. Giamarellos-Bourboulis EJ, Raftogiannis M, Antonopoulou A, Baziaka F, Koutoukas P, Savva A, et al. Effect of the Novel Influenza A (H1N1) Virus in the Human Immune System. PLOS ONE. 2009; 4(12):e8393. https://doi.org/10.1371/journal.pone.0008393 PMID: 20037642

45. Merekoulias G, Alexopoulos EC, Belezos T, Panagiotopoulou E, Jelastopulu DME. Lymphocyte to monocyte ratio as a screening tool for influenza. PLoS currents. 2010; 2:RRN1154–RRN. https://doi.org/10.1371/currents.RRN1154 PMID: 20383263.

46. Coşkun Ö, Avci IY, Sener K, Yaman H, Ogur R, Bodur H, et al. Relative lymphopenia and monocytosis may be considered as a surrogate marker of pandemic influenza a (H1N1). Journal of Clinical Virology. 2010; 47(4):388–9. https://doi.org/10.1016/j.jcv.2010.01.007 PMID: 20133186

47. Indavarapu A, Akinapelli A. Neutrophils to lymphocyte ratio as a screening tool for swine influenza. The Indian journal of medical research. 2011; 134(3):389–91. PMID: 21985824.

48. Shen H, Li B, Bai B, Hou J, Xu Z, Zhao M, et al. Laboratory features throughout the disease course of influenza A (H1N1) virus infection. Clin Lab. 2013; 59(3–4):337–42. PMID: 23724623.

49. Kurt-Jones EA, Mandell L, Whitney C, Padgett A, Gosselin K, Newburger PE, et al. Role of toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. Blood. 2002; 100(5):1860–8. PMID: 12176910.

50. Antal-Szalmas P, Strijp JA, Weersink AJ, Verhoef J, Van Kessel KP. Quantitation of surface CD14 on human monocytes and neutrophils. J Leukoc Biol. 1997; 61(6):721–8. https://doi.org/10.1002/jlb.61.6.721 PMID: 9201263.

51. Baumann CL, Aspalter IM, Sharif O, Pichlmair A, Blüml S, Grebien F, et al. CD14 is a coreceptor of Toll-like receptors 7 and 9. The Journal of Experimental Medicine. 2010; 207(12):2689–701. https://doi.org/10.1084/jem.20101111 PMID: 21078886

52. Fox A, Hoa LNM, Horby P, van Doorn HR, Trung NV, Ha NH, et al. Severe Pandemic H1N1 2009 Infection Is Associated with Transient NK and T Deficiency and Aberrant CD8 Responses. PLOS ONE. 2012; 7(2):e31535. https://doi.org/10.1371/journal.pone.0031535 PMID: 22363665

53. Chun J-K, Cha BH, Uh Y, Kim HY, Kim YK, Kwon W, et al. The Association of Lymphopenia with the Clinical Severity in the Korean Children Admitted to the Hospital with Pandemic (H1N1) 2009 Infection. Infect Chemother. 2011; 43(1):36–41.

54. de Freitas DN, Isaia HA, Henzel A, Simao E, Gassen RB, Rodrigues LC. Comparative study of lymphocytes from individuals that were vaccinated and unvaccinated against the pandemic 2009–2011 H1N1 influenza virus in Southern Brazil. Rev Soc Bras Med Tro. 2015; 48(5):514–23. https://doi.org/10.1590/0037-8682-0163-2015 PubMed PMID: WOS:000364431200003. PMID: 26516959

55. Wilkinson TM, Li CKF, Chui CSC, Huang AKY, Perkins M, Liebner JC, et al. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. Nature Medicine. 2012; 18:274. https://doi.org/10.1038/nm.2612 https://www.nature.com/articles/nm.2612#supplementary-information. PMID: 22286307

56. Zhou X, McElhaney JE. Age-related changes in memory and effector T cells responding to influenza A/H3N2 and pandemic A/H1N1 strains in humans. Vaccine. 2011; 29(11):2169–77. https://doi.org/10.1016/j.vaccine.2010.12.029 PMID: 21353149.

57. Zhao Y, Zhang Y-H, Denney L, Young D, Powell TJ, Peng Y-C, et al. High Levels of Virus-Specific CD4 + T Cells Predict Severe Pandemic Influenza A Virus Infection. American Journal of Respiratory and Critical Care Medicine. 2012; 186(12):1292–7. https://doi.org/10.1164/rccm.201207-1245OC PMID: 23087026.

58. Cheroutre H. Starting at the Beginning: New Perspectives on the Biology of Mucosal T Cells. 2004; 22 (1):217–46. https://doi.org/10.1146/annurev.immunol.22.012703.104522 PMID: 15032579.

59. Cawthon AG, Lu H, Alexander-Miller MA. Peptide Requirement for CTL Activation Reflects the Sensitivity to CD3 Engagement: Correlation with CD8αβ Versus CD8αα Expression. The Journal of Immunology. 2001; 167(5):2577–84. https://doi.org/10.4049/jimmunol.167.5.2577 PMID: 11509598

60. Van Oers NSC, Teh SJ, Garvin AM, Forbush KA, Perlmutter RM, Teh HS. CD8 Inhibits Signal Transduction through the T Cell Receptor in CD4-CD8- Thymocytes from T Cell Receptor Transgenic Mice Reconstituted with a Transgenic CD8α Molecule. Journal of Immunology. 1993; 151(2):777–90.

61. Denning TL, Granger S, Mucida D, Graddy R, Leclercq G, Zhang W, et al. Mouse TCRαβ+/CD8αα Intraepithelial Lymphocytes Express Genes That Down-Regulate Their Antigen Reactivity and Suppress Immune Responses. 2007; 178(7):4230–9. https://doi.org/10.4049/jimmunol.178.7.4230 PMID: 17371979

62. Cawthon AG, Alexander-Miller MA. Optimal Colocalization of TCR and CD8 as a Novel Mechanism for the Control of Functional Avidity. The Journal of Immunology. 2002; 169(7):3492–8. https://doi.org/10.4049/jimmunol.169.7.3492 PMID: 12244138

63. Walker LJ, Marrinan E, Muenchhoff M, Ferguson J, Kloverpris H, Cheroutre H, et al. CD8αα Expression Marks Terminally Differentiated Human CD8+ T Cells Expanded in Chronic Viral Infection. Frontiers in immunology. 2013; 4:223–. https://doi.org/10.3389/fimmu.2013.00223 PMID: 23964274.

64. Cheroutre H, Lambolez F. Doubting the TCR Coreceptor Function of CD8αα. Immunity. 2008; 28 (2):149–59. https://doi.org/10.1016/j.immuni.2008.01.005 PMID: 18275828

65. Konno A, Okada K, Mizuno K, Nishida M, Nagaoki S, Toma T, et al. CD8αα memory effector T cells descend directly from clonally expanded CD8α+βhigh TCRαβ T cells in vivo. 2002; 100(12):4090–7. https://doi.org/10.1182/blood-2002-04-1136 %J Blood

66. Roden AC, Morice WG, Hanson CA. Immunophenotypic Attributes of Benign Peripheral Blood γδ T Cells and Conditions Associated With Their Increase. Archives of Pathology & Laboratory Medicine. 2008; 132(11):1774–80. https://doi.org/10.1043/1543-2165-132.11.1774 PMID: 18976014.

67. Lahn M. The role of γδ T cells in the airways. Journal of Molecular Medicine. 2000; 78(8):409–25. https://doi.org/10.1007/s001090000123 PMID: 11097110

68. Lahn M, Kanehiro A, Takeda K, Joetham A, Schwarze J, Köhler G, et al. Negative regulation of airway responsiveness that is dependent on γδ T cells and independent of αβ T cells. Nature Medicine. 1999; 5:1150. https://doi.org/10.1038/13476 PMID: 10502818

69. Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan P-L, et al. Phosphoantigen-Expanded Human γδ T Cells Display Potent Cytotoxicity against Monocyte-Derived Macrophages Infected with Human and Avian Influenza Viruses. The Journal of Infectious Diseases. 2009; 200(6):858–65. https://doi.org/10.1086/605413 PMID: 19656068

70. Qin G, Liu Y, Zheng J, Ng IHY, Xiang Z, Lam K-T, et al. Type 1 responses of human Vγ9Vδ2 T cells to influenza A viruses. Journal of virology. 2011; 85(19):10109–16. https://doi.org/10.1128/JVI.05341-11 PMID: 21752902.

71. Piet B, de Bree GJ, Smids-Dierdorp BS, van der Loos CM, Remmerswaal EBM, von der Thüsen JH, et al. CD8+ T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung. The Journal of Clinical Investigation. 2011; 121(6):2254–63. https://doi.org/10.1172/JCI44675 PMID: 21537083

72. Hong MJ, Gu BH, Madison MC, Landers C, Tung HY, Kim M, et al. Protective role of γδ T cells in cigarette smoke and influenza infection. Mucosal Immunology. 2017; 11:894. https://doi.org/10.1038/mi.2017.93 https://www.nature.com/articles/mi201793#supplementary-information. PMID: 29091081

2.3 Publication III

Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain

Jane Hühr*, **Alexander Schäfer***, Theresa Schwaiger, Laura Zani, Julia Sehl, Thomas C. Mettenleiter, Sandra Blome, Ulrike Blohm
*, joint first authors

Transboundary and Emerging Diseases

doi: 10.1111/tbed.13678

June 2020

Received: 19 March 2020 | Revised: 4 June 2020 | Accepted: 5 June 2020

DOI: 10.1111/tbed.13678

ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain

Jane Hühr[1] | Alexander Schäfer[1] | Theresa Schwaiger[2] | Laura Zani[3] | Julia Sehl[4] | Thomas C. Mettenleiter[5] | Sandra Blome[6] | Ulrike Blohm[1]

[1]Institute of Immunology, Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany

[2]ViraTherapeutics GmbH, Innbruck, Austria

[3]Institute of Epidemiology, Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany

[4]Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[5]President, Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany

[6]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany

Correspondence
Ulrike Blohm, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, D - 17493 Greifswald – Insel Riems, Germany.
Email: Ulrike.Blohm@fli.de

Funding information
This work was funded by the Friedrich-Loeffler-Institut African Swine Fever Virus Research Network 'ASF-ResNet'.

Abstract

Since African swine fever (ASF) first appeared in the Caucasus region in 2007, it has spread rapidly and is now present in numerous European and Asian countries. In Europe, mainly wild boar populations are affected and pose a risk for domestic pigs. In Asia, domestic pigs are almost exclusively affected. An effective and safe vaccine is not available, and correlates of protection are far from being understood. Therefore, research on immune responses, immune dysfunction and pathogenesis is mandatory. It is acknowledged that T cells play a pivotal role. Thus, we investigated T-cell responses of domestic pigs and wild boar upon infection with the highly virulent ASF virus (ASFV) strain 'Armenia08'. For this purpose, we used a flow cytometry-based multicolour analysis to identify T-cell subtypes (cytotoxic T cells, T-helper cells, γδ T cells) and their functional impairment in ASFV-infected pigs. Domestic pigs showed lymphopaenia, and neither in the blood nor in the lymphoid organs was a proliferation of $CD8^+$ effector cells observed. Furthermore, a T-bet-dependent activation of the remaining CD8 T cells did not occur. In contrast, a T-cell response could be observed in wild boar at 5 days post-inoculation in the blood and in tendency also in some organs. However, this cytotoxic response was not beneficial as all wild boars showed a severe acute lethal disease and a higher proportion died spontaneously or was euthanized at the humane endpoint.

KEYWORDS

African swine fever virus, domestic pig, experimental infection, T-cell response, wild boar

1 | INTRODUCTION

African swine fever (ASF) is one of the most devastating diseases of domestic pigs and wild boar with lethality rates up to 100% (Kleiboeker, 2002; Penrith & Vosloo, 2009). The ASF virus (ASFV), a large double-stranded DNA virus, is the only known member of the *Asfarviridae* family (Alonso et al., 2018; Dixon et al., 2005). Since its onset in the Caucasus and Russia in 2007, ASF has spread widely and now affects several European countries. In autumn 2018, the virus also reached China, the world's largest pig producer, and spread subsequently to several Asian countries (Dixon, Sun, & Roberts, 2019). Within Europe, the abundant wild boar population plays an important role in the maintenance of ASFV. Wild boars serve as a reservoir for ASFV and present a risk factor for the

Jane Hühr and Alexander Schäfer should be considered joint first author.

introduction into the domestic pig population (Jori & Bastos, 2009; Pietschmann et al., 2016).

To date, no effective vaccine is available. Moreover, previous studies demonstrated dysregulated immune responses, which are still poorly understood. ASFV encodes several factors that mediate immune modulation and evasion (Dixon, Islam, Nash, & Reis, 2019). Multiple genes in the ASFV genome encode for type I interferon and apoptosis inhibitors during early stages of infection (Dixon, Islam, et al., 2019). However, infected macrophages might also induce TNF-α-mediated apoptosis in bystander lymphocytes during later stages (Dixon, Islam, et al., 2019).

Professional antigen-presenting cells (APCs), such as macrophages and dendritic cells, are the main targets for ASFV (Franzoni, Dei Giudici, & Oggiano, 2018). Consequently, the ability of APCs to present antigen could be disturbed and the induction of an effective immune response might be impaired (Franzoni, Graham, et al., 2018; Galindo & Alonso, 2017; Sanchez-Torres et al., 2003). Additionally, reduction in lymphocyte counts because of virus-mediated apoptosis is a well-known characteristic during ASFV infections (Dixon, Sanchez-Cordon, Galindo, & Alonso, 2017; Oura, Powell, Anderson, & Parkhouse, 1998; Ramiro-Ibanez, Ortega, Ruiz-Gonzalvo, Escribano, & Alonso, 1997). Besides, little is known about the mechanisms of the immune response against ASFV. Cytokines and chemokines are important factors orchestrating the immune system's response against viral infections. For ASF, an involvement of cytokines (TNF-α, IFN-α, IL-12, IL-1β) in immunopathology, especially tissue destruction, is described (Lacasta et al., 2015). Additionally, some authors found a correlation of protection against ASFV infections with an increase in IFN-γ, although the cellular source remained unknown (King et al., 2011; Sanchez-Cordon, Montoya, Reis, & Dixon, 2018). However, other authors could not confirm the correlation of these responses (Carlson et al., 2016). The cytokine profile of infected macrophages differs depending on the ASFV strain virulence (Franzoni et al., 2017). Unfortunately, the number of in vivo studies on ASFV infection, which could further investigate those questions, is limited (King et al., 2003; O'Donnell et al., 2015; Reis et al., 2016). Whether antibodies generated against ASFV proteins are neutralizing remains a matter of controversy. It was demonstrated that antibodies alone cannot protect from ASFV. Moreover, they are not predictive for disease outcome (Escribano, Galindo, & Alonso, 2013; Neilan et al., 2004; Onisk et al., 1994; Schlafer, Mebus, & McVicar, 1984; Zsak, Onisk, Afonso, & Rock, 1993).

T cells—especially $CD8\alpha^+$ T cells—play an important role in protective immunity against ASFV (Oura, Denyer, Takamatsu, & Parkhouse, 2005). After exposure to the avirulent ASFV strain OUR/T88/3, pigs were depleted of $CD8\alpha^+$ lymphocytes with monoclonal anti-CD8 antibodies. A subsequent challenge with homologous but virulent OUR/T88/1 revealed that depleted animals suffered from severe acute ASF and died, whereas undepleted animals showed only mild clinical signs and survived. Since CD8α is expressed on different T-cell populations such as cytotoxic T cells (CTL), γδ T cells, NK cells, invariant T cells or memory helper T cells (Saalmüller, Pauly, Hohlich, & Pfaff, 1999; Schäfer et al., 2019; Yang & Parkhouse, 1997), it remains unclear which of these subpopulations might mediate the described protection. Most of the findings on the immune response against ASFV were obtained from in vitro studies, often limited to the blood of infected animals. As antigen contact, antigen presentation and T-cell activation take place in lymphoid organs, investigation of these lymphoid tissues is indispensable. Furthermore, only little is known about the kinetics of the immune response, neither in blood nor in lymphoid organs, and whether T cells other than cytotoxic $CD8^+$ cells are involved in the immune response. Finally, it remains to be elucidated whether the disease is due to a failure to mount protective immunity or even displays an immunopathologic process. These gaps in knowledge highlight the necessity to understand ASFV modulation of immune responses, especially since increased efforts to develop new vaccine technologies still have not been successful. The aim of this study was to investigate the significance of the cellular immune response in more detail, in order to clarify which cell components are affected during ASFV infection. Therefore, we have chosen a comparative approach in which we inoculated domestic pigs and wild boar with the highly virulent ASFV 'Armenia08' in order to investigate the phenotype of different T-cell populations in blood and lymphatic organs as well as their kinetics and functionality in the course of the infection.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The study included 16 German landrace pigs of both sexes aged three months at the start of the trial and 16 subadult/adult European wild boars aged 1–2 years. All wild boars were clinically unremarkable and were treated with anti-parasitics. Domestic pigs were obtained from a commercial pig farm, and the wild boars were provided by wildlife parks in Mecklenburg-Western Pomerania. For the experiment, the animals were transferred into the high containment facilities of the Friedrich-Loeffler-Institut (L3+). After one week of acclimatization, 12 domestic pigs and 12 wild boars were oronasally inoculated with 2 ml cell culture supernatant containing $10^{6.25}$ haemadsorbing units (HAU)/ml of ASFV 'Armenia08'. Four domestic pigs and wild boars each were separated prior to infection, remained untreated and served as controls.

Clinical signs of domestic pigs and wild boars were recorded daily over the course of the trial. Rectal temperatures were only measured in domestic pigs in order to avoid frequent immobilization of wild boars. Fever was defined as a body temperature over 40°C for at least two consecutive days. Based on the adapted scoring system by Mittelholzer *et al.* (Mittelholzer, Moser, Tratschin, & Hofmann, 2000), the clinical score was evaluated as previously described (Petrov, Forth, Zani, Beer, & Blome, 2018). It comprised the parameters liveliness, bearing, breathing, gait, skin, eyes, faeces and feed uptake, reaching from 0 (asymptomatic) to 3 points each

(severe). The sum of the points was recorded as a clinical score. Fifteen score points were defined as humane endpoint. Five and 7 days post-infection (dpi), four domestic pigs and four wild boars were euthanized by intracardial injection of pentobarbital (Release, Wirtschaftsgenossenschaft deutscher Tierärzte) after deep anaesthesia with tiletamine/zolazepam (Zoletil ®, Virbac) and xylazine (Rompun® 2%, Bayer HealthCare). Necropsy was performed on all animals. For immunological investigations and to assess levels of viraemia, blood, lymphoid tissue from spleen, gastro-hepatic lymph nodes (ghLN, *Lymphonodi hepatici* or *gastrici*) and liver were collected.

2.2 | Virus detection

For virus back titration of the culture supernatant used for infection, the haemadsorption test (HAT) was performed by endpoint titration on macrophages derived from peripheral blood monocytic cells of healthy donor pigs as previously described (Pietschmann et al., 2015).

All tissue samples were homogenized using a TissueLyser II (Qiagen) at 30 Hz for 3 min in a 2-ml reaction tube in the presence of one 5-mm stainless steel bead and 300 µl sterile PBS. For qPCR, viral DNA was extracted from tissue samples using the NucleoMag VET Kit (Macherey-Nagel) and KingFisher® extraction platform (Thermo Scientific). The qPCR analysis was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) according to the assay previously described (King et al., 2003), with slight modifications (addition of a heterologous internal control). Genome copy numbers were estimated using an in-house standard. Standard was prepared as followed: A culture of peripheral blood mononuclear cell (PBMC)-derived macrophages was infected with ASFV strain 'Armenia08' and incubated at 37°C in a humidified atmosphere containing 5% CO2 for 72 hr. Following a freeze–thaw cycle, the cell culture supernatant was collected and cleared by centrifugation at 650 x g for 10 min. Viral nucleic acids were extracted from the supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Subsequently, the DNA concentration was determined by spectrophotometry using a NanoDrop 2000c (Thermo Fisher Scientific, Schwerte, Germany) and the exact number of DNA molecules was calculated using open-access online tools (http://molbiol.edu.ru/eng/scripts/01_07.html).

2.3 | Haematological analyses

Differential leucocyte counts were determined from fresh EDTA blood samples using an automated haematological analyzer (ProCyte Dx Hematology Analyzer, IDEXX). The data were used to calculate the total number of lymphocyte subpopulations using the frequencies obtained by flow cytometry.

2.4 | Preparation of single-cell suspensions

Single-cell suspensions from spleen and ghLN were generated by mechanically disrupting tissue with a steel strainer. Lymphocytes from liver tissue were isolated after perfusion with ice-cold PBS-EDTA. Subsequently, perfused regions were minced and resuspended in PBS-EDTA supplemented with 100 µM $CaCl_2$ and digested with collagenase D (1 mg/ml; Sigma-Aldrich) for 40 min at 37°C. The remaining tissue was removed via short centrifugation, and the cell pellet was resuspended in PBS-EDTA and used for flow cytometry.

2.5 | Flow cytometry

At indicated time points, 50 µl whole blood and 50 µl single-cell suspensions (corresponding to approximately 1×10^6 cells) of spleen, ghLN and liver were stained for flow cytometric analyses. All incubation steps with monoclonal antibodies (mAbs) targeting extracellular antigens were carried out for 15 min at 4°C in the dark. Between each antibody staining, a washing step was performed. Before intracellular labelling, erythrocytes in blood samples were lysed with red blood cell lysis buffer (1.55 M NH_4Cl, 100 mM $KHCO_3$, 12.7 mM Na_4EDTA, pH 7.4, in *Aqua destillata*). Subsequently, samples were fixed and permeabilized with the True-Nuclear Transcription Factor Buffer Set (BioLegend, USA) according to the manufacturer's instructions. All incubation steps for intracellular staining were carried out for 30 min at 4°C in the dark. Antibodies and conjugates used for flow cytometry are shown in Table 1. Appropriate controls (isotype and fluorescence minus one, FMO) were used throughout the establishment of the stainings. Anti-pig antibodies were used for wild boar as well, because both subspecies (wild boar, *Sus scrofa scrofa*, and domestic pig, *Sus scrofa domesticus*) exhibit high genetic consensus (Groenen et al., 2012). Moreover, anti-pig antibodies have successfully been used by other groups (Beltran-Beck et al., 2014; Cabezon et al., 2017). Flow Cytometer BD FACS Canto II with FACS DIVA Software (BD Bioscience) was used for all analyses. Living lymphocytes were gated based on their forward-scatter (FSC) and side-scatter (SSC) properties and further separated into different T-cell subpopulations. At least 10,000 live, single lymphocytes were acquired.

2.6 | Statistical analysis

GraphPad Prism7 (GraphPad Software Inc.) was used for statistical analysis and graph creation. Normality was verified using the Shapiro–Wilk test. To investigate statistically significant differences between infected and uninfected animals, ordinary one-way ANOVA with Dunnett's correction for multiple comparisons was used. Since both species displayed different cell frequencies even before the infection, differences between both species during the study period

TABLE 1 Antibodies used in this study

Marker	Clone	Isotype	Conjugate	Source	Dilution
CD2	MSA4	Mouse IgG2a	—	In-house	1:100
CD3ε	PPT3	Mouse IgG1	PE	Southern Biotech	1:500
CD3ε	PPT3	Mouse IgG1	—	In-house	
CD4a	74-12-4	Mouse IgG2b	PerCp-Cy5.5	BD	1:100
CD8a	76-2-11	Mouse IgG2a	FITC	Southern Biotech	1:100
CD8b	PG164A	Mouse IgG2a	—	In-house	1:1,000
CD79a	HM47	Mouse IgG1	PE	ebioscience	1:200
CD172a	74-22-15	Mouse IgG1	—	In-house	1:100
EOMES	WD1928	Mouse IgG1	PE	Invitrogen	1:50
Foxp3	236A/E7	Mouse IgG1	Biotin	ebioscience	1:200
gdTCR	PPT16	Mouse IgG2b	—	In-house	1:100
Ki-67	B56	Mouse IgG1	BV421	BD Biosciences	1:40
Perforin	dG9	Mouse IgG2b	Alexa 647	BioLegend	1:20
T-bet	4B10	Mouse IgG1	APC	BioLegend	1:500
IgG1	RMG1–1	Rat IgG	APC-Cy7	BioLegend	1:250
IgG1	RMG1–1	Rat IgG	BV421	BioLegend	1:400
IgG2a	Polyclonal	Goat IgG	APC-Cy7	Southern Biotech	1:250
IgG2a	Polyclonal	Goat IgG	APC	Jackson Immuno	1:1,000
IgG2b	Polyclonal	Goat IgG	PE-Cy7	Southern Biotech	1:400
Streptavidin	—	—	BV510	BioLegend	1:1,000

were not tested. Survival was analysed using the Mantel–Cox test. Each dot represents one animal with a bar indicating mean. Statistical significance was defined as $p < .05$ (*).

3 | RESULTS

3.1 | Clinical course

Back titration of the virus suspension used for inoculation verified the administered titre of $1 \times 10^{6.25}$ HAU/ml (data not shown). All tissue samples from inoculated domestic pigs and wild boars were positive for ASFV genome in qPCR. Subsequent to oronasal inoculation, domestic pigs developed severe clinical signs. Parallel to the onset of fever 5 dpi, loss of appetite, general depression and huddling were observed. Haemorrhages at the tips of the ears and lower legs of domestic pigs as well as conjunctivitis occurred in the final phase of the experiment. Similar signs of disease were observed in infected wild boars (depression, loss of appetite). Comparison of clinical signs among domestic pigs and wild boars was hampered by the fact that wild boar conceal disease by minimized sickness behaviour more than domestic pigs (Tizard, 2008). Moreover, assessment of clinical signs, that is fever, in wild boar, would have required to narcotize the animals. Such severe manipulations would have distorted the overall outcome and were, therefore, omitted. It has to be taken into account that survival kinetics alone do not represent the clinical course in the animals, especially because 4 animals each were randomly selected for necropsy 5 dpi and 7 dpi. Given the small group size, it cannot be ruled out that some of the animals euthanized for necropsy would have survived the infection. Still, early lethality indicated a severe disease course in wild boars. One wild boar died spontaneously overnight from 5 dpi to 6 dpi, two more from 6 dpi to 7 dpi. Of the domestic pigs, three were euthanized at the humane endpoint 8 dpi. Overall, there was no significant difference in the survival of domestic pigs and wild boar (data not shown). Due to the progressing clinical signs and the associated insufficient quality of the blood and tissue sample material, it was not possible to analyse all samples at every scheduled time. Nevertheless, data from remaining animals were integrated into the study to show trends. During necropsy, typical ASF lesions were observed in all animals, with increasing severity from 5 dpi to 7 dpi (data not shown). Lesions were highly variable and included cutaneous reddening, haemorrhages in lymph nodes, diffuse renal cortical and medullar haemorrhages or petechiae in kidneys, splenomegaly, lung oedema and gastritis. The remaining animals were kept to investigate later time points but had to be euthanized 8 dpi and were thus not included in this study.

3.2 | T-cell responses in peripheral blood

Because ASF is often associated with lymphopaenia and leucocyte apoptosis, we investigated which cell populations are directly affected and whether there are differences between domestic pigs and wild boars. In domestic pigs, the total number of leucocytes did not change during infection. However, a decrease in $CD3^+$ (T cells)

and CD79a$^+$ (B cells) lymphocytes 5 dpi and an increase in monocytes 7 dpi were observed (Figure 1 upper panel). In contrast to previous studies in domestic pigs (Dixon et al., 2017; Oura et al., 1998; Ramiro-Ibanez et al., 1997), we did not detect a decrease in lymphocyte counts in wild boars. Lymphocyte counts increased temporarily at 5 dpi, while total leucocytes and monocytes increased over the study period (Figure 1 lower panel). In line with higher lymphocyte counts, T and B cells of wild boars increased on 5 dpi in contrast to domestic pigs (Figure 1 lower panel). Besides, we detected no changes in the numbers of circulating granulocytes in domestic pigs (Figure 1 upper panel) but granulocytosis in wild boar (Figure 1 lower panel).

Studying the main subpopulations of T cells (αβ T cells and γδ T cells) revealed a tendency to decrease in total number of αβ T cells (CD3$^+$γδTCR$^-$) of domestic pigs (Figure 2 left panels). In wild boars, however, αβ T cells were temporarily increased at 5 dpi. The total amount of γδ T cells (CD3$^+$γδTCR$^+$) decreased in both, domestic pigs and wild boars, during infection (Figure 2 right panels). The αβ T cells were further divided into three subsets, CD4$^+$ helper T cells (CD3$^+$CD4$^+$CD8$^-$), double-positive (DP) T cells (CD3$^+$CD4$^+$CD8$^+$) and CD8$^+$ effector T cells (CD3$^+$CD4$^-$CD8$^+$). Analysis of these αβ T-cell subpopulations showed that the total number of CD4$^+$ T cells of domestic pigs decreased. In wild boars, on the other hand, first an increase at 5 dpi with a subsequent decrease at 7 dpi, even below their initial level, was observed. This reaction has to be viewed with caution as at 7 dpi only one animal could be investigated. The total number of DP T cells increased significantly in domestic pigs but remained unchanged in wild boars during the experiment. Numbers of CD8$^+$ effector T cells decreased in domestic pigs but displayed a temporary increase at 5 dpi in wild

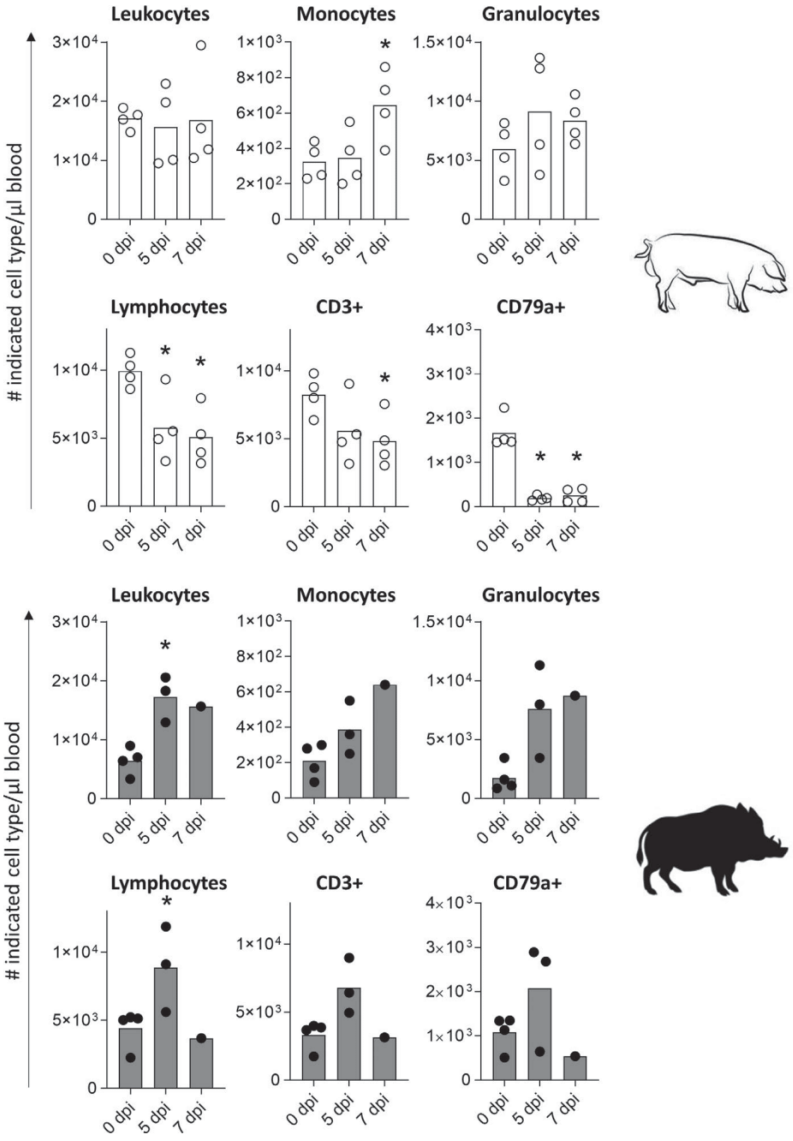


FIGURE 1 T cells in blood of domestic pigs decreased, while in wild boars increasing numbers of T cells occurred. Total number of leucocytes, lymphocytes, subdivided into T cells (CD3$^+$) and B cells (CD79a$^+$), monocytes and granulocytes from blood of domestic pigs (upper panel, open circles, white bars) and wild boars (lower panel, closed circles, grey bars) upon infection with ASFV 'Armenia08'. At indicated time points, pigs were euthanized and blood was collected. Blood counts were determined by using the IDEXX ProCyte Dx Haematology Analyzer. To calculate the total number of leucocyte subpopulations, the flow cytometry data were compared to data obtained from the blood counting device. Each point represents the value of a single pig, while the horizontal lines represent the means for the designated time points. *$p < .05$

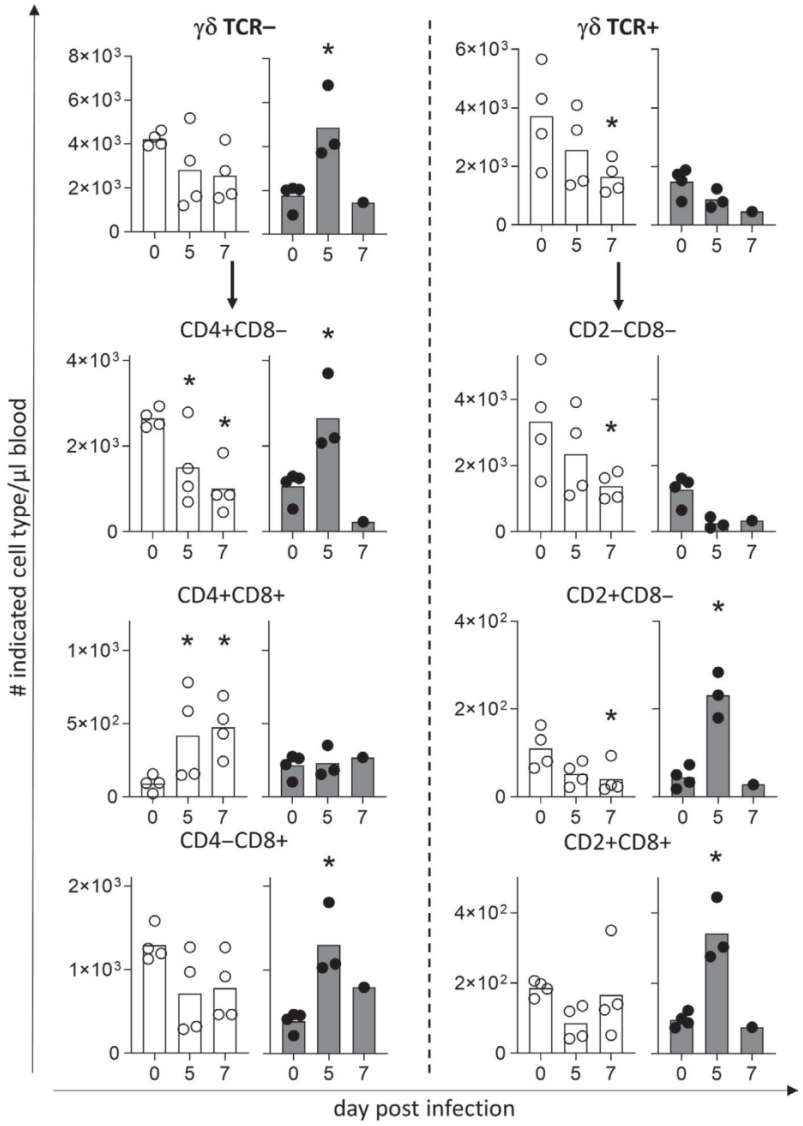


FIGURE 2 Distribution and phenotype of T-cell subsets in blood were different between domestic pigs and wild boars. Distribution and phenotype of different T-cell subsets from domestic pigs (open circles, white bars) and wild boars (closed circles, grey bars) upon ASFV 'Armenia08' infection. T cells from blood (Figure 2), ghLN (Figure 3), spleen (Figure 4) and liver (Figure 5) were analysed by flow cytometry. At indicated time points, pigs were euthanized and lymphocytes of indicated tissues were isolated. Lymphocytes were gated on $CD3^+$ T cells and grouped into γδ TCR^- (αβ T cells, left panels) and γδ TCR^+ (γδ T cells, right panels) T cells. αβ T cells were subdivided into T-helper cells ($CD4^+CD8^-$), double-positive cells ($CD4^+CD8^+$) and effector T cells ($CD4^-CD8^+$). γδ T cells were subdivided into naïve ($CD2^-CD8^-$), activated ($CD2^+CD8^-$) and effector ($CD2^+CD8^+$) cells. Each point represents the value of a single pig, while the horizontal lines represent the means for the designated time points. *$p < .05$

boars. In addition, all γδ T-cell subpopulations, namely naive ($CD2^-$ $CD8^-$), activated ($CD2^+CD8^-$) and effector ($CD2^+CD8^+$) γδ T cells of domestic pigs, decreased during infection, although effector γδ T cells recovered to pre-infection levels 7 dpi. This was in contrast to the increasing numbers of activated and effector γδ T cells in wild boars 5 dpi (Figure 2).

3.3 | Distribution and phenotype of T-cell subsets in lymphoid organs

To investigate the complex cellular immune response to ASFV, we also analysed phenotype and activation status of T cells as well as their distribution in lymphoid organs. We focused on spleen and regionally affected lymph nodes, ghLN, because they are well described as pathomorphologically conspicuous, as well as the liver, an organ with importance in innate and adaptive immunity.

With regard to αβ T cells of domestic pigs, total frequencies of αβ T cells in ghLN (Figure 3) and spleen remained constant (Figure 4). In the liver, a slight decrease in total frequency was detected over the course of infection on 7 dpi (Figure 5). In wild boars, a decrease in frequency of αβ T cells in ghLN was observed (Figure 3). In spleen (Figure 4) of wild boars, αβ T cells increased in frequency from 5 dpi, while they only peaked 5 dpi but returned to baseline at 7 dpi in the liver (Figure 5).

The distribution of the three aforementioned αβ T-cell subsets was analysed more closely. In domestic pigs, frequencies of $CD4^+$ T cells decreased 7 dpi in ghLN (Figure 3) and in spleen from 5 dpi on (Figure 4). In the liver, we observed an increase in the frequency of $CD4^+$ T cells 5 dpi, but 7 dpi the frequencies decreased (Figure 5).

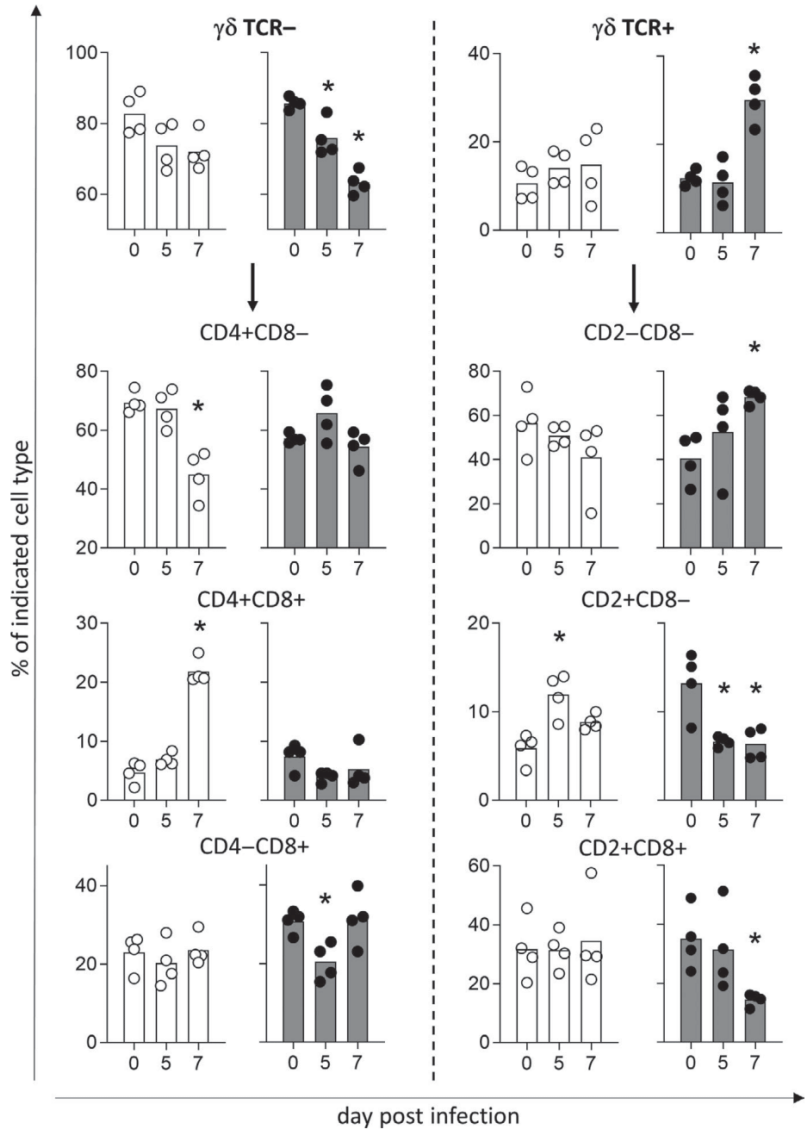


FIGURE 3 Distribution and phenotype of T-cell subsets in ghLN were different between domestic pigs and wild boar. For details, see figure legend of Figure 2

Frequencies of DP T cells in all investigated tissues of domestic pigs inversely mirrored $CD4^+$ T-cell frequencies, with an increase in all investigated organs. The percentage of $CD8^+$ T cells of domestic pigs did not change over time in ghLN (Figure 3) and spleen (Figure 4), while in the liver this subpopulation slightly dropped 5 dpi and normalized again 7 dpi (Figure 5). In wild boars, we detected a temporary decrease in $CD8^+$ T cells 5 dpi in ghLN (Figure 3), a decrease in $CD4^+$ T cells with a corresponding increase in $CD8^+$ T cells 7 dpi in the spleen (Figure 4) and heightened frequencies of $CD4^+$ T cells with a loss of DP T cells 5 dpi in the liver (Figure 5).

Furthermore, total frequencies of γδ T cells in ghLN, spleen and liver of domestic pigs remained unchanged during infection (Figures 3–5); additionally, only minor changes in frequencies of subpopulations of γδ T cells were detected. In ghLN, we detected an increase in activated γδ T cells. In liver and spleen, frequencies of effector γδ T cells tended to increase, with a corresponding loss of activated γδ T cells. In wild boars, total frequencies of γδ T cells were rather the opposite of αβ T cells: they increased in ghLN 7 dpi (Figure 3), whereas in spleen they decreased over time (Figure 4). Of note, increasing frequencies of γδ T cells in ghLN were mainly due to increasing frequencies of naïve γδ T cells. However, in the spleen, effector γδ T cells accumulated over the course of infection. In the liver, frequencies of γδ T-cell subpopulations did not change over time.

3.4 | Proliferative activity of T cells in lymphoid organs

To clarify whether the increasing frequencies in subpopulations of αβ T cells and γδ T cells were caused by infiltration

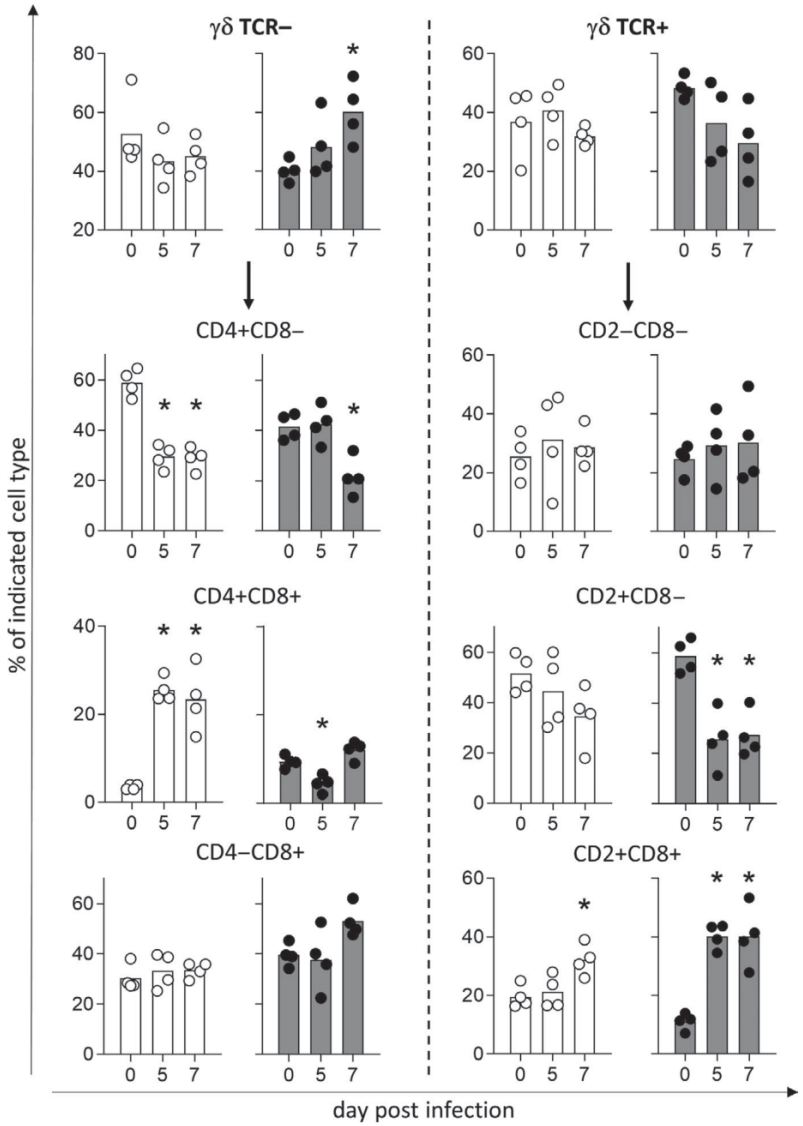


FIGURE 4 Distribution and phenotype of T-cell subsets in spleen were different between domestic pigs and wild boar. For details, see figure legend of Figure 2

or proliferation, we investigated the proliferative activity by Ki-67 detection of freshly isolated lymphocytes (Figure 6). During the course of infection, there were no changes in the expression of Ki-67 in αβ T cells in all investigated tissues of domestic pigs.

In contrast, DP T cells of wild boars in ghLN as well as in the spleen and liver showed an increase from 10% to more than 70% in Ki-67 expressing DP T cells at 5 dpi. Elevated frequencies of Ki-67 expressing DP T cells remained in ghLN and liver, while they declined to basal levels in the spleen. Moreover, we detected slightly increased frequencies of $CD4^+$ and $CD8\alpha\beta^+$ T cells exclusively in the liver of infected wild boars over the course of infection. Additionally, proliferating γδ T cells were only observed in ghLN and the liver of wild boars 5 dpi.

3.5 | Cytotoxic T-cell responses in lymphoid organs

We further analysed the expression of perforin as an effector mechanism of T cells. To determine whether there is any correlation between proliferative activity and expression of effector molecules, perforin in $CD8^+$ and DP αβ T cells and γδ T-cell populations in ghLN, spleen and liver were analysed (Figure 7). We detected a reduced perforin expression in T cells of domestic pigs 5 dpi in almost all investigated tissues; only $CD8\alpha\alpha^+$ T cells in spleen increased perforin expression over time. Especially, $CD8\alpha\beta^+$ T cells displayed a nearly complete loss of perforin in all tissues at 5 dpi, recovering at 7 dpi. The consumption of perforin at 5 dpi was also seen in wild boars; however, it was less pronounced with 50% of $CD8\alpha\beta^+$ T cells still expressing perforin. The loss of perforin expression was also detected

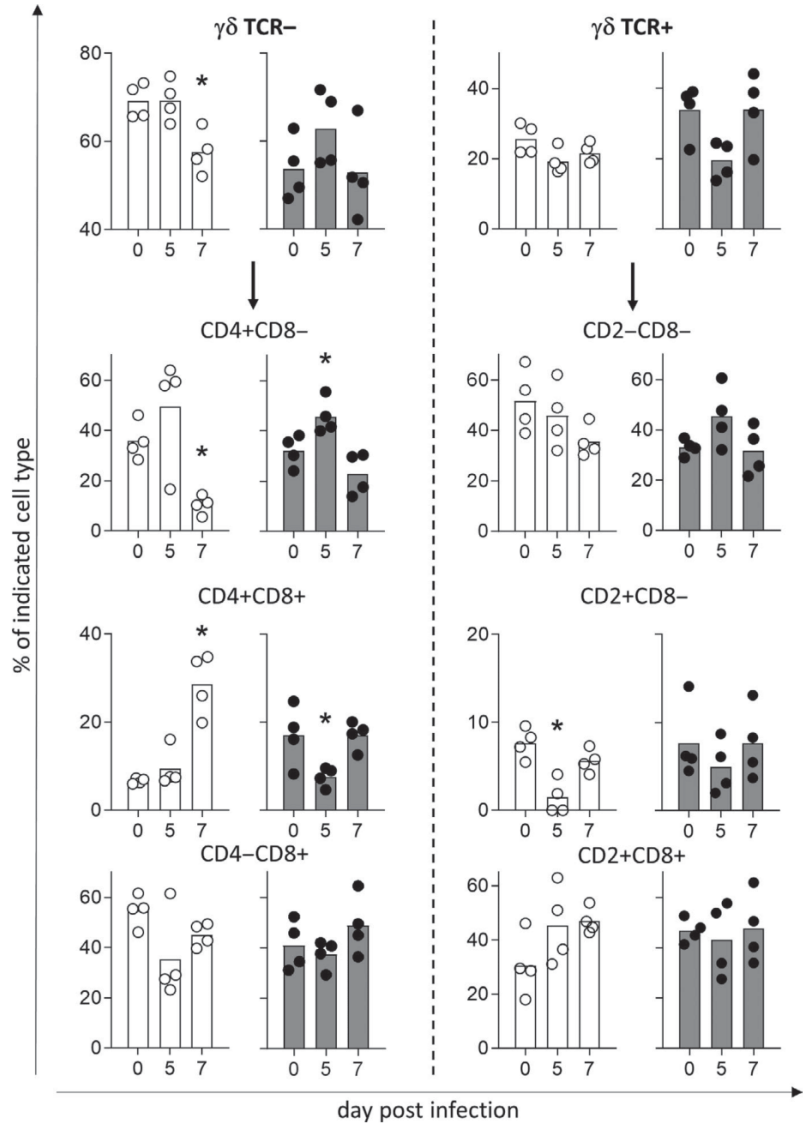


FIGURE 5 Distribution and phenotype of T-cell subsets in liver were different between domestic pigs and wild boar. For details, see figure legend of Figure 2

in DP T cells of domestic pigs, while DP T cells of wild boars in contrast increased perforin expression. The same was essentially true for $CD8^+$ γδ T cells.

3.6 | T-helper cell differentiation in lymphoid organs

The expression of T-bet in $CD8^+$ and $CD4^+$ T cells was investigated as a marker for activation.

T-bet expression of $CD8^+$ T cells (Figure 8a) from blood in domestic pigs decreased at 5 dpi but returned to basal expression levels at 7 dpi. In spleen and ghLN, no changes in the frequency of T-bet-expressing $CD8^+$ T cells were detectable. In contrast, in wild boars, the frequency of T-bet-expressing $CD8^+$ T cells increased especially in the blood over the course of infection, although to a lesser extent than in domestic pigs. T-bet expression of $CD4^+$ T cells ($CD3^+CD8^-$, Figure 8b) increased significantly 7 dpi in peripheral blood, ghLN and spleen of domestic pigs, whereas T-bet expression in $CD4^+$ T cells of wild boar was hardly detectable at any time point during the study period.

3.7 | Regulatory T-cell responses in blood and lymphoid organs

Further, the expression of FoxP3, as a marker for regulatory T cells (Käser, Gerner, & Saalmüller, 2011), was investigated to determine the induction of a regulatory immune response (Figure 9). High numbers of $FoxP3^+$ T cells in domestic pigs occurred pre-dominantly in blood and spleen, and to a lesser extent in ghLN at 7 dpi. In wild boars, regulatory T cells appeared at 5 dpi, again primarily in blood and spleen.

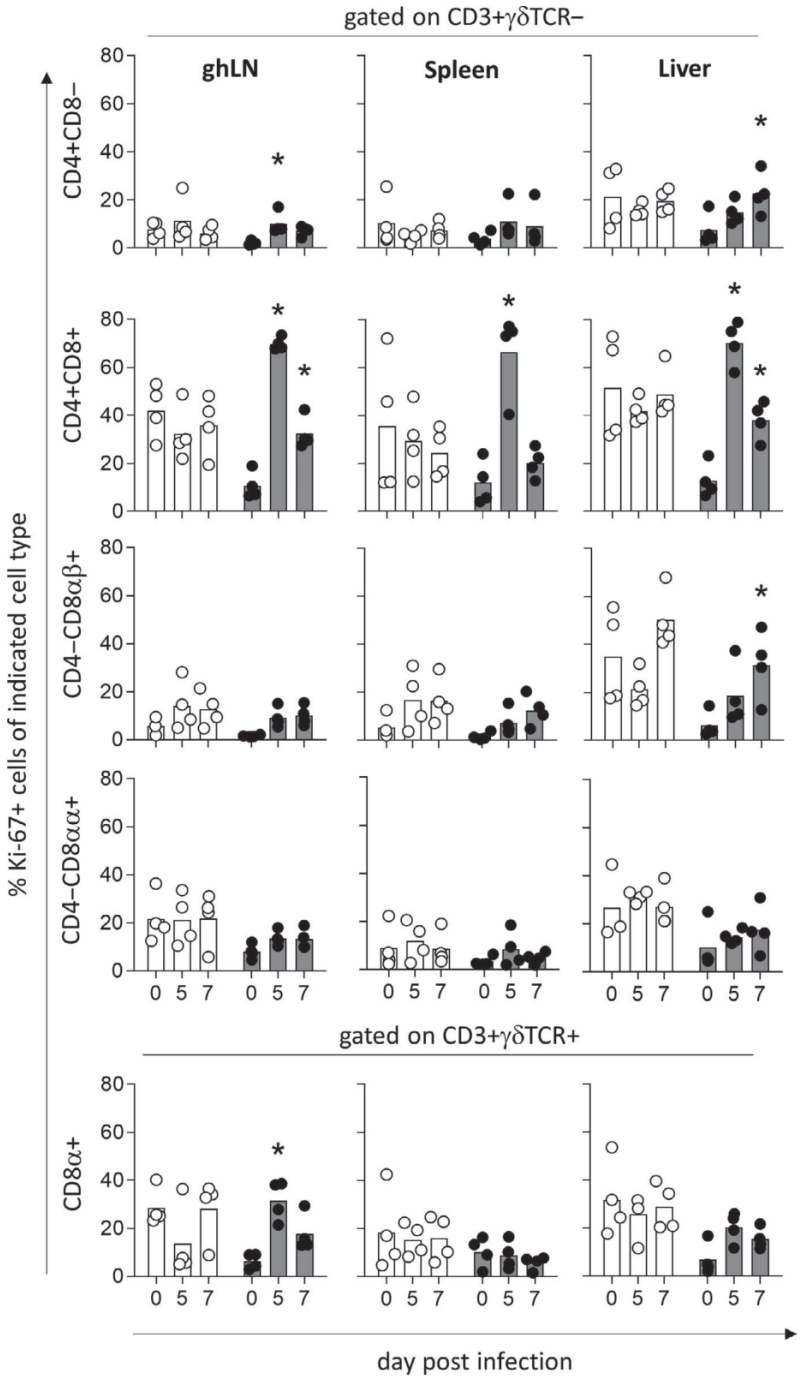


FIGURE 6 Proliferative activity of T cells from domestic pigs appeared to be impaired in contrast to T cells from wild boars. Proliferative activity of different T-cell subsets was measured by Ki-67 expression in ghLN, spleen and liver. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. Domestic pigs (open circles, white bars) and wild boars (closed circles, grey bars). *$p < .05$

4 | DISCUSSION

The major focus of our study was to characterize the T-cell-mediated responses in ASFV-infected domestic pigs compared with wild boars. CD8α$^+$ T cells, consisting of cytotoxic T cells (CTL), γδ T cells, NK cells, invariant T cells or memory helper T cells (Saalmüller et al., 1999; Schäfer et al., 2019; Yang & Parkhouse, 1997), have been shown to be of pivotal importance for anti-viral immunity after priming with homologous but attenuated ASFV strains (Oura et al., 2005). Furthermore, macrophages are described to be the main target cells and replication site of ASFV (Franzoni, Dei Giudici, et al., 2018; Gomez-Villamandos, Bautista, Sanchez-Cordon, & Carrasco, 2013). Therefore, we hypothesized that during infections with virulent ASFV strains, T-cell

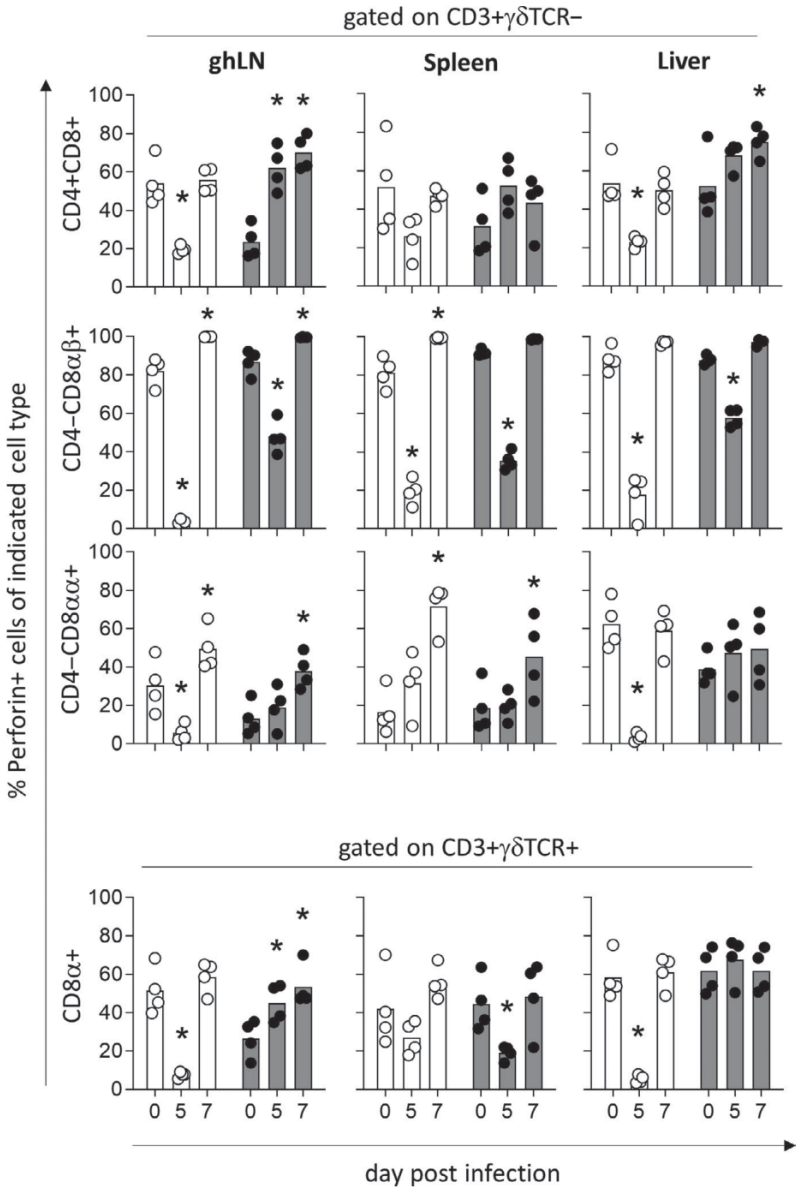


FIGURE 7 ASFV led to an impaired perforin expression of T cells in domestic pigs, while wild boar T cells appeared to counteract. Cytotoxic activity of different T-cell subsets was measured by the expression of perforin in ghLN, spleen and liver at indicated time points. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. Domestic pigs (open circles, white bars), wild boars (closed circles, grey bars). *$p < .05$

responses might be impaired due to insufficient antigen presentation of infected macrophages.

During an acute viral infection, MHC class I-restricted antigen presentation leads to T-box transcription factor TBX21 (T-bet)-dependent differentiation of naïve $CD8^+$ T cells into CTL. This activation is characterized by cell proliferation, expression of pro-inflammatory cytokines (e.g. IFN-γ or IL-2) and upregulation of effector molecules such as perforin and granzyme B (Barry & Bleackley, 2002; Cobb et al., 2009; Intlekofer et al., 2005; Pearce et al., 2003). Our results show that this protective immune response does not occur in virulent ASFV infection. However, we detected an enormous decrease in perforin expression in $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells, pre-dominantly in domestic pigs.

There are various possible explanations for the observed loss of perforin expression. The transforming growth factor (TGF)-β induces regulatory T-cell (Treg) responses (Chen et al., 2003) but has also been shown to reduce perforin expression in cytotoxic cells (Thomas & Massague, 2005). Moreover, TGF-β impairs proliferation of antigen-reactive lymphocytes by cell cycle arrest (Tiemessen et al., 2003). This is in line with our findings, but the underlying mechanisms require further investigation. Release of pro-inflammatory cytokines might result in a polyclonal activation, which ultimately leads to loss of perforin due to consumption and T-cell exhaustion. Exhausted T cells are usually thought to occur after multiple antigen encounters during chronic infections (Kurachi, 2019), but T cells with an exhaustion-like phenotype have been found during

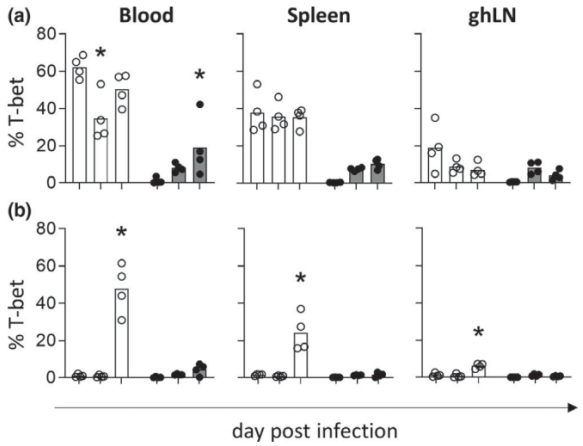


FIGURE 8 CD8$^+$ T cells downregulated T-bet expression and CD4$^+$ T cells differentiated in Th1 cells in domestic pigs but not wild boars. Expression of T-bet in (a) CD8$^+$ and (b) CD8- T cells of blood, spleen and ghLN of domestic pigs (open circles, white bars) and wild boar (closed circles, grey bars) is shown for indicated time points. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. *p < .05

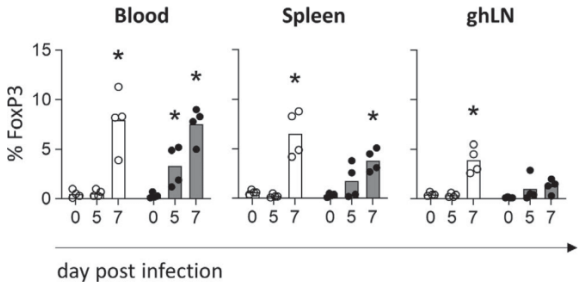


FIGURE 9 FoxP3$^+$ regulatory T cells were induced in domestic pigs and wild boars after ASFV infection. Expression of FoxP3 in CD3$^+$ γδ TCR$^-$ CD8$^-$ CD4$^+$ T cells of blood, spleen and ghLN of domestic pigs (open circles) and wild boar (closed circles) is shown for the indicated time points. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. *p < .05

acute infections in humans and mice (Erickson et al., 2015). These cells displayed characteristics of both effector and exhausted T cells and are thought to minimize tissue damage and immunopathology (Erickson et al., 2015). Since T-cell exhaustion is Eomes-dependent (Li, He, Hao, Ni, & Dong, 2018), this hypothesis has to be investigated in future studies. A more technical explanation supporting the consumption hypothesis is based on the antibody clone used in this study. Clone dG9 has been shown to detect perforin in granula only, missing detection of newly synthesized and immediately secreted perforin molecules (Hersperger, Makedonas, & Betts, 2008). Finally, it is also possible that the cytotoxic response switched from perforin-mediated to Fas/FasL-mediated killing (Meiraz, Garber, Harari, Hassin, & Berke, 2009). Since antibodies for other porcine cytotoxic proteins are missing, this switch remains undetectable.

In contrast to domestic pigs, we found T-bet$^+$CD8$^+$ T cells in blood and increasing frequencies of T-bet$^+$CD8$^+$ T cells in spleen and ghLN of wild boars. Even though a decrease in perforin$^+$CD8αβ$^+$ T cells in wild boars occurred in ghLN and in the liver, it was only half as pronounced as in domestic pigs. Moreover, we even observed an increase in perforin$^+$CD8αα$^+$ T cells in these organs over the time of infection, indicating a functional activation. Taken together, CD8$^+$ T cells in both species did not proliferate upon ASFV infection, but in contrast to domestic pigs, CD8$^+$ T cells from wild boars developed an activated phenotype with cytotoxic potential in the course of infection. However, this was measured by T-bet expression only, and classical activation markers such as CD69 are still missing. Activation could also be shown using in vitro assays to determine the release of IFN-γ by CD8$^+$ and CD4$^+$CD8$^+$ T cells in response to ASFV, as increasing numbers of IFN-γ-producing cells have been shown to correlate with protection (Takamatsu et al., 2013). Moreover, this could investigate functional differences between domestic pigs and wild boars. The detected cytotoxic T-cell response in wild boar was not accompanied by a milder disease course or elimination of virus. This phenomenon needs further investigation.

Another important component for cellular immune responses are MHC class II-restricted CD4$^+$ T-helper (Th) cells. Activated CD4$^+$ Th cells differentiate into different functional subsets, depending on cytokines from the antigenic environment (Gerner et al., 2015). Upon ASFV infection, we observed a strong increase in T-bet$^+$CD4$^+$ T cells in blood and lymphoid organs of domestic pigs 7 dpi, demonstrating a strong cytotoxic Th1 response (Szabo et al., 2000). However, the proliferation of CD4$^+$ T cells could not be detected, indicating that ASFV infection in domestic pigs causes activation but not expansion of pro-inflammatory Th1 cells. Whether these cells sufficiently stimulate CTL and macrophages has to be determined. In wild boars, there was no increase in T-bet expression of CD4$^+$ T cells. A disturbed or non-existent differentiation of Th1 cells further inhibits downstream activation of macrophages and CTL and anti-viral immune responses (Pritchard, Kedl, & Hunter, 2019). However, since Eomes and T-bet show functional redundancy (Intlekofer et al., 2008), lack of T-bet expression alone does not necessarily indicate a non-functioning Th1 response.

Pigs are known to hold a significant amount of CD4$^+$CD8$^+$ double-positive (DP) T cells (Saalmüller, Reddehase, Buhring, Jonjic, & Koszinowski, 1987). We observed an increase in DP T-cell frequency with a corresponding decrease in CD4$^+$ T-cell frequency in peripheral blood and lymphoid organs of domestic pigs, but no proliferative activity or proper perforin expression. Strikingly, we detected an enormous loss of perforin expression 5 dpi in DP T cells, which was restored two days later. DP T cells in wild boar showed a high proliferative activity. DP T cells are playing a distinct role in porcine immune responses (Okutani, Tsukahara, Kato, Fukuta, & Inoue, 2018; Saalmüller, Hirt, & Reddehase, 1989; Zuckermann, 1999) and are often described as memory or effector T cells (Denyer, Wileman, Stirling, Zuber, & Takamatsu, 2006; Gerner, Kaser, & Saalmüller, 2009; Saalmüller, Werner, & Fachinger, 2002; Zuckermann, 1999). Since only ASFV-naïve pigs

were infected and the trial ended 8 dpi, a memory response can be excluded. Previous studies demonstrated CD8α upregulation on porcine CD4 T cells after various in vitro stimulations (Reutner et al., 2013; Saalmüller et al., 1987, 2002). Other studies, using in vitro re-stimulation with viruses or viral peptides, showed proliferation or cytokine production in DP T cells but not conventional $CD4^+$ T cells (Blanco et al., 2000; Lefevre et al., 2012; Reutner et al., 2013; Saalmüller et al., 2002). DP T cells are capable of expressing perforin and granzyme (Chung et al., 2018; Herndler-Brandstetter, Schwanninger, & Grubeck-Loebenstein, 2007; Suni et al., 2001), also indicating a role as cytotoxic effector cells. Together, these studies indicate an activation-dependent expression of CD8α on $CD4^+$ T cells and underline the effector functions of DP T cells.

γδ T cells are often described not only as a bridge between the innate and adaptive immune system but also as cytotoxic effector cells (Charerntantanakul & Roth, 2006; Gerner et al., 2009; Takamatsu et al., 2006; Takamatsu, Denyer, & Wileman, 2002). Previously, it has been shown that γδ T cells of ASFV immune pigs were able to present viral antigen (Takamatsu et al., 2006). Moreover, higher frequencies of circulating γδ T cells correlated with increased survival in an infection with moderately virulent ASFV (Post, Weesendorp, Montoya, & Loeffen, 2017). However, the results of our study suggest that γδ T cells are also affected by highly virulent ASFV. In both species, the numbers of γδ T cells decreased during infection. We did not see any correlation between γδ T-cell frequencies and survival in our study. This might be explained by the higher virulence of the ASFV strain in our study and age differences of the animals compared with previously published reports (Post et al., 2017). Furthermore, there was no change in γδ T-cell subsets in domestic pigs. In contrast, in wild boars, the remaining γδ T cells in blood and spleen differentiated into $CD2^+CD8^-$ and $CD2^+CD8^+$ cells, respectively. Previous studies described $CD2^+CD8^+$ γδ T cells as terminally differentiated effector cells with cytotoxic and anti-viral effector functions (Lopez Fuertes et al., 1999; Stepanova & Sinkora, 2012; Takamatsu et al., 2006; Thielke et al., 2003), indicating a strong pro-inflammatory response. Interestingly, porcine $CD2^+CD8^+$ cells have also been discussed as regulatory cells (Wen et al., 2012) and their appearance correlates with the increase in $FoxP3^+CD4^+$ Tregs in wild boars. This might indicate an anti-inflammatory response in these animals. $CD2^-CD8^-$ and $CD2^+CD8^-$ γδ T cells are thought to elicit pro-inflammatory responses (Wen et al., 2012). These cells were found pre-dominantly in lymph nodes of infected wild boars and might suggest pro-inflammatory responses at the site of infection. In general, the diverse subsets and functions of porcine γδ T cells need further research.

$FoxP3^+$ Tregs are pivotal immune regulators and are needed to prevent immunopathology and tissue damage (Veiga-Parga, Sehrawat, & Rouse, 2013). Tregs appeared earlier in wild boars than in domestic pigs, which might be the reason for the non-development of $CD4^+$ Th1 cells and lack of activation of CTL. This is in line with recent findings by Sanchez-Cordon et al. Here, they showed that increased Treg frequencies and heightened levels of regulatory IL-10 after immunization with an attenuated ASFV strain impair anti-viral responses after virulent ASFV challenge (Sanchez-Cordon, Jabbar, Chapman, Dixon, & Montoya, 2020). Treg responses during acute viral infections are multifaceted. During human dengue fever, Tregs have been shown to proliferate and function normally. Still, because their frequency was too low, they were unable to reduce immunopathology (Lühn et al., 2007), demonstrating that pathological hyper-inflammation is not necessarily associated with impaired Treg functions (Boer, Joosten, & Ottenhoff, 2015). Mild disease, however, was associated with increased numbers of Tregs (Lühn et al., 2007). Therefore, the mere appearance of Tregs during ASFV infection does not exclude immunopathology. Moreover, during acute West Nile fever virus infection in mice, Tregs facilitated enhanced memory T-cell formation at the cost of prolonged antigen presence (Graham, Da Costa, & Lund, 2014). In the case of ASFV infection, prolonged presence of viruses might cause a higher immunopathology and, eventually, lethality. On the other hand, during acute herpes simplex virus infection in mice, Tregs have been suggested as pivotal orchestrators for the migration of inflammatory cells to and production of pro-inflammatory cytokines at the site of infection, thereby facilitating protective immunity (Lund, Hsing, Pham, & Rudensky, 2008). In general, the influence of Tregs on the clinical course and immune response during acute ASFV infection needs further investigation.

Taken together, the experimental ASFV infection with the highly virulent 'Armenia08' strain led to severe clinical courses in domestic pigs and wild boars. The immune response failed in both species and most animals died latest 8 dpi, although a few animals might have survived the infection. Apparently, the ASFV infection suppressed the proliferation of $CD8^+$ T cells, $CD4^+$ T cells and γδ T cells. However, in wild boars we detected $CD8^+$ T cells and γδ T cells with an activated phenotype and partial cytotoxic potential. No activation, measured by T-bet, was observed in domestic pigs. The reaction of DP T cells after ASFV infection was striking in both species: domestic pigs reacted with a differentiation of $CD4^+$ T cells into DP T cells. However, their proliferation remained impaired. The DP T cells of wild boars showed massive proliferative activity and increased perforin expression. Tregs appeared in both species but earlier in wild boars. Still, the immune responses in domestic pigs and wild boars were not able to counteract ASFV infection. Whether the outcome was caused by an inefficient or excessive immune response has to be determined in future studies, for which our study paves the way.

ACKNOWLEDGEMENTS

The authors thank Stefanie Knöfel and Silke Rehbein for excellent technical assistance in the laboratory. For exceptional care of animals and support during trial, we thank the animal caretakers Thomas Möritz, Steffen Brenz, Harald Manthei and Frank Klipp. Furthermore, for outstanding autopsy assistance we thank Christian Loth.

ETHICAL APPROVAL

In this study, all applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration. The animal experiment was approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V), under reference number LALLF 7221.3–1.1-064/17.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SB and UB conceived and designed the experiments. JH, LZ, JS, TS and SB acquired animal samples. JH and AS processed samples. JH, AS, TS and UB analysed and interpreted the data. JH, AS, TS, TCM and UB prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Alexander Schäfer https://orcid.org/0000-0002-4258-0651
Laura Zani https://orcid.org/0000-0001-5263-8389
Sandra Blome https://orcid.org/0000-0001-5465-5609
Ulrike Blohm https://orcid.org/0000-0003-4597-248X

REFERENCES

Alonso, C., Borca, M., Dixon, L., Revilla, Y., Rodriguez, F., Escribano, J. M., & Ictv Report, C. (2018). ICTV Virus Taxonomy Profile: Asfarviridae. *Journal of General Virology*, *99*(5), 613–614. https://doi.org/10.1099/jgv.0.001049

Barry, M., & Bleackley, R. C. (2002). Cytotoxic T lymphocytes: All roads lead to death. *Nature Reviews Immunology*, *2*(6), 401–409. https://doi.org/10.1038/nri819

Beltrán-Beck, B., de la Fuente, J., Garrido, J. M., Aranaz, A., Sevilla, I., Villar, M., … Gortazar, C. (2014). Oral vaccination with heat inactivated Mycobacterium bovis activates the complement system to protect against tuberculosis. *PLoS One*, *9*(5), e98048. https://doi.org/10.1371/journal.pone.0098048

Blanco, E., McCullough, K., Summerfield, A., Fiorini, J., Andreu, D., Chiva, C., … Sobrino, F. (2000). Interspecies major histocompatibility complex-restricted Th cell epitope on foot-and-mouth disease virus capsid protein VP4. *Journal of Virology*, *74*(10), 4902–4907. https://doi.org/10.1128/jvi.74.10.4902-4907.2000

Boer, M. C., Joosten, S. A., & Ottenhoff, T. H. (2015). Regulatory T-Cells at the interface between human host and pathogens in infectious diseases and vaccination. *Frontiers in Immunology*, *6*, 217. https://doi.org/10.3389/fimmu.2015.00217

Cabezón, O., Muñoz-González, S., Colom-Cadena, A., Pérez-Simó, M., Rosell, R., Lavín, S., … Ganges, L. (2017). African swine fever virus infection in Classical swine fever subclinically infected wild boars. *BMC Veterinary Research*, *13*(1), 227. https://doi.org/10.1186/s12917-017-1150-0

Carlson, J., O'Donnell, V., Alfano, M., Velazquez Salinas, L., Holinka, L., Krug, P., … Borca, M. (2016). Association of the host immune response with protection using a live attenuated african swine fever virus model. *Viruses*, *8*(10), 291. https://doi.org/10.3390/v8100291

Charerntantanakul, W., & Roth, J. A. (2006). Biology of porcine T lymphocytes. *Animal Health Research Reviews*, *7*(1–2), 81–96. https://doi.org/10.1017/S1466252307001235

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., … Wahl, S. M. (2003). Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *Journal of Experimental Medicine*, *198*(12), 1875–1886. https://doi.org/10.1084/jem.20030152

Chung, C. J., Cha, S.-H., Grimm, A. L., Ajithdoss, D., Rzepka, J., Chung, G., … Ho, C.-S. (2018). Pigs that recover from porcine reproduction and respiratory syndrome virus infection develop cytotoxic CD4(+)CD8(+) and CD4(+)CD8(-) T-cells that kill virus infected cells. *PLoS One*, *13*(9), e0203482. https://doi.org/10.1371/journal.pone.0203482

Cobb, D., Guo, S., Lara, A. M., Manque, P., Buck, G., & Smeltz, R. B. (2009). T-bet-dependent regulation of CD8+ T-cell expansion during experimental Trypanosoma cruzi infection. *Immunology*, *128*(4), 589–599. https://doi.org/10.1111/j.1365-2567.2009.03169.x

Denyer, M. S., Wileman, T. E., Stirling, C. M., Zuber, B., & Takamatsu, H. H. (2006). Perforin expression can define CD8 positive lymphocyte subsets in pigs allowing phenotypic and functional analysis of natural killer, cytotoxic T, natural killer T and MHC un-restricted cytotoxic T-cells. *Veterinary Immunology and Immunopathology*, *110*(3–4), 279–292. https://doi.org/10.1016/j.vetimm.2005.10.005

Dixon, L. K., Escribano, J. M., Martins, C., Rock, D. L., Salas, M. L., & Wilkinson, P. J. (2005). Asfarviridae. In C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, & L. A. Ball (Eds.), *Virus Taxonomy. VIII. Report of the ICTV* (pp. 135–143). London: Elsevier, Academic Press. Virology Journal, 2. doi:10.1186/1743-422x-2-64.

Dixon, L. K., Islam, M., Nash, R., & Reis, A. L. (2019). African swine fever virus evasion of host defences. *Virus Research*, *266*, 25–33. https://doi.org/10.1016/j.virusres.2019.04.002

Dixon, L. K., Sanchez-Cordon, P. J., Galindo, I., & Alonso, C. (2017). Investigations of Pro- and anti-apoptotic factors affecting african swine fever virus replication and pathogenesis. *Viruses*, *9*(9), 241. 10.3390/v9090241

Dixon, L. K., Sun, H., & Roberts, H. (2019). African swine fever. *Antiviral Research*, *165*, 34–41. https://doi.org/10.1016/j.antiviral.2019.02.018

Erickson, J. J., Lu, P., Wen, S., Hastings, A. K., Gilchuk, P., Joyce, S., … Williams, J. V. (2015). Acute viral respiratory infection rapidly induces a $CD8^+$ T cell exhaustion-like phenotype. *The Journal of Immunology*, *195*(9), 4319–4330. https://doi.org/10.4049/jimmunol.1403004

Escribano, J. M., Galindo, I., & Alonso, C. (2013). Antibody-mediated neutralization of African swine fever virus: Myths and facts. *Virus Research*, *173*(1), 101–109. https://doi.org/10.1016/j.virusres.2012.10.012

Franzoni, G., Dei Giudici, S., & Oggiano, A. (2018). Infection, modulation and responses of antigen-presenting cells to African swine fever viruses. *Virus Research*, *258*, 73–80. https://doi.org/10.1016/j.virusres.2018.10.007

Franzoni, G., Graham, S. P., Giudici, S. D., Bonelli, P., Pilo, G., Anfossi, A. G., … Oggiano, A. (2017). Characterization of the interaction of African swine fever virus with monocytes and derived macrophage subsets. *Veterinary Microbiology*, *198*, 88–98. https://doi.org/10.1016/j.vetmic.2016.12.010

Franzoni, G., Graham, S. P., Sanna, G., Angioi, P., Fiori, M. S., Anfossi, A., … Oggiano, A. (2018). Interaction of porcine monocyte-derived dendritic cells with African swine fever viruses of diverse virulence. *Veterinary Microbiology*, *216*, 190–197. https://doi.org/10.1016/j.vetmic.2018.02.021

Galindo, I., & Alonso, C. (2017). African swine fever virus: A review. *Viruses*, *9*(5), 103. https://doi.org/10.3390/v9050103

Gerner, W., Kaser, T., & Saalmüller, A. (2009). Porcine T lymphocytes and NK cells–an update. *Developmental and Comparative Immunology*, *33*(3), 310–320. https://doi.org/10.1016/j.dci.2008.06.003

Gerner, W., Talker, S. C., Koinig, H. C., Sedlak, C., Mair, K. H., & Saalmüller, A. (2015). Phenotypic and functional differentiation of porcine alphabeta T cells: Current knowledge and available tools. *Molecular Immunology*, *66*(1), 3–13. https://doi.org/10.1016/j.molimm.2014.10.025

Gomez-Villamandos, J. C., Bautista, M. J., Sanchez-Cordon, P. J., & Carrasco, L. (2013). Pathology of African swine fever: The role of monocyte-macrophage. *Virus Research*, *173*(1), 140–149. https://doi.org/10.1016/j.virusres.2013.01.017

Graham, J. B., Da Costa, A., & Lund, J. M. (2014). Regulatory T cells shape the resident memory T cell response to virus infection in the tissues. *The Journal of Immunology*, *192*(2), 683–690. https://doi.org/10.4049/jimmunol.1202153

Groenen, M. A. M., Archibald, A. L., Uenishi, H., Tuggle, C. K., Takeuchi, Y., Rothschild, M. F., … Schook, L. B. (2012). Analyses of pig genomes provide insight into porcine demography and evolution. *Nature*, *491*(7424), 393–398. https://doi.org/10.1038/nature11622

Herndler-Brandstetter, D., Schwanninger, A., & Grubeck-Loebenstein, B. (2007). CD4+ CD8+ T cells in young and elderly humans. Comment on Macchia I, Gauduin MC, Kaur A, Johnson RP. Expression of CD8alpha identifies a distinct subset of effector memory CD4 T lymphocytes. Immunology 2006; 119:232–42. *Immunology*, *120*(3), 292–294. https://doi.org/10.1111/j.1365-2567.2006.02542.x

Hersperger, A. R., Makedonas, G., & Betts, M. R. (2008). Flow cytometric detection of perforin upregulation in human CD8 T cells. *Cytometry A*, *73*(11), 1050–1057. https://doi.org/10.1002/cyto.a.20596

Intlekofer, A. M., Banerjee, A., Takemoto, N., Gordon, S. M., DeJong, C. S., Shin, H., … Reiner, S. L. (2008). Anomalous type 17 response to viral infection by CD8$^+$ T cells lacking T-bet and eomesodermin. *Science*, *321*(5887), 408–411. https://doi.org/10.1126/science.1159806

Intlekofer, A. M., Takemoto, N., Wherry, E. J., Longworth, S. A., Northrup, J. T., Palanivel, V. R., … Reiner, S. L. (2005). Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nature Immunology*, *6*(12), 1236–1244. https://doi.org/10.1038/ni1268

Jori, F., & Bastos, A. D. (2009). Role of wild suids in the epidemiology of African swine fever. *EcoHealth*, *6*(2), 296–310. https://doi.org/10.1007/s10393-009-0248-7

Käser, T., Gerner, W., & Saalmüller, A. (2011). Porcine regulatory T cells: Mechanisms and T-cell targets of suppression. *Developmental and Comparative Immunology*, *35*(11), 1166–1172. https://doi.org/10.1016/j.dci.2011.04.006

King, D. P., Reid, S. M., Hutchings, G. H., Grierson, S. S., Wilkinson, P. J., Dixon, L. K., … Drew, T. W. (2003). Development of a TaqMan (R) PCR assay with internal amplification control for the detection of African swine fever virus. *Journal of Virological Methods*, *107*(1), 53–61. https://doi.org/10.1016/S0166-0934(02)00189-1

King, K., Chapman, D., Argilaguet, J. M., Fishbourne, E., Hutet, E., Cariolet, R., … Takamatsu, H.-H. (2011). Protection of European domestic pigs from virulent African isolates of African swine fever virus by experimental immunisation. *Vaccine*, *29*(28), 4593–4600. https://doi.org/10.1016/j.vaccine.2011.04.052

Kleiboeker, S. B. (2002). Swine fever: Classical swine fever and African swine fever. *The Veterinary Clinics of North America. Food Animal Practice*, *18*(3), 431–451. https://doi.org/10.1016/S0749-0720(02)00028-2

Kurachi, M. (2019). CD8(+) T cell exhaustion. *Seminars in Immunopathology*, *41*(3), 327–337. https://doi.org/10.1007/s00281-019-00744-5

Lacasta, A., Monteagudo, P. L., Jiménez-Marín, Á., Accensi, F., Ballester, M., Argilaguet, J., … Rodríguez, F. (2015). Live attenuated African swine fever viruses as ideal tools to dissect the mechanisms involved in viral pathogenesis and immune protection. *Veterinary Research*, *46*, 135. https://doi.org/10.1186/s13567-015-0275-z

Lefevre, E. A., Carr, B. V., Inman, C. F., Prentice, H., Brown, I. H., Brookes, S. M., … Charleston, B. (2012). Immune responses in pigs vaccinated with adjuvanted and non-adjuvanted A(H1N1)pdm/09 influenza vaccines used in human immunization programmes. *PLoS One*, *7*(3), e32400. https://doi.org/10.1371/journal.pone.0032400

Li, J., He, Y., Hao, J., Ni, L., & Dong, C. (2018). High levels of Eomes promote exhaustion of anti-tumor CD8(+) T cells. *Frontiers in Immunology*, *9*, 2981. https://doi.org/10.3389/fimmu.2018.02981

Lopez Fuertes, L., Domenech, N., Alvarez, B., Ezquerra, A., Dominguez, J., Castro, J. M., & Alonso, F. (1999). Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. *Virus Research*, *64*(1), 33–42. https://doi.org/10.1016/s0168-1702(99)00073-8

Lühn, K., Simmons, C. P., Moran, E., Dung, N. T. P., Chau, T. N. B., Quyen, N. T. H., … Rowland-Jones, S. (2007). Increased frequencies of CD4+ CD25(high) regulatory T cells in acute dengue infection. *Journal of Experimental Medicine*, *204*(5), 979–985. https://doi.org/10.1084/jem.20061381

Lund, J. M., Hsing, L., Pham, T. T., & Rudensky, A. Y. (2008). Coordination of early protective immunity to viral infection by regulatory T cells. *Science*, *320*(5880), 1220–1224. https://doi.org/10.1126/science.1155209

Meiraz, A., Garber, O. G., Harari, S., Hassin, D., & Berke, G. (2009). Switch from perforin-expressing to perforin-deficient CD8(+) T cells accounts for two distinct types of effector cytotoxic T lymphocytes in vivo. *Immunology*, *128*(1), 69–82. https://doi.org/10.1111/j.1365-2567.2009.03072.x

Mittelholzer, C., Moser, C., Tratschin, J. D., & Hofmann, M. A. (2000). Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. *Veterinary Microbiology*, *74*(4), 293–308. https://doi.org/10.1016/S0378-1135(00)00195-4

Neilan, J. G., Zsak, L., Lu, Z., Burrage, T. G., Kutish, G. F., & Rock, D. L. (2004). Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology*, *319*(2), 337–342. https://doi.org/10.1016/j.virol.2003.11.011

O'Donnell, V., Holinka, L. G., Krug, P. W., Gladue, D. P., Carlson, J., Sanford, B., … Borca, M. V. (2015). African swine fever virus Georgia 2007 with a deletion of virulence-associated gene 9GL (B119L), when administered at low doses, leads to virus attenuation in swine and induces an effective protection against homologous challenge. *Journal of Virology*, *89*(16), 8556–8566. https://doi.org/10.1128/JVI.00969-15

Okutani, M., Tsukahara, T., Kato, Y., Fukuta, K., & Inoue, R. (2018). Gene expression profiles of CD4/CD8 double-positive T cells in porcine peripheral blood. *Animal Science Journal*, *89*(7), 979–987. https://doi.org/10.1111/asj.13021

Onisk, D. V., Borca, M. V., Kutish, G., Kramer, E., Irusta, P., & Rock, D. L. (1994). Passively transferred African swine fever virus antibodies protect swine against lethal infection. *Virology*, *198*(1), 350–354. https://doi.org/10.1006/viro.1994.1040

Oura, C. A., Denyer, M. S., Takamatsu, H., & Parkhouse, R. M. (2005). In vivo depletion of CD8$^+$ T lymphocytes abrogates protective immunity to African swine fever virus. *Journal of General Virology*, *86*(Pt 9), 2445–2450. https://doi.org/10.1099/vir.0.81038-0

Oura, C. A. L., Powell, P. P., Anderson, E., & Parkhouse, R. M. E. (1998). The pathogenesis of African swine fever in the resistant bushpig. *Journal of General Virology*, *79*, 1439–1443. https://doi.org/10.1099/0022-1317-79-6-1439

Pearce, E. L., Mullen, A. C., Martins, G. A., Krawczyk, C. M., Hutchins, A. S., Zediak, V. P., … Reiner, S. L. (2003). Control of effector CD8$^+$

T cell function by the transcription factor Eomesodermin. *Science*, *302*(5647), 1041–1043. https://doi.org/10.1126/science.1090148

Penrith, M. L., & Vosloo, W. (2009). Review of African swine fever: Transmission, spread and control. *Journal of the South African Veterinary Association*, *80*(2), 58–62.

Petrov, A., Forth, J. H., Zani, L., Beer, M., & Blome, S. (2018). No evidence for long-term carrier status of pigs after African swine fever virus infection. *Transboundary and Emerging Diseases*, *65*(5), 1318–1328. https://doi.org/10.1111/tbed.12881

Pietschmann, J., Guinat, C., Beer, M., Pronin, V., Tauscher, K., Petrov, A., … Blome, S. (2015). Course and transmission characteristics of oral low-dose infection of domestic pigs and European wild boar with a Caucasian African swine fever virus isolate. *Archives of Virology*, *160*(7), 1657–1667. https://doi.org/10.1007/s00705-015-2430-2

Pietschmann, J., Mur, L., Blome, S., Beer, M., Perez-Sanchez, R., Oleaga, A., & Sanchez-Vizcaino, J. M. (2016). African swine fever virus transmission cycles in Central Europe: Evaluation of wild boar-soft tick contacts through detection of antibodies against Ornithodoros erraticus saliva antigen. *BMC Veterinary Research*, *12*, 1. https://doi.org/10.1186/s12917-015-0629-9

Post, J., Weesendorp, E., Montoya, M., & Loeffen, W. L. (2017). Influence of age and dose of african swine fever virus infections on clinical outcome and blood parameters in pigs. *Viral Immunology*, *30*(1), 58–69. https://doi.org/10.1089/vim.2016.0121

Pritchard, G. H., Kedl, R. M., & Hunter, C. A. (2019). The evolving role of T-bet in resistance to infection. *Nature Reviews Immunology*, *19*(6), 398–410. https://doi.org/10.1038/s41577-019-0145-4

Ramiro-Ibanez, F., Ortega, A., Ruiz-Gonzalvo, F., Escribano, J. M., & Alonso, C. (1997). Modulation of immune cell populations and activation markers in the pathogenesis of African swine fever virus infection. *Virus Research*, *47*(1), 31–40. https://doi.org/10.1016/s0168-1702(96)01403-7

Reis, A. L., Abrams, C. C., Goatley, L. C., Netherton, C., Chapman, D. G., Sanchez-Cordon, P., & Dixon, L. K. (2016). Deletion of African swine fever virus interferon inhibitors from the genome of a virulent isolate reduces virulence in domestic pigs and induces a protective response. *Vaccine*, *34*(39), 4698–4705. https://doi.org/10.1016/j.vaccine.2016.08.011

Reutner, K., Leitner, J., Müllebner, A., Ladinig, A., Essler, S. E., Duvigneau, J. C., … Gerner, W. (2013). CD27 expression discriminates porcine T helper cells with functionally distinct properties. *Veterinary Research*, *44*, 18. https://doi.org/10.1186/1297-9716-44-18

Saalmüller, A., Hirt, W., & Reddehase, M. J. (1989). Phenotypic discrimination between thymic and extrathymic CD4-CD8$^-$ and CD4$^+$CD8$^+$ porcine T lymphocytes. *European Journal of Immunology*, *19*(11), 2011–2016. https://doi.org/10.1002/eji.1830191107

Saalmüller, A., Pauly, T., Hohlich, B. J., & Pfaff, E. (1999). Characterization of porcine T lymphocytes and their immune response against viral antigens. *Journal of Biotechnology*, *73*(2–3), 223–233. https://doi.org/10.1016/S0168-1656(99)00140-6

Saalmüller, A., Reddehase, M. J., Buhring, H. J., Jonjic, S., & Koszinowski, U. H. (1987). Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. *European Journal of Immunology*, *17*(9), 1297–1301. https://doi.org/10.1002/eji.1830170912

Saalmüller, A., Werner, T., & Fachinger, V. (2002). T-helper cells from naive to committed. *Veterinary Immunology and Immunopathology*, *87*(3–4), 137–145. https://doi.org/10.1016/S0165-2427(02)00045-4

Sanchez-Cordon, P. J., Jabbar, T., Chapman, D., Dixon, L. K., & Montoya, M. (2020). Absence of long-term protection in domestic pigs immunized with attenuated African swine fever virus isolate OURT88/3 or BeninDeltaMFG correlates with increased levels of regulatory T cells and IL-10. *Journal of Virology*, https://doi.org/10.1128/JVI.00350-20

Sanchez-Cordon, P. J., Montoya, M., Reis, A. L., & Dixon, L. K. (2018). African swine fever: A re-emerging viral disease threatening the global pig industry. *The Veterinary Journal*, *233*, 41–48. https://doi.org/10.1016/j.tvjl.2017.12.025

Sanchez-Torres, C., Gomez-Puertas, P., Gomez-del-Moral, M., Alonso, F., Escribano, J. M., Ezquerra, A., & Dominguez, J. (2003). Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection. *Archives of Virology*, *148*(12), 2307–2323. https://doi.org/10.1007/s00705-003-0188-4

Schäfer, A., Hühr, J., Schwaiger, T., Dorhoi, A., Mettenleiter, T. C., Blome, S., … Blohm, U. (2019). Porcine invariant natural killer t cells: functional profiling and dynamics in steady state and viral infections. *Frontiers in Immunology*, *10*, 1380. https://doi.org/10.3389/fimmu.2019.01380

Schlafer, D. H., Mebus, C. A., & McVicar, J. W. (1984). African swine fever in neonatal pigs: Passively acquired protection from colostrum or serum of recovered pigs. *American Journal of Veterinary Research*, *45*(7), 1367–1372.

Stepanova, K., & Sinkora, M. (2012). The expression of CD25, CD11b, SWC1, SWC7, MHC-II, and family of CD45 molecules can be used to characterize different stages of gammadelta T lymphocytes in pigs. *Developmental and Comparative Immunology*, *36*(4), 728–740. https://doi.org/10.1016/j.dci.2011.11.003

Suni, M. A., Ghanekar, S. A., Houck, D. W., Maecker, H. T., Wormsley, S. B., Picker, L. J., … Maino, V. C. (2001). CD4(+)CD8(dim) T lymphocytes exhibit enhanced cytokine expression, proliferation and cytotoxic activity in response to HCMV and HIV-1 antigens. *European Journal of Immunology*, *31*(8), 2512–2520. https://doi.org/10.1002/1521-4141(200108)31:8<2512:AID-IMMU2512gt;3.0.CO;2-M

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., & Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*, *100*(6), 655–669. https://doi.org/10.1016/s0092-8674(00)80702-3

Takamatsu, H.-H., Denyer, M. S., Lacasta, A., Stirling, C. M. A., Argilaguet, J. M., Netherton, C. L., … Rodríguez, F. (2013). Cellular immunity in ASFV responses. *Virus Research*, *173*(1), 110–121. https://doi.org/10.1016/j.virusres.2012.11.009

Takamatsu, H. H., Denyer, M. S., Stirling, C., Cox, S., Aggarwal, N., Dash, P., … Barnett, P. V. (2006). Porcine gammadelta T cells: Possible roles on the innate and adaptive immune responses following virus infection. *Veterinary Immunology and Immunopathology*, *112*(1–2), 49–61. https://doi.org/10.1016/j.vetimm.2006.03.011

Takamatsu, H. H., Denyer, M. S., & Wileman, T. E. (2002). A sub-population of circulating porcine gammadelta T cells can act as professional antigen presenting cells. *Veterinary Immunology and Immunopathology*, *87*(3–4), 223–224.

Thielke, K. H., Hoffmann-Moujahid, A., Weisser, C., Waldkirch, E., Pabst, R., Holtmeier, W., & Rothkotter, H. J. (2003). Proliferating intestinal gamma/delta T cells recirculate rapidly and are a major source of the gamma/delta T cell pool in the peripheral blood. *European Journal of Immunology*, *33*(6), 1649–1656. https://doi.org/10.1002/eji.200323442

Thomas, D. A., & Massague, J. (2005). TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell*, *8*(5), 369–380. https://doi.org/10.1016/j.ccr.2005.10.012

Tiemessen, M. M., Kunzmann, S., Schmidt-Weber, C. B., Garssen, J., Bruijnzeel-Koomen, C. A., Knol, E. F., & van Hoffen, E. (2003). Transforming growth factor-beta inhibits human antigen-specific CD4$^+$ T cell proliferation without modulating the cytokine response. *International Immunology*, *15*(12), 1495–1504. https://doi.org/10.1093/intimm/dxg147

Tizard, I. (2008). Sickness behavior, its mechanisms and significance. *Animal Health Research Reviews*, *9*(1), 87–99. https://doi.org/10.1017/S1466252308001448

Veiga-Parga, T., Sehrawat, S., & Rouse, B. T. (2013). Role of regulatory T cells during virus infection. *Immunological Reviews*, *255*(1), 182–196. https://doi.org/10.1111/imr.12085

Wen, K., Bui, T., Li, G., Liu, F., Li, Y., Kocher, J., & Yuan, L. (2012). Characterization of immune modulating functions of gammadelta T cell subsets in a gnotobiotic pig model of human rotavirus infection. *Comparative Immunology, Microbiology and Infectious Diseases*, *35*(4), 289–301. https://doi.org/10.1016/j.cimid.2012.01.010

Yang, H., & Parkhouse, R. M. (1997). Differential expression of CD8 epitopes amongst porcine CD8-positive functional lymphocyte subsets. *Immunology*, *92*(1), 45–52. https://doi.org/10.1046/j.1365-2567.1997.00308.x

Zsak, L., Onisk, D. V., Afonso, C. L., & Rock, D. L. (1993). Virulent African swine fever virus isolates are neutralized by swine immune serum and by monoclonal antibodies recognizing a 72-kDa viral protein. *Virology*, *196*(2), 596–602. https://doi.org/10.1006/viro.1993.1515

Zuckermann, F. A. (1999). Extrathymic CD4/CD8 double positive T cells. *Veterinary Immunology and Immunopathology*, *72*(1–2), 55–66. https://doi.org/10.1016/S0165-2427(99)00118-X

How to cite this article: HührJ, Schäfer A, Schwaiger T, et al. Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain. *Transbound Emerg Dis*. 2020;00:1–17. https://doi.org/10.1111/tbed.13678

2.4 Publication IV

T-cell responses in domestic pigs and wild boar upon infection with the moderately virulent African swine fever virus strain "Estonia2014"

Alexander Schäfer, Laura Zani, Jutta Pikalo, Jane Hühr, Julia Sehl, Thomas C. Mettenleiter, Angele Breithaupt, Sandra Blome, Ulrike Blohm

Transboundary and Emerging Diseases

doi: 10.22541/au.159986510.05288008
(Authorea Preprint)

submitted: September 2020

Transboundary and Emerging Diseases - submission proof

T-cell responses in domestic pigs and wild boar upon infection with the moderately virulent African swine fever virus strain "Estonia2014"

Journal:	*Transboundary and Emerging Diseases*
Manuscript ID	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Schäfer, Alexander; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Zani, Laura; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Pikalo, Jutta; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Hühr, Jane; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Sehl, Julia; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Mettenleiter, Thomas; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Breithaupt, Angele; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Blome, Sandra; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health Blohm, Ulrike; Friedrich-Loeffler-Institute Federal Research Institute for Animal Health
Subject Area:	Immune system, Virus, Pathogenesis

Transboundary and Emerging Diseases - submitted manuscript

T-cell responses in domestic pigs and wild boar upon infection with the moderately virulent African swine fever virus strain "Estonia2014"

Running title: T-cell responses in ASFV-Estonia infected pigs

Alexander Schäfer, Laura Zani, Jutta Pikalo, Jane Hühr, Julia Sehl, Thomas C. Mettenleiter, Angele Breithaupt, Sandra Blome, Ulrike Blohm§

All: Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany

ORCIDs:

Alexander Schäfer, 0000-0002-4258-0651

Laura Zani, 0000-0001-5263-8389

Jutta Pikalo, 0000-0003-3502-4240

Jane Hühr, 0000-0002-2235-9618

Julia Sehl, 0000-0002-3884-1943

Angele Breithaupt, 0000-0002-6373-5923

Sandra Blome, 0000-0001-5465-5609

Ulrike Blohm, 0000-0003-4597-248X

§Corresponding author:

Ulrike Blohm
Friedrich-Loeffler-Institut
Federal Research Institute for Animal Health
Südufer 10
D - 17493 Greifswald – Insel Riems
Germany
Phone: +49 38351 7 1543; E-mail: Ulrike.Blohm@fli.de

Summary

Infection with African swine fever virus (ASFV) causes a highly lethal hemorrhagic disease in domestic and Eurasian wild pigs. Thus, it is a major threat to pig populations worldwide and a cause of substantial economic losses. Recently, less virulent ASFV strains emerged naturally, which showed higher experimental virulence in wild boar than in domestic pigs. The reason for this difference in disease progression and outcome is unclear but likely involves different immunological responses. Unfortunately, besides the importance of $CD8\alpha^+$ lymphocytes, little is known about the immune responses against ASFV in suids. Against this background, we used a multicolor flow cytometry platform to investigate the T-cell responses in wild boar and domestic pigs after infection with the moderately virulent ASFV strain "Estonia2014" in two independent trials. $CD4^-/CD8\alpha^+$ and $CD4^+/CD8\alpha^+$ αβ T-cell frequencies increased in both subspecies in various tissues, but $CD8\alpha^+$ γδ T cells differentiated and responded in wild boar only. Proliferation in $CD8\alpha^+$ T cells was found 10 days *post infectionem* only. Frequencies of T-bet^+ T cells increased in wild boar but not in domestic pigs. Of note, we found a considerable loss of perforin expression in cytotoxic T cells, 5 and 7 dpi. Both subspecies established a regulatory T-cell response 10 dpi. In domestic pigs, we show increasing levels of ICOS+ and $CD8\alpha^+$ invariant Natural Killer T cells. These disparities in T-cell responses might explain some of the differences in disease progression in wild boar and domestic pigs and should pave the way for future studies.

Keywords: African swine fever virus, experimental infection, domestic pig, wild boar, T cell response, iNKT cells

1 Introduction

African Swine Fever (ASF) remains a major threat to wild and domestic pig populations (Pikalo, Zani, Hühr, Beer, & Blome, 2019). The causative agent, ASF virus (ASFV), is a large dsDNA virus and the only member in the genus *Asfivirus* of the *Asfarviridae* family (C. Alonso et al., 2018). In its geographic origin in sub-Saharan Africa, ASFV is transmitted by soft ticks of the *Ornithodoros* genus and circulates in other members of the *Suidae*, warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus larvatus*) (Gaudreault, Madden, Wilson, Trujillo, & Richt, 2020). While ASFV infection causes major disease in domestic pigs and wild boar with profound manifestations and high lethality, it is clinically inapparent in its reservoir hosts, i.e., African wild suids (C. A. L. Oura, Powell, Anderson, & Parkhouse, 1998). In European and Asian countries, the abundant wild boar populations serve as a reservoir for ASFV and present a risk for ASFV introduction into domestic pig holdings (Jori & Bastos, 2009; Pietschmann et al., 2016). Its near-global panzootic spread already caused death of millions of pigs in commercial and private farms and also led to major economic challenges (Mason-D'Croz et al., 2020).

At present, little is known about the role of the host's immune response against ASFV. Once infection in a mammalian host is established, ASFV has a distinct cell tropism for myeloid cells. It replicates in monocytes and macrophages but has also been found in granulocytes (Carrasco et al., 1996; Colgrove, Haelterman, & Coggins, 1969; Gomez-Villamandos, Bautista, Sanchez-Cordon, & Carrasco, 2013). The pivotal role of lymphocytes, presumably $CD8\alpha^+$ T cells in particular, has been demonstrated by antibody-dependent depletion of $CD8\alpha^+$ cells after priming with an avirulent ASFV strain, which resulted in the loss of protection after homologous challenge (C. A. Oura, Denyer, Takamatsu, & Parkhouse, 2005).

Recently, we showed that immune responses of domestic pigs and wild boar fail to clear an infection with the highly virulent ASFV strain "Armenia08", although for different reasons (Hühr et al., 2020). Wild boar and domestic pigs showed comparable clinical signs with substantial fatalities within the first 8 days *post infectionem* (dpi). However, we found activated T cells and signs of a cytotoxic response only in wild boar but not in domestic pigs. Moreover, T-cell proliferation was impaired in domestic pigs, while $CD4^+/CD8\alpha^+$ T cells in wild boar showed considerable proliferation. Of note, we detected a significant loss of perforin expression in both subspecies 5 dpi in $CD8\alpha^+$ T cells. Still, neither response was beneficial for the final disease outcome (Hühr et al., 2020).

In the present study, we used a similar approach and investigated the immune responses of domestic pigs and wild boar after infection with the moderately virulent ASFV strain, "Estonia2014". ASFV "Estonia2014" has been shown to cause fatal disease in wild boar whereas different domestic pigs survived the experimental infection (Zani et al., 2018). A comparative approach with a focus on T-cell responses was used in this study to find a possible explanation for this phenomenon. Moreover, with these data we are able to compare immune responses against moderately virulent ASFV with those against highly virulent ASFV recently published (Hühr et al., 2020).

2 Material and Methods

2.1 Experimental design

Two independent animal trials were included in this study. Domestic pigs (*Sus scrofa domesticus*) were obtained from a commercial pig farm and wild boar (*Sus scrofa scrofa*) were provided by wildlife parks in Mecklenburg-Western Pomerania. For the infection experiments, domestic pigs and wild boar were transferred into the high containment facilities of the Friedrich-Loeffler-Institut (L3+) and were left for acclimatization for a week.

In both studies, domestic pigs and wild boar were oro-nasally inoculated with 2 ml cell culture supernatant containing $10^{5.25}$ haemadsorbing units (HAU)/ml of ASFV "Estonia2014". A clinical score was assessed daily, based on a previously described scoring system (Pietschmann et al., 2015). Rectal temperatures were measured each day in domestic pigs and at autopsy in wild boar. 6 domestic pigs and 7 wild boar were left untreated and served as controls. In trial 1, 12 German landrace pigs, 3 months of age, and 12 wild boar, 1-2 years of age, were used. 4 animals of each group were randomly chosen for autopsy 5 and 7 dpi. For trial 2, 11 German landrace pigs, 3 months of age, and 12 wild boar, 1-2 years of age, were used. Autopsies were done 4, 7, and 10 dpi with 3 animals of each group (see table 1). Results from control animals and animals investigated 7 dpi, respectively, were grouped for all applicable analyses. For scheduled autopsies or when animals reached the humane endpoint, animals were narcotized with tiletamine/zolazepam (Zoletil ®, Virbac) and xylazine (Rompun® 2%, Bayer HealthCare) and then euthanized by intracardial injection of pentobarbital (Release, Wirtschaftsgenossenschaft deutscher Tierärzte) or exanguination. For analysis of the immune

response, blood, lung, spleen, gastro-hepatic lymph node (ghLN, *Lymphonodi hepatici* or *gastrici*), as well as liver were collected.

2.2 Virus detection

Hemadsorption test (HAT) was performed for virus back-titration by endpoint titration on macrophages derived from peripheral blood monocytic cells of healthy donor pigs as previously described (Pietschmann et al., 2015).

Viral genome was detected in blood and tissues by real-time PCR (qPCR) using routine assays as previously described (Sehl et al., 2020).

2.4 Preparation of single cell suspensions

Single cell suspensions were prepared as described previously (Hühr et al., 2020; Schäfer et al., 2019). Briefly, spleen and ghLN were mechanically disrupted with a steel strainer. Liver sections were cleared of peripheral blood by perfusion with ice-cold PBS-EDTA before further cell extraction. Samples from lungs and perfused liver were then digested (collagenase IV (2 mg/ml; Biochrom), DNase I (0.1 mg/ml; Sigma-Aldrich)) for 1 h at 37°C in serum-free cell culture media (1:1 Ham's F12/IMDM). Tissue residuals were removed by short centrifugation. The cells were washed with serum-supplemented cell culture media (10% fetal calf serum) and used for flow cytometry.

2.5 Flow cytometry

At indicated time points, whole blood and single cell suspensions of spleen, ghLN, lung, and liver were stained for flow cytometric analyses. 50 µl whole blood and 50 µl single cell suspensions (approx. 1×10^6 leukocytes) were used for staining. To identify iNKT cells, whole blood was incubated with PBS57-loaded murine CD1d (mCD1d) tetramers at room temperature for 30 min in the dark as described previously (Schäfer et al., 2019). All further incubation steps with monoclonal antibodies (mAbs) targeting extracellular antigens were carried out for 15 min at 4°C in the dark. Between each antibody staining, a washing step was

performed. Before intracellular labeling, erythrocytes in blood samples were lysed with red blood cell lysis buffer (1.55 M NH_4Cl, 100 mM $KHCO_3$, 12.7 mM Na_4EDTA, pH 7.4, in *Aqua destillata*). Subsequently, samples were fixed and permeabilized with the True-Nuclear Transcription Factor Buffer Set (Biolegend, USA) according to the manufacturer's instructions. All incubation steps for intracellular staining were carried out for 30 min at 4°C in the dark. Antibodies and conjugates used for flow cytometry are shown in table 2. The mCD1d tetramer was obtained from the NIH Tetramer Core Facility.

Dead cells were excluded by FSC/SSC characteristics and using Zombie Aqua (Biolegend, USA). Single cells were identified by consecutive FSC-W vs. FSC-H and SSC-W vs. SSC-H gating. Live, single lymphocytes were further divided into $CD3^+$/γδ T cell receptor $(TCR)^-$ (αβ T cells), $CD3^+$/γδ TCR^+ (γδ T cells), and $CD3^+$/mCD1d $tetramer^+$ (iNKT cells). Further subpopulations were gated according to the markers described in the figures and text. At least 1×10^4 single, live αβ T cells were recorded. CD4 was not detectable in one of the control wild boar in trial 2, presumably because of a polymorphism in the *CD4* alleles (Eguchi-Ogawa et al., 2018). $CD4^+$ and $CD4^+/CD8\alpha^+$ cells from this animal were therefore not included in the analyses.

Flow Cytometer BD FACS Canto II with FACS DIVA Software (BD Bioscience, San Jos, CA) and FlowJo™ V10 for Windows (Becton, Dickinson and Company; 2019) were used for all analyses.

2.6 Statistical analysis

Statistical analyses and graph creation were done using GraphPad Prism8 (Graphpad Software Inc., USA). Normality was verified using Shapiro-Wilk test. To investigate statistically significant differences between infected and uninfected animals, ordinary one-way ANOVA with Dunnett's correction for multiple comparisons was used. Differences between both swine subspecies were not tested because of differences at the baseline level. Data obtained in trial 1 is indicated by circles (●), data obtained in trial 2 is indicated by squares (■). Each dot represents one animal with a bar indicating mean. Statistical significance was defined as $p < 0.05$ and was indicated with an asterisk (*).

3 Results

3.1 Clinical course

Back-titration of the virus suspension used for inoculation verified the administered titer of $1 \times 10^{5.25}$ HAU/ml per pig in both trials. All tissue samples from inoculated domestic pigs and wild boar were positive for ASFV genome in qPCR and gross pathology was comparable between trial 1 and 2 (data not shown). All animals developed signs of disease starting 4 dpi. Domestic pigs developed moderate disease but recovered until 10 dpi (maximum clinical score 5), whereas wild boar showed signs of moderate to severe disease until the end of the study (maximum clinical score 10.5) and thus, confirmed higher virulence of ASFV "Estonia2014" in wild boar. Details regarding viral load, histopathology, and analysis by electron microscopy of trial 2 can be found in a parallel publication (Sehl et al., 2020).

Briefly, macroscopic pathology, ASFV antigen-positive myeloid cells investigated by flow cytometry, and viral loads in the investigated tissues showed comparable results in wild boar and domestic pigs. In contrast, higher levels of ASFV antigen were found in wild boar by immunohistochemistry.

3.2 Altered T-cell subset frequencies in lymphoid tissue

$CD3^+$ T cells were subdivided into αβ T cells ($CD3^+$/ γδ TCR^-) and γδ T cells ($CD3^+$/ γδ TCR^-). αβ T cells in domestic pigs showed decreases 5 dpi in blood, spleen, lung, and liver with corresponding increased γδ T-cell frequencies (Figure 1A, B). The T-cell frequencies in domestic pigs returned to control levels 10 dpi, except for αβ T-cell increases in the liver (Figure 1A). In wild boar, we detected temporarily increased αβ T-cell frequencies in blood and spleen 4 dpi but no other changes to baseline level (Figure 1A) and largely unaffected γδ T-cell frequencies over the study period, except for corresponding decreases in γδ T-cell frequencies in blood and spleen (Figure 1B). No changes were detected in the gastro-hepatic lymph nodes (ghLN, *Lymphonodi hepatici* or *gastrici*) of both subspecies.

3.3 CD8α⁺ T-cell subsets responded differently in domestic pigs and wild boar

CD8α⁺ lymphocytes are known mediators of immunity during ASFV infection (C. A. Oura et al., 2005). Being the largest group of CD8α⁺ lymphocytes, we investigated CD8α⁺ T-cell frequencies among αβ T cells. Frequencies of CD4⁻/CD8α⁺ αβ T cells increased in blood, spleen, lungs, and liver of domestic pigs (Figure 2A). The increase was most prominent in spleen, lung, and liver of infected animals and was significant 5 or 7 dpi until the end of the trial. In the blood, we detected increased frequencies of CD8α⁺ αβ T cells 10 dpi only. CD4⁻/CD8α⁺ αβ T cells in wild boar demonstrated a comparable course, except for the lung. There were no frequency alterations of CD4⁻/CD8α⁺ αβ T cells in ghLN of both subspecies (Figure 2A).

Frequencies of CD4⁺/CD8α⁺ double positive (DP) αβ T cells showed a similar but less pronounced pattern (Figure 2B). Domestic pigs had elevated levels of DP T cells 5 and 7 dpi in spleen and liver, correlating with increased levels of CD4⁻/CD8α⁺ αβ T cells in the same samples (Figure 2A, B). We also found increased frequencies of DP αβ T cells in the lungs and ghLN 7 dpi. There were no significant changes in the blood. In wild boar, we found increased DP αβ T-cell frequencies 5 dpi in the liver, 5 and 7 dpi in the lungs and 7 dpi in the blood (Figure 2B). Alterations in both subspecies were only temporary, as DP T cell frequencies returned to baseline levels in all animals 10 dpi.

γδ T cells are also known to express CD8α upon activation and differentiation into CD2⁺/ CD8α⁺ effector cells (Sedlak, Patzl, Saalmüller, & Gerner, 2014). We did not detect any changes in effector γδ T-cell frequencies in the investigated tissues in domestic pigs (Figure 3). In contrast, effector γδ T-cell frequencies in wild boar increased in spleen 5 and 7 dpi, and 7 dpi in lung. Most pronounced and persistent increases were found in the liver of infected animals 4 to 7 dpi (Figure 3). Comparable to DP αβ T cells, the changes were not permanent and effector γδ T-cell levels returned to control levels 10 dpi.

We also analyzed Ki-67 expression as a marker for proliferation in the second trial. We found pronounced proliferation of CD8αα⁺ and CD8αβ⁺ T cells in domestic pigs as well as in wild boar 10 dpi (Figure 4A, B). Proliferating cells were primarily found 10 dpi, but elevated frequencies of Ki-67⁺ CD8αα⁺ and CD8αβ⁺ T cells were also found 7 dpi in the spleen of domestic pigs. Regarding CD4⁺ αβ T cells, we found significantly increased frequencies of proliferating CD4⁺/CD8α⁻ T cells only in the spleen of both suid species 7 dpi. Moreover, DP T cells in domestic pigs proliferated significantly 7 and 10 dpi, again only in spleen (Supplemental figure

1). In contrast, we did not find Ki-67$^+$, proliferating γδ T cells in domestic pigs or wild boar (data not shown).

3.4 Perforin levels were decreased in cytotoxic T cells

To investigate the ability of cytotoxic lymphocytes to clear virus-infected cells, we analyzed the expression of one of the major cytotoxic effector molecules, perforin, in cytotoxic lymphocyte populations, CD8αα$^+$ and CD8αβ$^+$ αβ T cells, and effector γδ T cells (Figure 5).

In contrast to all other investigated tissues, perforin expression in CD8αα$^+$ T cells was increased 7 dpi in blood and spleen of domestic pigs (Figure 5A, B). Strikingly, it was reduced 4 dpi and virtually undetectable 5 dpi in the liver and ghLN of domestic pigs (Figure 5C, D). In wild boar, perforin expression decreased 5 dpi in the blood, but was elevated above control levels 7 and 10 dpi in blood, spleen, liver, and ghLN (Figure 5A-D).

CD8αβ$^+$ T cells showed a similar but more pronounced course with a significantly decreased expression of perforin 4 and 5 dpi in domestic pigs as well as wild boar in all investigated tissues (Figure 5A-D). Compared to the perforin expression in uninfected controls, the perforin loss in CD8αβ$^+$ T cells was even more distinct than in CD8αα$^+$ T cells. Perforin expression was back to control levels 7 dpi in all animals. In domestic pigs, another significant decrease of perforin occurred 10 dpi. This second reduction was less pronounced in wild boar.

CD8α$^+$ effector γδ T cells showed a perforin expression comparable to CD8αα$^+$ T cells. In domestic pigs, the perforin expression was increased 7 dpi in the blood (Figure 5A) but remained virtually unchanged in the spleen (Figure 5B). Wild boar showed a reduced perforin expression 5 dpi in blood and spleen. However, this effect was less pronounced as for the other T-cell populations and returned to control levels 7 and 10 dpi (Figure 5A, B). In the liver and ghLN, we detected slight perforin decreases in ghLN 10 dpi but no other significant expression changes in wild boar (Figure 5C, D). In contrast, perforin expression in domestic pigs showed a significant decrease 5 dpi in the liver, and 4 and 5 dpi in ghLN. After the cells regained their perforin expression levels 7 dpi, there was another loss 10 dpi (Figure 5C, D).

3.5 T-bet-dependent T-cell activation was found in wild boar but not domestic pigs

Expression of the T-box transcription factor TBX21 (T-bet) in αβ and γδ T cells was investigated as a marker for activation. Domestic pigs had relatively high levels of T-bet$^+$ cells even before the infection and thus, there was no further activation measurable over the course of the study. In blood and spleen, T-bet expression was decreased below baseline levels (Supplemental figure 2). In contrast, we detected increased frequencies of T-bet$^+$ CD4$^-$/CD8α$^+$ αβ T cells in spleen, lung, and liver of infected wild boar 10 dpi (Figure 6). Moreover, we found heightened proportions of T-bet$^+$ CD4$^+$/CD8α$^-$ αβ T cells 10 dpi and of all differentiation states of γδ T cells 7 and 10 dpi in the lung of infected wild boar (Figure 6).

3.6 Regulatory T cells were induced in both subspecies

FoxP3$^+$ cells among CD4$^+$/CD8α$^-$ and CD4$^+$/CD8α$^+$ T cells were analyzed to identify regulatory T-cell responses (Käser, Gerner, & Saalmüller, 2011). In spleen, lung, and ghLN, Treg frequencies increased significantly 7 or 10 dpi in domestic pigs and wild boar, with the highest frequencies in the lungs of both subspecies (Figure 7). Interestingly, although Tregs were induced earlier in domestic pigs, Treg frequencies reached higher levels in wild boar. Of note, there were more DP Tregs than CD4$^+$/CD8α$^-$ Tregs in both subspecies (Figure 7).

3.7 iNKT cells in domestic pigs were activated

During infection with highly virulent ASFV “Armenia08”, we described iNKT-cell frequency fluctuations in disease-affected tissues (Schäfer et al., 2019). In trial 2 of this study, we investigated iNKT-cell frequency and expression of activation markers (CD25, ICOS, and MHC II) and maturation markers (CD4 and CD8α) on iNKT cells of domestic pigs according to previous findings (Schäfer et al., 2019). We did not find changes in iNKT-cell frequency or CD25 expression (Supplemental figure 3). ICOS was upregulated on iNKT cells in blood, spleen, and ghLN 7 and 10 dpi (Figure 8A). Moreover, frequencies of CD4$^-$/CD8α$^+$ and CD4$^+$/CD8α$^+$ iNKT cells increased in the blood of infected animals and to a lesser extent also in the spleen, lung, and liver (Figure 8A, B). Of note, MHC II was downregulated on iNKT cells in the blood 4 dpi

and in the liver 10 dpi (Figure 8D). We were not able to identify an iNKT-cell population in wild boar and thus could not investigate iNKT-cell responses in those animals.

4 Discussion

The porcine T-cell response against ASFV infection is largely not understood. Moreover, knowledge about the responses of susceptible *Suidae* subspecies outside of Africa, domestic pigs (*Sus scrofa domesticus*) and wild boar (*Sus scrofa scrofa*), is scarce. Similar to our first approach to analyze these differences upon infection with the highly virulent ASFV strain "Armenia08" (Hühr et al., 2020), we used a multicolor flow cytometry platform to investigate T-cell responses in domestic pigs and wild boar after infection with the moderately virulent ASFV isolate, "Estonia2014".

The most fundamental characteristics of an ongoing immune response are local or systemic alterations in the composition of leukocytes in tissues affected by disease. As one of the few known parameters of protection against ASFV infection, the importance of (cytotoxic) $CD8\alpha^+$ lymphocytes has been demonstrated, although to a limited extent only. It has been shown that isolated porcine PBMC, after *in vivo* priming with virulent ASFV, were able to specifically lyse ASFV-infected cells *in vitro* (Norley & Wardley, 1984). This was a first indication of virus-specific cytotoxicity. However, PBMCs were not differentiated and protection was not investigated. SLA I- and CD8-dependent lysis of ASFV-infected target cells by PBMCs from ASFV-immune minipigs (Martins, Lawman, Scholl, Mebus, & Lunney, 1993) and specific lysis of isolated $CD8\alpha^+$ but not $CD4^+$ T cells (F. Alonso, Domínguez, Viñuela, & Revilla, 1997) has also been shown. In another study, antibody-dependent depletion of $CD8\alpha^+$ cells *in vivo* in domestic pigs primed with the low virulent ASFV strain "OUR/T88/3" resulted in loss of protection after homologous challenge with the virulent ASFV strain "OUR/T88/1" (C. A. Oura et al., 2005). We could show that the CD8α response in wild boar and domestic pigs during infection with moderately virulent ASFV is based primarily on increases of $CD4^-/CD8\alpha^+$ and to a lesser extent of $CD4^+/CD8\alpha^+$ (DP) T cells. Interestingly, in our previous study with the highly virulent ASFV "Armenia08", the CD8α response was primarily based on DP T cells (Hühr et al., 2020). $CD4^+/CD8\alpha^+$ DP T cells are often described to possess memory functions (Gerner, Kaser, & Saalmüller, 2009). ASFV-specific memory responses can be excluded during experimental ASFV infection with naïve pigs. However, ASFV-specific responses might play a

role in field infections since serological evidence for previous ASFV infections was found in hunted animals (Nurmoja et al., 2017). On the other hand, DP memory T cells in the spleen might be activated independent of their cognate antigen by release of IL-15 or IL-18 (Soudja, Ruiz, Marie, & Lauvau, 2012). Porcine DP T cells are also described to exhibit effector functions, like cytotoxic responses or cytokine production (Gerner et al., 2009). However, in contrast to CD8αα$^+$ or CD8αβ$^+$ αβ T cells, we only found proliferating DP T cells in the spleen but not in other tissues. Moreover, the pronounced loss of perforin in other cytotoxic T-cell populations was not found in DP T cells. This might indicate that DP T cells are orchestrators of systemic responses but do not take part in antiviral responses in disease-affected tissue during moderately virulent ASFV infection.

Pigs belong to a group of mammals with relatively high frequencies of γδ T cells. They can exert effector functions like cytokine production and cytotoxicity, and are even able to present antigens to other lymphocytes (Sedlak et al., 2014). The main effector population is characterized as CD2$^+$/CD8α$^+$ (Sedlak et al., 2014). In the present study, we found pronounced increases of effector γδ T-cell frequencies in spleen, lung, and liver of infected wild boar but not domestic pigs. Moreover, we detected T-bet-dependent activation of γδ T cells in wild boar only. This is in line with our previous findings during highly virulent ASFV infection, where wild boar were found to have a considerably stronger bias for γδ T-cell responses (Hühr et al., 2020). This indicates a profound dissimilarity in the antiviral responses of both subspecies and might give an explanation for their different disease severity and survival. Of note, this is in contrast to previous findings, where higher frequencies of circulating γδ T cells correlated with increased survival of infection with moderately virulent ASFV, independent of age or virus dose (Post, Weesendorp, Montoya, & Loeffen, 2017). There might be some explanations for this discrepancy. First, we detected increased lethality and heightened frequencies of effector γδ T cells in wild boar, while the aforementioned study used domestic pigs. While we were also not able to detect changes of γδ T-cell frequencies in domestic pigs, this might be caused by different ASFV strains used. Finally, the numbers of survivors that showed correlations with γδ T-cell levels in the study by Post et al. were relatively small. This underlines the need for in-depth research not only during ASFV infection in general but also for the differences between wild boar and domestic pigs. Moreover, since infection of professional antigen-presenting cells alter their function (Gomez-Villamandos et al., 2013), it would be of interest to investigate whether γδ T cells take part in the antigen presentation during ASFV infection.

We found regulatory T cells (Tregs) in both subspecies but higher frequencies in wild boar. The role of Tregs during ASFV infection is largely unexplored. However, previous studies showed that Tregs might present a way of viral immune evasion because they were able to inhibit specifically antiviral responses (Sánchez-Cordón, Jabbar, Chapman, Dixon, & Montoya, 2020). Higher percentages of Tregs in wild boar might therefore be an explanation for their higher disease burden in this study and lethality previously observed (Zani et al., 2018). In a parallel study by Sehl *et al.* using histopathology from tissues of trial 2 of this study, domestic pigs but not wild boar showed lymphohistiocytic interstitial pneumonia even 10 dpi (Sehl et al., 2020). This might be a sign for prolonged pro-inflammatory responses in domestic pigs in contrast to wild boar. This is in line with our findings of higher Treg frequencies in wild boar. Moreover, this indicates that pro-inflammatory responses are able to counteract ASFV infection, as long as they are not downregulated too early.

A porcine T-cell population that is still not well understood is invariant Natural Killer T (iNKT) cells. We could previously show that iNKT-cell frequencies significantly increased in some tissues during infection with highly virulent ASFV (Schäfer et al., 2019). Although at the time we were not able to investigate effector mechanisms or surface markers on iNKT cells *ex vivo*, our study provided first evidence that iNKT cells participate in the antiviral response during ASFV infection. The fact, that we were unable to find changes in the general iNKT-cell frequency might be explained by the less virulent ASFV strain in this study. Nevertheless, activation of iNKT cells was shown by significantly increased frequencies of $ICOS^+$ iNKT cells. ICOS is an essential protein for iNKT-cell activation, homeostasis, and survival (Gleimer, Boehmer, & Kreslavsky, 2012). Some studies correlated ICOS expression on iNKT cells with pro-inflammatory Th1 responses (Akbari et al., 2008; Kaneda et al., 2005), while others described it as a marker of effector iNKT cells (Burmeister et al., 2008). Increased expression of CD8α and CD4, as previously established markers of maturation of porcine iNKT cells (Schäfer et al., 2019), underlines these findings. Interestingly, a role for NKT cells has previously been suggested, as $CD3^+/CD4^-/CD8\alpha^+/CD5^\pm/CD6^-/CD11b^+/CD16^+$ cells expanded after *in vitro* stimulation of porcine PBMC with ASFV (Denyer, Wileman, Stirling, Zuber, & Takamatsu, 2006). However, even though we and others could show that the phenotype of iNKT cells differs from that finding (Schäfer et al., 2019), the significant alterations in iNKT-cell frequency in our first study and our findings in this study support the notion that iNKT cells take part in the antiviral response against ASFV.

Besides analysis of the cellular composition of leukocytes in the investigated tissues, effector functions are also pivotal to understand the underlining immune mechanisms. Perforin is one of the major lytic molecules used by cytotoxic lymphocytes to kill target cells (Prager & Watzl, 2019). Instead of direct cell lysis, cytotoxic lymphocytes can also induce apoptosis in their target cells by death receptor-mediated pathways using Fas ligand (FasL) or TRAIL (Prager & Watzl, 2019). The significant and partially complete loss of perforin 4 to 5 dpi in this study resembled the observed loss of perforin we found during infection with the highly virulent ASFV strain "Armenia08" (Hühr et al., 2020). However, the perforin decrease was more pronounced during infection with highly virulent ASFV, especially on a systemic level, i.e. in the spleen. There are various explanations for the perforin loss observed in both studies. Perforin-mediated killing is thought to be the major pathway in the early cytotoxic response but can be switched to Fas/FasL-mediated apoptosis induction with a complete loss of perforin expression on RNA and protein level during the course of infection (Meiraz, Garber, Harari, Hassin, & Berke, 2009). An effector molecule switch might be an explanation for the observations in our study, however, this is not directly detectable because antibodies against porcine FasL are still missing. Still, there are some lines of evidence suggesting this might be the case. Expression of viral homologues of the mammalian anti-apoptotic protein Bcl-2 (Afonso, Neilan, Kutish, & Rock, 1996) and also prevention of apoptosis by these viral homologues has been shown for ASFV strains (Galindo, Hernaez, Díaz-Gil, Escribano, & Alonso, 2008). Bcl-2 is also known to preferentially inhibit perforin-mediated apoptosis but less Fas/FasL-mediated apoptosis, depending on the cellular target (Sutton, Vaux, & Trapani, 1997). A switch from perforin-mediated to Fas/FasL-mediated cytotoxic responses might therefore be beneficial and protective and would be in line with the lower disease severity and heightened survival of domestic pigs. Wild boar, in contrast, had higher levels of perforin^{+} lymphocytes on average, indicating that they might not have switched the cytotoxic pathway or at least not to the extent that domestic pigs did. Given that inhibition of perforin has been shown to protect from tissue damage during viral hepatitis (Welz et al., 2018), this might also be an explanation for the more severe inflammation and tissue degradation in the liver of infected wild boar (Sehl et al., 2020). On the other hand, it cannot be excluded that we missed newly synthesized and immediately secreted perforin, which is not detected by antibody clone dG9 used in this study (Hersperger, Makedonas, & Betts, 2008). However, missing detection due to immediate secretion would still hint to a strong cytotoxic response. In this case, the

cytotoxic response would have been higher in domestic pigs because the perforin loss was detected earlier and more pronounced than in wild boar. Therefore, it can be hypothesized that the more pronounced and earlier response in domestic pigs was beneficial and protective at least during infection with moderately virulent ASFV. Wild boar, in contrast, might not have been able to counter the infection because of their impaired response, eventually leading to death as observed in previous studies (Zani et al., 2018). Which explanation holds true, switch of cytotoxic pathways or earlier and stronger cytotoxic response, has to be investigated in future studies. It still has to be kept in mind that cytotoxic responses are not only beneficial but might also cause immunopathology and thus, contribute to disease burden (Duan & Thomas, 2016).

In summary, we described the first comparative analysis of immune responses of wild boar and domestic pigs during moderately virulent ASFV infection. While more severe in wild boar, domestic pigs showed signs of moderate disease and recovery of all animals. Overall, we found comparable courses of immunity in both subspecies. Both developed a heavily $CD8\alpha^+$-biased response with proliferation of $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ cells. However, although their αβ T-cell responses were largely similar, wild boar developed a more pronounced effector γδ T-cell response. We also found only small signs of T-bet-dependent activation predominately in lungs and liver of wild boar but none in domestic pigs. Moreover, we found a distinct loss of perforin in cytotoxic T cells in domestic pigs and to a lesser extent also in wild boar, similar to previous results during infection with highly virulent ASFV “Armenia08”. Tregs appeared in higher levels in wild boar. Finally, we were able show the first description of functional iNKT-cell responses during ASFV infection. With this data, our study paves the way for further in-depth analyses of porcine immunity towards ASFV.

Acknowledgements

The authors thank Silke Rehbein, Cathleen Klement, and Stefanie Knöfel for excellent technical assistance in the laboratory. For exceptional care of animals and support during the trials, we thank the animal caretakers Thomas Möritz, Steffen Brenz, Harald Manthei, and Frank Klipp. Furthermore, we thank Christian Loth for outstanding autopsy assistance. We are grateful to the NIH Tetramer Core Facility for providing the mCD1d tetramer.

Ethics statement

All applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration in the present study. The animal experiments were approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) under reference numbers LALLF 7221.3-1.1-064/17 and LALLF 7221.3-2-011/19.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceived and designed experiments: TCM, AB, SB, UB. Acquired animal samples: LZ, JP, JS, JH, AB, SB, UB. Sample processing: AS, JP, JH. Data analysis and interpretations: AS, UB. Manuscript preparation: AS, TCM, SB, UB. All authors reviewed and approved the final version of the manuscript.

Funding

This work was funded through the Friedrich-Loeffler-Institut African Swine Fever Virus Research Network 'ASF-ResNet'.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

References

Afonso, C. L., Neilan, J. G., Kutish, G. F., & Rock, D. L. (1996). An African swine fever virus Bc1-2 homolog, 5-HL, suppresses apoptotic cell death. *Journal of Virology*, *70*(7), 4858–4863. https://doi.org/10.1128/JVI.70.7.4858-4863.1996

Akbari, O., Stock, P., Meyer, E. H., Freeman, G. J., Sharpe, A. H., Umetsu, D. T., & DeKruyff, R. H. (2008). Icos/icosl interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *Journal of Immunology (Baltimore, Md. : 1950)*, *180*(8), 5448–5456. https://doi.org/10.4049/jimmunol.180.8.5448

Alonso, C., Borca, M., Dixon, L., Revilla, Y., Rodriguez, F., Escribano, J. M., & Ictv, R. C. (2018). Ictv Virus Taxonomy Profile: Asfarviridae. *The Journal of General Virology*, *99*(5), 613–614. https://doi.org/10.1099/jgv.0.001049

Alonso, F., Domínguez, J., Viñuela, E., & Revilla, Y. (1997). African swine fever virus-specific cytotoxic T lymphocytes recognize the 32 kDa immediate early protein (vp32). *Virus Research*, *49*(2), 123–130. https://doi.org/10.1016/S0168-1702(97)01459-7

Burmeister, Y., Lischke, T., Dahler, A. C., Mages, H. W., Lam, K.-P., Coyle, A. J., . . . Hutloff, A. (2008). Icos controls the pool size of effector-memory and regulatory T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *180*(2), 774–782. https://doi.org/10.4049/jimmunol.180.2.774

Carrasco, L., Gómez-Villamandos, J. C., Bautista, M. J., Martín de las Mulas, J., Villeda, C. J., Wilkinson, P. J., & Sierra, M. A. (1996). In vivo replication of African swine fever virus (Malawi '83) in neutrophils. *Veterinary Research*, *27*(1), 55–62.

Colgrove, G. S., Haelterman, E. O., & Coggins, L. (1969). Pathogenesis of African swine fever in young pigs. *American Journal of Veterinary Research*, *30*(8), 1343–1359.

Denyer, M. S., Wileman, T. E., Stirling, C. M., Zuber, B., & Takamatsu, H. H. (2006). Perforin expression can define CD8 positive lymphocyte subsets in pigs allowing phenotypic and functional analysis of natural killer, cytotoxic T, natural killer T and MHC un-restricted cytotoxic T-cells. *Vet Immunol Immunopathol*, *110*(3-4), 279–292. https://doi.org/10.1016/j.vetimm.2005.10.005

Duan, S., & Thomas, P. G. (2016). Balancing Immune Protection and Immune Pathology by CD8(+) T-Cell Responses to Influenza Infection. *Frontiers in Immunology*, *7*, 25. https://doi.org/10.3389/fimmu.2016.00025

Eguchi-Ogawa, T., Matsubara, T., Toki, D., Okumura, N., Ando, A., Kitagawa, H., & Uenishi, H. (2018). Distribution of the CD4 Alleles in Sus scrofa Demonstrates the Genetic Profiles of Western Breeds and Miniature Pigs. *Animal Biotechnology*, *29*(3), 227–233. https://doi.org/10.1080/10495398.2017.1367691

Galindo, I., Hernaez, B., Díaz-Gil, G., Escribano, J. M., & Alonso, C. (2008). A179l, a viral Bcl-2 homologue, targets the core Bcl-2 apoptotic machinery and its upstream BH3 activators with selective binding restrictions for Bid and Noxa. *Virology*, *375*(2), 561–572. https://doi.org/10.1016/j.virol.2008.01.050

Gaudreault, N. N., Madden, D. W., Wilson, W. C., Trujillo, J. D., & Richt, J. A. (2020). African Swine Fever Virus: An Emerging DNA Arbovirus. *Frontiers in Veterinary Science*, *7*, 215. https://doi.org/10.3389/fvets.2020.00215

Gerner, W., Kaser, T., & Saalmüller, A. (2009). Porcine T lymphocytes and NK cells--an update. *Dev Comp Immunol*, *33*(3), 310–320. https://doi.org/10.1016/j.dci.2008.06.003

Gleimer, M., Boehmer, H. von, & Kreslavsky, T. (2012). Plzf Controls the Expression of a Limited Number of Genes Essential for NKT Cell Function. *Frontiers in Immunology*, *3*, 374. https://doi.org/10.3389/fimmu.2012.00374

Gomez-Villamandos, J. C., Bautista, M. J., Sanchez-Cordon, P. J., & Carrasco, L. (2013). Pathology of African swine fever: the role of monocyte-macrophage. *Virus Res*, *173*(1), 140–149. https://doi.org/10.1016/j.virusres.2013.01.017

Hersperger, A. R., Makedonas, G., & Betts, M. R. (2008). Flow cytometric detection of perforin upregulation in human CD8 T cells. *Cytometry a*, *73*(11), 1050–1057. https://doi.org/10.1002/cyto.a.20596

Hühr, J., Schäfer, A., Schwaiger, T., Zani, L., Sehl, J., Mettenleiter, T. C., . . . Blohm, U. (2020). Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain. *Transbound Emerg Dis.* Advance online publication. https://doi.org/10.1111/tbed.13678

Jori, F., & Bastos, A. D. (2009). Role of wild suids in the epidemiology of African swine fever. *Ecohealth*, *6*(2), 296–310. https://doi.org/10.1007/s10393-009-0248-7

Kaneda, H., Takeda, K., Ota, T., Kaduka, Y., Akiba, H., Ikarashi, Y., . . . Okumura, K. (2005). Icos costimulates invariant NKT cell activation. *Biochemical and Biophysical Research Communications*, *327*(1), 201–207. https://doi.org/10.1016/j.bbrc.2004.12.004

Käser, T., Gerner, W., & Saalmüller, A. (2011). Porcine regulatory T cells: Mechanisms and T-cell targets of suppression. *Developmental and Comparative Immunology*, *35*(11), 1166–1172. https://doi.org/10.1016/j.dci.2011.04.006

Martins, C. L., Lawman, M. J., Scholl, T., Mebus, C. A., & Lunney, J. K. (1993). African swine fever virus specific porcine cytotoxic T cell activity. *Archives of Virology*, *129*(1-4), 211–225. https://doi.org/10.1007/BF01316896

Mason-D'Croz, D., Bogard, J. R., Herrero, M., Robinson, S., Sulser, T. B., Wiebe, K., . . . Godfray, H. C. J. (2020). Modelling the global economic consequences of a major African swine fever outbreak in China. *Nature Food*, *1*(4), 221–228. https://doi.org/10.1038/s43016-020-0057-2

Meiraz, A., Garber, O. G., Harari, S., Hassin, D., & Berke, G. (2009). Switch from perforin-expressing to perforin-deficient CD8(+) T cells accounts for two distinct types of effector cytotoxic T lymphocytes in vivo. *Immunology*, *128*(1), 69–82. https://doi.org/10.1111/j.1365-2567.2009.03072.x

Norley, S. G., & Wardley, R. C. (1984). Cytotoxic lymphocytes induced by African swine fever infection. *Research in Veterinary Science*, *37*(2), 255–257.

Nurmoja, I., Petrov, A., Breidenstein, C., Zani, L., Forth, J. H., Beer, M., . . . Blome, S. (2017). Biological characterization of African swine fever virus genotype II strains from north-eastern Estonia in European wild boar. *Transbound Emerg Dis*, *64*(6), 2034–2041. https://doi.org/10.1111/tbed.12614

Oura, C. A., Denyer, M. S., Takamatsu, H., & Parkhouse, R. M. (2005). In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J Gen Virol*, *86*(Pt 9), 2445–2450. https://doi.org/10.1099/vir.0.81038-0

Oura, C. A. L., Powell, P. P., Anderson, E., & Parkhouse, R. M. E. (1998). The pathogenesis of African swine fever in the resistant bushpig. *Journal of General Virology*, *79*, 1439–1443. https://doi.org/10.1099/0022-1317-79-6-1439

Pietschmann, J., Guinat, C., Beer, M., Pronin, V., Tauscher, K., Petrov, A., . . . Blome, S. (2015). Course and transmission characteristics of oral low-dose infection of domestic pigs and European wild boar with a Caucasian African swine fever virus isolate. *Archives of Virology*, *160*(7), 1657–1667. https://doi.org/10.1007/s00705-015-2430-2

Pietschmann, J., Mur, L., Blome, S., Beer, M., Perez-Sanchez, R., Oleaga, A., & Sanchez-Vizcaino, J. M. (2016). African swine fever virus transmission cycles in Central Europe: Evaluation of wild boar-soft tick contacts through detection of antibodies against Ornithodoros erraticus saliva antigen. *BMC Vet Res*, *12*, 1. https://doi.org/10.1186/s12917-015-0629-9

Pikalo, J., Zani, L., Hühr, J., Beer, M., & Blome, S. (2019). Pathogenesis of African swine fever in domestic pigs and European wild boar - Lessons learned from recent animal trials. *Virus Res*, *271*, 197614. https://doi.org/10.1016/j.virusres.2019.04.001

Post, J., Weesendorp, E., Montoya, M., & Loeffen, W. L. (2017). Influence of Age and Dose of African Swine Fever Virus Infections on Clinical Outcome and Blood Parameters in Pigs. *Viral Immunology*, *30*(1), 58–69. https://doi.org/10.1089/vim.2016.0121

Prager, I., & Watzl, C. (2019). Mechanisms of natural killer cell-mediated cellular cytotoxicity. *Journal of Leukocyte Biology*, *105*(6), 1319–1329. https://doi.org/10.1002/JLB.MR0718-269R

Sánchez-Cordón, P. J., Jabbar, T., Chapman, D., Dixon, L. K., & Montoya, M. (2020). Absence of Long-Term Protection in Domestic Pigs Immunized with Attenuated African Swine Fever Virus Isolate

OURT88/3 or BeninΔMGF Correlates with Increased Levels of Regulatory T Cells and Interleukin-10. *Journal of Virology*, *94*(14). https://doi.org/10.1128/JVI.00350-20

Schäfer, A., Hühr, J., Schwaiger, T., Dorhoi, A., Mettenleiter, T. C., Blome, S., . . . Blohm, U. (2019). Porcine Invariant Natural Killer T Cells: Functional Profiling and Dynamics in Steady State and Viral Infections. *Front Immunol*, *10*, 1380. https://doi.org/10.3389/fimmu.2019.01380

Sedlak, C., Patzl, M., Saalmüller, A., & Gerner, W. (2014). CD2 and CD8alpha define porcine gammadelta T cells with distinct cytokine production profiles. *Dev Comp Immunol*, *45*(1), 97–106. https://doi.org/10.1016/j.dci.2014.02.008

Sehl, J., Pikalo, J., Schäfer, A., Franzke, K., Pannhorst, K., Elnagar, A., . . . Breithaupt, A. (2020). *Comparative Pathology of Domestic Pigs and Wild Boar Infected with the Moderately Virulent African Swine Fever Virus Strain "Estonia 2014".* https://doi.org/10.20944/preprints202007.0747.v1

Soudja, S. M.'H., Ruiz, A. L., Marie, J. C., & Lauvau, G. (2012). Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity*, *37*(3), 549–562. https://doi.org/10.1016/j.immuni.2012.05.029

Sutton, V. R., Vaux, D. L., & Trapani, J. A. (1997). Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes. *Journal of Immunology (Baltimore, Md. : 1950)*, *158*(12), 5783–5790.

Welz, M., Eickhoff, S., Abdullah, Z., Trebicka, J., Gartlan, K. H., Spicer, J. A., . . . Kastenmüller, W. (2018). Perforin inhibition protects from lethal endothelial damage during fulminant viral hepatitis. *Nature Communications*, *9*(1), 4805. https://doi.org/10.1038/s41467-018-07213-x

Zani, L., Forth, J. H., Forth, L., Nurmoja, I., Leidenberger, S., Henke, J., . . . Blome, S. (2018). Deletion at the 5'-end of Estonian ASFV strains associated with an attenuated phenotype. *Scientific Reports*, *8*(1), 6510. https://doi.org/10.1038/s41598-018-24740-1

Tables

Table 1 Experimental design.

	day	0	1	2	3	4	5	6	7	8	9	10	Controls
Trial 1	WB	Infection					4		4				4
	DP						4		4				4
Trial 2	WB					3			3			3	3
	DP					3			3			3	2

WB, wild boar; DP, domestic pigs

Table 2 Antibodies used in this study.

Marker	Clone	Isotype	Conjugate	Source	Dilution
CD2	MSA4	mouse IgG2a	—	in-house	1:100
CD3ε	PPT3	mouse IgG1	APC	Southern Biotech	1:500
CD3ε	PPT3	mouse IgG1	PE	Southern Biotech	1:500
CD4a	74-12-4	mouse IgG2b	PerCp-Cy5.5	BD	1 :100
CD8a	76-2-11	mouse IgG2a	FITC	Southern Biotech	1:100
CD8b	PG164A	mouse IgG2a	—	in-house	1:1000
Foxp3	FJK-16s	mouse IgG1	PE-Cy7	ebioscience	1:200
gdTCR	PPT16	mouse IgG2b	—	in-house	1:100
Ki-67	B56	mouse IgG1	BV421	BD Biosciences	1:40
Perforin	dG9	mouse IgG2b	Alexa 647	Biolegend	1:20
T-bet	4B10	mouse IgG1	APC	Biolegend	1:500
IgG2a	polyclonal	Goat IgG	APC-Cy7	Southern Biotech	1:250
IgG2b	polyclonal	Goat IgG	PE	Southern Biotech	1:400
IgG2b	polyclonal	Goat IgG	PE-Cy7	Southern Biotech	1:400

Figure legends

Figure 1 T-cell subset variations in various tissues upon moderately virulent ASFV infection. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. $CD3^+$ lymphocytes were subdivided into γδ T cell receptor $(TCR)^-$ αβ T cells and γδ TCR^+ γδ T cells. Frequency of (**A**) αβ and (**B**) γδ T cells in blood, spleen, lung, liver, and ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Figure 2 Increasing frequencies of $CD8\alpha^+$ αβ T cells. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. $CD3^+$ αβ T cells were analyzed for CD4 and CD8α expression. Frequency of (**A**) $CD4^-/CD8\alpha^+$ αβ T cells and (**B**) $CD4^+/CD8\alpha^+$ αβ T cells in blood, spleen, lung, liver, and ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Figure 3 Increasing frequencies of $CD8\alpha^+$ effector γδ T cells in wild boar. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. $CD3^+$ γδ T cells were analyzed for CD8α expression. Frequency of $CD8\alpha^+$ γδ T cells (**A**) spleen, (**B**) lung, (**C**) liver, and (**D**) ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Figure 4 Increasing frequencies of proliferating $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ αβ T cells. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. $CD8\alpha^+$ αβ T cells were subdivided based on their expression of the CD8β chain. Frequency of (**A**) $CD8\alpha\alpha^+$ and (**B**) $CD8\alpha\beta^+$ T cells in blood, spleen, lung, and liver. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$.

Figure 5 Loss of perforin expression in cytotoxic T cells. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes were isolated. (**A**) CD8αα$^+$ and CD8αβ$^+$ αβ T cells, and (**B**) CD2$^+$/CD8α$^+$ effector γδ T cells were investigated for perforin expression in the indicated tissues. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Figure 6 T-bet-dependent T-cell activation in wild boar. At the indicated time points, wild boar were euthanized and lymphocytes were isolated. (**A**) CD4$^+$/CD8α$^-$, CD4$^-$/CD8α$^+$, and CD4$^+$/CD8α$^+$ αβ T cells (from left to right) as well as (**B**) activated and effector γδ T cells were analyzed for their expression of T-bet. T-bet expression in domestic pigs for comparison can be found in Supplemental figure 2. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Figure 7 FoxP3$^+$ regulatory T cells appeared in domestic pigs and wild boar. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. CD4$^+$/CD8α$^-$ or CD4$^+$/CD8α$^+$ αβ T cells were analyzed for their expression of FoxP3. Frequency of (**A**) FoxP3$^+$/CD4$^+$/CD8α$^-$ and (**B**) FoxP3$^+$/CD4$^+$/CD8α$^+$ T cells in blood, spleen, and ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Figure 8 Activation and differentiation of iNKT cells in domestic pigs. At the indicated time points, domestic pigs were euthanized and lymphocytes of the respective tissues were isolated. CD3$^+$ mCD1d tetramer$^+$ iNKT cells were analyzed by flow cytometry. Frequency of iNKT cells positive for (**A**) ICOS, (**B**) MHC II, (**C**) CD8α, and (**D**) CD4/CD8α. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Supplemental figure 1 Proliferation of $CD4^+/CD8\alpha^-$ and $CD4^+/CD8\alpha^+$ αβ T cells in the spleen. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. Frequency of (**A**) $CD4^+/CD8\alpha^-$ and (**B**) $CD4^+/CD8\alpha^+$ T cells in spleen. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$.

Supplemental figure 2 No evidence of T-bet-dependent activation in domestic pigs. At the indicated time points, domestic pigs (orange) were euthanized and lymphocytes of the respective tissues were isolated. Frequency of $T\text{-}bet^+$ cells in (**A**) blood, (**B**) spleen, (**C**) liver, and (**D**) ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

Supplemental figure 3 Frequency and CD25 expression of iNKT cells in domestic pigs. At the indicated time points, domestic pigs were euthanized and lymphocytes of the respective tissues were isolated. $CD3^+$ mCD1d $tetramer^+$ iNKT cells were analyzed by flow cytometry. Frequency of iNKT cells (**A**) of $CD3^+$ cells and (**B**) positive for CD25. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

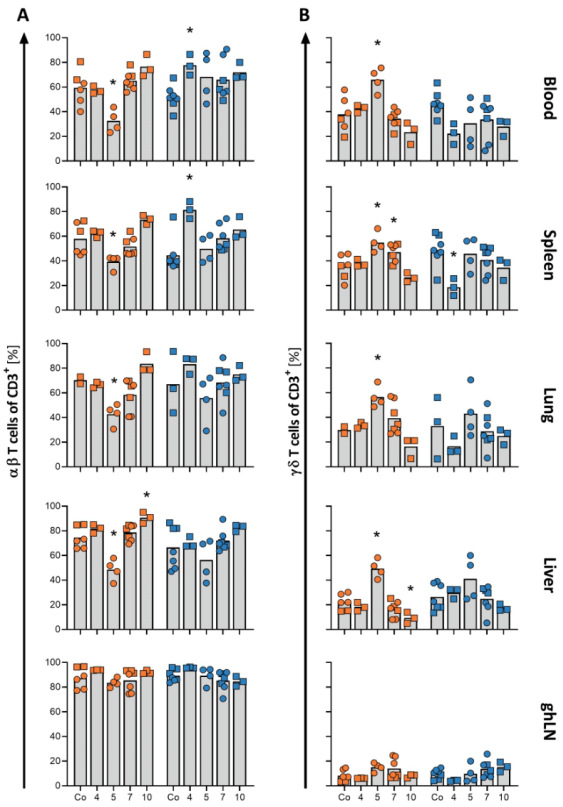


Figure 1 T-cell subset variations in various tissues upon moderately virulent ASFV infection. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. CD3+ lymphocytes were subdivided into γδ T cell receptor (TCR)– αβ T cells and γδ TCR+ γδ T cells. Frequency of (A) αβ and (B) γδ T cells in blood, spleen, lung, liver, and ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

190x275mm (300 x 300 DPI)

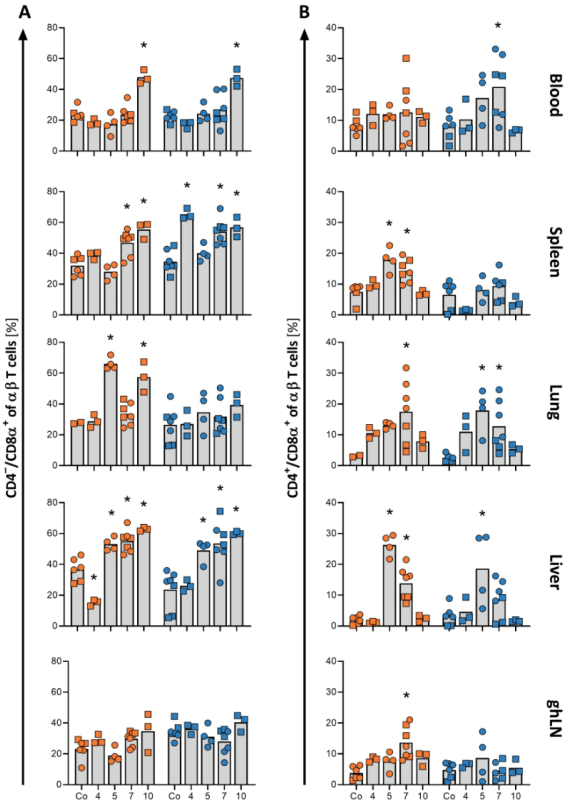


Figure 2 Increasing frequencies of CD8α+ αβ T cells. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. CD3+ αβ T cells were analyzed for CD4 and CD8α expression. Frequency of (A) CD4−/CD8α+ αβ T cells and (B) CD4+/CD8α+ αβ T cells in blood, spleen, lung, liver, and ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

190x275mm (300 x 300 DPI)

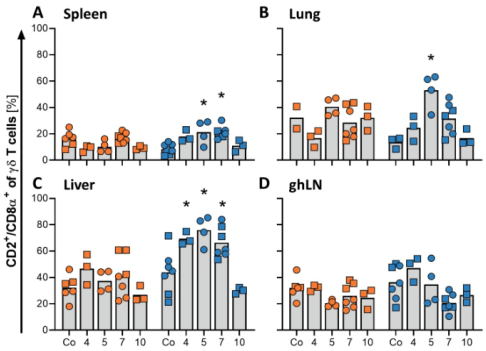


Figure 3 Increasing frequencies of CD8α+ effector γδ T cells in wild boar. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. CD3+ γδ T cells were analyzed for CD8α expression. Frequency of CD8α+ γδ T cells (A) spleen, (B) lung, (C) liver, and (D) ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

190x275mm (300 x 300 DPI)

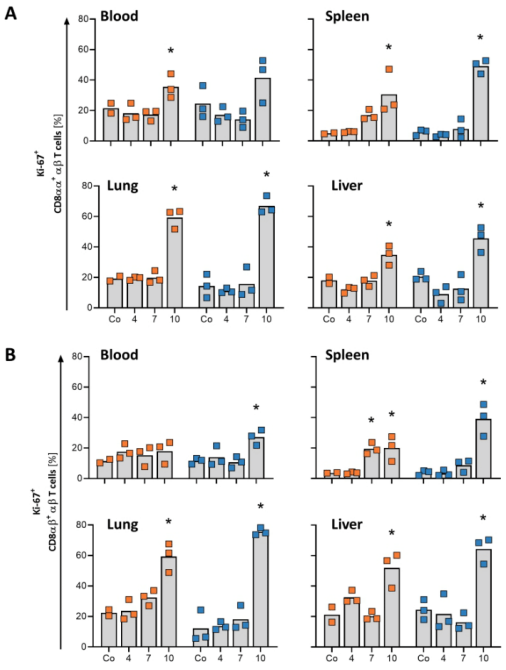


Figure 4 Increasing frequencies of proliferating CD8αα+ and CD8αβ+ αβ T cells. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. CD8α+ αβ T cells were subdivided based on their expression of the CD8β chain. Frequency of (A) CD8αα+ and (B) CD8αβ+ T cells in blood, spleen, lung, and liver. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$.

190x275mm (300 x 300 DPI)

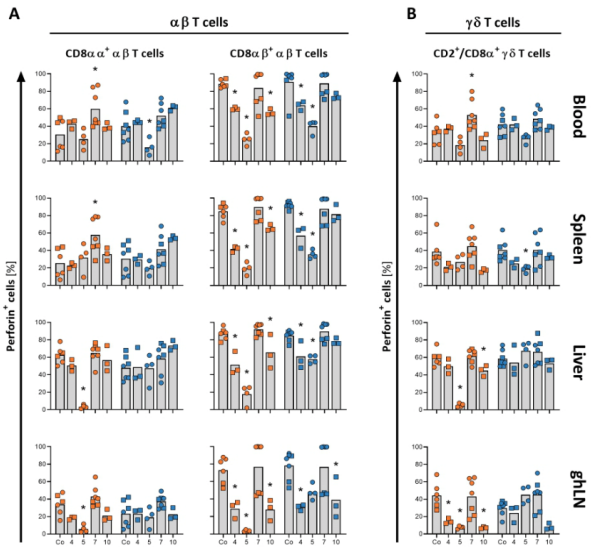


Figure 5 Loss of perforin expression in cytotoxic T cells. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes were isolated. (A) CD8αα+ and CD8αβ+ αβ T cells, and (B) CD2+/CD8α+ effector γδ T cells were investigated for perforin expression in the indicated tissues. Each point represents data from a single pig while bars represent the means for the designated time points. Data from trial 1 (●), data from trial 2 (■). *, $p < 0.05$. ghLN, gastro-hepatic lymph node

190x275mm (300 x 300 DPI)

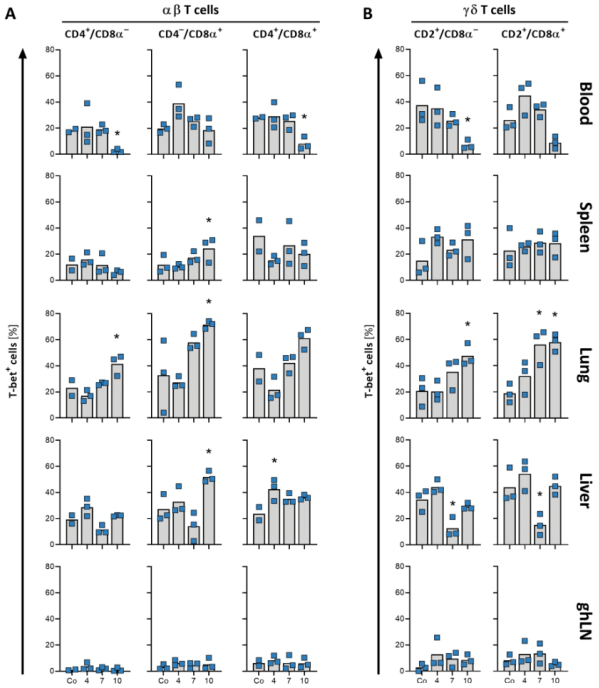


Figure 6 T-bet-dependent T-cell activation in wild boar. At the indicated time points, wild boar were euthanized and lymphocytes were isolated. (A) CD4+/CD8α−, CD4−/CD8α+, and CD4+/CD8α+ αβ T cells (from left to right) as well as (B) activated and effector γδ T cells were analyzed for their expression of T-bet. T-bet expression in domestic pigs for comparison can be found in Supplemental figure 2. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

190x275mm (300 x 300 DPI)

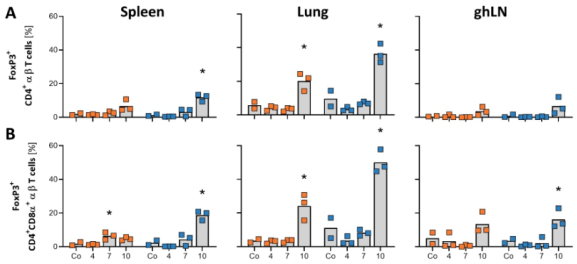


Figure 7 FoxP3+ regulatory T cells appeared in domestic pigs and wild boar. At the indicated time points, domestic pigs (orange) and wild boar (blue) were euthanized and lymphocytes of the respective tissues were isolated. CD4+/CD8α− or CD4+/CD8α+ αβ T cells were analyzed for their expression of FoxP3. Frequency of (A) FoxP3+/CD4+/CD8α− and (B) FoxP3+/CD4+/CD8α+ T cells in blood, spleen, and ghLN. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

190x275mm (300 x 300 DPI)

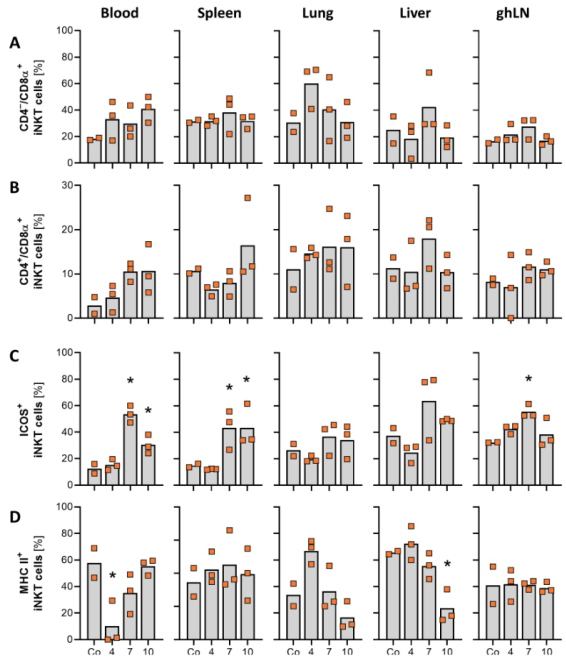
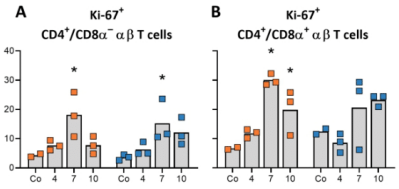
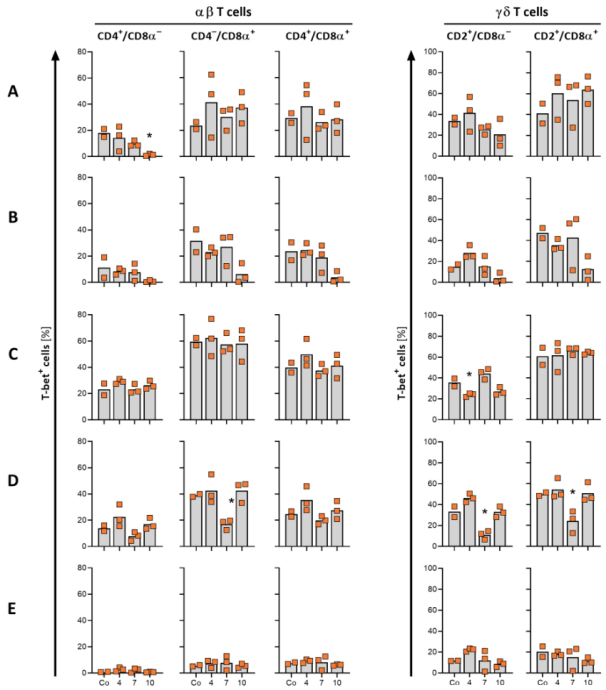


Figure 8 Activation and differentiation of iNKT cells in domestic pigs. At the indicated time points, domestic pigs were euthanized and lymphocytes of the respective tissues were isolated. CD3+ mCD1d tetramer+ iNKT cells were analyzed by flow cytometry. Frequency of iNKT cells positive for (A) ICOS, (B) MHC II, (C) CD8α, and (D) CD4/CD8α. Each point represents data from a single pig while bars represent the means for the designated time points. *, $p < 0.05$. ghLN, gastro-hepatic lymph node

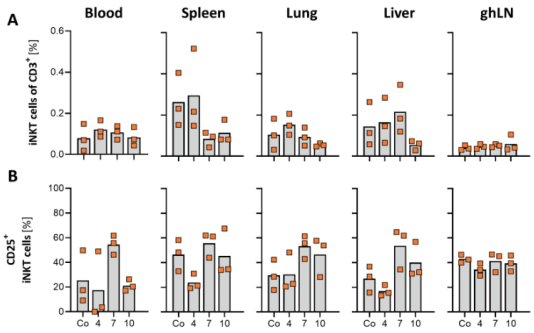
190x275mm (300 x 300 DPI)



190x275mm (300 x 300 DPI)



190x275mm (300 x 300 DPI)



190x275mm (300 x 300 DPI)

2.5 Publication V

Comparative Pathology of Domestic Pigs and Wild Boar Infected with the Moderately Virulent African Swine Fever Virus Strain "Estonia 2014"

Julia Sehl, Jutta Pikalo, **Alexander Schäfer**, Kati Franzke, Katrin Pannhorst, Ahmed Elnagar, Ulrike Blohm, Sandra Blome, Angele Breithaupt

Pathogens

doi: 10.3390/pathogens9080662

August 2020

pathogens

MDPI

Article

Comparative Pathology of Domestic Pigs and Wild Boar Infected with the Moderately Virulent African Swine Fever Virus Strain "Estonia 2014"

Julia Sehl, Jutta Pikalo, Alexander Schäfer, Kati Franzke, Katrin Pannhorst, Ahmed Elnagar, Ulrike Blohm, Sandra Blome * and Angele Breithaupt

Friedrich-Loeffler-Institut, 17493 Greifswald-Insel Riems, Germany; Julia.Sehl@fli.de (J.S.); Jutta.Pikalo@fli.de (J.P.); alexander.schaefer@fli.de (A.S.); kati.franzke@fli.de (K.F.); Katrin.Pannhorst@fli.de (K.P.); Ahmed.Elnagar@fli.de (A.E.); Ulrike.Blohm@fli.de (U.B.); Angele.Breithaupt@fli.de (A.B.)

* Correspondence: Sandra.Blome@fli.de

Received: 29 July 2020; Accepted: 13 August 2020; Published: 16 August 2020

Abstract: Endemically infected European wild boar are considered a major reservoir of African swine fever virus in Europe. While high lethality was observed in the majority of field cases, strains of moderate virulence occurred in the Baltic States. One of these, "Estonia 2014", led to a higher number of clinically healthy, antibody-positive animals in the hunting bag of North-Eastern Estonia. Experimental characterization showed high virulence in wild boar but moderate virulence in domestic pigs. Putative pathogenic differences between wild boar and domestic pigs are unresolved and comparative pathological studies are limited. We here report on a kinetic experiment in both subspecies. Three animals each were euthanized at 4, 7, and 10 days post infection (dpi). Clinical data confirmed higher virulence in wild boar although macroscopy and viral genome load in blood and tissues were comparable in both subspecies. The percentage of viral antigen positive myeloid cells tested by flow cytometry did not differ significantly in most tissues. Only immunohistochemistry revealed consistently higher viral antigen loads in wild boar tissues in particular 7 dpi, whereas domestic pigs already eliminated the virus. The moderate virulence in domestic pigs could be explained by a more effective viral clearance.

Keywords: African swine fever virus; virulence; pathology; wild boar; domestic pig; macroscopy; histopathology; immunology

1. Introduction

African swine fever (ASF) is a notifiable disease and one of the most important and serious threats to the pig industry today causing relevant global economic consequences [1]. The ASF virus (ASFV) belongs to the genus *Asfivirus* in the *Asfarviridae* family. The virus affects all species of the *Suidae* but only domestic pigs and Eurasian wild boar develop signs of a hemorrhagic fever like illness. Warthogs are considered reservoir hosts and do not display noticeable clinical signs. The same is probably true for other African wild suids. In Africa, the virus is endemic in sub-Saharan countries where it is vectored, especially in the sylvatic cycle with warthogs, by soft ticks of the genus *Ornithodorus* [2].

In 2007, ASF was introduced into Georgia and has then become a large-scale epidemic involving different European countries [3]. In 2018, the disease was detected in East Asia where it is progressively spreading [4–6].

Since (i) ASF established self-sustaining cycles, independent of ticks, and (ii) wild boar densities increased in the last decades, the wild boar is of particular importance in the spread of ASFV in Europe [7]. The ASFV strains circulating in Europe belong to the p72 genotype II, which are generally

highly virulent in both domestic pigs and wild boar and cause acute disease with almost 100% lethality in animals of all ages and sexes [8,9]. Experimental infection reveals an incubation time of approximately 3–5 days. Typical clinical findings include high fever, dullness, and anorexia, but also comprise vomiting, bloody diarrhea, reddening of the skin, respiratory disorders, abortion, or stillbirth as well as neurological signs [5,10–12]. Animals die within 7–13 days pi commonly showing enlarged, hemorrhagic lymph nodes, reddening of tonsils, splenomegaly, petechial hemorrhages in different organs such as the kidney, colon, or urinary bladder as well as lung, and gall bladder wall edema (reviewed in [13]).

Subacute and chronic forms are also reported, which result from infection with moderately or low virulent virus strains. Infected animals can survive and even recover from the disease [14]. Such an attenuated phenotype was reported for an Estonian ASFV strain. In 2014, ASF has affected the Estonian wild boar population, and subsequently spread to domestic pig holdings [15]. Mortality and morbidity was reported to be variable: in the south of Estonia, mortality was high in wild boar while in contrast, in the northeast of the country, mortality was strikingly low and anti-ASFV antibodies were detected in hunted animals [16]. Domestic pigs in general showed only unspecific clinical signs, typical hemorrhagic fever was less frequently reported. Severe disease was primarily present in pregnant and nursing sows [15]. An initial animal trial with a north-eastern Estonia Isolate (Ida-Viru region) in European wild boar resulted in acute, severe disease and clinical signs typical for acute ASF. All but one boar succumbed to the infection. The survivor recovered completely, showed high antibody titers and did not establish a carrier state as evidenced by the lack of transmission to sentinels [16]. A follow-up characterization of the isolate obtained from the survivor was conducted in potbelly-type minipigs and domestic pigs. In total, 75% survived, showing transient infection with mild clinical signs [17]. In contrast, all wild boar, which were subsequently inoculated with an isolate obtained from a surviving domestic, died. In a further comparative trial with domestic pigs and wild boar (with predetermined days of necropsy), only three suckling wild boar piglets recovered while all domestic pigs showed only transient fever (Hühr et al., unpublished data).

Although to some extent inconsistent with field observations, there is cumulative evidence that the north-eastern Estonia isolate is moderately virulent and attenuated in domestic pigs, but still highly virulent in adult wild boar. Genome sequence analysis revealed a 14.5 kilobase pair deletion at the 5'end of viral DNA, which is responsible for the attenuated phenotype in domestic pigs [17]. Comparative pathological studies on ASFV isolates in domestic pigs and wild boar are limited on precise, systematic and semi-quantitative evaluations of macroscopic and/or histopathologic lesions [18] as well as target cell identification. Thus, information on host factors for virulence is scarce.

We therefore aimed to compare the early pathogenesis of ASFV "Estonia 2014" in domestic pigs and wild boar. In a kinetic study, we investigated the clinical outcome for correlation with the macroscopic and histopathologic lesion, the viral antigen distribution and the cellular response to ASFV infection. For this purpose, we inoculated nine domestic pigs and nine wild boar with ASFV "Estonia 2014" [17] and monitored daily for clinical signs using a harmonized scoring system. Three animals each were euthanized and analyzed at 4, 7, and 10 days post infection (dpi).

2. Results

2.1. Clinical Disease

Clinical scores of animals were assessed based on the protocol published by Pietschmann et al. (2015). Following oronasal inoculation, the incubation period was 4 days in both subspecies. The animals showed general depression, anorexia, curved back, ataxia, respiratory distress, and increased recumbency. In brief, wild boar revealed higher clinical scores (up to score 10.5) than domestic pigs (up to score 5) and remaining domestic pigs recovered until day 10 (score 0), whereas wild boar still presented with apparent moderate disease (score 6–7) (Figure 1).

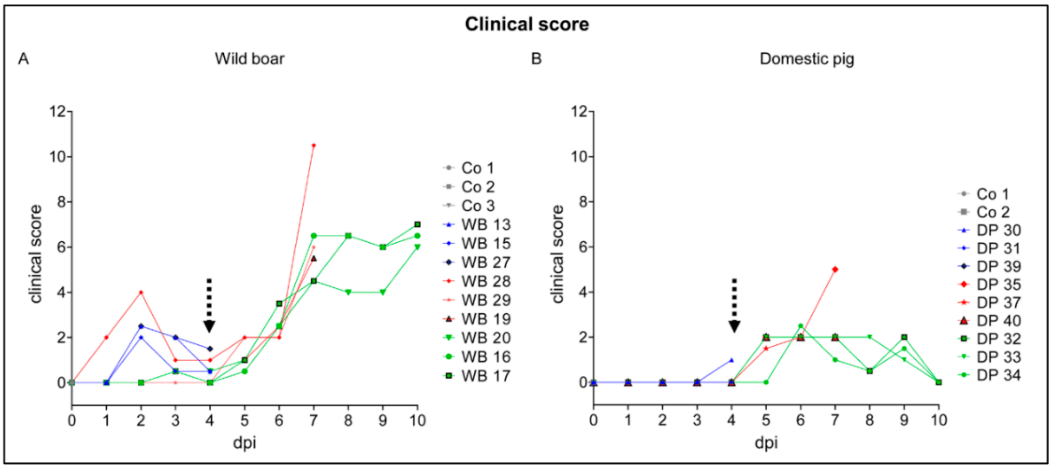


Figure 1. Clinical scores of animals were assessed based on the protocol published by Pietschmann et al. (2015). Clinical scores of wild boar (**A**) and domestic pigs (**B**) were determined daily over the study period. The dotted arrow marks the onset of African swine fever (ASF)-related clinical scores. Co = control, WB = wild boar, DP = domestic pig.

In detail, the clinical score of 5/12 wild boar (animals #13, #15, #17, #27, #28) was affected by signs not related to the infection from the very beginning. Hierarchic encounters led to lameness, slightly affected liveliness, posture, breathing, and laying (Figure 1A). These animals were evaluated with maximum 4 points before ASF became clinically apparent. Starting at 4 dpi, clinical signs typical for ASF were noted (animals #13, #15, #20, #27, #28) and affected all animals from day 5 onwards. The maximum clinical score of 10.5 was reached at 7 dpi (wild boar #28). The wild boar at 10 dpi had clinical scores from 6 to 7 points.

At 4 dpi, domestic pig #30 showed diarrhea (score 1), all other pigs were normal (Figure 1B). The clinical score in domestic pigs increased from day 4 to day 7 pi to maximum 5 points mainly affecting liveliness, position, breathing, feed intake, and walk. Until day 10 pi all remaining domestic pigs clinically recovered. Additionally, body temperature was measured daily in domestic pigs, fever (>40.0 °C) was recorded starting at day 6 pi, lasting until day 10 with up to 40.6 °C. Temperature profiles from 0 to 10 dpi of individual domestic pigs are shown in Supplementary Table S1. Domestic pigs and wild boar, which served as control animals, were clinically healthy.

2.2. Viral Genome Load in Blood and Tissues

Prior to inoculation, all animals were tested negative for ASF viral antigen, viral genome, and virus using lateral flow assays (LFD), ASFV-specific qPCR, and hemadsorption test (HAT), respectively. The back titration of the inoculated virus calculated according to Spearman and Kärber [19,20] verified the administered titer of $1 \times 10^{5.25}$ hemadsorbing units (HAU) per mL per pig.

The viral genome load in blood and tissue samples was tested by qPCR for correlation with the clinical course (Figure 2). Blood cells were further investigated by flow cytometry and the frequency of $p72^{+}$ myeloid subsets was determined. In correlation with the onset of clinical disease, ASFV genome was detectable from day 4 pi onwards in blood and tissues. The genome load in the blood peaked with comparable amounts in both subspecies on day 7 (wild boar: $10^{4.74}$ genome copies per µL, domestic pigs: $10^{4.35}$ genome copies per µL) and only slightly declined on day 10 pi (wild boar: $10^{4.41}$ genome copies per µL, domestic pigs: $10^{3.95}$ genome copies per µL). The highest ASFV genome loads in tissues were detected in the spleen ($10^{2.8}$–$10^{4.9}$ genome copies per µL), followed by the liver ($10^{1.9}$–$10^{4.2}$ genome copies per µL) and lung (10^{1}–$10^{4.1}$ genome copies per µL) and much lesser in the inguinal lymph node (10^{0}–$10^{3.4}$ genome copies per µL). Viral genome loads did not differ significantly at 4 dpi between wild boar and domestic pigs, but were slightly higher in wild boar compared to

domestic pigs at 7 dpi. Although the ASFV genome loads decreased at 10 dpi in all tissues of both subspecies, it was still above 10^3 genome copies per µL. At this time wild boar revealed significantly higher viral genome loads in the blood and liver.

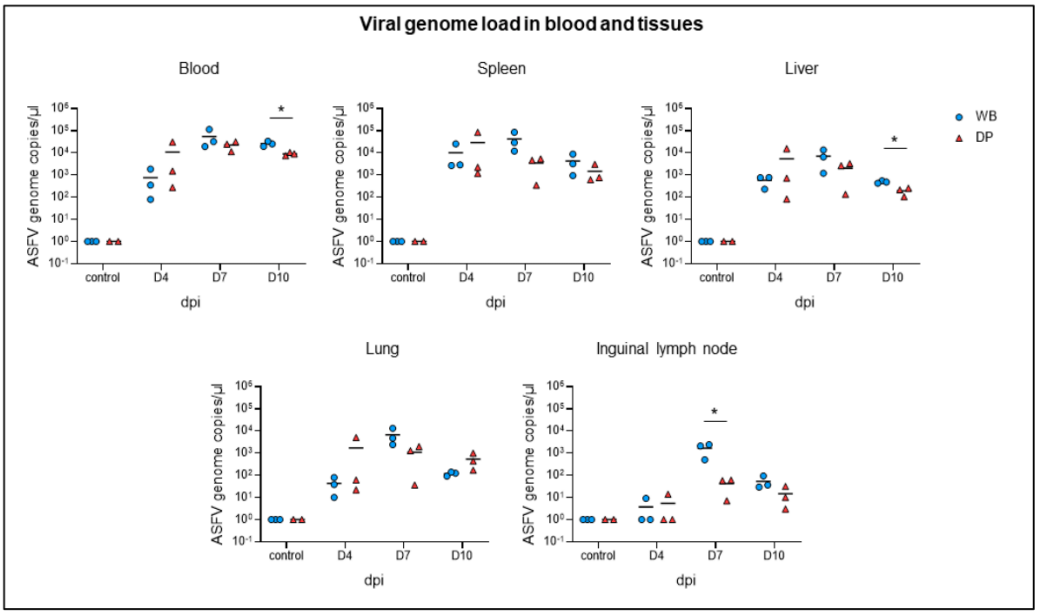


Figure 2. Detection of viral genome in blood and tissues (spleen, liver, lung, inguinal lymph node) of wild boar and domestic pigs by qRT-PCR. WB = wild boar, DP = domestic pig, * $p < 0.05$.

Flow cytometric analyses of blood samples showed, that the relative proportions of $FSC^{hi}/SSC^{hi}/CD172^{+}/CD14^{+}$ cells (granulocytes), $FSC^{med}/SSC^{low}/CD172^{+/}CD14^{+}$ cells (monocytes), and $FSC^{med}/SSC^{low}/CD172^{+}/CD14^{-}$ cells (dendritic cells) stayed within the range seen in control animals throughout the experiment. The proportion of $p72^{+}$ cells did not differ significantly between wild boar and domestic pigs. The relative amount of $p72^{+}$ monocytes and dendritic cells was below 20% at day 4 pi in both subspecies, slightly increased at day 7 pi but markedly peaked at day 10 pi with up to 90% of monocytes and approximately 40% of dendritic cells affected. There were no significant differences in the p72 mean fluorescence intensity (MFI) in both subspecies at day 10 pi (wild boar: DC 737 ± 68, monocytes 887 ± 38; domestic pig: DC 730 ± 132, monocytes 819 ± 107). The percentage of $p72^{+}$ granulocytes was high from the beginning in wild boar (40%) and domestic pigs (60%) and continuously increased until day 10 pi (up to 98% in both subspecies). The p72 MFI was higher in granulocytes compared to other myeloid populations but did not differ significantly between the two subspecies (wild boar: 1221 ± 67; domestic pig: 1408 ± 163).

2.3. Gross Pathology

Full autopsy was conducted on all animals. Standardized gross scoring was performed on the following organs based on Galindo-Cardiel et al. [21]: tonsils, spleen, lymph nodes (hepatogastric, renal and popliteal), lung (cranial and caudal lobes), liver with gall bladder, and kidneys. Gross lesions were generally mild to moderate and were detected in wild boar and domestic pigs from day 4 pi on. The severity of lesions increased over time in both subspecies but did not differ markedly.

Slightly enlarged, hemorrhagic lymph nodes were present on 4 dpi in 2/3 wild boar but not in domestic pigs. At 7 dpi 2/3 domestic pigs began to show mild to moderate enlargement of lymph nodes whereas only 1/3 wild boar was affected. At day 10 pi all domestic pigs and 2/3 wild boar showed mainly moderate enlargement. Lymph node hemorrhages, particularly affecting the hepatogastric and renal lymph node, were found in 1/3 wild boar at day 4 pi. All domestic pigs had hemorrhages at day 7.

At 10 dpi, 2/3 wild boar and all domestic pigs showed hemorrhages. Besides mild enlargement, mild hemorrhages were only rarely observed in the popliteal lymph node over the study period (4/9 wild boar, 4/9 domestic pig).

Moderate petechiae (cortico-medullar pattern) mainly affecting the renal cortex occurred at day 10 pi in all wild boar and in 2/3 domestic pigs. Gross appearance was largely comparable in both subspecies even though the amount of petechiae varied.

In the lung, up to moderate multifocal to coalescing consolidated areas were observed in 2/3 domestic pigs at 7 dpi, mainly affecting the cranial lobes (Figure 3A). On day 10 pi all domestic pigs revealed pulmonary lesions while only one wild boar showed a small consolidated area in the cranial lobe (Figure 3B).

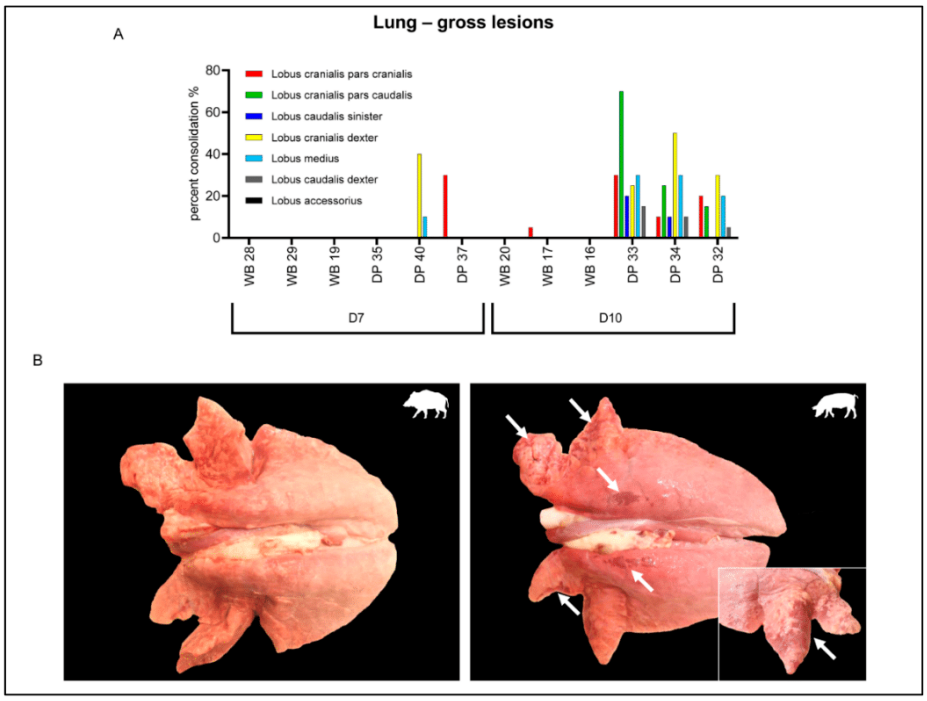


Figure 3. Gross lesions in the lung of wild boar and domestic pigs. (**A**) Macroscopic scoring of percentage pulmonary consolidation in lung lobes occurring at 7 and 10 days post infection (dpi) in wild boar and domestic pigs. (**B**) Lung lesions of WB and DP at day 10 pi. Arrows indicate consolidated areas. WB = wild boar, DP = domestic pig.

Occasional findings included moderate diffuse hemorrhagic gastritis in a wild boar at day 7 pi, edema of the gall bladder wall in a domestic pig at 10 dpi, severe multifocal myocardial necrosis and hemorrhage in a domestic pig on day 10, and intratonsillar abscesses in wild boar at day 7 and 10 pi. All findings were confirmed by histopathology. No lesions were found in the spleen. Macroscopically, no changes were observed in the control animals.

2.4. Histopathological, Immunological, and Electron Microscopical Analysis

From days 4, 7, and 10 pi hematoxylin-eosin stained sections of the spleen, hepatogastric and popliteal lymph node, palatine tonsil, bone marrow, lung, liver with gall bladder, kidney, cerebrum, and cerebellum were analyzed and scored semi-quantitatively based on Galindo-Cardiel et al. [21]. T-cell subsets of the immune cell infiltrates detected in the spleen, lung, liver, and hepatogastric lymph node were further investigated by flow cytometry. Additionally, immunohistochemistry was performed to identify and semi-quantify p72-positive target cells and cells undergoing apoptosis.

To gain deeper insights in putative target cells within the myelomonocytic cell lineage, the presence of the ASFV protein p72 was tested in myeloid cells by flow cytometry. In addition, representative tissue sections of the spleen, lung, and liver from wild boar and domestic pigs were investigated for cell-specific presence of ASFV particles by electron microscopy at indicated time points.

2.4.1. Spleen

Although splenic gross lesions, like splenomegaly or infarction, were not evident, histopathological changes were present (Figure 4). Whereas hematoxylin eosin-staining revealed no specific differences between wild boar and domestic pigs, immunohistochemistry yielded markedly different results (Figure 5A,B). In detail, apoptosis of lymphocytes in the white pulp was up to moderately present in control animals as well as at 4 dpi. Apoptosis slightly increased at day 7 and became less at day 10 (Figure 4A). Apoptosis of lymphoid cells was confirmed by active caspase-3 labelling (Figure 4B, see immunohistochemistry in the inset). In correlation with the onset of clinical disease, apoptosis and necrosis of myelomonocytic cells in the red pulp were mild at 4 dpi, mild to severe at 7 dpi, and mild to moderate at day 10 (Figure 4C). Additionally, myelomonocytic cells of the red pulp appeared slightly swollen (hypertrophic) at day 4 in single wild boar and domestic pigs and became more prevalent at 7 and 10 dpi in wild boar and domestic pigs (Figure 4D, inset). Immunohistochemistry identified viral antigen abundantly (score 3) in myelomonocytic cells, in particular in the red pulp at day 4 pi in all animals (Figure 5A). At day 7 pi a high amount (score 3) of positive cells was still detectable in wild boar while viral antigen load markedly decreased in domestic pigs (score 0–2). At day 10 pi scattered positive cells (score 1) were detectable in one domestic pig. ASFV p72 antigen detection is illustrated in Figure 5B.

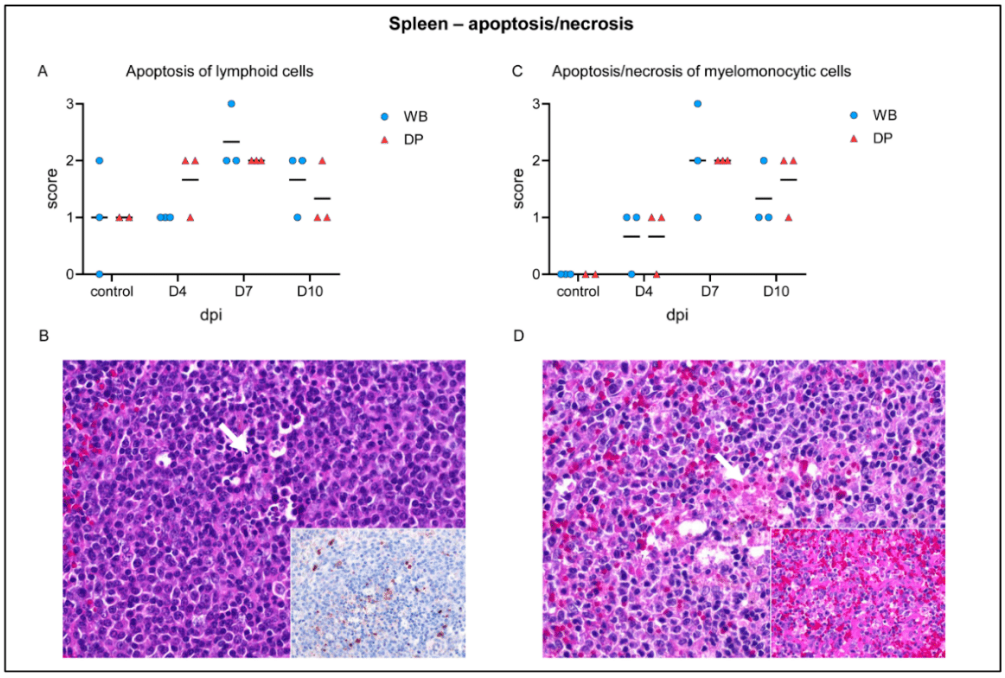


Figure 4. Apoptotic and necrotic changes in the spleen of wild boar and domestic pigs. (**A**) Microscopical scoring of apoptosis of lymphoid cells. (**B**) Pyknosis and karrhyorhexis (arrow) of lymphoid cells and multifocal caspase-3-labelled cells (inset) in a domestic pig at day 4 pi. (**C**) Microscopical scoring of apoptosis/necrosis of myelomonocytic cells. (**D**) Focal coagulative necrosis in the spleen (arrow) and multifocal hyperplastic dendritic cells in a wild boar at day 7 pi. (inset). WB = wild boar, DP = domestic pig, median as horizontal line.

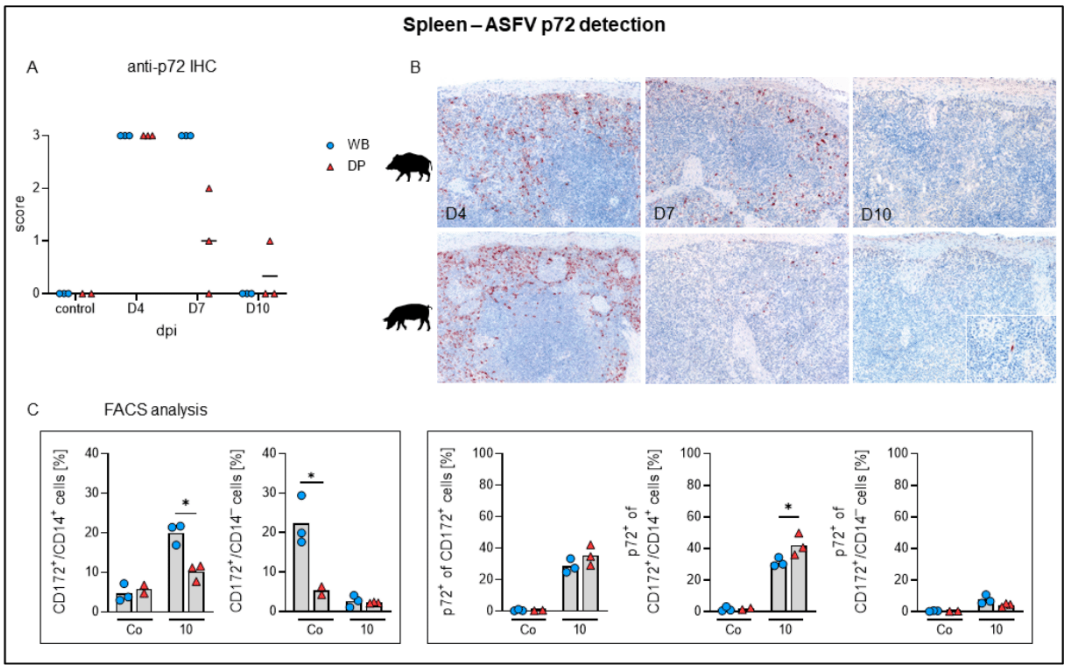


Figure 5. ASFV p72 detection in the spleen of wild boar and domestic pigs. (**A**) Microscopical scoring of p72 positive cells in the spleen. (**B**) Representative sections of anti-p72 immunohistochemistry in wild boar and domestic pigs at days 4, 7, and 10 pi. (**C**) Frequencies of $CD172^+/CD14^+$ monocytes and $CD172^+/CD14^-$ dendritic cells among live leukocytes (left panels) and frequencies of $p72^+$ monocytes and $p72^+$ dendritic cells (right panels) in the spleens of investigated animals. WB = wild boar, DP = domestic pig, IHC = immunohistochemistry, * $p < 0.05$, median as horizontal line (**A**) or bar (**C**).

In contrast to p72 antigen detection by immunohistochemistry, flow cytometric analysis showed that the proportion of infected $CD172^+/CD14^+$ monocytes was moderate at 10 dpi in both species (mean 30–40%) while there were only few $p72^+$ $CD172^+/CD14^-$ dendritic cells in the spleen (Figure 5C). Electron microscopy confirmed infection of monocytes/macrophages showing abundant vacuoles and intracytoplasmic ASFV particles (details not shown).

2.4.2. Lymph Nodes

Lymphocytolysis (syn. apoptosis) in lymphoid follicles of the hepatogastric as well as popliteal lymph node, was mildly present in control animals. Mild to moderate lymphoid apoptosis was consistently present in both lymph nodes of both subspecies, except for one wild boar showing severe lymphocytolysis in the hepatogastric lymph node. Apoptosis and necrosis, affecting the perifollicular cortex and paracortex was found in infected animals at varying degree, starting at 4 dpi. The hepatogastric lymph node was generally more consistently and more severely affected compared to the peripheral, popliteal lymph node (details not shown).

Up to moderate ASFV antigen was detected at day 4 pi in single wild boar and domestic pig in both lymph nodes (Figure 6A,C). Particularly in wild boar, the amount of $p72^+$ cells increased markedly but was low in domestic pigs by day 7 pi, shown in Figure 6B,D. At day 10 pi, viral antigen was undetectable in hepatogastric lymph nodes or only slightly found in single animals in the popliteal lymph node. Flow cytometric analysis yielded only few monocytes and dendritic cells in the hepatogastric lymph node from which primarily monocytes were p72-positive (details not shown).

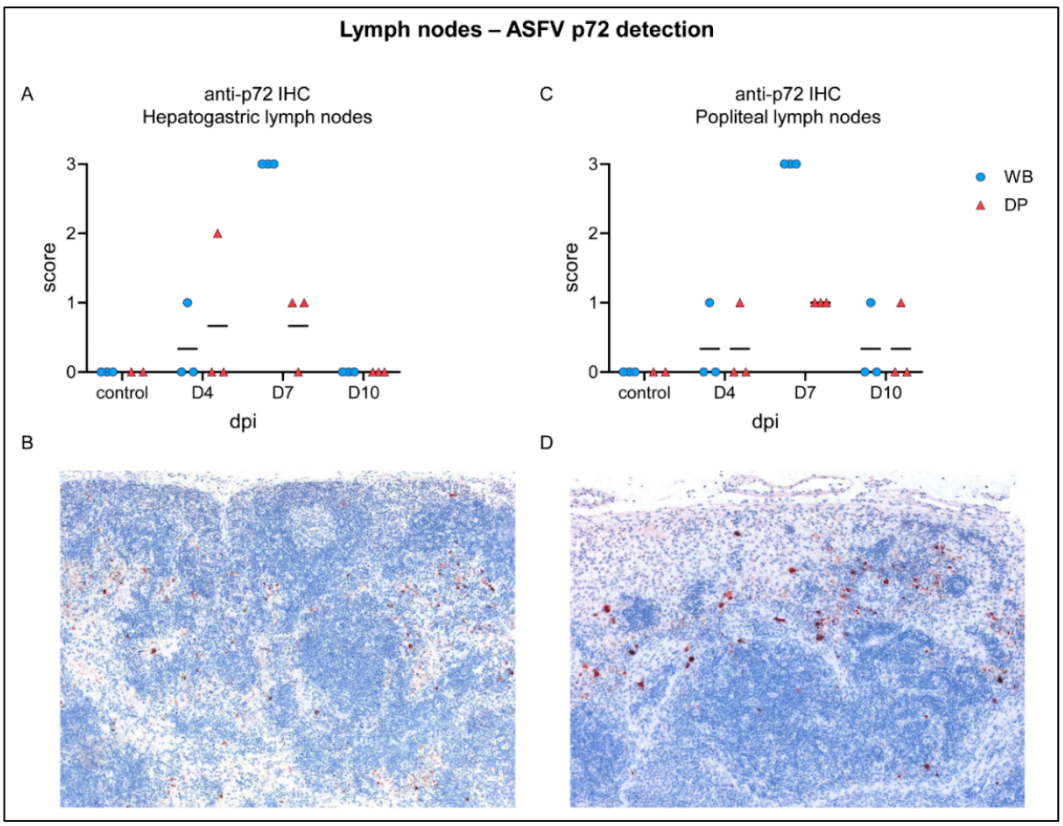


Figure 6. ASFV p72 antigen detection in lymph nodes of wild boar and domestic pigs. (**A**) Microscopical scoring of p72 positive cells in the hepatogastric lymph nodes. (**B**) Section of the hepatogastric lymph node from a wild boar showing abundant p72 postive cells at day 7 pi. (**C**) Microscopical scoring of p72 positive cells in the popliteal lymph node. (**D**) Section of the popliteal lymph node from a WB showing abundant p72 positive cells at day 7 pi. WB = wild boar, DP = domestic pig, median as horizontal line.

Further, histopathology confirmed macroscopically recorded hemorrhages in the hepatogastric and popliteal lymph nodes, and additionally congestion was found.

2.4.3. Palatine Tonsil

Apoptosis of lymphoid follicle cells was consistently detected in infected but also in control animals. Mild congestion and only very mild necrosis was observed at 7 dpi in two wild boar and one domestic pig. In all infected and non-infected animals, tonsillary crypts were filled with variable amount of viable and degenerate neutrophilic granulocytes. This was particularly pronounced in two wild boar from day 7 and one wild boar from day 10 pi. The crypt epithelium as well as myelomonocytic cells were slightly positive for ASFV antigen in one domestic pig at 4 dpi. All infected animals from day 7 pi revealed a high amount (score 1–3) of p72 antigen in the tonsil, most abundantly in wild boar. In one wild boar, the mucosal epithelium was ASFV antigen positive. At day 10 pi only one wild boar but all domestic pigs showed scattered p72-labelling in the crypt epithelium and myelomonocytic cells (details not shown).

2.4.4. Bone Marrow

The bone marrow revealed no histopathological changes, but immunohistochemistry showed p72-positive myeloid cells, including megakaryocytes. High amounts of viral antigen were detected in

all wild boar at day 7 pi while all domestic pigs showed fewer positive cells. At day 10 pi, only few positive cells were present in two wild boar (details not shown).

2.4.5. Lung

Histopathology identified interstitial inflammation in the lungs, mainly affecting the cranial lung lobes, characterized by infiltrating lymphocytes and few macrophages. Detailed evaluation revealed for wild boar mild to moderate interstitial pneumonia at 4 dpi in 2/3 animals, and in all wild boar at 7 dpi. No inflammation was detectable at 10 dpi. Domestic pigs also exhibited mild pneumonia at 4 dpi in 2/3 animals, and mild to moderate inflammation at 7 dpi. In contrast to wild boar, moderate to severe pneumonia was found at 10 dpi (Figure 7A,B). In addition, interstitial macrophages appeared more prominent in single animals at day 4 pi, which particularly increased in wild boar at day 7 pi and was present until the end of the study in both groups. Of note, mild infiltrates were also found in one control wild boar and domestic pig. Mild alveolar edema was only occasionally detected, but was severe in wild boar #28 euthanized at day 7 pi with the highest clinical score (score 10.5). Further unspecific findings included pulmonary congestion, found in the majority of controls and infected animals.

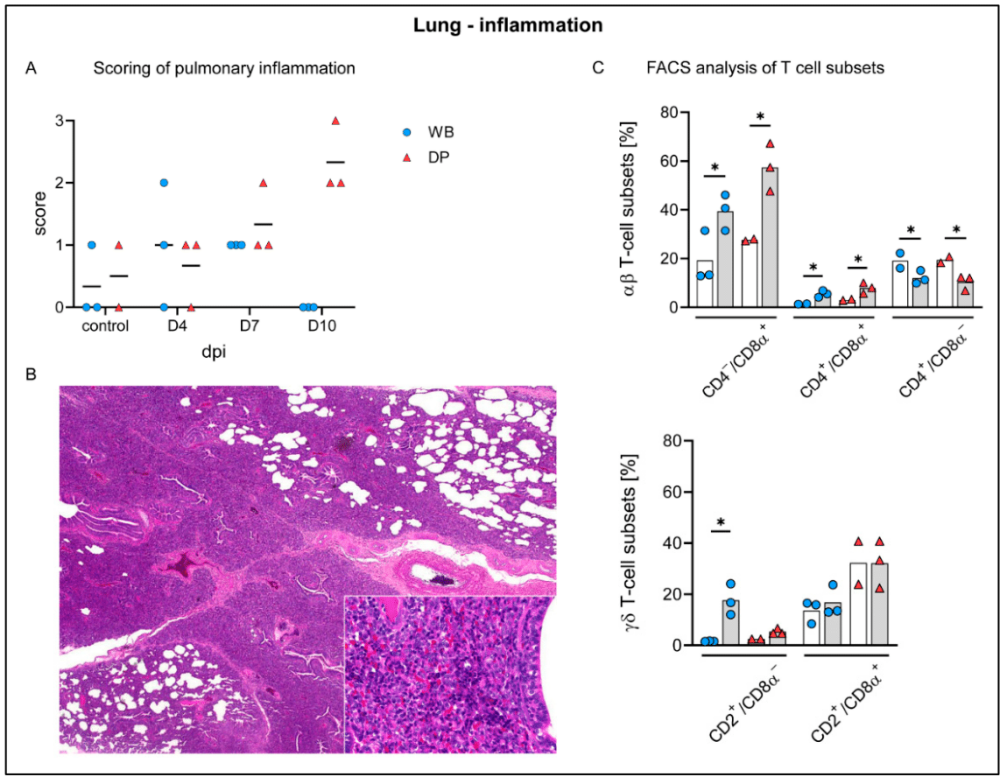


Figure 7. Pulmonary inflammation in wild boar and domestic pigs. (**A**) Microscopical scoring of pulmonary inflammation. (**B**) Section of lung tissue from a domestic pig showing severe interstitial pneumonia with mainly infiltrating lymphocytes and histiocytes (inset) at day 10 pi. (**C**) Frequency of αβ and γδ T-cell subsets in the lung of control animals (white bars) and wild boar and domestic pigs at 10 dpi (grey bars). WB = wild boar, DP = domestic pig, * $p < 0.05$, median as horizontal line (**A**) or bars (**C**).

To identify the phenotype of infiltrating cells and compare the responses in wild boar and domestic pigs, flow cytometric analysis was performed on control and day 10 lung tissues. The overall response at day 10 was comparable in both subspecies. We detected a significant increase of CD8α$^+$ αβ T

cells in both subspecies. Moreover, $CD4^+/CD8\alpha^+$ αβ T cells increased significantly in wild boar and domestic pigs but considerably less pronounced, while $CD4^+$ αβ T cells showed a corresponding decrease (Figure 7C). The frequencies of activated γδ T cells were significantly elevated in wild boar but showed only a small increase in domestic pigs. Effector γδ T-cell frequencies were not altered in both subspecies (Figure 7C).

By immunohistochemistry, ASFV antigen was detected multifocally in mononuclear cells in all animals starting at day 4 pi, where the number of positive cells was comparably low in wild boar and domestic pigs (Figure 8A). At day 7 pi viral antigen was found abundantly (score 3) in all wild boar whereas all lungs of domestic pigs revealed markedly lower amounts (score 1) of positive cells. At day 10 pi antigen detection yielded similar scores for wild boar and domestic pigs (score 1–2). Figure 8B shows representative anti-p72 labelled lung sections from day 4 to 10 pi. Immunological investigation demonstrated that at 10 dpi most $CD172^+/CD14^+$ monocytes in the lungs were p72-positive, while only around 50% of the dendritic cells were positive for viral p72. Moreover, there were no changes in the frequencies of lung monocytes in both subspecies. Of note, wild boar showed significantly higher dendritic cell frequencies than domestic pigs 10 dpi (Figure 8C). By electron microscopy, ASFV particles were identified in pulmonary intravascular macrophages/monocytes (PIM) (Figure 9).

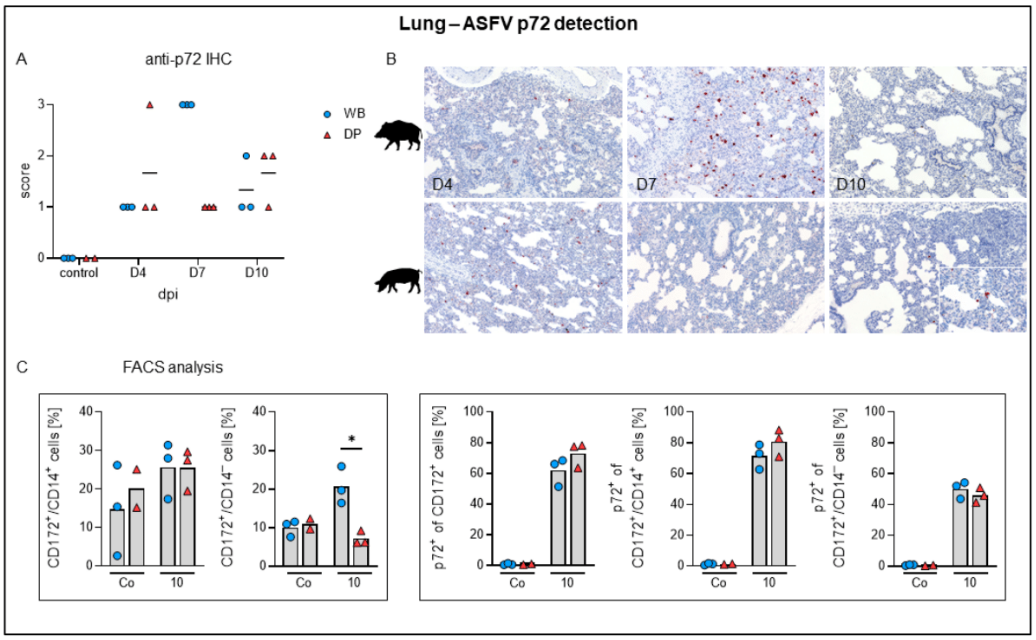


Figure 8. ASFV p72 antigen detection in the lung of wild boar and domestic pigs. (**A**) Microscopical scoring of p72 positive cells in the lung. (**B**) Representative sections of the lung in wild boar and domestic pigs stained with a rabbit anti-p72 serum at days 4, 7, and 10 pi. (**C**) Frequencies of $CD172^+/CD14^+$ monocytes and $CD172^+/CD14^-$ dendritic cells among live leukocytes (left panels) and frequencies of $p72^+$ monocytes and $p72^+$ dendritic cells (right panels) in the lungs of investigated animals. * $p < 0.05$, median as horizontal line (**A**) or bar (**C**).

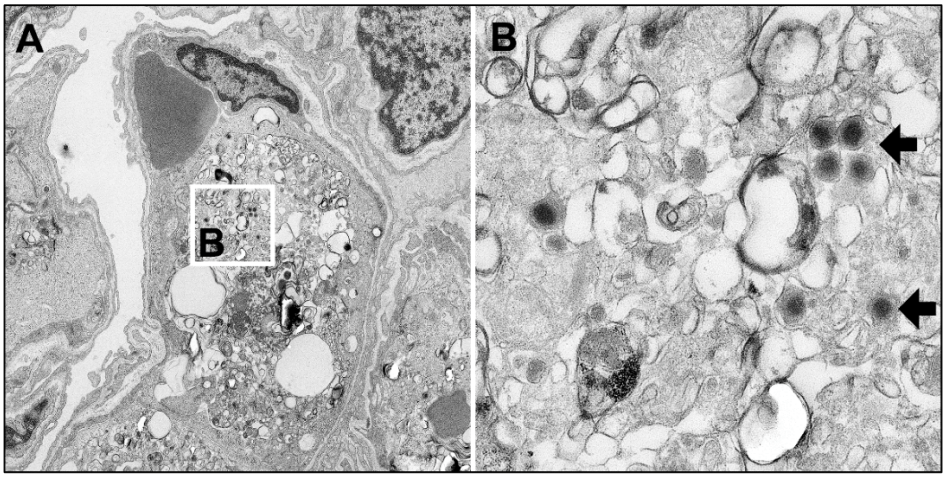


Figure 9. Electron-micrograph of an ASFV-infected pulmonary intravascular macrophage (PIM). (**A**) PIM (center) with multiple virions found in the cytoplasm (white square) (**B**) Detail of figure A showing typical electron-dense virions (arrows).

2.4.6. Liver

Histopathology identified lymphoid infiltrates mainly affecting the hepatic sinuses, increasing over time in both subspecies (Figure 10A,B). Starting on 4 dpi in 2/3 domestic pigs, sinusoidal infiltrates were consistently present in wild boar and domestic pigs at 7 (mild to moderate) and 10 dpi (moderate). Flow cytometry revealed infiltrating lymphocytes to be mainly $CD8\alpha^+$ and to a lesser extent also $CD4^+/CD8\alpha^+$ $\alpha\beta$ T cells, which increased significantly in wild boar and domestic pigs compared to controls. $CD2^+/CD8^-\gamma\delta$ T-cell frequencies were also elevated in both subspecies (Figure 10C). In addition, microscopic investigation found Kupffer cell degeneration, characterized by swelling and detachment, as well as necrosis. Starting mildly at day 4 pi in one domestic pig, degeneration and necrosis was found more frequently and more severe in wild boar compared to domestic pigs and lasted until day 10 pi (Figure 11A,B). The degeneration of Kupffer cells correlated with a clear drop of $CD172^{low}/CD14^+$ cells in the liver at day 10 pi in wild boar and domestic pigs, as found in the flow cytometric analyses (Figure 11C). Immunohistochemistry demonstrated viral antigen in all wild boar and domestic pigs at 4 dpi mainly in sinusoid-lining cells, indicative for Kupffer cells and/or endothelium (score 1–3) but also in few hepatocytes until day 7 pi (data not shown). Whereas all wild boar exhibited abundant (score 3) viral antigen, 2/3 domestic pigs showed only few positive sinusoid lining cells (score 1) at 7 dpi. No $p72^+$ cells were detected at 10 dpi in both subspecies (Figure 12A). Flow cytometry showed increasing proportions of $p72^+$ $CD172^+/CD14^+$ monocytes (up to 70%), $CD172^+/CD14^-$ dendritic cells (up to 40%), and $CD172^{low}/CD14^+$ putative Kupffer cells (up to 20%) over time. We found higher amounts of $p72^+$ myeloid cells in wild boar compared to domestic pigs, especially given the fact that the frequencies of monocytes and dendritic cells were significantly higher in wild boar than in domestic pigs 10 dpi (Figure 12B). However, given that there were only few Kupffer cells left, the majority of $p72^+$ cells consisted of monocytes. The infection of Kupffer cells was also confirmed by electron microscopy (Figure 12C). Congestion of the liver was detected in all infected and control animals, but was slightly more severe at day 4 pi.

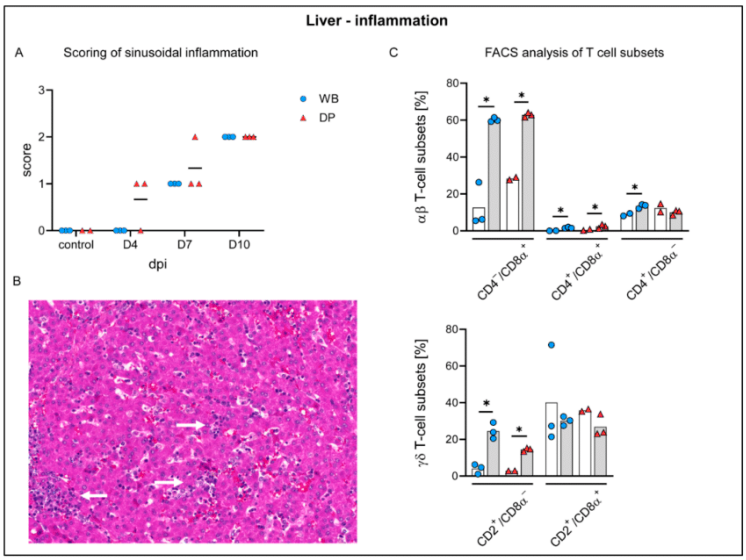


Figure 10. Hepatic inflammatory reaction against ASFV infection in wild boar and domestic pigs. (**A**) Microscopical scoring of sinusoidal inflammation. (**B**) Liver section of a domestic pig showing moderate lymphocytic infiltration (arrow). (**C**) Frequency of αβ and γδ T-cell subsets in the liver of WB and DP in controls (white bars) and 10 dpi (grey bars). WB = wild boar, DP = domestic pig, * $p < 0.05$, median as horizontal line (**A**) or bar (**C**).

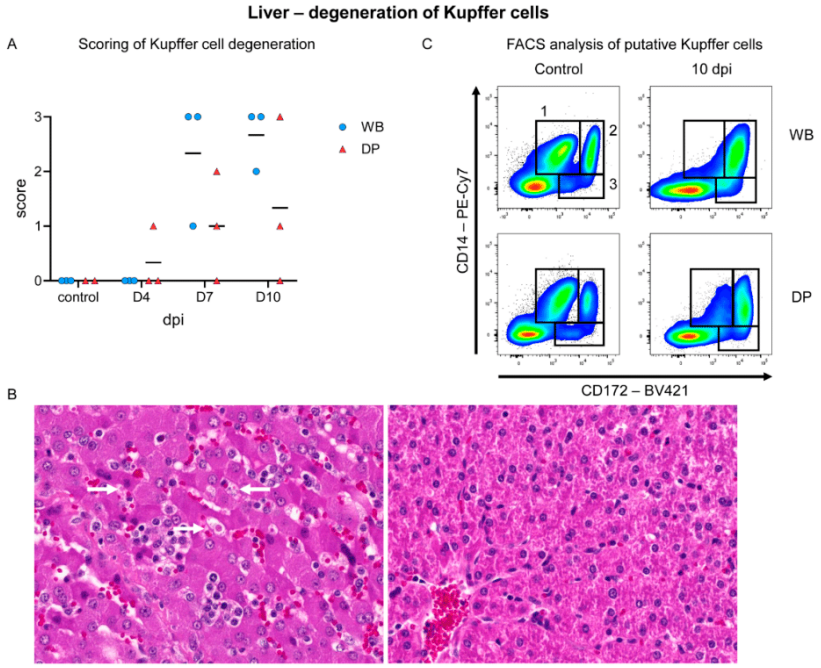


Figure 11. Kupffer cell degeneration in the liver of wild boar and domestic pigs. (**A**) Microscopical scoring of Kupffer cell degeneration in the liver. (**B**) Representative sections of an ASFV infected liver with marked Kupffer cell swelling/degeneration (arrow) in a wild boar 10 dpi (left) and a liver of a control animal (right). (**C**) Identification of myeloid cell populations in the liver by co-expression of CD14 and CD172. (**1**) $CD14^{+}/CD172^{low}$, (**2**) $CD14^{+}/CD172^{+}$, and (**3**) $CD14^{-}/CD172^{+}$ cells were identified as putative liver macrophages or Kupffer cells, monocytes, and dendritic cells, respectively. WB = wild boar, DP = domestic pig, median as horizontal line (**A**).

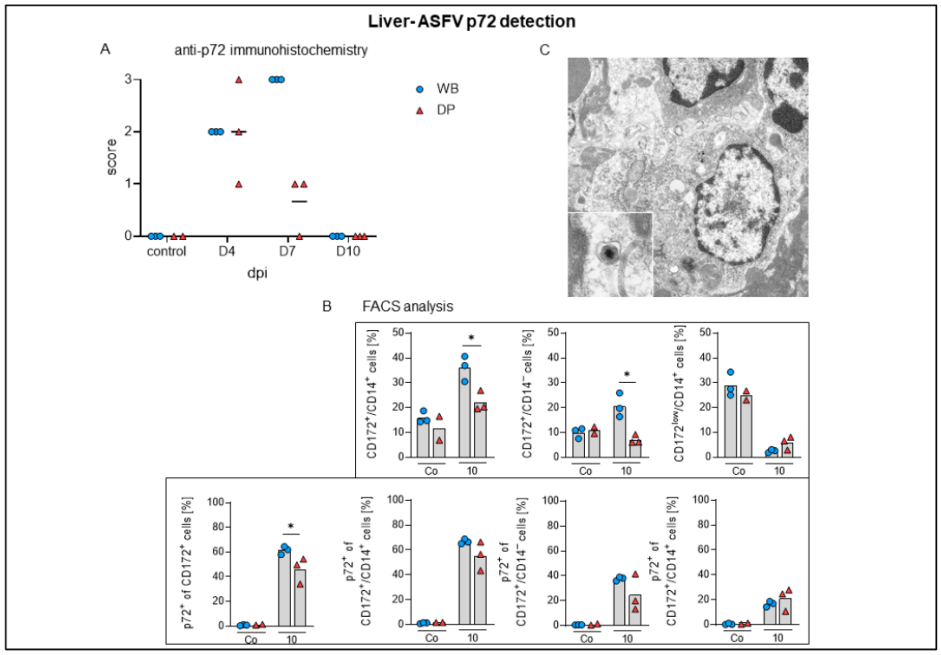


Figure 12. ASFV p72 antigen detection in the liver of wild boar and domestic pigs. (**A**) Microscopical scoring of p72 positive cells in the liver. (**B**) Frequencies of $CD172^+/CD14^+$ monocytes, $CD172^+/CD14^-$ dendritic cells, and $CD172^{low}/CD14^+$ putative Kupffer cells among live leukocytes (upper panels) and frequencies of $p72^+$ monocytes, $p72^+$ dendritic cells, and $p72^+$ putative Kupffer cells (lower panels) in the liver of investigated animals. (**C**) Electron micrograph of a Kupffer cell with intracytoplasmic ASF virions (inset). WB = wild boar, DP = domestic pig, * $p < 0.05$, median as horizontal line.

2.4.7. Kidney

In addition to macroscopically recorded petechial hemorrhages, congestion of the cortex and medulla were observed in nearly all of the infected, but also in the control animals. Mild interstitial lymphocytic infiltration confined to the cortex was found in one control wild boar and in the majority of infected wild boar. ASFV antigen was up to moderately detected in the cortical and medullary interstitium at day 7 in 3/3 wild boar and 1/3 domestic pigs, and mildly at 10 dpi in 3/3 wild boar only (details not shown).

2.4.8. Brain

Sections of the cerebrum at the level of the hippocampus revealed no histopathological changes, but viral antigen was detected in few cortical glial cells in one wild boar at day 7 pi, in two wild boar and one domestic pig at day 10.

The cerebellar section was evaluated at the level of the pons. Although histopathological changes were absent, immunohistochemistry revealed focal antigen positive cells in the granule cell layer and choroid plexus in one wild boar at day 7 pi and positive glial cells in one wild boar at day 10 pi (details not shown).

3. Discussion

Differences in the disease course and in susceptibility to ASFV infection between domestic pigs and European wild boar have been described, indicating differences in the pathogenic mechanisms in the subspecies [18] but mostly exclude histopathology and target cell identification. In the present study, we compared domestic pigs and wild boar infected with ASFV "Estonia 2014" that previously showed an attenuated phenotype in domestic pigs but still high virulence in adult wild boar [17].

To gain more insights on host factors for virulence, we analyzed three domestic pigs and three wild boar each on 4, 7, and 10 dpi in a kinetic trial. We scored clinical signs, gross pathological changes and histological lesions, as well as antigen distribution by immunohistochemistry. Blood and tissue samples were examined for viral genome load by qPCR. Electron microscopy as well as flow cytometry were performed to further evaluate changes observed pathomorphologically.

Clinical scoring revealed ASF-related disease starting 4 dpi. Wild boar showed much higher clinical scores than domestic pigs. Whereas domestic pigs fully recovered until day 10, wild boar still exhibited moderate disease. This mild clinical picture and subsequent recovery of domestic pigs reflects experimental data obtained by Zani et al. [17] with this virus strain. The very same study confirmed a more severe clinical course in wild boar, with two female 2-year old wild boar found dead at 8 and 9 dpi and 6–8-month-old piglets were euthanized at days 16 and 17 pi. This indicates that virulence might be age-related, although the number of animals used was low and it is generally assumed that age-dependence of clinical signs does not exist for highly virulent ASFV strains [22]. Since our study focused on early lesions and viral antigen distribution and thus lasted 10 days only, we cannot exclude that the wild boar might have reached the humane endpoint later on or could have recovered completely.

ASFV genome was detectable from day 4 pi onwards in blood and tissue samples. The viral genome load in tissues is most likely dependent on the viral load in the blood since the values for both were comparably high over the study period. However, no correlation with the overall clinical course was found based on (i) the lack of marked pathological differences between wild boar and domestic pigs, in particular at 10 dpi, (ii) absence of clinical disease at 10 dpi in domestic pigs with persistent high viral loads in blood and tissues with more than 10^3 genome copies per µL, and (iii) almost comparable genome loads in tissues of wild boar at days 4 and 10 pi but a more severe disease at 10 dpi. Flow cytometric analyses of whole blood samples showed that the relative proportions of granulocytes, monocytes, and dendritic cells did not change throughout the experiment and that frequencies of $p72^+$ cells did not differ between wild boar and domestic pigs. In line with previous observations in granulocytes of domestic pigs inoculated with highly virulent ASFV [23] viral antigen was abundantly found in our study. It remains unclear whether the viral antigen in granulocytes derives from productive infection, as it has been shown for highly virulent ASFV strains [23], or phagocytosis of cell debris and erythrocytes with viral attachments, or a combination of both. Given that erythrocytes carry the vast majority of infectious particles in the blood [24], it seems likely that cells with high phagocytosis activity and possible viral replication, i.e., granulocytes, have a higher MFI than other myeloid cell subsets. Moreover, since granulocyte identification was conducted by FSC vs. SSC gating, we cannot rule out that large macrophages might have been found in the granulocyte gate. Given that up to 50% of all live blood leukocytes were granulocytes and most of them were positive for the viral protein p72, the major antigen load in the leukocyte fraction of the blood was found in granulocytes throughout the whole study period. We also provide evidence that cells of the myeloid lineage are affected by ASFV infection in various tissues. It has been shown that ASFV is able to infect and modulate dendritic cells in vitro [25,26], however, in vivo evidence was scarce [27,28]. The ASFV protein p72 was found in dendritic cells in virtually all tissues investigated. ASFV infection of dendritic cells is thought to impair their function [29] but this requires further investigation.

The role and impact of neutralizing antibodies upon ASFV infection are still controversially discussed, and full neutralization could not be shown in recent studies with convalescent animals (Petrov et al. 2018). However, the production of efficiently neutralizing antibodies in domestic pigs could have influenced the disease outcome and explain convalescence in domestic pigs in our study as it has been described after infection with moderately virulent strains [30].

Although ASFV is an obligate pathogen for both wild boar and domestic pigs, potential genetic differences among and across the two subspecies should be considered as they could influence the susceptibility to ASFV and thus disease outcome [31]. Different clinical signs, immunological responses, and pathological outcomes are also known for highly pathogenic porcine reproductive and

respiratory syndrome virus infection in wild boar and domestic pigs [32]. So far, genetic discrimination between domestic pigs and wild boar has been challenging since there have been only few genetic markers identified to distinguish between domestic pigs and wild boar [33], and even immunogenetic investigation of domestic pigs and wild boar could not identify any statistically significant differences in allele frequency and heterozygosity across SNPs [34]. However, very recently SLA-1 diversity was investigated in different domestic pig breeds showing significant distribution of SLA-1 alleles among pig breeds which could explain differences in the immune response [35]. This has not been done for wild boar so far and underlines the necessity of further investigation.

In accordance with earlier studies, macroscopic changes did not generally differ between domestic pigs and wild boar [36]. At 4 dpi, changes were generally mild and gradually became more severe until 10 dpi. In particular, typical hemorrhages in lymph nodes and kidneys were found as already described [16,17].

Apoptosis of lymphoid cells (syn: lymphocytolysis) could be confirmed for the spleen and lymph nodes [37–41] but did not differ between wild boar and domestic pigs. Apoptosis/necrosis in the splenic red pulp as well as in perifollicular and paracortical regions of lymph nodes were also present to the same extent in wild boar and domestic pigs, though not as severe as after infection with highly-virulent strains found in the spleen [42]. Immunohistochemistry revealed much more viral antigen positive cells in the splenic red pulp as well as in the lymph nodes at day 7 pi in wild boar compared to domestic pigs, indicating differences in the pace of viral clearance. Higher numbers of viral antigen positive cells in wild boar were also present in the palatine tonsil, although pathohistological changes were rather mild and largely similar in domestic pigs and wild boar.

Pulmonary consolidation was present in almost all domestic pigs at day 7 and 10 pi (up to 70% affected lung lobe), but only in one wild boar (<5% affected). Histopathology identified up to severe lymphohistiocytic interstitial pneumonia, partially also affecting macroscopically normal lungs of both subspecies. Pneumonic lesions were particularly associated with viral antigen labelled cells but those were also found disseminated in otherwise unaffected lung tissue. At day 7 pi viral antigen was found abundantly in wild boar, but only minimally in domestic pigs, again indicating an impaired virus elimination in wild boar. Pneumonia is described in domestic pigs after field and experimental infection [43,44] and can be either associated with ASF or secondary infections [45]. Supporting the histopathologic findings, flow cytometry of T cell subsets in lung tissue showed an immune response mainly driven by $CD8\alpha^+$ $\alpha\beta$ T cells and $CD2^+/CD8\alpha^-$ activated $\gamma\delta$ T cells. A comparable T-cell response was also found in the liver. $CD8\alpha^+$ T cells are known to be pivotal mediators of the anti-ASFV response [46]. Recently, it has been shown that highly virulent ASFV infections induce different responses in wild boar and domestic pigs [47]. Wild boar mounted a cytotoxic Th1 response, while the T-cell response in domestic pigs seemed to be impaired. Neither response was beneficial as all animals showed signs of severe disease and most succumbed to the infection [47]. During the moderately virulent ASFV infection in the present study, the pulmonary and hepatic inflammations seem to be driven mainly by cytotoxic $CD8\alpha^+$ $\alpha\beta$ T cells in both subspecies. A distinct response of cytotoxic T cells might cause the tissue damage and cell degeneration observed in this study, indicating a possible role for immunopathologic processes in ASF pathogenesis which urges further research.

We also observed an increase in the number and size of pulmonary macrophages which is fully consistent with the findings shown by Carrasco et al. [48] who found proliferation particularly of pulmonary intravascular macrophages (PIM) in response to intracytoplasmic immunocomplexes after ASFV infection. In the present study, the infection of PIM was demonstrated in wild boar by electron microscopy, one of the main target cells of ASF in the lung of domestic pigs [49,50].

In the liver, lymphocytic infiltrates were initially observed in the sinuses only in domestic pigs, but the values in domestic pigs and wild boar increased and converged until day 10. Since p72 antigen was no longer detectable with immunohistochemistry at day 7 pi in domestic pigs, but was still present in wild boar, again ASFV clearing seems to be more effective in domestic pigs. Kupffer cell degeneration, necrosis, and loss was obvious in both subspecies although slightly more severe

in wild boar. This finding is typical for ASF as reported by Carrasco et al. [48] and correlated with flow cytometry results in the present study, showing a clear drop of $CD172^{low}/CD14^{+}$ cells beginning day 7 pi in wild boar and domestic pigs.

Higher numbers of viral antigen positive cells in wild boar were also present in the kidney and brain although pathohistological changes were rather mild and largely similar in domestic pigs and wild boar.

In summary, we confirmed that ASFV "Estonia 2014" is more virulent in wild boar than in domestic pigs. However, autopsy as well as routine histopathology failed to identify significant differences in the lesion profile and could not identify the cause for the more pronounced disease in wild boar. Further, the viral genome load in tissues is most likely attributed to the viral load in the blood and is thus not suitable to evaluate affected target tissue and cells. The viral genome load also remains almost unchanged over time, again not reflecting the clinical course. Using flow cytometry, we could show that cells of the myeloid lineage, including monocytes, dendritic cells, granulocytes, and myeloid cells in the liver, contain viral antigen in various tissues. Even though we could not find distinct differences in target cells or virus tropism between wild boar and domestic pigs, immunohistochemistry showed striking differences at 7 dpi: while antigen detection in wild boar peaked at day 7 in main target tissues, i.e., spleen, liver, and lung, the domestic pigs have already started to eliminate the virus. This raises the question of whether in the early phase of the disease, the delayed viral clearing in wild boar is relevant for the virulence of ASFV Estonia. To address this, particularly immunologic-mechanistical and functional insights will be important to shed light in this field. In this respect, comparative cytokine and large-scale immune cell profile analysis in domestic pigs and wild boar are needed to explain the different phenotypes after infection with ASFV "Estonia 2014".

4. Materials and Methods

4.1. Study Design

In the animal experiment, all applicable animal welfare regulations including EU Directive 2010/63/EC and institutional guidelines were taken into consideration. The animal experiment was approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference number 7221.3-2-011/19.

The present study was carried out for detailed pathological analysis of disease dynamics of domestic pigs and wild boar infected with an attenuated Estonian ASFV isolate ("Estonia 2014"). To this end, an ASFV-positive macrophage culture supernatant was prepared to a final titer of approximately $1 \times 10^{5.25}$ hemadsorbing units (HAU) per mL. Domestic pigs and wild boar were inoculated oro-nasally. The dilutions were based on an end-point virus titration of the original material on macrophages derived from peripheral blood monocytic cells (PBMCs). Upon application, back titration was carried out to confirm the administered virus-containing dose.

4.2. Cells

For the preparation of PBMC-derived macrophages blood was collected from healthy domestic donor pigs that are kept in the quarantine stable at the Friedrich-Loeffler-Institut (FLI). Briefly, PBMCs were obtained from EDTA-anticoagulated blood using Pancoll animal density gradient medium (PAN Biotech, Aidenbach, Germany). PBMCs were grown in RPMI-1640 cell culture medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was supplied with amphotericin B, streptomycin, and penicillin to avoid bacterial and fungal growth. To facilitate maturation of macrophages, GM-CSF (granulocyte macrophage colony-stimulating factor; Biomol, Hamburg, Germany) was added to the cell culture medium with a concentration at 2 ng/mL.

4.3. Virus

The Estonian virus "Estonia 2014" isolate belongs to genotype II with a deletion at the 5′ end (GenBank Accession number LS478113.1) that corresponds to an attenuated phenotype [17]. The virus was grown on PBMCs and the supernatant was used for the animal trial with a final titer of $1 \times 10^{5.25}$ HAU per mL. The titer was confirmed by back titration of the inoculum.

4.4. Animal Experiment

The study included 12 6–8-month-old European wild boar and 11 10-week-old domestic pigs of mixed sex. Wild boar were obtained from three different wildlife parks, domestic pigs from one commercial fattening farm. The animals were housed in groups in the high containment facility of the FLI (L3+) prior to infection. All animals were individually ear-tagged. The animals were fed a commercial pig food with corn and hay-cob supplement and had access to water ad libitum. After an acclimatization period of 1 week, the animals were inoculated oro-nasally with 2 mL of $1 \times 10^{5.25}$ HAU per mL. On day 0, three wild boar and two domestic pigs were euthanized and used as control animals. On days 4, 7, and 10, three animals from each group (domestic pigs and wild boar) were euthanized and submitted to necropsy. The animals were deeply anaesthetized with tiletamine/zolazepam (Zoletil®, Virbac, Carros cedex, France), ketamin (Ketamin 10%, Medistar, Ascheberg, Germany) and xylazine (Xylavet 2%, CP Pharma, Burgdorf, Germany) and subsequently killed by bleeding.

4.4.1. Clinical Scoring

Clinical parameters of all animals were assessed daily based on a harmonized scoring system as previously described [51]. The sum of the points was recorded as the clinical score (CS) that was also used to define humane endpoints prior to the experiment. The body temperature was measured daily in the domestic pigs and at day 10 pi in wild boar. Blood samples were collected at day 0 before inoculation and before euthanasia on days 4, 7, and 10 post infection.

4.4.2. Tissue Sample Collection

Full autopsy was performed on all animals and tissue samples including the spleen, hepatogastric and popliteal lymph nodes, palatine tonsil, bone marrow, lung (cranial and caudal lobe), liver with gall bladder, kidney, and brain (cerebellum and cerebrum) were taken for histopathological investigation. For electron microscopy, lung, spleen, and liver were collected. Blood (EDTA, serum, citrate) and swab samples (cotton swab, genotube, prime store) were taken for further analysis (data not shown).

4.4.3. Pathomorphological and Electron Microscopical Analysis

Gross Pathology and Macroscopic Scoring

During necropsy all animals were scored based to the gross protocol published by Galindo-Cardiel et al. [21] with slight modifications as follows. Organ lesions were generally scored on an ordinal scale ranging from 0 to 3 with normal (0), mild (1), moderate (2), or severe (3). In addition, lymphatic tissue including the spleen, palatine tonsil, and lymph nodes were examined for hyperplasia (size increased), hemorrhage, and necrosis and scored accordingly. Hemorrhages in the kidney were evaluated with normal (0), petechiae (1), ecchymoses (2), and diffuse hemorrhage (3). The percentage distribution of pulmonary consolidation was determined for *the lobus cranialis sinister pars cranialis, lobus cranialis sinister pars caudalis, lobus caudalis sinister, lobus cranialis dexter, lobus medius, lobus caudalis dexter and lobus accessorius*. Tissue samples were fixed in 10% neutral-buffered formalin for at least 3 weeks.

Histopathology and Immunohistochemistry

Representative sections of each fixed organ sample were cut and embedded in paraffin wax. Bone tissue was decalcified for at least 3 days in Formical 2000 (Decal, Tallman, New York, NY, USA).

Embedded sections were cut at 2–3 µm thick slices, mounted on Super-Frost-Plus-slides (Carl Roth GmbH, Karlsruhe, Germany), and stained with hematoxylin-eosin.

For immunohistochemistry, paraffin-embedded sections were dewaxed and rehydrated through ascending concentrations of ethyl alcohol. Sections were rinsed with deionized water (A. dest.) and treated with 3% of hydrogen peroxide (Merck, Darmstadt, Germany) for 10 min to block intrinsic peroxidases. After washing with A. dest. sections were demasked with Tris/EDTA buffer (10 mmol/L Tris and 1mmol/L EDTA, pH = 8.95) for 20 min, 700 W in a microwave. Afterwards sections were gradually cooled down and transferred into coverplates (Thermo Fisher Scientific GmbH, Schwerte, Germany). Sections were rinsed with Tris-buffered saline (TBS) and incubated with undiluted normal goat serum for 30 min to block unspecific binding sites prior to incubation with the primary antibody. Sections were then treated for 1 h with a rabbit polyclonal primary antibody against the major capsid protein p72 of ASFV (diluted in TBS 1:1600). To investigate apoptosis a rabbit antiserum against active caspase-3 (Promega, Madison, WI, USA; 1:200, diluted in TBS) was used. Following washing with TBS sections were incubated with a secondary, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; diluted in TBS in 1:200) for 30 min. An ABC-kit (Vector; diluted in TBS 1:200, 30 min) was used providing the conjugated horseradish peroxidase which was followed by an incubation with AEC-substrate (DAKO, Hamburg, Germany) for 10 min to visualize positive reactions. The reaction was stopped with ionized water. Finally, sections were counterstained with hematoxylin for 2 min, rinsed in ionized water for 10 min and mounted with Aquatex (Merck).

Semi-Quantitative Scoring

A Zeiss AXIO Scope A1 microscope equipped with 2.5×, 10×, 20×, and 40× objectives was used for brightfield microscopial analysis of histological specimen.

Based on the protocol by Galindo-Cardiel et al. [21] sections were semi-quantitatively scored with some adaptations as follows: normal (0), mild (1), moderate (2), severe (3). Scored pathohistological changes of each organ are listed in Table 1. For evaluation, the post-examination masking approach was used [52] to avoid missing subtle or unexpected infection-related changes. An initial evaluation with full access to all study-related information and slides was made to determine the scoring and identify the main lesions. Subsequently, slides were masked and scores were assigned.

Table 1. Histopathological changes in tissues investigated in domestic pigs and wild boar.

Organ	Lesion (Scored with Normal (0), Mild (1), Moderate (2), Severe (3)				
Spleen	Apoptosis of Lymphoid Cells (Syn. Lymphocytolysis)	Apoptosis/Necrosis in the Red Pulp	Hyperplasia/Hypertrophy of Myelomonocytic Cells (i.e., Dendritic Cells, Monocytes, Macrophages)		
Liver with gall bladder	Congestion/hemorrhage	Sinusoidal inflammatory infiltrates	Degeneration/necrosis and loss of Kupffer cells		Hyperplasia of bile duct epithelium
Lymph nodes	Congestion/hemorrhage	Apoptosis of lymphoid cells	Apoptosis/necrosis, perifollicular cortex and paracortex	Presence of tingible body macrophages	Hyperplasia of lymphocytes
Palatine tonsil	Congestion/hemorrhage	Apoptosis of lymphoid cells	Apoptosis/necrosis, perifollicular cells	Crypt abscessation	
Lung	Congestion/hemorrhage	Edema	Inflammation	Hyperplasia/hypertrophy of alveolar and interstitial monocytes/macrophages	
Kidney	Congestion/hemorrhage		Inflammatory infiltrates		
Brain	Inflammatory infiltrates		Neuronal degeneration	Gliosis	
Bone marrow	Necrosis				

Immunohistochemically stained sections were scored for viral antigen distribution and scored on a 0–3 scale. The most affected area per sample sections was scored with no antigen (0), < 1–3 positive cells (1), 4–15 cells (2), and >16 cells (3) per high (40×) power field. Cells showing fine granular cytoplasmic labelling were considered positive; chromogen aggregations without cellular association were not counted.

4.4.4. Electron Microscopy

To confirm viral infection and to identify ASFV-infected cells, electron microscopy was conducted. Tissue samples were cut (1 mm^3) and fixed overnight in 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate pH = 7.2 and 300 mosmol (SERVA Electrophoresis, Heidelberg, Germany). Then, 1% aqueous OsO_4 was used for post fixation and 2.5% uranyl acetate in ethanol for *en bloc* staining (SERVA Electrophoresis. After a stepwise dehydration in ethanol the samples were cleared in propylene oxide and infiltrated with Glycid Ether 100 (SERVA Electrophoresis. For polymerization, samples were filled in flat embedding molds and incubated for 3 days at 60 °C.

Semithin sections of resin embedded samples were stained with 1% Toluidine Blue O in aqueous sodium borate (Carl Roth) on a hot plate. The POI was trimmed, and prepared ultrathin sections were transferred to formvar coated nickel grids (slot grids; Plano, Wetzlar, Germany). All grids were counterstained with uranyl acetate and lead citrate before examination with a Tecnai Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV.

4.4.5. Flow Cytometry

Whole blood or leukocytes isolated from the indicated organs were investigated by flow cytometry. Single cell suspensions of tissue samples were prepared as described previously [47]. In total, 50 µL whole blood or 50 µL single cell suspension (approx. 1×10^6 cells) was used for each staining. Incubation steps with monoclonal antibodies were carried out at 4 °C for 15 min in the dark for extracellular staining. Cells were fixed and permeabilized using the True Nuclear Transcription Factor Buffer Set (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. For intracellular staining after fixation, cells were incubated at 4 °C for 30 min in the dark. Erythrocytes were lysed before fixation with red cell lysis buffer (1.55 M NH_4Cl, 100 mM $KHCO_3$, 12.7 mM Na_4EDTA, pH = 7.4, in *Aqua destillata*).

The following antibodies were used in this study: anti-pig CD3ε-APC (clone PPT3, 1:500), anti-pig CD8α-FITC (clone 76-2-11, 1:100), anti-pig CD4-PerCP (clone 74-12-4, 1:100), anti-pig γδ T cell receptor (TCR, IgG2b, clone PPT16, 1:100), anti-pig CD2 (IgG2a, clone MSA4, 1:100), anti-pig CD172α (IgG1, clone 74-22-15; 1:100), anti-pig CD14 (IgG2b, clone MIL2; 1:500), anti-pig CD163-PE (clone 2A10/11; 1:100), and anti-vp72-ASP (IgG2a, clone 18BG3; 1:100). For detection of unconjugated primary antibodies, the following secondary antibodies were used: anti-mouse IgG1-BV421 (clone RMG1-1, 1:400), anti-mouse IgG2b-PE-Cy7 (goat polyclonal, Southern Biotech, 1:400), anti-mouse IgG2a-APC-Cy7 (goat polyclonal, Southern Biotech, 1:250), and anti-mouse IgG2a-APC (goat polyclonal, dianova, 1:500). CD4 was not detectable in one of the control wild boars in trial 2, probably because of a polymorphism in the *CD4* alleles [53]. $CD4^+$ and $CD4^+/CD8\alpha^+$ cells from this animal were therefore not included in the analyses.

Dead cells were identified using Zombie Aqua (Biolegend) and excluded from subsequent analyses. Doublets were excluded by FSC-H vs. FSC-A gating. In all tissues, FSC^{med}/SSC^{low} were subdivided into $CD172^+/CD14^+$ monocytes/macrophages and $CD172^+/CD14^-$ dendritic cells. $FSC^{hi}/SSC^{hi}/CD172^+/CD14^+$ cells in the blood were identified as granulocytes. The gating strategy is shown in Supplementary Figure S1. In the liver, a population of $CD172^{low}/CD14^+$ cells resembled liver macrophages or Kupffer cells. $FSC^{low}/SSC^{low}/CD3^+$ lymphocytes were differentiated into αβ T cells ($CD3^+/\gamma\delta$ TCR^-) and γδ T cells ($CD3^+/\gamma\delta$ TCR^+). Analysis of co-expression of CD4 and CD8α was used to identify subpopulations of αβ T cells. Activated and effector γδ T cells were identified as $CD2^+/CD8\alpha^-$ and $CD2^+/CD8\alpha^+$, respectively. Flow Cytometer BD FACS Canto II with FACS DIVA Software (BD Bioscience, San Jos, CA) and FlowJo™ V10 for Windows (Becton, Dickinson and Company; 2019) were used for all analyses.

4.4.6. Processing of Blood Samples

Serum samples were centrifuged at 2150× *g* for 20 min at 20 °C and EDTA blood was aliquoted and stored at −80 °C until further use. Tissue samples, which were collected during necropsy, were stored at –80 °C. qPCRs were performed after homogenization in 1 mL phosphate-buffered saline (PBS) using a metal bead TissueLyser II (Qiagen GmbH, Hilden, Germany). The supernatant was used for virus isolation (hemadsorption tests).

ASFV Antigen and Genome Detection

For rapid antigen detection Lateral Flow device INgezim.ASFCROM Antigeno 11.ASF.K42 (Ingenasa) was used with blood and serum. For qPCR, viral nucleic acid was extracted using the NucleoMag Vet Kit (Machery-Nagel, Düren, Germany) and the KingFisher® extraction platform (Thermo Fisher Scientific, Waltham, MA USA). qPCRs were performed according to the protocols published by King et al. [54] and Tignon et al. [55] with slight modifications. All PCRs were performed using a C1000™ thermal cycler from BIO-RAD (Hercules, CA, USA), with the CFX96™ Real-Time System. Results of both qPCRs were recorded as genome copies per µL.

To detect ASFV in serum, blood and tissue samples a hemadsorption test (HAT) was performed using PBMC-derived macrophages according to slightly modified standard procedures [56]. The samples were tested in quadruplicate.

4.4.7. Statistical Analysis

GraphPad Prism 8 (Graphpad Software Inc., San Diego, CA, USA) was used for statistical analyses and graph creation. Statistically significant differences were investigated by multiple *t*-tests with Holm–Sidak's correction for multiple comparisons. Statistical significance was defined as $p < 0.05$ and indicated with an asterisk (*).

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/8/662/s1, Figure S1: Gating strategy for the identification of infected myeloid cells by flow cytometry., Table S1: Temperature profiles of control and infected domestic pigs from 0 to 10 dpi.

Author Contributions: Conceptualization, A.B., S.B., J.S., J.P. and U.B.; methodology, J.S., A.B., J.P., A.S., A.E., K.P., K.F. and U.B.; formal analysis, J.S., A.B., J.P., A.S., U.B. and K.F.; investigation, J.S., A.B., J.P., A.S., U.B., S.B. and K.F.; resources, S.B.; writing—original draft preparation, J.S., A.B., A.S., U.B. and K.F.; writing—review and editing, S.B., A.B. and J.S.; visualization, J.S., A.S., J.P. and K.F.; supervision, S.B. and A.B.; project administration, S.B.; funding acquisition, S.B.. All authors have read and agreed to the published version of the manuscript.

Funding: This publication is partly based upon work from COST Action 15116 ASF-STOP supported by the European Cooperation in Science and Technology and was partially funded by the FLI ASF research network.

Acknowledgments: For expert technical assistance we thank Silvia Schuparis, Ulrike Kleinert, Robin Brandt, Silke Rehbein, Christian Loth, and the animal caretakers who were involved in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mason-D'Croz, D.; Bogard, J.R.; Herrero, M.; Robinson, S.; Sulser, T.B.; Wiebe, K.; Willenbockel, D.; Godfray, H.C.J. Modelling the global economic consequences of a major African swine fever outbreak in China. *Nat. Food* **2020**, *1*, 221–228. [CrossRef]
2. Burrage, T.G. African swine fever virus infection in Ornithodoros ticks. *Virus Res.* **2013**, *173*, 131–139. [CrossRef]
3. Gogin, A.; Gerasimov, V.; Malogolovkin, A.; Kolbasov, D. African swine fever in the North Caucasus region and the Russian Federation in years 2007–2012. *Virus Res.* **2013**, *173*, 198–203. [CrossRef]
4. Li, L.; Ren, Z.; Wang, Q.; Ge, S.; Liu, Y.; Liu, C.; Liu, F.; Hu, Y.; Li, J.; Bao, J.; et al. Infection of African swine fever in wild boar, China, 2018. *Transbound. Emerg. Dis.* **2019**, *66*, 1395–1398. [CrossRef]

5. Zhao, D.; Liu, R.; Zhang, X.; Li, F.; Wang, J.; Zhang, J.; Liu, X.; Wang, L.; Zhang, J.; Wu, X.; et al. Replication and virulence in pigs of the first African swine fever virus isolated in China. *Emerg. Microbes Infect.* **2019**, *8*, 438–447. [CrossRef] [PubMed]
6. Yoo, D.; Kim, H.; Lee, J.Y.; Yoo, H.S. African swine fever: Etiology, epidemiological status in Korea, and perspective on control. *J. Vet. Sci.* **2020**, *21*, e38. [CrossRef] [PubMed]
7. Frant, M.; Wozniakowski, G.; Pejsak, Z. African Swine Fever (ASF) and Ticks. No Risk of Tick-mediated ASF Spread in Poland and Baltic States. *J. Vet. Res.* **2017**, *61*, 375–380. [CrossRef] [PubMed]
8. Gallardo, C.; Fernandez-Pinero, J.; Pelayo, V.; Gazaev, I.; Markowska-Daniel, I.; Pridotkas, G.; Nieto, R.; Fernandez-Pacheco, P.; Bokhan, S.; Nevolko, O.; et al. Genetic variation among African swine fever genotype II viruses, eastern and central Europe. *Emerg. Infect. Dis.* **2014**, *20*, 1544–1547. [CrossRef] [PubMed]
9. Chapman, D.A.; Darby, A.C.; Da Silva, M.; Upton, C.; Radford, A.D.; Dixon, L.K. Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus. *Emerg. Infect. Dis.* **2011**, *17*, 599–605. [CrossRef] [PubMed]
10. Guinat, C.; Reis, A.L.; Netherton, C.L.; Goatley, L.; Pfeiffer, D.U.; Dixon, L. Dynamics of African swine fever virus shedding and excretion in domestic pigs infected by intramuscular inoculation and contact transmission. *Vet. Res.* **2014**, *45*, 93. [CrossRef]
11. Howey, E.B.; O'Donnell, V.; de Carvalho Ferreira, H.C.; Borca, M.V.; Arzt, J. Pathogenesis of highly virulent African swine fever virus in domestic pigs exposed via intraoropharyngeal, intranasopharyngeal, and intramuscular inoculation, and by direct contact with infected pigs. *Virus Res.* **2013**, *178*, 328–339. [CrossRef] [PubMed]
12. Gabriel, C.; Blome, S.; Malogolovkin, A.; Parilov, S.; Kolbasov, D.; Teifke, J.P.; Beer, M. Characterization of African swine fever virus Caucasus isolate in European wild boars. *Emerg. Infect. Dis.* **2011**, *17*, 2342–2345. [CrossRef] [PubMed]
13. Pikalo, J.; Zani, L.; Hühr, J.; Beer, M.; Blome, S. Pathogenesis of African swine fever in domestic pigs and European wild boar-Lessons learned from recent animal trials. *Virus Res.* **2019**, *271*, 197614. [CrossRef] [PubMed]
14. Gallardo, M.C.; Reoyo, A.T.; Fernandez-Pinero, J.; Iglesias, I.; Munoz, M.J.; Arias, M.L. African swine fever: A global view of the current challenge. *Porcine Health Manag.* **2015**, *1*, 21. [CrossRef]
15. Nurmoja, I.; Motus, K.; Kristian, M.; Niine, T.; Schulz, K.; Depner, K.; Viltrop, A. Epidemiological analysis of the 2015-2017 African swine fever outbreaks in Estonia. *Prev. Vet. Med.* **2018**. [CrossRef]
16. Nurmoja, I.; Petrov, A.; Breidenstein, C.; Zani, L.; Forth, J.H.; Beer, M.; Kristian, M.; Viltrop, A.; Blome, S. Biological characterization of African swine fever virus genotype II strains from north-eastern Estonia in European wild boar. *Transbound. Emerg. Dis.* **2017**, *64*, 2034–2041. [CrossRef]
17. Zani, L.; Forth, J.H.; Forth, L.; Nurmoja, I.; Leidenberger, S.; Henke, J.; Carlson, J.; Breidenstein, C.; Viltrop, A.; Höper, D.; et al. Deletion at the 5'-end of Estonian ASFV strains associated with an attenuated phenotype. *Sci. Rep.* **2018**, *8*, 6510. [CrossRef]
18. Sanchez-Cordon, P.J.; Nunez, A.; Neimanis, A.; Wikstrom-Lassa, E.; Montoya, M.; Crooke, H.; Gavier-Widen, D. African Swine Fever: Disease Dynamics in Wild Boar Experimentally Infected with ASFV Isolates Belonging to Genotype I and II. *Viruses* **2019**, *11*. [CrossRef]
19. Kärber, G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn-Schmiedeb. Arch. Exp. Pathol. Pharmakol.* **1931**, *162*, 480–483. [CrossRef]
20. Spearman, C. The method of 'right and wrong cases' ('Constant stimuli') without Gauss's formulae. *Br. J. Psychol. 1904–1920* **1908**, *2*, 227–242. [CrossRef]
21. Galindo-Cardiel, I.; Ballester, M.; Solanes, D.; Nofrarias, M.; Lopez-Soria, S.; Argilaguet, J.M.; Lacasta, A.; Accensi, F.; Rodriguez, F.; Segales, J. Standardization of pathological investigations in the framework of experimental ASFV infections. *Virus Res.* **2013**, *173*, 180–190. [CrossRef]
22. Schulz, K.; Staubach, C.; Blome, S. African and classical swine fever: Similarities, differences and epidemiological consequences. *Vet. Res.* **2017**, *48*, 84. [CrossRef]
23. Carrasco, L.; Gomez-Villamandos, J.C.; Bautista, M.J.; Martin de las Mulas, J.; Villeda, C.J.; Wilkinson, P.J.; Sierra, M.A. In vivo replication of African swine fever virus (Malawi '83) in neutrophils. *Vet. Res.* **1996**, *27*, 55–62. [PubMed]

24. Genovesi, E.V.; Knudsen, R.C.; Whyard, T.C.; Mebus, C.A. Moderately virulent African swine fever virus infection: Blood cell changes and infective virus distribution among blood components. *Am. J. Vet. Res.* **1988**, *49*, 338–344. [PubMed]
25. Franzoni, G.; Graham, S.P.; Sanna, G.; Angioi, P.; Fiori, M.S.; Anfossi, A.; Amadori, M.; Dei Giudici, S.; Oggiano, A. Interaction of porcine monocyte-derived dendritic cells with African swine fever viruses of diverse virulence. *Vet. Microbiol.* **2018**, *216*, 190–197. [CrossRef] [PubMed]
26. Golding, J.P.; Goatley, L.; Goodbourn, S.; Dixon, L.K.; Taylor, G.; Netherton, C.L. Sensitivity of African swine fever virus to type I interferon is linked to genes within multigene families 360 and 505. *Virology* **2016**, *493*, 154–161. [CrossRef]
27. Gregg, D.A.; Schlafer, D.H.; Mebus, C.A. African swine fever virus infection of skin-derived dendritic cells in vitro causes interference with subsequent foot-and-mouth disease virus infection. *J. Vet. Diagn. Investig.* **1995**, *7*, 44–51. [CrossRef]
28. Gregg, D.A.; Mebus, C.A.; Schlafer, D.H. Early infection of interdigitating dendritic cells in the pig lymph node with African swine fever viruses of high and low virulence: Immunohistochemical and ultrastructural studies. *J. Vet. Diagn. Investig.* **1995**, *7*, 23–30. [CrossRef]
29. Franzoni, G.; Graham, S.P.; Dei Giudici, S.; Oggiano, A. Porcine Dendritic Cells and Viruses: An Update. *Viruses* **2019**, *11*. [CrossRef]
30. Escribano, J.M.; Galindo, I.; Alonso, C. Antibody-mediated neutralization of African swine fever virus: Myths and facts. *Virus Res.* **2013**, *173*, 101–109. [CrossRef]
31. Netherton, C.L.; Connell, S.; Benfield, C.T.O.; Dixon, L.K. The Genetics of Life and Death: Virus-Host Interactions Underpinning Resistance to African Swine Fever, a Viral Hemorrhagic Disease. *Front. Genet.* **2019**, 10. [CrossRef] [PubMed]
32. Do, T.D.; Park, C.; Choi, K.; Jeong, J.; Vo, M.K.; Nguyen, T.T.; Chae, C. Comparison of pathogenicity of highly pathogenic porcine reproductive and respiratory syndrome virus between wild and domestic pigs. *Vet. Res. Commun.* **2015**, *39*, 79–85. [CrossRef] [PubMed]
33. Lorenzini, R.; Fanelli, R.; Tancredi, F.; Siclari, A.; Garofalo, L. Matching STR and SNP genotyping to discriminate between wild boar, domestic pigs and their recent hybrids for forensic purposes. *Sci. Rep.* **2020**, *10*, 3188. [CrossRef] [PubMed]
34. Chen, S.; Gomes, R.; Costa, V.; Santos, P.; Charneca, R.; Zhang, Y.P.; Liu, X.H.; Wang, S.; Bento, P.; Nunes, J.L.; et al. How immunogenetically different are domestic pigs from wild boars: A perspective from single-nucleotide polymorphisms of 19 immunity-related candidate genes. *Immunogenetics* **2013**, *65*, 737–748. [CrossRef] [PubMed]
35. Le, M.T.; Choi, H.; Lee, H.; Le, V.C.Q.; Ahn, B.; Ho, C.-S.; Hong, K.; Song, H.; Kim, J.-H.; Park, C. SLA-1 Genetic Diversity in Pigs: Extensive Analysis of Copy Number Variation, Heterozygosity, Expression, and Breed Specificity. *Sci. Rep.* **2020**, *10*, 743. [CrossRef] [PubMed]
36. Salguero, F.J. Comparative Pathology and Pathogenesis of African Swine Fever Infection in Swine. *Front. Vet. Sci.* **2020**, *7*, 282. [CrossRef]
37. Zakaryan, H.; Cholakyans, V.; Simonyan, L.; Misakyan, A.; Karalova, E.; Chavushyan, A.; Karalyan, Z. A study of lymphoid organs and serum proinflammatory cytokines in pigs infected with African swine fever virus genotype II. *Arch. Virol.* **2015**, *160*, 1407–1414. [CrossRef]
38. Oura, C.A.; Powell, P.P.; Parkhouse, R.M. African swine fever: A disease characterized by apoptosis. *J. Gen. Virol.* **1998**, *79*, 1427–1438. [CrossRef]
39. Gomez-Villamandos, J.C.; Hervas, J.; Mendez, A.; Carrasco, L.; Martin de las Mulas, J.; Villeda, C.J.; Wilkinson, P.J.; Sierra, A. Experimental African swine fever: Apoptosis of lymphocytes and virus replication in other cells. *J. Gen. Virol.* **1995**, *76*, 2399–2405. [CrossRef]
40. Carrasco, L.; de Lara, F.C.; Martin de las Mulas, J.; Gomez-Villamandos, J.C.; Perez, J.; Wilkinson, P.J.; Sierra, M.A. Apoptosis in lymph nodes in acute African swine fever. *J. Comp. Pathol.* **1996**, *115*, 415–428.
41. Salguero, F.J.; Sanchez-Cordon, P.J.; Nunez, A.; Fernandez de Marco, M.; Gomez-Villamandos, J.C. Proinflammatory cytokines induce lymphocyte apoptosis in acute African swine fever infection. *J. Comp. Pathol.* **2005**, *132*, 289–302. [CrossRef] [PubMed]
42. Pikalo, J.; Schoder, M.E.; Sehl, J.; Breithaupt, A.; Tignon, M.; Cay, A.B.; Gager, A.M.; Fischer, M.; Beer, M.; Blome, S. The African swine fever virus isolate Belgium 2018/1 shows high virulence in European wild boar. *Transbound. Emerg. Dis.* **2020**, *67*, 1654–1659. [CrossRef] [PubMed]

43. Samkange, A.; Mushonga, B.; Mudimba, D.; Chiwome, B.A.; Jago, M.; Kandiwa, E.; Bishi, A.S.; Molini, U. African Swine Fever Outbreak at a Farm in Central Namibia. *Case Rep. Vet. Med.* **2019**, *2019*. [CrossRef] [PubMed]
44. Moulton, J.E.; Pan, I.C.; Hess, W.R.; DeBoer, C.J.; Tessler, J. Pathologic features of chronic pneumonia in pigs with experimentally induced African swine fever. *Am. J. Vet. Res.* **1975**, *36*, 27–32.
45. Brown, V.R.; Bevins, S.N. A Review of African Swine Fever and the Potential for Introduction into the United States and the Possibility of Subsequent Establishment in Feral Swine and Native Ticks. *Front. Vet. Sci.* **2018**, *5*, 11. [CrossRef]
46. Oura, C.A.L.; Denyer, M.S.; Takamatsu, H.; Parkhouse, R.M.E. In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J. Gen. Virol.* **2005**, *86*, 2445–2450. [CrossRef]
47. Hühr, J.; Schäfer, A.; Schwaiger, T.; Zani, L.; Sehl, J.; Mettenleiter, T.C.; Blome, S.; Blohm, U. Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain. *Transbound. Emerg. Dis.* **2020**. [CrossRef]
48. Carrasco, L.; Fernandez, A.; Gomez Villamandos, J.C.; Mozos, E.; Mendez, A.; Jover, A. Kupffer cells and PIMs in acute experimental African swine fever. *Histol. Histopathol.* **1992**, *7*, 421–425.
49. Oura, C.A.; Powell, P.P.; Parkhouse, R.M. Detection of African swine fever virus in infected pig tissues by immunocytochemistry and in sity hybridisation. *J. Virol. Methods* **1998**, *72*, 205–217. [CrossRef]
50. Carrasco, L.; de Lara, F.C.; Gomez-Villamandos, J.C.; Bautista, M.J.; Villeda, C.J.; Wilkinson, P.J.; Sierra, M.A. The pathogenic role of pulmonary intravascular macrophages in acute African swine fever. *Res. Vet. Sci.* **1996**, *61*, 193–198. [CrossRef]
51. Pietschmann, J.; Guinat, C.; Beer, M.; Pronin, V.; Tauscher, K.; Petrov, A.; Keil, G.; Blome, S. Course and transmission characteristics of oral low-dose infection of domestic pigs and European wild boar with a Caucasian African swine fever virus isolate. *Arch. Virol.* **2015**, *160*, 1657–1667. [CrossRef] [PubMed]
52. Meyerholz, D.K.; Beck, A.P. Fundamental Concepts for Semiquantitative Tissue Scoring in Translational Research. *ILAR Journal* **2019**, *59*, 13–17. [CrossRef] [PubMed]
53. Eguchi-Ogawa, T.; Matsubara, T.; Toki, D.; Okumura, N.; Ando, A.; Kitagawa, H.; Uenishi, H. Distribution of the CD4 Alleles in Sus scrofa Demonstrates the Genetic Profiles of Western Breeds and Miniature Pigs. *Anim. Biotechnol.* **2018**, *29*, 227–233. [CrossRef] [PubMed]
54. King, D.P.; Reid, S.M.; Hutchings, G.H.; Grierson, S.S.; Wilkinson, P.J.; Dixon, L.K.; Bastos, A.D.; Drew, T.W. Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *J. Virol. Methods* **2003**, *107*, 53–61. [CrossRef]
55. Tignon, M.; Gallardo, C.; Iscaro, C.; Hutet, E.; Van der Stede, Y.; Kolbasov, D.; De Mia, G.M.; Le Potier, M.F.; Bishop, R.P.; Arias, M.; et al. Development and inter-laboratory validation study of an improved new real-time PCR assay with internal control for detection and laboratory diagnosis of African swine fever virus. *J. Virol. Methods* **2011**, *178*, 161–170. [CrossRef]
56. Carrascosa, A.L.; Bustos, M.J.; de Leon, P. Methods for growing and titrating African swine fever virus: Field and laboratory samples. *Curr. Protoc. Cell Biol.* **2011**, *26*. [CrossRef]

3 Own Contributions to Publications

Publication I

Porcine Invariant Natural Killer T Cells: Functional Profiling and Dynamics in Steady State and Viral Infections. **Alexander Schäfer**, Jane Hühr, Theresa Schwaiger, Anca Dorhoi, Thomas C. Mettenleiter, Sandra Blome, Charlotte Schröder and Ulrike Blohm.
Frontiers in Immunology, doi: 10.3389/fimmu.2019.01380

Alexander Schäfer	Design of the study; Sample Preparation; Investigation; Data Analysis; Visualization; Original Draft and Correction of the Manuscript
Jane Hühr	Sample Acquisition; Correction of the Manuscript
Theresa Schwaiger	Sample Acquisition; Correction of the Manuscript
Anca Dorhoi	Data Analysis; Correction of the Manuscript
Thomas C. Mettenleiter	Funding Acquisition; Project Administration; Supervision; Correction of the Manuscript
Sandra Blome	Sample Acquisition; Correction of the Manuscript
Charlotte Schröder	Sample Acquisition; Correction of the Manuscript
Ulrike Blohm	Design of the study; Funding Acquisition; Data Analysis; Supervision; Correction of the Manuscript

Publication II

Experimental H1N1pdm09 infection in pigs mimics human seasonal influenza infections.
Theresa Schwaiger, Julia Sehl, Claudia Karte, **Alexander Schäfer**, Jane Hühr, Thomas C. Mettenleiter, Charlotte Schröder, Bernd Köllner, Reiner Ulrich, Ulrike Blohm.
PLoS ONE, doi: 10.1371/journal.pone.0222943

Theresa Schwaiger	Design of the study; Sample Acquisition; Investigation; Data Analysis; Visualization; Original Draft and Correction of the Manuscript
Julia Sehl	Sample Acquisition; Investigation; Data Analysis; Original Draft and Correction of the Manuscript
Claudia Karte	Veterinarian Care; Sample Acquisition; Correction of the Manuscript
Alexander Schäfer	Sample Preparation; Data Analysis; Correction of the Manuscript
Jane Hühr	Sample Acquisition; Correction of the Manuscript
Thomas C. Mettenleiter	Funding Acquisition; Project Administration; Correction of the Manuscript
Charlotte Schröder	Veterinarian Care; Sample Acquisition
Bernd Köllner	Funding Acquisition; Sample Acquisition
Reiner Ulrich	Design of the study; Sample Acquisition; Investigation; Supervision; Correction of the Manuscript
Ulrike Blohm	Design of the study; Funding Acquisition; Investigation; Data Analysis; Supervision; Visualization; Original Draft and Correction of the Manuscript

Publication III

Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain. Jane Hühr*, **Alexander Schäfer***, Theresa Schwaiger, Laura Zani, Julia Sehl, Thomas C. Mettenleiter, Sandra Blome, Ulrike Blohm. *, joint first authors
Transboundary and Emerging Diseases, doi: 10.1111/tbed.13678

Jane Hühr*	Sample Acquisition; Investigation; Original Draft and Correction of the Manuscript
Alexander Schäfer*	Sample Preparation; Investigation; Data Analysis; Visualization; Original Draft and Correction of the Manuscript
Theresa Schwaiger	Sample Acquisition; Correction of the Manuscript
Laura Zani	Veterinarian Care; Sample Acquisition; Correction of the Manuscript
Julia Sehl	Sample Acquisition; Correction of the Manuscript
Thomas C. Mettenleiter	Supervision; Correction of the Manuscript
Sandra Blome	Design of the study; Funding Acquisition; Veterinarian Care; Sample Acquisition; Supervision; Correction of the Manuscript
Ulrike Blohm	Design of the study; Sample Acquisition; Data Analysis; Supervision; Correction of the Manuscript

Publication IV

T-cell responses in domestic pigs and wild boar upon infection with the moderately virulent African swine fever virus strain "Estonia2014". **Alexander Schäfer**, Laura Zani, Jutta Pikalo, Jane Hühr, Julia Sehl, Thomas C. Mettenleiter, Angele Breithaupt, Sandra Blome, Ulrike Blohm. *Transboundary and Emerging Diseases*, submitted: September 2020, doi: 10.22541/au.159986510.05288008 (Authorea Preprint)

Alexander Schäfer	Sample Preparation; Investigation; Data Analysis; Visualization; Original Draft and Correction of the Manuscript
Laura Zani	Veterinarian Care; Investigation; Data Analysis; Sample Acquisition; Correction of the Manuscript
Jutta Pikalo	Veterinarian Care; Investigation; Data Analysis; Sample Acquisition; Correction of the Manuscript
Jane Hühr	Sample Acquisition; Correction of the Manuscript
Julia Sehl	Sample Acquisition; Correction of the Manuscript
Thomas C. Mettenleiter	Supervision; Correction of the Manuscript
Angele Breithaupt	Design of the study; Sample Acquisition; Correction of the Manuscript
Sandra Blome	Design of the study; Veterinarian Care; Sample Acquisition; Investigation; Supervision; Correction of the Manuscript
Ulrike Blohm	Design of the study; Sample Acquisition; Investigation; Data Analysis; Supervision; Original Draft and Correction of the Manuscript

Publication V

Comparative Pathology of Domestic Pigs and Wild Boar Infected with the Moderately Virulent African Swine Fever Virus Strain "Estonia 2014". Julia Sehl, Jutta Pikalo, **Alexander Schäfer**, Kati Franzke, Katrin Pannhorst, Ahmed Elnagar, Ulrike Blohm, Sandra Blome, Angele Breithaupt. *Pathogens*, doi: 10.3390/pathogens9080662

Julia Sehl	Sample Acquisition; Pathological Investigation; Data Analysis; Original Draft and Correction of the Manuscript
Jutta Pikalo	Veterinarian Care; Sample Acquisition; Clinical Investigation; Data Analysis; Correction of the Manuscript
Alexander Schäfer	Sample Preparation; Flow Cytometry Investigation; Data Analysis; Original Draft and Correction of the Manuscript
Kati Franzke	Sample Acquisition; Electron Microscopy Investigation; Data Analysis; Correction of the Manuscript
Katrin Pannhorst	Sample Acquisition; Correction of the Manuscript
Ahmed Elnagar	Sample Acquisition; Correction of the Manuscript
Ulrike Blohm	Design of the study; Sample Acquisition; Correction of the Manuscript
Sandra Blome	Design of the study; Veterinarian Care; Sample Acquisition; Investigation; Supervision; Correction of the Manuscript
Angele Breithaupt	Design of the study; Sample Acquisition; Investigation; Data Analysis; Supervision; Original Draft and Correction of the Manuscript

In Agreement (Paper I to V)

.....

Place, Date

.....

Prof. Dr. Dr. h. c. T.C. Mettenleiter

.....

Alexander Schäfer

4 Results and Discussion

During Infection, Time Is Everything

Viral infections are fought by two distinct but cooperating arms of the host's immune system. The innate system identifies conserved pathogen structures by innate receptors, in a process that allows for rapid detection and responses. Thereby, it impairs viral spread, but does not achieve viral clearance in every case. Adaptive immune responses use clonally distributed receptors with high diversity to detect individual structures on pathogens, and thus, protect the host substantially more targeted. However, these responses need days to weeks to fully unfold. Viruses, on the other hand, are fast growing and spread easily between host cells. Time, therefore, is a vital good in viral infections. Conventional adaptive responses against viral infections were analyzed in **papers II-IV**, whereby pigs were investigated as biomedical models for human IAV infections (**paper II**) and as natural hosts for ASFV infections (**paper III-V**). To effectively protect the host during infections with fast acting pathogens, several fast reacting lymphocyte populations evolved that close the gap between the two arms. One of these bridging populations is iNKT cells, which remained rather enigmatic in pigs yet. Their characteristics were investigated in **paper I**.

Porcine iNKT cells in steady state and viral infection (Paper I)

Porcine iNKT cells have been identified several years ago but expression of surface markers, functional capacities and involvement in host defense have not been thoroughly investigated. In contrast, the pivotal roles of iNKT-cell responses during infections in humans and mice have been described long ago [153]. Therefore, the first major task of this thesis was to characterize porcine iNKT cells in greater detail.

The widespread detection system to investigate iNKT cells is antigen-loaded CD1d tetramers, commonly used in humans and mice [168, 177, 178] but also in pigs [191-195]. Fluorochrome-labelled CD1d tetramers allow the implementation of multicolor flow cytometry for the investigation of rare cells like iNKT cells, which enables researchers to analyze high cell numbers in order to accumulate a reasonable number of cells of interest. In **paper I**, we characterized porcine iNKT cells by multicolor flow cytometry. The most accessible sample is peripheral blood, which was used as the first tissue in the investigations. A major advantage of our newly established protocol is that the cells did not need to be cultivated prior to analysis. Human or murine iNKT cells are often expanded *in vitro* before they are further investigated.

However, this may change their phenotype and functional properties [171, 237], whereas our analysis of freshly isolated authentic iNKT cells produces less biased results.

The mean frequency of peripheral iNKT cells in naïve domestic pigs was 0.5%, mirroring the frequency in both human [237] and murine [238] blood. Peripheral iNKT cells displayed a mostly CD8α$^+$ or DN phenotype with a minor DP subset but no distinct CD4$^+$ population. This was in accordance with results from other groups [201, 203] and was also similar to human but contrary to murine iNKT cells [171]. Porcine CD8α$^+$ iNKT cells have previously been shown to be the main IFNγ producer among iNKT cells after *in vitro* activation [201]. Moreover, CD8α is also upregulated on γδ T cells [119, 122] and CD4$^+$ αβ T cells during activation and differentiation [191]. Earlier studies described porcine iNKT cells as CD4$^-$/CD8α$^+$ or DN [201, 203], which has been discussed as a substantial difference to human iNKT cells [196]. However, we could show that porcine iNKT cells express CD4, but only when stimulated. This was underlined by the fact that activated (CD4$^-$/CD8α$^+$ and CD4$^+$/CD8α$^+$) porcine iNKT cells expressed higher levels of classical activation markers, i.e., CD25, ICOS, and MHC II. Thus, we proposed expression of CD8α and, even more, CD4 as a marker for activation of porcine iNKT cells. This also disproves the assumption that porcine and human iNKT cells differ significantly in this instance and instead indicates central similarities. Given the importance of co-receptor expression for activation and effector functions, the predominant expression of CD8α might be of even greater significance.

One of the key characteristics of innate T cells, such as iNKT cells, is their effector and memory phenotype after exiting the thymus without need for further activation or antigen contact [169, 239, 240]. We were able to show that peripheral porcine iNKT cells display an antigen-experienced phenotype, evidenced by lack of CD45RA expression. CD45RA is a splice variant of the CD45 gene with high molecular weight expressed on naïve cells. Splice variants with lower molecular weight (and thus, loss of CD45RA expression) are expressed after antigen contact and on memory T cells [241]. In line with this, we showed significantly higher frequencies of cells expressing markers generally associated with activation, i.e., CD5 [242], CD25 (IL-2Rα [243]), ICOS [244], and MHC II [245] among porcine iNKT cells in comparison to cTC. This demonstrated that porcine iNKT cells have a pre-activated phenotype in peripheral blood in healthy individuals, analogous to iNKT cells in other species [169, 239, 240].

Activation and effector functions of murine cTC are regulated by ICOS, especially during the induction of humoral responses [244]. ICOS expression in murine iNKT cells is also regulated by the central iNKT-cell transcription factor PLZF [246], is pivotal for their homeostasis and peripheral survival [247], and is a major co-stimulator for activation [248]. Our data indicate a central role of ICOS for porcine iNKT cells as well. Not only did virtually all iNKT cells express ICOS, its expression level further increased upon antigenic activation in iNKT cells but not cTC.

ICOS expression is also a hallmark of cells vital for the induction and maturation of humoral responses, T_{FH} cells [113]. Moreover, it has been shown that B cells express the iNKT-cell ligand CD1d, and interactions of B cells with iNKT cells are sufficient to induce antibody production and maturation even in the absence of classical Th cells [249]. iNKT cells with a similar phenotype as T_{FH} cells, termed $iNKT_{FH}$ cells, have been found in mice and they also shape humoral responses in a T_{FH}-like manner [250]. A recent study provided similar evidence for porcine iNKT cells: Renu *et al.* found that addition of αGC, and thus activation of iNKT cells, to an intranasally administered, inactivated IAV vaccine induced B-cell activation and improved mucosal antibody levels [195]. This indicates that $iNKT_{FH}$ cells are also present in pigs. Given that the most efficient B-cell helpers in humans are $CD4^+$ iNKT cells [251], the possibility of B-cell help by porcine iNKT cells is further supported by the upregulation of CD4 and MHC II on iNKT cells upon antigenic activation that we demonstrated, because activated $CD4^+$/MHC II^+ iNKT cells in pigs might provide T-cell help even in the absence of cTC. However, the molecular mechanisms how porcine iNKT cells also provide B-cell help and shape humoral responses need to be investigated in further studies.

Another important protein found on the surface of porcine iNKT cells is MHC II, which is typically limited to APCs. However, T cells from many species, including humans, dogs, cattle, and rats, but not mice, are able to synthesize MHC II, especially after activation [245]. Porcine T cells are also known to express MHC II [9], especially when they acquire memory status [252] or differentiate after antigen contact [253]. Expression of MHC II is functionally intertwined with expression of CD4. Blocking CD4 with mAbs decreased the amount of IFNγ and IL-4 produced by activated human iNKT cells, also in the absence of MHC II^+ APC [254]. Therefore, $CD4^+$/MHC II^+ iNKT cells regulate activation by interaction of CD4 and MHC II on different iNKT cells *in trans* [254]. These results further indicate striking similarities between porcine and human iNKT cells and demonstrate the advantage of pigs over mice as biomedical models.

Similarities to other species were also found based on the expression analysis of PLZF and lineage-specific transcription factors. Investigation of naïve porcine iNKT cells revealed three subpopulations in peripheral blood: PLZF$^+$/T-bet$^+$ iNKT1, PLZF^{hi}/T-bet$^-$ iNKT2, and a minor fraction of PLZF^{low}/T-bet$^-$ cells, termed non-iNKT1/2. These subsets were partially similar to murine ones [161], indicating that iNKT cells have conserved developmental and functional pathways. However, as of now it is not possible to further distinguish these subsets because additional mAbs are not yet available for pigs. Proper differentiation was verified in stimulation experiments with αGC, a known potent inducer of iNKT1 responses [255]. Upon stimulation, the majority of porcine iNKT cells differentiated into T-bet$^+$/ICOS$^+$/perforin$^+$ cells and produced IFNγ, all indicative of a Th1-biased activation [247, 248].

In **paper I**, we also presented a first insight into the kinetics of porcine iNKT cells during viral infections *in vivo*. We found elevated iNKT-cell frequencies in blood, BAL, lung, and spleen, and a significant increase in lung LN 4 and 7 dpi during subclinical IAV infection. In pigs with severe ASF after infection with highly virulent ASFV "Armenia2008", iNKT-cell frequencies increased significantly in blood, liver, lung, and liver LN 5 dpi. We did not find changes in iNKT-cell frequencies but signs of iNKT-cell activation in corresponding tissues during infection with moderately virulent ASFV "Estonia2014" (**paper IV**). Significant changes in iNKT-cell frequencies and upregulation of activation markers suggest that iNKT cells take part in antiviral immunity during IAV and ASFV infections. The *in vivo* responses were generally less pronounced than those found in *in vitro* studies. This is caused by different potencies of the stimulants. Experimental activation induced by αGC is more potent than by natural ligands or cytokines [161], as αGC has high affinity to the iNKT-cell TCR and a long half-life [255]. However, neither IAV nor ASFV are known to produce any glycolipids, indicating that TCR-driven activation does not take place during these infections. This leaves cytokines or endogenous glycolipids as inducers of iNKT-cell activation instead of pathogen-derived antigens [188]. An expansion of iNKT cells has also been shown in IAV-infected mice and humans [222, 256], and among human PBMCs stimulated with viral TLR ligands or UV-inactivated HHV-1 [257]. The authors concluded that detection of viral danger signals induced expression of type I IFNs, resulting in iNKT-cell activation [257]. Type I IFNs were detected in pigs during infection with IAV [258, 259] as well as ASFV [260, 261]. Therefore, an activating milieu is probably present in infected pigs. *In vitro*, we were only able to induce significant levels of activation with IAV but not ASFV. This can be explained by the experimental setup. We infected $CD172^+$ monocytes and used the supernatant to stimulate freshly isolated PBMCs. At least during infection with ASFV, DCs are considered the main producers of type I IFNs [260], which explains the lack of activation with ASFV-primed supernatant. Therefore, it can be hypothesized that activation of porcine iNKT cells during viral infections is driven by cytokines, presumably type I IFNs. This needs further *in vivo* verification.

In summary, in **paper I** we provided a detailed characterization of porcine iNKT cells at steady-state and during viral infections. Peripheral porcine iNKT cells primarily expressed CD8α but not CD4 on their surface and exhibited an antigen-experienced memory phenotype. They were divided into three functional subsets by differential expression analysis of PLZF and T-bet. Antigenic activation resulted in significant proliferation, differentiation into a Th1-like state, and induced expression of effector proteins, i.e., perforin and IFNγ. Finally, we demonstrated *in vivo* and *in vitro* response kinetics during infections with IAV and ASFV. These results provided the most detailed insight into porcine iNKT cells yet and also evidence of considerable homologies between human and porcine, and to a lesser extent murine, iNKT cells.

Pigs as biomedical models for infectious diseases (Paper II)

IAV infections remain a significant threat not only to human but also porcine populations and are thought to pave the way for secondary, often fatal, bacterial infections, especially in vulnerable populations, like the elderly or immunocompromised patients [262]. These co-infections come with increased morbidity and lethality, especially in pandemic influenza [263]. IAV facilitate secondary infections in different ways. Induced type I IFN expression impairs antibacterial responses in general [264] and T-cell responses in particular [265]. Additionally, bacterial receptors are increasingly expressed during and after IAV infections or become more accessible, especially in the lower respiratory tract [266]. Both pigs and humans are susceptible for IAV but also for related potential elicitors of secondary infections, e.g., *Streptococcus suis* and *Streptococcus pneumoniae*, respectively. Moreover, pigs and humans are equally affected by these bactoviral coinfections [267, 268]. Besides, physiologic similarities between humans and pigs allow for investigations of experimental porcine infections with human pathogens, e.g., *Streptococcus pneumoniae* [269].

However, in order to understand co-infections, we need basic data about monocausal infections because ongoing T-cell responses are still largely not understood. Various immune cells contributing to antiviral responses have been identified in previous investigations, but translation of results from murine studies to human patients remains challenging [270]. Thus, the use of a new biomedical model, the pig, has been suggested [262]. In **paper II**, we investigated porcine immune responses against H1N1pdm09 infection along the route of entry and in disease-affected tissue. Since studies of the immune response against IAV infections in pigs have been done before [212-219], we used a new experimental approach. First, we compared local responses in individual pigs dissected at specific days after infection. Second, we investigated the systemic response in a separate group, where the same animals were tested repeatedly, in order to describe individual lymphocyte kinetics.

Infected pigs in our study showed no clinical signs of disease. IAV infections often cause rather mild clinical signs in pigs, which has also been shown for infections with H1N1pdm09 [219, 271, 272]. This is consistent with studies showing no elevated levels of pro-inflammatory cytokines in BAL fluid of pigs after infection with H1N1pdm09 [273, 274]. Human IAV infections vary considerably, probably because the heterogeneity in the human population, regarding age, infectious dose, and infecting strains, is significantly higher than in experimental infections of pigs. However, consistent with our findings, studies estimated that a major proportion of human IAV infections are also asymptomatic [275]. IAV antigen was largely restricted to the nose and lungs, and to a lesser extent to the trachea of infected pigs and was only detectable until 7 dpi. Distribution and replication sites of IAV in humans, as well as their contribution to viral dissemination and infectivity, are not well understood. Recent studies in

primary human cell cultures and another model species, ferrets, indicate the nasal epithelium as the major source of viral transmission [276], which is in line with our findings.

On a systemic level, we detected a mild lymphopenia, which was driven by αβ T cells, γδ T cells, and B cells and lasted until 7 dpi. Lymphopenia is a common symptom of human IAV infections [277-279], and has even been proposed as a possible screening tool for patients [280, 281]. Neutrophilia, increased numbers of neutrophils, are often found in humans [277], but were not observed in our study. However, we detected an increase of $CD14^+$ granulocytes, which is indicative of granulocyte activation [282]. Moreover, we found proliferating cells among DP αβ T cells, $CD8\alpha^+$ αβ T cells, and activated γδ T cells. Proliferation was more pronounced after the first infection. This is also comparable to human IAV infections [283]. Together, these systemic findings correlated well with human IAV infections, especially given the fact that the exact time of infection in humans is usually unknown and thus, changes are difficult to attribute to a certain period after infection.

Locally, we detected an influx of αβ T cells, especially in nose and BAL 4 to 7 dpi. The overall response was heavily dominated by $CD8\alpha\alpha^+$ αβ T cells, which were the predominant subset in nose, BAL, and lung. In lung LN, the distribution even reversed from $CD8\alpha\beta^+$ to $CD8\alpha\alpha^+$ 4 to 7 dpi. Both subsets displayed increased perforin expression 4 dpi in all tissues but most prominent in the nose, indicative of an ongoing cytotoxic response. γδ T cells were considerably less frequent than αβ T cells in the investigated tissues, but still displayed an activated phenotype and increased perforin expression, especially in the nose. Responses after the second infection were generally similar but less pronounced. Since we found a rapid influx of cytotoxic effector cells at the site of viral entry, it can be assumed that the proliferating lymphocytes detected systemically migrated to the sites of inflammation. This is supported by data from human infections, where proliferating cells in the peripheral blood have been shown to exert cytotoxic properties [283]. Although significantly more frequent in porcine than in human blood, γδ T-cell frequencies in the lungs of both species are comparable [284]. Moreover, cytotoxic responses were also found in human γδ T cells during *in vitro* IAV experiments [285, 286].

Increased CD8αα expression is suggestive of regulatory responses [94, 287]. $CD8\alpha\alpha^+$ T cells emerge from $CD8\alpha\beta^+$ T cells that downregulated the CD8 β-chain, which results in reduced activation [93, 288]. Therefore, we hypothesize that IAV infection in pigs induces broad cytotoxic and inhibitory T-cell responses at the local level in parallel. This effectively clears the infection while maintaining tissue integrity and without excessive tissue damage. Of note, the local immune responses found in our study correlate well with the postulated predominant IAV distribution in the upper respiratory tract, i.e., nasal epithelium [276]. Distinct cytotoxic immune responses in the nose suggest that it is the primary site of IAV replication in pigs as well. Future studies should investigate whether IAV transmission in pigs is also largely

dependent on replication in the nose, to further corroborate the findings in a human-like model.

In conclusion, in **paper II** we demonstrated substantial similarities between human and porcine infections with H1N1pdm09. Viral antigen and inflammation were limited to nose, trachea, and lung. Although we found systemic lymphocyte proliferation, cytotoxic responses exerted by CD8αα$^+$ αβ T cells and activated γδ T cells were restricted to the route of viral entry. These responses efficiently cleared both the first and second infection, whereby responses after the second infection were less pronounced. These results closely mirror characteristics of human IAV infections. Therefore, pigs, as new biomedical models, offer a promising way to understand pathogenic processes and immune responses during IAV infection.

Pigs as natural hosts for viral infections (Paper III–V)

In the last part of the presented thesis, we investigated porcine T-cell responses during ASFV infections for which pigs are the only known natural hosts. Since ASFV was introduced from its sub-Saharan African origin into numerous European and Asian countries, it caused outbreaks with many million fatalities. The immune response in susceptible species is far from understood and thus, vaccines are not available. Therefore, we investigated porcine T-cell responses against highly and moderately virulent ASFV strains, "Armenia2008" (**paper III**) and "Estonia2014" (**paper IV**), respectively, to gain insights into the immune processes involved in anti-ASFV responses. Besides domestic pigs, wild boar are also susceptible to ASFV infection and contribute to viral spread in Europe and Asia [61-63]. Therefore, we opted for an experimental approach in which not only the two virus strains but also the two hosts were examined. Moreover, we applied a combined analysis platform of clinical, histopathological, immunological, and electron microscopical investigation techniques to compare "Estonia2014" infections in both subspecies (**paper V**).

The importance of CD8α$^+$ lymphocytes for protective immunity against ASFV infection has been shown previously by Oura *et al.* [227]. A strong CD8α-biased response was also found in our studies, but with different qualities. During infection with highly virulent "Armenia2008", the response in domestic pigs was dominated by DP αβ T cells, while infection with moderately virulent "Estonia2014" induced a response of CD8α$^+$ and DP αβ T cells. In contrast, we found a strong bias towards γδ T cells in wild boar during both infections. γδ T cells in wild boar differentiated into CD2$^+$/CD8α$^+$ effector cells, while there was no response of γδ T cells in domestic pigs. With these results, our studies give a first evidence-based indication, which CD8α$^+$ T-cell population is responsible for the antiviral effects: In domestic pigs, antiviral effects seem to be orchestrated mainly by CD8α$^+$ and DP αβ T cells, while wild boar have a response

dominated by γδ T cells. This is particularly interesting because a previous study found a positive correlation between γδ T-cell frequencies and survival of ASFV infection in a rather small sample of domestic pigs [236]. Our results challenge this correlation because wild boar had higher disease severity and lethality although they had higher γδ T-cell frequencies [80]. Porcine γδ T cells, especially when differentiated into CD8α$^+$ effector cells, exert mainly pro-inflammatory functions [289], which might explain the more severe disease progression in wild boar. These findings correlated with increased Kupffer cell degeneration in the liver of infected wild boar in a comparative pathological analysis in **paper V**. Besides, there were only minor differences between domestic pigs and wild boar on a histopathological level. Domestic pigs showed higher and prolonged inflammation in the lung, while wild boar demonstrated more severe degeneration of Kupffer cells in the liver. However, there were no differences in the viral loads in investigated tissues.

The specific antiviral mechanisms remain to be elucidated. It is unknown whether the bias towards γδ T cells is a general immune characteristic of wild boar or if it is specific for viral or even ASFV infections. Although domestic pigs and Eurasian wild boar have high genetic consensus [290], little is known about potential differences at steady state or in response to infections. Wild boar are used as a model species for infections with members of the *Mycobacterium tuberculosis* complex [291-293] and Classical Swine Fever Virus [294]. However, these studies usually focus on expression analysis of immune-related genes [295, 296] or survival of vaccinated animals after challenge infection [291, 292]. Leukocytes are rarely mostly superficially analyzed, e.g., for the CD4/CD8α ratio [292]. Our data, therefore, give a first broad insight into composition and functional aspects of the wild boar immune system. However, even this comparison is hampered by the fact that wild boar and domestic pigs in our studies were not age matched. Nevertheless, comparative studies of wild and laboratory mice suggest that there are major differences to be expected. Wild mice were found to have significantly higher frequencies of effector and effector memory T cells, which also secreted significantly more IFNγ and IL-4 [297]. Their higher immune heterogeneity and variability at the genetic level make wild mice more comparable to humans [6]. Similar findings can be expected if domestic pigs and wild boar were compared, but this has yet not been done systematically.

One surprising, reproducible finding was a near-complete loss of the cytotoxic effector molecule perforin in cytotoxic T-cell subsets in both studies and both suids. The loss was most pronounced in domestic pigs and during highly virulent "Armenia2008" infection. Cytotoxic molecules like perforin or FasL are usually induced in T cells in pro-inflammatory environments to directly lyse target cells or induce apoptosis [104]. Several mechanisms behind the observed loss of perforin are possible. Lysis by perforin might be switched to Fas/FasL-

mediated induction of apoptosis during ongoing immune responses, which is accompanied by a loss of perforin even on mRNA level [298]. ASFV strains are known to express functional viral homologues of the anti-apoptotic protein Bcl-2 [299, 300], which preferentially inhibits perforin-mediated lysis [301]. A switch to FasL-mediated killing would circumvent this viral immune evasion and also explain the loss of perforin. Since perforin levels and clinical scores were lower in domestic pigs, it can be hypothesized that they shifted their cytotoxic response, eventually leading to viral clearance. Wild boar, on the other hand, had higher remaining perforin levels, which might be efficiently counteracted by ASFV, ultimately leading to impaired viral clearance, higher clinical scores, and increased fatalities. This is also in line with more severe liver damage in ASFV-infected wild boar [302], as perforin inhibition is tissue-protective during viral hepatitis [303]. Another possible explanation concerns the mAb clone, dG9, which only detects vesicle-associated perforin [304]. If newly synthesized perforin is not detected, this indicates a pronounced cytotoxic response especially in domestic pigs and to a lesser extent in wild boar. Since domestic pigs survive infection with moderately virulent "Estonia2014", a strong cytotoxic response seems beneficial, while the lower response in wild boar renders them vulnerable to ASF. This might also explain why wild boar succumb even to the moderately virulent ASFV infection [80]. Cytotoxic responses can also be inhibited by TGFβ secreted by Tregs, which we found in infections with highly and moderately virulent ASFV [305]. Finally, loss of effector molecules could be explained by T-cell exhaustion. T-cell exhaustion is usually found in chronic infections but has also been shown during acute infections. High antigen loads in infected tissue and continuous TCR activation lead to exhausted T cells, which loose effector functions [306]. However, the mechanistic background of the observed loss of perforin has to be elucidated in future studies.

Moreover, we revealed CD8α$^+$ iNKT cells as the principal subpopulation during steady state and further induced expression of CD8α$^+$ on iNKT cells during activation in **paper I**. In the study by Oura *et al.*, iNKT cells were therefore also depleted. Expansion of T cells with an innate phenotype after *in vitro* stimulation of porcine PBMCs with ASFV indicated a role for iNKT-cell responses [107]. Although the actual iNKT-cell phenotype differs from the described one, our results also suggest a contribution of iNKT cells to anti-ASFV immunity. Besides the *in vivo* kinetics in **paper I**, we also described activated iNKT cells in blood, spleen, and ghLN during infection with moderately virulent ASFV in **paper IV**. We found higher frequencies of iNKT cells positive for ICOS, demonstrating highly activated cells [244, 248]. Our findings in **paper I** suggest a differentiation into antiviral iNKT1 cells. ICOS expression might also indicate T_{FH}-like responses, which induce humoral responses and, eventually, production of antibodies [113]. In line with this assumption, ICOS$^+$ iNKT cells were mainly found 7 to 10 dpi in spleen and LN of infected pigs, at an appropriate time and in tissues known for the presence of

antibody-producing B cells [84, 102]. However, functional details have to be elucidated in future studies.

In summary, in **papers III and IV** we presented the first temporospatial description of T-cell responses against highly and moderately virulent ASFV infections in domestic pigs and wild boar. Domestic pigs displayed T-cell responses dominated by $CD8\alpha^+$ and DP αβ T cells during moderately and highly virulent ASFV infections, respectively. Wild boar, on the other hand, mounted a distinct γδ T-cell response. The implications remain unknown as of yet. **Paper V** correlated histopathological findings with immunological analyses during moderately virulent ASFV infection.

5 SUMMARY AND OUTLOOK

Viral diseases are a threat to bacteria and enormous animals alike. Vaccines are available against several viruses. However, for some viruses, like ASFV, we still lack vaccines, while for others, like IAV, they are not as effective as we need them to be. To a large extent, this is because we do not fully understand the mechanisms conferring antiviral immunity. To improve our understanding of antiviral immunity, we used a model species that is in many immunological aspects closer to humans than the widely used laboratory mice, pigs. In this thesis, pigs were investigated as a potential biomedical model species for viral respiratory infections in humans and as a natural host for viral infections. Both approaches provide valuable insights into aspects of porcine immunology that can either be used as the foundation for translational research or for the design of targeted therapeutics and vaccines for pigs.

Insights into fundamental characteristics of the porcine immune system form the basis for translational studies. **Paper I** pioneered a detailed characterization of porcine iNKT cells. To make pigs and porcine iNKT cells more available for scientific investigations, we established multicolor flow cytometry analysis platforms that allow for a more detailed investigation of these cells than previously possible. We found porcine iNKT cells circulating in peripheral blood to be a rare population among $CD3^+$ lymphocytes that displays a pre-activated effector state and can be divided into at least three functional subsets. Upon antigenic activation, they proliferated rapidly, secreted pro-inflammatory cytokines, and exerted cytotoxicity. Moreover, we provided first evidence for a role of iNKT cells in porcine IAV and ASFV infections, which we investigated in more detail in **paper IV**. Central characteristics, i.e., phenotype and functional properties, exhibit a high degree of similarity between humans and pigs. Moreover, differences between human and murine iNKT cells are more pronounced than between humans and pigs.

Based on the results obtained in **paper II**, the established biomedical model could be used for further studies of infectious respiratory diseases. IAV infections pave the way for secondary co-infections with increased morbidity and lethality. These bactoviral co-infections are a threat to both pigs and humans. The shared susceptibility as well as homologies on the physiological and immunological level make pigs exceptionally suitable animal models for studies of these infections. **Paper I and II** can also be interpreted under translational aspects. Activation of iNKT cells in porcine vaccination studies showed promising results. Based on these and our findings, this might be a suitable approach for humans as well. Along with other studies, our results suggest that pigs might be a well-suited large animal model for research in infectious diseases. This is true especially for respiratory infections, such as seasonal IAV infections, for

which pigs are natural hosts and contribute to viral spread and emergence as "mixing vessels", which can result in pandemic strains like H1N1pdm09. We could show that porcine iNKT cells as well as the antiviral responses of cTC against H1N1pdm09 in pigs are comparable to human cells and processes. The increased implementation of pigs in basic and applied research might enable an improved translation of scientific knowledge to human and veterinary medicine.

In two further studies, **papers III and IV**, we investigated T-cell responses during a viral infection, ASF, for which pigs are the only natural hosts. Immune responses were similar after highly and moderately virulent ASFV infection in domestic pigs and wild boar, respectively. However, they differed between both species. Antiviral immunity in domestic pigs was predominantly exerted by αβ T cells, $CD8\alpha^+$ and DP αβ T cells, while the response in wild boar was dominated by γδ T cells, mainly $CD8\alpha^+$ effector cells. Since wild boar show a higher disease severity and lethality, even during infection with moderately virulent ASFV "Estonia2014", a shift to γδ T cells seems to be detrimental. In contrast, domestic pigs survive infections with moderately virulent ASFV "Estonia2014", which indicates that $CD8\alpha^+$ or DP αβ T cells confer protection at least in infections with non-highly virulent ASFV strains. Interestingly, in **paper V** we found higher and prolonged inflammation in domestic pigs, correlating with increased T-cell influx. However, histopathological analyses revealed no direct explanation for the differences in disease progression and lethality in domestic pigs and wild boar. These findings require further studies to elucidate the underlying mechanisms.

The lack of basic data about immunological differences between domestic pigs and wild boar hampers attempts to understand immunity against ASFV. We found differences between both suid subspecies already at steady state and even more prominent during ASFV infections in **papers III-V**. Most apparently, T-cell responses in wild boar were heavily biased towards γδ T cells, while immune responses in domestic pigs were based on αβ T cells. However, information about even basic characteristics, like the composition, phenotypes, and functional qualities of wild boar's immune system, is missing. Therefore, essential baseline data must be obtained in order to adequately assess changes in future studies.

Analyses like these reveal major advantages of pigs as a biomedical model. On the one hand, similar to conventional model species, researchers can investigate every tissue at any desired time. Tissue from human patients is often scarce or not at all available, so models that can be investigated at specific times after infection are needed. On the other hand, results obtained in pigs are more comparable to humans than data from murine studies. Moreover, pigs are susceptible to similar pathogens as humans and experimental infections can be investigated without the need for major genetic manipulations. However, there are also limitations of the porcine model system. Analysis tools are not as advanced as they are for mice, especially in terms of availability of mAbs or genetically modified organisms. Still, given the major

advantages that become more and more obvious, efforts should be made to make pigs more applicable for basic and translational research. In addition, findings derived from pigs can be used for the species itself. Pigs are a major livestock species and new treatments, or vaccines could also be used for them. Therefore, this research could eventually also improve animal welfare.

In summary, the presented thesis significantly enhanced our knowledge of porcine immune processes for cTC in general and iNKT cells in particular. Results were obtained both at steady state and in the context of IAV and ASFV infections, and thus, made pigs more available as a model for future research. The use of multicolor flow cytometry provided a broad overview of the ongoing immune reactions and enables further, more wide-ranging studies that can also address open questions in even more complex infection scenarios.

REFERENCES

1. Cooper, D.K.C., B. Ekser, and A.J. Tector, *A brief history of clinical xenotransplantation.* Int J Surg, 2015. **23**(Pt B): p. 205-210.
2. Meurens, F., et al., *The pig: a model for human infectious diseases.* Trends Microbiol, 2012. **20**(1): p. 50-7.
3. Dawson, H.D., *Comparative assessment of the pig, mouse, and human genomes: A structural and functional analysis of genes involved in immunity,* in *The Minipig in Biomedical Research*, P.A. McAnulty, Dayan, A., Hastings, K.H., Ganderup, N.-C., Editor. 2011, Taylor & Francis Group: CRC Press. p. 321–341.
4. Rothkötter, H.J., *Anatomical particularities of the porcine immune system--a physician's view.* Dev Comp Immunol, 2009. **33**(3): p. 267-72.
5. Saalmüller, A. and W. Gerner, *The immune system of swine*, in *Encyclopedia of immunology*, M.J.H. Ratcliffe, Editor. 2016, Elsevier: Amsterdam. p. 538–548.
6. Masopust, D., C.P. Sivula, and S.C. Jameson, *Of Mice, Dirty Mice, and Men: Using Mice To Understand Human Immunology.* J Immunol, 2017. **199**(2): p. 383-388.
7. Engel, P., et al., *CD Nomenclature 2015: Human Leukocyte Differentiation Antigen Workshops as a Driving Force in Immunology.* J Immunol, 2015. **195**(10): p. 4555-63.
8. Dawson, H.D. and J.K. Lunney, *Porcine cluster of differentiation (CD) markers 2018 update.* Res Vet Sci, 2018. **118**: p. 199-246.
9. Gerner, W., et al., *Phenotypic and functional differentiation of porcine αβ T cells: current knowledge and available tools.* Mol Immunol, 2015. **66**(1): p. 3-13.
10. Rajao, D.S. and A.L. Vincent, *Swine as a model for influenza A virus infection and immunity.* ILAR J, 2015. **56**(1): p. 44-52.
11. Flint, J., et al., *Molecular Biology.* 4th ed. Principles of Virology. Vol. 1. 2015, Washington, DC: ASM Press.
12. Shaw, M.L. and P. Palese, *Orthomyxoviridae: The viruses and their replication.*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2013, Lippincott Williams & Wilkins: Phalidelphia. p. 1691–1740.
13. Domingo, E. and J.J. Holland, *RNA virus mutations and fitness for survival.* Annu Rev Microbiol, 1997. **51**: p. 151-78.
14. Mostafa, A., et al., *Zoonotic Potential of Influenza A Viruses: A Comprehensive Overview.* Viruses, 2018. **10**(9).
15. Tong, S., et al., *A distinct lineage of influenza A virus from bats.* Proc Natl Acad Sci U S A, 2012. **109**(11): p. 4269-74.
16. Short, K.R., et al., *One health, multiple challenges: The inter-species transmission of influenza A virus.* One Health, 2015. **1**: p. 1-13.
17. Cauldwell, A.V., et al., *Viral determinants of influenza A virus host range.* J Gen Virol, 2014. **95**(Pt 6): p. 1193-1210.
18. Parry, R., et al., *Divergent Influenza-Like Viruses of Amphibians and Fish Support an Ancient Evolutionary Association.* Viruses, 2020. **12**(9).

19. Shi, M., et al., *The evolutionary history of vertebrate RNA viruses.* Nature, 2018. **556**(7700): p. 197-202.
20. Fabian, P., et al., *Influenza virus in human exhaled breath: an observational study.* PLoS One, 2008. **3**(7): p. e2691.
21. Mubareka, S., et al., *Transmission of influenza virus via aerosols and fomites in the guinea pig model.* J Infect Dis, 2009. **199**(6): p. 858-65.
22. Carrat, F., et al., *Time lines of infection and disease in human influenza: a review of volunteer challenge studies.* Am J Epidemiol, 2008. **167**(7): p. 775-85.
23. Böttcher-Friebertshäuser, E., et al., *The hemagglutinin: a determinant of pathogenicity.* Curr Top Microbiol Immunol, 2014. **385**: p. 3-34.
24. Imai, M. and Y. Kawaoka, *The role of receptor binding specificity in interspecies transmission of influenza viruses.* Curr Opin Virol, 2012. **2**(2): p. 160-7.
25. Suzuki, Y., et al., *Sialic acid species as a determinant of the host range of influenza A viruses.* J Virol, 2000. **74**(24): p. 11825-31.
26. Nelli, R.K., et al., *Comparative distribution of human and avian type sialic acid influenza receptors in the pig.* BMC Vet Res, 2010. **6**: p. 4.
27. Morens, D.M. and J.K. Taubenberger, *Part 1. Chapter 3. The 1918 Influenza Pandemic: A Still-Mysterious Litmus Test for Pandemic Prevention and Control*, in *Preparing for Pandemics in the Modern World*, C.C. Blackburn, Editor. 2020, Texas A&M University Press: College Station.
28. Taubenberger, J.K. and D.M. Morens, *1918 Influenza: the mother of all pandemics.* Emerg Infect Dis, 2006. **12**(1): p. 15-22.
29. Morens, D.M. and A.S. Fauci, *Emerging Pandemic Diseases: How We Got To COVID-19.* Cell, 2020.
30. Garten, R.J., et al., *Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans.* Science, 2009. **325**(5937): p. 197-201.
31. Smith, G.J., et al., *Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic.* Nature, 2009. **459**(7250): p. 1122-5.
32. Trovao, N.S. and M.I. Nelson, *When Pigs Fly: Pandemic influenza enters the 21st century.* PLoS Pathog, 2020. **16**(3): p. e1008259.
33. Janke, B.H., *Influenza A virus infections in swine: pathogenesis and diagnosis.* Vet Pathol, 2014. **51**(2): p. 410-26.
34. Nelson, M.I., et al., *Global migration of influenza A viruses in swine.* Nat Commun, 2015. **6**: p. 6696.
35. Ito, T., et al., *Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.* J Virol, 1998. **72**(9): p. 7367-73.
36. Scholtissek, C., et al., *The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses.* Virology, 1985. **147**(2): p. 287-94.
37. Trebbien, R., L.E. Larsen, and B.M. Viuff, *Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs.* Virol J, 2011. **8**: p. 434.

38. Gray, G.C., D.W. Trampel, and J.A. Roth, *Pandemic influenza planning: shouldn't swine and poultry workers be included?* Vaccine, 2007. **25**(22): p. 4376-81.
39. Park, M.S., et al., *Animal models for the risk assessment of viral pandemic potential.* Lab Anim Res, 2020. **36**: p. 11.
40. Zhang, X., et al., *Tissue tropisms opt for transmissible reassortants during avian and swine influenza A virus co-infection in swine.* PLoS Pathog, 2018. **14**(12): p. e1007417.
41. Schicker, R.S., et al., *Outbreak of Influenza A(H3N2) Variant Virus Infections Among Persons Attending Agricultural Fairs Housing Infected Swine - Michigan and Ohio, July-August 2016.* MMWR Morb Mortal Wkly Rep, 2016. **65**(42): p. 1157-1160.
42. Nelson, M.I., et al., *Continual Reintroduction of Human Pandemic H1N1 Influenza A Viruses into Swine in the United States, 2009 to 2014.* J Virol, 2015. **89**(12): p. 6218-26.
43. Dixon, L.K., J. M. Escribano, C. Martins, D. L. Rock, M. L. Salas, and P.J. Wilkinson, *Asfarviridae. In: Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (eds), Virus Taxonomy. VIII. Report of the ICTV, pp. 135–143. Elsevier, Academic Press, London.* Virology Journal, 2005. **2**.
44. Alonso, C., et al., *ICTV Virus Taxonomy Profile: Asfarviridae.* J Gen Virol, 2018. **99**(5): p. 613-614.
45. Galindo, I. and C. Alonso, *African Swine Fever Virus: A Review.* Viruses, 2017. **9**(5).
46. Dixon, L.K., et al., *African swine fever virus replication and genomics.* Virus Res, 2013. **173**(1): p. 3-14.
47. Bastos, A.D., et al., *Genotyping field strains of African swine fever virus by partial p72 gene characterisation.* Arch Virol, 2003. **148**(4): p. 693-706.
48. Salas, M.L. and G. Andres, *African swine fever virus morphogenesis.* Virus Res, 2013. **173**(1): p. 29-41.
49. Schloer, G.M., *Polypeptides and structure of African swine fever virus.* Virus Res, 1985. **3**(4): p. 295-310.
50. Gaudreault, N.N., et al., *African Swine Fever Virus: An Emerging DNA Arbovirus.* Frontiers in veterinary science, 2020. **7**: p. 215.
51. Oura, C.A.L., et al., *The pathogenesis of African swine fever in the resistant bushpig.* Journal of General Virology, 1998. **79**: p. 1439–1443.
52. Pikalo, J., et al., *Pathogenesis of African swine fever in domestic pigs and European wild boar - Lessons learned from recent animal trials.* Virus Res, 2019. **271**: p. 197614.
53. Mason-D'Croz, D., et al., *Modelling the global economic consequences of a major African swine fever outbreak in China.* Nature Food, 2020. **1**(4): p. 221–228.
54. Dixon, L.K., et al., *African Swine Fever Epidemiology and Control.* Annu Rev Anim Biosci, 2020. **8**: p. 221-246.
55. Dixon, L.K., H. Sun, and H. Roberts, *African swine fever.* Antiviral Res, 2019. **165**: p. 34-41.
56. Gogin, A., et al., *African swine fever in the North Caucasus region and the Russian Federation in years 2007-2012.* Virus Res, 2013. **173**(1): p. 198-203.
57. Li, L., et al., *Infection of African swine fever in wild boar, China, 2018.* Transbound Emerg Dis, 2019. **66**(3): p. 1395-1398.

58. Yoo, D., et al., *African swine fever: Etiology, epidemiological status in Korea, and perspective on control.* J Vet Sci, 2020. **21**(2): p. e38.
59. Zhao, D., et al., *Replication and virulence in pigs of the first African swine fever virus isolated in China.* Emerg Microbes Infect, 2019. **8**(1): p. 438-447.
60. Friedrich-Loeffler-Institut. *First case of African swine fever in wild boar in Germany.* 2020 September 10, 2020 September 14, 2020]; Available from: https://www.fli.de/en/news/short-messages/short-message/first-case-of-african-swine-fever-in-wild-boar-in-germany/.
61. Frant, M., G. Wozniakowski, and Z. Pejsak, *African Swine Fever (ASF) and Ticks. No Risk of Tick-mediated ASF Spread in Poland and Baltic States.* J Vet Res, 2017. **61**(4): p. 375-380.
62. Jori, F. and A.D. Bastos, *Role of wild suids in the epidemiology of African swine fever.* Ecohealth, 2009. **6**(2): p. 296–310.
63. Pietschmann, J., et al., *African swine fever virus transmission cycles in Central Europe: Evaluation of wild boar-soft tick contacts through detection of antibodies against Ornithodoros erraticus saliva antigen.* BMC Vet Res, 2016. **12**: p. 1.
64. Guinat, C., et al., *Transmission routes of African swine fever virus to domestic pigs: current knowledge and future research directions.* Vet Rec, 2016. **178**(11): p. 262-7.
65. Rowlands, R.J., et al., *African swine fever virus isolate, Georgia, 2007.* Emerg Infect Dis, 2008. **14**(12): p. 1870-4.
66. Franzoni, G., S. Dei Giudici, and A. Oggiano, *Infection, modulation and responses of antigen-presenting cells to African swine fever viruses.* Virus Res, 2018. **258**: p. 73-80.
67. Carrillo, C., et al., *Long-term persistent infection of swine monocytes/macrophages with African swine fever virus.* J Virol, 1994. **68**(1): p. 580-3.
68. Gomez-Villamandos, J.C., et al., *Pathology of African swine fever: the role of monocyte-macrophage.* Virus Res, 2013. **173**(1): p. 140–149.
69. Gregg, D.A., C.A. Mebus, and D.H. Schlafer, *Early infection of interdigitating dendritic cells in the pig lymph node with African swine fever viruses of high and low virulence: immunohistochemical and ultrastructural studies.* Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 1995. **7**(1): p. 23–30.
70. Carrasco, L., et al., *In vivo replication of African swine fever virus (Malawi '83) in neutrophils.* Veterinary research, 1996. **27**(1): p. 55–62.
71. Lau, S.K., P.G. Chu, and L.M. Weiss, *CD163: a specific marker of macrophages in paraffin-embedded tissue samples.* Am J Clin Pathol, 2004. **122**(5): p. 794-801.
72. Sanchez-Torres, C., et al., *Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection.* Arch Virol, 2003. **148**(12): p. 2307-23.
73. Lithgow, P., et al., *Correlation of cell surface marker expression with African swine fever virus infection.* Vet Microbiol, 2014. **168**(2-4): p. 413-9.

74. Popescu, L., et al., *Genetically edited pigs lacking CD163 show no resistance following infection with the African swine fever virus isolate, Georgia 2007/1.* Virology, 2017. **501**: p. 102-106.
75. Holmes, N., *CD45: all is not yet crystal clear.* Immunology, 2006. **117**(2): p. 145-55.
76. Rowlands, R.J., et al., *The CD2v protein enhances African swine fever virus replication in the tick vector, Ornithodoros erraticus.* Virology, 2009. **393**(2): p. 319-28.
77. Blome, S., K. Franzke, and M. Beer, *African swine fever - A review of current knowledge.* Virus Res, 2020. **287**: p. 198099.
78. Chapman, D.A.G., et al., *Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates.* J Gen Virol, 2008. **89**(Pt 2): p. 397-408.
79. Blome, S., C. Gabriel, and M. Beer, *Modern adjuvants do not enhance the efficacy of an inactivated African swine fever virus vaccine preparation.* Vaccine, 2014. **32**(31): p. 3879-82.
80. Zani, L., et al., *Deletion at the 5'-end of Estonian ASFV strains associated with an attenuated phenotype.* Sci Rep, 2018. **8**(1): p. 6510.
81. Nurmoja, I., et al., *Biological characterization of African swine fever virus genotype II strains from north-eastern Estonia in European wild boar.* Transbound Emerg Dis, 2017. **64**(6): p. 2034–2041.
82. Iwasaki, A., E.F. Foxman, and R.D. Molony, *Early local immune defences in the respiratory tract.* Nat Rev Immunol, 2017. **17**(1): p. 7-20.
83. Rosendahl Huber, S., et al., *T cell responses to viral infections - opportunities for Peptide vaccination.* Front Immunol, 2014. **5**: p. 171.
84. Abbas, A.K., A.H.H. Lichtmann, and S. Pillai, *Cellular and Molecular Immunology.* 9th ed. 2017: Elsevier.
85. Zarnitsyna, V.I., et al., *Estimating the diversity, completeness, and cross-reactivity of the T cell repertoire.* Front Immunol, 2013. **4**: p. 485.
86. Legut, M., D.K. Cole, and A.K. Sewell, *The promise of γδ T cells and the γδ T cell receptor for cancer immunotherapy.* Cell Mol Immunol, 2015. **12**(6): p. 656-68.
87. Arstila, T.P., et al., *A direct estimate of the human αβ T cell receptor diversity.* Science, 1999. **286**(5441): p. 958-61.
88. Qi, Q., et al., *Diversity and clonal selection in the human T-cell repertoire.* Proc Natl Acad Sci U S A, 2014. **111**(36): p. 13139-44.
89. Laydon, D.J., C.R. Bangham, and B. Asquith, *Estimating T-cell repertoire diversity: limitations of classical estimators and a new approach.* Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1675).
90. Charerntantanakul, W. and J.A. Roth, *Biology of porcine T lymphocytes.* Anim Health Res Rev, 2006. **7**(1-2): p. 81-96.
91. Käser, T., W. Gerner, and A. Saalmüller, *Porcine regulatory T cells: Mechanisms and T-cell targets of suppression.* Developmental and Comparative Immunology, 2011. **35**(11): p. 1166–1172.
92. Denyer, M.S., et al., *Perforin expression can define CD8 positive lymphocyte subsets in pigs allowing phenotypic and functional analysis of natural killer, cytotoxic T, natural*

killer T and MHC un-restricted cytotoxic T-cells. Vet Immunol Immunopathol, 2006. **110**(3-4): p. 279–292.

93. Cheroutre, H. and F. Lambolez, *Doubting the TCR coreceptor function of CD8αα.* Immunity, 2008. **28**(2): p. 149-59.
94. Denning, T.L., et al., *Mouse TCRαβ+CD8αα intraepithelial lymphocytes express genes that down-regulate their antigen reactivity and suppress immune responses.* J Immunol, 2007. **178**(7): p. 4230-9.
95. Konno, A., et al., *CD8αα a memory effector T cells descend directly from clonally expanded CD8α+βhigh TCRαβ T cells in vivo.* Blood, 2002. **100**(12): p. 4090-7.
96. Walker, L.J., et al., *CD8αα Expression Marks Terminally Differentiated Human CD8+ T Cells Expanded in Chronic Viral Infection.* Front Immunol, 2013. **4**: p. 223.
97. Saalmüller, A., et al., *Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes.* Eur J Immunol, 1987. **17**(9): p. 1297–1301.
98. Reutner, K., et al., *CD27 expression discriminates porcine T helper cells with functionally distinct properties.* Vet Res, 2013. **44**: p. 18.
99. Lefevre, E.A., et al., *Immune responses in pigs vaccinated with adjuvanted and non-adjuvanted A(H1N1)pdm/09 influenza vaccines used in human immunization programmes.* PLoS One, 2012. **7**(3): p. e32400.
100. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision.* Nat Rev Immunol, 2002. **2**(5): p. 309-22.
101. Waschbisch, A., et al., *Analysis of CD4+ CD8+ double-positive T cells in blood, cerebrospinal fluid and multiple sclerosis lesions.* Clin Exp Immunol, 2014. **177**(2): p. 404-11.
102. Murphy, K. and C. Weaver, *Janeway's Immunobiology.* 9th ed. 2016: Garland Publishing.
103. Van Laethem, F., A.N. Tikhonova, and A. Singer, *MHC restriction is imposed on a diverse T cell receptor repertoire by CD4 and CD8 co-receptors during thymic selection.* Trends Immunol, 2012. **33**(9): p. 437-41.
104. Prager, I. and C. Watzl, *Mechanisms of natural killer cell-mediated cellular cytotoxicity.* J Leukoc Biol, 2019. **105**(6): p. 1319-1329.
105. de Bruin, T.G., et al., *Cytolytic function for pseudorabies virus-stimulated porcine CD4+ CD8dull+ lymphocytes.* Viral Immunol, 2000. **13**(4): p. 511-20.
106. de Bruin, T.G., et al., *Discrimination of different subsets of cytolytic cells in pseudorabies virus-immune and naive pigs.* J Gen Virol, 2000. **81**(Pt 6): p. 1529-37.
107. Takamatsu, H.H., et al., *Cellular immunity in ASFV responses.* Virus Res, 2013. **173**(1): p. 110-21.
108. Mousset, C.M., et al., *Comprehensive Phenotyping of T Cells Using Flow Cytometry.* Cytometry A, 2019. **95**(6): p. 647-654.
109. Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment.* Cell, 2000. **100**(6): p. 655–669.

110. Ebner, F., et al., *A novel lineage transcription factor based analysis reveals differences in T helper cell subpopulation development in infected and intrauterine growth restricted (IUGR) piglets.* Dev Comp Immunol, 2014. **46**(2): p. 333-40.
111. Ivanov, II, et al., *The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
112. Singh, B., et al., *Modulation of autoimmune diseases by interleukin (IL)-17 producing regulatory T helper (Th17) cells.* Indian J Med Res, 2013. **138**(5): p. 591-4.
113. Jogdand, G.M., S. Mohanty, and S. Devadas, *Regulators of Tfh Cell Differentiation.* Front Immunol, 2016. **7**: p. 520.
114. Ugolini, M., et al., *Recognition of microbial viability via TLR8 drives TFH cell differentiation and vaccine responses.* Nat Immunol, 2018. **19**(4): p. 386-396.
115. Walker, L.S. and D.M. Sansom, *The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses.* Nat Rev Immunol, 2011. **11**(12): p. 852-63.
116. Gondek, D.C., et al., *Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism.* J Immunol, 2005. **174**(4): p. 1783-6.
117. Saalmüller, A., W. Hirt, and M.J. Reddehase, *Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes.* Eur J Immunol, 1989. **19**(11): p. 2011–2016.
118. Hirt, W., A. Saalmüller, and M.J. Reddehase, *Distinct γ/δ T cell receptors define two subsets of circulating porcine CD2-CD4-CD8- T lymphocytes.* Eur J Immunol, 1990. **20**(2): p. 265-9.
119. Sedlak, C., et al., *CD2 and CD8α define porcine γδ T cells with distinct cytokine production profiles.* Dev Comp Immunol, 2014. **45**(1): p. 97–106.
120. Takamatsu, H.H., et al., *Porcine γδ T cells: possible roles on the innate and adaptive immune responses following virus infection.* Vet Immunol Immunopathol, 2006. **112**(1-2): p. 49–61.
121. Holtmeier, W. and D. Kabelitz, *γδ T cells link innate and adaptive immune responses.* Chem Immunol Allergy, 2005. **86**: p. 151-183.
122. Stepanova, K. and M. Sinkora, *The expression of CD25, CD11b, SWC1, SWC7, MHC-II, and family of CD45 molecules can be used to characterize different stages of γδ T lymphocytes in pigs.* Dev Comp Immunol, 2012. **36**(4): p. 728-40.
123. Stepanova, K. and M. Sinkora, *Porcine γδ T lymphocytes can be categorized into two functionally and developmentally distinct subsets according to expression of CD2 and level of TCR.* J Immunol, 2013. **190**(5): p. 2111–2120.
124. Sedlak, C., et al., *IL-12 and IL-18 induce interferon-γ production and de novo CD2 expression in porcine γδ T cells.* Dev Comp Immunol, 2014. **47**(1): p. 115-22.
125. Roden, A.C., W.G. Morice, and C.A. Hanson, *Immunophenotypic attributes of benign peripheral blood γδ T cells and conditions associated with their increase.* Arch Pathol Lab Med, 2008. **132**(11): p. 1774-80.
126. Kadivar, M., et al., *CD8αβ+ γδ T Cells: A Novel T Cell Subset with a Potential Role in Inflammatory Bowel Disease.* J Immunol, 2016. **197**(12): p. 4584-4592.

127. Kalyan, S. and D. Kabelitz, *Defining the nature of human γδ T cells: a biographical sketch of the highly empathetic.* Cell Mol Immunol, 2013. **10**(1): p. 21-9.
128. Pistoia, V., et al., *Human γδ T-Cells: From Surface Receptors to the Therapy of High-Risk Leukemias.* Front Immunol, 2018. **9**: p. 984.
129. Bonneville, M., R.L. O'Brien, and W.K. Born, *γδ T cell effector functions: a blend of innate programming and acquired plasticity.* Nat Rev Immunol, 2010. **10**(7): p. 467-78.
130. Chien, Y.H., C. Meyer, and M. Bonneville, *γδ T cells: first line of defense and beyond.* Annu Rev Immunol, 2014. **32**: p. 121-55.
131. Morita, C.T., R.A. Mariuzza, and M.B. Brenner, *Antigen recognition by human γδ T cells: pattern recognition by the adaptive immune system.* Springer Semin Immunopathol, 2000. **22**(3): p. 191-217.
132. Adams, E.J., Y.H. Chien, and K.C. Garcia, *Structure of a γδ T cell receptor in complex with the nonclassical MHC T22.* Science, 2005. **308**(5719): p. 227-31.
133. Deseke, M. and I. Prinz, *Ligand recognition by the γδ TCR and discrimination between homeostasis and stress conditions.* Cell Mol Immunol, 2020. **17**(9): p. 914-924.
134. Bluestone, J.A., et al., *Structure and specificity of T cell receptor γδ on major histocompatibility complex antigen-specific CD3+, CD4-, CD8- T lymphocytes.* J Exp Med, 1988. **168**(5): p. 1899-916.
135. Houlden, B.A., et al., *A TCR γδ cell recognizing a novel TL-encoded gene product.* Cold Spring Harb Symp Quant Biol, 1989. **54 Pt 1**: p. 45-55.
136. Spada, F.M., et al., *Self-recognition of CD1 by γδ T cells: implications for innate immunity.* J Exp Med, 2000. **191**(6): p. 937-48.
137. Wu, J., V. Groh, and T. Spies, *T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial γδ T cells.* J Immunol, 2002. **169**(3): p. 1236-40.
138. Sciammas, R., et al., *Unique antigen recognition by a herpesvirus-specific TCR-γδ cell.* J Immunol, 1994. **152**(11): p. 5392-7.
139. Halary, F., et al., *Shared reactivity of Vδ2neg γδ T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells.* J Exp Med, 2005. **201**(10): p. 1567-78.
140. Le Nours, J., et al., *A class of γδ T cell receptors recognize the underside of the antigen-presenting molecule MR1.* Science, 2019. **366**(6472): p. 1522-1527.
141. Sutton, C.E., et al., *Interleukin-1 and IL-23 induce innate IL-17 production from γδ T cells, amplifying Th17 responses and autoimmunity.* Immunity, 2009. **31**(2): p. 331-41.
142. Girardi, M., et al., *Regulation of cutaneous malignancy by γδ T cells.* Science, 2001. **294**(5542): p. 605-9.
143. Qin, G., et al., *Phosphoantigen-expanded human γδ T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses.* J Infect Dis, 2009. **200**(6): p. 858-65.
144. Conti, L., et al., *Reciprocal activating interaction between dendritic cells and pamidronate-stimulated γδ T cells: role of CD86 and inflammatory cytokines.* J Immunol, 2005. **174**(1): p. 252-60.

145. Devilder, M.C., et al., *Potentiation of antigen-stimulated Vγ9Vδ2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation.* J Immunol, 2006. **176**(3): p. 1386-93.
146. Edwards, S.C., et al., *A population of proinflammatory T cells coexpresses αβ and γδ T cell receptors in mice and humans.* J Exp Med, 2020. **217**(5).
147. Olin, M.R., et al., *γδ T-lymphocyte cytotoxic activity against Mycobacterium bovis analyzed by flow cytometry.* J Immunol Methods, 2005. **297**(1-2): p. 1-11.
148. de Bruin, M.G., et al., *Establishment and characterization of porcine cytolytic cell lines and clones.* Vet Immunol Immunopathol, 1997. **59**(3-4): p. 337-47.
149. Yang, H. and R.M. Parkhouse, *Differential expression of CD8 epitopes amongst porcine CD8-positive functional lymphocyte subsets.* Immunology, 1997. **92**(1): p. 45–52.
150. Lee, J., et al., *Gammadelta T cells in immunity induced by Mycobacterium bovis bacillus Calmette-Guerin vaccination.* Infect Immun, 2004. **72**(3): p. 1504-11.
151. Brandes, M., K. Willimann, and B. Moser, *Professional antigen-presentation function by human γδ T Cells.* Science, 2005. **309**(5732): p. 264-8.
152. Takamatsu, H.H., M.S. Denyer, and T.E. Wileman, *A sub-population of circulating porcine γδ T cells can act as professional antigen presenting cells.* Vet Immunol Immunopathol, 2002. **87**(3-4): p. 223–224.
153. Crosby, C.M. and M. Kronenberg, *Invariant natural killer T cells: front line fighters in the war against pathogenic microbes.* Immunogenetics, 2016. **68**(8): p. 639-48.
154. Cole, S.L., et al., *Involvement of the 4-1BB/4-1BBL pathway in control of monocyte numbers by invariant NKT cells.* J Immunol, 2014. **192**(8): p. 3898-907.
155. D'Andrea, A., et al., *Neonatal invariant Vα24+ NKT lymphocytes are activated memory cells.* Eur J Immunol, 2000. **30**(6): p. 1544-50.
156. Park, S.H., et al., *Unaltered phenotype, tissue distribution and function of Vα14(+) NKT cells in germ-free mice.* Eur J Immunol, 2000. **30**(2): p. 620-5.
157. Stetson, D.B., et al., *Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function.* J Exp Med, 2003. **198**(7): p. 1069-76.
158. Kovalovsky, D., et al., *The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions.* Nat Immunol, 2008. **9**(9): p. 1055-64.
159. Savage, A.K., et al., *The transcription factor PLZF directs the effector program of the NKT cell lineage.* Immunity, 2008. **29**(3): p. 391-403.
160. Zhang, S., et al., *Zbtb16 (PLZF) is stably suppressed and not inducible in non-innate T cells via T cell receptor-mediated signaling.* Sci Rep, 2015. **5**: p. 12113.
161. Crosby, C.M. and M. Kronenberg, *Tissue-specific functions of invariant natural killer T cells.* Nature Reviews Immunology, 2018.
162. Benlagha, K., et al., *A thymic precursor to the NK T cell lineage.* Science, 2002. **296**(5567): p. 553-5.
163. Constantinides, M.G. and A. Bendelac, *Transcriptional regulation of the NKT cell lineage.* Curr Opin Immunol, 2013. **25**(2): p. 161-7.

164. Lee, Y.J., et al., *Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells.* Nat Immunol, 2013. **14**(11): p. 1146-54.
165. McNab, F.W., et al., *The influence of CD1d in postselection NKT cell maturation and homeostasis.* J Immunol, 2005. **175**(6): p. 3762-8.
166. Strong, B.S., et al., *Extrinsic allospecific signals of hematopoietic origin dictate iNKT cell lineage-fate decisions during development.* Sci Rep, 2016. **6**: p. 28837.
167. Watarai, H., et al., *Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines.* PLoS Biol, 2012. **10**(2): p. e1001255.
168. Gumperz, J.E., et al., *Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining.* J Exp Med, 2002. **195**(5): p. 625-36.
169. Lee, P.T., et al., *Distinct functional lineages of human V(α)24 natural killer T cells.* J Exp Med, 2002. **195**(5): p. 637-41.
170. Moreira-Teixeira, L., et al., *Proinflammatory environment dictates the IL-17-producing capacity of human invariant NKT cells.* J Immunol, 2011. **186**(10): p. 5758-65.
171. Garner, L.C., P. Klenerman, and N.M. Provine, *Insights Into Mucosal-Associated Invariant T Cell Biology From Studies of Invariant Natural Killer T Cells.* Front Immunol, 2018. **9**: p. 1478.
172. Hill, T.M., et al., *CD1d-Restricted Natural Killer T Cells*, in *eLS*. 2016, John Wiley & Sons, Ltd: Chichester. p. 1-27.
173. Brigl, M. and M.B. Brenner, *CD1: antigen presentation and T cell function.* Annu Rev Immunol, 2004. **22**: p. 817-90.
174. Uldrich, A.P., et al., *NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge.* J Immunol, 2005. **175**(5): p. 3092-3101.
175. Van Der Vliet, H.J., et al., *Effects of α-galactosylceramide (KRN7000), interleukin-12 and interleukin-7 on phenotype and cytokine profile of human Vα24+ Vβ11+ T cells.* Immunology, 1999. **98**(4): p. 557-63.
176. Liu, Y., et al., *A modified α-galactosyl ceramide for staining and stimulating natural killer T cells.* J Immunol Methods, 2006. **312**(1-2): p. 34-9.
177. Benlagha, K., et al., *In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers.* J Exp Med, 2000. **191**(11): p. 1895-903.
178. Sidobre, S. and M. Kronenberg, *CD1 tetramers: a powerful tool for the analysis of glycolipid-reactive T cells.* J Immunol Methods, 2002. **268**(1): p. 107-21.
179. Looringh van Beeck, F.A., et al., *Functional CD1d and/or NKT cell invariant chain transcript in horse, pig, African elephant and guinea pig, but not in ruminants.* Mol Immunol, 2009. **46**(7): p. 1424-31.
180. Bendelac, A., *Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes.* J Exp Med, 1995. **182**(6): p. 2091-6.
181. Uldrich, A.P., et al., *CD1d-lipid antigen recognition by the γδ TCR.* Nat Immunol, 2013. **14**(11): p. 1137-45.
182. Kumar, A., et al., *Natural Killer T Cells: An Ecological Evolutionary Developmental Biology Perspective.* Front Immunol, 2017. **8**: p. 1858.

183. Brigl, M., et al., *Mechanism of CD1d-restricted natural killer T cell activation during microbial infection.* Nat Immunol, 2003. **4**(12): p. 1230-7.
184. Cohen, N.R., et al., *Innate recognition of cell wall β-glucans drives invariant natural killer T cell responses against fungi.* 2011. **10**(5): p. 437-50.
185. Albacker, L.A., et al., *Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity.* Nat Med, 2013. **19**(10): p. 1297-304.
186. Paget, C., et al., *Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids.* Immunity, 2007. **27**(4): p. 597-609.
187. Nagarajan, N.A. and M. Kronenberg, *Invariant NKT cells amplify the innate immune response to lipopolysaccharide.* J Immunol, 2007. **178**(5): p. 2706-13.
188. Tyznik, A.J., et al., *Cutting edge: the mechanism of invariant NKT cell responses to viral danger signals.* J Immunol, 2008. **181**(7): p. 4452-6.
189. Wesley, J.D., et al., *NK cell-like behavior of Vα14i NK T cells during MCMV infection.* PLoS Pathog, 2008. **4**(7): p. e1000106.
190. Brigl, M., et al., *Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection.* J Exp Med, 2011. **208**(6): p. 1163-77.
191. Artiaga, B.L., et al., *Adjuvant effects of therapeutic glycolipids administered to a cohort of NKT cell-diverse pigs.* Vet Immunol Immunopathol, 2014. **162**(1-2): p. 1-13.
192. Artiaga, B.L., et al., *α-Galactosylceramide protects swine against influenza infection when administered as a vaccine adjuvant.* Sci Rep, 2016. **6**: p. 23593.
193. Artiaga, B.L., et al., *Rapid control of pandemic H1N1 influenza by targeting NKT-cells.* Sci Rep, 2016. **6**: p. 37999.
194. Dwivedi, V., et al., *Adjuvant effects of invariant NKT cell ligand potentiates the innate and adaptive immunity to an inactivated H1N1 swine influenza virus vaccine in pigs.* Vet Microbiol, 2016. **186**: p. 157-63.
195. Renu, S., et al., *Intranasal delivery of influenza antigen by nanoparticles, but not NKT-cell adjuvant differentially induces the expression of B-cell activation factors in mice and swine.* Cell Immunol, 2018.
196. Yang, G., J.A. Richt, and J.P. Driver, *Harnessing Invariant NKT Cells to Improve Influenza Vaccines: A Pig Perspective.* Int J Mol Sci, 2017. **19**(1).
197. Eguchi-Ogawa, T., et al., *Analysis of the genomic structure of the porcine CD1 gene cluster.* Genomics, 2007. **89**(2): p. 248-61.
198. Yang, G., et al., *Targeted disruption of CD1d prevents NKT cell development in pigs.* Mamm Genome, 2015. **26**(5-6): p. 264-70.
199. Yang, G., et al., *Next Generation Sequencing of the Pig αβ TCR Repertoire Identifies the Porcine Invariant NKT Cell Receptor.* J Immunol, 2019. **202**(7): p. 1981-1991.
200. Renukaradhya, G.J., et al., *Functional invariant NKT cells in pig lungs regulate the airway hyperreactivity: a potential animal model.* J Clin Immunol, 2011. **31**(2): p. 228-39.
201. Yang, G., et al., *Characterizing porcine invariant natural killer T cells: A comparative study with NK cells and T cells.* Dev Comp Immunol, 2017. **76**: p. 343-351.

202. Montoya, C.J., et al., *Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11.* Immunology, 2007. **122**(1): p. 1-14.
203. Thierry, A., et al., *Identification of invariant natural killer T cells in porcine peripheral blood.* Vet Immunol Immunopathol, 2012. **149**(3-4): p. 272-9.
204. Podack, E.R. and G.P. Munson, *Killing of Microbes and Cancer by the Immune System with Three Mammalian Pore-Forming Killer Proteins.* Front Immunol, 2016. **7**: p. 464.
205. Chiu, C. and P.J. Openshaw, *Antiviral B cell and T cell immunity in the lungs.* Nat Immunol, 2015. **16**(1): p. 18-26.
206. Kim, H.M., et al., *Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs.* J Virol, 2008. **82**(9): p. 4265-74.
207. Schulman, J.L. and Kilbourn.Ed, *Induction of Partial Specific Heterotypic Immunity in Mice by a Single Infection with Influenza a Virus.* Journal of Bacteriology, 1965. **89**(1): p. 170-&.
208. Yetter, R.A., W.H. Barber, and P.A. Small, *Heterotypic Immunity to Influenza in Ferrets.* Infection and Immunity, 1980. **29**(2): p. 650-653.
209. Seo, S.H., M. Peiris, and R.G. Webster, *Protective cross-reactive cellular immunity to lethal A/Goose/Guangdong/1/96-Like H5N1 influenza virus is correlated with the proportion of pulmonary CD8(+) T cells expressing gamma interferon.* Journal of Virology, 2002. **76**(10): p. 4886-4890.
210. Hiremath, J., et al., *Entrapment of H1N1 Influenza Virus Derived Conserved Peptides in PLGA Nanoparticles Enhances T Cell Response and Vaccine Efficacy in Pigs.* Plos One, 2016. **11**(4).
211. Khatri, M., et al., *Swine Influenza H1N1 Virus Induces Acute Inflammatory Immune Responses in Pig Lungs: a Potential Animal Model for Human H1N1 Influenza Virus.* Journal of Virology, 2010. **84**(21): p. 11210-11218.
212. Heinen, P.P., E.A. de Boer-Luijtze, and A.T.J. Bianchi, *Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection.* Journal of General Virology, 2001. **82**: p. 2697-2707.
213. Pomorska-Mol, M., I. Markowska-Daniel, and K. Kwit, *Immune and acute phase response in pigs experimentally infected with H1N2 swine influenza virus.* FEMS Immunol Med Microbiol, 2012. **66**(3): p. 334-42.
214. Pomorska-Mol, M., et al., *Immune and inflammatory response in pigs during acute influenza caused by H1N1 swine influenza virus.* Arch Virol, 2014. **159**(10): p. 2605-14.
215. Sinkora, M., et al., *The comparative profile of lymphoid cells and the T and B cell spectratype of germ-free piglets infected with viruses SIV, PRRSV or PCV2.* Vet Res, 2014. **45**: p. 91.
216. Tungatt, K., et al., *Induction of influenza-specific local CD8 T-cells in the respiratory tract after aerosol delivery of vaccine antigen or virus in the Babraham inbred pig.* PLoS Pathog, 2018. **14**(5): p. e1007017.
217. Talker, S.C., et al., *Magnitude and kinetics of multifunctional CD4+ and CD8beta+ T cells in pigs infected with swine influenza A virus.* Vet Res, 2015. **46**: p. 52.

218. Talker, S.C., et al., *Influenza A Virus Infection in Pigs Attracts Multifunctional and Cross-Reactive T Cells to the Lung.* J Virol, 2016. **90**(20): p. 9364-82.
219. Lange, E., et al., *Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs.* Journal of General Virology, 2009. **90**: p. 2119-2123.
220. Mair, K.H., et al., *Porcine CD3(+)NKp46(+) Lymphocytes Have NK-Cell Characteristics and Are Present in Increased Frequencies in the Lungs of Influenza-Infected Animals.* Front Immunol, 2016. **7**: p. 263.
221. Rajao, D.S., et al., *Pigs with Severe Combined Immunodeficiency Are Impaired in Controlling Influenza A Virus Infection.* J Innate Immun, 2017. **9**(2): p. 193-202.
222. De Santo, C., et al., *Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans.* J Clin Invest, 2008. **118**(12): p. 4036-48.
223. Paget, C., et al., *Potential role of invariant NKT cells in the control of pulmonary inflammation and CD8+ T cell response during acute influenza A virus H3N2 pneumonia.* J Immunol, 2011. **186**(10): p. 5590-602.
224. Ho, L.P., et al., *Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection.* Eur J Immunol, 2008. **38**(7): p. 1913-22.
225. Ishikawa, H., et al., *IFN-γ production downstream of NKT cell activation in mice infected with influenza virus enhances the cytolytic activities of both NK cells and viral antigen-specific CD8+ T cells.* Virology, 2010. **407**(2): p. 325-32.
226. Gu, W., et al., *Unaltered influenza disease outcomes in swine prophylactically treated with alpha-galactosylceramide.* Dev Comp Immunol, 2020. **114**: p. 103843.
227. Oura, C.A., et al., *In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus.* J Gen Virol, 2005. **86**(Pt 9): p. 2445-50.
228. Saalmüller, A., et al., *Characterization of porcine T lymphocytes and their immune response against viral antigens.* Journal of Biotechnology, 1999. **73**(2-3): p. 223–233.
229. Martins, C.L., et al., *African swine fever virus specific porcine cytotoxic T cell activity.* Archives of Virology, 1993. **129**(1-4): p. 211–225.
230. Norley, S.G. and R.C. Wardley, *Investigation of porcine natural-killer cell activity with reference to African swine-fever virus infection.* Immunology, 1983. **49**(4): p. 593-7.
231. Norley, S.G. and R.C. Wardley, *Cytotoxic lymphocytes induced by African swine fever infection.* Research in veterinary science, 1984. **37**(2): p. 255–257.
232. Netherton, C.L., et al., *Identification and Immunogenicity of African Swine Fever Virus Antigens.* Frontiers in Immunology, 2019. **10**: p. 1318.
233. Canals, A., et al., *Analysis of T lymphocyte subsets proliferating in response to infective and UV-inactivated African swine fever viruses.* Vet Microbiol, 1992. **33**(1-4): p. 117-27.
234. Casal, I., E. Vinuela, and L. Enjuanes, *Synthesis of African swine fever (ASF) virus-specific antibodies in vitro in a porcine leucocyte system.* Immunology, 1987. **62**(2): p. 207-13.
235. Sánchez-Cordón, P.J., et al., *Absence of Long-Term Protection in Domestic Pigs Immunized with Attenuated African Swine Fever Virus Isolate OURT88/3 or*

BeninΔMGF Correlates with Increased Levels of Regulatory T Cells and Interleukin-10. Journal of Virology, 2020. **94**(14).

236. Post, J., et al., *Influence of Age and Dose of African Swine Fever Virus Infections on Clinical Outcome and Blood Parameters in Pigs.* Viral immunology, 2017. **30**(1): p. 58–69.
237. Chan, A.C., et al., *Ex-vivo analysis of human natural killer T cells demonstrates heterogeneity between tissues and within established CD4(+) and CD4(-) subsets.* Clin Exp Immunol, 2013. **172**(1): p. 129-37.
238. Lee, Y.J., et al., *Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response.* Immunity, 2015. **43**(3): p. 566-78.
239. Kim, C.H., B. Johnston, and E.C. Butcher, *Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among Vα24(+)Vβ11(+) NKT cell subsets with distinct cytokine-producing capacity.* Blood, 2002. **100**(1): p. 11-6.
240. Sandberg, J.K., N. Bhardwaj, and D.F. Nixon, *Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Vα24 NKT cell compartment.* Eur J Immunol, 2003. **33**(3): p. 588-96.
241. Mahnke, Y.D., et al., *The who's who of T-cell differentiation: human memory T-cell subsets.* Eur J Immunol, 2013. **43**(11): p. 2797-809.
242. Domingues, R.G., et al., *CD5 expression is regulated during human T-cell activation by alternative polyadenylation, PTBP1, and miR-204.* Eur J Immunol, 2016. **46**(6): p. 1490-503.
243. Waldmann, T.A., *The multi-subunit interleukin-2 receptor.* Annu Rev Biochem, 1989. **58**: p. 875-911.
244. Dong, C., et al., *ICOS co-stimulatory receptor is essential for T-cell activation and function.* Nature, 2001. **409**(6816): p. 97-101.
245. Holling, T.M., E. Schooten, and P.J. van Den Elsen, *Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men.* Hum Immunol, 2004. **65**(4): p. 282-90.
246. Gleimer, M., H. von Boehmer, and T. Kreslavsky, *PLZF Controls the Expression of a Limited Number of Genes Essential for NKT Cell Function.* Front Immunol, 2012. **3**: p. 374.
247. Akbari, O., et al., *ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival.* J Immunol, 2008. **180**(8): p. 5448-56.
248. Kaneda, H., et al., *ICOS costimulates invariant NKT cell activation.* Biochem Biophys Res Commun, 2005. **327**(1): p. 201-7.
249. Lang, M.L., *The importance of B cell CD1d expression for humoral immunity.* Expert Rev Vaccines, 2014. **13**(11): p. 1275-8.
250. Chang, P.P., et al., *Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses.* Nat Immunol, 2011. **13**(1): p. 35-43.
251. Zeng, S.G., et al., *Human invariant NKT cell subsets differentially promote differentiation, antibody production, and T cell stimulation by B cells in vitro.* J Immunol, 2013. **191**(4): p. 1666-76.

252. Saalmüller, A., T. Werner, and V. Fachinger, *T-helper cells from naive to committed.* Vet Immunol Immunopathol, 2002. **87**(3-4): p. 137–145.
253. Talker, S.C., et al., *Phenotypic maturation of porcine NK- and T-cell subsets.* Dev Comp Immunol, 2013. **40**(1): p. 51-68.
254. Thedrez, A., et al., *CD4 engagement by CD1d potentiates activation of CD4+ invariant NKT cells.* Blood, 2007. **110**(1): p. 251-8.
255. Cerundolo, V., et al., *Harnessing invariant NKT cells in vaccination strategies.* Nature Reviews Immunology, 2009. **9**: p. 28.
256. Paget, C., et al., *Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages.* J Biol Chem, 2012. **287**(12): p. 8816-29.
257. Raftery, M.J., et al., *Viral danger signals control CD1d de novo synthesis and NKT cell activation.* Eur J Immunol, 2008. **38**(3): p. 668-79.
258. Barbe, F., et al., *Role of IFN-alpha during the acute stage of a swine influenza virus infection.* Res Vet Sci, 2010. **88**(1): p. 172-8.
259. Van Reeth, K., S. Van Gucht, and M. Pensaert, *Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs.* Viral Immunol, 2002. **15**(4): p. 583-94.
260. Golding, J.P., et al., *Sensitivity of African swine fever virus to type I interferon is linked to genes within multigene families 360 and 505.* Virology, 2016. **493**: p. 154-61.
261. Karalyan, Z., et al., *Interferon status and white blood cells during infection with African swine fever virus in vivo.* Vet Immunol Immunopathol, 2012. **145**(1-2): p. 551-5.
262. Siemens, N., et al., *Port d'Entree for Respiratory Infections - Does the Influenza A Virus Pave the Way for Bacteria?* Front Microbiol, 2017. **8**: p. 2602.
263. Morens, D.M., J.K. Taubenberger, and A.S. Fauci, *Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness.* J Infect Dis, 2008. **198**(7): p. 962-70.
264. Lee, B., et al., *Influenza-induced type I interferon enhances susceptibility to gram-negative and gram-positive bacterial pneumonia in mice.* Am J Physiol Lung Cell Mol Physiol, 2015. **309**(2): p. L158-67.
265. Nakamura, S., K.M. Davis, and J.N. Weiser, *Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice.* J Clin Invest, 2011. **121**(9): p. 3657-65.
266. McCullers, J.A. and K.C. Bartmess, *Role of neuraminidase in lethal synergism between influenza virus and Streptococcus pneumoniae.* J Infect Dis, 2003. **187**(6): p. 1000-9.
267. Rudd, J.M., et al., *Lethal Synergism between Influenza and Streptococcus pneumoniae.* J Infect Pulm Dis, 2016. **2**(2).
268. Saade, G., et al., *Coinfections and their molecular consequences in the porcine respiratory tract.* Vet Res, 2020. **51**(1): p. 80.
269. de Greeff, A., et al., *Pneumococcal colonization and invasive disease studied in a porcine model.* BMC Microbiol, 2016. **16**: p. 102.

270. Rynda-Apple, A., K.M. Robinson, and J.F. Alcorn, *Influenza and Bacterial Superinfection: Illuminating the Immunologic Mechanisms of Disease.* Infect Immun, 2015. **83**(10): p. 3764-70.
271. Brookes, S.M., et al., *Replication, Pathogenesis and Transmission of Pandemic (H1N1) 2009 Virus in Non-Immune Pigs.* Plos One, 2010. **5**(2).
272. Itoh, Y., et al., *In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses.* Nature, 2009. **460**(7258): p. 1021-U110.
273. Gauger, P.C., et al., *Vaccine-associated enhanced respiratory disease does not interfere with the adaptive immune response following challenge with pandemic A/H1N1 2009.* Viral Immunol, 2013. **26**(5): p. 314-21.
274. Gauger, P.C., et al., *Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus.* Vet Pathol, 2012. **49**(6): p. 900-12.
275. Leung, N.H., et al., *The Fraction of Influenza Virus Infections That Are Asymptomatic: A Systematic Review and Meta-analysis.* Epidemiology, 2015. **26**(6): p. 862-72.
276. Richard, M., et al., *Influenza A viruses are transmitted via the air from the nasal respiratory epithelium of ferrets.* Nat Commun, 2020. **11**(1): p. 766.
277. Chen, W.W., et al., *[Changes and analysis of peripheral white blood cells and lymphocyte subsets for patients with pandemic influenza A virus (H1N1) infection].* Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 2010. **24**(5): p. 331-3.
278. Cunha, B.A., F.M. Pherez, and P. Schoch, *Diagnostic importance of relative lymphopenia as a marker of swine influenza (H1N1) in adults.* Clin Infect Dis, 2009. **49**(9): p. 1454-6.
279. Giamarellos-Bourboulis, E.J., et al., *Effect of the novel influenza A (H1N1) virus in the human immune system.* PLoS One, 2009. **4**(12): p. e8393.
280. Coskun, O., et al., *Relative lymphopenia and monocytosis may be considered as a surrogate marker of pandemic influenza a (H1N1).* J Clin Virol, 2010. **47**(4): p. 388-9.
281. Merekoulias, G., et al., *Lymphocyte to monocyte ratio as a screening tool for influenza.* PLoS Curr, 2010. **2**: p. RRN1154.
282. Antal-Szalmas, P., et al., *Quantitation of surface CD14 on human monocytes and neutrophils.* J Leukoc Biol, 1997. **61**(6): p. 721-8.
283. Wilkinson, T.M., et al., *Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans.* Nat Med, 2012. **18**(2): p. 274-80.
284. Lahn, M., *The role of γδ T cells in the airways.* J Mol Med (Berl), 2000. **78**(8): p. 409-25.
285. Piet, B., et al., *CD8(+) T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung.* J Clin Invest, 2011. **121**(6): p. 2254-63.
286. Qin, G., et al., *Type 1 responses of human Vγ9Vδ2 T cells to influenza A viruses.* J Virol, 2011. **85**(19): p. 10109-16.
287. Cawthon, A.G. and M.A. Alexander-Miller, *Optimal colocalization of TCR and CD8 as a novel mechanism for the control of functional avidity.* J Immunol, 2002. **169**(7): p. 3492-8.

288. Konno, A., et al., *CD8αα memory effector T cells descend directly from clonally expanded CD8αβ high TCRαβ T cells in vivo.* Blood, 2002. **100**(12): p. 4090-7.
289. Wen, K., et al., *Characterization of immune modulating functions of γδ T cell subsets in a gnotobiotic pig model of human rotavirus infection.* Comp Immunol Microbiol Infect Dis, 2012. **35**(4): p. 289-301.
290. Groenen, M.A., et al., *Analyses of pig genomes provide insight into porcine demography and evolution.* Nature, 2012. **491**(7424): p. 393-8.
291. Ballesteros, C., et al., *First data on Eurasian wild boar response to oral immunization with BCG and challenge with a Mycobacterium bovis field strain.* Vaccine, 2009. **27**(48): p. 6662-8.
292. Beltran-Beck, B., et al., *Oral vaccination with heat inactivated Mycobacterium bovis activates the complement system to protect against tuberculosis.* PLoS One, 2014. **9**(5): p. e98048.
293. Lastra, J.M., et al., *Expression of immunoregulatory genes in peripheral blood mononuclear cells of European wild boar immunized with BCG.* Vet Microbiol, 2009. **134**(3-4): p. 334-9.
294. Cabezon, O., et al., *African swine fever virus infection in Classical swine fever subclinically infected wild boars.* BMC Vet Res, 2017. **13**(1): p. 227.
295. Bergman, I.M., et al., *European wild boars and domestic pigs display different polymorphic patterns in the Toll-like receptor (TLR) 1, TLR2, and TLR6 genes.* Immunogenetics, 2010. **62**(1): p. 49-58.
296. Chen, S., et al., *How immunogenetically different are domestic pigs from wild boars: a perspective from single-nucleotide polymorphisms of 19 immunity-related candidate genes.* Immunogenetics, 2013. **65**(10): p. 737-48.
297. Abolins, S., et al., *The comparative immunology of wild and laboratory mice, Mus musculus domesticus.* Nat Commun, 2017. **8**: p. 14811.
298. Meiraz, A., et al., *Switch from perforin-expressing to perforin-deficient CD8(+) T cells accounts for two distinct types of effector cytotoxic T lymphocytes in vivo.* Immunology, 2009. **128**(1): p. 69-82.
299. Afonso, C.L., et al., *An African swine fever virus Bc1-2 homolog, 5-HL, suppresses apoptotic cell death.* J Virol, 1996. **70**(7): p. 4858-63.
300. Galindo, I., et al., *A179L, a viral Bcl-2 homologue, targets the core Bcl-2 apoptotic machinery and its upstream BH3 activators with selective binding restrictions for Bid and Noxa.* Virology, 2008. **375**(2): p. 561-72.
301. Sutton, V.R., D.L. Vaux, and J.A. Trapani, *Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes.* J Immunol, 1997. **158**(12): p. 5783-90.
302. Sehl, J., et al., *Comparative Pathology of Domestic Pigs and Wild Boar Infected with the Moderately Virulent African Swine Fever Virus Strain "Estonia 2014".* Pathogens, 2020. **9**(8).
303. Welz, M., et al., *Perforin inhibition protects from lethal endothelial damage during fulminant viral hepatitis.* Nat Commun, 2018. **9**(1): p. 4805.

304. Hersperger, A.R., G. Makedonas, and M.R. Betts, *Flow cytometric detection of perforin upregulation in human CD8 T cells.* Cytometry A, 2008. **73**(11): p. 1050-7.
305. Thomas, D.A. and J. Massague, *TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance.* Cancer Cell, 2005. **8**(5): p. 369-80.
306. Erickson, J.J., et al., *Acute Viral Respiratory Infection Rapidly Induces a CD8+ T Cell Exhaustion-like Phenotype.* J Immunol, 2015. **195**(9): p. 4319-30.

APPENDIX

Curriculum vitae

Publications

Scientific presentations

Publications

Publications included in this thesis

2020

Schäfer, A., Zani, L., Pikalo, J., Hühr, J., Sehl, J., Mettenleiter, T.C., Breithaupt, A., Blome, S., Blohm, U.
T-cell responses in domestic pigs and wild boar upon infection with the moderately virulent African swine fever virus strain "Estonia2014".
Transboundary and Emerging Diseases, in review

Hühr, J.*, **Schäfer, A.***, Schwaiger, T., Zani, L., Sehl, J., Mettenleiter, T.C., Blome, S., Blohm, U. *, joint first authors
Impaired T cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain.
Transboundary and Emerging Diseases, doi: 10.1111/tbed.13678

Sehl, J., Pikalo, J., **Schäfer, A.**, Franzke, K., Pannhorst, K., Elnagar, A., Blohm, U., Blome, S., Breithaupt, A.
Comparative pathology of domestic pigs and wild boar infected with the moderately virulent African swine fever virus strain "Estonia 2014".
Pathogens, doi: 10.3390/pathogens9080662

2019

Schäfer, A., Hühr, J., Schwaiger, T., Dorhoi, A., Mettenleiter, T.C., Blome, S., Schröder, C., Blohm, U.
Porcine invariant Natural Killer T cells: functional profiling and dynamics in steady state and viral infections.
Frontiers in Immunology, doi: 10.3389/fimmu.2019.01380

Schwaiger, T., Sehl, J., Karte, C., **Schäfer, A.**, Hühr, J., Mettenleiter, T.C., Schröder, C., Köllner, B., Ulrich, R., Blohm, U.
Experimental H1N1pdm09 infection in pigs mimics human seasonal influenza infections.
PLoS ONE, doi: 10.1371/journal.pone.0222943

Publications not included in this thesis

2020

Gierse, L.C., Meene, A., Schultz, D., Schwaiger, T., Karte, C., Schröder, C., Wang, H., Wünsche, C., Methling, K., Kreikemeyer, B., Fuchs, S., Bernhardt, J., Becher, D., Lalk, M., **KoInfekt Study Group**, Urich, T., Riedel, K. A.
Multi-Omics Protocol for Swine Feces to Elucidate Longitudinal Dynamics in Microbiome Structure and Function.
Microorganisms, doi: 10.3390/microorganisms8121887

Mostafa, A., Blaurock, C., Scheibner, D., Müller, C., Blohm, U., **Schäfer, A.**, Gischke, M., Salaheldin, A.H., Nooh, H.Z., Ali, M.A., Breithaupt, A., Mettenleiter, T.C., Pleschka, S., Abdelwhab, E.M.
Genetic incompatibilities and reduced transmission in chickens may limit the evolution of reassortants between H9N2 and panzootic H5N8 clade 2.3.4.4 avian influenza virus showing high virulence for mammals
Virus Evolution, doi: 10.1093/ve/veaa077

2019

Schultz, D., Methling, K., **KoInfekt Study Group**, Rothe, M., Lalk, M.
Eicosanoid Profile of Influenza A Virus Infected Pigs.
Metabolites, doi: 10.3390/metabo9070130

2018

Ugolini, M., Gerhard, J., Burkert, S., Jensen, K.J., Georg, P., Ebner, F., Volkers, S., Thada, S., Dietert, K., Bauer, L., **Schäfer, A.**, […], Jungersen, G., Schumann, R.R., Suttorp, N., Sander, L.E.
Recognition of microbial viability via TLR8 drives T-FH cell differentiation and vaccine responses.
Nature Immunology, doi: 10.1038/s41590-018-0068-4

Scientific presentations

2020	Talk	**Schäfer, A.**, Sake, H.J., Petersen, B., Blohm, U. Immunological consequences of a beta-2-microglobulin knockout in domestic pigs. 24th DGfI-Symposium "*Infection and Immune Defense*", Castle Rothenfels
2019	Poster	**Schäfer, A.**, Sake, H.J., Petersen, B., Blohm, U. Immune phenotyping of beta-2-microglobulin knockout pigs. *8th Junior Scientist Symposium*, Friedrich-Loeffler-Institut, Jena
	Talk	**Schäfer, A.**, Schwaiger, T., Dorhoi, A., Mettenleiter, T.C., Schröder, C., Blohm, U. Functional profiling and dynamics of naïve porcine invariant Natural Killer T cells. 12th International Veterinary Immunology Symposium, Seattle, WA, USA ***Awarded with: The AAI Young Investigator Award of the American Association of Immunologists***
	Talk	**Schäfer, A.**, Hühr, J., Schwaiger, T., Dorhoi, A., Mettenleiter, T.C., Blome, S., Schröder, C., Blohm, U. Porcine invariant Natural Killer T cells: functional profiling and dynamics in steady state and viral infections. 23rd DGfI-Symposium "*Infection and Immune Defense*", Castle Rothenfels
2018	Talk/ Poster	**Schäfer, A.**, Hühr, J., Schwaiger, T., Dorhoi, A., Mettenleiter, T.C., Blome, S., Schröder, C., Blohm, U. Guardians at the gate: Characterization of porcine invariant Natural Killer T cells. *7th Junior Scientist Symposium*, Friedrich-Loeffler-Institut, Greifswald
	Talk	**Schäfer, A.**, Schröder, C., Köllner, B., Schwaiger, T., Dorhoi, A., Blohm, U. Guardians at the gate: Porcine invariant T cells in bactoviral (co-)infections in swine. 22nd DGfI-Symposium "*Infection and Immune Defense*", Castle Rothenfels
2017	Poster	**Schäfer, A.**, Zeun, J., Schröder, C., Köllner, B., Schwaiger, T., Blohm, U. The gate and its keepers: Responses of epithelial cells and invariant T cells in bactoviral co-infections in swine. 1st International Conference on Respiratory Pathogens (ICoRP), Rostock

EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

……..	……..
Ort, Datum	Alexander Schäfer

ACKNOWLEDGEMENT

Science is teamwork. The studies in this thesis could not have been carried out, and thus the thesis itself could not have been written, had it not been for the many people who have contributed in numerous ways. Here, I want to thank these people:

I am extremely grateful to **Prof. Dr. Dr. h.c. Thomas C. Mettenleiter** for his supervision and his service as first referee. Being as busy as he is, it is not self-evident to invest so much time in his students and to have an open door for discussions and supportive suggestions.

I am deeply indebted to **Dr. Ulrike Blohm** for her supervision, uncounted discussions, and helpful comments during my studies. Thank you for the chance to contribute to so many projects, but also for your constant support! "Killing ideas" has been a lot of fun with you!

I am also grateful to my numerous colleagues in the KoInfekt project at the FLI, mainly to **Dr. Theresa Schwaiger** for all her effort for this project and the animal trials, but also to Dr. Dirk Höper, Claudia Karte, Dr. Charlotte Schröder, and Dr. Bernd Köllner.

For all their efforts during the animal studies, I am thankful to **PD Dr. Sandra Blome**, **Dr. Jutta Pikalo**, **Dr. Angele Breithaupt, Dr. Reiner Ulrich** and **Dr. Julia Sehl**, **Dr. Laura Zani**, and **Dr. Kati Franzke**.

Our studies have also established collaborations, which resulted in great ideas, experiments, and papers. This includes but is not limited to: **Prof. Sven Hammerschmidt** and **Dr. Nikolai Siemens**, **Prof. Leif Erik Sander**, **Prof. Anca Dorhoi**, **Dr. El-Sayed Abdelwhab, and Dr. Gang Pei** together with their respective teams, and also all collaborating labs in the **KoInfekt** project.

I also thank all those helpful hands in the lab, stable or in the necropsy halls, e.g.: **Stefanie Knöfel**, **Silke Rehbein**, **Cathleen Klement**, **Jane Hühr, Luise Hartmann, Luise Hohensee**, **Bärbel Hammerschmidt**, **Thomas Möritz, Lukas Steinke**, **Ralf Redmer**, **Christian Loth**, and all the other colleagues, technicians, veterinarians, and animal care takers at the FLI.

The thesis was thankfully funded by the European Union (European Social Fund), Excellence Initiative Mecklenburg-Western Pomerania.

Many thanks to **Gini and Tami** for encouraging discussions, helpful tips, and latest lab gossip!

Mein größter Dank gilt **meinen Eltern**. Ohne euch wäre ich nicht, wer und wo ich bin. Vielen Dank für eure Unterstützung in all den Jahren!