Myeloid cells' response to infection with
Staphylococcus aureus and *Streptococcus pyogenes*

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"The Guide says there is an art to flying", said Ford, "or rather a knack. The knack lies in learning how to throw yourself at the ground and miss."

Douglas Adams, *Life, the Universe and Everything*

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LIST OF SCIENTIFIC PAPERS AND MANUSCRIPTS

The following scientific papers/manuscripts are included in this thesis:

I.   **Lea A. Tölken**, Georgi I. Wassilew, Daniel Grolimund, Timm Weitkamp, Bernhard Hesse, Anastasia Rakow, Nikolai Siemens#, Janosch Schoon#; Cobalt and Chromium Ions Impair Macrophage Response to *Staphylococcus aureus* Infection; accepted for publication in ACS Biomaterials Science & Engineering, 06. December 2023; # equal contribution

II. **Lea A. Tölken**, Antje D. Paulikat, Lana H. Jachmann, Alexander Reder, Manuela Gesell Salazar, Laura M. Palma Medina, Stephan Michalik, Uwe Völker, Mattias Svensson, Anna Norrby-Teglund, Katharina J. Hoff, Michael Lammers, and Nikolai Siemens; Reduced Interleukin-18 Secretion by Human Monocytic Cells in Response to Infections with Hyper-Virulent *Streptococcus pyogenes*; Submitted to Journal of Biomedical Science, 08. December 2023

III. Patience Shumba, Thomas Sura, Kirsten Moll, Bhavya Chakrakodi, **Lea A Tölken**, Jörn Hoßmann, Katharina J Hoff, Ole Hyldegaard, Michael Nekludov, Mattias Svensson, Per Arnell, Steinar Skrede; INFECT Study Group; Anna Norrby-Teglund, Nikolai Siemens; Neutrophil-derived Reactive Agents Induce a Transient SpeB Negative Phenotype in *Streptococcus pyogenes*, J Biomed Sci 30, 52 (2023). https://doi.org/10.1186/s12929-023-00947-x

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LIST OF ABBREVIATIONS

ALTR	Adverse local tissue reaction
APC	Antigen presenting cell
CA-MRSA	Community acquired MRSA
CD	Cluster of differentiation
cDC	Classical/conventional DC
CLSM	Confocal laser scanning microscopy
CoCrMo	Cobalt Chromium Molybdenum
CovR/S	Control of virulence R/S
CXCL	C–X–C motif chemokine ligand
DAMP	Danger associated molecular pattern
DC	Dendritic cell
FSC	Forward scatter
GAS	Group A streptococcus
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSDMA	Gasdermin A
GSDMD	Gasdermin D
HLA-DR	Human leukocyte antigen
HMGB1	High motility group box 1
IFN	Interferon
IL	Interleukin
IVIG	Intravenous immunoglobulin
MHC	Major histocompatibility complex
moDC	Monocyte-derived DC
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MRSA	Methicillin-resistant *Staphylococcus aureus*
NET	Neutrophil extracellular trap
NF	Necrotizing fasciitis
NLRP3	NLR family pyrin domain containing 3
NSTI	Necrotizing skin and soft tissue infection
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid DC
PVL	Panton Valentin Leukocidin
PRR	Pattern recognition receptor
RopB	Regulator of Protease
ROS	Reactive oxygen species
SCV	Small colony variant
SLO	Streptolysin O
SLS	Streptolysin S
SpeB	Streptococcal pyrogenic exotoxin B
SSC	Sideward scatter
STSS	Streptococcal toxic shock syndrome
TcR	T cell receptor
THY	Todd-Hewitt broth supplemented with 1.5% (w/v) yeast extract
TNF	Tumor necrosis factor
TLR	Toll like receptor

1. SUMMARY

During infections, innate immune cells are crucial for initiating a pro-inflammatory immune response and clearing the invading pathogen. Delay in pathogen clearance or initiation of an immune response due to impaired functionality of immune cells can result in devastating consequences. The cellular compartment of the innate immune system comprises an array of specialized cell types: Macrophages are tissue-resident professional phagocytes that clear cellular debris, pathogens, and foreign objects. Dendritic cells (DCs) are immune sentinels specialized in antigen uptake and subsequent T cell priming. They are primary sources of cytokines in response to infection. Neutrophils are efficient effector cells that respond rapidly to infection and clear bacteria by different mechanisms. If effector mechanisms of these cells are affected by either bacterial or other factors, infections might not be resolved and can spread throughout the host.

Cobalt-chromium-molybdenum biomaterial is widely used in arthroplasty. Implant-derived wear particles and ions lead to macrophage-driven adverse local tissue reactions: Such reactions have been linked to an increased risk of periprosthetic joint infection after revision arthroplasty. While metal-induced cytotoxicity is well characterized in human macrophages, direct effects on their functionality remain elusive. In Paper I, we show that local peri-implant tissue is exposed to Co and Cr *in situ*. Influx of macrophages is also evident. Exposure of isolated human monocytes/macrophages to Cr^{3+} *in vitro* had only minor effects. However, exposure of monocytes/macrophages to pathologic concentrations of Co^{2+} significantly impaired both phenotype and functionality. High concentrations of Co^{2+} induced loss of surface markers, including CD14 and CD16. Both Co^{2+} and Cr^{3+} impaired macrophage responses to *Staphylococcus aureus* infection. Co^{2+}-exposed macrophages, in particular, showed decreased phagocytic activity. These findings demonstrate the immunosuppressive effects of locally elevated metal ions on the innate immune response.

Streptococcus pyogenes (group A streptococcus, GAS) causes a variety of diseases ranging from mild to severe necrotizing soft tissue infections (NSTIs). In the host environment hyper-virulent GAS variants carrying mutations within the genes encoding for control of virulence (Cov)R/S two component system are enriched. This adaptation is associated with loss of SpeB secretion. In Paper II, we show that *in vitro* infections with hyper-virulent GAS variants harboring dysfunctional CovR/S suppress secretion of IL-8 and IL-18 by human monocytic cells. This phenotype was mediated by a caspase-8 dependent mechanism. Knockout of streptococcal SLO in a GAS strain carrying functional CovR/S even increased secretion of IL-1β and IL-18 by moDCs. Of 67 fully sequenced GAS NSTI isolates, 28 contained *covS* or *covR*

mutations that rendered the TCS dysfunctional. However, no differences in systemic IL-8 and IL-18 were detected in these patients.

GAS isolates recovered from patients often display a mixed phenotype, consisting of SpeB positive (SpeB$^+$) and SpeB negative (SpeB$^-$) clones. Irreversible loss of SpeB expression is often caused by loss of function mutations in regulatory components (CovR/S, RopB). Loss of SpeB is often associated with hyper-virulence. In Paper III, we show that the host environment induces transiently abrogated secretion of SpeB by GAS. Tissue inflammation, neutrophil influx, and degranulation correlated with increased frequencies of SpeB$^-$ GAS clones. Isolates recovered from tissue expressed but did not secrete SpeB, which was reversible. Neutrophil-derived ROS were identified as the main factor responsible for abrogated SpeB secretion. Hyper-virulent SpeB$^-$ clones also exhibit better survival within and induce excessive degranulation of neutrophils.

2. ZUSAMMENFASSUNG

Während einer Infektion sind die Zellen des innaten Immunsystems unabdingbar für die schnelle Initiation einer proinflammatorischen Immunantwort und die Bekämfung des eindringenden Erregers. Eine verspätete Reaktion oder eine Beeinträchtigung der Funktionalität der Immunzellen kann verheerende Folgen haben. Das innate Immunsystem besteht aus einer Vielzahl spezialisierter Effektorzellen mit spezifischen Aufgaben. Makrophagen sind geweberesidente Zellen, welche Fremdkörper, Zelltrümmer und eindringende Pathogene phagozytieren und abtöten. Dendritische Zellen (DCs) sind spezialisiert auf die Präsentation von Antigenen für T Zellen und deren Aktivierung. Zu diesem Zweck sekretieren DCs auch eine Vielzahl von Zytokinen. Neutrophile sind effiziente Effektorzellen, die schnell auf Infektionen reagieren und Bakterien über verschiedene Wege abtöten. Wenn die Mechanismen dieser Zellen durch bakterielle oder anderweitige Faktoren gestört oder beeinflusst werden, können entstehende Infektionen eventuell nicht rechtzeitig eingedämmt werden und die Erreger breiten sich im Körper aus.

Kobalt-Chrom-Molybdän (CoCrMo) Legierungen sind gängige Endoprothesenmaterialien, welche häufig in der Arthoprosthetik zum Einsatz kommen. Durch Abnutzungsvorgänge vom Implantat freigesetzte Partikel und Ionen können zu adversen lokalen Gewebsreaktionen führen. Diese werden häufig durch Makrophagen verursacht und gehen mit einem erhöhten Risiko von periprosthetischen Infektionen einher. Metallinduzierte Zytotoxizität gegenüber humanen Makrophagen ist ein gut charakterisiertes Phänomen. Wohingegen die Auswirkungen von Metallexposition auf Funktion und Phänotyp von Makrophagen noch unbekannt sind. In Paper I wird gezeigt, dass das periprosthetische Gewebe gegenüber Co und Cr exponiert ist. Außerdem wurden Areale mit verstärkter Einwanderung von Makrophagen beobachtet. Exposition gegenüber Cr^3 hatte nur geringe Effekte auf den Phänotyp der Makrophagen, während Exposition gegenüber Co^{2+} Phänotyp und Funktion stark beeinträchtigte. Pathologische Konzentrationen von Co^{2+} induzierten den Verlust von Oberflächenexpression bestimmter Marker (CD14, CD16). Sowohl Co^{2+} als auch Cr^{3+} verhinderten Aktivierung von Makrophagen nach Infektion mit *Staphylococcus aureus*. Diese Ergebnisse zeigen, dass lokal erhöhte Konzentrationen von Metallionen immunsuppressive Effekte haben und die Funktion innater Immunzellen beeinträchtigen.

Streptococcus pyogenes (GAS, Gruppe A Streptokokken) verursacht eine Vielzahl humaner Erkrankungen. Diese reichen von milden, bis hin zu schweren nekrotisierenden Weichteilinfektionen (NSTI). Innerhalb des Wirtes können hypervirulente GAS-Klone entstehen. Dies geschieht häufig durch Mutationen der für das Zweikomponentensystem

CovR/S kodierenden Gene *covS* und *covR*. Dies geht oft mit Verlust der Sekretion der Protease SpeB einher. In Paper II wird gezeigt, dass hypervirulente GAS Stämme die Sekretion von Interleukin (IL)-8 und IL-18 durch humane monozytäre Zellen supprimieren. Dieser Phänotyp wurde durch die Initiator-Caspase-8 vermittelt. Deletion des Zytolysins SLO in einem Stamm mit funktionalem CovR/S führte außerdem zu erhöhter Sekretion von IL-1β und IL-18 durch infizierte DCs. In 67 sequenzierten GAS Isolaten aus NSTI Patienten konnten 28 potenziell inaktivierende Mutationen von *covS* oder *covR* gefunden werden. Allerdings konnten im Plasma der Patienten keine Unterschiede der systemischen Konzentrationen von IL-8 und IL-18 festgestellt werden.

GAS Patientenisolate zeigen oft einen gemischten Phänotyp, bestehend aus SpeB positiven (SpeB$^+$) und SpeB$^-$ Klonen. Irreversibler Verlust der Sekretion von SpeB wird oft durch Mutationen in regulatorischen Elementen (CovR/S, RopB) verursacht, welche mit Hypervirulenz verbunden sind. In Paper III wird gezeigt, dass die Wirtsumgebung zu einem transienten Verlust der Sekretion von SpeB durch GAS führt. Inflammation des Gewebes, Einwanderung von neutrophilen und neutrophile Degranulation korrelierten mit dem vermehrten auftreten SpeB$^-$ GAS Klone. Aus Gewebe isolierte GAS exprimierten SpeB, während sie dieses jedoch nicht sekretieren. Von Neutrophilen produzierte reaktive Sauerstoffspezies waren der Hauptfaktor, welcher zum Verlust der SpeB Sekretion durch GAS führte. Außerdem verursachten hypervirulente SpeB$^-$ Klone exzessive Degranulierung von Neutrophilen und konnten intrazellulär replizieren.

3. BACKGROUND

3.1 Innate Immune System

The innate immune system is the first line of defense against invading pathogens. Its primary function is to sense pathogens and rapidly initiate an inflammatory response. It consists of different components that have various functions: epithelial surfaces, e.g., skin or mucosal surfaces, function as barriers to prevent pathogens from entering the host. If such barriers are breached, acellular components like the complement system are in place to detect and kill bacteria. The cellular components of the innate immune system comprise an array of either circulating or tissue-resident specialized cell types. These are equipped to fight invading pathogens and, if necessary, initiate a specific, adaptive immune response (1–3).

3.1.1 Sensing of Pathogens & Inflammasome Activation

In general, innate immune cells sense conserved molecular patterns signaling danger (Pathogen/danger-associated molecular patterns (DAMP/PAMPs)) via specific pattern recognition receptors (PRRs). Recognition of PAMPs by their respective receptors induces activation of intracellular signaling cascades, which ultimately result in initiation of a proinflammatory immune response (4). This includes production of cytokines, chemokines like interleukin (IL)-8, and antimicrobial proteins.

Inflammasomes represent additional mechanisms to sense invading pathogens. This process eventually leads to release of strongly proinflammatory cytokines IL-1β and IL-18. While most cell types can produce both cytokines, myeloid cells, especially macrophages and dendritic cells (DCs), are the primary sources of both cytokines (5,6). In contrast to other eukaryotic cells, cells of monocytic origin do not require a priming step before inflammasome activation. In short, recognition of PAMPs and DAMPs induces assembly of inflammasomes. These are cytosolic multiprotein complexes consisting of adaptor proteins, e.g., ASC, that link caspase-1 with sensor proteins like NLR family pyrin domain containing 3 (NLRP3). Upon activation, these proteins oligomerize, and caspase-1 autocatalytically activates itself. Active caspase-1 processes the precursors of gasdermin D (GSDMD), pro-IL-1β, and pro-IL-18 into their respective mature forms. The N-terminal products of GSDMD form pores in the cell membrane, through which the mature cytokines IL-1β and IL-18 are released (7). IL-1β induces a range of proinflammatory effects, including production of proinflammatory cytokines and activation of macrophage and neutrophil effector functions (5). IL-18, together with IL-12, induces Th1 polarization and secretion of interferon (IFN)-γ (7,8). Other caspases were also shown to process and mediate release of mature IL-1β and IL-18, including caspase-4/5 (7) and caspase-8 (9–13).

3.2.1 Myeloid Cells

Myeloid cells are a heterogeneous group of different innate immune cells. They can be broadly grouped into monocytes, granulocytes (neutrophils, eosinophils, and mast cells), macrophages, and DCs. All of these cell types are derived from a common progenitor that differentiates into the respective cell types in the bone marrow (14).

3.2.1.1 Macrophages & Monocytes

Macrophages, e.g. alveolar macrophages, Langerhans cells and Kupffer cells, are tissue-resident cells specialized in phagocytosis and clearance of pathogens, cellular debris, and other foreign objects. In their function as sessile immune sentinels, macrophages monitor their immediate surroundings in epithelial and mucosal surfaces (15,16). Phagocytosis is a multi-step process (**Figure 1**), starting with recognition of a pathogen/foreign object via PRRs, followed by engulfment of the particle. Then, the phagosome is formed, which matures and eventually fuses with lysosomes, generating the phagolysosome. Inside the phagolysosome, acidic pH, reactive oxygen species (ROS), and antimicrobial compounds kill pathogens or degrade other foreign objects (17). Macrophages show a high degree of plasticity: they can respond to infection by clearing pathogens and secreting cytokines and chemokines, showing an overall pro-inflammatory phenotype. In addition, macrophages are critically involved in tissue homeostasis and wound healing, where they show a more anti-inflammatory phenotype. Different factors like cytokines determine the phenotype, which can also be changed during an infection (15,16,18). If tissue-resident macrophages are depleted, circulating monocytes are recruited and differentiate into macrophages (15,16).
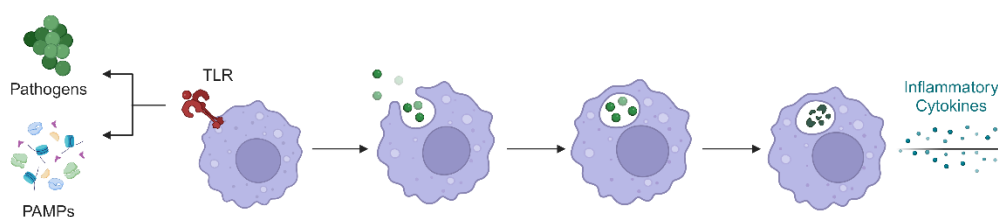
**Figure 1 Phagocytosis by macrophages**

Phagocytosis is a multi-step process. First, the phagocyte recognizes PAMPs or DAMPs via TLRs. The particle/pathogen is engulfed by the phagocyte cell membrane, and the phagosome is formed. After maturation of the phagosome and fusion with the lysosome, acidic pH and antimicrobial peptides/enzymes kill the pathogen or degrade other PAMPs/DAMPs. If a pathogen is detected, the secretion of inflammatory cytokines recruits further immune cells to the site of infection. (Figure created with BioRender)

Monocytes are highly abundant, circulating cells that are recruited to the site of inflammation via chemokine gradients. The existing pro-inflammatory conditions induce differentiation of

monocytes into macrophage- or DC-like cells (15,16). These cell types exhibit further pro-inflammatory phenotypes and produce high levels of cytokines and chemokines (16,19).

3.2.1.2 Dendritic Cells

DCs are mobile sentinel leukocytes involved in control of infections and tolerance (7). Their primary function is to initiate and regulate an adaptive immune response upon encountering a pathogen. Recognition and phagocytosis of pathogens induce processing of antigens and activation of DCs. To induce a pathogen-specific adaptive immune response, DCs migrate to secondary lymphoid organs and present the processed antigens to naïve T cells via MHC molecules. This process is visualized in **Figure 2**. Thus, DCs bridge the innate and adaptive arm of the immune system.
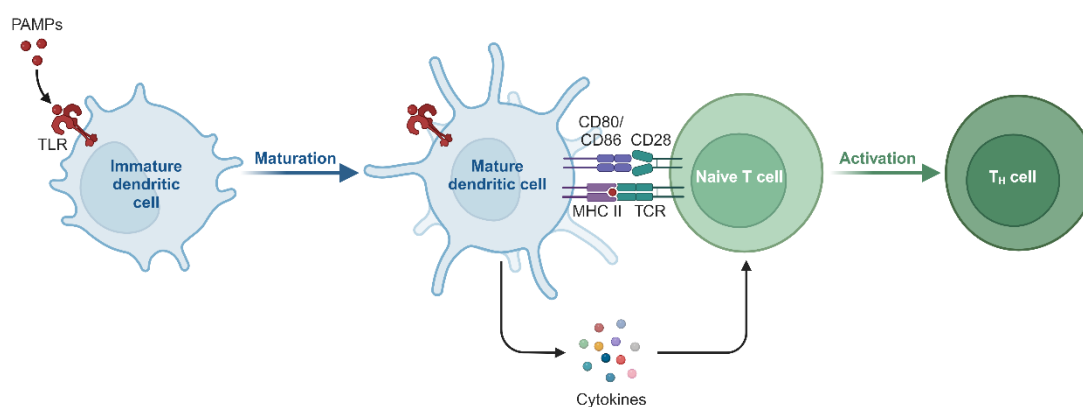
Figure 2 DC maturation and presentation of antigens to T cells

Immature DCs recognize PAMPs via e.g. TLRs. Subsequently, they mature and present the processed antigens to T cells via e.g. MHC II molecules. In addition, they increase the surface expression of co-stimulatory molecules, e.g., CD80/CD86, which interact with receptors on T cells, e.g., CD28. By secretion of different cytokines, DCs shape the direction of T_H cell differentiation. Presentation of antigens, co-stimulation, and cytokines lead to T cell activation. (Figure was created with BioRender)

As the most potent type of APCs, DCs are located throughout the whole body: they are present in blood and lymphoid as well as non-lymphoid tissues (20,21). DCs are a heterogeneous cell population and can be generally divided into two major subgroups: conventional or classical DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs can be further divided into cDC1 and cDC2 subsets (20,21). cDC1 and cDC2 are also functionally distinct: while cDC1s specialize in cross-presentation of antigens to $CD8^+$ T cells via MHCI, cDC2s mainly present antigens to $CD4^+$ T cells via MHCII (20–23). pDCs are mainly located in human blood and tonsils. They are specialized in sensing viral pathogens and are characterized by rapid and excessive production of type I and III interferons in response to activating stimuli (23,24). Monocyte-derived DCs (moDCs) are another subgroup of DCs that may arise during inflammatory conditions (25–27). Since circulating DCs are scarce, much of the knowledge about DC function and response to infection was obtained by working with moDCs. They can be

generated *in vitro* by culturing human monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 for several days. The obtained cells share certain traits with cDCs, e.g., maturing in response to infections, secretion of proinflammatory cytokines, and limited presentation of antigens (21–23,28).

3.2.1.3 Neutrophils

Neutrophils are the most abundant type of circulating leukocytes, making up 50-70% of total blood leukocytes. Due to their short lifespan, they are continuously released from the bone marrow (29). Neutrophils are among the first immune cells recruited to the site of infection. They are recruited via a multi-step process, encompassing binding to endothelial layers and transmigrating to connecting tissues via chemokine gradients (29–31). Once at the site of infection, neutrophils fight the invading pathogen using multiple antimicrobial mechanisms (32). Three main effector mechanisms are in place to fight infections:

i. Degranulation: Neutrophils possess intracellular granules containing an array of different antimicrobial proteins and proteases. These granules, namely azurophilic, specific, tertiary granules, and secretory vesicles, are ready to use but differ in content. Some components are only present in one type of granule, e.g., myeloperoxidase (MPO) in azurophilic granules, while other components are present in multiple granules, e.g., resistin (33,34). During degranulation, granules fuse with the cell membrane and release their contents into the extracellular milieu. Antimicrobial peptides like LL-37 or defensins directly kill the pathogen, while enzymes like lysozyme attack the bacterial cell wall or proteases degrade key virulence factors and toxins (35). Degranulation is a primary effector mechanism for neutrophils and, therefore, tightly controlled by different signaling pathways. However, excessive neutrophil degranulation is associated with different disease pathologies, including inflammatory conditions like septic shock (36). In addition to antimicrobial compounds, neutrophil granules also contain pre-formed cytokines (37,38).

ii. Neutrophil extracellular traps (NETs): NET formation is characterized by release of decondensed chromatin and granule proteins into the extracellular space by activated neutrophils. The chromatin traps bacteria, while the granule contents like LL-37 and histones kill the bacteria. Although this process helps to clear pathogens, excessive NET formation was shown to be involved in sepsis (39).

iii. Phagocytosis and oxidant killing: This pathway includes uptake and killing of bacteria using ROS and reactive nitrogen species. ROS are generated via the NADPH oxidase or the myeloperoxidase-hydrogen peroxide system (40). Hydrogen peroxide (H_2O_2) is generated via spontaneous or dismutase-driven conversion of a superoxide

intermediate (41). H_2O_2 can then be further converted to hydroxyl radicals or used as a substrate for generation of hypochlorous acid (HOCl) by MPO (42). These ROS are then used to kill bacteria.

3.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive opportunistic pathogen, persistently colonizing the gut, nasopharynx, and skin of up to one-third of human population (43,44). Clinical manifestations of *S. aureus* disease range from mild skin and throat infections to severe, life-threatening diseases like necrotizing skin and soft tissue infections (NSTIs) or necrotizing pneumonia. (45) The increasing prevalence of antibiotic resistant strains, especially methicillin-resistant *S. aureus* (MRSA) strains, represent a significant healthcare concern (46). Since the mid-1990s, an increasing number of non-hospital associated MRSA infections, so-called community-acquired MRSA infections (CA-MRSA), have been registered. In contrast to hospital acquired infections, this type of infection also affects healthy individuals and is associated with more severe outcomes (47,48).

S. aureus infection is partially driven by secretion of a wide array of virulence factors (49). Pore-forming toxins like Panton Valentine Leukocidin (PVL), alpha-hemolysin, LukAB, and LukED are known to target innate immune cells via receptor-mediated lysis (50–55). In addition, *S. aureus* is able to internalize and persist in various cell types, including professional phagocytes, epithelial, and endothelial cells (56). The cytoplasmic environment induces a switch to small-colony variants (SCV), which are characterized by slow metabolism and antibiotic tolerance (57). In the form of SCVs, *S. aureus* is able to persist and cause recurrent infections (58,59).

3.2.1 Periprosthetic Joint Infections

Joint replacement surgeries comprise successful orthopedic procedures to treat patients suffering from chronic pain and degenerative joint diseases (60). A projection based on age, gender, socioeconomic status, and year indicates increasing numbers of total joint replacements and corresponding implant revisions until 2030 (61). Tissue inflammation is a common complication of such procedures, often caused by implant-derived wear products. In severe cases, such wear products can induce adverse local tissue reactions (ALTRs). These include tissue necrosis and pseudotumor formation and can lead to implant revision (62,63). ALTRs are mainly driven by the innate immune response, characterized by extensive macrophage infiltration of peri-prosthetic tissue. However, lymphocyte infiltration is also evident. The infiltrating immune cells create a strongly proinflammatory environment, induced

by wear particle- and metal ion-mediated inflammasome activation as well as induction of necrosis (62,64–67). In clinical context, implant-derived metal ions and tissue inflammation are associated with early implant revisions (68–71), which occur in 7 to 15% of total hip or knee replacements (61).

Periprosthetic joint infections (PJIs) are another complication of arthroplasty. *S. aureus* is among the most prevalent causative pathogens (45,72–78). Simultaneous with increasing numbers of joint replacements and revisions, the incidence of PJIs has increased over past decades (79–81). Common symptoms include pain, swelling, and erythema of the affected joint (74). The majority of PJIs occur within 1 year of initial surgery and are considered low-grade infections, meaning a low bacterial load is present within the tissue (74). Exposure of bone marrow and peri-implant tissue to implant-derived wear products is believed to create an environment favorable for PJIs (79,82). Treatment of PJIs includes revision surgery with partial or complete exchange or explantation of the implant (83), debridement of necrotic/infected tissue, and prolonged antibiotic treatment (84–87).

3.3 *Streptococcus pyogenes*

Streptococcus pyogenes is a β-hemolytic, Gram-positive part of the commensal human microbiome (88). It is also referred to as group A streptococcus (GAS), based on the presence of Lancefield group A polysaccharide in the cell wall (89). However, GAS can also cause a broad spectrum of human diseases, ranging from mild superficial infections like pharyngitis to severe, life-threatening conditions like necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) (90). In addition, post-streptococcal sequelae like rheumatic fever, rheumatic heart disease, or acute glomerulonephritis can occur following streptococcal infections (91). Host and bacterial factors can contribute to a switch from colonizing to pathogenic phenotype (91,92). GAS has emerged as a significant cause of morbidity and mortality, especially in low-income countries (91). GAS are estimated to cause disease in 18.1 million people and to cause approx. 500,000 deaths annually (91). From an epidemiological standpoint, GAS serotypes are classified based on the expression of different variants of the *emm*-gene, encoding for the highly abundant surface M-protein (93). So far, more than 200 *emm*-types have been identified, which are globally distributed (93). Depending on the geographical location, certain *emm*-types, e.g., *emm1* or *emm3* in industrial countries, are frequently associated with invasive disease (91). Recently, an increase in invasive GAS infections caused by a highly virulent *emm1*-clone, termed M1$_{UK}$, was reported in several countries (94,95). Despite the long history of GAS research, no commercial vaccine is currently available (96).

3.3.1   Necrotizing Skin and Soft Tissue Infections

NSTIs are rare, severe, and rapidly progressing diseases. These infections are associated with high morbidity and mortality (97,98). Based on microbial etiology, three types of NSTIs can be distinguished: type I NSTIs are of polymicrobial origin, affecting mainly older individuals with underlying conditions (99,100). Type II NSTIs are monomicrobial infections, primarily affecting young and previously healthy individuals (99,101). GAS are among the most common pathogens, causing monomicrobial NSTIs (98,102), accounting for up to 31% of NSTI cases (98,102). Type III infections account for less than 1% of cases and are caused by *Vibrio* species, typically confined to warm coastal areas (99).

Delayed diagnosis can lead to multiple organ failure, a symptom of STSS, further complicating the treatment of NSTIs. Initial symptoms are non-specific, including fever, nausea, soft tissue edema, erythema, and sudden onset of severe pain (101). Treatment of NSTIs comprises extensive tissue debridement and intravenous antibiotic treatment, often a combination of a β-lactam like penicillin and clindamycin (103–106). Other therapies considered in treatment of NSTIs are admission of intravenous immunoglobulin (IVIG) (103) and hyperbaric oxygen therapy (107).

3.3.2   GAS Virulence Mechanisms

The course of human GAS infection is a multifactorial process involving bacterial and human factors. GAS produce a multitude of different virulence factors mediating tissue invasion, immune evasion, and bacterial survival. The composition and amount of virulence factors depend on M-type and environmental factors, meaning not all factors are present under all conditions (108–110). In this section, the most prominent virulence factors and regulation of virulence factor production are summarized. The main virulence mechanisms are visualized in **Figure 3**.
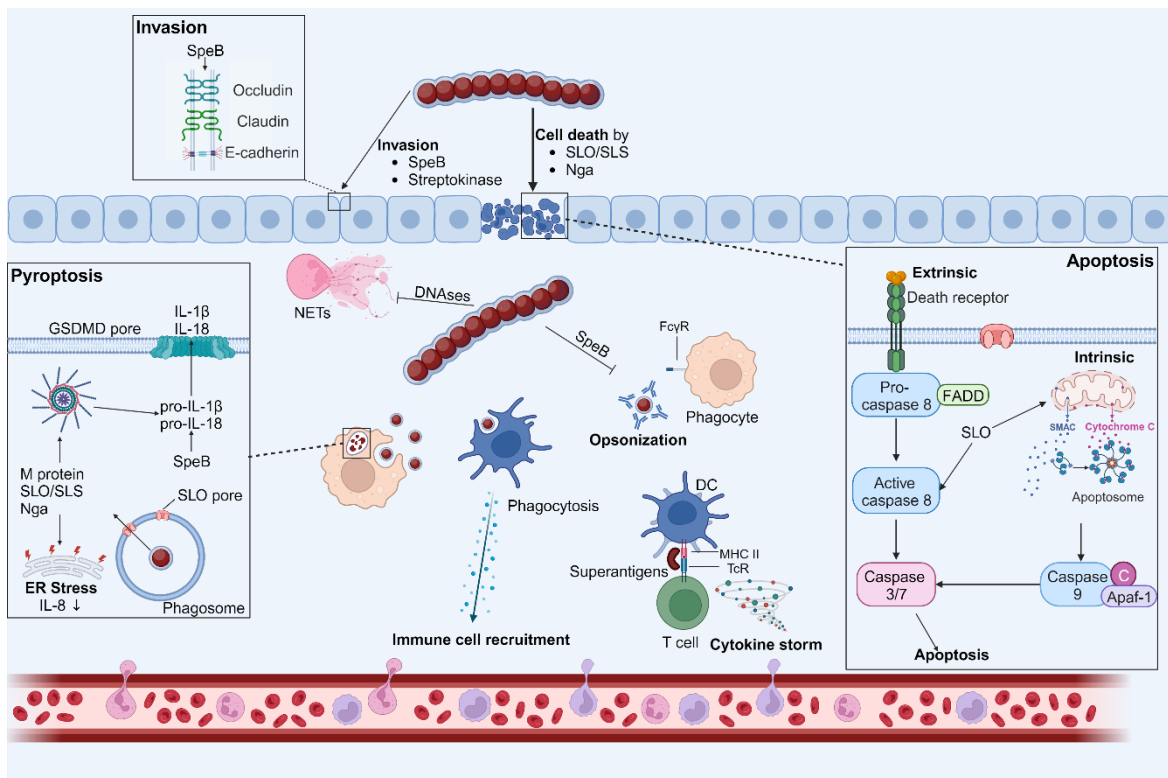
Figure 3 GAS virulence mechanisms

GAS possesses a plethora of different virulence factors, which help to invade host tissues/cells and evade the immune system. The cysteine protease SpeB can cleave cell junction proteins, granting the bacteria access to deeper tissue layers. The cytolysins SLO and SLS can induce apoptosis of epithelial cells, which disrupts the barrier function. GAS can escape from NETs by secreting DNAses that degrade the DNA backbone. SpeB inhibits opsonization and subsequent phagocytosis by degrading antibodies. Superantigens induce pathological secretion of cytokines (cytokine storm). SLO mediates escape from phagosomes. SLO and Nga can induce ER stress and inflammasome activation, leading to release of IL-1β and IL-18. (Figure was created with BioRender, adapted from (96))

GAS virulence is tightly regulated. One of the main regulators is the two component system (TCS) CovR/S (control of virulence, also known as CsrRS (capsule synthesis regulon)). It consists of the membrane-associated sensor kinase CovS and the DNA-binding response regulator CovR. CovS activates and autophosphorylates upon sensing activating stimuli, e.g., pH acidification, high bacterial cell density, and high salt concentrations (111–113). Phosphorylated CovS then transfers the phosphate to position D53 at bound CovR, which induces dissociation from CovS (112). Phosphorylated CovR binds to DNA and either induces or represses transcription of target genes (114,115). Overall, CovR/S was found to regulate expression of up to 15% of the *S. pyogenes* genome (116). Dysfunctional CovR/S allows GAS to adopt a hyper-virulent phenotype. Such clones are frequently isolated from patients and in mice experiments, indicating a central role for CovR/S in GAS pathogenesis (117–122).

CovR/S controls production of several key virulence factors. It acts as a positive regulator of *speB* transcription. SpeB (streptococcal pyrogenic exotoxin B) is a highly conserved cysteine protease that is involved in many infectious processes. In addition to CovR/S, SpeB expression

is regulated by the global regulator of protease (RopB) (123). Despite its name, SpeB is neither an exotoxin nor pyrogenic (124,125). During *in vitro* culture of GAS, SpeB is produced and secreted as an inactive 42 kDa zymogen that autocatalytically cleaves itself into a 28 kDa active enzyme (126,127). SpeB cleaves host, as well as bacterial proteins. Known target proteins on the host side are extracellular matrix proteins (128), epithelial junction proteins (129), complement components (130), and the antimicrobial peptide LL-37 (131). Streptococcal proteins cleaved by SpeB include the M protein, the DNase Sda1, and streptolysin O (SLO), among others (132–135). This range of targets on both bacterial and host sites enables SpeB to modify the response to infection. SpeB has direct immunomodulatory effects by attenuating autophagy and improving intracellular bacterial survival (136) or impairing recruitment of immune cells by degrading chemokines (137). This protease also displays direct pro-inflammatory effects by cleaving inactive pro-IL-1β into its mature form (138). Moreover, pro-IL-18 and pro-IL-36γ, which are secreted by keratinocytes, are also cleaved to their respective mature forms by SpeB (139,140). In addition, SpeB is known to induce caspase-independent induction of pyroptosis by cleaving GSDMA. (141,142). Although the presence of SpeB at early stages of infection is beneficial for the course of infection, SpeB-negative isolates are frequently isolated from patients (117,143,144). This phenotype is often observed due to dysfunctional CovR/S.

For most virulence factors, CovR/S acts as a transcriptional repressor. It represses production of, e.g., the capsule synthesis operon, M-protein, SLO, and SpyCEP (145). The hyaluronic acid capsule of *S. pyogenes* was shown to confer protection from phagocytes (146,147) and mediate initial adherence to skin and pharynx epithelial cells (148). The chemical composition of the capsule is identical to human hyaluronic acid, enabling GAS to avoid detection by immune cells. However, capsule production does not seem essential for GAS virulence since isolates completely lacking the *hasABC* operon, responsible for capsule synthesis, or harboring inactivating mutations of *hasAB* were reported (149–152). The M-protein was shown to bind host plasminogen/plasmin or fibrinogen (88) and histones, conferring resistance to killing by the innate immune compartment (153). It also mediates adherence to host cells (154–156), and some M-types induce activation of the NLRP3 inflammasome (157,158). Hyper-virulent strains display a mucoid colony morphology and increased surface abundance of the M protein (159).

The clinical significance of the switch to a hyper-virulent phenotype is underscored by the finding that >50% of strains isolated from STSS patients possessed *covR/S* mutations, while only 2% of noninvasive isolates did (160,161). STSS is characterized by rapidly progressing multi-organ failure and is associated with high morbidity and mortality (162). Streptococcal superantigens are potent exotoxins and are involved in the pathogenesis of STSS (163).

Superantigens can crosslink the β-chain of the TcR and the MHC II molecules of APCs without prior antigen processing. This induces uncontrolled, non-antigen-specific activation and proliferation of up to 20% of total T cells (164,165). Excessive release of cytokines was shown to contribute to STSS and scarlet fever (94,164). Superantigens, especially SpeA, were shown to be essential during the initial phase of infection, enhancing bacterial colonization (166,167). In addition, superantigens induce activation and cytokine release of human mucosal-associated invariant T (MAIT) cells, which was shown to contribute to the pathological cytokine storm during STSS (168). Hypervirulent GAS variants often display increased production of superantigens (159,169).

STSS isolates were also found to produce increased levels of SLO (160,170), which is the case in hyper-virulent variants, as functional CovR/S represses transcription of *slo* (122). SLO and streptolysin S (SLS) are pore-forming toxins and are widely distributed in GAS clinical isolates. SLO is a cholesterol-dependent cytolysin that mediates host cell lysis via binding multiple glycan receptors (171). SLS is a small non-immunogenic peptide that accumulates at the bacterial surface and causes eukaryotic membranes to collapse (172,173). Both toxins induce cell death of a wide range of host cells, including innate immune cells (172,174,175). In addition, both toxins were shown to induce activation of the NLRP3 inflammasome in human macrophages (158). *Slo* is co-expressed with *nga*, a NAD glycohydrolase (NADase) (176). The physical interaction of SLO and Nga was shown to co-stabilize the two toxins after secretion (177,178). In addition, Nga facilitates the binding of SLO to the cell membrane, promoting SLO pore formation. Translocation of Nga to the host cell cytosol is dependent on SLO (179). In the cytosol, it depletes energy stores and induces cell death (180,181). The combined action of both toxins contributes to bacterial pathogenesis (182) by mediating escape from phagolysosomal killing (183,184) and disrupting host cell protein synthesis and trafficking (185,186). Moreover, it was recently shown that NADase activity suppresses release of type I IFNs by macrophages, promoting inflammation and severe disease (187). Increased expression of SLO and Nga is associated with increased disease severity and emergence of new, highly virulent strains (109,151,188).

3.3.3 Interaction of GAS with Neutrophils and DCs

Severe GAS infections, e.g., NSTIs, are characterized by high bacterial loads and extensive immune cell infiltration, including neutrophils (189,190). Extensive neutrophil degranulation and release of proteolytic enzymes are associated with substantial tissue damage. Several GAS virulence factors were shown to induce neutrophil degranulation, including the M protein (189–191). GAS can escape NETs, mediated by DNases (192). So far, six different DNases have been described, and all sequenced clinical isolates encode for at least one DNase (193).

DNases release trapped bacteria from NETs by degrading the DNA framework (194,195). They also impair recognition of bacteria by immune cells via TLR9 (196,197). In addition, GAS can interfere with neutrophil recruitment by degrading the chemokine IL-8 using the cell envelope serine protease SpyCEP (198–200). SLO was shown to promote bacterial resistance to phagocytic killing by impairing neutrophil oxidative burst (201). SLS was shown to impair neutrophil recruitment and opsonophagocytic killing of bacteria by targeting neurons, causing invasive disease (202).

Little is known about the interaction of GAS and DCs. One study has identified DCs as an essential part of defense against GAS infection in a mouse model (203). In general, DCs were shown to mature and secrete IL-12 in response to GAS infection (204,205). However, both capsule and SLO were shown to interfere with DC maturation and, in the case of SLO, induce significant cell death (206,207).

3.4 Study Objectives

The innate immune system, particularly myeloid cells, is crucial for detecting invading pathogens and rapidly initiating a pro-inflammatory response. However, different factors, both host and bacterial, can affect key effector mechanisms. Metal-based implants are widely used in arthroplasty and are well-known factors affecting immune cells' function and phenotype. Both *S. aureus* and GAS are Gram-positive human commensals that can cause severe disease. GAS, in particular, have developed multiple mechanisms to evade and manipulate the human immune system. Delay or even prevention of myeloid cell activation could severely affect the outcome of infection. Understanding the underlying disease mechanisms and contributions of individual virulence factors provides targets to improve existing therapies/materials or develop new ones.

This thesis aims to characterize how implant-derived metal ions and bacterial phenotype switches impact and modulate the innate immune systems' response to infection.

The specific objectives are:

i. To investigate how exposure of monocytes/macrophages to implant-derived Co^{2+} and Cr^{3+} ions impacts the cellular response to *S. aureus* infection (Paper I)

ii. To characterize the cytokine response of moDCs to infections with virulent and hyper-virulent GAS strains (Paper II)

iii. To identify the mechanism responsible for the SpeB negative phenotype in GAS observed in patient tissue (Paper III)

4. RESEARCH APPROACH

The detailed experimental methods are described in the methods section of the corresponding papers. Selected methods are presented below.

4.1 Ethics Statement

All studies were conducted in accordance with the Helsinki Declaration. Patient samples were retrieved from the INFECT patient cohort consisting of patients with NSTI (INFECT study; ClinicalTrials.gov, NCT01790698). The patient biopsies and plasma were obtained at surgical interventions, and GAS infections were primarily identified by routine diagnostics. Patient enrolment and sample analyses were approved by the regional Ethical Review Board at the National Committee on Health Research Ethics in Copenhagen (Ref. No. 1211709), the regional ethics committee in Gothenburg (Ref. No. 930-12), the regional ethics committee Vest, Norway (REK, Ref. No. 325786), and the regional Ethics Committee in Stockholm (Ref. No. 2012/2110-31/2).

Peri-implant soft tissue samples were collected during aseptic arthroplasty implant revisions at the University Medicine Greifswald. The ethical research committee at the University Medicine Greifswald approved the study (BB 178/20). All patients gave written informed consent to participate in this study.

Blood samples from healthy volunteers or buffy coats of blood provided by the blood bank at the University Medicine Greifswald were used. The buffy coats were provided anonymously. The ethical research committee at the University Medicine Greifswald approved the study (Ref. No. BB 014/14 and Ref. No. BB 006/18). All experiments were carried out in accordance with the approved guidelines.

4.2 Bacterial Strains

The GAS strains (5448, 5626, 8003, and 8157) were isolated from NSTI patients in Canada (208,209). The strains 2002 and 2006 were obtained during the INFECT study (210,211). In addition, two hyper-virulent strains harboring dysfunctional CovR/S, MGAS5005 (159) and 5448AP (118,212) were used. All strains were grown in Todd-Hewitt broth supplemented with 1.5% (w/v) yeast extract (THY) at 37°C and 5% CO_2 for 16-18 h.

S. aureus strain 6005 (213) was grown overnight at 37°C in casein hydrolysate and yeast extract medium with agitation (214).

4.3 Isolation of Human Myeloid Cells and Infection

Myeloid cells are critical first responders to infection. While macrophages are resident cells, DCs are highly mobile, migrating cells. Both cell types are difficult to obtain from peripheral blood but can be generated from monocytes under inflammatory conditions. *In vitro*, this is achieved by isolating monocytes from peripheral blood and culturing them in presence of cytokines for several days. The obtained macrophages or DCs were shown to respond to infections similar to their tissue-resident counterparts (7,15,16,215). Neutrophils are among the first cells recruited to the site of infection. Since neutrophils have a short lifespan, they were freshly isolated from whole blood of healthy donors and immediately infected with GAS. The general experimental approach of Paper I and Paper II is shown below (**Figure 4**).
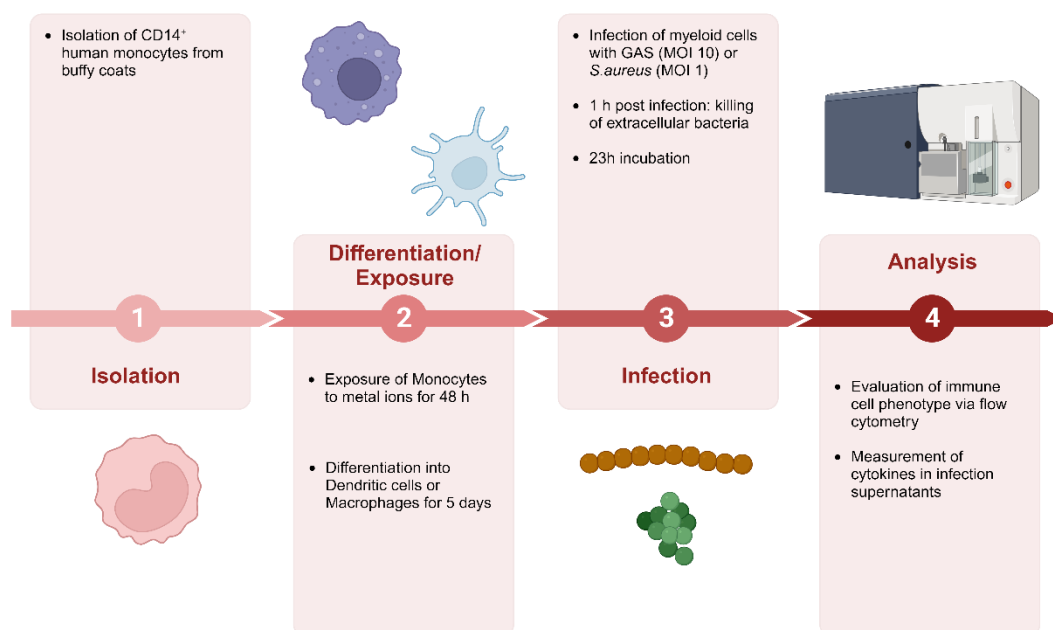
Figure 4 General experimental approach for Paper I and Paper II.

CD14$^+$ monocytes were isolated from human buffy coats via positive selection. The obtained monocytes were either exposed to Co^{2+} or Cr^{3+} ions or a combination of both for 48 h, or they were used to generate moDCs or monocyte-derived macrophages. After 24 h of metal exposure, monocytes/macrophages were infected with *S. aureus* (MOI 1), while moDCs and macrophages were infected with GAS (MOI 10) after 5 d of differentiation. In both cases, extracellular bacteria were killed by addition of antibiotics 1 h post-infection. To ensure full maturation of cells, they were incubated for additional 23 h. To evaluate immune cell phenotype post-infection, the cells were stained for different surface markers and analyzed via flow cytometry. In case of GAS infections, the cytokine response was evaluated using a bead-based, flow cytometric assay. (Figure was created with BioRender)

4.3.1 Isolation of Human Monocytes, Exposure to Metal Ions & Infection with *S. aureus*

Macrophages were shown to contribute to tissue pathology of ALTRs. However, the influence of metal exposure on the cellular phenotype and response to infections is still unclear. Paper I aimed to elucidate the impact of metal exposure on monocyte/macrophage phenotype and their response to infection. To analyze metal ion-mediated effects on human monocytes/macrophages, monocytes were isolated from buffy coats using a bead-based positive selection approach. Cobalt (II) chloride hexahydrate and chromium (III) chloride hexahydrate were dissolved in human AB serum and incubated overnight at 37 °C to mimic *in vivo* formation of biomacromolecule-ion-complexes. Isolated monocytes were exposed to metal salt solutions for 48 h. Metal-stimulated monocytes/macrophages were infected with *S. aureus* 6005 at a multiplicity of infection (MOI) of 1 for 1 h to mimic initial low-grade infections. Extracellular bacteria were killed by addition of antibiotics. For flow cytometry, infected monocytes were incubated for additional 23 h. For assessing intracellular bacterial counts, monocytes were infected as described above. 1 h after addition of antibiotics, cells were washed and lysed, and intracellular bacteria were plated on blood agar plates.

4.3.2 Isolation of Human Monocytes, Generation of moDCs & Infection with GAS

DCs are essential for resolving infections and initiation of an adaptive immune response. However, little is known about interactions of GAS and DCs. Paper II aimed to analyze the impact of hyper-virulent GAS variants on the cytokine response of human monocytic cells. To generate moDCs and monocyte-derived macrophages, human monocytes were isolated from buffy coats using a bead-based positive selection approach. The moDCs were generated by culturing monocytes for 5 d in presence of GM-CSF and IL-4. The monocyte-derived macrophages were generated by culturing monocytes in presence of only GM-CSF for 7 d. Monocytes, monocyte-derived macrophages, or moDCs were infected with GAS strains at a MOI 10 for 1 h. Next, extracellular bacteria were killed by addition antibiotics. To ensure full maturation in response to infection, cells were incubated for additional 23 h. Supernatants were collected from all cell types and the moDCs were directly prepared for flow cytometry. For assessing intracellular bacterial survival kinetics, moDCs were infected as described above. After addition of antibiotics, the cells were washed, lysed, and intracellular bacteria plated on blood agar plates.

4.3.3   Isolation of human neutrophils and infection with GAS

NSTIs are characterized by extensive immune cell infiltration, including neutrophils (189,190). Extensive neutrophil degranulation is associated with increased tissue pathology. Paper III aimed to characterize the impact of SpeB⁻ phenotype GAS on the neutrophil response. Human primary neutrophils were isolated from healthy donors using density gradient centrifugation on Polymorphprep. After isolation, neutrophils were immediately infected with GAS at a MOI 10 for 1 h. Extracellular bacteria were killed by addition of antibiotics. At indicated time points (Paper III), neutrophils were washed lysed, and intracellular bacteria were plated on casein agar plates to assess SpeB secretion. Neutrophil degranulation was assessed by secretome analysis of supernatants post-infection with GAS.

4.4 Analysis of Immune Cell Phenotype Post-Infection

Myeloid cells can undergo drastic phenotypic changes upon encountering a pathogen. MoDCs will mature in response to infection, meaning they stop antigen uptake and increase expression of surface markers essential for T cell stimulation (HLA-DR, CD80, CD83, CD86, CD40) (7). Monocytes and macrophages also express a distinct array of surface markers that changes in response to infection (19). To evaluate the cellular response to infection of both metal-exposed and infected monocytes/macrophages and infected moDCs, expression of surface markers was assessed via flow cytometry. First, dead cells were labeled, and unspecific binding of immunoglobulins was blocked by addition of an Fc-blocking agent. Cells were incubated with titrated amounts of monoclonal antibodies directed against different surface and intracellular molecules. Cells were washed between each staining step and fixed. Data were acquired using a FACSAriaIII flow cytometer. The respective gating strategies for monocytes/macrophages (Paper I; Fig. 5A) and moDCs (Paper II; Fig. 5B) are shown in **Figure 5**.
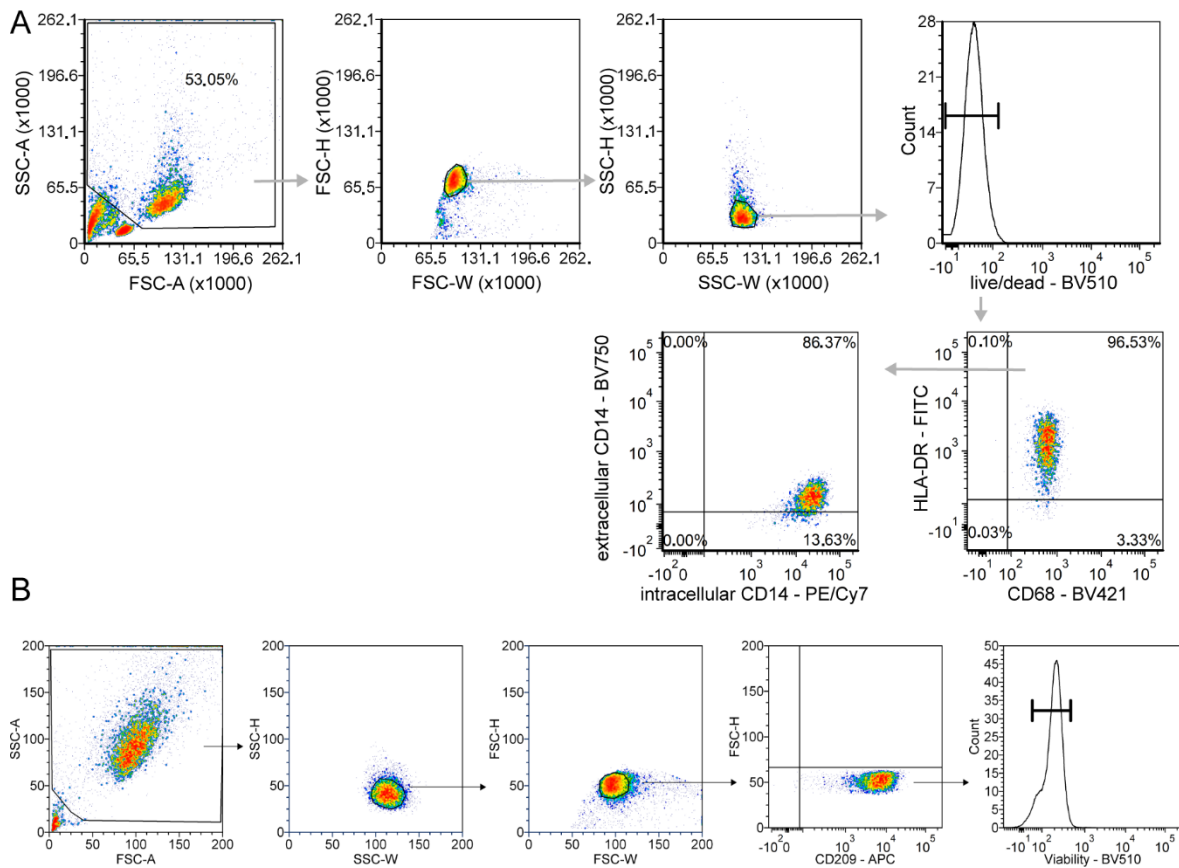
Figure 5 Gating strategies used to identify human myeloid cells

(A) Monocytes/Macrophages: doublets were excluded by consecutive gating of FSC-H/FSC-W and SSC-H/SSC-W. Dead cells were excluded by using the Zombie Aqua™ Fixable Viability Kit. Monocytes/macrophages were selected based on the expression of HLA-DR and CD68. Macrophage subsets were further distinguished based on expression of intracellular and extracellular CD14. Subsequently, the macrophage subsets were analyzed for positivity and surface expression of CD16, CD38, HLA-DR, and PD-L1. (B) MoDCs: doublets were excluded by consecutive gating of FSC-H/FSC-W and SSC-H/SSC-W. MoDCs were selected based on the expression of the specific moDC marker DC-SIGN (CD209). Dead cells were excluded by using the Zombie Aqua™ Fixable Viability Kit. moDCs were analyzed for positivity and expression of different costimulatory surface markers, including CD40, CD80, CD83, CD86, and HLA-DR.

4.5 Cytokine Secretion in Response to GAS Infection

In addition to presenting antigens and clearing pathogens, myeloid cells secrete cytokines to shape the T cell response. DCs were shown to be the primary sources of IL-12 (7), while macrophages are potent producers of tumor necrosis factor (TNF), IL-6, and IL-8 (19). Moreover, myeloid cells are the main producers of inflammasome-derived Il-1β and IL-18 (7). Therefore, supernatants of the infected cells were collected, and cytokine concentrations were measured using a bead-based multiplex assay. Data were acquired with a FACSAria III flow cytometer.

5. RESULTS & DISCUSSION

5.1 Cobalt and Chromium Ions Impair Macrophage Response to *Staphylococcus aureus* Infection

CoCrMo alloys are biomaterials widely used in arthroplasty. Development of ALTRs due to implant-derived wear particles and metal ions is associated with an increased risk for revision arthroplasty and PJI. ALTRs are characterized by macrophage infiltration (62). Metallic wear particles and ions, in particular Co^{2+} and Cr^{3+}, were shown to induce cytotoxicity (216) and interfere with macrophage migration (217) and activation (63). However, little is known about the effects of exposure to Co^{2+} and Cr^{3+} ions on human primary macrophages and its subsequent influence on the cellular response to bacterial infection.

5.1.1 Local Metal Exposure and Macrophage Infiltration Are Evident in Patient Tissue

Local exposure of peri-implant tissue to Co^{2+} and Cr^{3+} ions (217) and macrophage infiltration (62) are well-accepted phenomena in arthroplasty. Local exposure to Co and Cr was evident in all biopsies used in this study (Paper I, Figs. 1,2). To evaluate macrophage infiltration, confocal laser scanning microscopy (CLSM) was performed. This revealed highly auto-fluorescent metal particles distributed in the tissue (Paper I, Fig. 3A), as well as dense areas of macrophage infiltration (Paper I, Fig. 3B). These results confirmed local exposure of macrophages to implant-derived metallic wear particles and metal ions.

5.1.2 Exposure to Metal Ions Induces Loss of Surface Markers

To evaluate how exposure to pathological concentrations of Co^{2+} and Cr^{3+} ions impacts monocyte/macrophage phenotype, primary human monocytes were isolated. Subsequently, cells were exposed to Co^{2+} and/or Cr^{3+} ions for 48 h, and the cell phenotype was analyzed via flow cytometry. In all conditions, monocytes differentiated into macrophages (Paper I, Fig. S1), and exposure to metal ions did not significantly reduce macrophage viability (Paper I, Fig. 4A). Analysis of macrophage phenotype revealed an almost complete loss of surface CD14 when cells were exposed to high concentrations of Co^{2+} (Paper I, Fig. 4B). However, solely CD14$^+$ macrophages were detected via CLSM (Paper I, Fig. 3B). Since immunostaining of tissue biopsies requires permeabilization steps, distinction between surface-associated and intracellular CD14-staining was not possible via this method. Therefore, *in vitro* metal-exposed monocytes/macrophages were stained for intracellular CD14. In all conditions, the entire population stained positive for intracellular CD14 (Paper I, Fig. 4C and S2). In addition, expression of the FCγRIII receptor CD16 was analyzed, showing an almost complete loss of surface CD16 when cells were exposed to high concentrations of Co^{2+} (Paper I, Fig. 4D). Metal

ions, especially Co^{2+}, were shown to induce intracellular oxidative stress (218). Loss of surface CD14, paired with detectable intracellular CD14, suggests that high concentrations of Co^{2+} induce ER or Golgi stress. Induction of both pathways can lead to disruption of intracellular protein trafficking, which interferes with immune cell functionality (219). Tissue-resident macrophages readily phagocytose implant-derived wear particles. However, due to their inorganic nature, such particles cannot be fully degraded, and the phagolysosomal acidic pH even leads to release of additional ions from those particles (218). The released ions can diffuse to the cytosol and induce oxidative stress and misfolding of proteins, resulting in ER stress induction via the unfolded protein response (218).
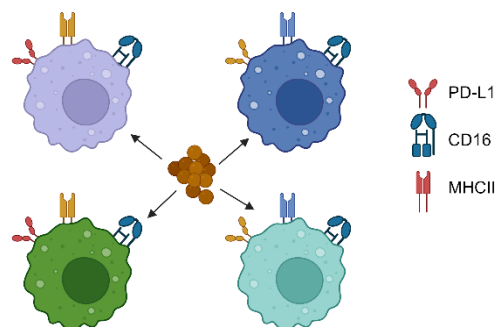
Figure 6 Effects of metal exposure on macrophage phenotype

Human primary monocytes were left untreated (purple) or exposed to Co^{2+} (blue), Cr^{3+} (green) or Co^{2+}/Cr^{3+} (turquoise). In all conditions, monocytes differentiated to macrophages. Macrophage phenotype and response to infection were analyzed. PD-L1, CD16, and MHC II are included in this figure. Blue indicates downregulated, yellow indicates unchanged, and red indicates upregulated expression of surface markers in response to infection. (Figure created with BioRender)

Since previous studies have reported activation of macrophages in response to stimulation with metal ions (220,221), surface expression of activation marker CD38, antigen-presenting HLA-DR, and checkpoint inhibitor PD-L1 were assessed. Exposure to metal ions did not cause macrophage activation on a cellular level, as shown by lack of CD38 expression (Paper I, Fig. 5A) and unchanged expression of PD-L1 (Paper I, Fig. 5C). In addition, a decrease of HLA-DR expression was detected in macrophages exposed to high concentrations of Co^{2+} (Paper I, Fig. 5B). In the mentioned studies, macrophage activation was analyzed by expression of cytokine mRNAs (*IL1B, CXCL8*) (220) and release of cytokines (IL-1β, CXCL8) (221), both referred to as indicative of macrophage activation. However, we did not detect any signs of macrophage activation on a cellular level, as shown by the lack of upregulation of activation markers (CD38, HLA-DR, PD-L1). Expression of HLA-DR was even downregulated after exposure to high concentrations of Co^{2+}, which was also confirmed on transcript level (Paper I, Fig. S4E, **Figure 6**). These results point to metal ion-mediated immuno-suppressive effects but not activation. However, induction of ER stress was shown to induce inflammasome

activation and release of IL-1β, suggesting cytokine release by induction of stress responses rather than activation of macrophages (219).

5.1.3 Exposure to Metal Ions Impairs Macrophages' Response to Infection

Next, we assessed the metal-exposed macrophages' response to infection. Since *S. aureus* is a common cause of PJIs, cells were infected with methicillin-susceptible *S. aureus* strain 6005. To mimic peri-implant tissue conditions, the cells were continuously exposed to metal ions. Since exposure of macrophages to high concentrations of Co^{2+} led to an almost complete loss of surface CD16 (Paper I, Fig. 6D), we also assessed the phagocytic capacity of metal-exposed cells. Here, macrophages exposed to high concentrations of Co^{2+} showed reduced uptake of *S. aureus*, as compared to the other conditions (Paper I, Fig. 6A, **Figure 6**). In all other conditions, macrophages showed reduced expression of surface CD16 post-infection (Paper I, Fig. 6D). CD16 is a FcγIII receptor that facilitates phagocytosis of opsonized bacteria (222). As the experiments were carried out in presence of human serum, opsonization of bacteria is likely to occur in this setting. *S. aureus* is a common colonizer among humans (43,44), and antibodies directed against staphylococcal proteins can readily be detected in sera of healthy people (223). The almost complete loss of surface CD16 provides a probable cause for the reduced phagocytosis. Binding of the antibodies Fc region to CD16 induces activation of several signaling cascades, ultimately leading to engulfment of the receptor-bound antibody and bacteria within a phagosome (224). In addition, Fcγ receptors can take a more anti-inflammatory role triggered by binding of free IgG and IVIG. Anti-inflammatory effects include dampening ROS response and reduced influx of immune cells to inflamed tissue (224,225). Although speculative, reduced phagocytosis could delay bacterial clearance in a tissue setting, while loss of anti-inflammatory effector functions could aggravate tissue damage by excessive immune cell activation. If bacteria are not cleared, they could proliferate and spread to surrounding tissue, as well as enter biofilm formation. Cases of biofilm formation have been described in PJI patients (74,226).

In addition, cellular activation of macrophages was analyzed in response to infection. Metal ions completely suppressed activation of infected macrophages, shown by lack of expansion of CD38+ cells (Paper I, Fig. 7A). In addition, macrophages exposed to high concentrations of Co^{2+} showed diminished upregulation of PD-L1 expression in response to infection (Paper I, Fig. 7C, **Figure 6**). PD-L1 is part of the inhibitory immune checkpoint receptor set PD-1/PD-L1. Activation of this pathway inhibits T cell activation and provides a mechanism to prevent excessive, pathologic T cell activation (227). Therapeutics targeting this pathway are used in treatment of several forms of cancer (227). Reduced PD-L1 expression in response to activating stimuli might provide an additional explanation for the pro-inflammatory environment

in ALTRs. Stimulation of T cells by clinical *S. aureus* isolates leads to activation and surface expression of PD-1, the receptor for PD-L1 (228). Interaction of PD-1 and PD-L1 inhibits excessive T cell activation and cytokine secretion. Thus, if inhibition of T cells via the PD-1/PD-L1 axis is reduced, uncontrolled T cell activation and extensive secretion of cytokines are likely to contribute to ALTR pathology.

In conclusion, these findings provide insights into the biological side effects of a widely used biomaterial. Development of ALTRs as a consequence of local and systemic exposure to implant-derived wear particles and metal ions is well-studied. Understanding the detailed mechanisms of the local tissue response and the consequences for the immune response is critical to improving existing biomaterials as well as developing new materials. This study shows that exposure to metal ions significantly interferes with essential effector functions of monocytes/macrophages. These results provide an explanation for the increased risk of PJIs following revision surgeries. In this very active field of study, this study might help to re-assess the use of CoCrMo in considering the possible long-term effects of implant-derived wear products. In the future, analysis of *ex vivo* samples regarding macrophage phenotype and response to infection should be considered.

5.2 Hyper-virulent *Streptococcus pyogenes* Impairs Interleukin-18 Response in Human Monocytic Cells

Myeloid cells are primary sources of cytokines and chemokines upon detecting invading pathogens. The composition of cytokines/chemokines determines which immune cells are recruited and shapes the subsequent T cell response needed to clear the infection. However, bacteria, including GAS, have developed several ways to circumvent the host response. In severe infections, GAS were shown to adopt a hyper-virulent phenotype to avoid clearance and influence the immune response.

5.2.1 GAS Harboring Dysfunctional CovR/S Suppress IL-8 and IL-18 Secretion in Myeloid Cells

Hyper-virulent GAS strains were shown to induce an excessive pro-inflammatory response, which is often associated with STSS (160). Myeloid cells, especially DCs, are primary sources for the IL-1 family cytokines IL-1β and IL-18 (7). Both cytokines are released via the inflammasome axis and are closely linked to NSTIs and development of STSS (168,229–231). To determine whether virulent and hyper-virulent GAS strains induce differential cytokine responses by moDCs, cells were infected with either four strains harboring functional CovR/S (CovR/S$^+$) or four strains harboring dysfunctional CovR/S (CovR/S$^-$). While secretion of IL-1β was unaffected by GAS phenotype (Paper II, Fig. 1A), moDCs infected with hyper-virulent strains secreted significantly reduced levels of IL-18 (Paper II, Fig. 1B) and IL-8 (Paper II, Fig. 1C). A similar phenotype was observed in infections of monocytes and monocyte-derived macrophages, showing that it is not restricted to moDCs (Paper II, Fig.2).

In addition to cytokine secretion. DC maturation is characterized by expression of co-stimulatory surface markers to provide activating signals for T cells. Therefore, moDC maturation in response to GAS infection was assessed. MoDCs remained viable (Paper II, Fig. 3B) and readily matured in response to infections, irrespective of GAS phenotype (Paper II, Fig. 3A-G). In addition, analysis of whole proteome of infected moDCs did not reveal significant global differences between CovR/S$^+$ and CovR/S$^-$ infection (Paper II, Fig. 3H). This suggests that, although hyper-virulent strains lead to abrogated secretion of IL-8 and IL-18, overall maturation of moDCs is not affected.

5.2.2 Caspase-8 Inhibition Restores Secretion of IL-8 and IL-18 by MoDCs in CovR/S$^-$ Infections

Hyper-virulent strains exhibit increased production of the cell-envelope protease SpyCEP (122), which has been shown to reduce levels of IL-8 in GAS infections (120,198). Thus, we focused on elucidating the mechanism responsible for abrogated IL-18 secretion in moDCs.

Pro-IL-18 contains predicted cleavage sites for caspase-1 and caspase-3 (232). Processing of IL-18 by caspase-3 yields biologically inactive fragments (233). To determine whether inhibition of caspase-3 or its activator caspase-8 would restore secretion of IL-18 by moDCs, cells were pretreated with specific caspase inhibitors and subsequently infected with a hyper-virulent strain. Secretion of IL-1β was not affected by caspase inhibition (Paper II, Fig. 4A-B). Caspase-8 and pan-caspase inhibition restored secretion of IL-18 (Paper II, Fig. 4C) and IL-8 in response to CovR/S⁻ infections (Paper II, Fig. 4D). Next, caspase-8 activity was measured in infected moDCs. Infections with a CovR/S⁻ strain activated caspase-8 in moDCs, while infections with the CovR/S⁺ strain did not (Paper II, Fig. 5C). Caspase-8 is involved in many cellular processes. It was shown to participate in necroptosis as part of the ripoptosome, which ultimately leads to activation of NF-κB and a pro-inflammatory response (234). In addition, caspase-8 can substitute for loss of caspase-1 in pyroptosis, resulting in release of both mature IL-1β and IL-18 (10,12,13). This protease is dysregulated in many cancer cells, emphasizing its central role in cellular signaling (234). Caspase-8 undergoes post-translational modifications depending on cellular function and activating stimulus. Such modifications shape and shift its activity and affinity for different target molecules (234,235). Although speculative, CovR/S⁺ and CovR/S⁻ strains might induce differential post-translational modification of caspase-8. This could prevent activation in infections with CovR/S⁺ strains and induce cleavage of IL-18 in infections with CovR/S⁻ strains, potentially yielding inactive IL-18. Alternatively, another protease downstream of caspase-8 could cleave IL-18 in CovR/S⁻ infections of moDC.

5.2.3 Impact of Dysfunctional CovR/S on Systemic Cytokine Levels in NSTI Patients

Elevated levels of IL-18 were detected in plasma and saliva in pharyngitis human challenge trial participants, suggesting a role for IL-18 in human GAS infections (236). In addition, the IL-12/IL-18 axis (168) is closely linked to NSTIs and STSS. Therefore, the CovR/S phenotype of GAS isolates collected during the INFECT study was determined and correlated to previously measured plasma concentrations of IL-8, IL-1β, and IL-18 (231). Matching of CovR/S phenotype to plasma cytokine concentrations of respective patients revealed equal systemic concentrations of IL-18, irrespective of CovR/S status (Paper II, Fig. 6A-C). In addition, patients were grouped based on clinical presentation, with and without septic shock. No differences in systemic concentrations of IL-18 were detected between patient groups (Paper II, Fig. 6F). The main function of IL-18 is induction of IFN-γ secretion by T cells (8). IFN-γ is one of the primary driving forces of STSS development (163). In STSS, MAIT cells were the major contributors to IFN-γ production in response to GAS (168). Reduced local IL-18 concentrations could impair or delay activation of MAIT cells and corresponding secretion of IFN-γ. As IFN-γ primes macrophages for phagocytosis and induces an overall more pro-

inflammatory phenotype (237), abrogated secretion could improve bacterial survival. However, hyper-virulent GAS were shown to produce increased amounts of superantigens (228), potentially negating the effects of reduced IL-18.

5.2.4   Secretion of IL-1β and IL-18 by MoDCs Is Increased in Infections with 5448Δ*slo*

SLO was previously shown to interfere with maturation of murine DCs (207) and to induce caspase activation (175). As its expression is under CovR/S control, we hypothesized that SLO is involved in suppressing the secretion of IL-18 by moDCs. Since we were not successful in constructing a *slo* mutant in 5448AP genetic background, experiments were performed with 5448Δ*slo*. Here, we hypothesized that infections of moDCs with 5448Δ*slo* would further increase the IL-18 response compared to wild-type infections. Both strains induced secretion of equal levels of IL-8 (Paper II, Fig. 7F). However, concentrations of IL-1β (Paper II, Fig. 7G) and IL-18 (Paper II, Fig. 7H) were significantly higher in infections of moDCs with 5448Δ*slo* as compared to wild-type infections. Our data suggests that SLO is involved in suppression/reduction of cytokine secretion by moDCs via an unknown mechanism. This cytolysin was shown to induce ER stress in murine cells (186). As described in section 5.1.2, ER stress impacts intracellular protein production and trafficking (219). Monocytic cells possess intracellular stores of pro-IL-1β and pro-IL-18 (238,239). However, *de novo* synthesis and release of both cytokines would be affected during ER stress, preventing release of additional cytokines. In this way, excessive secretion of SLO would i) lyse host phagocytes, ii) interfere with cytokine/chemokine secretion, and iii) inhibit/delay recruitment of additional phagocytes. Thus, this cytolysin provides different immune evasion mechanisms.

Data on GAS-DC interaction is scarce and mainly derived from work with murine models. Cytokines/chemokines derived from myeloid cells are crucial effector molecules in infections. They are involved in recruitment of immune cells and induction of an adaptive immune response. Through tissue passage, GAS can adopt a hyper-virulent phenotype mediated by mutations of genes encoding the CovR/S TCS. Here, we show that *in vitro* infections of monocytic cells with such hyper-virulent strains result in abrogated release of IL-8 and IL-18. This phenotype was reversible upon inhibition of caspase-8. In conclusion, this study provides insights into the interaction of hyper-virulent GAS strains with human moDCs. While we did not observe an impact of GAS phenotype on systemic concentrations of IL-18, local effects of abrogated IL-18 secretion are still unknown and require further studies.

5.3 Neutrophil-derived Reactive Agents Induce a Transient SpeB Negative Phenotype in *Streptococcus pyogenes*

GAS isolated from NSTI patient tissue biopsies frequently display a mixed phenotype, consisting of SpeB positive (SpeB$^+$) and SpeB negative (SpeB$^-$) colonies (210). This phenotype can stem from genetic mutations in regulatory components, giving rise to hyper-virulent clones (117,212,240) or other still unknown mechanisms. SpeB itself is involved in many infectious processes: it can degrade cytokines, immunoglobulins, or complement factors to evade phagocytosis while also degrading bacterial factors like the M-protein or SLO to avoid detection (96). The aim of Paper III was to further characterize the hyper-virulent GAS phenotype, with a focus on SpeB$^-$ phenotype.

5.3.1 Loss of SpeB in GAS Recovered from Tissue Biopsies Is Reversible

To characterize the phenotype of GAS recovered from tissue biopsies in regards to SpeB secretion, colonies were plated on casein agar. Presence of clear hydrolysis zones around the colonies indicates secretion of active SpeB. Biopsies frequently contained a mixture of both SpeB$^+$ and SpeB$^-$ clones (Paper III, Fig. 1A). Loss of SpeB secretion has been associated with genetic mutations of transcriptional regulators *covR/S* or *ropB*, giving rise to hyper-virulent clones, or *speB* itself (116,123,240). Whole genome sequencing revealed that some strains contained mutations in *covS* or *ropB* (Paper III, Fig. S2-4). However, passage of SpeB$^-$ clones without mutational inactivation of *covS* or *ropB* in THY restored SpeB secretion (Paper III, Fig. 1B), indicating that the host environment induces this transient phenotype.

5.3.2 Neutrophil-derived ROS Induce SpeB$^-$ Phenotype

Correlation of SpeB$^-$ phenotype with inflammatory markers, IL-8 and HMGB1, as well as markers for neutrophil infiltration and degranulation in tissue biopsies revealed positive correlation of all markers with bacterial load and increasing numbers of SpeB$^-$ clones (Paper III, Fig. 1C-F). HMGB1 is a nuclear protein that is released by necrotic or injured cells. It was shown to be highly expressed in tissue biopsies of NSTI patients and serves as a chemoattractant for neutrophils (189,241). To confirm that a neutrophil-derived factor induces the transient SpeB$^-$ phenotype, isolated human neutrophils were infected with four different GAS strains showing stable SpeB phenotypes. Colonies recovered from neutrophil infections showed a mixed phenotype (Paper III, Fig. 2D-F), confirming that a neutrophil-derived factor induces transient SpeB negativity. Single colony proteomics and gene expression analysis of SpeB$^-$ colonies revealed transcription of *speB* and higher intracellular abundance of SpeB as compared to SpeB$^+$ colonies, suggesting impaired secretion of SpeB (Paper III, Fig. 3).

Neutrophils have different mechanisms to kill bacteria. To analyze the impact of oxidative burst on SpeB secretion, GAS strain 5448 was exposed to different concentrations of ROS, namely H_2O_2 and HOCl. Exposure to sub-lethal concentrations of both species induced a switch to SpeB$^-$ phenotype in 30-70% of colonies (Paper III, Fig. 4A-D). To confirm involvement of ROS in the SpeB phenotype switch in neutrophils, infections were repeated in presence of an MPO inhibitor. MPO is the primary source of HOCl in neutrophils (42). The observed effects on SpeB secretion were time- and concentration-dependent: low concentrations of the inhibitor led to initially higher killing and fewer SpeB$^-$ colonies. However, this was reversed with continued infection, as shown by less killing and more SpeB$^+$ colonies. Higher concentrations of the inhibitor led to more killing and more colonies remaining SpeB$^+$ (Paper III, Fig. 4E-F). In general, ROS can induce oxidation of the functional cysteine in cysteine proteases, rendering them non-functional (242). To prove that ROS did not affect SpeB activity, SpeB activity was assessed in presence/absence of H_2O_2. No effect of H_2O_2 on SpeB activity was observed (Paper III, Fig. 4H-J).

To confirm a role for MPO *in vivo*, NSTI patient tissue biopsies with known SpeB background were stained for MPO and GAS: Although comparable bacterial loads were detected in tissue biopsies of infections with SpeB$^+$ and SpeB$^-$ clones, higher levels of MPO were detected in biopsies of infections with SpeB$^-$ clones (Paper III, Fig. 5A-C). In a clinical context, the release of MPO is associated with hyper-inflammation and high levels of MPO were found in patients suffering from sepsis (243–245). Overall, this shift in SpeB phenotype points to neutrophils as potential bacterial reservoirs when SpeB secretion is impaired, or MPO is inhibited.

5.3.3 Increased Neutrophil Degranulation in Infections with Hyper-virulent 5448AP

Neutrophil infections with stable SpeB$^-$ strain 5448AP led to continuous intracellular bacterial replication (Paper III, Fig. S13A), which was also observed for strain 8003 (Paper III, Fig. 2D). Both strains have *covR/S* mutations, inactivating the TCS (118) (Paper III, Fig. S2-4). SpeB negativity was also associated with higher neutrophil degranulation, as shown in proteomic analysis of neutrophil secretome. SpeB$^-$ 5448AP induced a higher release of granule proteins as compared to SpeB$^+$ 5448 (Paper III, Fig. 6A-H). While lack of SpeB seems beneficial in terms of bacterial survival inside neutrophils, increased release of antimicrobial granule contents and enzymes can cause further tissue damage. In addition, inactivating mutations of *covR/S* and the associated loss of SpeB are also associated with impaired neutrophil recruitment, as shown by suppressed secretion of IL-8 in 5448AP infections of other myeloid cells (Paper II, Fig. 2). Hyper-inflammation and extensive tissue damage in NSTIs are partially derived from influx and excessive degranulation of neutrophils (48, 49). Reduced IL-8 levels due to activity of SpyCEP were shown to reduce neutrophil recruitment. In addition, hyper-

virulent GAS strains were previously shown to have improved intracellular survival in neutrophils and induce excessive degranulation (50, 51). This indicates, that hyper-virulent GAS not only impair recruitment of neutrophils, but also influence how neutrophils respond to GAS.

In conclusion, the role of SpeB in GAS pathogenesis remains controversial. On the one hand, SpeB provides protection from the immune system by degrading antimicrobial compounds like LL-37 (209) and impairing phagocytosis by degrading C3b (246) and immunoglobulins (247). On the other hand, SpeB also cleaves the precursors of IL-1β (240,248) and IL-18 (139) into their respective mature forms. These cytokines have potent proinflammatory effects and induce an immune response. The majority of isolates recovered from infections encode for *speB* and lack of active SpeB secretion is, in most cases, associated with mutational inactivation of transcriptional regulators (249). This study has shown that GAS adopt a transient SpeB⁻ phenotype, to improve survival within neutrophils. Neutrophil-derived ROS drive loss of SpeB secretion, and inhibition of MPO reverses the phenotype switch. These insights into GAS fitness in the soft tissue might provide a new potential target for the treatment of NSTIs.

6. CONCLUSIONS

This thesis aimed to elucidate and provide a better understanding of factors influencing myeloid cells' response to infections. Myeloid cells are crucial first responders to infections. In addition to clearing the pathogen, they secrete cytokines to recruit further immune cells, initiate an effective adaptive immune response and eventually resolve the infection. Metal-based implants are widely used in arthroplasty and are well-known factors affecting immune cells' function and phenotype. Both *S. aureus* and GAS are Gram-positive human commensals that can cause severe disease. GAS, in particular, have developed multiple mechanisms to evade and manipulate the human immune system. Understanding the underlying disease mechanisms and contributions of individual virulence factors provides targets to improve existing therapies/materials or develop new ones.

Paper I

CoCrMo alloys are biomaterials widely used in arthroplasty. They have been linked to the development of ALTRs, which are characterized by tissue necrosis, extensive inflammation, and macrophage infiltration. Macrophages are tissue-resident cells that phagocytose foreign particles, including implant-derived wear particles, in order to clear them. While inflammation caused by implant-derived particles is well studied, the effects of metal ions on the cellular phenotype and functionality of monocytes/macrophages remain elusive. In this study, isolated human monocytes were exposed to pathologic concentrations of Co^{2+} or Cr^{3+} ions *in vitro,* and cell phenotype, as well as response to infection, were analyzed:

  i.   Local peri-implant tissue is exposed to Co and Cr with evident macrophage infiltration
  ii.   Exposure to high concentrations of Co^{2+} induces differentiation to macrophages with a metal-specific imprint
  iii. Co^{2+} or Cr^{3+} do not induce activation of exposed macrophages
  iv. Co^{2+} and Cr^{3+} ions inhibit macrophage response to *S. aureus* infection

These findings demonstrate that implant-derived metal ions impact macrophage phenotype and functionality. While exposure to Cr^{3+} had only minor effects on macrophage phenotype, exposure to Co^{2+} led to almost complete loss of surface CD14 and CD16, as well as decreased HLA-DR expression. In addition, exposure to Co^{2+} impaired phagocytosis of *S. aureus*. Both metal ions inhibit cellular activation of macrophages.

Paper II

GAS can adopt a hyper-virulent phenotype within the host. This is generally mediated by dysfunctional CovR/S. DCs are key components of the host's immune response. They present antigens and secrete cytokines to shape the T cell response. As knowledge on the DC-GAS interaction is scarce, this study aimed to investigate the cytokine response to hyper-virulent GAS variants. Therefore, moDCs were infected with different GAS variants, and cytokine profiling was performed:

i.   GAS harboring dysfunctional CovR/S suppress IL-8 and IL-18 secretion by human monocytic cells

ii.   DC maturation is not impaired by hyper-virulent GAS

iii.   Caspase-8 inhibition restores secretion of IL-8 and IL-18 in moDCs infected with hyper-virulent GAS

iv.   Infections of moDCs with 5448AP result in higher caspase-8 activity

v.   Dysfunctional CovR/S has no impact on systemic IL-18 in NSTI patients

vi.   Secretion of IL-1β and IL-18 by moDCs is increased in infections with a SLO knockout

These results show that infections with GAS strains harboring dysfunctional CovR/S result in abrogated secretion of IL-8 and IL-18 by monocytic cells. This suppression is mainly mediated by a caspase-8-dependent mechanism. While dysfunctional CovR/S had no impact on systemic IL-18 concentrations in NSTI patients, the effects on local tissue concentrations are still unknown and require further studies.

Paper III

The secreted streptococcal protease SpeB is highly conserved and well-studied virulence factor. SpeB retains a controversial role in pathogenesis and severity of GAS infections. In NSTI patient biopsies as well as *in vitro* experiments, it was shown that:

i.   The host environment induces transient loss of SpeB secretion

ii.   Reversible loss of SpeB proteolytic activity is associated with tissue pathology and inflammation

iii.   ROS derived from neutrophils drive the transient SpeB$^-$ phenotype

iv.   Presence of MPO is enhanced in tissue biopsies with increased SpeB$^-$ variants

v.   SpeB$^-$ variants induce higher neutrophil degranulation than SpeB$^+$ strains

Irreversible loss of SpeB secretion is commonly caused by mutation of *covR/S* or *ropB.* These results show that GAS can transiently abrogate SpeB secretion in response to neutrophil-derived ROS. SpeB$^-$ variants induce extensive neutrophil degranulation and replicate

intracellularly. Extensive neutrophil degranulation drives hyper-inflammatory conditions and tissue damage, both hallmarks of NSTIs. MPO inhibition partially restored secretion of SpeB and improved killing of GAS by neutrophils.

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8. APPENDIX

i. 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 selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Greifswald, 08.12.2023   ...……………………………

Lea Tölken

ii. Curriculum Vitae

Personal Details

Name:	Lea Alessandra Tölken
Date of birth:	February 08, 1996
Place of birth:	Bremerhaven

Education

02/2020 to present	**PhD student** at the Interfaculty Institute for Genetics and Functional Genome Research, Department of Molecular Genetics and Infection Biology; Research Group "Infectious Diseases"
10/2017 to 08/2020	Master of Science in Biochemistry, University of Greifswald
	Master Thesis: Group G streptococcal defense mechanisms in skin infections
08/2018 to 12/2018	Visiting student at the University of Manitoba, Winnipeg, MB; Canada
10/2014 to 11/2017	Bachelor of Science in Biochemistry, University of Greifswald
	Bachelor Thesis: Influence of fatty acids on the cardiolipin-composition and proliferation of human T cells
08/2006 to 07/2014	General university entrance qualification at the Gymnasium Wesermünde, Bremerhaven

Additional Skills

Languages	German (native), English (fluent in speaking and writing)
Certificates	Qualification for laboratory animal experiments acc. to FELASA category B

Greifswald, 08.12.2023   ...……………………………

                                                              Lea Tölken

iii.   Publications and conference contributions

a)   Papers and Manuscripts Not Included in this Thesis

Antje D. Paulikat, **Lea A. Tölken**, Lana H. Jachmann, Gerhard Burchhardt, Sven Hammerschmidt, Nikolai Siemens; *Streptococcus pneumoniae* Impairs Maturation of Human Dendritic Cells and Consequent Activation of CD4⁺ T Cells via Pneumolysin; J Innate Immun; 2 September 2022; 14 (5): 569–580. https://doi.org/10.1159/000522339

Fabian Cuypers, Alexander Schäfer, Sebastian B. Skorka, Surabhi Surabhi, **Lea A. Tölken,** Antje D. Paulikat, Thomas P. Kohler, Saskia A. Otto, Thomas C. Mettenleiter, Sven Hammerschmidt, Ulrike Blohm, Nikolai Siemens; Innate immune responses at the asymptomatic stage of influenza A viral infections of *Streptococcus pneumoniae* colonized and non-colonized mice.; Scientific Reports. 2021;11(1):20609. https://doi.org/10.1038/s41598-021-00211-y

Lea A. Tölken#, Antje D. Paulikat#, Fabian Cuypers, Sebastian B. Skorka, Sven Hammerschmidt, Nikolai Siemens; Cytokine Profiling in Influenza A Virus and Staphylococcal (Co-)Infections. Infectious Microbes & Diseases 4(4):p 161-167, December 2022. | DOI: 10.1097/IM9.0000000000000108. # equal contribution

Lea A. Tölken#, Janine V. Neufend#, Oddvar Oppegaard, Karen Methling, Kirsten Moll, Sylvio Redanz, Miriam M.D. Katsburg, Murtadha Q. Ali, Patience Shumba, Bernd Kreikemeyer, Steinar Skrede, Marcus Fulde, Anna Norrby-Teglund, Michael Lalk, Bård R. Kittang, Nikolai Siemens; Streptokinase reduces *Streptococcus dysgalactiae* subsp. *equisimilis* biofilm formation; submitted to BMC Microbiology; # equal contribution

b)   Conference Contributions

International Joint Meeting Infection Biology and Antibiotics 2022; oral presentation: Hyper-virulent *Streptococcus pyogenes* impairs interleukin-18 response in human immune cells of myeloid lineage

21st Lancefield International Symposium for Streptococci and Streptococcal Diseases 2022, Stockholm; poster presentation: Hyper-virulent *Streptococcus pyogenes* impairs interleukin-18 response in human immune cells of myeloid lineage

2nd Summer School on "Infection Biology, Greifswald; oral presentation: Hyper-virulent *Streptococcus pyogenes* impairs interleukin-18 response in human immune cells of myeloid lineage

5th German Pneumococcal & Streptococcal Symposium 2023, Hannover; oral presentation: Hyper-virulent *Streptococcus pyogenes* impairs interleukin-18 response in human immune cells of myeloid lineage via a caspase-mediated mechanism

iv.   Danksagung

Wie dem einen oder anderen aufgefallen sein könnte bin ich kein Freund vieler Worte. Mein Dank gilt allen, die mir in den letzten vier Jahren zur Seite gestanden haben! Besonders bei folgenden Leuten möchte ich mich von Herzen bedanken:

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Bei allen Mitarbeitern der Abteilung für molekulare Genetik und Infektionsbiologie für ihre Hilfsbereitschaft und Unterstützung während der letzten vier Jahre.

Bei meinen Mit-Doktoranden, die jederzeit ein offenes Ohr hatten und mir immer mit Rat und Tat zur Seite gestanden haben. Danke für Kaffeepausen, mehr oder weniger wissenschaftliche Diskussionen und einfach witzige Unterhaltungen, die meine Zeit hier unvergesslich gemacht haben.

Bei meiner Familie und meinen Freunden, die mir jederzeit zur Seite gestanden haben. Danke für eure immerwährende Geduld und eure bedingungslose Unterstützung während dieser Zeit.

<div align="center">Danke!</div>

PAPER I

Cobalt and Chromium Ions Impair Macrophage Response to *Staphylococcus aureus* Infection

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Author Contributions:

Conceptualization: **LAT**, NS, JS; Data curation: **LAT**, NS, JS; Formal analysis: **LAT**, NS, JS; Funding acquisition: NS, GIW, JS; Investigation: **LAT**, NS, JS, BH, TW, AR, DG; Methodology: NS, JS, BH, TW, DG; Project administration: NS, JS; Resources: GIW, NS, JS; Software: GIW, NS, JS; Supervision: NS, JS; Validation: **LAT**, GIW, AR, NS; JS; Visualization: **LAT**, NS, JS; Writing - original draft: **LAT**, NS, JS; Writing - review & editing: **LAT**, GIW, AR, NS, JS, TW, BH, DG.

..…………………………

Prof. Dr. Nikolai Siemens

..…………………………

Lea Tölken

Cobalt and chromium ions impair macrophage response to *Staphylococcus aureus* infection

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Abstract

Cobalt-chromium-molybdenum (CoCrMo) alloys are routinely used in arthroplasty. CoCrMo wear particles and ions derived from arthroplasty implants lead to macrophage-driven adverse local tissue reactions, which have been linked to an increased risk of periprosthetic joint infection after revision arthroplasty. While metal-induced cytotoxicity is well characterized in human macrophages, direct effects on their functionality have remained elusive. Synchrotron radiation x-ray microtomography and x-ray fluorescence mapping indicated that peri-implant tissues harvested during aseptic revision of different arthroplasty implants are exposed to Co and Cr in situ. Confocal laser scanning microscopy revealed that macrophage influx is predominant in patient tissue. While in vitro exposure to Cr^{3+} had only minor effects on monocytes/macrophage phenotype, pathologic concentrations of Co^{2+} significantly impaired both, monocytes/macrophage phenotype and functionality. High concentrations of Co^{2+} led to a shift in macrophage subsets and loss of surface markers including CD14 and CD16. Both, Co^{2+} and Cr^{3+} impaired macrophage responses to *Staphylococcus aureus* infection and particularly Co^{2+}-exposed macrophages showed decreased phagocytic activity. These findings demonstrate the immunosuppressive effects of locally elevated metal ions on the innate immune response and supports further investigations, including studies exploring whether Co^{2+} and Cr^{3+} or CoCrMo alloys per se expose the patients to a higher risk of infections post revision arthroplasty.

Keywords: macrophages, *Staphylococcus aureus*, arthroplasty, cobalt, chromium, periprosthetic joint infection

1. Introduction

Arthroplasty is a successful surgical procedure for the treatment of patients suffering from chronic pain due to degenerative joint disease. Complications that usually occur in the late postoperative course due to tissue inflammation caused by material degradation and release or infections lead to revisions of implants. Cobalt-chromium-molybdenum (CoCrMo) metal alloys are metallic biomaterials widely used in arthroplasty. Multiple studies have indicated adverse effects of wear particles and metal ions released from CoCrMo alloys. These adverse local tissue reactions (ALTRs), such as tissue necrosis and pseudotumor formation, are characterized by lymphocyte and macrophage infiltration [1]. Two specific types of necrosis occur in ALTRs, namely, (i) necrosis predominantly through death of macrophages and (ii) tissue necrosis characterized by cell death of stromal elements [2]. *In vitro* data indicate that macrophage necrosis and not apoptosis is the predominant type of cell death following exposure to high concentrations of Co^{2+} [3]. Widely accepted mechanisms posit that phagocytosis of arthroprosthetic metal particles activates the NLRP3 inflammasome and that the cellular damage caused by metal ions result in the release of proinflammatory cytokines, subsequently giving rise to the inflammatory responses observed in ALTRs [4-6]. The proinflammatory environment has been held responsible for the differentiation of macrophages to bone resorbing osteoclasts and their activation, hence for the onset of periprosthetic osteolysis [7]. In clinical context, exposure to Co^{2+} and Cr^{3+} has been associated with early implant revision and increased rates of secondary revision, due to aseptic implant loosening and/or periprosthetic joint infections (PJI) [8-13]. Factors such as metal-induced cytotoxicity as well as ALTRs are believed to create an environment favorable to bacterial infections [14-15].

Previous studies have shown that peri-implant tissues, including bone marrow, are exposed to Co- and Cr-containing particles [2, 16-18] and that these metals are present in the particulate as well

as in the dissociated state [19]. The comparison between *in vitro* corrosion and tribocorrosion showed that the dissolution of Co occurred primarily in tribocorrosive conditions, suggesting that the mechanism responsible for particle release affects their Co content [20]. *Ex vivo* analyses of the elemental composition of CoCrMo particles in periprosthetic bone marrow have revealed that Co content depends on their size [21]. The comparison of elemental ratios of CoCrMo-derived particles with the composition of the implanted bulk alloy showed that particle degradation results in the release of Co ions [21], reaching local pathologic concentrations exceeding 10 µg/mL [19]. Besides CoCr alloy particles, trivalent Cr salts and oxides have been shown to be the predominant state of Cr in periprosthetic tissue collected during revisions of metal-on-metal hip implants [18]. Notably, Co has only been found co-localized with Cr in the elemental states, indicating that dissociated Co is released during the wear process or due to dealloying by passive or cell mediated degradation processes [17-18, 21]. Consequently, dissociated Co and Cr can cross cellular barriers leading to systemic exposure [22-24].

Besides ALTRs, PJIs are devastating complications of arthroplasty, requiring revision surgery with partial or total exchange or explantation of the implant, debridement of necrotic and infected tissues, and effective antimicrobial treatment [25-28]. The incidence of PJIs is constantly increasing during the last decades. However, certain referral centers, particularly in Scandinavia, are reporting that the incidence appears to have levelled out after 2010 [14, 29-30]. *Staphylococcus aureus* is one of the major causes of bone and joint infections [31-32] including PJIs [33-35]. Being held responsible for up to 28% of PJIs, makes it one of the most prevalent pathogens in revision arthroplasty [36-38].

As part of the innate immune axis, macrophages are of crucial importance in initiating immediate response to highly inflammatory conditions and particularly infections. Tissue-resident macrophages are the first responders. They are involved in tissue-homeostasis, clearance of

cellular debris as well as pathogens, and metallic particles, among others. [39-40]. As a result of inflammation, the limited number of resident macrophages is depleted, circulating monocytes influx into the tissue and differentiate to macrophages [41]. High influx of macrophages is commonly detected in peri-implant tissue, especially in ALTRs, and macrophages often co-localize with metal particles [2]. Metal particles as well as ions have been shown to impact macrophage viability [42], migration [43], and activation [1]. However, the pathogen-macrophage interplay following metal exposure has remained unknown.

Thus, the aims of this study were to evaluate whether exposure to Co and Cr and subsequent macrophage influx is evident at the time of aseptic arthroplasty implant revision and to analyze the impact of Co and Cr ions on human primary monocytes/macrophages including markers associated with infections and the ability of these cells to respond to subsequent *S. aureus* infection. We show that macrophages change their phenotype post Co^{2+} exposure. More strikingly, Co^{2+} affect expression of various surface markers and impair overall macrophage response to *S. aureus* infection. In contrast, Cr^{3+} does not significantly affect monocyte/macrophage phenotype, but the response to infection.

2. Materials and Methods

2.1 Patient recruitment and sampling

The independent ethics committee (IEC) of the University Medicine Greifswald approved the study (BB 178/20 and BB 014/14) in accordance with the World Medical Association Declaration of Helsinki. All patients gave written informed consent to participate in this study. All experiments were carried out in accordance with the approved guidelines. Peri-implant soft tissue samples were collected during aseptic arthroplasty implant revisions. Buffy coats obtained from healthy blood donors were anonymously provided by the blood bank at the University Medicine Greifswald.

2.2 Synchrotron radiation x-ray computed tomography

Peri-implant soft tissue samples were fixed in phosphate buffered saline (PBS, BIO SELL) containing 4 % v/v formaldehyde (Carl Roth). Following overnight fixation, the specimens were placed in 2 ml screw-top tubes filled with PBS containing 100 U/mL penicillin and 1 µg/mL streptomycin (both Gibco). The specimens were scanned using Synchrotron radiation micro-computed tomography (SR-µCT) at the Anatomix beamline at the Synchrotron SOLEIL; the beamline and its instrumentation are described elsewhere [44-45]. In brief, the voxel size was 3.07 μm^3, the central energy of the filtered white X-ray beam was around 40 keV. Each scan involved a slight offset of the rotation axis in relation to the detector field of view, aiming to enlarge the diameter of the reconstructed volume. For each scan, between 3,000 and 4,000 projections were acquired, each with an exposure time of 50 ms per projection. For the reconstruction of tomographic data, the standard pipeline implemented at the beamline was used; it is based on the PyHST2 framework. A Paganin phase-retrieval filter with a kernel length ("Paganin length" in PyHST2) of 138 µm was applied to facilitate data analysis.

Further data analysis and visualization of the tomographic reconstructions was performed using the AVIZO software (Thermo Fisher Scientific). For the segmentation of metallic particles, the 3D images were thresholded selecting a value above the gray-values corresponding to mineralized tissue. Subsequently, only particles larger than 4 voxels were considered as metal particles.

2.3 Synchrotron radiation x-ray fluorescence mapping

Parts of the fixed specimens (see 2.2) were dehydrated for 24 h in each step by an ascending ethanol gradient (70%, 80%, 96%, and 100%). After completing dehydration by incubation with xylene (Th. Geyer) for 3 h, the specimens were embedded in paraffin and 10 µm sections were prepared. The sections were placed between two pieces of a 4 µm thin ultralene foil (Spex SamplePrep) and mounted on custom-made sample holders. Synchrotron radiation micro x-ray fluorescence (SR-µXRF) mapping was performed at the microXAS beamline of the Swiss Light Source (Paul Scherrer Institute). Monochromatic excitation radiation of 9.8 keV (above Zn K-edge) was selected using a double crystal monochromator equipped with Si(111) crystals. The primary beam was focused by two orthogonal reflective mirrors ('Kirkpatrick-Baez geometry') down to a spot size of 1 µm x 2 µm (h x v). XRF spectra were collected using silicon drift detectors systems. Chemical images were recorded in 'on-the-fly' mode, with a pixel size of 20 µm x 20 µm to obtain overviews for further selection of regions of interest to be scanned with a pixel size of 2 µm x 2 µm. XRF spectra obtained for each individual pixel were deconvoluted using the software PyMCA [46].

2.4 Histology, Immunostaining and laser scanning microscopy

The paraffin embedded tissue samples used for sectioning and subsequent SR-XRF analyses were additionally sectioned for histology (5 µm) and for immunostaining (10 µm). The sections were mounted on glass slides, dried overnight at 60 °C and dewaxed with xylene prior to

rehydration by ethanol gradient (100%, 96%, 80%, 70%) and water. The section adjacent to the section used for immunostaining were stained with hematoxylin and eosin (both Carl Roth) by the "progressive method" [47]. For immunostaining, antigen retrieval was performed by heating the slides in a microwave submersed in citrate buffer (pH 6.0, Sigma Aldrich) until boiling followed by 10 min at a sub-boiling temperature (95-98 °C). Tissue sections were permeabilized with 1% saponin in PBS for 30 min at room temperature. Sections were blocked using 2.5% fetal calf serum (Gibco) and 2.5% normal goat serum (Abcam) in PBS for 30 min at room temperature. Sections were stained overnight at 4 °C using titrated amounts of fluorophore-labeled antibodies. Sections were washed with PBS containing 1% saponin between each staining step. The following antibodies were used for immunofluorescence analysis (clone, fluorophore; all BioLegend): CD68 (Y1/82A, AlexaFlour647), CD45 /2D1, AlexaFlour700), CD14 (HCD14, AlexaFlour594) and HLA-DR (L243, AlexaFlour488). The staining was visualized using a Leica DMi8 CS Premium confocal laser scanning microscope (Leica).

2.5 Isolation of human monocytes and in vitro metal exposure

Human monocytes were isolated from buffy coats using CD14 S-pluriBead antihuman beads (PluriSelect) according to manufacturer's instructions. Cobalt (II) chloride hexahydrate and chromium (III) chloride hexahydrate (both Sigma Aldrich) were dissolved in human AB serum (PAN-Biotech) at a stock concentration of 10 mg×mL^{-1} Co^{2+} or 10 mg/mL Cr^{3+} and 10 mg×mL^{-1} Co^{2+} plus 10 mg×mL^{-1} Cr^{3+} and incubated overnight at 37 °C. Pre-incubation was performed to emulate in vivo formation of ion complexes of biomacromolecules. Isolated monocytes were exposed to metal salt solutions for 48 h at 37 °C and 5% CO$_2$ (final concentrations: 1 µg×mL^{-1} and 10 µg×mL^{-1} or of each in combination). Pure human AB serum served as control.

2.6 Bacterial strain

S. aureus strain 6005 [48] was grown overnight at 37 °C in casein hydrolysate and yeast extract medium with agitation [49]. To mimic initial low grade infections, a multiplicity of infection (MOI) of 1 was used.

2.7 Infection of monocytes/macrophages

Stimulated monocytes were infected with *S. aureus* 6005 at a MOI of 1 for 1 h. Extracellular bacteria were killed by addition of antibiotics (400 µg×mL^{-1} gentamicin/ 2 µg×mL^{-1} lysostaphin, both Sigma Aldrich). For flow cytometry, infected monocytes were incubated for an additional 23 h at 37 °C and 5% CO_2. For assessing intracellular bacterial counts, monocytes were infected as described above. One hour after addition of antibiotics, cells were washed, lysed, and intracellular bacteria were plated on blood agar plates (Oxoid).

2.8 Flow cytometry

Dead cells were stained using the Zombie Aqua Fixable Viability Kit (BioLegend) according to the manufacturer's instructions. Human TruStain FcX (BioLegend) was used according to the manufacturer's instructions to block unspecific binding of immunoglobulins. Extracellular markers were stained by incubating the cells with titrated amounts of monoclonal antibodies at 4 °C for 30 min. Cells were fixed and permeabilized using Cyto-Fast Fix/Perm Buffer Set (BioLegend). Intracellular markers were stained using titrated amounts of monoclonal antibodies at 4 °C for 30 min. Cells were washed between each staining step. Antibodies and clones directed against the following markers were used (clone, fluorophore; all BioLegend): CD14 (63D3, BV750 or HCD14, PE/Cy7), CD16 (B73.1, PE/Dazzle594), HLA-DR (LN3, FITC), CD38 (HB-7, PerCP), CD86 (BU63, PE/Cy7), PD-L1 (29E.2A3, APC) and CD68 (Y1/82A, BV421). The gating strategy is shown in the Supporting Information. (Figure S1). Data were acquired using a

FACSAriaIII flow cytometer and FACS DIVA 8.0 Software (both BD Biosciences) and analyzed using FCS Express 7 Software (De Novo Software).

2.8 Quantitative reverse transcription PCR analysis

Total RNA was isolated using RiboPure RNA purification Kit (Ambion) according to manufacturer's guidelines. cDNA synthesis was performed using the Superscript first-strand synthesis system for RT-PCR (Invitrogen). The real-time PCR amplification was performed with iTaq Universal SYBR Green Supermix kit (Biorad) using a StepOnePlus sequence detection system (Applied Biosystems). The levels of β-actin transcription (h_beta-actin_for: 5'-CTCTTCCAGCCTTCCTTCCT-3', h_beta-actin_rev: 5'-AGCACTGTGTTGGCGTACAG-3' (both Eurofins)) were used for normalization. The following commercially available primers were used (Name (Cat.#); all Qiagen): Hs_HLA_DRA_1_SG (QT00089383), Hs_CD68_1_SG (QT00037184), Hs_PPARG_1_SG (QT00029841), Hs_STAT1_1_SG (QT00074123), Hs_IRF5_1_SG (QT00092736).

2.10 Statistics

Statistical significance of differences was determined using the Kruskal-Wallis test with Dunn's multiple comparison posttest. Data plotting and exploratory analyses were performed using GraphPad Prism version 8. A *p* value below 0.05 was considered statistically significant. The analyses included all samples and data, and individual values are presented for all data points. The PyMCA software was used to perform RGB imaging, heatmap imaging, and peak spectra analyzes of the XRF data[46].

3. Results

3.1 Local metal exposure and macrophage influx in patient tissue

To evaluate whether patients are locally exposed to Co- and Cr-containing degradation products, peri-implant tissue samples were collected during aseptic revision surgery of hip, knee and ankle arthroplasty implants (n=3) (Figure 1A,B).

Histological analysis of the tissue samples revealed ALTR indicated by immune cell infiltration accompanied by numerous metal particles (Figure 1C). Spatially resolved multi-element mapping by SR-µXRF showed local exposure in all three cases analyzed, characterized by Co- and Cr-containing particles of heterogeneous elemental composition (Figure 1D). The highest Co and Cr signals were detected in peri-implant tissue collected during revision total hip arthroplasty, with minor Co content i.e. only few regions indicating co-localized Co and Cr, pointing towards local Co dissolution. XRF-spectra of the soft tissue sample, harvested during revision total knee arthroplasty of an exposed versus non-exposed volume indicated arthroprosthetic degradation products characterized by co-localized Co and Cr. The soft tissue sample harvested during revision ankle arthroplasty was characterized by bone residues indicated by high calcium levels. Co-localized Co and Cr were found to be abundant in soft tissue surrounding osteolytic bone.

Since elemental analyses using SR-µXRF are limited to only a small region of interest, parts of the tissue samples were additionally scanned using SR-µCT to evaluate whether metal debris is distributed throughout the tissue samples. These analyses indicated that exposure to particles of different sizes is evident (Figure 2). Segmentation of micron-sized particles (>116 µm^3) revealed a particle count of 842/250 mm^3, 1435/250 mm^3, and 104/250 mm^3 in peri-implant tissue collected during revision surgery of a hip, knee, and ankle arthroplasty implant, respectively.

Taken together, local Co and Cr exposure was evident at the time of revisions of standard hip, knee, and ankle arthroplasty implants and metal debris are distributed throughout the analyzed peri-implant tissue samples.

To confirm that infiltrating immune cells are macrophages, sections of nine patients (n=9), including the tree mentioned above, were immuno-stained (CD45 – leukocyte common antigen; CD68 – mainly expressed by macrophages; CD14 – mainly expressed by monocytic cells) and visualized via confocal laser scanning microscopy (CLSM). Highly auto-fluorescent metal particles were detected in tissues of all patients (Figure 3A). Areas with extensive macrophage infiltration were evident in seven out of nine patient tissues analyzed (Figure 3B). All macrophages stained positive for CD14 (Figure 3B). In many cases, macrophages and metal particles co-localized, indicating potential phagocytosis (Figure 3B, lower panel).

3.2 Exposure of monocytes to metal ions initiates macrophage differentiation with a metal-specific imprint

After proving that local exposure to Co and Cr and infiltration of macrophages at the time of aseptic revision of various arthroplasty implants is evident in peri-implant tissue, we next evaluated the effects of Co and Cr ion exposure on human monocytes/macrophages. To this end, primary monocytes were exposed to 1 or 10 $\mu g \times mL^{-1}$ of Co^{2+}, Cr^{3+} or both metal ions for 48 h and cell phenotype was analyzed via flow cytometry. In all conditions, monocytes differentiated to macrophages ($CD68^+HLA-DR^+$; Fig. S1). Although the viability of cells dropped following 48 h exposure to high concentrations of Co^{2+}, it did not reach statistical significance compared to non-exposed cells (Figure 4A). Subsequent analyses of surface-expressed CD14, a co-receptor of TLR4, allowed for discrimination of two subsets: $CD68^+CD14^+$ and $CD68^+CD14^-$ cells

(Figure 4B). Exposure of monocytes to 10 µg×mL^{-1} Co^{2+} alone or Co^{2+}/Cr^{3+} combination resulted in a shift from CD68$^+$CD14$^+$ to mainly CD68$^+$CD14$^-$ subset (Figure 4B). The subset distribution remained unchanged in all other conditions as compared to untreated cells (Figure 4B). Notably, this result was in contrast to the tissue analyses showing exclusively CD14$^+$ macrophages (Figure 3B). Therefore, the two subsets were further analyzed for presence of intracellular CD14. Irrespective of the prior analyses dividing macrophages in CD68$^+$CD14$^+$ and CD68$^+$CD14$^-$ populations, all cells stained positive for intracellular CD14 (Figures 4C and S2). Therefore, these two subsets are referred to as CD68$^+$CD14^{do+} (CD14 double positive) and CD68$^+$CD14^{in+} (intracellular CD14$^+$) in the following. However, both subsets were characterized by reduced expression of intracellular CD14 (Figure S2). Next, expression of CD16 (FCγRIII) was analyzed. Exposure to high concentrations of Co^{2+} resulted in an almost complete loss of CD16 on the cell surface (Figure 4D).

Next, stimulatory potential of metal ions towards macrophages was assessed. No activation of macrophages (CD38) in response to any condition was observed (Figure 5A). Irrespective of the macrophage subset, surface expression of HLA-DR was significantly decreased in response to high Co^{2+} concentrations, including co-exposure to Co^{2+}/Cr^{3+} (Figure 5B). In contrast, PD-L1 expression remained unaffected in all conditions (Figure 5C).

3.3 Cobalt and chromium ions inhibit macrophage response to *Staphylococcus aureus* infections

Next, the responsiveness of metal exposed macrophages towards subsequent *S. aureus* infections was assessed. Since *S. aureus* is a common cause of PJIs, a MSSA strain 6005 was used. In brief, human primary monocytes were exposed to metal ions for 24 h, subsequently infected with 6005 for 1 h, extracellular bacteria were killed, and macrophage responsiveness was analyzed

after 23 additional hours of infection. To match the peri-implant tissue environment, metal exposure continued through the entire series of infections.

First, phagocytic activity was analyzed by plating intracellular bacteria. Macrophages exposed to 10 $\mu g \times mL^{-1}$ Co^{2+} alone or in combination with Cr^{3+} showed significantly reduced bacterial phagocytosis (Figure 6A). To exclude potential metal ion mediated bactericidal effects, 6005 strain was directly exposed to 10 $\mu g \times mL^{-1}$ of metal ions. No effects were observed (Figure S3).

Subsequently, macrophage phenotype was analyzed in response to infections. Overall, no additional *S. aureus*-mediated cytotoxicity towards macrophages was noted (Figure 6B). Furthermore, the subsets distribution ($CD68^{+}CD14^{do+}$ and $CD68^{+}CD14^{in+}$) followed the previously observed metal-mediated phenotype and remained unaffected in all infectious conditions (Figure 6C). Irrespective of infections, general exposure of cells to high concentrations of Co^{2+} resulted in almost complete loss of CD16 on the cell surface. Of note, infected macrophages exposed to low concentrations of Co^{2+} showed comparable reduction of CD16 expression (Figure 6D). A similar trend was observed in infections of macrophages with prior Cr^{3+} treatment (Figure 6D).

In addition, the activation of macrophages was analyzed. While expansion of $CD38^{+}$ cells was noted in response to infections of naïve cells, all metal ions completely suppressed this activation, irrespective of the type or concentration used (Figure 7A). Overall, no upregulation of HLA-DR expression in response to infection was detected (Figure 7B). In contrast, infection of macrophages led to an upregulation of PD-L1 expression in almost all conditions (Figure 7C). This effect was diminished in infected macrophages which were exposed to 10 $\mu g \times mL^{-1}$ of Co^{2+} alone or in combination with Cr^{3+} (Figure 6c).

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Next, gene expression of different transcription factors and surface markers involved in macrophage activation in response to infection was analyzed. Since mostly single stimulations with high metal ion concentrations, and particularly Co^{2+}, had pronounced effects on macrophage responses, only 10 µg×mL^{-1} of Co^{2+} or Cr^{3+} prior to infection were used. *CD68* mRNA expression, indicative of monocytes to macrophages differentiation, was confirmed in all conditions (Figure S4A). The pro-inflammatory transcriptional factor *STAT1* was upregulated in *S. aureus* infections, while expression of anti-inflammatory *PPARγ* was reduced. In contrast, metal-exposed macrophages expressed similar levels of both transcriptional factors as compared to the unstimulated controls (Figure S4B-C). *IRF5* expression was unaffected in all conditions tested (Figure S4D). In congruence with the flow cytometry analyses, *HLA-DR* mRNA levels were reduced particularly in response to Co^{2+} stimulations irrespective of infections (Figure S4E).

4. Discussion

Metal particles and ions derived from orthopedic implants containing CoCrMo can cause ALTRs [2, 50]. Tissue necrosis and influx of lymphocytes and macrophages are regularly observed in neoplastic tissue specimens from patients undergoing aseptic arthroplasty implant revision. Macrophages are crucial first-responders to infection and arthroprosthetic particles. They clear pathogens and debris and recruit additional immune cells through release of chemoattractants. Metal particles and ions were shown to significantly affect macrophage viability [51]. Here, we show that macrophages infiltrate peri-implant tissue of Co/Cr-exposed patients and change their phenotype post Co^{2+} exposure. More strikingly, Co^{2+} affect expression of various infection-associated surface markers and impair overall macrophage response to *S. aureus* infection. In contrast, Cr^{3+} does not significantly affect macrophage phenotype, but the response to infection. The SR-μXRF and SR-μCT analyses presented here clearly demonstrate that peri-implant tissues, obtained at the time of revision of different implants, are exposed to Co and Cr. In earlier studies, we demonstrated that Co and Cr in peri-implant tissue and fluid obtained during hip arthroplasty implant revisions are present in the particulate and also in the dissociated state [19, 52], with concentrations of up to 25 μg×mL^{-1} of Cr and 7 μg×mL^{-1} of Co in periprosthetic tissue and 9 μg×mL^{-1} of Cr and 15 μg×mL^{-1} of Co in synovial fluid. These data and other studies emphasize not only that the concentrations used for cell stimulations in the present study emulate a clinically relevant exposure scenario but also that Co and Cr released from arthroplasty implants are characterized by a multitude of physicochemical properties [16-18, 21, 53], resulting in an interplay of biologically active metal ions and immunogenic particles [16, 54-55]. Dense metal aggregation commonly results in tissue inflammation characterized by influx of macrophages and lymphocytes [2]. Detailed CLSM analyses of patient tissue identified mainly macrophages (CD45$^+$CD68$^+$ cells),

which often co-localized with metal particles. Subsequently, we determined the phenotype and functionality of macrophages in a series of *in vitro* experiments using human primary cells. Most studies focus on cytotoxicity of Co and Cr ions and particles towards different cell types, *e.g.* fibroblasts, lymphocytes and different monocytic cell lines [1]. Especially Co was shown to be cytotoxic and necrosis was identified as a predominant type of cell death [42, 56]. While Co^{2+} induced cytolytic events in THP1 macrophages, Cr^{3+} did not [51]. This is in line with our results on human primary monocytes/macrophages showing that high concentrations of Co^{2+} are cytotoxic and Cr^{3+} are not. Characterization of macrophages revealed a substantial shift of subsets from $CD68^+CD14^{do+}$ to $CD68^+CD14^{in+}$ in response to high Co^{2+} concentrations. The later completely lacked the surface deposition of CD14.

Macrophages phagocytose metal particles. The acidic pH in the phagolysosome leads to release of Co^{2+} from Co particles, due to increased solubility of this element at low pH [57]. Consequently, Co^{2+} induce oxidative stress and reactive oxygen species (ROS) production in macrophages [51, 58]. Excessive ROS production is in turn linked to impaired macrophage migration [43]. Although speculative, Co^{2+}-mediated stress might impair intracellular protein transport from the ER to Golgi apparatus, leading to a loss of surface CD14 as well as other markers, including the observed lack of CD16 in our experimental setup, as intracellular stores are not transported to the cell membrane anymore [59].

Studies have shown that THP1 and U937 monocytic cell lines upregulate transcription of genes encoding for cytokines/chemokines (*IL1B*, *CXCL8*) [51], or release cytokines (IL-6, TNFα, IFN-γ) [60] in response to metal exposure. Consequently, this phenomenon has been referred to as "activation". In contrast to those two studies, we did not observe activation of macrophages in response to metal ions, since none of the key cellular markers analyzed (CD38, HLA-DR, PD-L1)

were upregulated. Even down-regulation of HLA-DR in response to high Co^{2+} concentrations was noted, which was confirmed on mRNA level. This immunosuppression is further confirmed in infection experiments under constant metal ion exposure. Co^{2+} and Cr^{3+} severely impaired the cellular response to infection. While *S. aureus* infection of naïve cells resulted in expansion of $CD38^+$ cells, all metal conditions suppressed this effect. In addition, no upregulation of HLA-DR was observed and PD-L1 up-regulation was exclusively impaired when high concentrations of Co^{2+} were used. PD-L1 is a checkpoint inhibitor that binds to PD-1 on T cells. Interaction of these molecules controls T cell activation by preventing excessive proliferation [40]. Our surface markers data is further supported by mRNA expression analyses of transcriptional factors. STAT1, a pro-inflammatory transcriptional regulator, expression was exclusively upregulated in *S. aureus* infections without prior metal exposure, while PPARγ, which is rather suppressed under infectious conditions, was down-regulated. In contrast, mRNA expression of these regulators in metal exposed cells remained at the levels of unstimulated controls. Therefore, our data support the concept that local exposure to Co^{2+} and Cr^{3+} in human peri-implant tissues suppresses critical immune functions of professional phagocytes and therefore predisposes patients to an increased risk of peri-implant infections, as seen in patients with ALTR [13, 15, 25-27]. This hypothesis is further supported by our observation that high concentrations of Co^{2+} resulted in lack of CD16 deposition on macrophage surface. CD16 is a FcγIII receptor that facilitates phagocytosis of bacteria [61]. Both, lack of surface associated CD14 as well as CD16 potentially explain the observed reduced bacterial phagocytosis. Of note, *S. aureus* growth itself was not affected by the metal concentrations, suggesting that it might form biofilms in PJIs [34-35, 62-64]. PJIs occur in up to 4% of revision arthroplasties [25]. It was reported that the presence of a foreign body and local metal release reduce the infectivity dose of *S. aureus* [65]. Furthermore, a limited number of animal studies revealed

reduced phagocytic and bactericidal activities of tissue infiltrating granulocytes induced by non-phagocytosable foreign bodies, while circulating neutrophils retained the activities [66-67]. Our *in vitro* data support these findings and underline the need for additional investigations analyzing *ex vivo* metal-mediated local immunosuppressive effects and their consequences for potential local infections. In addition, further prospective clinical studies are warranted.

5. Conclusions

In conclusion, our results provide evidence that exposure to Co- and Cr-ions inhibits essential immune functions of professional phagocytes. This finding potentially explains the increased risk of PJIs following revision arthroplasty. CoCrMo is a metallic biomaterial constantly used in orthopedic surgery. Degradation of this applied metallic biomaterial leading to systemic and local metal exposure and ALTR is a clinically well-known scenario. Based on our data, a risk assessment for PJI, especially for infections with *S. aureus*, after the use of CoCrMo-containing arthroplasty implants in prospectively designed studies seems indicated. In addition, *in vitro* studies on Co and Cr in the particulate state and *ex vivo* studies on the classical pro- and anti-inflammatory phenotype as well as functionality of macrophages locally exposed to metal ions and particles should be performed. Our data support a better understanding of the local tissue response after material degradation and add weight to the risk and benefit evaluation of CoCrMo routinely used in arthroplasty. Possible long-term effects of metal-based biomaterial degradation including the risk of infection should be considered.
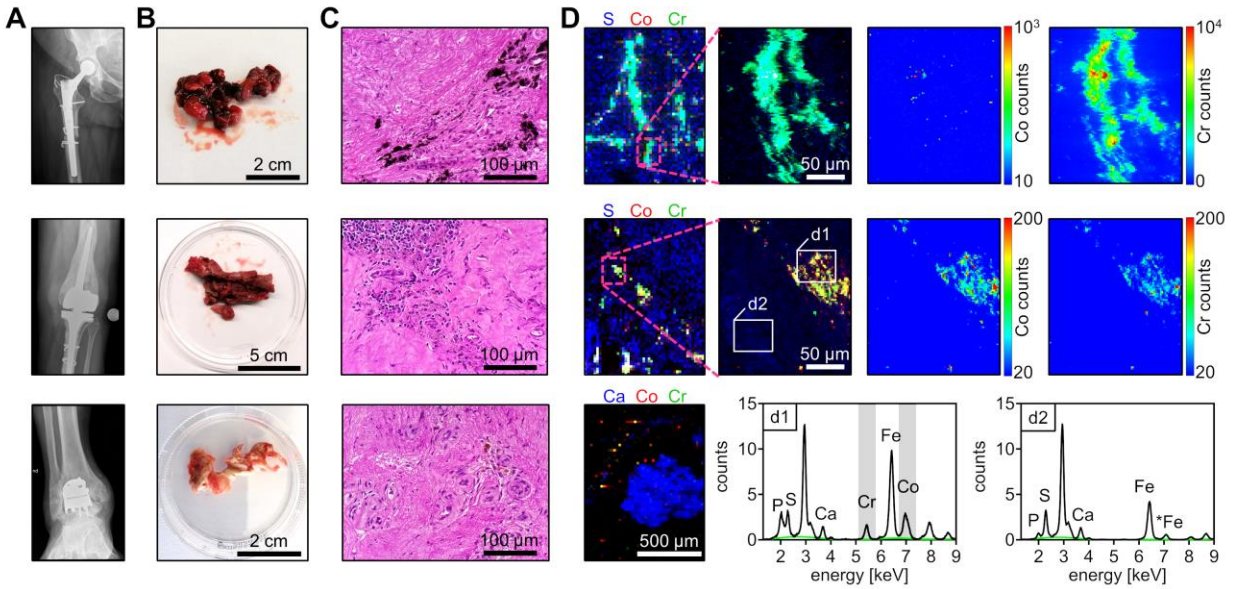
Figure 1. Histological evaluation and multi-element detection of peri-implant tissue collected from three patients undergoing arthroplasty implant revision. The respective samples, tissue stains and XRF maps are arranged horizontally. (A) From top to bottom: Anteroposterior radiographs taken before revision of hip, knee and ankle arthroplasty implants. (B) Peri-implant soft tissue samples. (C) Hematoxylin and eosin staining of tissue sections. (D) RGB imaging, heatmaps of the Co and Cr signals, and spectra of XRF maps of tissue sections at 20 µm steps (left) and 2 µm steps (right). RGB images (2 µm steps) depict the same region depicted by heatmaps.
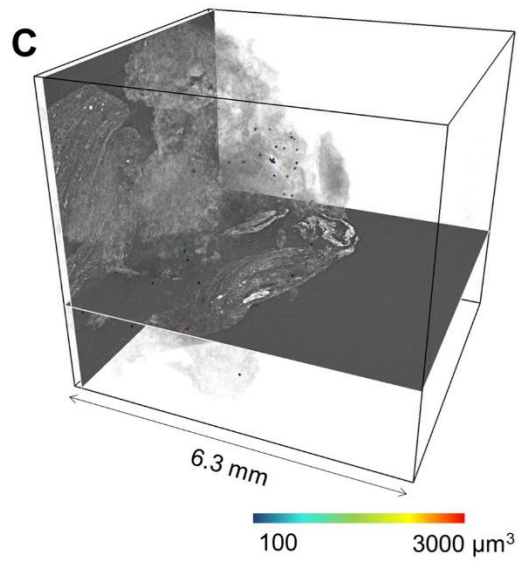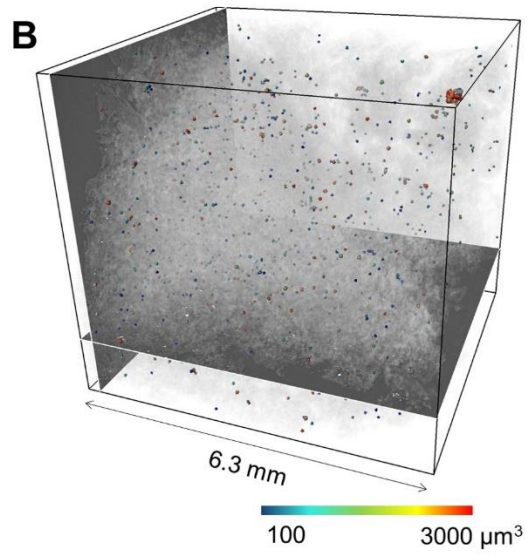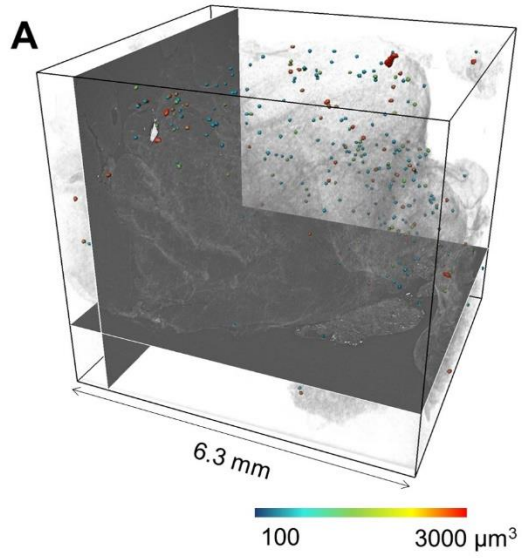
Figure 2. Renderings of SR-µCT data and segmentation of micron-sized debris in peri-implant tissue samples collected during revision surgery of a hip arthroplasty implant (A), knee arthroplasty implant (B) and ankle arthroplasty implant (C). The colors of the debris particles and the color bars indicate the volume of each particle. Please note that for the visualization, the particles have been dilated using a 3D spherical structuring element of 5 voxels in diameter to enhance the visibility of the particle distribution. The gray transparent cloud-like features are soft-tissue structures.
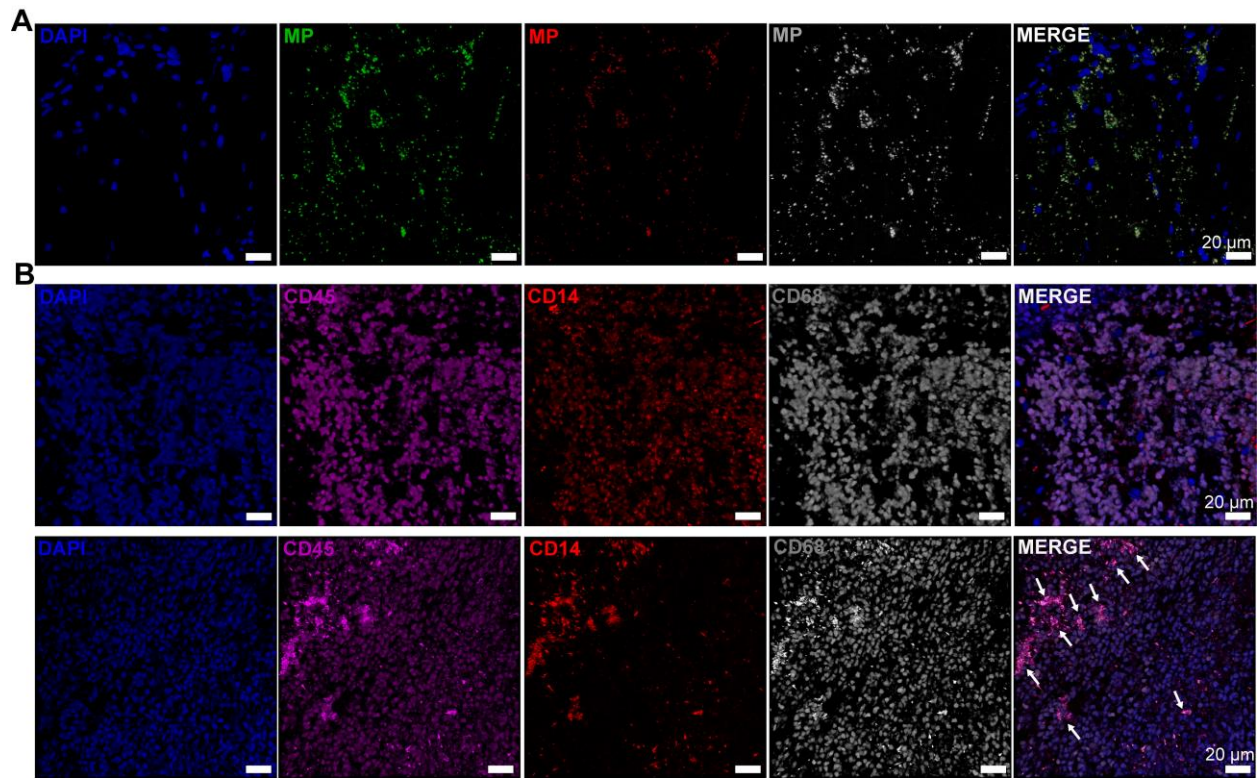
Figure 3. Macrophage infiltration in peri-implant tissue visualized by positive CD45/CD68/CD14 staining and CLSM. (A) Representative reconstruction of CLSM micrographs visualizing highly auto-fluorescent metal particles (MP) and (B) macrophages (n=9). Arrows indicate metal particles associated with macrophages.
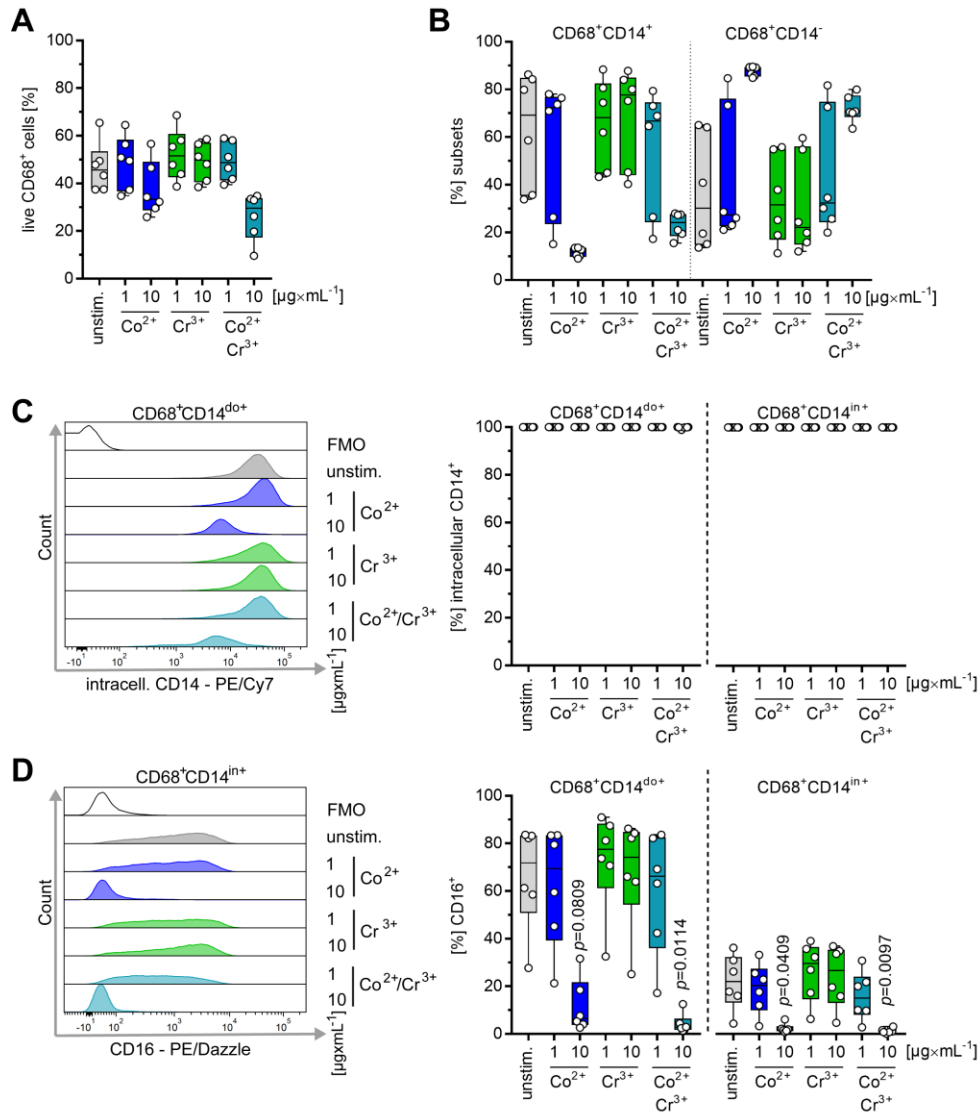
Figure 4. Metal-induced imprint of macrophage populations. Human primary monocytes were exposed to 1 or 10 $\mu g \times mL^{-1}$ of Co^{2+}, Cr^{3+} alone or in combination for 48 h. Macrophage viability (A) and subsets (B-D) were analyzed via flow cytometry (n=6). Representative histograms are shown in C and D (left panels). (B) Macrophage subsets were evaluated based on intracellular CD68 and extracellular CD14 positivity. (C) Detailed assessment of the subsets was performed based on frequencies of cells, which stained positive for intracellular CD14 ($CD14^+$) and (D) extracellular CD16 ($CD16^+$). The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor. The level of significance was determined using Kruskal-Wallis test with Dunn's posttest. FMO, fluorescence minus one; unstim., unstimulated control.
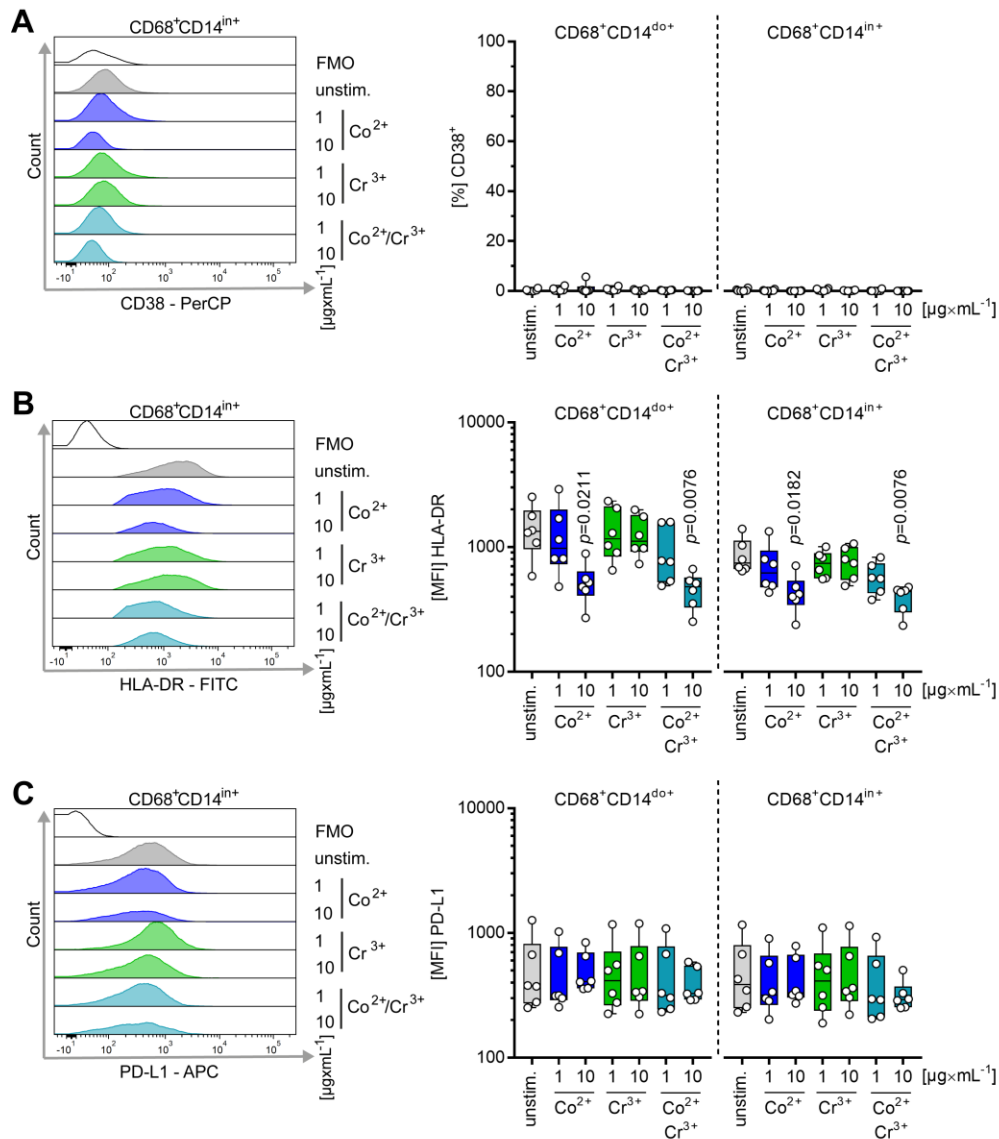
Figure 5. Metal-exposure decreases surface-expression of HLA-DR on macrophages. Human primary monocytes were exposed to 1 or 10 μg×mL^{-1} of Co^{2+}, Cr^{3+} alone or in combination for 48 h and macrophage subsets (A-C) was analyzed via flow cytometry (n=6). Representative histograms are shown in A-C (left panels). The macrophage response to infection and metal exposure was evaluated based on frequencies of CD38$^+$ (A) as well as expression of HLA-DR (B) and PD-L1 (C). The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor. The level of significance was determined using Kruskal-Wallis test with Dunn's posttest. FMO, fluorescence minus one; unstim., unstimulated control.
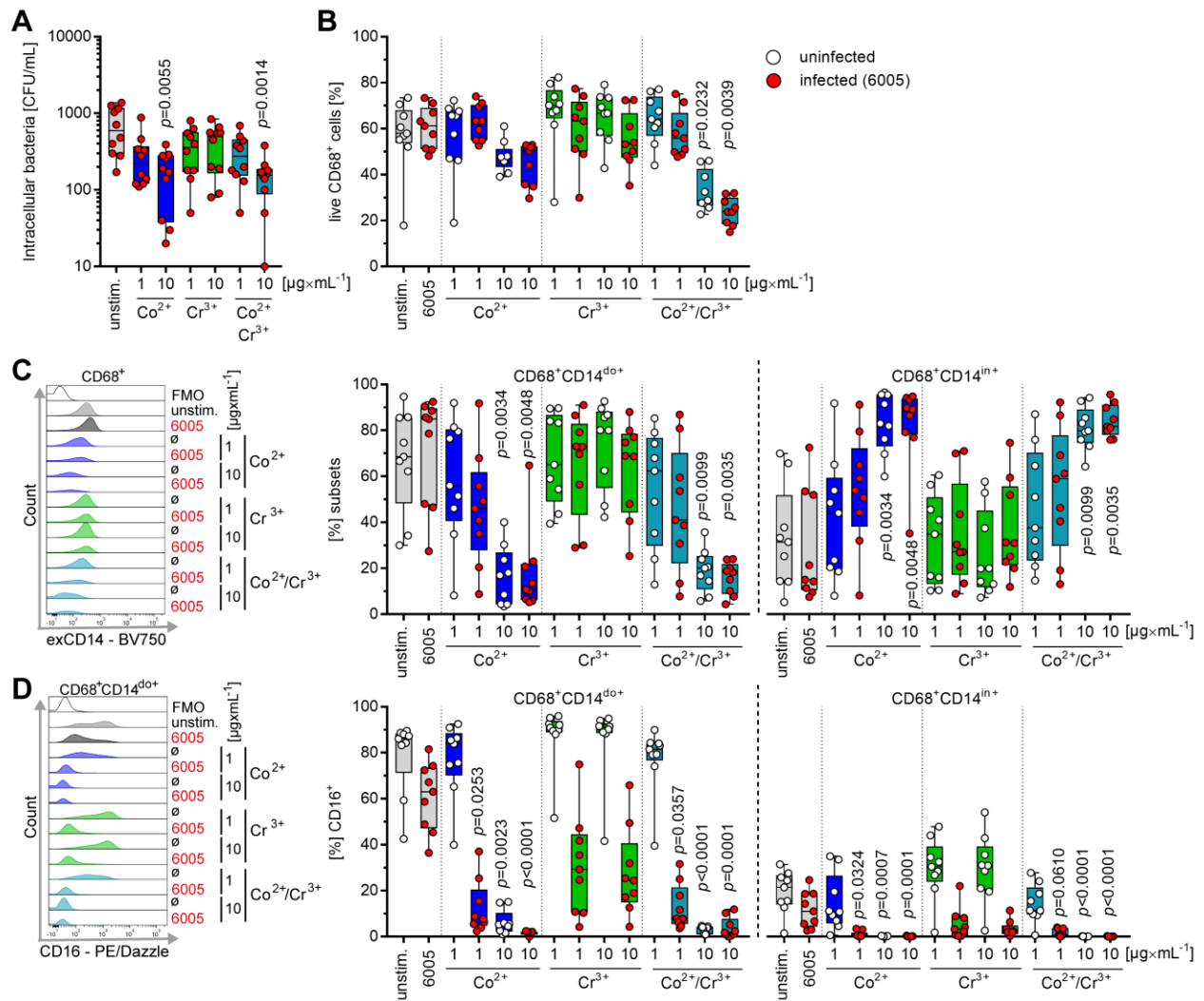
Figure 6. Reduced phagocytosis of bacteria in Co^{2+}-exposed cells. Human macrophages were exposed to 1 or 10 µg×mL^{-1} of Co^{2+} or Cr^{3+} alone or in combination for 24 h. Metal-exposed cells were infected with *S. aureus* 6005 (MOI 1). Extracellular bacteria were killed by substituting the media with antibiotics. Viable intracellular bacteria were determined 2 h post infection (A). Macrophage viability (B) and subsets (C-D) were analyzed via flow cytometry after a total of 48 h of metal treatment with 24 h of infection (n=10). Representative histograms are shown in left panels of C and D. (C) Analyses of macrophage subsets in response to infections. (D) Frequencies of CD16 positive cells in response to infections. The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor. The level of significance was determined using Kruskal-Wallis test with Dunn's posttest. FMO, fluorescence minus one; unstim., unstimulated control.
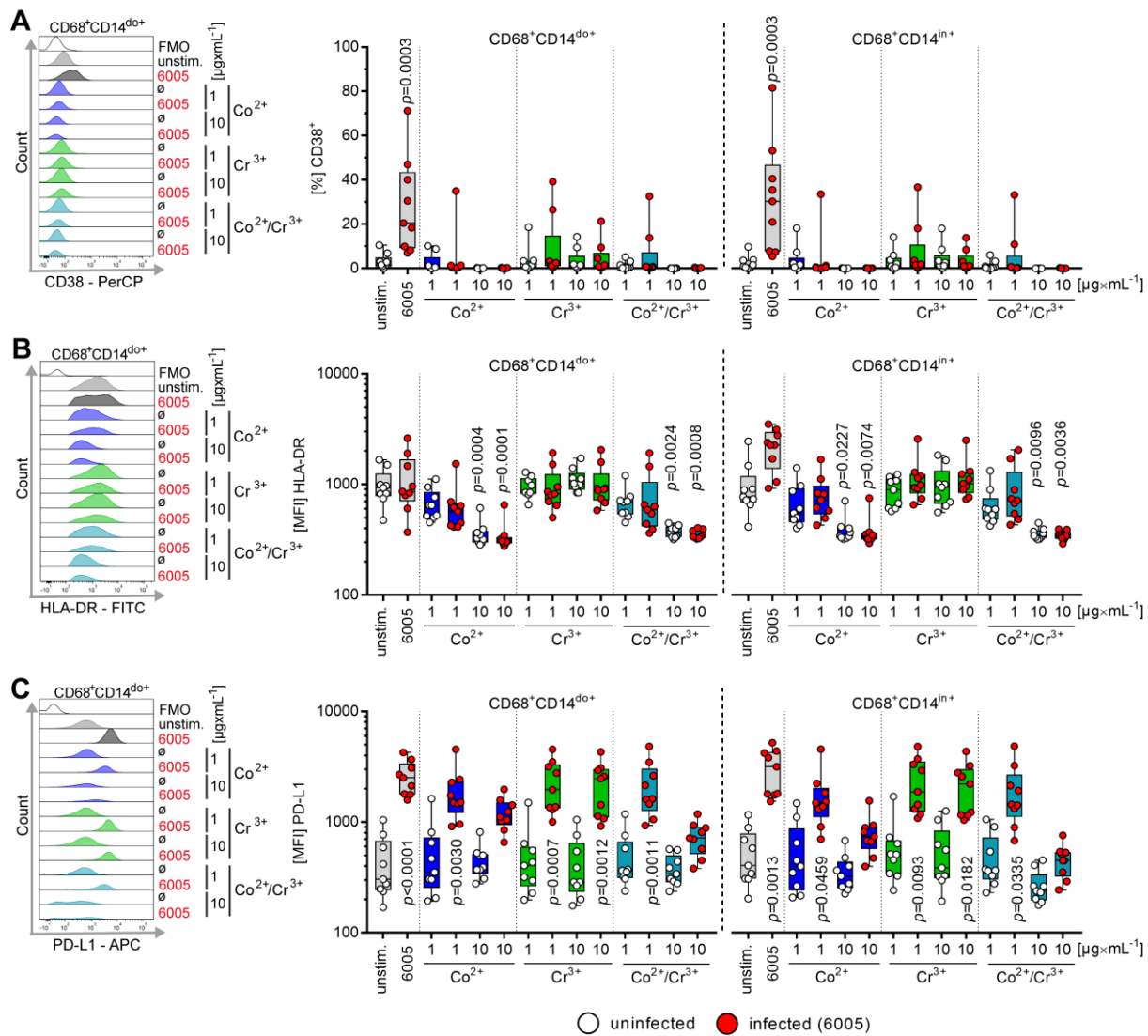
Figure 7. Metal-exposed cells are less responsive to *S. aureus* infections. Human monocytes were exposed to 1 or 10 µg×mL^{-1} of Co^{2+} or Cr^{3+} alone or in combination for 24 h and metal-exposed cells were infected with *S. aureus* 6005 (MOI 1). Macrophage phenotype (A-C) was analyzed via flow cytometry after a total of 48 h of metal treatment, which included 24 h of infection (n=10). Representative histograms are shown in left panels of A-C. The phenotype was evaluated based on frequencies of CD38$^+$ (A) as well as expression of HLA-DR (B) and PD-L1 (C) on macrophage surface. The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor. The level of significance was determined using Kruskal-Wallis test with Dunn's posttest. FMO, fluorescence minus one; unstim., unstimulated control.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge:

Extended results: Figure S1. Gating strategy used to identify human monocytes/macrophages; Figure S2. Intracellular CD14 expression in macrophages; Figure S3. The impact of metal ions on *Staphylococcus aureus* vitality; Figure S4. Gene expression analyses in macrophages.

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Conceptualization: LAT, NS, JS; Data curation: LAT, NS, JS; Formal analysis: LAT, NS, JS; Funding acquisition: NS, GIW, JS; Investigation: LAT, NS, JS, BH, TW, AR, DG.; Methodology: NS, JS, BH, TW, DG; Project administration: NS, JS; Resources: GIW, NS, JS; Software: GIW, NS, JS; Supervision: NS, JS; Validation: LAT, GIW, AR, NS; JS; Visualization: LAT, NS, JS; Writing - original draft: LAT, NS, JS; Writing - review & editing: LAT, GIW, AR, NS, JS, TW, BH, DG.

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Notes

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ABBREVIATIONS

ALTR, adverse local tissue reaction; CLSM, confocal laser scanning microscopy; CoCrMo, Cobalt-chromium-molybdenum; FMO, fluorescence minus one; PBS, phosphate buffered saline, PJI, periprosthetic joint infection; SR-µCT, synchrotron radiation micro-computed tomography; SR-µXRF, synchrotron radiation micro x-ray fluorescence analyses

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Cobalt and chromium ions impair macrophage response to *Staphylococcus aureus* infection

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Supporting Information

Cobalt and chromium ions impair macrophage response to *Staphylococcus aureus* infection

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Figure S1. Gating strategy used to identify human monocytes/macrophages. Doublets were excluded by consecutive gating of FSC-H/FSC-W and SSC-H/SSC-W. Dead cells were excluded by using the Zombie Aqua™ Fixable Viability Kit. Monocytes/macrophages were selected based on the expression of HLA-DR and CD68. Macrophage subsets were further distinguished based on expression of intracellular and extracellular CD14.
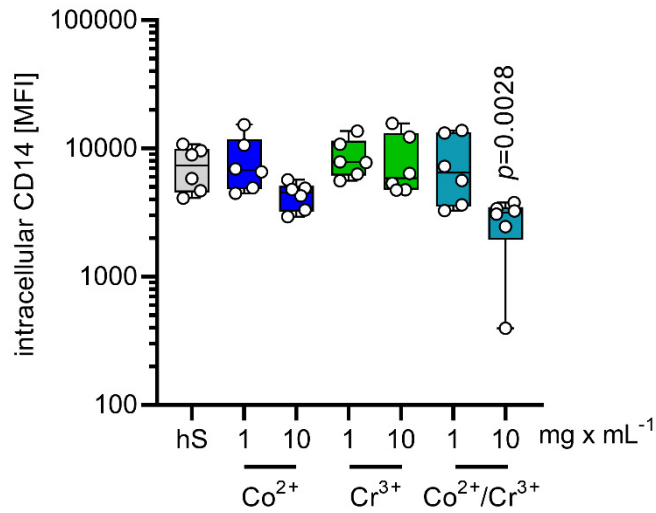
Figure S2. Intracellular CD14 expression in macrophages. Human primary monocytes were exposed to 1 or 10 µg×mL^{-1} of Co^{2+}, Cr^{3+} alone or in combination for 48 h and expression of intracellular CD14 was analyzed via flow cytometry (n=6). The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor. The level of significance was determined using Kruskal-Wallis test with Dunn's posttest. FMO, fluorescence minus one; unstim., unstimulated control.
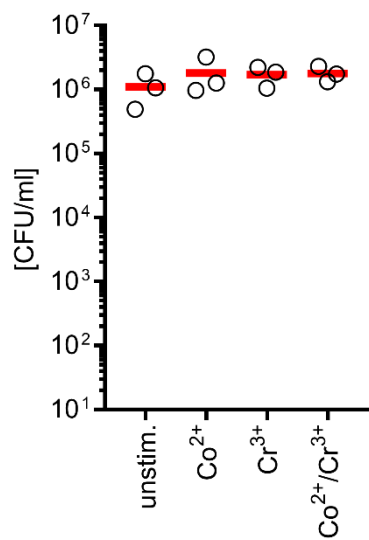
Figure S3. The impact of 10 µg×mL^{-1} of indicated metal ions on *Staphylococcus aureus* vitality. In short, 1x10^6 bacteria were incubated with 10 µg×mL^{-1} of indicated metal ions for 1 h at 37°C. Viable bacteria were plated on blood agar plates (n=3). unstim., unstimulated control.
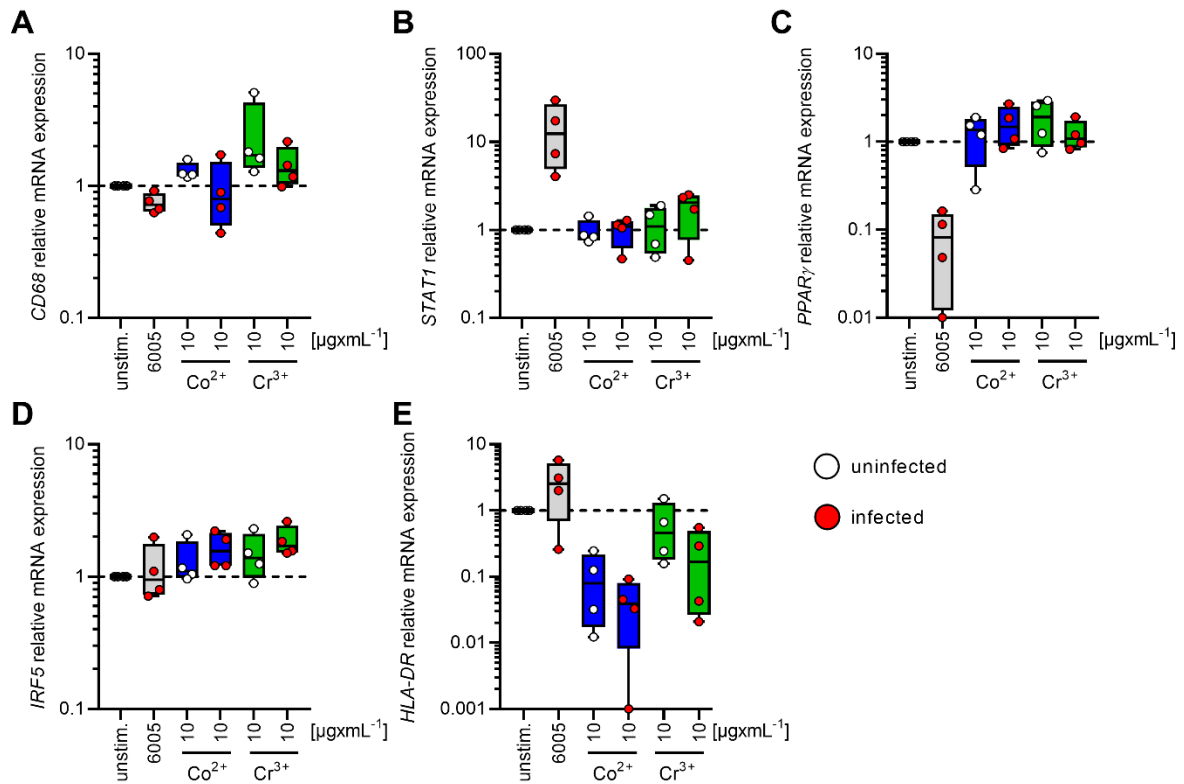
Figure S4. Gene expression analyses in macrophages. Human primary monocytes were exposed to 10 µg×mL^{-1} of Co^{2+} or Cr^{3+} for 48 h. Metal-exposed cells were infected with *S. aureus* 6005 (MOI 1). Extracellular bacteria were killed by substituting the media with antibiotics. Gene expression of genes encoding for *CD68* (A), *STAT1* (B), *PPARγ* (C), *IRF5* (D), and *HLA-DR* (E) are shown. The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor (n=4). unstim., unstimulated control.

Reduced Interleukin-18 Secretion by Human Monocytic Cells in Response to Infections with Hyper-virulent *Streptococcus pyogenes*

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Abstract

Background *Streptococcus pyogenes* (group A streptococcus, GAS) causes a variety of diseases ranging from mild superficial infections of the throat and skin to severe invasive infections, such as necrotizing soft tissue infections (NSTIs). Tissue passage of GAS often results in mutations within the genes encoding for control of virulence (Cov)R/S two component system leading to a hyper-virulent phenotype. Dendritic cells (DCs) are innate immune sentinels specialized in antigen uptake and subsequent T cell priming. This study aimed to analyze cytokine release by DCs in response to wild-type and natural *covR/S* mutant infections.

Methods Human primary monocytes, macrophages, and monocyte-derived (mo)DCs were used. DC maturation and release of pro-inflammatory cytokines in response to infections with wild-type and *covR/S* mutants were assessed via flow cytometry.

Results *In vitro* infections of moDCs and other monocytic cells with natural GAS *covR/S* mutants resulted in reduced secretion of IL-8 and IL-18 as compared to wild-type infections. In contrast, moDC maturation remained unaffected. Inhibition of caspase-8 restored secretion of both molecules. Knock-out of streptolysin O in GAS strain with unaffected CovR/S even further elevated the IL-18 secretion by moDCs. Of 67 fully sequenced NSTI GAS isolates, 28 harbored mutations resulting in dysfunctional CovR/S. However, analyses of plasma IL-8 and Il-18 levels did not correlate with presence or absence of such mutations.

Conclusions Our data demonstrate that strains, which harbor *covR/S* mutations interfere with IL-18 response in monocytic cells by utilizing the caspase-8 axis. Future experiments aim to identify the underlying mechanism and consequences for NSTI patients.

Keywords: *Streptococcus pyogenes*, dendritic cells, interleukin-18, CovR/S, necrotizing soft tissue infection

Background

Streptococcus pyogenes (group A streptococcus, GAS) causes a variety of human diseases ranging from mild superficial to severe invasive infections, including necrotizing soft tissue infections (NSTIs). Globally, invasive GAS infections are estimated to cause approx. 500,000 deaths annually [12]. NSTIs encompass necrosis of any layer of the skin and soft tissue compartment and are often associated with systemic toxicity. Despite prompt antibiotic treatment, intensive care, and extensive surgical intervention [2], the morbidity and mortality of such infections remain substantial [39,56]. While microbial etiologies of NSTIs are diverse, GAS are among the main causative pathogens of monomicrobial NSTIs [10,39]. In a recent Scandinavian prospective multicenter study INFECT, 31% of surgically confirmed NSTI cases were caused by GAS [11,39].

In the host, GAS can adopt a hyper-virulent phenotype, often mediated by inactivating mutations within genes encoding for the two component system (TCS) control of virulence (Cov)R/S [17,20,57,58,65]. Strains possessing covR/S mutations are frequently isolated from patients suffering from invasive GAS infections [5,26,39,60]. In mouse studies, GAS hyper-virulence is characterized by induction of excessive inflammation through increased superantigenic activity [6]. However, immunosuppressive effects, including impaired neutrophil responses, are also reported [62]. The CovR/S system consists of the histidine kinase CovS and the response regulator CovR. CovS senses environmental signals including pH acidification [18,25]. Upon activation, CovS phosphorylates CovR at D53. Phosphorylated CovR binds DNA and activates or represses transcription of several target genes [18,25]. CovR/S regulates up to 15% of the GAS genome and is a transcriptional suppressor of several key virulence factors, including the hyaluronic acid capsule, M protein, and cholesterol-dependent cytolysin streptolysin O (SLO). Consequently, inactivating mutations in covR/S result in higher expression of these virulence factors, among others [14,57].

Dendritic cells (DCs) are sentinel leukocytes and proficient antigen presenting cells (APCs). They bridge the innate and adaptive arms of the immune system by presenting processed antigens to T cells [54]. After ingestion of pathogens, DCs mature, migrate to the secondary lymph nodes, and present antigens to T cells via major histocompatibility complex (MHC) class II molecules [55]. In addition, DCs express co-stimulatory molecules and secrete cytokines to shape the direction of T cell priming and differentiation [41]. Only limited data exists on DC-GAS interactions. In murine models, it was shown that DCs are essential to limit GAS spread to the lymph nodes [38]. Furthermore, in vitro studies have

3

70 demonstrated that GAS inhibit DC maturation in a capsule- and/or SLO-dependent manner and induce

71 significant cell death [15,35]. In addition, the streptococcal DNase Sda1 was found to impair

72 plasmacytoid dendritic cell recruitment by reducing IFN-1 levels at the tissue site of infection [34].

73 DCs and other myeloid cells of monocytic lineage are major sources of cytokine/chemokine production

74 for an appropriate response to infections. In addition to interleukin (IL)-8, a potent neutrophil

75 chemoattractant, DCs secrete IL-1β and IL-18. Both IL-1-family cytokines are stored intracellularly as

76 inactive precursors. Processing and release occur upon activation of the NLRP3 inflammasome. The

77 associated active executioner caspase-1 cleaves the precursors into the respective mature cytokines.

78 Subsequently, the release is mediated through caspase-1 processed gasdermin D pores [23]. The

79 secreted GAS cysteine protease SpeB was shown to cleave pro-IL-1β and pro-IL-18 into their respective

80 mature forms [28,36]. Both cytokines have strong pro-inflammatory effects and play crucial roles in

81 invasive GAS infections. IL-1β network was identified as a key network involved in susceptibility to GAS

82 NSTIs [13]. IL-18 is crucial for activation of mucosa associated invariant T (MAIT) cells and subsequent

83 interferon (IFN)-γ secretion [43,66]. Excessive MAIT cell activation through IL-12/IL-18 was linked to

84 pathological cytokine storm underlying STSS [19], which is a common complication of NSTIs [44,46].

85 To date, data on GAS-DCs interactions are scarce and mainly derived from murine studies. Thus, we

86 investigated the cytokine response of human monocyte-derived (mo)DCs and other cells to infections

87 with either wild-type GAS or natural *covR/S* mutants. All cells of monocytic lineage infected with *covR/S*

88 mutants secreted significantly less IL-8 and IL-18 as compared to wild-type infections. Inhibition of host

89 initiator caspase-8 restored the secretion of both cytokines in response to infections. Validation of

90 systemic plasma IL-18 levels in patients did not show differences based on bacterial CovR/S

91 assignment.

Methods

Bacterial and strains

The following GAS strains were used: NSTI/STSS strains 5448, 5626, 8003, and 8157 (provided by Donald E. Low, Mount Sinai Hospital, Toronto, Canada) [27,33], 2002 and 2006 from the INFECT cohort [51], MGAS5005 [57], 5448AP [65], 5448Δ*emm1* [37], and 5448Δ*slo* [61]. All strains were cultured in Todd-Hewitt broth (Carl Roth) supplemented with 1.5% (w/v) yeast extract (Carl Roth) at 37°C. Hemolytic activity of GAS stationary culture supernatants was assessed as previously described [35].

Isolation of human monocytes and generation of dendritic cells and macrophages

Human monocytes were isolated from buffy coats using CD14 S-pluriBead antihuman beads (PluriSelect) or EasySep Human CD14 Positive Selection Kit II (Stemcell technologies) according to manufacturer's instructions. The moDCs were generated by culturing monocytes for 5 d in RPMI1640 (Cytivia) medium supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS; Thermo Fisher), 89 ng×mL^{-1} GM-CSF, and 22 ng×mL^{-1} IL-4 (both Immunotools). Medium was exchanged on day 3.

The monocyte-derived macrophages were generated by culturing monocytes in RPMI1640 (Cytivia) medium supplemented with 10% (v/v) heat inactivated FCS (Thermo Fisher) and 25 ng×mL^{-1} GM-CSF (Immunotools) for 7 d. Medium was exchanged on days 3 and 5.

Infections of myeloid cells

All infections were performed in RPMI1640 complete media. 1×10^5 monocytes, 2×10^5 monocyte-derived macrophages, or 1×10^5 moDCs were infected with GAS at a multiplicity of infection (MOI) 10 for 1 h. Next, extracellular bacteria were killed by addition of RPMI1640 containing antibiotics (100 µg×mL^{-1} streptomycin, 100 IU×mL^{-1} penicillin G (Hyclone)). After a total of 23 h infection, supernatants were collected and stored at -80°C until further analyses. The moDCs were directly prepared for flow cytometry.

117 For assessing intracellular bacterial survival kinetics, 2×10^5 moDCs were infected, as described above.

118 After addition of antibiotics, the cells were washed, lysed, and intracellular bacteria plated on blood agar

119 plates (Oxoid).

120 For inhibition studies, moDCs were pre-treated with inhibitors targeting different caspases: caspase-3

121 (Cas3/7-Inhibitor I (CAS 220509-74-0), Sigma Aldrich, 500 nM; Ac-DEVD-cho (Invivogen), 40 µM),

122 caspase-8 (z-IETD-fmk (Invivogen), 40 µM), or pan-caspase inhibitor (z-VAD-fmk, (Invivogen), 40 µM).

123 moDCs were pre-treated with the respective inhibitors for 1 h at 37°C and subsequently infected as

124 described above. Inhibition of caspases remained through the entire course of infections. DMSO served

125 as solvent control.

126

127 **Flow cytometry**

128 Dead cells were labeled using the Zombie Aqua Fixable Viability Kit (BioLegend). Unspecific binding of

129 immunoglobulins was blocked by using Human TruStain FcX (BioLegend) according to the

130 manufacturer's instructions. Incubations of cells with titrated amounts of monoclonal antibodies were

131 carried out for 30 min at 4°C in the dark. Cells were washed between each staining step and fixed using

132 the Cyto-Fast Fix/Perm Buffer Set (BioLegend). Antibodies and clones directed against the following

133 markers were used (target, clone, fluorochrome, all Bio-Legend): CD209 (9E9A8, APC), CD209 (9E9A8,

134 BV421), CD40 (5C3, Alexa Fluor700), CD80 (2D10, BV711), CD86 (BU63, PE/Cyanine7), CD83

135 (HB15e, BV421) and HLA-DR (L243, FITC). The gating strategy is shown in Figure S1. Data were

136 acquired using a FACSAria III flow cytometer and FACS DIVA 8.0 Software (both BD Biosciences, San

137 Jose, CA, USA) and analyzed using FCS Express 7 Software (De Novo Software).

138

139 **Cytokine measurements**

140 Cytokine concentrations in cell culture supernatants were measured via LEGENDPlex human

141 inflammation panel (13-plex) kit or custom panel (3-plex: IL-1β, IL-8, IL18) (both BioLegend) according

142 to the manufacturer's instructions. Data were acquired with a FACSAria III flow cytometer using FACS

143 DIVA Software (both BD Bioscience) and analyzed using LEGENDPlex software (BioLegend).

144 Furthermore, previously published cytokine levels in acute plasma from NSTI patients from the infect

145 cohort [46] was reanalyzed.

146

Caspase activity measurements

148 Caspase 8 activity was measured using the Caspase-Glo® 8 Assay Systems (Promega) according to

149 manufacturer's instructions. Caspase 3 activity was measured using the Magic Red Caspase 3/7 Kit

150 (BioRad) according to manufacturer's instructions.

151

Bioinformatic analysis

153 Analyses of CovR and CovS sequences were performed as described previously [51]. In brief, genes

154 were identified via BLAST (v. 2.9.0+) using a custom Python script. Mutations were automatically

155 identified using Python (Reference sequence CovS: WP_002991036.1, CovR: WP_002991052.1).

156 Predictions of CovR/S functionality (Table S1) were based on literature review (Table S2) or predicted

157 protein structures using AlphaFold2 [29,30]. Previously published patient cytokine data [46] and whole

158 genome sequences [60] of the GAS strains were retrieved from the INFECT cohort and reanalyzed.

159

Proteomic Analysis

161 Infected moDC pellets were resuspended in 100 µl Tris-HCl (5 mM pH 7.4 containing 5% SDS) and

162 immediately disrupted using a Dismembrator (Retsch) at 2,600 rpm for 3 min (in a 4.8 ml Teflon vessel

163 precooled in liquid nitrogen with an 8 mm diameter steel ball). Next, the cell powder was resuspended

164 with 400 µl of preheated (95°C) Tris-HCl buffer (5 mM, pH 7.4) and the viscous lysate was transferred

165 into a fresh 1.5 ml low bind pre-lubricated Eppendorf tube and shaken for 1 min at 95°C and 1,400 rpm.

166 The lysate was cooled to RT and 2 µl of a 1 M $MgCl_2$ stock solution (final conc. 4 mM $MgCl_2$) was added.

167 Next, 1 µl of a 1:100 diluted benzonase (Pierce Universal Nuclease; Pierce) stock solution (final 0.005

168 U/µl) was added and mixed by short vortexing. The samples were incubated in an ultrasonic bath at RT

169 for 5 min until the viscous lysate was liquefied by complete degradation of DNA and RNA. The raw

170 lysates were centrifuged (17,000 g; RT; 30 min). After centrifugation, the protein lysate was transferred

171 into a fresh 1.5 ml low bind pre-lubricated Eppendorf tube, and the pelleted cell debris was discarded.

172   The protein concentration of the samples was determined using the Micro BCA Protein Assay Kit

173   following the manufacturer's protocol (Pierce). Samples were stored at -80°C until further use. Sample

174   preparation for mass spectrometry measurements was performed using the SP3 protocol as previously

175   described [8].

176   LC-MS/MS analyses of tryptic peptide solutions were carried out on a reverse phase HPLC

177   chromatography system (Ultimate 3000 nano-LC system, Thermo Fisher Scientific) coupled to a Q

178   Exactive™ Exploris 480 mass spectrometer (Thermo Fisher Scientific) in data-independent acquisition

179   mode. Data analyses were performed in Spectronaut version 17 (Biognosys AG), based on a database

180   search against a Uniprot/Swissprot database limited to 20,375 human entries (version 2022_01) using

181   trypsin/P digest rule with maximum number of two missed cleavages, methionine oxidation as variable

182   modifications (for details see Table S3). Further DIA-MS data analysis was carried out in R (R Version

183   4.2.0) using the tidyverse (version 1.3.1), factomineR (version 2.4.0) and PECA package (version

184   1.32.0) with a paired reproducibility-optimized test statistic (ROTS) [50,59] as described by Cuypers *et*

185   *al* [16].

186

187   **Statistics**

188   Statistical significance of differences was determined using Kruskal-Wallis test with Dunn's multiple

189   comparison posttest or Mann-Whitney *U* test. Statistics were performed using GraphPad Prism version

190   8. A *p* value less than 0.05 was considered significant.

Results

Reduced release of IL-8 and IL-18 by myeloid cells in response to infections with GAS harboring dysfunctional CovR/S

To assess the pro-inflammatory response by moDCs, cells were infected with four GAS strains harboring functional CovR/S (CovR/S+; 2006, 5448, 5626, 8157) or four *covS* mutant strains carrying non-functional CovR/S (CovR/S-; 2002, MGAS5005, 5448AP, 8003) and IL1β, IL-8 as well as IL-18 release in response to infections was measured. The sequences as well as CovR/S functionality of the respective strains were previously assessed [51] (Table S1) and confirmed via SpeB proteolytic activity on casein agar plates (Fig. S2). While moDCs readily secreted equal amounts of IL-1β in response to all infections (Fig. 1A), IL-18 as well as IL-8 levels were significantly less in infections with CovR/S- as compared to CovR/S+ strains (Fig. 1B-C).

To evaluate whether (i) this observation is restricted to moDCs and (ii) to exclude potential impact of genomic variations between the GAS strains, moDCs, monocytes, and monocyte-derived macrophages were infected with *emm*1 STSS isolate 5448 and its corresponding *covS* mutant 5448AP [65] and the release of multiple cytokines in response to infections was assessed (Fig. 2, Fig. S3). Irrespective of the CovR/S variant used for infections, all three cell types released comparable levels of IFN-γ, tumor necrosis factor (TNF)-α, MCP-1, IL-6, IL-10, IL-12p70, and IL-23 (Fig. 2A-C; Fig. S3). Notably, of the two inflammasome-related cytokines, only IL-1β release was equally induced (Fig. 2D). In contrast, IL-18 secretion in 5448AP infections remained at the levels of uninfected controls (Fig. 2E). Furthermore, similar results were noted for IL-8 release (Fig. 2F).

Infected dendritic cells clear intracellular bacteria and mature irrespective of GAS CovR/S phenotype

Next, we assessed whether 5448AP infections would also have an effect on DC maturation. Since CovR/S inactivation has been reported to increase surface abundance of M-protein [58], an *emm*-knockout strain was included in this series of experiments. MoDCs were infected with 5448, 5448AP, or 5448Δ*emm1* and bacterial intracellular survival (Fig. S4) and moDC maturation were assessed (Fig. 3A). Irrespective of the strain used, moDCs remained viable (Fig. 3B) and eliminated all bacteria within 24 h of total infection time. No differences in GAS uptake were noted (Fig. S4). Furthermore,

220 moDCs readily matured in all infectious conditions to the levels of LPS stimulations, characterized by

221 increased frequencies of CD80 and CD83 positive cells as well as upregulation of CD40, CD86, and

222 HLA-DR on the surface (Fig. 3A-G).

223 To assess potential differences on a global level, whole proteome of moDCs infected with 5448 or

224 5448AP was quantitatively profiled by mass spectrometry at 6 and 24 h post infection. Uninfected

225 moDCs at respective time points served as controls. In total, 5857 protein groups were identified. For

226 quantification, 4801 protein groups identified by the presence of two or more peptides and observed in

227 at least half of the experiments for each condition were used. Principal component analysis (PCA)

228 revealed that uninfected controls (6 and 24 h) and both 6 h infections with 5448 and 5448AP clustered

229 together (Fig. 3H). Furthermore, rather a donor specific proteome signature with no obvious impact of

230 infections was noted at 6 h (Fig. S5; Table S4) suggesting that 6 h of infection are too short to induce

231 maturation. In contrast, the moDC proteomes of both 24 h infections were different from uninfected

232 moDC and separated in the PCA (Fig. 3H). Statistical analyses revealed a general infection specific

233 signature. However, no major differences were observed between 5448 and 5448AP infections (Fig. S6,

234 Table S4). Furthermore, IL-18 as well as IL-8 were not detected within moDCs (Table S4).

235

236 **Caspase-8 inhibition restores secretion of IL-8 and IL-18 by moDCs in 5448AP infections**

237 Since reduction of IL-8 levels in GAS infections by action of either SpyCEP [63,68] or Nga [45] is well

238 established, we further focused on IL-18 secretion by moDCs. The IL-18 amino acid sequence contains

239 predicted cleavage sites for executioner proteases, including caspase-1 and caspase-3 [49]. Processing

240 of IL-18 by caspase-3 was shown to yield biologically inactive fragments [48]. To investigate whether

241 inhibition of caspase-3 or its upstream initiator caspase-8 would restore the moDCs ability to secrete IL-

242 18 (and potentially IL-8), moDCs were pre-treated with specific inhibitors of caspase-3, caspase-8, or a

243 pan-caspase inhibitor. Subsequently, pre-treated moDCs were infected with 5448AP, and multiple

244 inflammatory cytokines were measured in supernatants. No cytokine secretion in response to inhibitors

245 or its solvent, DMSO, was noted (Fig. S7). Caspase-3, caspase-8, or pan-caspase inhibition did not alter

246 secretion of IFN-α2, IFN-γ, MCP-1, IL-6, IL-10, IL-12p70 or IL-23, as elevated levels of these cytokines

247 were detected in all infection conditions (Fig. 4, Fig. S7). Furthermore, IL-1β secretion in response to all

248 5448AP infections remained at the levels of 5448 infections (Fig. 4A-B). Notably, in the presence of

249   caspase-8 as well as pan-caspase inhibitors, the levels of IL-18 increased in response to 5448AP

250   infections, reaching the levels of 5448 infection control (Fig. 4A, 4C). A similar trend was also observed

251   for IL-8 (Fig. 4A, 4D), particularly when caspase-8 inhibitor was used.

252

253   **Infections of moDCs with 5448AP result in higher caspase-8 activity**

254   Since caspase-8 inhibition restored the secretion of IL-8 and IL-18 in response to 5448AP infections, its

255   intracellular abundance was assessed in moDCs. Executioner caspase-3 was also analyzed.

256   Irrespective of the strain used for the infections, moDCs equally responded by upregulating the

257   expression of both caspases at 24 h post infection (Fig. 5A-B; Table S4). However, total protein

258   abundance does not reflect its activity. Therefore, moDCs were infected with both strains and caspase-

259   8 activity was assessed at different time points of infection. Luminescence-based measurement over a

260   period of 8 h revealed a stable increase of caspase-8 activity in response to 5448AP infections (Fig. 5C)

261   In contrast, 5448 infections rather suppressed caspase-8 activity. Assessment of its activity in several

262   donors confirmed the initial result. While 5448AP infections activated caspase-8, 5448 infections did not

263   (Fig. 5D). Downstream assessment of executioner caspase-3 activity via flow cytometry showed that

264   only a negligible percentage of infected moDCs were positive for active caspase-3 (Fig. 5E).

265

266   **Impact of dysfunctional CovR/S on systemic cytokine levels in NSTI patients**

267   As both, the IL-1β network [13] and the IL-12/IL-18 axis [19] are closely linked to NSTIs and STSS, the

268   CovR/S phenotype of GAS isolates collected during the INFECT study was determined and correlated

269   to previously measured plasma concentrations of IL-8, IL-1β, and IL-18 [46]. In total, *covS* and *covR*

270   genes of 67 isolates were analyzed and amino acid sequences were predicted (Table S1). The majority

271   of mutations leading to amino acid substitution or truncated proteins were identified in CovS (Table S1).

272   To assess the functionality of different CovR/S variants, literature review (Table S2) and protein

273   structure predictions using AlphaFold2 (Fig. S8) were performed [29,30]. Twenty-eight strains were

274   predicted to harbor dysfunctional CovR/S (Table S1, Fig. S8; please refer to the figure legend of Fig. S8

275   for detailed description of the functionality assignment). Next, the CovR/S phenotype was matched to

276   plasma cytokine concentrations of respective patients. Irrespective of functional or dysfunctional CovR/S

277   of the infective GAS, equal systemic concentrations of IL-8, IL-1β, and IL-18 (Fig. 6A-C) were detected

278 in patient's plasma. As the IL-12/IL-18 axis is also linked to development of STSS, patients were grouped

279 based on clinical presentation, with and without septic shock. These analyses revealed that patients

280 suffering from septic shock have significantly higher systemic IL-8 or IL-1β plasma levels if they are

281 infected with CovR/S$^+$ or CovR/S$^-$ strains, respectively (Fig. 6D-E). No differences in systemic IL-18

282 levels between patient groups were observed (Fig. 6F).

283

284 **Secretion of IL-1β and IL-18 by moDCs is increased in infections with 5448Δ*slo***

285 SLO is a known inducer of apoptotic program in myeloid cells, which requires activation of caspase-8

286 [61]. Since SLO was previously shown to (i) impact maturation of murine DCs, (ii) to interfere with

287 caspases [15,35], and (iii) its expression is under CovR/S control [57], we hypothesized that SLO might

288 be responsible for impaired secretion of IL-18 by moDCs. First, hemolytic activity of stationary culture

289 supernatants of 5448 and 5448AP was measured. This revealed significantly higher hemolytic activity

290 of 5448AP supernatants as compared to 5448 (Fig. 7A). Several attempts to construct a *slo*-knock-out

291 in 5448AP background was unsuccessful. We therefore hypothesized that infections of moDCs with

292 5448Δ*slo* strain would further increase IL-18 response as compared to 5448 wild-type infections.

293 Therefore, cells were infected with 5448 and its corresponding 5448Δ*slo*. Deletion of SLO had no

294 obvious impact on moDC viability (Fig. 7B). Furthermore, moDCs matured in response to all infections

295 characterized by increased frequencies of CD83$^+$ (Fig. C) and CD86$^+$ (Fig. 7D) cells as well as increased

296 expression of surface HLA-DR (Fig. 7E). Analyses of cytokines revealed an equal IL-8 response

297 (Fig. 7F). In contrast, IL-1β (Fig. 7G) and IL-18 (Fig. 7H) secretion were significantly higher in 5448Δ*slo*

298 as compared to wild-type infections.

Discussion

Myeloid cells are crucial responders to infections, responsible for pathogen clearance, recruitment of other immune cells, and shaping the T cell response through secretion of a plethora of cytokines and chemokines. They are the primary sources of IL-1 family cytokines IL-1β and IL-18 as well as potent producers of the main human neutrophil chemoattractant IL-8 [23]. Bacteria, including GAS, have developed multiple phenotypic and genetic adaptations to evade the host immune system effector mechanisms. In this study, we show that infections with GAS strains harboring dysfunctional CovR/S result in reduced secretion of IL-8 and IL-18 by monocytic cells. This phenomenon is mainly mediated by a caspase-8-dependent mechanism.

GAS have developed several ways to adapt to the host environment. Enhanced mutational rates in the *covR/S* genes often mediate the switch to a hyper-virulent phenotype [65]. CovS senses environmental signals and phosphorylates the bound response regulator CovR. Phosphorylated CovR dissociates from CovS, binds to its target DNA sequences, and either activates or represses transcription of target genes [18,25]. Not every mutation in *covR* or *covS* will lead to dysfunctional TCS [47]. However, loss of function mutations are often associated with increased virulence [17] due to de-repression of transcription of several virulence factors, which is particularly pronounced in *emm1* strains [6,24,32]. Of 67 strains analyzed in this study, 28 isolates possessed mutations, which were experimentally validated or predicted to negatively affect the functionality of the TCS resulting in a hyper-virulent phenotype. In such strains, increased levels of key virulence factors, including the capsule, M protein, SpyCEP, and Nga were reported [57,58].

The cell-envelope protease SpyCEP was shown to cleave human IL-8, resulting in decreased local levels and consequently impaired neutrophil recruitment to the site of infection [53,63,68]. In addition, induction of ER stress and Golgi fragmentation by the NAD-glycohydrolase Nga was shown to reduce secretion of IL-8 by GAS-infected macrophages [45]. Furthermore, Nga activity inhibits type I interferon production in macrophages via suppression of STING signaling, which also correlates with increased disease severity in NSTI patients [42]. The results of this study are in line with previous observations. However, our data indicates that streptococcal SpyCEP and Nga are not the only factors responsible for the abrogated IL-8 secretion, as inhibition of initiator caspase-8 restored IL-8 release by moDCs. This suggests that particularly host factors play also a role in the observed phenotype.

13

328   Strains harboring dysfunctional CovR/S were also shown to produce increased levels of SLO [31,35,58].

329   SLO forms pores in membranes of phagocytes [67], mediates bacterial escape from phagosomes, and

330   induces macrophage death [61]. In addition, SLO is essential for Nga translocation across the plasma

331   membrane into the host cell cytosol. Consequently, Nga depletes cellular energy stores and interferes

332   with cytokine secretion [42,45]. STSS isolates were shown to produce significantly more SLO than

333   strains derived from non-invasive infections, emphasizing a crucial role for this cytolysin in invasive

334   diseases [7,26]. Much of the knowledge about DC-GAS interactions is derived from work with murine

335   cells or prolonged infection times. In such settings, DCs infected with a CovR/S dysfunctional and high-

336   SLO secreting 5448AP strain showed impaired maturation and significant cell death [15,35]. Our results

337   are in stark contrast to these previous findings mostly due to the following reasons: (i) short initial

338   infections which were not exceeding one hour and (ii) immediate depletion of extracellular bacterial

339   supply through antibiotic treatment. Even in such setting, IL-18 secretion by infected monocytic cells

340   was impaired. SLO is a known inducer of apoptotic program through activation of the initiator caspase-

341   8 [61]. Although speculative, increased production of SLO by phagocytosed GAS might lead to the

342   observed increased activation of caspase-8. Consequently, caspase-8 or a downstream-protease other

343   than caspase-3 might cleave IL-18, which results in the observed abrogated IL-18 secretion. Although

344   we were not able to construct a *slo*-knockout in 5448AP background, our data supports the concept that

345   SLO interferes with the cytokine axis, since moDCs infected with 5448Δ*slo* secreted high amounts of

346   IL-1β and IL-18, even exceeding the levels of wild-type infections.

347   The role of the IL-1β network in NSTIs is well established [13]. Furthermore, the IL-12/IL-18 axis has

348   been linked to the pathological MAIT cell activation and development of STSS [19,44,46]. Although we

349   did not detect differences in systemic IL-18 levels in patients, local tissue concentrations might differ

350   due to a mix of GAS clones, which are usually recovered from NSTI tissue biopsies [52]. Reduced local

351   IL-18 levels could potentially delay activation of resident or recruited MAIT cells characterized by

352   reduced IFN-γ production. Nonetheless, hyper-virulent GAS harboring *covR/S* mutations produce

353   increased amounts of superantigens [58], which might potentially negate the IL-18 effect. In general,

354   myeloid cells are the primary sources of IL-1β and IL-18 [23] and elevated levels of both cytokines were

355   found locally and systemically in a pharyngitis human challenge trial as well as increased frequencies

356   of circulating monocytes and DCs [3]. In our *in vitro* experimental setup, strains harboring dysfunctional

357   CovR/S exclusively suppressed IL-18 release by myeloid cells. Notably, these two cytokines are

commonly considered to be processed and released together. Thus, little is known about their differential processing. Canonical maturation and release are mediated by inflammasome-associated executioner caspase-1. However, other non-canonical inflammasomes as well as proteases can process both cytokines [66]. In addition, cell death pathways mediate caspase-1 and/or caspase-8 dependent release of IL-1β [64] and caspase-8 is able to substitute for loss of caspase-1 dependent processing of IL-18 [4,9,21,22,40]. Furthermore, cleavage of pro- and mature IL-18 via caspase-3 was shown to yield biologically inactive fragments [1,48]. In this study, inhibition of caspase-8 restored secretion of IL-18 in infections with hyper-virulent GAS, while inhibition of caspase-3 did not. This suggests that either caspase-8 or an unknown factor downstream is responsible for this effect, which is also supported by the fact that caspase-8 activity was exclusively increased in 5448AP infections.

Conclusions

Cytokines/chemokines derived from myeloid cells are crucial effector molecules in infections. Through tissue passage, GAS adopt a hyper-virulent phenotype mediated by mutations of genes encoding the CovR/S TCS. Here, we show that *in vitro* infections of monocytic cells with such hyper-virulent strains result in abrogated release of IL-8 and IL-18. To our knowledge, this is the first report that provides evidence of IL-18 suppression by hyper-virulent GAS strains in cells of monocytic lineage and warrants further experimental studies to identify host as well as bacterial factors which are used to interfere with the immune response.

Abbreviations

GAS: Group A streptococcus; NSTI: necrotizing skin and soft tissue infection; TCS: two component system; STSS: streptococcal toxic shock syndrome; SLO: streptolysin O; (mo)DC: (monocyte-derived) dendritic cell; IL: interleukin; IFN: interferon; MAIT: mucosa associated invariant T cell; CD: cluster of differentiation

Supplementary information

Additional file 1: Figures S1-8; Tables S2, S3, S5; Table legends S1, S4

Additional file 2: Tables S1, S4

Declarations

Patient material and blood samples

This study reanalyzed previously published plasma cytokine levels and whole genome sequences of GAS strains retrieved from the INFECT patient cohort consisting of patients with NSTI (INFECT study; ClinicalTrials.gov, NCT01790698).

Buffy coats of blood provided by the blood bank at the University Medicine Greifswald were used. The buffy coats were provided anonymously. The ethical research committee at the University Medicine Greifswald approved the study (Ref. No. BB 014/14). All experiments were carried out in accordance with the approved guidelines.

Consent for publication

Not applicable

Availability of data and materials

All data associated with this study are presented in the manuscript and supplementary material. Whole genome sequencing data of GAS strains are available at the European Nucleotide Archive (ENA) under

402   the reference number PRJNA524111. The mass spectrometry proteomics data have been deposited to

403   the Mass Spectrometry Interactive Virtual Environment (MassIVE) with the dataset identifier

404   MSV000093585.

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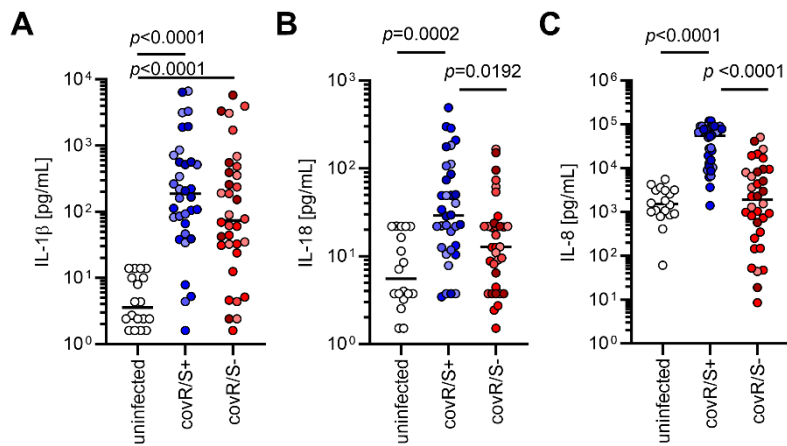
Fig. 1 MoDCs infected with GAS strains harboring dysfunctional CovR/S secrete less IL-8 and IL-18. MoDCs were infected with four strains possessing functional CovR/S (5448, 5626, 2006, 8157) or four strains possessing non-functional CovR/S (5448AP, 2002, 5005, 8003) as assessed by sequence analyses and SpeB proteolytic assay. The concentrations of IL-1β (**A**), IL-18 (**B**), and IL-8 (**C**) were measured in supernatants of (un)infected moDCs. Each dot represents one independent experiment with cells from one donor (n≥8). Horizontal lines denote median values. The level of significance was determined using Kruskal-Wallis test with Dunn's post test. Different shades of color represent infections with different strains.
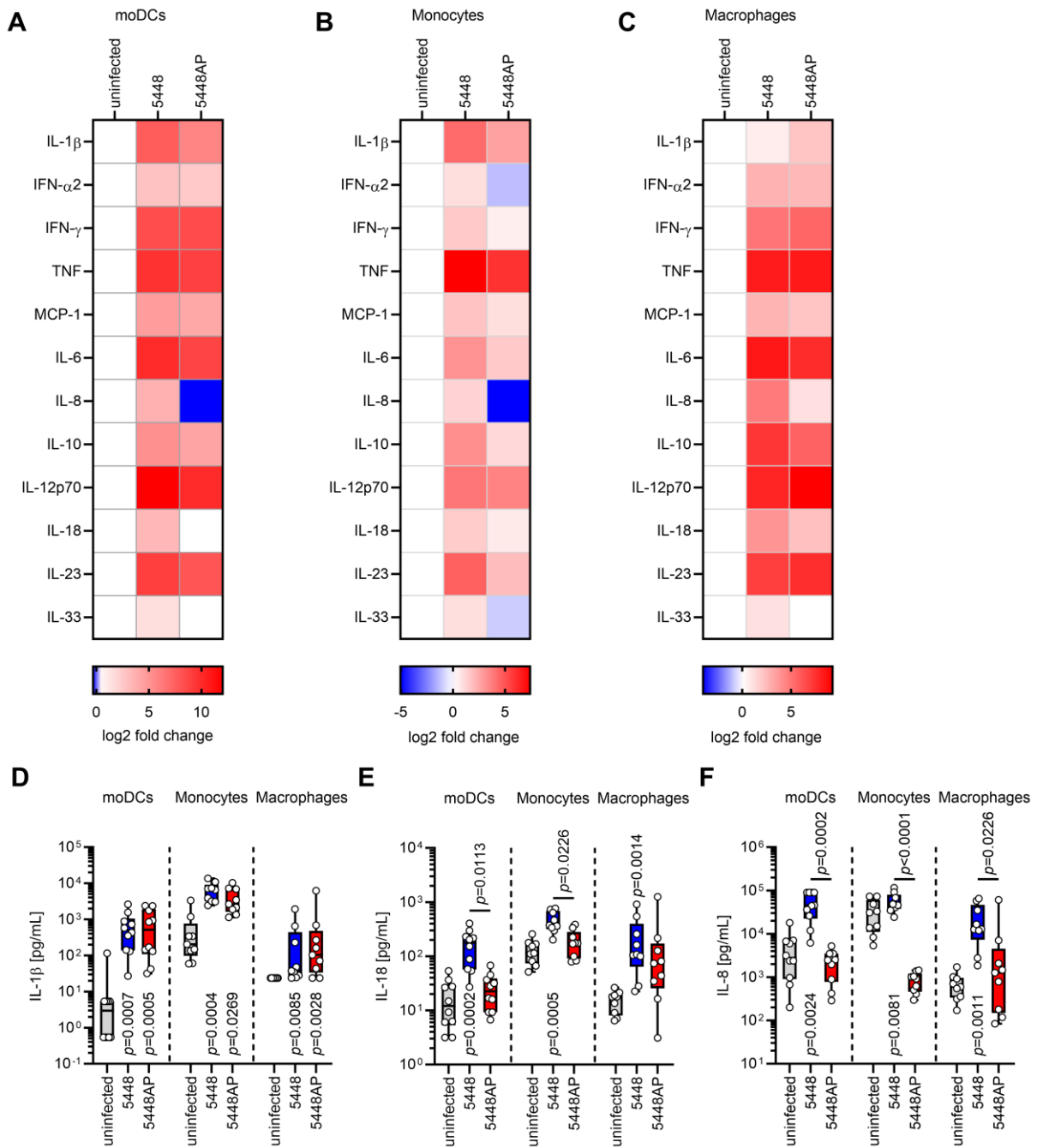
Fig. 2 Monocytic cells infected with 5448AP secrete less IL-18 and IL-8. MoDCs (**A**), monocytes (**B**), or monocyte-derived macrophages (**C**) were infected with 5448 or 5448AP and cytokine secretion was measured via a multiplex assay (n≥8). The heatmaps represent the log2 fold change of cytokine concentration in relation to the uninfected controls (**A-C**). Original data are displayed in **D-F** and Fig. S3. Original data of IL-1β (**D**), IL-18 (**E**), and IL-8 (**F**) concentration in supernatants of (un)infected moDCs, monocytes, and monocyte-derived macrophages. The data in (D-F) are displayed as box plots. Each dot represents one independent experiment with cells from one donor (n≥8). The level of significance was determined using Kruskal-Wallis test with Dunn's posttest.

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677
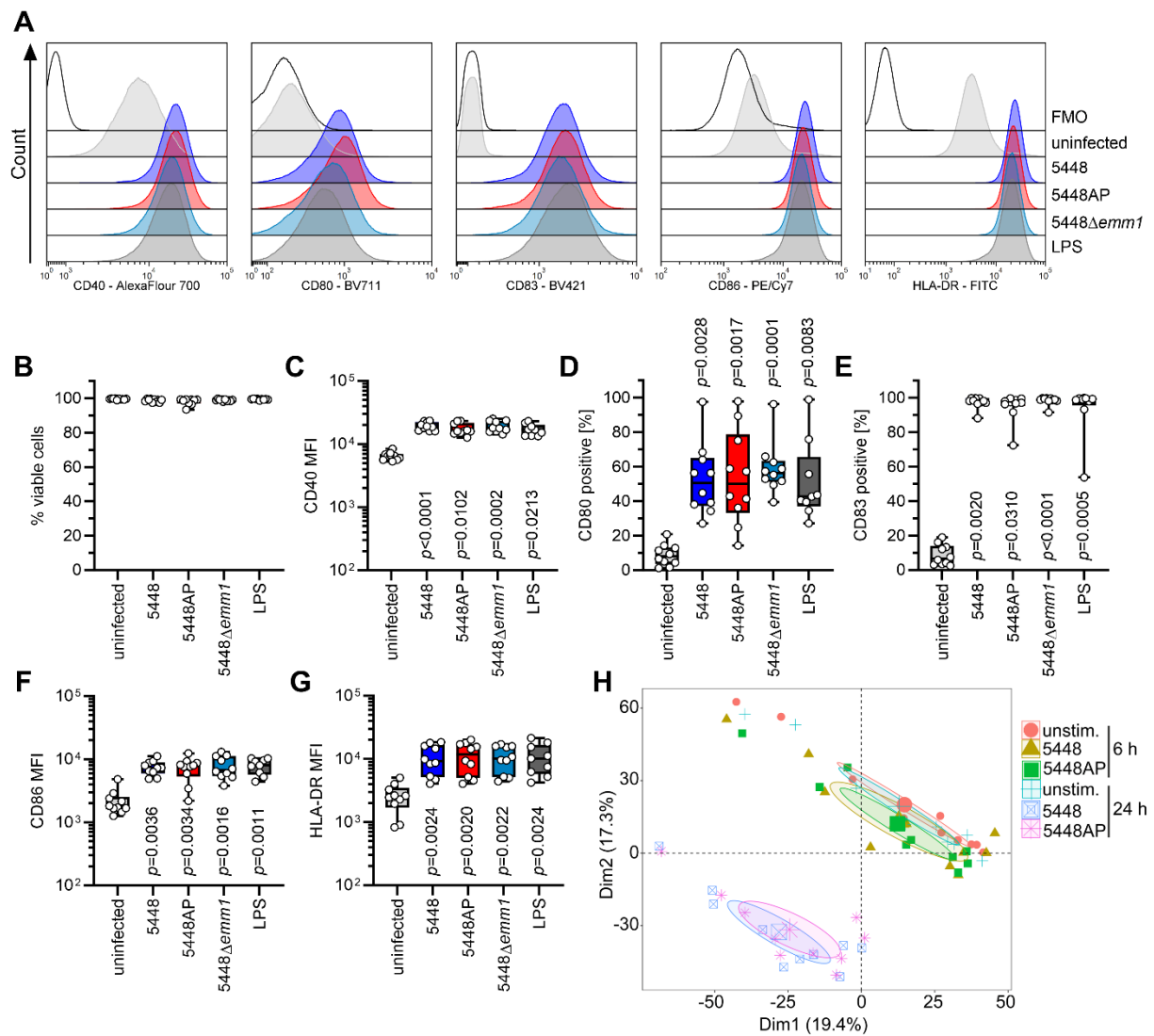
Fig. 3 5448AP does not impair DC maturation. MoDC were infected with 5448, 5448AP, or 5448Δ*emm1*
for 1 h. Extracellular bacteria were killed by substituting the media with antibiotics for additional 23 h.
MoDC phenotype (**A, C-G**) and viability (**B**) were evaluated via flow cytometry. Representative
histograms for each marker are shown in (**A**). The maturation process was evaluated by assessing the
expression of CD40 (**B**), frequencies of CD80+ (**D**) and CD83+ (**E**) cells as well as CD86 (**F**) and HLA-
DR expression (**G**). (**H**) Principal component analysis of intracellular proteins 6 h and 24 h post infections
with indicated strains. Each dot represents one donor (n=10). The ellipses indicate the calculated 95%
probability region for a bivariate normal distribution with an average center of groups. The data in (B-G)
are displayed as box plots. Each dot represents one independent experiment with cells from one donor
(n=10). The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest.
FMO, fluorescence minus one; MFI, mean fluorescence intensity.

25
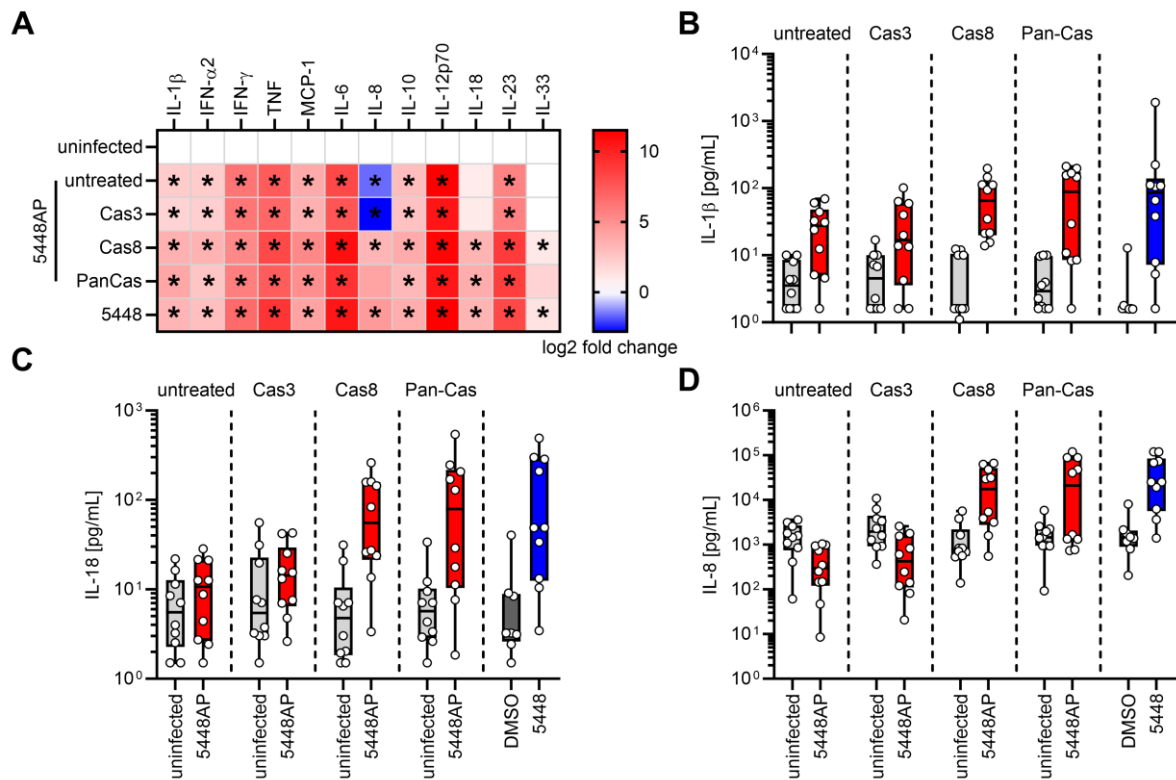
Fig. 4 Inhibition of caspase-8 restores IL-18 and IL-8 secretion in 5448AP infections. MoDCs were treated with caspase inhibitors (caspase-3: Cas3/7-Inhibitor I, Ac-DEVD-cho; caspase-8: z-IETD-fmk, pan-capase: z-VAD-fmk) and subsequently infected with 5448AP. Cytokine secretion by moDCs was measured via a multiplex assay. (**A**) The heatmap represents the log2 fold change of cytokine concentration in relation to the respective uninfected controls. Original data are displayed in **B-D** and Fig. S7. Original data of IL-1β (**B**), IL-18 (**C**), and IL-8 (**D**) concentration in supernatants of (un)infected moDCs. The data in (B-D) are displayed as box plots. Each dot represents one independent experiment with cells from one donor (n=10). A separate uninfected control with the respective inhibitor treatment was performed for each 5448AP infection. Untreated: indicates 5448AP infection without inhibitors. Each infection was compared to its respective uninfected controls. The level of significance was determined using Mann-Whitney *U*-test. Exact statistical analysis is displayed in Table S5.
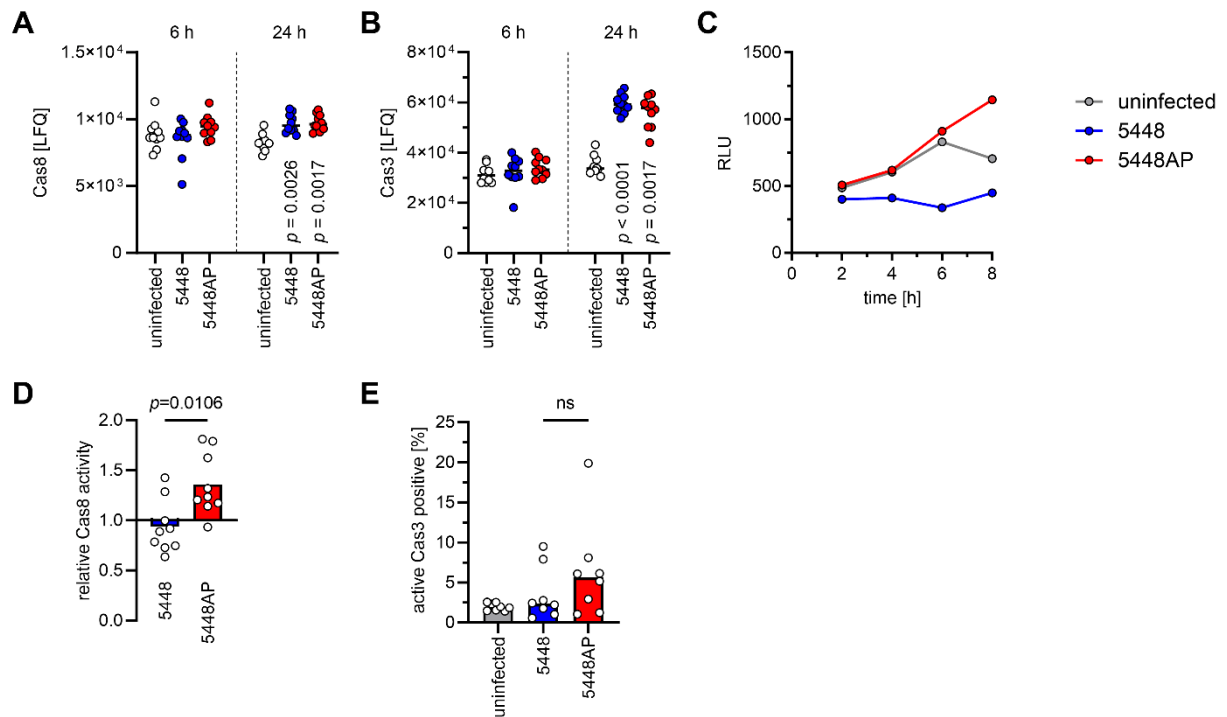
Fig. 5 5448AP-infected moDCs exhibit high caspase-8 activity. MoDCs were infected with 5448 or 5448AP. Relative abundance of caspase-8 (**A**) and caspase-3 (**B**) 6 h and 24 h post infection. Original calculations are displayed in Table S3. (**C**) Caspase-8 activity of (un)infected moDCs as measured at indicated time points by luminescence. (**D**) Relative caspase-8 activity at 8 h post infection. (**E**) Frequencies of caspase-3+ moDCs 8 h post infection. Each dot represents one independent experiment with cells from one donor (n≥8). Horizontal lines in (A, B) denote median values. Bars in (D-E) denote mean values. The level of significance was determined using Kruskal-Wallis test with Dunn's posttest in (A, B, E) or Mann-Whitney *U*-test in (D). LFQ, label-free quantification intensities; RLU, relative light units.

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711
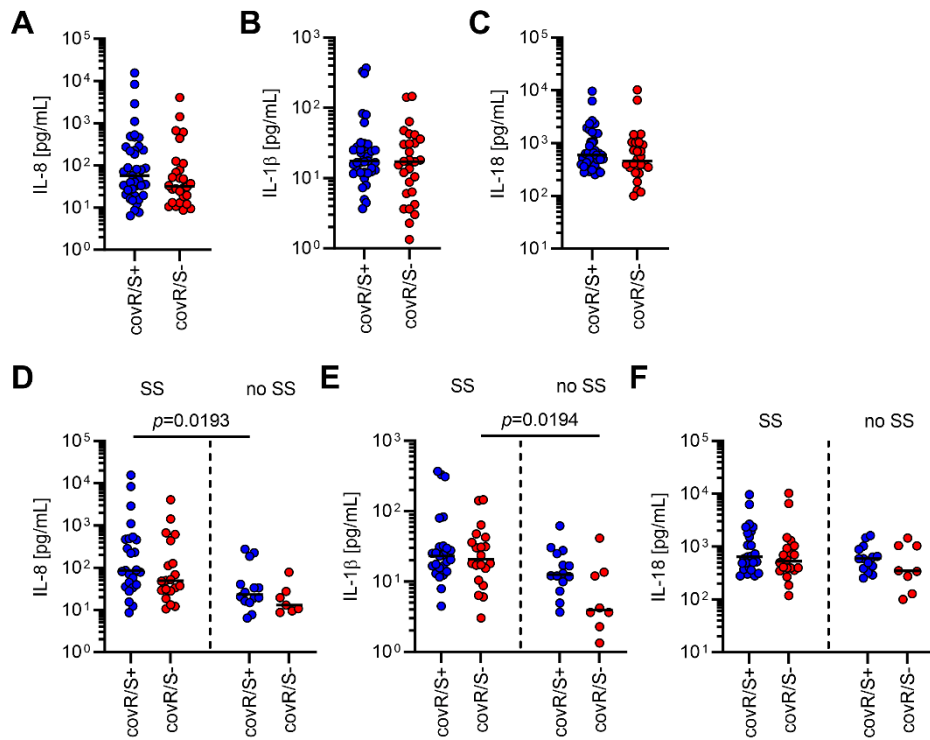
Fig. 6 Cytokine/chemokine levels in plasma of NSTI patients. Levels of IL-8 (**A**), IL-1β (**B**), and IL-18 (**C**) were previously determined in plasma of NSTI patients [46] and reanalyzed. Comparison of IL-8 (**D**), IL-1β (**E**), and IL-18 (**F**) plasma concentrations between septic shock and no septic shock. Each dot represents data from one patient. Horizontal lines denote median values. The level of significance was determined using Kruskal-Wallis test with Dunn's posttest. SS, septic shock.

717
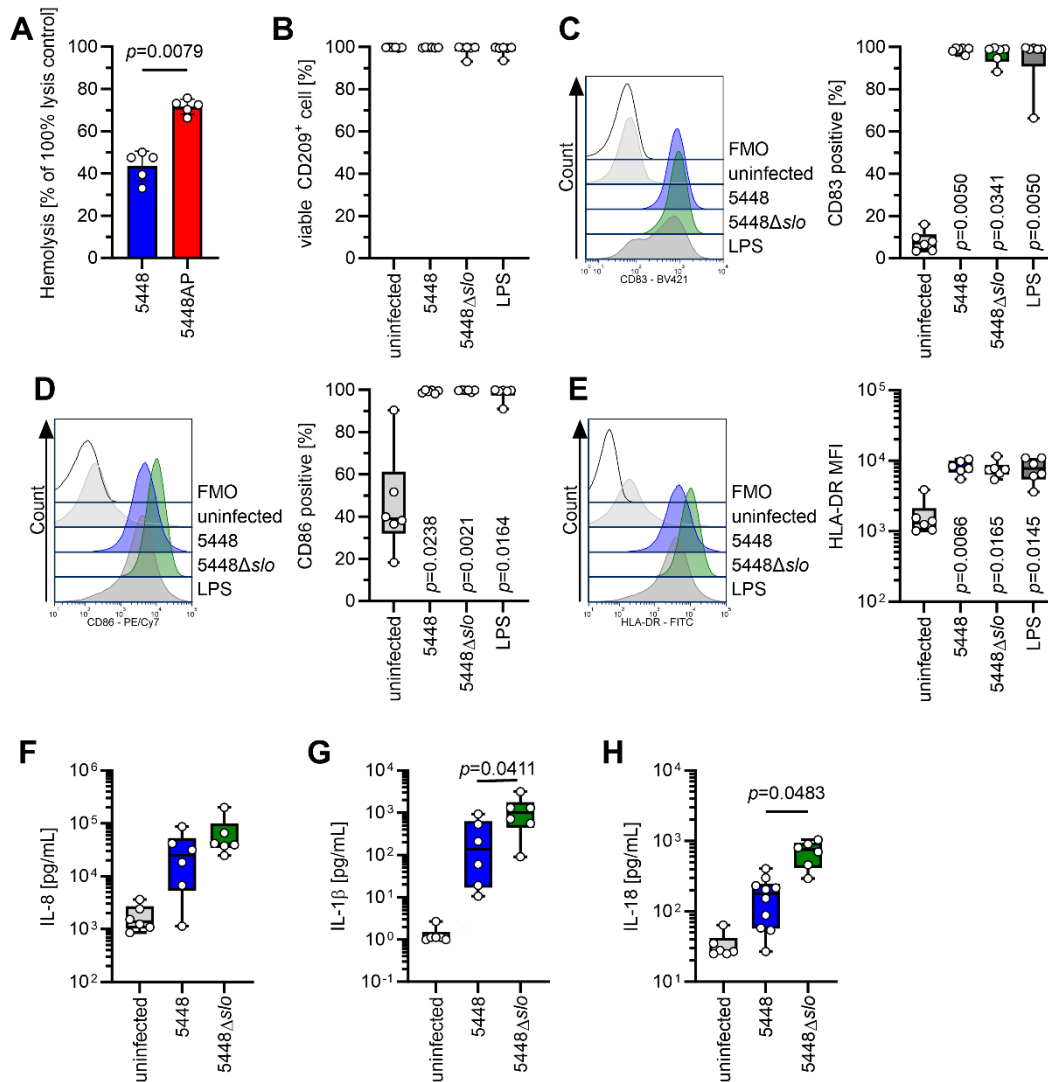
Fig. 7 Knock-out of SLO in 5448 leads to elevated secretion of IL-1β and IL-18 by moDCs. (**A**) SLO hemolytic activity in supernatants of 5448 and 5448AP strains. MoDCs were infected with 5448 or 5448Δ*slo* and moDC viability (**B**) and phenotype (**C-E**) were evaluated via flow cytometry. The maturation process was evaluated by assessing the frequencies of CD83⁺ (**C**) and CD86⁺ (**D**) cells as well as expression of HLA-DR (**E**). Representative histograms for each marker are shown in each respective left panel. The concentrations of IL-8 (**F**), IL-1β (**G**), and IL-18 (**H**) were measured in supernatants of (un)infected moDCs. The data in (B-H) are displayed as box plots. Each dot represents one independent experiment with cells from one donor (n≥6). The level of significance was determined using Kruskal-Wallis test with Dunn's posttest. FMO, fluorescence minus one; MFI, mean fluorescence intensity.

Additional File 1

Reduced interleukin-18 secretion by human monocytic cells in response to infections with hyper-virulent *Streptococcus pyogenes*

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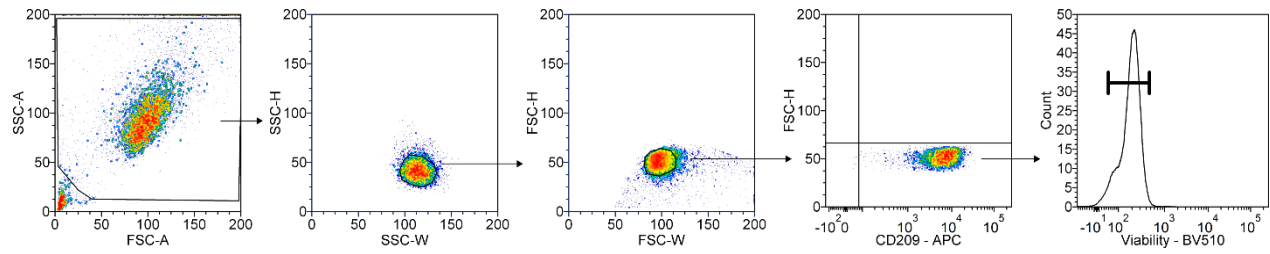
Fig. S1 Gating strategy used to identify human moDCs. Doublets were excluded by consecutive gating of FSC-H/FSC-W and SSC-H/SSC-W. MoDCs were selected based on the expression of the specific moDC marker DC-SIGN (CD209). Dead cells were excluded by using the Zombie Aqua™ Fixable Viability Kit.
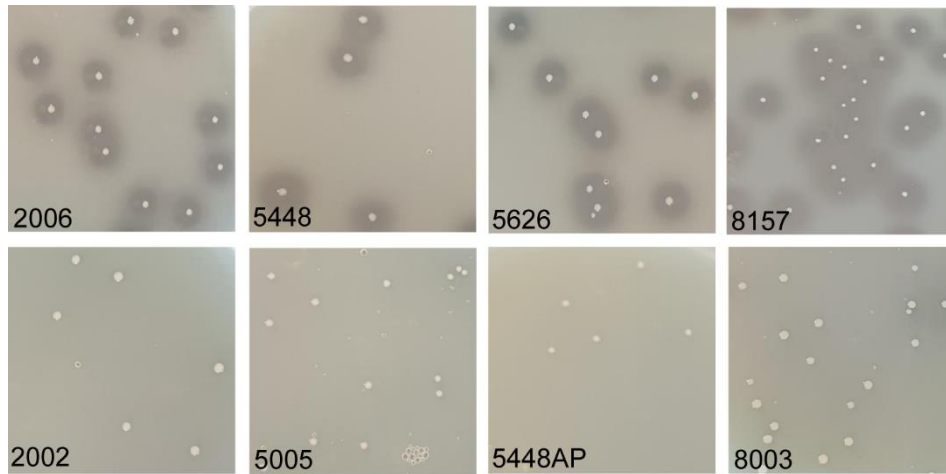
Fig. S2 CovR/S TCS functionality as assessed via SpeB proteolytic activity on casein agar plates. Presence of hydrolysis zones around the colonies was categorized as CovR/S$^+$. Lack of hydrolysis zones was categorized as CovR/S$^-$.
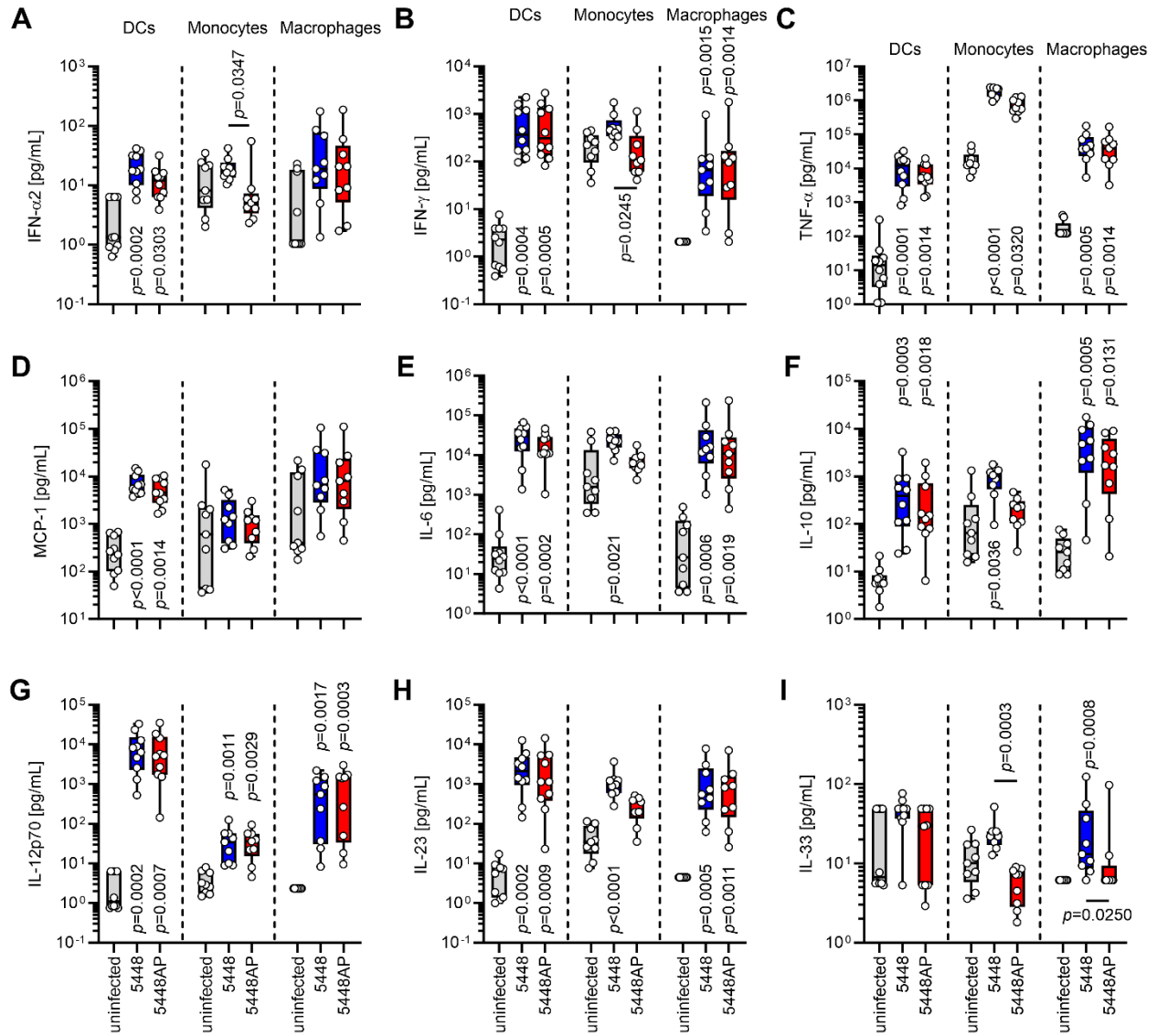
Fig. S3 MoDCs, monocytes, or monocyte-derived macrophages were infected with 5448 or 5448AP and cytokine secretion by myeloid cells was measured via a multiplex assay (n≥8). Displayed are concentrations of IFN-α2 (A), IFN-γ (B), TNF-α (C), MCP-1 (D), IL-6 (E), IL-10 (F), IL-12p70 (G), IL-23 (H), and IL-33 (I) in supernatants of (un)infected cells. The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor (n≥8). The level of significance was determined using Kruskal-Wallis test with Dunn's posttest.
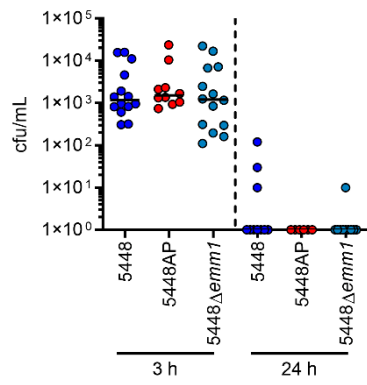
Fig. S4 MoDCs eliminate intracellular GAS. Viable intracellular bacteria were determined at indicated time points post infections. Each dot represents one independent experiment with cells from one donor (n≥10). Horizontal lines denote median value.
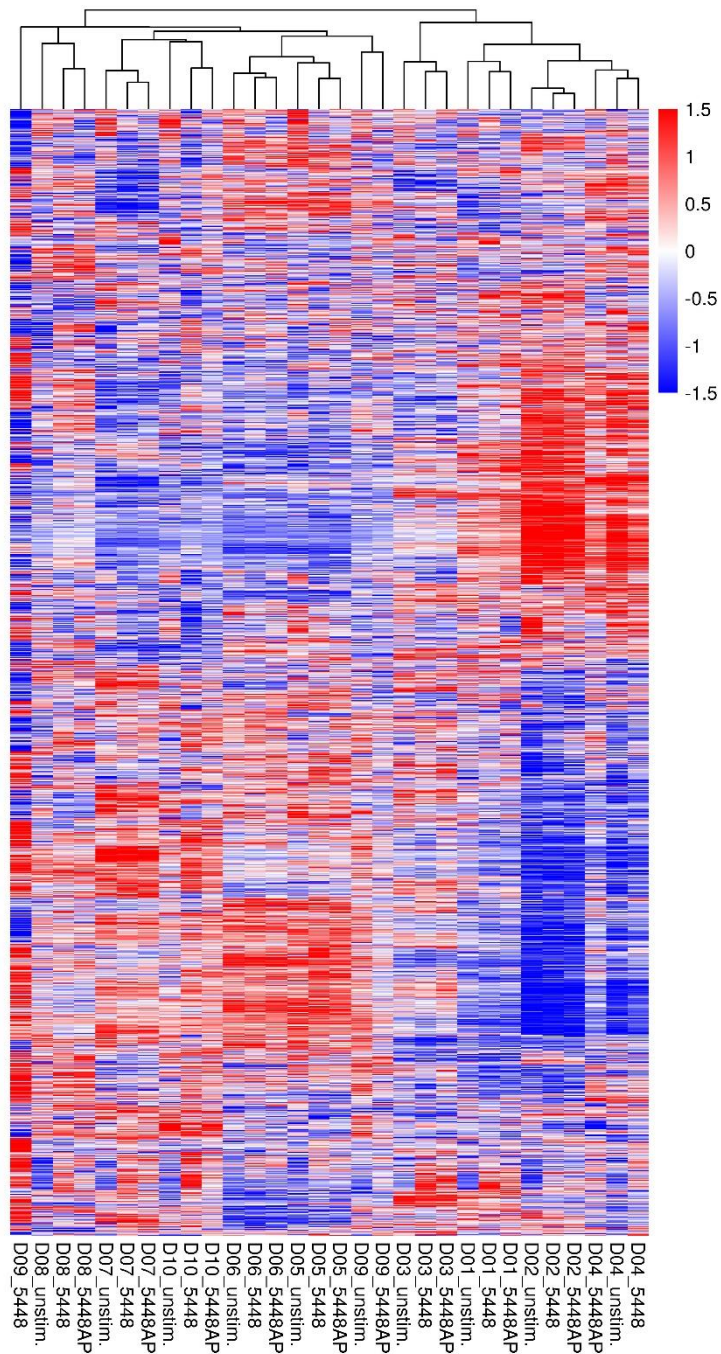
Fig. S5 Heatmap (z-scored) depicting protein intensities (maxLFQ) in moDCs 6 h post infection. Each column represents one condition for one respective donor (n=10). In total, 5857 protein groups were identified. For quantification 4801 protein groups, identified with two or more peptides and found in 50% of the individual condition replicates, were used.

Fig. S6 Heatmap (z-scored) depicting protein intensities (maxLFQ) in moDCs 24 h post infection. Each column represents one condition for one respective donor (n=10). In total, 5857 protein groups were identified. For quantification 4801 protein groups, identified with two or more peptides and found in 50% of the individual condition replicates, were used.
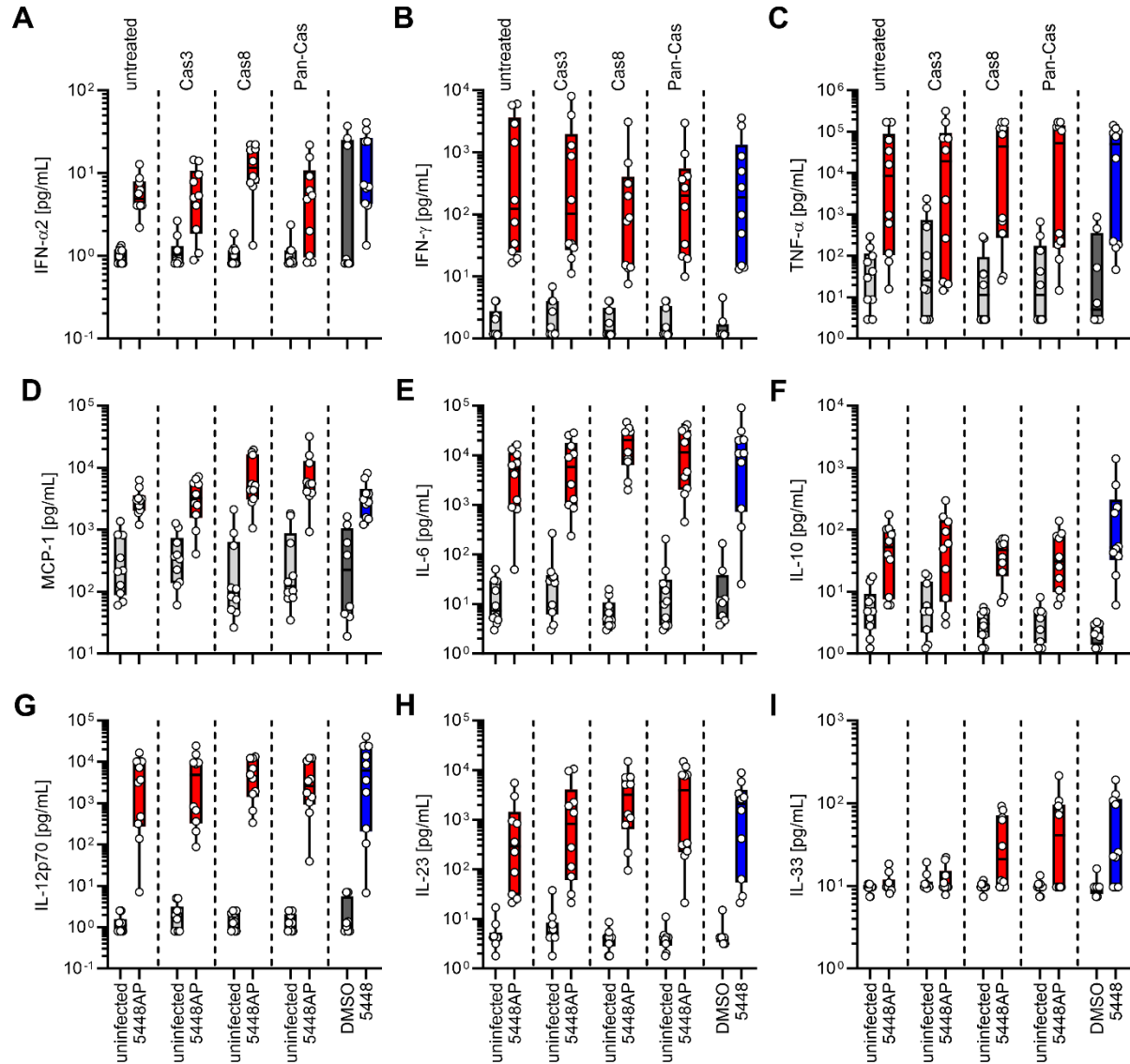
Fig. S7 MoDCs were treated with caspase inhibitors (caspase-3: Cas3/7-Inhibitor I, Ac-DEVD-cho; caspase-8: z-IETD-fmk, pan-capase: z-VAD-fmk) and subsequently infected with 5448AP. Cytokine secretion by moDCs was measured via a multiplex assay. The concentrations of IFN-α2 (A), IFN-γ (B), TNF-α (C), MCP-1 (D), IL-6 (E), IL-10 (F), IL-12p70 (G), IL-23 (H), and IL-33 (I) were measured in supernatants of (un)infected moDCs. The data are displayed as box plots. Each dot represents one independent experiment with cells from one donor (n=10). A separate uninfected control with the respective inhibitor treatment was performed for each 5448AP infection. Untreated: indicates 5448AP infection without inhibitors. Each infection was compared to its respective uninfected controls. The level of significance was determined using Mann-Whitney U-test. Exact *p*-values are displayed in Table S1.
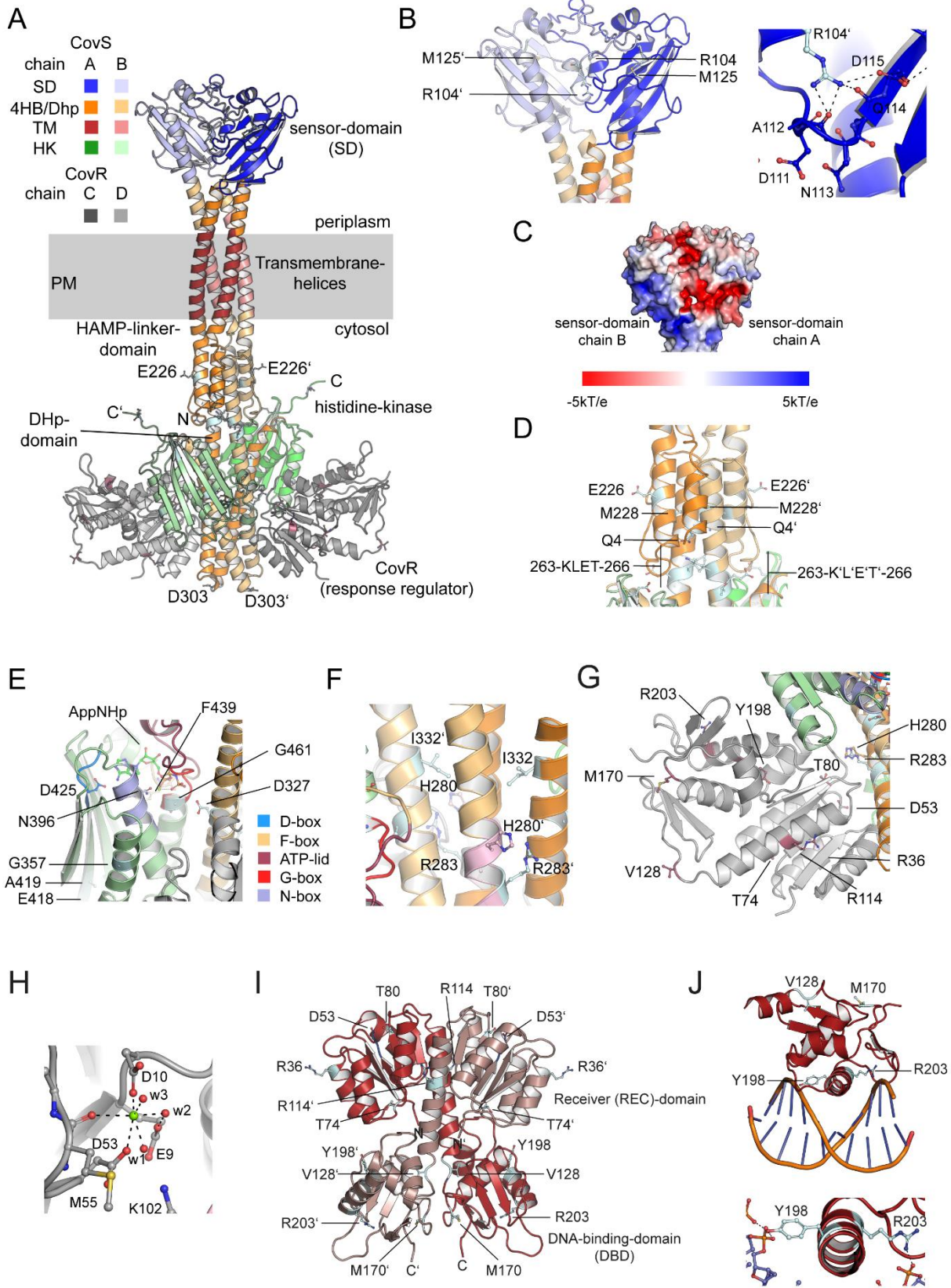
Fig. S8: Structural analyses of the overall fold of the CovS/CovR histidine kinase response regulator complex by AlphaFold2.[5,6]

(A) AlphaFold2 was used to predict the overall structure of the tetrameric CovR$_2$•2CovS complex. As described for two-component signaling systems, the histidine kinase CovS dimerizes. It consists of a periplasmic sensor-domain (SD), a section that contains the transmembrane helices, a HAMP (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and phosphatases)-linker-domain forming a four-helix bundle, a dimerization and histidine phosphotransfer (DHp)-domain containing the phosphorylated histidine residue (H280) and the histidine-kinase (HK)-domain containing the ATP-binding site. The response regulator CovR binds to the DHp-domain and HK-domain. Two molecules of CovS bind to a CovR-dimer. CovR is phosphorylated at an aspartate residue, D53, that induces dissociation from CovS to bind to the DNA. The structure shows that CovS is in a state incompatible with phosphorylation of H280 and transfer of the phosphoryl-group to D53, suggesting that activation of the sensor-domain by ligand binding, such as LL-37, or by a pH shift into the acidic range. The figure was created by PyMOL.[8]

(B) The sensor domain (SD) of the histidine kinase CovS dimerizes by formation of interactions of residues from chain A with residues of chain B. M125 and R104 were found to be mutated to isoleucine and histidine, respectively. Mutation of R104H would interfere with formation of several interactions. R104 from chain B/A forms an important salt bridge with the side chain of D115 of chain A/B, i.e. *in trans*, thereby interconnecting the dimer. Moreover, R104 of chain B/A forms hydrogen bonds to the main chain carbonyl of A112 and D111 of chain A/B, respectively. Asp115 is part of a large electrostatic network containing several acidic residues (right panel). The side chain of Asp115 shows an increased pK$_a$ value of 6.09 for chain A and of 5.85 for chain B, as determined by the APBS-PDB2PQR software suite.[7] This would allow alteration of the protonation state dependent of the pH and thereby to translate changes in the pH-value into altered conformations. It is postulated that CovS senses acidic shifts of the environmental pH to approximately pH 5.5. Upon protonation of the Asp115 side chains of chainA/B, formation of the salt bridge with R104 in chain B/A is abolished. This might result in alteration of the sensor-domain conformation that is further propagated into the cell, finally activating the histidine kinase activity. The impact of the mutation of M125I is less obvious. It might interfere with the conformation of the SD. The pK$_a$-values were determined by the APBS-PDB2PQR software suite.[7] The figure was created by PyMOL.[8]

(C) The surface of the sensor-domain contains a strong acidic patch. The electrostatic surface potential is shown from -5kT/e (negatively charged, red) to +5kT/e (positively charged, blue). D115 of chain A is part of this acidic patch visible in this representation. The acidic patch of chain B is on the opposite surface area. The figure was created by the APBS plugin in PyMOL.[8]

(D) Residues that are mutated in CovS cluster at the transition from the HAMP-domain to the DHp-domain. The residues E226, M228, and Q4 were found to be mutated, and patch 163-KLET-266 was found to be deleted. This area is important to translate the extracellular activation of the sensor-domain (SD) into the activation of the histidine kinase (HK). To this end, a conformational change might be elicited that results in the rotation of the ⍺-helices of the HAMP- and DHp-domains that bring H280 located in DHp-domain in proximity to the ATP-bound to the HK-domain. Mutation of residues in this area might impair the signal-transduction from the sensor-domain to finally activate the histidine kinase. The figure was created by PyMOL.[8]

(E) Structural organization of the histidine kinase domain. The CovS HK-domain shows all sequence motifs essential for an active histidine kinase. The D-box contains an aspartate, i.e. D425, that directly contacts the exocyclic amino group of the adenine base at C6, thereby creating specificity for adenine nucleotides. The F-box as a central element contains F438. The ATP-lid covers the ATP-molecule. The G-box contains several glycine side chains. This sequence element contacts the ⍺- and ⍺-phosphates and is similar to a P-loop described for other nucleotide binding proteins. It is needed for ATP-binding and forms an oxyanion hole to stabilize a negative charge occurring at the phosphate oxygen during catalysis. G461 within the G-box was found to be mutated to serine. This mutation might directly interfere with ATP/ADP-binding and might impair phosphoryl-group transfer. The stretch from E418 to K432 was found to be deleted. This will render the kinase inactive, as ATP/ADP-binding will be impaired and the HK-fold will be disrupted. Finally, the N-box contains a conserved asparagine, i.e. N396. N396 is involved in coordinating the bound Mg^{2+}-ion needed for nucleotide binding and to allow the nucleophilic attack of the activated H280 for phosphoryl-group transfer. Mg^{2+}-ATP was modelled by superimposing the CovS•CovR structure and the structure 4KP4 by PyMOL.[8] K399 of the N-box forms a salt bridge with D327 of the DHp-domain, thereby positioning the HK-domain in the conformation analyzed here. D327 within the DHp domain was found to be mutated to asparagine. Related histidine kinases have a glutamine at the analogous position. As asparagine and glutamine have similar physicochemical properties but are sterically different, an impact of CovS•CovR function cannot be excluded. Another mutation identified in the HK-domain is K498N at the C-terminus. This residue is solvent-exposed and might not directly affect CovS activity. K is a positively charged residue at physiological pH and might be important for solubility of CovS. Furthermore, lysines were known to be post-translationally modified, i.e. by lysine acetylation. Mutation of K498N would abolish this. The figure was created by PyMOL.[8].

(F) Closeup of the CovS DHp-domain showing position of I332 and the phosphoryl-group acceptor H280 and R283. I332 was found to be mutated to valine. Although this is an exchange of residues with similar physicochemical properties, both being hydrophobic

side chains, they are sterically different. As predicted by marcoil I332 is positioned at position "a" of the coiled-coil heptad repeat [2]. This means that it is directly involved in the formation of the interface of the coiled-coil structure. Exchange of I332 to V, therefore, might affect the coiled-coil structure. As a direct consequence this could affect the position of the phosphoryl-group acceptor H280 and E281 serving as hydrogen bond acceptor orienting and activating H280 for nucleophilic attack on the γ-phosphate of the ATP molecule. Notably, H280 shows a strongly reduced pK_a value of 2.09 (chain A)/2.10 (Chain B) compared to the free amino acid. This shows that H280 is deprotonated in this structural environment and able to act as a strong nucleophile during catalysis. R283 is postulated to be important for phosphoryl-group transfer by contacting the negatively-charged ATP-phosphoryl groups. Similar to an arginine-finger in small GTP-binding proteins, this might improve the nucleophilic attack of H280 on the γ-phosphate by neutralizing the negative charge emerging in the transition state of catalysis. Besides, R383 contacts H280. Deletion of R283 affects the pK_a of H280, shifting it to 5.24 (chain A)/5.18 (chain B). This shows that deletion of R283 decreases nucleophilicity of H280. R283 was found to be deleted in CovS variants. While AlphaFold2 structure predictions (not shown) suggest that deletion of R283 does not affect CovS structure, it will impair catalytic activity of HK. The pK_a-values were determined by the APBS-PDB2PQR software suite.[7] The figure was created by PyMOL.[8]

(G) Structure of the response-regulator CovR bound to histidine kinase CovS. Residues found to be mutated in CovR are shown in dark red. R36 and V128 are positioned towards the solvent. It was found that R36 is mutated to cysteine. Mutation of a solvent exposed residue to cysteine might affect protein function by formation of cysteine disulfide bonds, if occurring under non-reducing conditions. M170 is solvent exposed but might also play a structural role. i.e. as hydrophobic amino acid and by acting hydrogen bond acceptor via bridging water molecules not visible in this structural model. T80 was found to be mutated to alanine. T80 is located not too far away from D53, the phosphoryl-group acceptor site in CovR. The hydroxyl-group at the side chain of T80 might play a structural role, i.e. via formation of hydrogen bonds with bridging water molecule. This cannot be achieved by an alanine side chain. If this manifests in CovS•CovR activity needs to be evaluated. R203 and Y198 are located within the CovR DNA-binding helix. In the CovS-bound state R203 is solvent exposed and Y198 might play a structural role via formation of hydrogen bonds between the side chain hydroxyl and bridging water molecules. The figure was created by PyMOL.[8]

(H) Superposition of the AlphaFold2 CovR•CovS structure with the structure of the response regulator CheY (PDB: 2CHE) suggests that the response-regulator CovR binds Mg^{2+}. The Mg^{2+}-binding site is totally conserved. The Mg^{2+} is hexacoordinated by three water molecules (w1, w2, w3), the side chain of D110, the main chain carbonyl oxygen of M55 (N59 in CheY) and by the side chain of the phosphoryl-group acceptor D53. E9 positions w2 by formation of a hydrogen bond. K102 contacts D53 by formation of a salt bridge. During catalysis the carboxylate of D53 would act as a nucleophile and performs an in-line attack to the phosphoimidazol ring of H280 with H280 acting as a leaving group. It is suggested that the bound Mg^{2+} stabilizes the pentavalent bipyramidal transition state during phosphoryl-transfer reactions. In analogy to CheY, K102 in CovR might be dispensable for the phosphoryl-transfer reaction. However, it might bind to the phosphorylated D53 carboxylate thereby eliciting conformational changes. This might result in dissociation of CovR from CovS adopting a conformation that allows CovR to bind to the target DNA sequence. The figure was created by PyMOL[8].

(I) Overall conformation of the non-complexed, non-phosphorylated CovR dimer as predicted by AlphaFold2. CovR can be separated in a C-terminal DNA-binding domain (aa129-227) with the DNA-binding helix containing R203 and Y198 and a N-terminal receiver (REC)-domain (4-117) that contains D53 that is phosphorylated by the histidine kinase of CovS in order to act as a transcriptional regulator. The conformation shown here is not compatible with DNA-binding at both DNA-binding sites. The figure was created by PyMOL.[8]

(J) Closeup of the DNA-binding site of CovR in complex with DNA. CovR was superimposed with 1GXP. CovR inserts an α-helix into the major groove of the DNA. R203 and Y198 form contacts to the sugar-phosphate backbone. The side chain hydroxyl of Y198 forms a hydrogen bond with the phosphate of the DNA-backbone, R203 forms a salt bridge with the phosphate of the DNA-backbone. In this conformation, both residues would contribute to DNA-binding affinity but not to create sequence specificity. However, binding to DNA might alter the conformation and a crystal structure of CovS in complex with dsDNA would be needed to judge this. R203 was shown to be mutated to serine, which is not compatible with creating the interactions with DNA. Y198 was found to be deleted in CovR variants. Mutation of R203S will likely decrease binding affinity while possibly retaining some DNA-binding. However, deletion of Y198 will most likely result in variant not capable to bind DNA as all residues that mediate DNA-binding, i.e. R200, R203 and K205, are located C-terminally of Y198 and will not be properly oriented to allow DNA-binding. The figure was created by PyMOL[8]

Table S2 Literature review on previously characterized *covR/S* mutations

Amino acid substitutions	CovR/S phenotype	Reference
I332V in CovS	functional	[3]
G461S in CovS	dysfunctional	[4]
E226G in CovS	dysfunctional	[4]
R203S in CovR	dysfunctional	[1]
M1I in CovS	dysfunctional	[10]
T214P in CovS	dysfunctional	[10]
truncation from aa300 in CovS	dysfunctional	[12]
truncation from aa202 in CovS	dysfunctional	[11]

Table S3. Parameters for mass spectrometry

reversed phase liquid chromatography (RPLC)

instrument	Ultimate 3000 RSLC (Thermo Scientific)
trap column	75 μm inner diameter, packed with 3 μm C18 particles (Acclaim PepMap100, Thermo Scientific)
analytical column	Accucore 150-C18, (Thermo Fisher Scientific) 25 cm x 75 μm, 2,6 μm C18 particles, 150 Å pore size
buffer system	binary buffer system consisting of 0.1% acetic acid in HPLC-grade water (solvent A) and 100% ACN in 0.1% acetic acid (solvent B)
flow rate	300 nl/min
gradient	(0min-2% B 2min-5% B 10min-7% B 70min-25% B 75min-40% B 77min-90% B 83min-90% B 85min-2% B 95min-2%B
column oven temperature	40°C
Mass spectrometry	
instrument	Orbitrap Exploris 480
electrospray	Nanospray Flex™ Ion Source
operation mode	data-independent
Full Scan Properties	
MS scan resolution	120000
AGC target	3e6 (300%)
maximum ion injection time for the MS scan	60 ms
Scan range	350 to 1200 m/z
Microscans	1
Polarity	positive
RF Lens	50%
Spectra data type	profile
Dia Properties (MS2)	
Resolution	30,000

maximum ion injection time for the MS/MS scans	auto
Normalized AGC target	3E6
Spectra data type	profile
Microscans	1
isolation window	66
Isolation window width	13 m/z
Window overlay	2 m/z
Fixed first mass	200
HCD collision energy	30%

Table S5 Exact *p*-values of statistical analysis of cytokine secretion by moDCs after caspase-inhibition (n=10). A separate uninfected control with the respective inhibitor treatment was performed for each 5448AP infection. The level of significance was determined using Mann-Whitney *U*-test.

	untreated vs. 5448AP	Cas3 Inh vs. 5448AP	Cas8 Inh vs. 5448AP	PanCas Inh vs. 5448AP	untreated vs. 5448
IL-1β	*p*=0.0045	*p*=0.0385	*p*<0.0001	*p*=0.0055	*p*=0.0045
IFNα	*p*<0.0001	*p*=0.0019	*p*<0.0001	*p*=0.0094	*p*<0.0001
IFNγ	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001
TNF	*p*=0.0020	*p*=0.0286	*p*=0.0007	*p*=0.0036	*p*=0.0007
MCP-1	*p*<0.0001	*p*=0.0002	*p*<0.0001	*p*<0.0001	*p*<0.0001
IL-6	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001
IL-8	*p*=0.0068	*p*=0.0115	*p*=0.0068	n.s.	*p*=0.0003
IL-10	*p*=0.0014	*p*=0.0109	*p*<0.0001	*p*<0.0001	*p*=0.0321
IL-12p70	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001
IL-18	n.s.	n.s.	*p*=0.0014	*p*=0.0084	*p*=0.0021
IL-23	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001
IL-33	n.s.	n.s.	*p*=0.0159	n.s.	*p*=0.0027

Table legends for Additional File 2.

Table S1 CovR/S phenotype of GAS isolates recovered from the INFECT cohort. Type of infection (poly-/monomicrobial), involvement of septic shock, CovR/CovS mutations as identified by bioinformatics, and CovR/S functionality. Data was published in [9] and re-analyzed based on CovR/S functionality. CovR/S functionality was assessed by literature review (Table S2) and protein structure prediction (Fig. S8). Yellow color indicates dysfunctional CovR/S system.

Table S4 Statistical analyses of the abundance patterns of proteins of (un)infected moDCs 6 h and 24 h post indicated infections. Color code: blue: $\log_2$ fold change below 0; red: $\log_2$ fold change above 0.

References

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PAPER III

Neutrophil-derived Reactive Agents Induce a Transient SpeB Negative Phenotype in *Streptococcus pyogenes*

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Conceptualization: PS, ANT, NS; Methodology: PS, TS, JH, KJH, OH, MN, PA, SS, ANT, NS; Investigation: PS, TS, KM, BC, **LAT**, JH, KJH, OH, MN, MS, PA, SS, NS; Visualization: PS, TS, KM, BC, **LAT**, JH, KJH, NS; Funding acquisition: ANT, NS; Project administration: ANT, NS; Supervision: ANT, NS; Writing—original draft: PS, TS, ANT, NS; Writing—review & editing: PS, TS, BC, **LAT**, KM, JH, KJH, OH, MN, MS, PA, SS, ANT, NS.

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RESEARCH

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Neutrophil-derived reactive agents induce a transient SpeB negative phenotype in *Streptococcus pyogenes*

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Abstract

Background *Streptococcus pyogenes* (group A streptococci; GAS) is the main causative pathogen of monomicrobial necrotizing soft tissue infections (NSTIs). To resist immuno-clearance, GAS adapt their genetic information and/or phenotype to the surrounding environment. Hyper-virulent streptococcal pyrogenic exotoxin B (SpeB) negative variants caused by *cov*RS mutations are enriched during infection. A key driving force for this process is the bacterial Sda1 DNase.

Methods Bacterial infiltration, immune cell influx, tissue necrosis and inflammation in patient´s biopsies were determined using immunohistochemistry. SpeB secretion and activity by GAS post infections or challenges with reactive agents were determined via Western blot or casein agar and proteolytic activity assays, respectively. Proteome of GAS single colonies and neutrophil secretome were profiled, using mass spectrometry.

Results Here, we identify another strategy resulting in SpeB-negative variants, namely reversible abrogation of SpeB secretion triggered by neutrophil effector molecules. Analysis of NSTI patient tissue biopsies revealed that tissue inflammation, neutrophil influx, and degranulation positively correlate with increasing frequency of SpeB-negative GAS clones. Using single colony proteomics, we show that GAS isolated directly from tissue express but do not secrete SpeB. Once the tissue pressure is lifted, GAS regain SpeB secreting function. Neutrophils were identified as the main immune cells responsible for the observed phenotype. Subsequent analyses identified hydrogen peroxide and hypochlorous acid as reactive agents driving this phenotypic GAS adaptation to the tissue environment. SpeB-negative GAS show improved survival within neutrophils and induce increased degranulation.

Conclusions Our findings provide new information about GAS fitness and heterogeneity in the soft tissue milieu and provide new potential targets for therapeutic intervention in NSTIs.

Keywords *Streptococcus pyogenes*, Necrotizing soft tissue infections, SpeB, Neutrophils

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†Anna Norrby-Teglund and Nikolai Siemens contributed equally to this work.

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Introduction

Necrotizing soft tissue infections (NSTIs) are rapidly progressing infections of any layer of the skin or soft tissue. The infections are associated with significant morbidity and mortality [22, 39]. Extensive surgical interventions and even amputations are often required despite intensive care and prompt antibiotic therapy [1]. Based on the microbiological etiology, NSTIs are categorized into two main types: type 1 of polymicrobial and type 2 of monomicrobial nature [37]. *Streptococcus pyogenes* (group A streptococci [GAS]) is the major causative pathogen of NSTIs [3, 22]. GAS NSTIs are more frequent among younger individuals without comorbidities, preferably located to the extremities, and often complicated by streptococcal toxic shock syndrome (STSS) [4, 22, 41]. A recent Scandinavian prospective multicenter study called INFECT enrolled 409 surgically confirmed NSTI cases, among which 31% constituted GAS cases [4, 22].

Several studies have shown that GAS NSTIs are characterized by high bacterial load and a hyper-inflammatory response characterized by a massive infiltration of immune cells, including neutrophils and macrophages, and elevated levels of pro-inflammatory molecules [14, 15, 26, 27, 33, 42]. The ability of GAS to cause hyper-inflammation and tissue damage can be attributed to an arsenal of virulence factors. In light of neutrophil activation, several GAS virulence factors such as M-protein and phosphoglycerate kinase (PGK) have been identified as potent inducers of neutrophil activation and degranulation resulting in release of proteolytic enzymes and other effector molecules into the surrounding milieu and subsequent tissue damage [14, 15, 32, 33, 35, 38, 43]. Another important virulence factor is the streptococcal pyrogenic exotoxin B (SpeB), which is a cysteine protease targeting numerous important host substrates such as immunoglobulins, complement, antimicrobial peptides, and interleukin (IL)-1β [25, 37]. Notably, it also has endogenous substrates including several important virulence factors [37]. In the case of PGK, neutrophil activation is only evident in SpeB-negative variants [43]. Such variants have been ascribed hyper-virulent properties and reported to be enriched during infection through mutations in genes encoding CovR/S two-component system and stand-alone regulator RopB resulting in an irreversible loss of SpeB expression [13, 40].

Bacterial killing by neutrophils involves both non-oxidative and oxidative mechanisms. The latter of which is ensured through reactive oxygen species (ROS). ROS are generated via the NADPH oxidase and myeloperoxidase (MPO) systems [47]. Hydrogen peroxide (H_2O_2) is produced by spontaneous or dismutase driven conversion of superoxide. The subsequent MPO-catalysed oxidation of chlorine by H_2O_2 results in formation of hypochlorous acid (HOCl) [24]. MPO is highly abundant within azurophilic granules of neutrophils. Hence, HOCl can be found intracellularly within neutrophils as well as extracellularly [28].

The role of SpeB in NSTI remains elusive. In our previous report [33], we identified that NSTI patient tissue biopsies frequently contained a mixture of SpeB-positive (SpeB$^+$) and SpeB-negative (SpeB$^-$) clones as assessed by proteolytic assays. In this study, we further characterized these clones and through single colony proteomics, we show that the bacteria express but do not secrete SpeB. We further show that this phenotype is induced by the neutrophils, and particularly MPO-derived HOCl and its precursor, H_2O_2, resulting in SpeB-negative GAS clones that survive and multiply within neutrophils, induce excessive degranulation, and thereby contribute to tissue disruptive and hyper-inflammatory processes in NSTIs.

Methods

Bacterial strains

GAS 5448, 5626, 8003, and 8157 are NSTI isolates from Toronto, Canada (provided by Donald E. Low, Mount Sinai Hospital, Toronto, Canada) [16, 19]. All strains were cultured in Todd-Hewitt broth (Carl Roth) supplemented with 1.5% (w/v) yeast extract (Carl Roth) at 37 °C. GAS strains from the INFECT NSTI patients were recovered directly by culture from the tissue biopsies.

SpeB expression and protease activity analyses

The SpeB protease activity was determined as described previously [34]. Briefly, the bacteria were grown in THY (16–18 h), centrifuged, the supernatants were collected and filtered (pore size, 0.20 μm). Supernatants were incubated with 5 mM DTT for 30 min at 37 °C, after which *n*-benzoyl-proline-phenylalanine-arginine-*p*-nitroanilide hydrochloride (1 mM) and phosphate buffer (pH 6; 5 mM) were added and absorbance at 405 nm was detected. Experiments also included testing activity with addition of H_2O_2 (10 and 100 μM). Furthermore, SpeB secretion and activity were tested via casein digestion assay as described previously [21]. Serial dilutions of bacteria were plated on modified Columbia agar containing 3% (w/v) skim milk (both Sigma-Aldrich) following incubation under 37 °C and 5% CO_2 atmosphere for 24 h. SpeB$^+$ producers were characterized by a clearance zone around the colonies, whereas non-producers had no zone of clearance.

For Western blot analyses, bacterial supernatants were precipitated with 96% (v/v) ethanol over night at − 20 °C. Equal amounts of protein were incubated with sodium dodecyl sulfate loading buffer at 90 °C for 10 min. Proteins were separated using a 4–12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The

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membrane was blocked with 5% (w/v) dry casein powder in 0.05% (v/v) Tween-20 Tris-buffered saline. The following antibodies were used for detection: anti-SpeB antibody (Abcam) and secondary anti-rabbit IgG horseradish peroxidase linked Fab fragment (GE Healthcare).

Antimicrobial testing

1×10^6 CFU/ml of bacterial strains were exposed to different concentrations of clindamycin, benzylpenicillin (both Sigma-Aldrich), LL-37 (Invivogen), Lysozyme (Sigma-Aldrich), HNP-1 (Peprotech), MPO, resistin, HBP (R&D Systems), and H_2O_2 (Sigma-Aldrich) for 3 h and plated in serial dilutions on casein agar plates. CFUs were analyzed after 24 h of incubation at 37 °C and 5% CO_2.

Human cells, culture conditions, and infections

The human keratinocyte cell line N/TERT-1 (a gift from J. Rheinwald and the Cell Culture Core of the Harvard Skin Disease Research Centre, Boston, MA, USA) was cultured in EpiLife medium (Invitrogen). Primary normal human dermal fibroblasts (NHDFs) were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) FCS (Invitrogen). Human primary neutrophils were isolated from healthy donors using a density gradient centrifugation on Polymorphprep (Axis Shield). Neutrophil viability was assessed via trypan blue staining. After isolation, neutrophils were suspended in RPMI1640 medium (HyClone) supplemented with 10 mM L-glutamine, 25 mmol/l HEPES (all HyClone), and 5% (v/v) FCS. PBMCs were isolated from buffy coats by Lymphoprep (Axis-Shields) gradient centrifugation. Cells were allowed to adhere in cell culture flasks (Corning) for 30 min at 37 °C in serum free RPMI1640 media. The non-adherent cells were removed by washing with PBS (HyClone). Monocyte-derived macrophages were generated by culturing primary human monocytes isolated from peripheral blood of healthy donors in 6-well plates (Corning) for 9 days at a density of 1×10^6 cells/well. Monocytes were differentiated to pro-inflammatory macrophages for 7 days in cell culture media containing GM-CSF (25 ng/ml), followed by two additional days of incubation with LPS (100 ng/ml). Media was changed every 2–3 days. All cells were cultured under 37 °C and 5% CO_2 atmosphere.

All infections were performed at a multiplicity of infection (MOI) 10 in a final volume of 1 ml of the respective media. Adherence to and internalization into N/TERT-1 cells and NHDFs were quantified using the antibiotic protection assay [23]. Briefly, 24-well plates were inoculated with 2.5×10^5 N/TERT-1 or NHDFs/well without antibiotics. The cells were allowed to grow to confluence. For the assay, cells were washed and infected with GAS (MOI 10). Two hours after infection, the viable counts of

bacteria (colony-forming units, CFU) released from lysed cells were determined by plating on blood agar. For the assessment of bacterial internalization, 2 h after infection, the cells were washed with PBS and incubated with media supplemented with benzylpenicillin (20 µg ml^{-1}) and gentamicin (120 µg ml^{-1}) for additional 2 or 4 h. Subsequently, the cells were washed, lysed, and the CFU counts were determined as described above. For the assessment of SpeB release and activity, intracellular bacteria were plated on casein agar plates.

1×10^6 macrophages were infected for 2 h following 1 h of antibiotic treatment and 5×10^5 neutrophils were infected for 1 h following antibiotic treatment. Assessment of intracellular bacterial numbers was performed as described above. For MPO inhibition, different concentrations of 4-aminobenzoic hydrazide (Sigma-Aldrich) were used: 25, 50 and 100 µM. After isolation, neutrophils were suspended in RPMI media with MPO inhibitor and incubated at 37 °C for 30 min prior to infections. All infections were performed in the presence of the inhibitor. Bacterial survival was evaluated 1, 2 and 4 h post infections. For proteomics, all infections were performed in Hank's balanced salts solution (HBSS, Invitrogen). Neutrophil supernatants were collected and stored at −20 °C until analysis.

Immunostaining and analysis of tissue biopsies

Patient biopsies were cryosectioned (5–8 µm) using a MICROM cryostat HM 560 MV (Zeiss), fixed in 2% (v/v) formaldehyde or ice-cold acetone, and immunostained as previously described [33, 35]. The following antibodies were used for immunohistochemistry: anti-human HMGB1 (clone EPR3507; Abcam), anti-human IL-8 (clone NAP-1; Invitrogen), anti-human resistin (clone 184,305; R&D systems), and anti-human neutrophil-elastase (clone NP57; DAKO). Biotinylated secondary antibodies included goat anti-mouse IgG and goat anti-rabbit IgG (both from Vector Laboratories). The immunohistochemically stained sections were analyzed by acquired computerized image analysis (ACIA) [20]. The cell area was defined by the hematoxylin counterstaining, and the results are presented as percent positively stained area × mean intensity of positive staining.

The following antibodies were used for immunofluorescence analyses: anti-MPO (MAB3174; R&D), anti-GAS (9191; Abcam). Secondary antibodies included anti-mouse IgG AF488, anti-goat IgG AF594, and anti-rabbit IgG AF594 (all Invitrogen). IF-images were acquired with Olympus BXS1 microscope and Olympus cellSense software (Olympus) and/or Leica Stellaris 8 confocal laser scanning microscope (CLSM) and LasX software (Leica Microsystems). Four to six images were taken per biopsy. Analysis of the micrographs was performed in a

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two-step procedure, which included an automated recognition of GAS and MPO stainings followed by a calculation of the stained area (px^2). Each picture was manually checked and if necessary corrected after an automated recognition.

Whole genome sequencing and data processing

DNA extraction from GAS strains 5448, 5626, 8003, and 8157, whole genome sequencing, and analyses were performed as previously described [36]. Detailed description is provided in the Additional file 1.

Protein extraction, LC–MS/MS analyses, and data processing of single GAS colonies and neutrophil secretome

Neutrophil secretome was determined as previously described [8]. Detailed description of protein extraction, measurements, and analyses are provided in the Additional file 1.

Statistics

If not otherwise indicated, statistical significance of differences was determined using the 2-tailed Mann–Whitney *U* test. Multiple comparisons were done using Kruskal Wallis test with Dunn's post-test. Correlation analyses were determined using Spearman test. Statistics were performed using GraphPad Prism version 7 (GraphPad software). A *p*-value less than 0.05 was considered significant.

Results

Reversible loss of SpeB proteolytic activity by GAS is associated with tissue pathology and inflammation

Bacteria were isolated from tissue biopsies collected from GAS patients (Additional file 2: Table S1) through direct plating of tissue sections on casein agar plates to assess SpeB activity. These analyses confirmed previous observations that infected tissue harbors a mixture of SpeB$^+$ and SpeB$^-$ clones (Fig. 1A). The majority of successor generations of SpeB$^-$ clones returned to a wild-type-like SpeB$^+$ phenotype after cultivation in THY media (Fig. 1B and Additional file 1: Fig. S1). To confirm that the observed SpeB$^-$ phenotype is not linked to genetic variations within mutational hotspots (*covS*, *covR*, and *ropB*) or *speB*, comparative whole genome sequence analyses were performed. Although strains 2001, 2006, 6016, and 6018 were exclusively SpeB$^-$ post direct tissue isolation (Fig. 1A), no mutations in these strains were found. In general, the analyses of these hotspots revealed only minor variance on nucleotide and amino acid levels (Additional file 1: Fig. S2-S4). CovR amino acid sequences were 100% identical between all strains. In strain 2002, *covS* initiated with the rare start codon ATA [11] and a

frame shift causing single nucleotide deletion in position 381 was detected (Additional file 1: Fig. S2). Most of the mutations were found in strain 6028, *e.g.,* mutations within *covS* leading to several amino acid substitutions and a notable deletion of 45 nucleotides (Additional file 1: Figs. S2 and S3). Furthermore, single mutations leading to amino acid substitution within RopB were found in strains 2002, 5004, 6026, and 6028 (Additional file 1: Fig. S4). *SpeB* gene itself was unaffected. Additional exemplary sequence analyses of other TCS kinases/regulators identified rather *emm*-type specific mutations resulting in conserved amino acid substitutions, which would most likely not affect SpeB expression (Additional file 2: Table S2). These results suggest that in addition to irreversible loss of SpeB expression due to mutations in *covR/S* or *ropB* regions, reversible abrogation of SpeB expression at protein or activity level occurs in the tissue setting.

The observation of heterogeneity in SpeB proteolytic activity led us to explore how this related to tissue inflammation and damage. For this purpose, biopsies collected from repeated surgeries at different sites of infection (*e.g.*, fascia, soft tissue, and muscle) of 31 GAS NSTI patients were analyzed for the presence of markers of tissue necrosis [high-mobility-group-protein B1 (HMGB1)], inflammation (IL-8), neutrophil infiltration [neutrophil elastase (NE)], and neutrophil degranulation (resistin; Additional file 1: Fig. S5). Correlation analyses based on bacterial load and the percentage of SpeB$^-$ clones recovered from the respective tissue specimen with each of the markers were performed. All of the analyzed markers showed a positive correlation to bacterial load (Fig. 1C–F). Moreover, positive correlations between tissue necrosis, inflammation, and neutrophil infiltration as well as degranulation with increasing percentage of SpeB$^-$ clones, irrespective of their rise through genetic or phenotypic variations, were found (Fig. 1C–F).

Intracellular GAS recovered from neutrophils show reversible loss of SpeB proteolytic activity

Based on our data showing the mixed SpeB phenotype particularly in the tissue milieu, we sought to identify which factors might be driving this process. Due to increased variability in SpeB phenotype of freshly isolated GAS NSTI strains, four well-characterized strains of the two dominant NSTI *emm*1 and *emm*3 types (*emm*1 strains 5448 and 8157 and *emm*3 strains 5626 and 8003) were selected. These strains displayed stable SpeB$^+$ and SpeB$^-$ phenotypes, respectively. All colonies of 5448, 8157 and 5626 showed a clearance zone, while colonies of 8003 did not. (Fig. 2A). Analyses of supernatants from overnight cultures showed that SpeB was readily detectable in 5448, 8157, and 5626 (Fig. 2B,
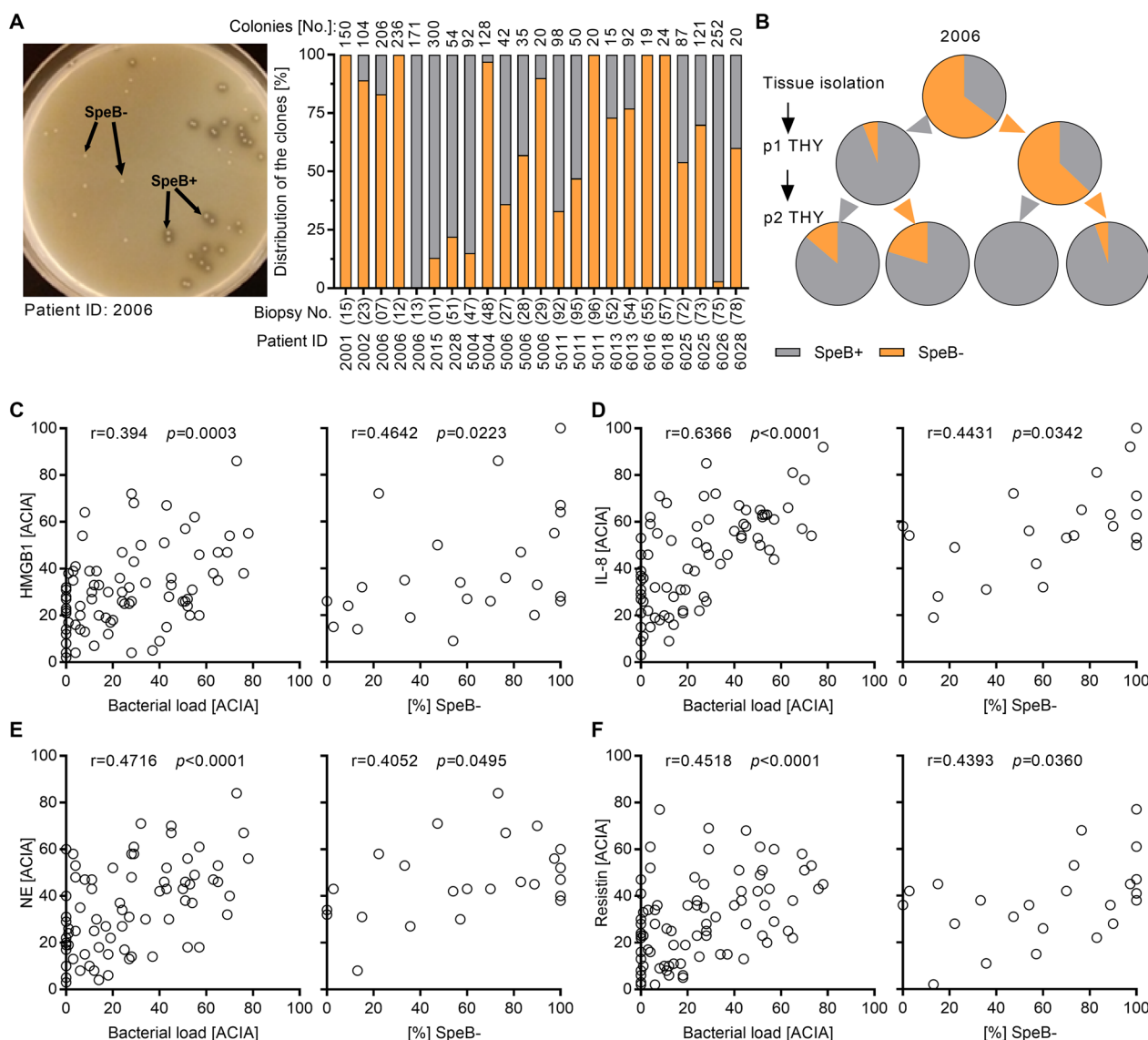
Fig. 1 Reversible loss of SpeB is associated with tissue pathology and inflammation. **A** Distribution of SpeB$^+$ and SpeB$^-$ GAS clones directly isolated from NSTI patient tissue biopsies (n = 23). **B** Percentage of SpeB$^+$ and SpeB$^-$ GAS clones after the passage in THY media (p1, passage 1; p2, passage 2). Representative analysis of 2006 GAS patient isolate is shown. **C**–**G** Correlation analysis of bacterial load (left panel; n = 81 biopsies) or percentage of SpeB$^-$ clones (right panel; n = 23 biopsies) with the presence of HMGB1 (**C**), IL-8 (**D**), infiltrating neutrophils (**E**), and resistin (**F**) in patient biopsies. Correlation was determined using Spearman test. Semiquantitative acquired computerized image analyses (ACIA) of immuno-histochemical staining were performed as described in the methods section. The cell area was defined by the hematoxylin counterstaining, and the results are presented as percent positively stained area × mean intensity of positive staining

Additional file 1: Fig. S8). This was further verified in a SpeB proteolytic assay (Fig. 2C). Next, the strains' whole genome were sequenced and comparative analyses with annotated genomes of M1GAS (SF370, *emm*1) and MGAS315 (*emm*3) were performed. In general, rather low numbers of sequence variants were identified within the genomes (Additional file 1: Fig. S6). Analyses of mutational hotspots responsible for loss of SpeB expression identified a mutation in *covS* of 8003

strain resulting in $T_{214}P$ substitution (Additional file 1: Fig. S7).

Next, using the four strains with stable SpeB$^+$ and SpeB$^-$ phenotype, different human skin and soft tissue cell types, including keratinocytes, primary fibroblasts and immune cells (primary macrophages and neutrophils) were infected with GAS strains followed by assessment of infectivity, as well as SpeB activity by single colonies recovered from the intracellular compartment.
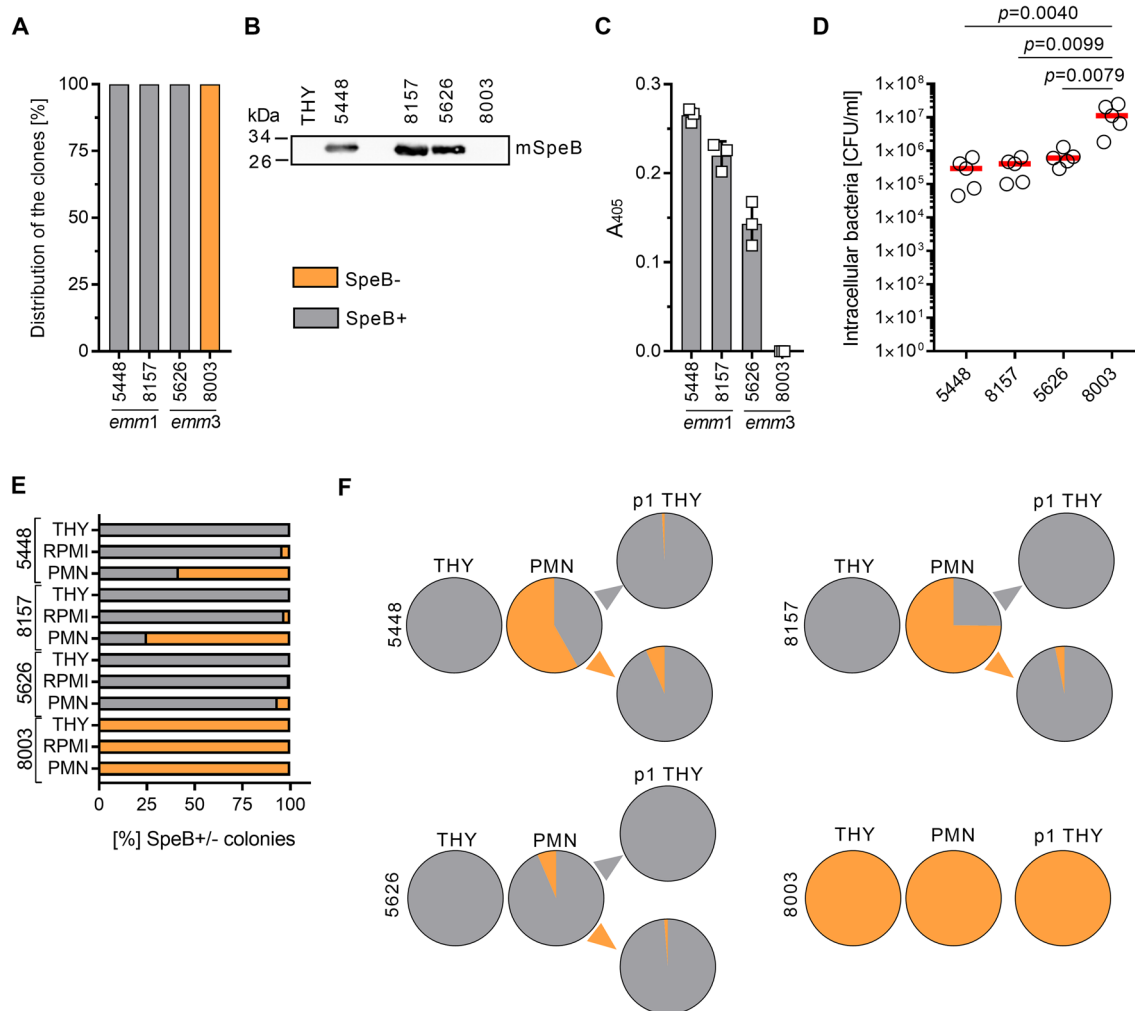
Fig. 2 Human neutrophils induce loss of SpeB in GAS. **A** Assessment of SpeB positivity/negativity of indicated strains via casein agar assay. Mean percentage from four independent experiments is shown (n=4). **B** Representative image of *Western*-Blot analyses (n=3; mSpeB, mature SpeB) and (**C**) SpeB activity assay (n=3) of bacterial supernatants. GAS were grown over-night (16 h) in THY and sterile-filtered supernatants were used for the analyses. Original blot is shown in Additional file 1: Fig. S8. Each dot in **C** represents one independent experiment. Bars denote mean values ± SD. **D** Intracellular bacterial counts after 1 h of neutrophil infection. Each dot represents an experiment with neutrophils from one donor. The horizontal lines denote median values (n=5). The level of significance was determined using Kruskal–Wallis test with Dunn's multiple comparison post-test. **E** Assessment of SpeB positivity/negativity of indicated strains recovered from primary human neutrophils shown in **D**. Mean percentage of SpeB⁺ and SpeB⁻ clones from five independent experiments are shown (n=5). Bacteria incubated in RPMI and THY media served as controls. **F** Distribution of SpeB⁺ and SpeB⁻ clones post neutrophil (PMN) and subsequent THY media passage. Displayed are mean [%] of five independent experiments (n=5)

Since clindamycin and benzylpenicillin are used to treat GAS NSTIs, control experiments with these two antibiotics were also performed (Additional file 1: Fig. S9). No differences in adherence to or internalization into keratinocytes and fibroblasts between the strains were detected (Additional file 1: Fig. S9A-B and D-E). In contrast, while the bacteria were multiplying within keratinocytes, reduced numbers of bacteria were recovered from fibroblasts over time. Only minor effects on SpeB of these two cell types were seen (Additional file 1:

Fig. S9C, F). Next, primary macrophages and neutrophils were infected and intracellular bacteria were recovered. No differences in bacterial numbers were seen in macrophage infections (Additional file 1: Fig. S9G). In contrast, significantly higher numbers of SpeB⁻ 8003 strain as compared to other three SpeB⁺ stains were recovered from neutrophils (Fig. 2D). Infection of both myeloid cell types resulted in loss of SpeB activity on casein agar (Fig. 2E and Additional file 1: Fig. S9H). However, this phenotype was more pronounced in *emm*1 strains,

where more than 50% of the colonies were SpeB⁻ when exposed to neutrophils (Fig. 2E). Subsequent passaging of single colonies from neutrophil experiments showed that the majority of successor generations of SpeB⁻ clones returned to a wild-type-like SpeB⁺ phenotype (Fig. 2F). Bacteria exposed to minimal inhibitory concentrations of antibiotics retained its original SpeB⁺ or SpeB⁻ phenotype (Additional file 1: Fig. S9I-K). These results demonstrate that the reversible loss of SpeB activity is seen among intracellular GAS, particularly in neutrophils, and consequently, might represent an adaptation strategy of GAS to the intracellular environment of neutrophils.

SpeB accumulates intracellularly in GAS as a response to intracellular environment of neutrophils

To verify if the SpeB⁻ phenotype is characterized by the transient loss of SpeB expression, an impaired secretion or loss of proteolytic activity a series of experiments was performed.

First, relative *speB* transcription in single colonies of the 5448 strain post neutrophil infection was determined. *SpeB* transcription was detected in both SpeB⁺ as well as in SpeB⁻ colonies, albeit weaker in the latter (Fig. 3A).

Second, the proteome of SpeB⁺ and SpeB⁻ single colonies recovered from intracellular bacteria in neutrophils was quantitatively profiled by mass spectrometry. Bacteria cultured in THY media resulting in 100% SpeB⁺ served as controls. In total, up-to 752 proteins were identified in single colonies (Additional file 2: Table S3). Principal component analysis showed that all three groups (THY vs. PMN SpeB⁺ vs. PMN SpeB⁻) separate from each other, suggesting that each of them is characterized by a distinct proteome profile (Fig. 3B). Next, protein expression patterns of SpeB⁻ colonies post neutrophil infections were compared either to SpeB⁺ from the same experiment or to colonies from THY control (Fig. 3C, D). Notably, SpeB was found in all samples. In fact, SpeB⁻ GAS colonies accumulated higher amounts of SpeB intracellularly, as compared to the SpeB⁺ colonies or THY controls (Fig. 3C, D). Alignment of detected peptides with the amino acid sequence of SpeB revealed that SpeB zymogene was exclusively present intracellularly (Additional file 1: Figure S10). In addition, other secreted proteins, including DNaseB and immunogenic secreted protein 2 (Isp2) and surface anchored antiphagocytic M1 protein as well as C5a peptidase (ScpA), were found in higher abundance in SpeB⁻ colonies. Identical experiments were performed with SpeB⁻ 8003 strain. 8003 remained SpeB⁻ post neutrophil challenge and SpeB was not detected (Additional file 1: Fig. S11 and Additional file 2: Table S4).

Third, we sought to determine which neutrophil-derived components might be responsible for the observed reversible SpeB⁻ phenotype. Therefore, an initial screen with strain 5448, which stably expresses and releases SpeB, was performed using antimicrobial peptides, lysozyme, and granule components (HBP and resistin). None of the tested agents had an impact on SpeB release/proteolytic activity by bacteria (Additional file 1: Fig. S12). Next, 5448 was exposed to different concentrations of hydrogen peroxide (H_2O_2). High concentrations of H_2O_2 (1 mM) caused a 1-log reduction in bacterial counts, while no effect on CFU were noted in the presence of 10–100 µM (Fig. 4A). Exposure of 5448 strain to these sub-lethal concentrations of H_2O_2 resulted in increased frequency of SpeB⁻ clones in up to 32% of the total GAS population (Fig. 4B). Since MPO converts the majority of available H_2O_2 intracellularly to HOCl, we next exposed strain 5448 to different concentrations of HOCl. Up to 70% of GAS did not secrete SpeB upon exposure to sub-lethal concentrations of HOCl (Fig. 4C, D). However, NaCl exposure, the source of Cl⁻, induced a similar bacterial phenotype although not to the same extent. To verify this result in a more complex setting, human primary neutrophils were infected with the 5448 strain in the presence of an MPO inhibitor and bacterial killing as well as SpeB positivity were assessed over time. To exclude potential masking effects due to the time-dependent delay in abrogated SpeB-secretion, 5448AP strain was used as a stable SpeB⁻ control. In the infection model, reduced numbers of 5448 strain were recovered from neutrophils one-hour post infection (Fig. 4E). However, increased bacterial survival was noticed over a period of the next three hours if low concentrations of MPO-inhibitor (0–50 µM) were used. Four hours post infections almost equal numbers of bacteria compared to the initial infection inoculum were recovered (Fig. 4E). High concentrations of MPO inhibitor (100 µM) reversed this effect and an increased killing of bacteria was observed over time (Fig. 4E). In congruence with those observations, the amount of SpeB⁻ clones significantly increased over the infection period. MPO-inhibition partially reversed this effect, particularly evident at 2 h post infections (Fig. 4F). In contrast, neutrophil infections with stable SpeB⁻ 5448AP strain were characterized by a continuous intracellular multiplication (Additional file 1: Fig. S13A). As expected, 5448AP remained SpeB⁻ over the entire period of infections (Fig. S13B). No MPO-inhibitor mediated effects on bacterial growth were observed (Additional file 1: Fig. S13C).

Finally, to assess whether the secretion or only the activity of SpeB were impaired by exposure of the bacteria to H_2O_2 within neutrophils or in liquid cultures, 5448 strain was (i) plated on casein agar containing H_2O_2 and (ii) SpeB presence as well as activity were determined in the presence/absence of H_2O_2 in bacterial supernatants.
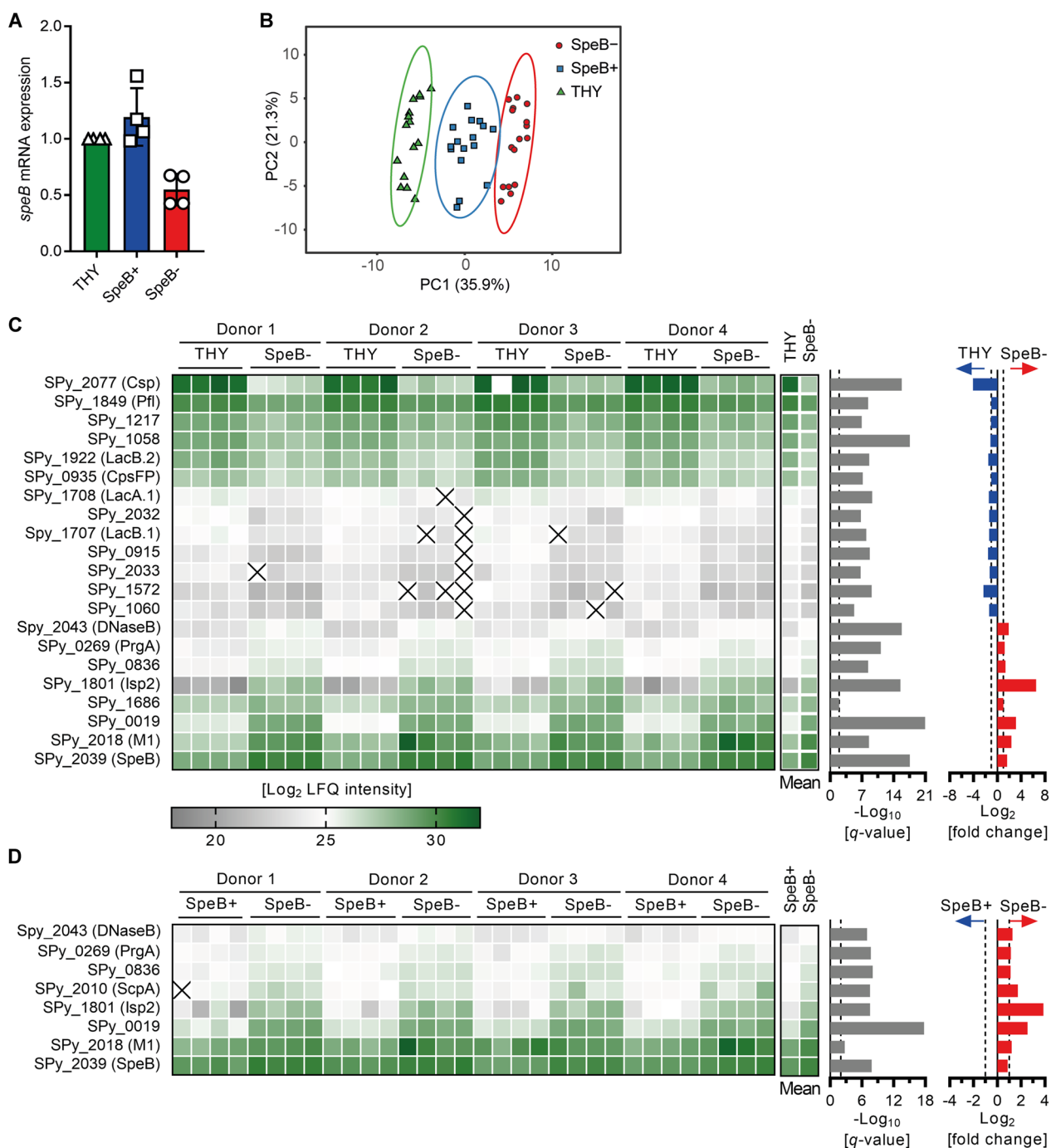
Fig. 3 Distinct single colony proteome profile of GAS post neutrophil infection. **A** Relative *speB* mRNA expression in 5448 single colonies post neutrophil infections. Each dot represents one colony (n = 4). Bars denote mean value ± SD. **B** Principal component analysis of the proteome of single SpeB⁻ and SpeB⁺ colonies post neutrophil infections. GAS incubated in THY media, which remained 100% SpeB⁺ were used as controls. Each dot represents proteome analysis of one GAS colony (n = 16). **C, D** Heat map of differentially expressed proteins on a single colony level. Displayed are Log₂ LFQ intensities (left panel), −Log₁₀ *q*-value (middle panel), and Log₂ fold change of the mean (right panel). Each column represents one single colony (four per condition and donor; n = 16 in total). **C** Protein abundance of THY vs. SpeB⁻ colonies and **D** SpeB⁺ vs. SpeB⁻ colonies are displayed (×, not detected)

These analyses revealed that H_2O_2 does not impair SpeB activity but secretion in a subpopulation of the bacteria as shown via casein agar plating revealing negative colonies as well as Western blot analysis showing reduced levels of SpeB post H_2O_2 treatment (Fig. 4G–J; Additional file 1: Fig. S14).

Enhanced neutrophil degranulation and presence of MPO in tissue biopsies with increased SpeB⁻ variants

Since MPO converts major amounts of H_2O_2 to HOCl, the presence of this molecule was determined in patient tissue biopsies based on the initial assessment of SpeB-positivity/negativity. Multiple tissue sections were stained for GAS and MPO, and analyzed microscopically (Fig. 5A, Additional file 1: Fig. S15). Confocal laser scanning microscopy (CLSM) and subsequent image analyses revealed that although equal bacterial load is detectable in both types of biopsies (SpeB⁻ vs. SpeB⁺), higher amounts of MPO were detected in biopsies associated with SpeB⁻ GAS clones (Fig. 5B, C). However, as evident by the micrographs, these biopsies are characterized by extensive necrosis resulting in a relatively diffuse DAPI staining (Fig. 5A). This is a characteristic of these infections and the biopsies represent tissue that are clinically indicated for surgical removal and will hence be associated with necrosis.

Both, *in vitro* as well as *ex vivo* analyses suggest that SpeB secretion is abrogated in GAS in response to H_2O_2 and HOCl, which subsequently triggers increased neutrophil activation resulting in enhanced tissue pathology. Therefore, we next assessed neutrophil activation on a global level. Secretome composition of neutrophils post 5448 infections was quantitatively profiled by mass spectrometry. Again, to exclude time-dependent delay in abrogated SpeB secretion of this strain, the SpeB⁻ 5448AP strain, which encodes truncated *covS* transcript effecting

expression of several virulence factors (Sda1, capsule, M1-protein) [45], was used as a control. To ensure that potential alterations in secretome composition and protein abundance were not caused by differences in cytotoxicity, LDH assay was performed. Although slightly increased cytolytic events were detected over time, no differences were seen between 5448 and 5448AP infections (Additional file 1: Fig. S16). Up to 495 secreted proteins/peptides were detected in neutrophil supernatants post infections (Additional file 2: Table S5), suggesting that both, SpeB⁺ as well as SpeB⁻ strains, activate neutrophils. However, granule content release was more pronounced in infections with SpeB⁻ 5448AP strain over time (Fig. 6A, B). Comparison of neutrophil degranulation in response to 5448 vs. 5448AP strain showed that 103 proteins/peptides were more abundant in neutrophil secretome of SpeB⁻ 5448AP infections (Fig. 6C–E). Among these, several tissue degrading enzymes, including neutrophil elastase (ELANE) and metalloproteases (MMP8, MMP9) were detected. PCA revealed that neutrophil secretome of both infections is distinct at 4 h post-infections (Fig. 6F). Subsequent pathway analyses by *Reactome* profiler showed that the SpeB⁻ 5448AP strain activates several neutrophil specific pathways to a higher extent as compared to the 5448, including degranulation and immune responses (Fig. 6G, Additional file 2: Table S6). Sub-analyses of neutrophil cellular compartments showed that protein/peptides of all four granules were released in higher abundance in response to the SpeB⁻ 5448AP infections (Fig. 6H).

Discussion

Phenotypic and genetic adaptations of GAS to the tissue environment are key survival mechanisms to establish a successful infection. In this study, we show that GAS reversibly abrogate SpeB secretion in the tissue setting

(See figure on next page.)

Fig. 4 H_2O_2 and HOCl impair SpeB secretion by GAS. **A** GAS 5448 strain was exposed to indicated concentration of hydrogen peroxide (H_2O_2) and bacterial numbers were determined 1 h post stimulations. Each dot represents one independent experiment. Lines denote mean values (n = 5). **B** Assessment of SpeB positivity/negativity of 5448 strain post H_2O_2 treatment as displayed in **A**. Mean percentage from five independent experiments is shown (n = 5). **C** GAS 5448 strain was exposed to indicated concentration of HOCl and bacterial numbers were determined 1 h post stimulations. Each dot represents one independent experiment. Lines denote mean values (n = 5). **D** Assessment of SpeB positivity/ negativity of 5448 strain post HOCl treatment as displayed in **C**. Mean percentage from five independent experiments is shown (n = 5). **A**–**D** Untreated bacteria in THY media, acidified NaCl or Na_3PO_4 served as controls. **E** Human primary neutrophils were infected with GAS strain 5448 and intracellular bacteria were determined by plating serial dilution of neutrophil lysates on casein agar plates post indicated time points. The MPO activity was inhibited by indicated concentrations of MPO inhibitor 30 min prior and during the entire infection period. Dots represent the median value ± range of independent experiments with five donors (n = 5). **F** Assessment of SpeB positivity/negativity of 5448 strain recovered from primary human neutrophils shown in **E**. Mean percentages of SpeB⁺ and SpeB⁻ clones from five independent experiments are shown (n = 5). **G** Representative images of GAS 5448 directly plated on casein agar containing indicated concentrations of H_2O_2 and **H** subsequent analyses. Mean percentage from four independent experiments is shown (n = 4). **I** SpeB activity assay (n = 4) of bacterial supernatants. GAS were grown over-night (16 h) in THY and sterile-filtered supernatants supplemented with indicated concentrations of H_2O_2 were used for the analyses. **J** Western blot (upper left panel), silver staining of the loading control (upper lower panel), and image analyses (right panel) of GAS 5448 supernatants post exposure to indicated concentrations of H_2O_2. Representative images and analysis of three independent experiments are shown (n = 3). The level of significance between the groups of experiments presented in **A**–**H** was determined using Kruskal Wallis test with Dunn's posttest. The level of significance in **J** was determined using Friedman test
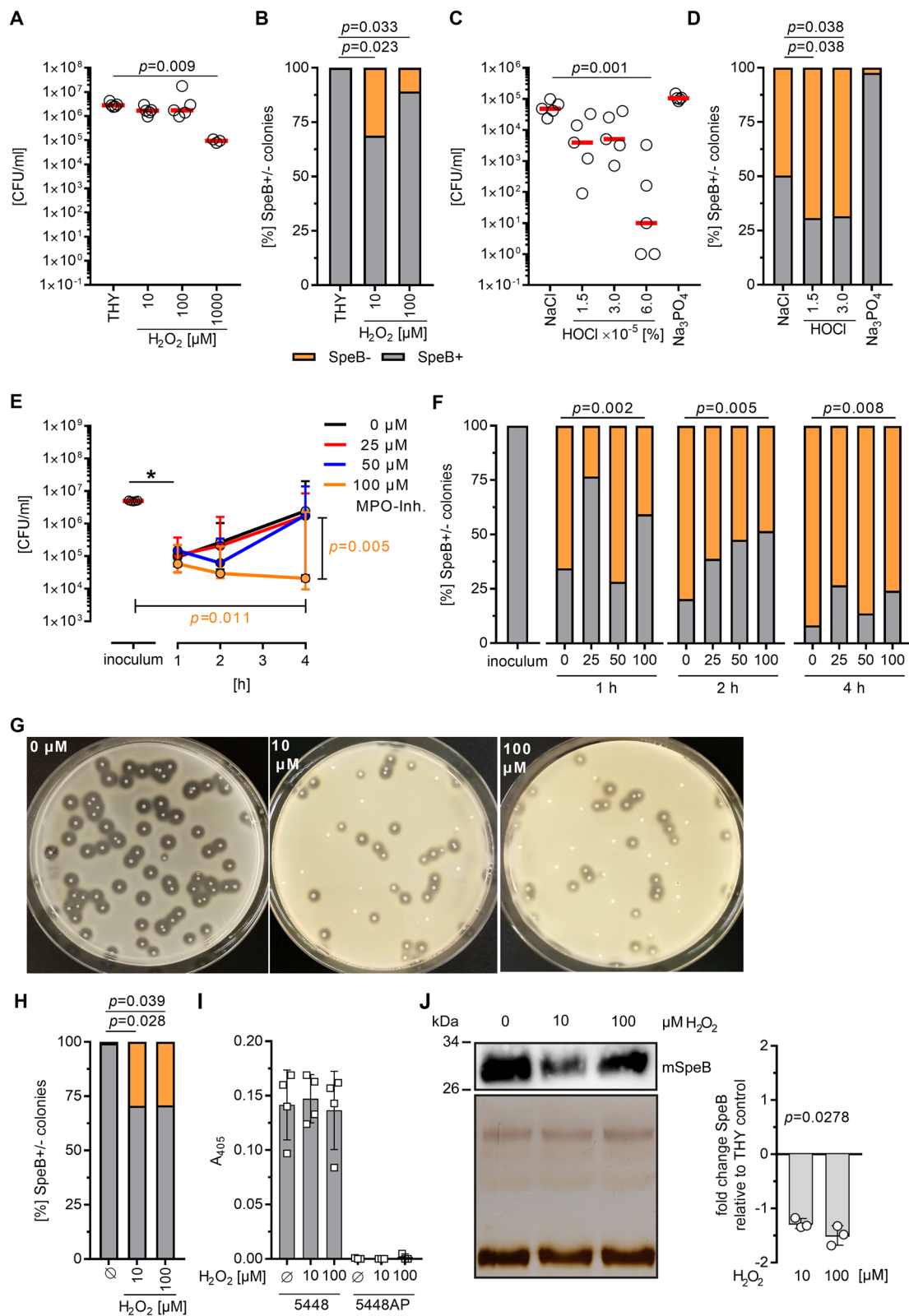
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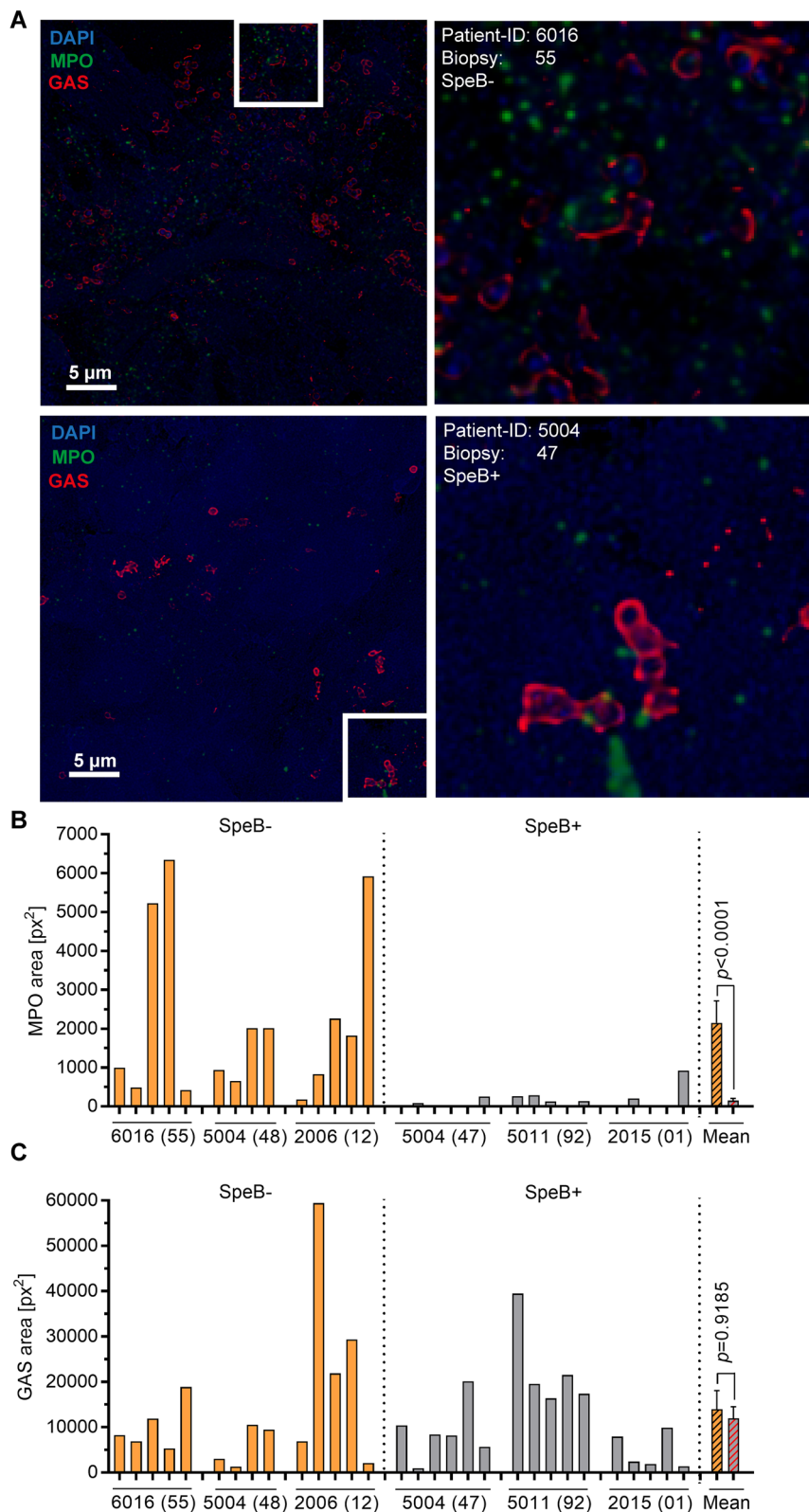
Fig. 5 Increased levels of MPO in patient tissue biopsies associated with SpeB⁻ GAS. **A** Representative immunofluorescence micrographs of the distribution of MPO in patient biopsies are shown. **B** Image analyses of MPO and **C** GAS stainings. Calculation of the stained area (px²) per analyzed micrograph is presented. Each bar within diagrams denotes analysis of one image from indicated tissue biopsies. Bars on the right of each diagram denote mean values ± SD. The level of significance between the groups was determined using 2-tailed Mann–Whitney *U* test
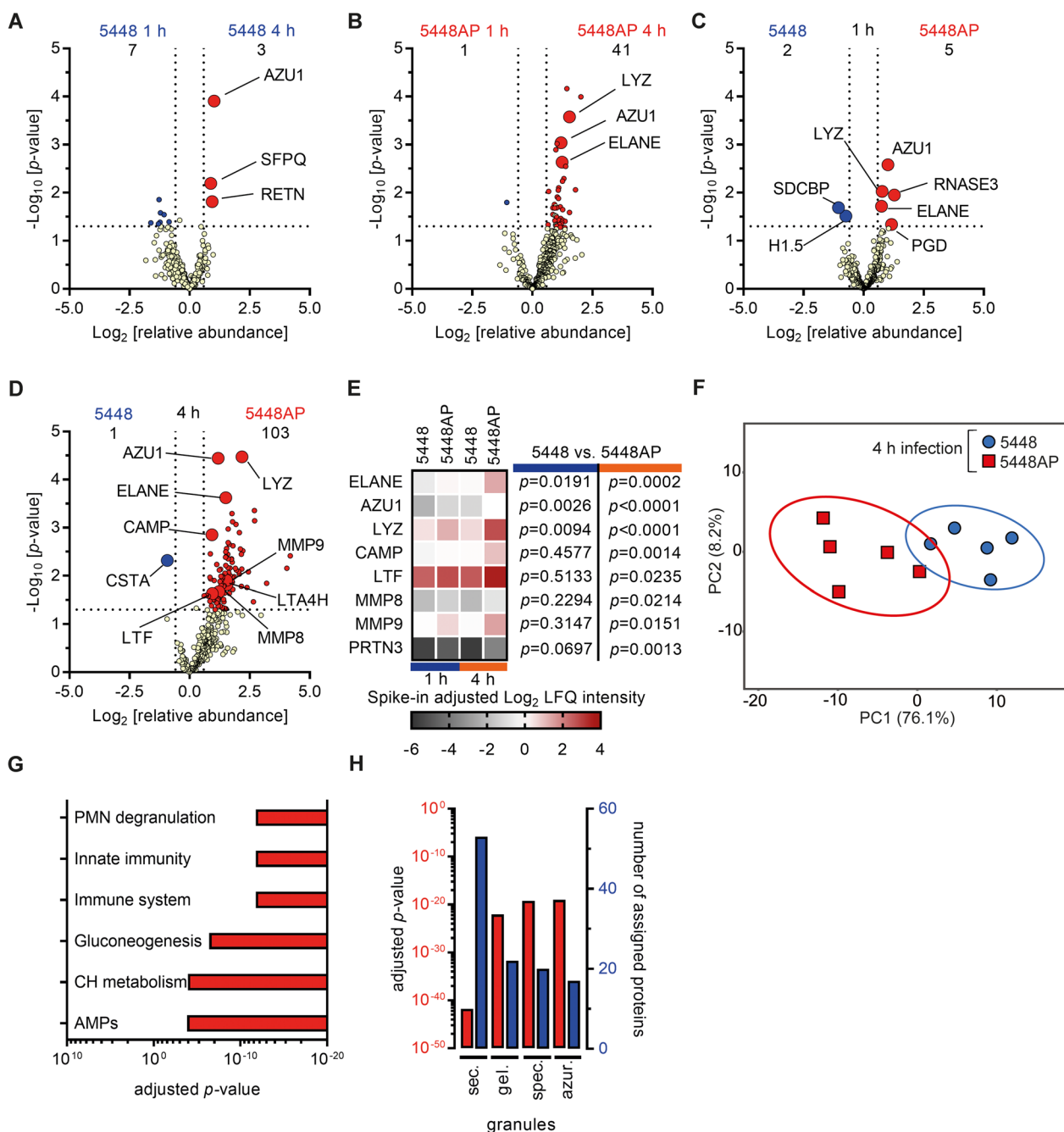
Fig. 6 Hyper-responsiveness of human neutrophils induced by SpeB-negative GAS. **A, B** Time-dependent and **C, D** strain-dependent comparison of secretome profiles of human neutrophils exposed to 5448 or 5448AP strains. Displayed are changes in relative abundance and *p*-values of the original analyses in Additional file 2: Table S5 (n = 5). **E** Heat map highlighting some of the significant differences of protein/peptide abundance in neutrophil secretomes from (**A**–**D**). **F** Principal component analysis of secreted proteins post 4 h of infection of indicated strains. Each dot represents one donor (n = 5). The ellipses indicate the calculated 95% probability region for a bivariate normal distribution with an average center of groups. **G** Top six upregulated pathways in 5448AP infections of primary neutrophils as compared to 5448 infections. Displayed are adjusted *p*-values as determined by functional profiling in the *Reactome* database (CH, carbohydrate; AMPs, antimicrobial peptides). **H** Cellular compartment analyses of protein/peptides found in higher abundance in neutrophil secretomes of 5448AP infections as compared to 5448 infections. Displayed are adjusted *p*-values (left red axis, red bars) and number of proteins (right blue axis, blue bars). Functional profiling was performed using *Reactome* database (sec., secretory; gel., gelatinase; spec., specific; azur., azurophilic)

resulting in improved survival of the bacteria and subsequently in higher tissue inflammation and pathology in NSTI.

Bacteria are highly adaptable species. Their genetic information and other structures are capable of alterations. Some of them are reversible when a particular pressure is lifted, whereas other alterations are maintained [7]. GAS have adapted several strategies to survive within the hostile environment in NSTIs [18, 37, 48]. One of them is the enhanced mutational rate within the *covR/S* and *ropB* genes resulting in SpeB-negative clones. Studies on these two mutational hotspots are inconclusive with respect to impact on virulence. Some studies show that these variants are less virulent, while others demonstrated that natural *covR/S* mutants are hyper-virulent [6, 45]. In humans, it was shown that SpeB is readily detectable in sera and tissues of NSTI patients [10, 16, 33] and a mixture of SpeB$^+$ and SpeB$^-$ GAS clones is usually seen [16, 33]. Here, we demonstrate that in addition to the irreversible loss of SpeB, due to genetic mutations, a subpopulation of GAS reversibly abrogates SpeB secretion in the tissue setting. This phenomenon is more pronounced in *emm*1 strains. Single colony proteomics showed that SpeB accumulates intracellularly and is not released. Once the tissue pressure is lifted, GAS regain the SpeB secreting function. Tissue necrosis, inflammation, neutrophil influx and degranulation are enhanced in patient biopsies associated with increased amount of SpeB$^-$ GAS clones. Our data further confirm previous observations that neutrophil activation and degranulation are the main hallmarks of GAS NSTIs [14–16, 38]. These pathologic processes seem to be further intensified in biopsies with higher frequencies of the SpeB$^-$ GAS. Notably, PGK, which has been demonstrated to be a potent activator of neutrophils, is highly susceptible to SpeB proteolysis and its activity can only be seen in SpeB$^-$ strains [43]. Also, Zhu et al. reported that loss of SpeB resulted in increased fitness of *emm*1 GAS in a non-human primate model of necrotizing myositis [48]. Furthermore, it can be noted that in the non-human primate model neutrophil activation and degranulation was associated with tissue pathology [48].

It is widely accepted that *covR/S* mutations and the consequent total loss of SpeB result in upregulated expression of several virulence factors, which are susceptible to SpeB degradation, including Sda1, M protein, PGK, and streptolysin O. All of them facilitate pathogen's escape from neutrophils and other immune cell types [17, 45]. Notably, GAS single colony proteomics demonstrated that in comparison to SpeB$^+$, SpeB$^-$ clones from the same neutrophil infections are characterized by enhanced accumulation of surface anchored M1 protein and C5a peptidase and the secreted virulence

factors DNaseB and Isp2. All of these factors are of crucial importance in immune system evasion and are implicated in hyper-inflammatory processes [32, 37]. In addition, Isp was recently reported to contribute to necrotizing myositis [18]. Therefore, we speculate that transient SpeB$^-$ clones might persist in the tissue for a longer period and subsequently contribute to excessive degranulation of neutrophils and hyper-inflammation. This fact is further supported by neutrophil secretome analyses. Increasing abundance of granule proteins was observed in 5448 infections over time. To rule out time-dependent effects, infections with stable hyper-virulent SpeB$^-$ 5448AP strain were performed. It should be emphasized that 5448AP harbors a global regulatory mutation which affects not only SpeB secretion. This strain is further characterized by an enhanced expression of antiphagocytic M1 protein and secretion of Sda1 and Streptokinase, among others [45]. In congruence with our data, it was shown that 5448AP strain limits production of ROS, thereby ensuring it survival [46]. In contrast to the report by Williams and colleagues [46], our analyses revealed that the SpeB$^-$ 5448AP strain activates neutrophils to a higher extent as compared to its parental SpeB$^+$ 5448 strain. In line with this, higher abundance of resistin and MPO was detected in biopsies associated with increased amounts of SpeB$^-$ GAS clones. This is in congruence with a previous report that in absence of SpeB, GAS trigger excessive neutrophil degranulation including the release of MPO [43]. MPO is associated with increased mortality in patients with sepsis [31] and a recent study demonstrated that GAS NSTIs patients had higher MPO levels in blood serum as compared to polymicrobial NSTIs. Moreover, patients presenting with STSS even significantly exceeded MPO levels of those who presented without shock [12].

Since our data suggested that neutrophils are the main cellular compartment responsible for SpeB-negativity, we subsequently analyzed the impact of neutrophil derived components on GAS phenotype. Among the tested effector molecules, H_2O_2 and HOCl, and Cl$^-$ were responsible for the reversible loss of SpeB secretion by GAS. Neutrophils phagocytose and subsequently kill bacteria primarily through the activity of NADPH oxidase and MPO [2]. In addition, MPO can be found extracellularly through degranulation or NET formation. Both, extracellular as well as intracellular MPO converts H_2O_2 to HOCl through oxidation of Cl$^-$ [2]. Moreover, increased survival of bacteria within neutrophils was associated with increasing percentage of SpeB$^-$ clones over time. Inhibition of MPO activity resulted in significantly reduced frequency of SpeB$^-$ GAS clones. However, there are certain limitations to this result: (i) NaCl, the source of Cl$^-$, also impaired SpeB secretion by GAS and (ii) inhibition

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of MPO in neutrophils only partially reversed the SpeB⁻ phenotype. At this stage, we cannot rule out that Cl⁻ itself can induce such bacterial phenotype. Nonetheless, HOCl is a less stable compound than H_2O_2 and therefore, bacterial killing will only be effective in a short time. In this case, inhibition of MPO most likely resulted in H_2O_2 accumulation within neutrophils and in the observed improved bacterial killing.

Production of fully active SpeB is a multistage process, which includes the involvement of trigger factor (RopA), the PPIase PrsA as well as cytoplasmic SpeB inhibitor Spi (reviewed in [5]). Furthermore, GAS encode highly conserved canonical Sec pathway [9], which components are enriched at the ExPortal microdomain [30]. It was shown that cationic antimicrobial peptides, including polymyxin B, preferentially target the ExPortal. Consequently, the anionic lipids and proteins are redistributed around the peripheral membrane, which is associated with the inhibition of toxin secretion, including SpeB [44]. Whether H_2O_2, HOCl or Cl⁻ interfere with the above-mentioned processes remains to be elucidated.

To our knowledge, this study is the first to show that in addition to the genetically induced loss of SpeB expression, GAS transiently abrogate SpeB secretion in response to neutrophil-derived H_2O_2 and HOCl. SpeB⁻ clones resist phagocytic killing resulting in enhanced neutrophil degranulation, which is associated with pronounced tissue inflammation and pathology. This finding provides further insight how bacterial persistence arises in the tissue setting. Further experimental studies are warranted to determine the molecular mechanism responsible for transient loss of SpeB secretion in a subpopulation of GAS.

Conclusions

Bacteria adapt their genetic information and phenotype to challenges they are facing during an infection. In severe GAS infections, hypervirulent SpeB-negative clones have been identified to arise due to *covR/S* and *ropB* mutations. Here we identify that such clones also arise through a transient phenotypic switch triggered by the neutrophil effector molecules hydrogen peroxide and hypochlorous acid. The resulting clones have abrogated SpeB secretion and trigger exacerbated neutrophil degranulation, which results in enhanced tissue pathology. Thus, this study identified a new virulence strategy by streptococci in severe tissue infections.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

GAS	Group A streptococci
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
MPO	Myeloperoxidase
NSTIs	Necrotizing soft tissue infections
SpeB	Streptococcal pyrogenic exotoxin B
STSS	Streptococcal toxic shock syndrome

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12929-023-00947-x.

Additional file 1. Figures S1-S16 and methods section.

Additional file 2. Tables S1-S6.

Availability of data and materials

All data associated with this study are presented in the paper and supplementary material. Whole genome sequencing data of GAS strains are available at the European Nucleotide Archive (ENA) under the reference number PRJNA 524111. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [29] partner repository with the dataset identifier PXD040160.

Declarations

Ethics approval and consent to participate

All studies were conducted in accordance with the Helsinki Declaration. Patient samples were retrieved from the INFECT patient cohort consisting of patients with NSTI (INFECT study; ClinicalTrials.gov, NCT01790698). The patient biopsies were obtained at surgical interventions and GAS infections were

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primarily identified by routine diagnostics. Patient enrolment and sample analyses were approved by the regional Ethical Review Board at the National Committee on Health Research Ethics in Copenhagen (Ref. No. 1211709), the regional ethics committee in Gothenburg (Ref. No. 930-12), the regional ethics committee Vest, Norway (REK, Ref. No. 325786), and the regional Ethics Committee in Stockholm (Ref. No. 2012/2110-31/2). Blood samples from healthy volunteers or buffy coats of blood provided by the blood bank at the University Medicine Greifswald were used. The buffy coats were provided anonymously. In case of healthy volunteers, donors were individuals well acquainted with the research conducted and written informed consent was obtained. The ethical research committee at the University Medicine Greifswald approved the study (Ref. No. BB 014/14 and Ref. No BB 006/18). All experiments were carried out in accordance with the approved guidelines.

Consent for publication
Not applicable.

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Publisher's Note

Supplemental Material

Neutrophil-derived reactive agents induce a transient SpeB negative phenotype in *Streptococcus pyogenes*

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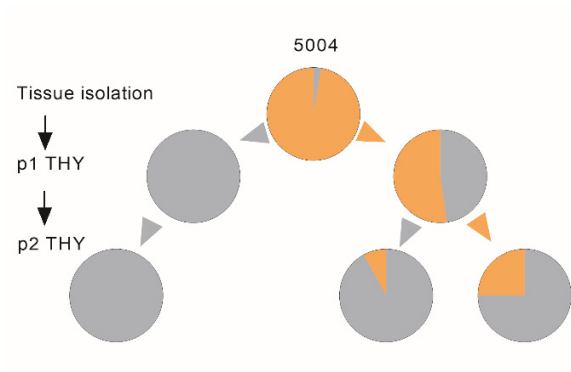
Fig. S1. Reversible loss of SpeB secretion after tissue recovery. Sections of the tissue biopsy of patient 5004 were cultured on casein agar plates.SpeB+ and SpeB- colonies were recovered and passaged in THY media over night. SpeB-positivity/-negativity were assessed via casein agar assay (SpeB+ [grey]; SpeB- [orange]).
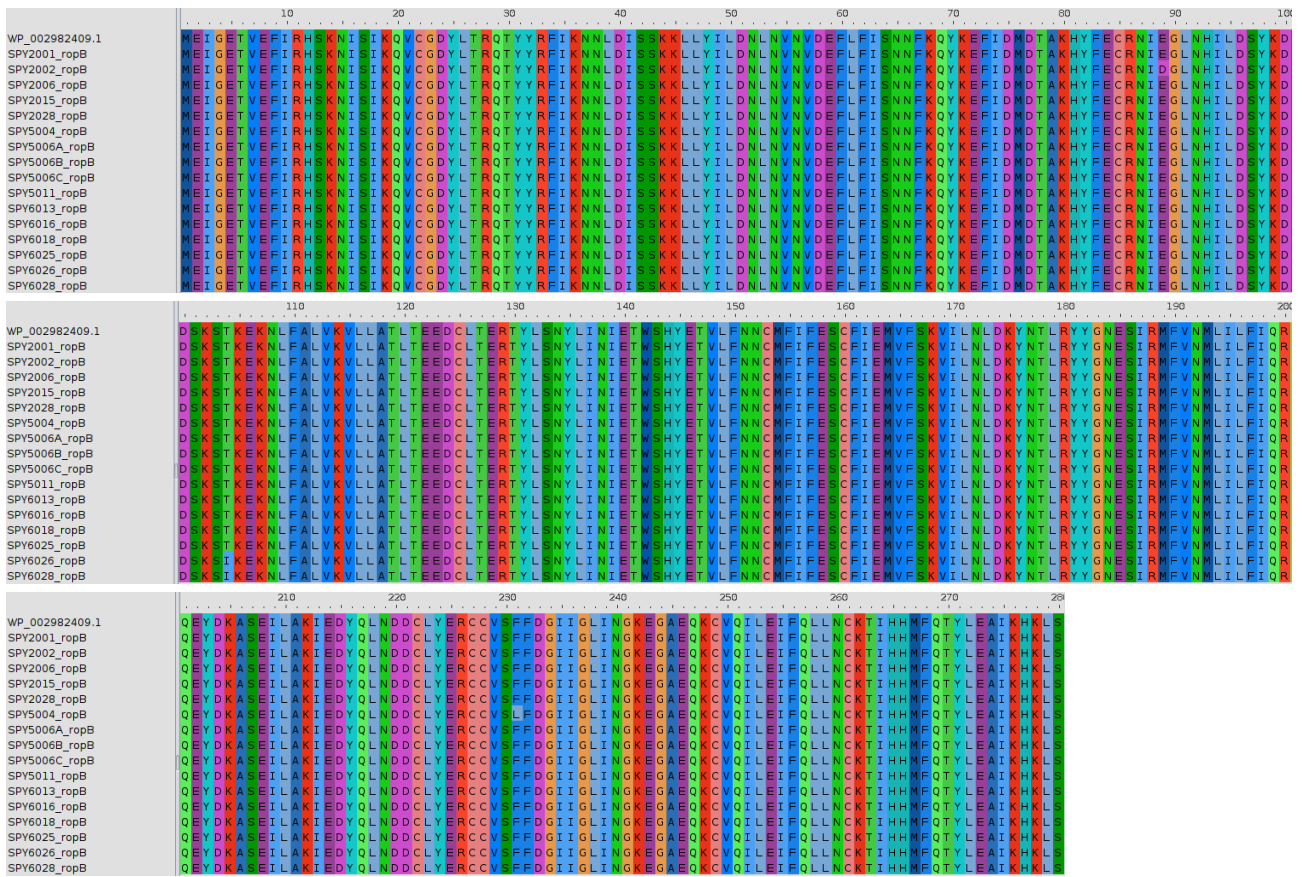
Fig. S2. *CovS* gene sequence alignment of indicated strains isolated from NSTI patient´s biopsies (Figure 1A).
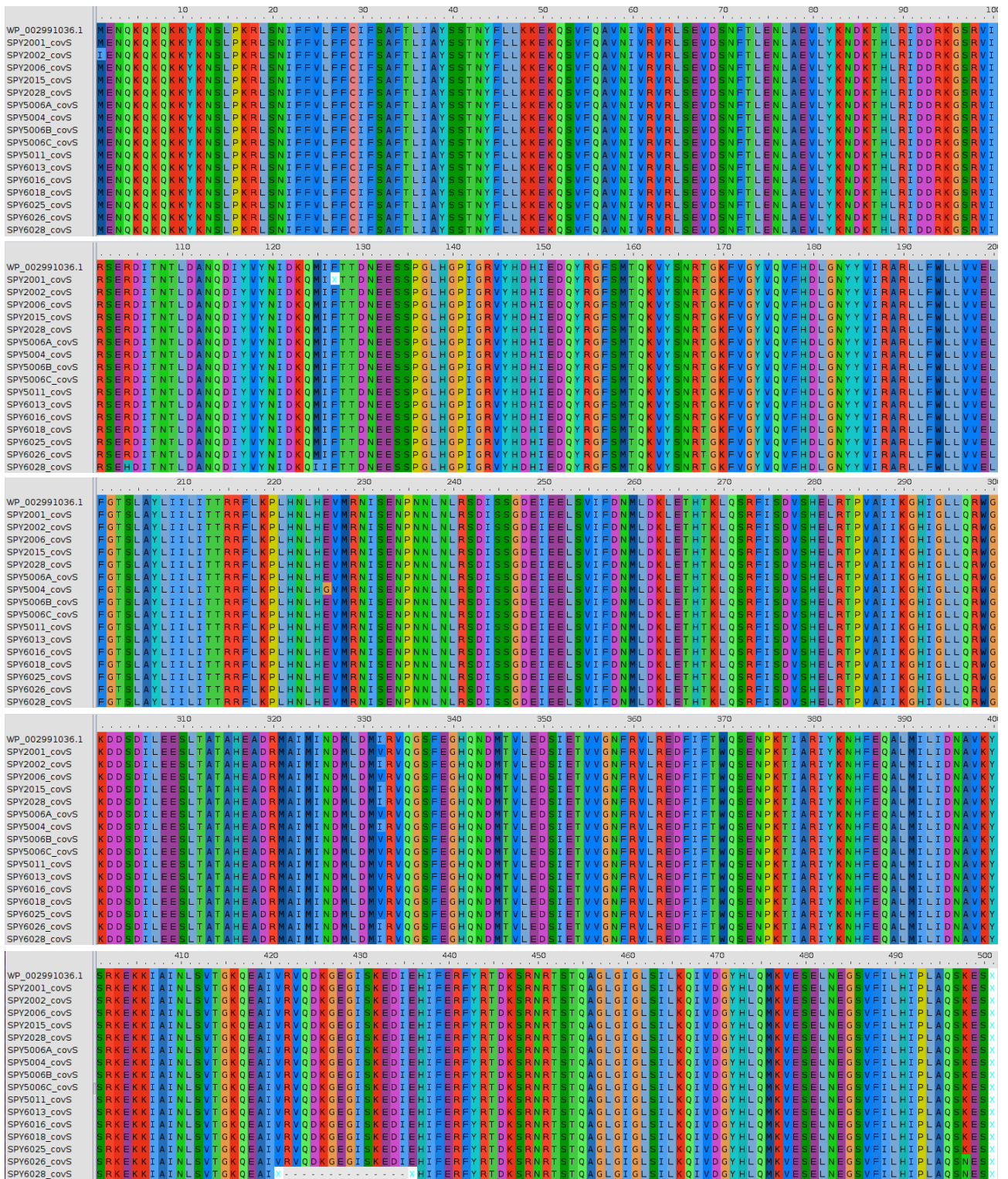
Fig. S3. CovS amino acid sequence alignment of indicated strains isolated from NSTI patient´s biopsies (Figure 1A).

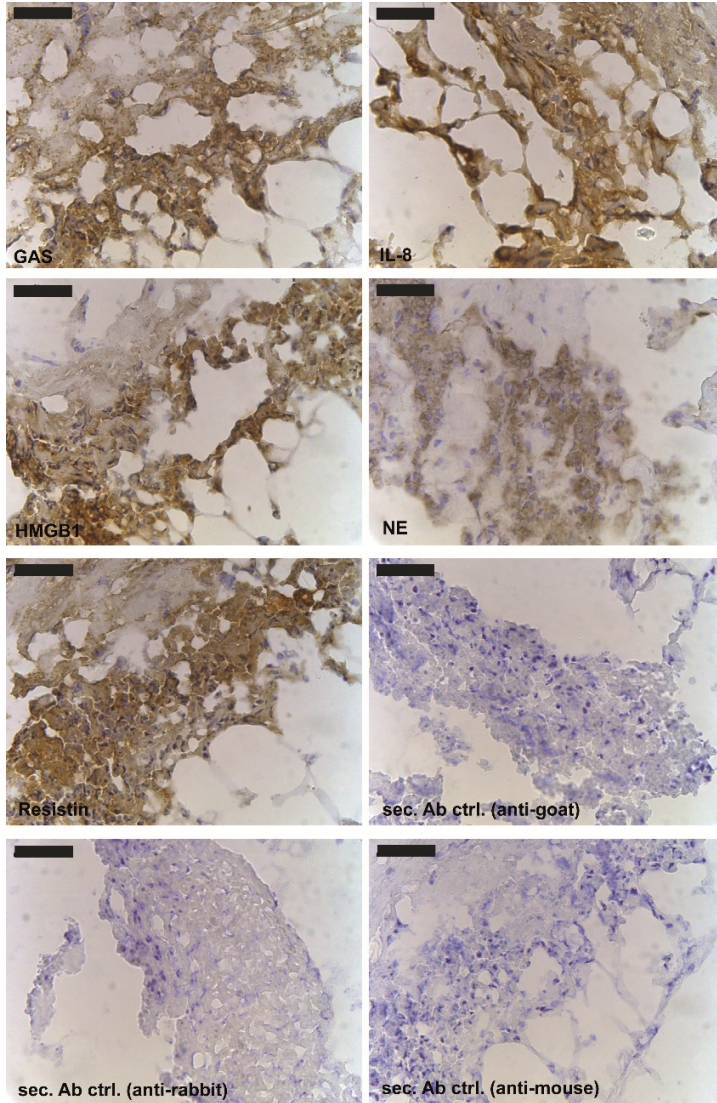**Fig. S4. RopB amino acid sequence alignment of indicated strains isolated from NSTI patient´s biopsies** (Figure 1A).

Fig. S5. Inflammation and phagocytic infiltration at the local site of infection. Representative immuno-histochemically stained tissue biopsies from GAS NSTI patients. (GAS, group A streptococcus; HMGB1, High-mobility group protein B1; NE, neutrophil elastase; black bars: 50 µm).
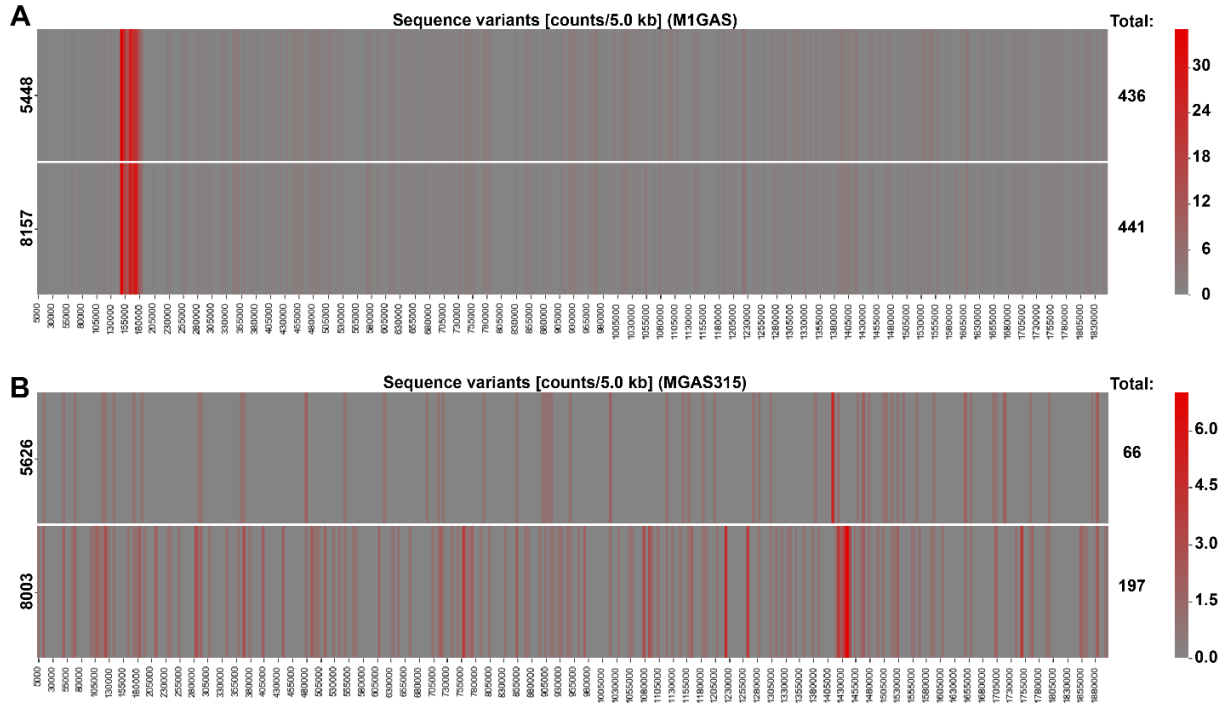
Figure S6. Whole genome sequencing analyses. Base differences per 5.0 kb based on the comparison of the whole genomes of the indicated strains with the annotated genomes of (**A**) M1GAS (*emm1* strains) and (**B**) MGAS315 (*emm3* strains).

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SPy_0336        MENQKQKQKKYKNSLPKRLSNIFFVLFFCIFSAFTLIAYSSTNYFLLKKEKQSVFQAVNI
SpyM3_0245      MENQKQKQKKYKNSLPKRLSNIFFVLFFCIFSAFTLIAYSSTNYFLLKKEKQSVFQAVNI
CNGIICF_01748   MENQKQKQKKYKNSLPKRLSNIFFVLFFCIFSAFTLIAYSSTNYFLLKKEKQSVFQAVNI
IKGFMBIA_01747  MENQKQKQKKYKNSLPKRLSNIFFVLFFCIFSAFTLIAYSSTNYFLLKKEKQSVFQAVNI
DNELCKEF_00023  MENQKQKQKKYKNSLPKRLSNIFFVLFFCIFSAFTLIAYSSTNYFLLKKEKQSVFQAVNI
LCNJLCPO_00752  MENQKQKQKKYKNSLPKRLSNIFFVLFFCIFSAFTLIAYSSTNYFLLKKEKQSVFQAVNI
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SpyM3_0245      VRVRLSEVDSNFTLENLAEVLYKNDKTHLRIDDRKGSRVIRSERDITNTLDANQDIYVYN
CNGIICF_01748   VRVRLSEVDSNFTLENLAEVLYKNDKTHLRIDDRKGSRVIRSERDITNTLDANQDIYVYN
IKGFMBIA_01747  VRVRLSEVDSNFTLENLAEVLYKNDKTHLRIDDRKGSRVIRSERDITNTLDANQDIYVYN
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CNGIICF_01748   LGNYYVIRARLLFWLLVVELFGTSLAYLIILITTRRFLKPLHNLHEVMRNISENPNNLNL
IKGFMBIA_01747  LGNYYVIRARLLFWLLVVELFGTSLAYLIILITTRRFLKPLHNLHEVMRNISENPNNLNL
DNELCKEF_00023  LGNYYVIRARLLFWLLVVELFGTSLAYLIILITTRRFLKPLHNLHEVMRNISENPNNLNL
LCNJLCPO_00752  LGNYYVIRARLLFWLLVVELFGTSLAYLIILITPRRFLKPLHNLHEVMRNISENPNNLNL
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LCNJLCPO_00752  RSDISSGDEIEELSVIFDNMLDKLETHTKLQSRFISDVSHELRTPVAIIKGHIGLLQRWG
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CNGIICF_01748   VRVQDKGEGISKEDIEHIFERFYRTDKSRNRTSTQAGLGIGLSILKQIVDGYHLQMKVES
IKGFMBIA_01747  VRVQDKGEGISKEDIEHIFERFYRTDKSRNRTSTQAGLGIGLSILKQIVDGYHLQMKVES
DNELCKEF_00023  VRVQDKGEGISKEDIEHIFERFYRTDKSRNRTSTQAGLGIGLSILKQIVDGYHLQMKVES
LCNJLCPO_00752  VRVQDKGEGISKEDIEHIFERFYRTDKSRNRTSTQAGLGIGLSILKQIVDGYHLQMKVES
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SPy_0336        ELNEGSVFILHIPLAQSKES           M1GAS    (SPyxxxx)
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CNGIICF_01748   ELNEGSVFILHIPLAQSKES           5448     (CNGIICF_xxxxx)
IKGFMBIA_01747  ELNEGSVFILHIPLAQSKES           8157     (IKGFMBIA_xxxxx)
DNELCKEF_00023  ELNEGSVFILHIPLAQSKES           5626     (DNELCKEF_xxxxx)
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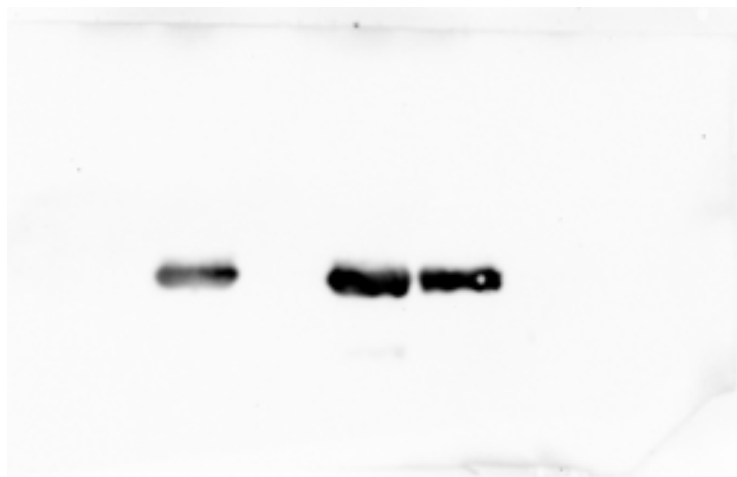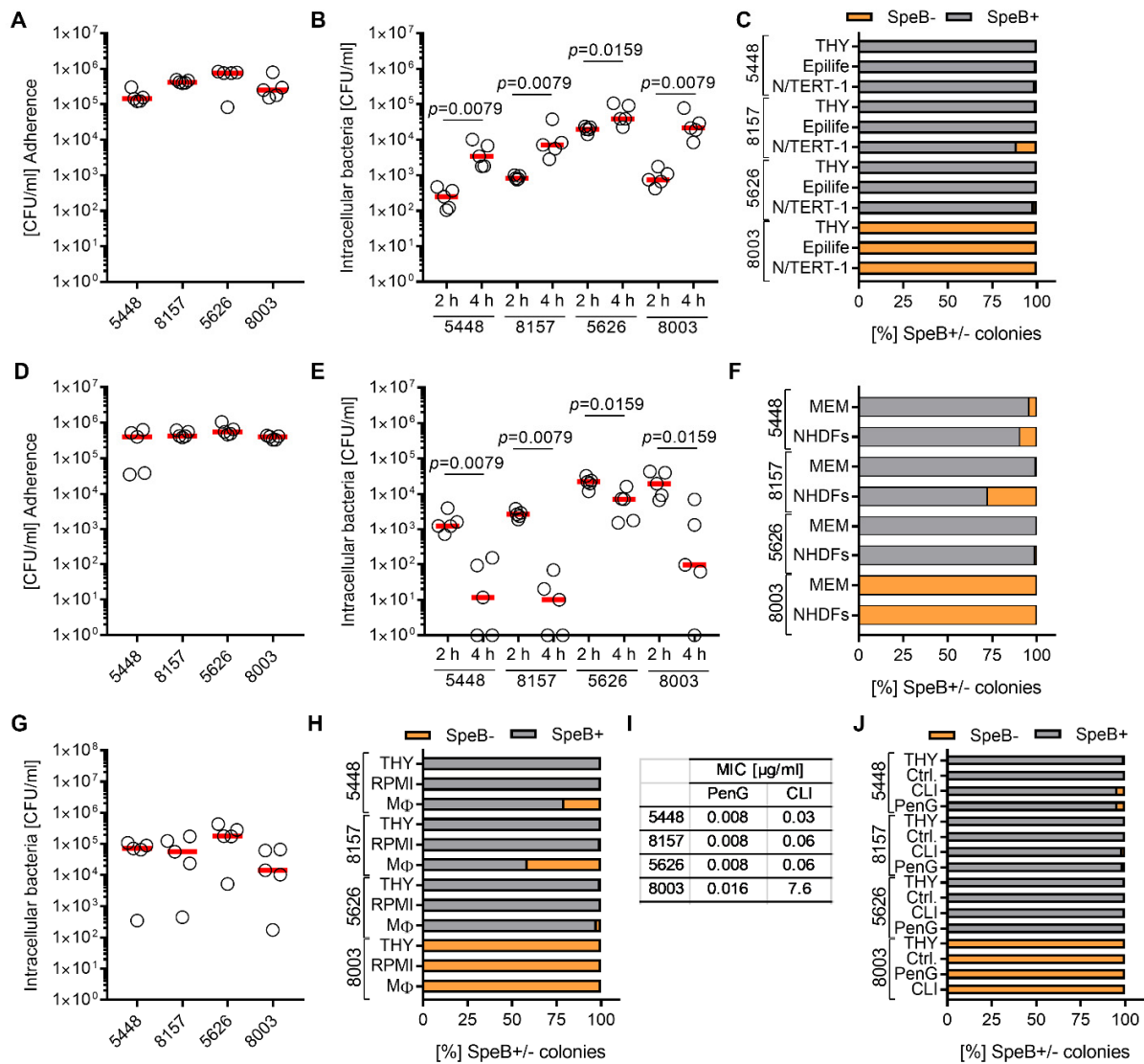
Fig. S7. Mutation within *covS* gene in 8003 strain results in T$_{214}$P substitution. Amino acid alignment of the indicated four GAS strains used in this study.

Fig. S8. Original SpeB blot as displayed in Fig. 2

Fig. S9. SpeB phenotype in GAS co-culture with keratinocytes, fibroblasts, and macrophages. Extracellular (**A**) and intracellular (**B**) bacterial numbers recovered from keratinocytes after indicated time points of infection. Each dot represents one independent experiment. The horizontal lines denote median values (n=5). (**C**) Assessment of SpeB positivity/negativity of indicated strains via casein agar assay recovered after 4 h of intracellular passage in keratinocytes. Mean percentage from five independent experiments is shown (n=5). Extracellular (**D**) and intracellular (**E**) bacterial numbers recovered from human primary fibroblasts after indicated time points of infection. Each dot represents one independent experiment. The horizontal lines denote median values (n=5). (**F**) Assessment of SpeB positivity/negativity of indicated strains via casein agar assay recovered after 2 h of intracellular passage in fibroblasts. Mean percentage from five independent experiments is shown (n=5). (**G**) Intracellular bacterial counts after 2 h of macrophage infection. Each dot represents an experiment with macrophages from one donor. The horizontal lines denote median values (n=5). (**H**) Assessment of SpeB positivity/negativity of indicated strains recovered from primary human macrophages shown in (**G**). Mean percentage of SpeB+ and SpeB- clones from five independent experiments are shown (n=5). Bacteria incubated in EpiLife, RPMI, and THY media served as controls. (**I**) Penicillin G (PenG) and clindamycin (CLI) minimal inhibitory concentrations (MICs) of indicated

GAS strains (n=4). (**K**) Distribution of SpeB+ and SpeB- clones post antibiotic treatment. Displayed are mean [%] of four independent experiments (n=4). The level of significance was determined using Kruskal-Wallis test with Dunn´s multiple comparison post-test.

A

```
1    MNKKKLGVRLLSLLALGGFVLANPVFADQNFARNEKEAKDSAITFIQKSAAIKAGARSAE
61   DIKLDKVNLGGELSGSNMYVYNISTGGFVIVSGDKRSPEILGYSTSGSFDANGKENIASF
121  MESYVEQIKENKKLDTTYAGTAEIKQPVVKSLLDSKGIHYNQGNPYNLLTPVIEKVKPGE
181  QSFVGQHAATGCVATATAQIMKYHNYPNKGLKDYTYTLSSNNPYFNHPKNLFAAISTRQY
241  NWNNILPTYSGRESNVQKMAISELMADVGISVDMDYGPSSGSAGSSRVQRALKENFGYNQ
301  SVHQINRGDFSKQDWEAQIDKELSQNQPVYYQGVGKVGGHAFVIDGADGRNFYHVNWGWG
361  GVSDGFFRLDALNPSALGTGGGAGGFNGYQSAVVGIKP
```

B

| SEQUENCE	START POSITION	END POSITION	LENGTH
DSAITFIQK	40	48	9
SAEDIKLDK	58	66	9
SPEILGYSTSGSFDANGK	97	114	18
ENIASFMESYVEQIK	115	129	15
ENIASFMESYVEQIKENK	115	132	18
KLDTTYAGTAEIK	133	145	13
LDTTYAGTAEIK	134	145	12
GIHYNQGNPYNLLTPVIEK	157	175	19
YHNYPNK	203	209	7
DYTYTLSSNNPYFNHPK	213	229	17
NLFAAISTR	230	238	9
QYNWNNILPTYSGR	239	252	14
ALKENFGYNQSVHQINR	291	307	17
ENFGYNQSVHQINR	294	307	14
QDWEAQIDK	313	321	9
QDWEAQIDKELSQNQPVYYQGVGK	313	336	24
ELSQNQPVYYQGVGK	322	336	15
VGGHAFVIDGADGR	337	350	14
LDALNPSALGTGGGAGGFNGYQSAVVGIKP	369	398	30

Fig. S10. SpeB single colony analysis. (**A**) Fasta sequence of SpeB-zymogene with SpeB tryptic peptides (green), detected by mass spectrometry in the single colony analysis. (**B**) SpeB tryptic peptides detected by mass spectrometry in the single colony analysis. The pro-domain is depicted in red whereas the sequence for mature SpeB is indicated in black.
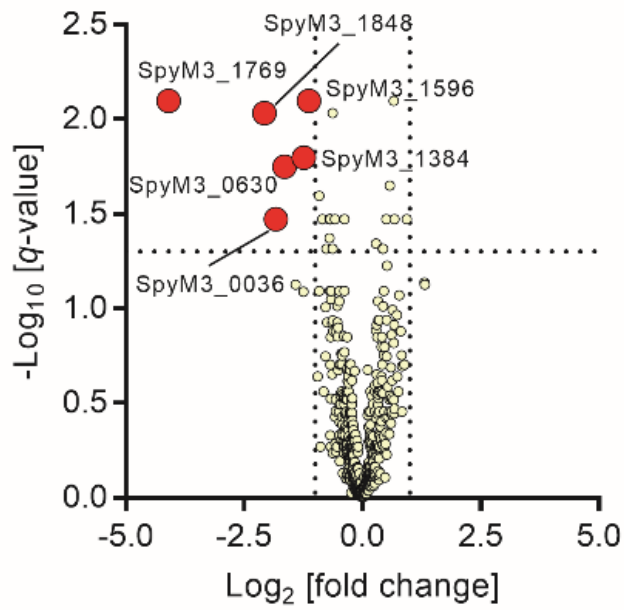
Fig. S11. Single colony proteome analysis of GAS 8003. Volcano plot displaying significant differences of single colony proteome post neutrophils infection compared to the THY control. Original data is displayed in Supplemental Table 3.
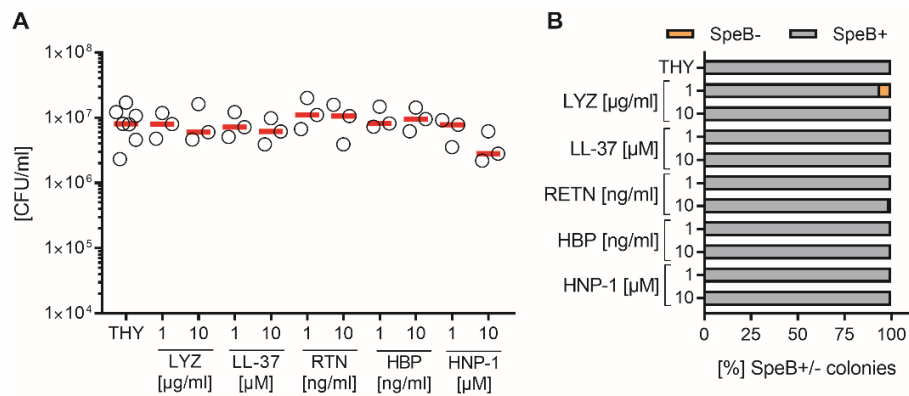
Fig. S12. The impact of neutrophil derived peptides/proteins on group A streptococcal SpeB secretion. 5448 GAS strain was exposed to indicated concentrations of proteins/peptides for 3 h and bacterial viability (**A**) and SpeB secretion (**B**) were assessed. (**A**) Each dot represents one independent experiment. The horizontal lines denote median values (n≥3). (**B**) Mean percentage from three independent experiments (shown in A) is displayed (LYZ, lysozyme; RTN, resistin; HBP, heparin binding protein; HNP-1, human neutrophil peptide 1).
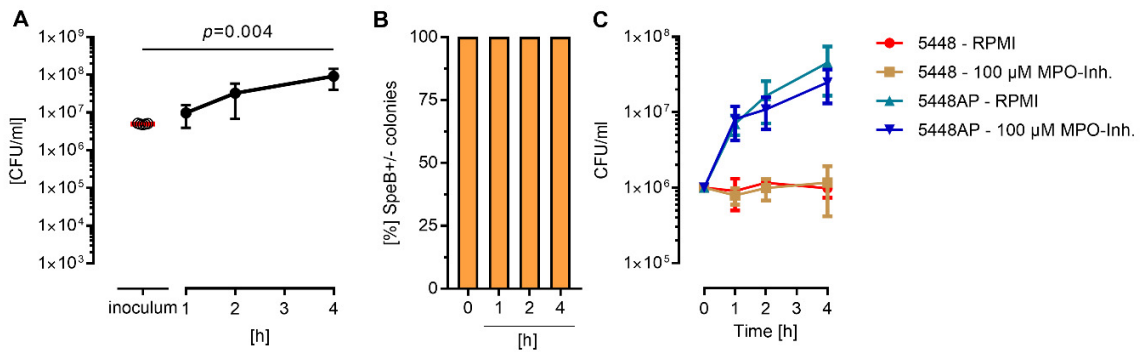
Fig. S13. 5448AP survives intracellular neutrophil passage. (*A*) Human primary neutrophils were infected GAS strain 5448AP and intracellular bacteria were determined by plating serial dilution of neutrophil lysates on casein agar plates post indicated time points. Dots represent the median value ±range of independent experiments with five donors (n=5). (*B*) Assessment of SpeB positivity/negativity of 5448AP strain recovered from primary human neutrophils shown in (*A*) (n=5). All 5448AP clones remained negative (orange). The level of significance between the groups of all experiments was determined using Kruskal Wallis test with Dunn´s posttest. (*C*) Control experiment confirming that 100 µM MPO-inhibitor had no effect on bacterial growth (n=3).
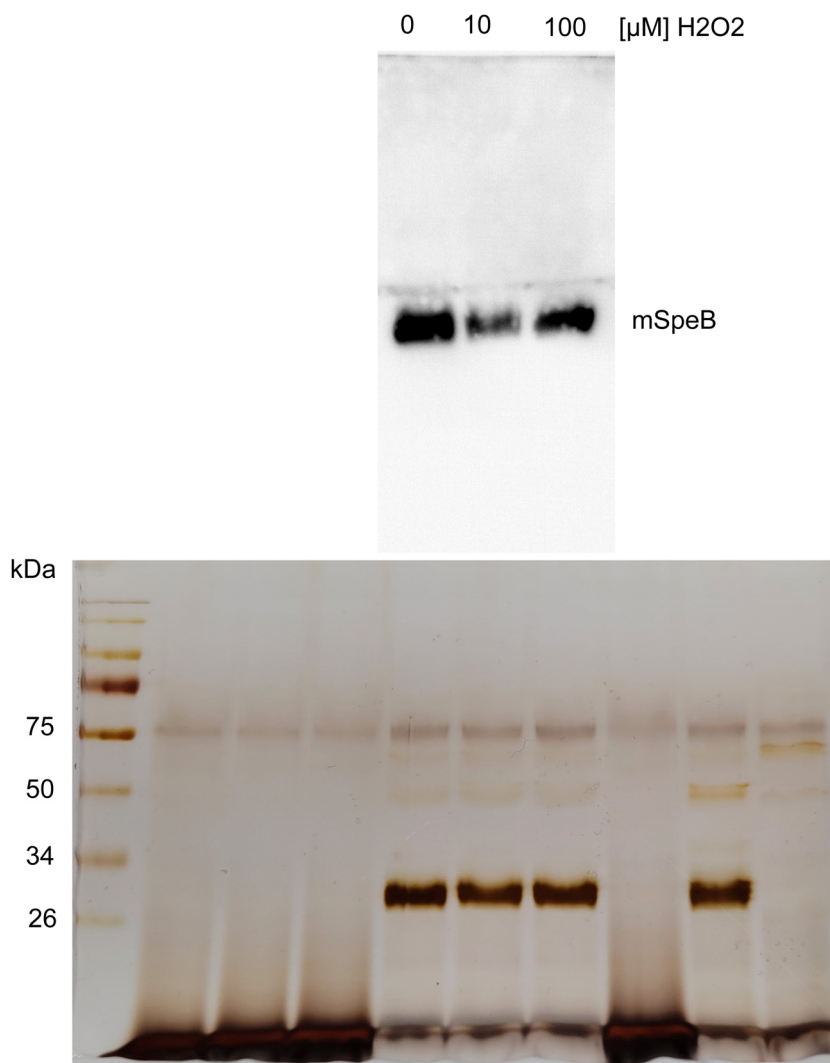
Fig. S14. Original Western blot (upper panel) and silver staining of the loading control (lower panel) of GAS 5448 supernatants post exposure to indicated concentrations of H_2O_2.
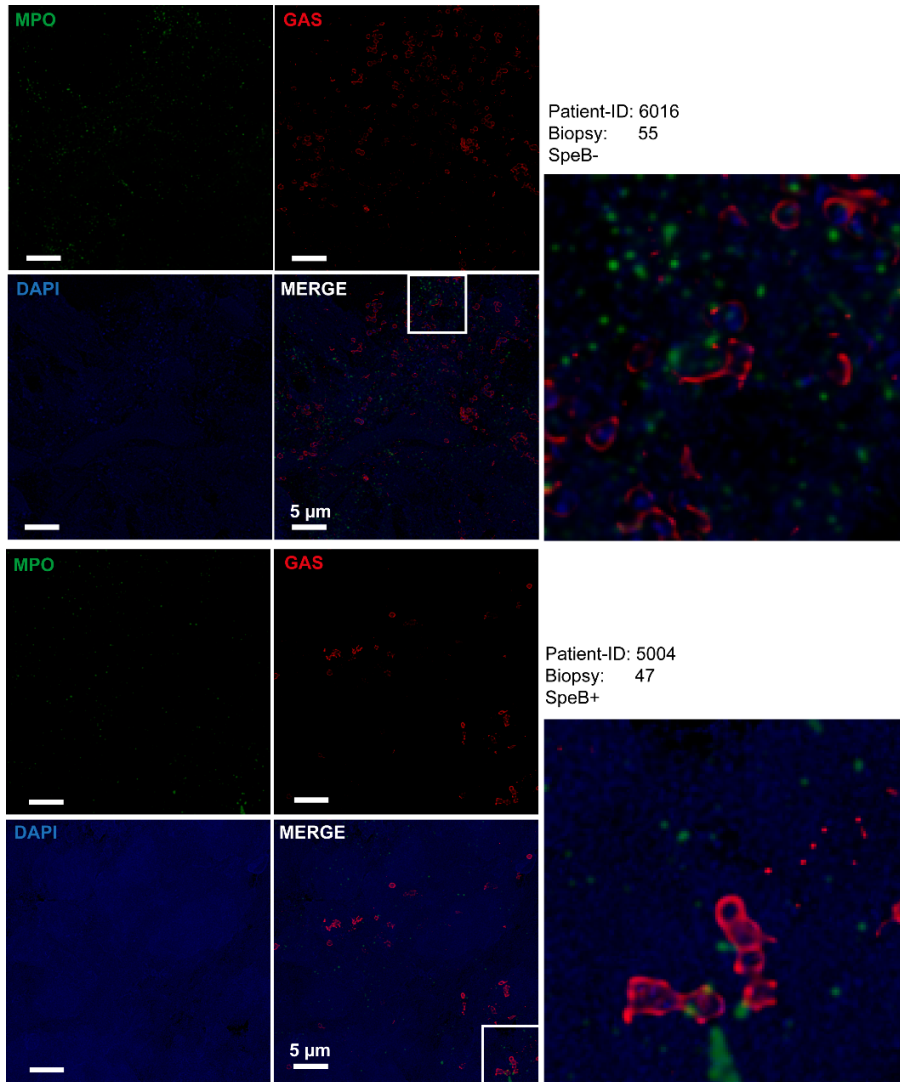
Fig. S15. Increased levels of MPO in patient tissue biopsies associated with SpeB⁻ GAS. Representative immunofluorescence micrographs of the distribution of MPO in patient biopsies.
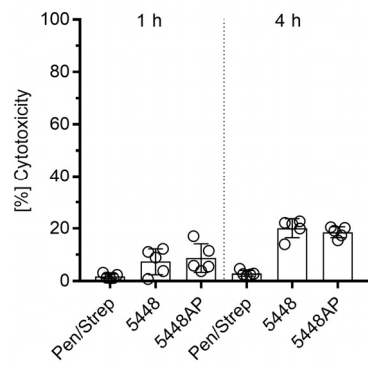
Fig. S16. Cytotoxicity induced by bacterial infections towards human primary neutrophils. Mean values ± s.d. from five donors (n=5) are shown. Each dot represents one donor.

Materials and Methods

Whole genome sequencing and data processing. DNA extraction from GAS strains 5448, 5626, 8003, and 8157, whole genome sequencing, and analyses were performed as previously described [14]. Briefly, Bacterial DNA was purified utilizing the DNeasy Blood & Tissue kit (Qiagen). Sequencing libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit (NEB). Genomes were sequenced using Illumina MiSeq (2x300 bp). Sequencing data are deposited in the European Nucleotide Archive (ENA database) under the BioProject PRJNA524111 [3]. Raw reads were processed in paired-end mode by fastq-mcf version 1.05. Processed reads were assembled using SPAdes (version 3.11.1) de novo genome assembler software [2] in careful mode. Contigs with a length less than 200 bp or average coverage lower than 15 were discarded. Average contig coverage was determined by first mapping processed reads to the assembled contigs utilizing Bowtie2 (version 2.3.2) determining the per base coverage using the SAMtools (version 1.8) [11] and calculating the mean coverage for each contig. All assemblies were annotated utilizing Prokka genome annotation software (version 1.4) [13]. Gene comparisons are based on orthologous genes identified by the Proteinortho software (version 5.16b) [8]. Processed reads of 5448 and 8157 strains were mapped to the *S. pyogenes* M1GAS (*emm*1) reference genome and of 5626 and 8003 strains to MGAS315 (*emm*3) genome using Bowtie2 [6]. Variants were called using mpileup part of BCFtools (version 1.8) [10]. Variants were filtered out by a minimum SNP and INDEL distance of 10 bp, variant quality of 30, coverage of 50, mapping quality of 40 and a Z-score of 1.96 [5].

The genomes of GAS strains (Figure 1) were retrieved from the European Nucleotide Archive in FASTA format [9]. As reference strain, assembly GCF_000006785.2_ASM678v2 from the National Center for Biotechnology was used. The gene that encodes *covS* corresponds to the protein WP_002991036.1, *covR* corresponds to protein WP_002991052.1, and *ropB* corresponds to WP_002982409.1 in the reference strain. The reference protein sequences for CovS, CovR and RopB were searched in the genomes of strains using NCBI-BLAST version 2.9.0+ [1,4]. In case of CovR and RopB, the protein sequence in reference strains was extracted from the first BLAST hit, respectively. For CovS, the BLAST output showed that SPY2001 has a frame-shift causing deletion. Thus, cdbfasta tools version 0.99 (https://github.com/gpertea/cdbfasta, last accessed on February 18th 2021) were used to excise the corresponding coding sequence of BLAST hits from the strain genomes. For CovS, we performed a multiple

protein sequence alignment with CLUSTAL version 2.1 [15]. Subsequently, a translation of the aligned sequences was performed with AliView version 1.26 [7].

Protein extraction, LC-MS/MS analyses, and data processing of single GAS colonies and neutrophil secretome. Single SpeB+ and/or SpeB- colonies, as assessed by casein agar assay, were transferred into tubes containing 200 µl 50 mM triethylammonium bicarbonate (TEAB) lysis buffer and 100 µl Lysing matrix B (MP Biomedicals). Bacteria were disrupted by bead beating (6 m/s, 5×20 s; 60 s ice incubation between the cycles) via FastPrep-24 5G (MP Biomedicals). Protein-containing supernatant was separated from bacterial debris via centrifugation (10 min, 10,000×g, 4°C). Protein concentrations were determined using BCA-assay (Thermo Fisher Scientific). 2.5 µg of total protein was reduced (5 mM Tris(2 carboxyethyl)phosphin [TCEP]; 45 min; 65°C), alkylated (10 mM iodoacetamide [IAA]; 15 min; room temperature; dark), and an in-solution digest with 250 ng of trypsin was performed (18 h; 37°C). Samples were concentrated and desalted via ZipTips (C18; Millipore), dried, and stored at -80°C until further use.

Samples were measured on a QExactive mass spectrometer coupled to an EASY nLC-1000 liquid chromatography system. Samples were loaded on an in-house packed column (ReproSil-Pur 120 C18-AQ, 3 µm) of 20 cm length and 100 µm inner diameter. Peptides were eluted by a non-linear 86 min gradient from 2% to 99% solvent B (acetonitrile with 0.1% acetic acid). The overview scans (MS1) covered a mass range of 300–1650 m/z at a resolution of 70,000 (at 200 m/z). The 12 most abundant precursors were selected for HCD fragmentation at NCE 27.5 with an AGC target of 1e5 and an under-fill ratio of 5%. Dynamic exclusion was set to 30 s, lock mass correction was enabled, and ions with unknown charge of one or higher than six were excluded from fragmentation.

MaxQuant version 1.6.10.43 and ncbi genome assembly data base id=233599 (M1GAS) and id=299846 (MGAS315) were used for protein identification. The minimal number of unique peptides per protein group was set to 2 to be considered as identified. Oxidation (M) was considered as a variable and carbamidomethyl (C) as a fixed modification. Data were analyzed using Perseus version 1.6.10.43. Quantitative values were log$_2$ transformed and the resulting values were filtered based on the following criteria: only identified by site, reverse, potential contamination, quantified in at least 75% of biological

replicates per group. Differential expression of bacterial proteins was determined via Student´s *t* test with Benjamini-Hochberg FDR correction. Proteins were considered to be significantly differentially expressed if *q*-value was <0.01 and $\log_2$ fold change was ≥1. The principle component analysis (PCA) of the scaled data was carried out using the ClustVis web tool [12].

Neutrophil secretome profiling: 500 µl neutrophil supernatant was reduced with TCEP (5 mM, 45 min, 65°C) and alkylated with IAA (10 mM, 20 min, room temperature, dark). Five microliters of SP3 beads (hydrophobic: Sera-Mag Speedbeads carboxylate-modified particles [GE Healthcare]; hydrophilic: Speedbead magnetic carboxylate-modified particles [GE Healthcare]) were added to the sample and acetonitrile added to a final concentration of 70% (v/v) and incubated in a thermomixer (5 min, 24 °C, 900 rpm). Tubes were placed in a magnetic rack to collect the beads and the supernatant was removed. Beads were washed with 80% (v/v) ethanol twice and air dried. Proteins were digested by adding 25 µl digestion buffer (50 mM TEAB) containing 100 ng of trypsin followed by 30 s sonication in a water bath to disaggregate the beads and an incubation at 37°C for 18 h. Beads were removed by centrifugation (20,000 g, 1 min) and by placing the tubes in a magnetic rack to transfer the supernatant to a glass vial. The supernatants were dried by vacuum centrifugation and peptides were reconstituted in 12 µl 0.1% acetic acid in water containing iRT peptides.

Generated tryptic peptides were analyzed by LC-MS/MS. Therefore, an EASY nLC 1000 (Thermo Fischer) was coupled to an QExactive mass spectrometer (Thermo Fisher). Peptides were loaded onto in house packed fused silica columns of 20 cm length and an inner diameter of 75 µm, filled with Dr. Maisch ReproSil Pur 120 C18-AQ 1.9 µm (Dr. Maisch). Peptides were eluted using a non-linear binary gradient of 86 min from 2% to 99% solvent B (0.1% acetic acid in acetonitrile) in solvent A (0.1% acetic acid). The overview scans (MS1) covered a mass range of 300–1650 m/z at a resolution of 70,000 (at 200 m/z). The 10 most abundant precursors were selected for HCD fragmentation at NCE 27.5 with an AGC target of 1e5 and an under-fill ratio of 10%. Dynamic exclusion was set to 30 s, lock mass correction was enabled, and ions with unknown charge, charge of one or higher than six were excluded from fragmentation.

MaxQuant version 1.6.17.0 and ncbi genome assembly data base id=233599 (M1GAS) and UniProt human reference proteome (downloaded 20190715) were used for protein identification. The minimal number of

unique peptides per protein group was set to 2 to be considered as identified. Oxidation (M) was considered as a variable and carbamidomethyl (C) as a fixed modification. Data were analyzed using Perseus version 1.6.14.0. Quantitative values were $\log_2$ transformed and the resulting values were filtered based on the following criteria: only identified by site, reverse, potential contamination, quantified in at least 4 of 5 biological replicates per group. LFQ intensities were normalized to the intensity of the spiked iRT. Differential expression of bacterial proteins was determined via Student´s t test with Benjamini-Hochberg FDR correction. Proteins were considered to be significantly differentially expressed if p-value was <0.05 and $\log_2$ fold change was ≥1.

Cytotoxicity LDH release assay. Cytotoxicity was determined by measurement of the LDH activity via CytoTox 96 Non-Radio Kit (Promega) according to manufacturer's guidelines.

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