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Chemotaxis-dependent homing of innate lymphoid cells in the context of pregnancy

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1. Introduction

1.1. Scientific background of the work

Pregnancy is a very specific period of time for the female human body that challenges the maternal immune system in multiple aspects. Remarkably, the number of pregnancy losses is relatively high. Wilcox *et al.* showed that the number of spontaneous abortions in clinically and biochemically proven pregnancies until the 28th week of pregnancy is up to 32 % (Wilcox & Horney, 1984), while the total number of spontaneous abortions after conception is estimated as high as 50 - 70 % (Beck et al., 1998; Wieacker et al., 2002; Wieacker P et al., 2005).

It has been shown that there is a huge diversity of parameters that have an impact on successful outcome of pregnancy, such as maternal age, uterine conditions, abortions in the past, genetic, endocrinological and immunological factors and maternal diseases such as autoimmune conditions or chronic diseases (Wieacker et al., 2002). As the impact of the immune system on successful conception and outcome of pregnancy has been extensively proven, the investigation of factors that lead to this high number of abortions is not only of scientific, but especially of clinical interest. Furthermore, the examination of the immune system during gestation will potentially have an impact on clinical problems of pregnancy including preterm birth, pre-eclampsia and intrauterine growth restriction in the future.

Innate lymphoid cells (ILCs) have an emerging role in immunology. Straddling properties of both the innate and adaptive immune system leads to their impact on health and disease. There has been plenty of investigation of ILCs in the gut, lung and skin, though the elucidation of their role in the reproductive system is still in its infancy.

Chemokines and chemokine receptors have been shown to be crucial for leukocyte homing and migration. As a finely balanced composition of immune cells at the fetal-maternal interface is a determining factor of successful pregnancy, mechanisms of their recruitment are of high interest. The effect of pregnancy and pregnancy hormones on ILC migration and certain chemokine receptors have thus been the aim of this work.

1.2. Pregnancy

After fertilization has taken place, the implantation is the first physical interaction between the conceptus and maternal immune system (Beck et al., 1998). It is accompanied by the decidualization of the uterine stroma and leads to the synthesis of beta-human chorionic gonadotropin (β-hCG), a pregnancy-supportive hormone, as it stimulates the corpus luteum to maintain progesterone production (Mihm et al., 2011).

The total duration of pregnancy is estimated as 280 days or 40 weeks, starting from the first day of the last menstrual period. Pregnancy is divided into three trimesters, each holding specific immunological properties (Pascual ZN & Langaker MD, 2022).

Since half of the fetus' genes are inherited by the father, it formally counts as a semi-allogeneic "foreign body". Therefore, the maternal immune system needs to be finely adjusted after implantation in order to ensure proper detection of paternal antigens, thus avoiding rejection of the blastocyst while providing sufficient protection from infections. This is not only crucial for natural conception but also other methods such as *in vitro* fertilization.

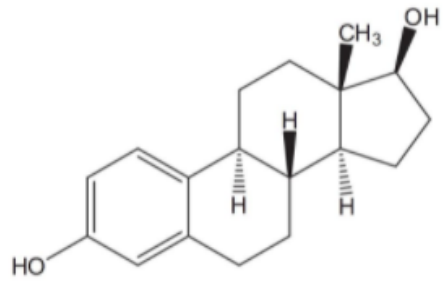
1.2.1. Pregnancy hormones

During the luteal phase of the menstrual cycle, following ovulation, the remaining follicle develops into the corpus luteum, which in turn mediates secretion of progesterone. The subsequently rising levels of progesterone induce decidualization and thereby provide endometrial receptivity for a potential pregnancy (Demayo & Lydon, 2020). If no fertilization occurs, the corpus luteum degenerates and progesterone levels fall, inducing the following menses. In case of a successful implantation, trophoblast cells start to secrete β-hCG, which sustains corpus luteum progesterone production, until after 10 weeks of gestation the placenta takes over (Pascual ZN & Langaker MD, 2022; Reed & Carr, 2018). Progesterone is an important steroid sex hormone for the establishment and maintenance of successful pregnancy (Arck et al., 2007). Progesterone as a steroid hormone acts through the Progesterone Receptor (PR), a nuclear receptor, of which different isotypes exist (Brosens et al., 2004). It is distributed abundantly throughout the female body and

apart from the gonads can be found in the nervous system, the GI-tract, bones and skin (Asavasupreechar et al., 2020; Guennoun et al., 2015).

In case of a pregnancy, the female body is exposed to much higher levels of these sex steroid hormones. Progesterone levels gradually increase during pregnancy, with a decrease around 10th week of pregnancy in response to the luteoplacental shift from 25 ng/mL (5th week of pregnancy) until a peak (202 ng/mL) is reached around term (Oertel et al., 1959; Shah & Nagarajan, 2013; Wiest, 1967; Yannone et al., 1968). Besides estrone (E1) and estriol (E3), estradiol (E2) is considered the predominant and most biologically active form of estrogens (Lowe et al., 2019). Its levels gradually rise during gestation, with a 30-fold increase at term compared with early pregnancy (Loriaux et al., 1972). As these female sex steroid hormones have immunomodulatory and various other properties, they are involved in the phenomenon of decreased disease activity of certain autoimmune diseases during pregnancy (Bhatia et al., 2014).

1)



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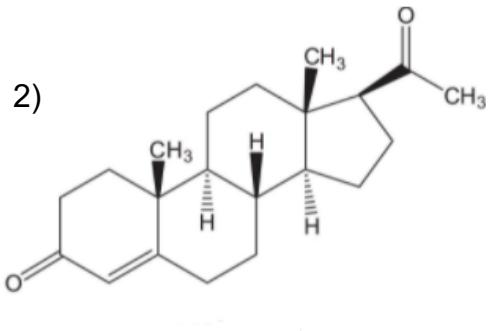

Figure 1: Structural formulas of 17β-estradiol (1) and progesterone (2), modified after Marques *et al*. (Marques et al., 2015)

1.3. The immune system

The immune system is responsible for detection and elimination of potential pathogens as well as the correct distinction between self and nonself in order to maintain integrity and individuality of the human organism. It can be divided into the innate or nonspecific and the adaptive or specific immune system (Baenkler et al., 2018).

1.3.1. The innate immune system

The innate or nonspecific immune system is the first one to react to pathogens and consists of physical barriers, defense mechanisms and general immune responses, including cellular, complement and inflammatory reactions. It is triggered by antigens or infectious foreign particles and creates a nonspecific general immune response that leads to lysis of cells. All cells that belong to the innate immune system are leukocytes and are derived from a pluripotent hematopoietic progenitor cell.

Phagocytes, macrophages and dendritic cells have different receptors, including Toll-like receptors (TLR) on their surface that allow for them to bind to bacteria, viruses, parasites or other infectious or non-infectious particles, inducing the process of phagocytosis.

Mast cells can be found in tissues and mucous surfaces and contain granules filled with histamine, heparin and enzymes. They can be activated via antigens, the complement system or physical stimuli. Upon activation, they release cytokines and their granules to generate an inflammatory cascade. These induce blood vessel dilation and cell trafficking towards the site of infection. Mast cells further mediate allergic reactions (Hiromatsu & Toda, 2003).

The group of granulocytes includes neutrophils, eosinophils and basophils. Neutrophils contain granules with enzymes and peptides in their cytoplasm that are toxic for bacteria and infectious particles when released. Eosinophils and Basophils are specialized in targeting parasites. They are also involved in allergic reactions. Eosinophils secrete toxic enzymes, such as peroxidase and thereby generate free radicals (Smith, 2014).

Natural Killer (NK) cells do not directly attack pathogens but have cytotoxic properties as they detect and destroy infected host cells by inducing programmed cell death or apoptosis. NK cells can detect these cells either by detection of MHC-molecules, by opsonization or cytokine-mediated (Waldhauer & Steinle, 2008). NK cells belong to the cytotoxic arm of ILCs. Non-cytotoxic ILCs, also called helper ILCs, exert their functions mainly through production of cytokines.

1.3.2. The adaptive immune system

The adaptive or specific immune system generates an acquired immunity, so it reacts based on antigen exposure and generates an immunological memory to adapt the immune response when exposed again. This can promote lifelong immunity. The adaptive immune response needs much longer for an adequate reaction, so the simultaneous activation of the innate immune system is also required.

Lymphocytes are the centre of the adaptive immune system. As a subpopulation of leukocytes, they derive from hematopoietic stem cells in the bone marrow and possess certain receptors to recognize specific antigens of the pathogen to subsequently fight it. Antigens, as molecules on the pathogen's surface, can be bound by antibodies and receptors conjugated with lymphocytes. Every antigen has an epitope, which depicts the exact binding spot of the lymphocyte receptor or antibody (Janeway et al., 2001).

Lymphocytes of the adaptive immune system are divided into two subgroups: T lymphocytes and B lymphocytes.

1.3.2.1 T lymphocytes

Once formed in the bone marrow, progenitor T cells migrate to the thymus gland to maturate. Here, they develop T cell receptors (TCR) and immunophenotype surface proteins, such as CD3, CD4 and CD8. Every T cell expresses CD3 and either CD4 or CD8. $CD4^+$ T cells are called helper cells (TH) as they promote the activation of other immune cells. Th1 cells are especially suited for fighting intracellular pathogens, while Th2 cells help with eliminating extracellular parasites. $CD4^+CD25^+FOXP3^+$ cells

are called regulatory T cells (Tregs) as they help with the distinction between self and foreign molecules, which is a crucial part of T cell activity. During maturation in the thymus, self-reactive T cells undergo apoptosis (negative selection) while adequately functional cells will further differentiate (positive selection) (Hof & Schlüter, 2019).

$CD8^+$ T cells are called cytotoxic cells as they destroy and remove pathogens and infected host cells via apoptosis. T cells can only recognize antigens that are attached to specific molecules on antigen-presenting cells (APC), such as dendritic cells (DC) and macrophages. These surface molecules are called Major Histocompatibility Complex (MHC) and can appear as MHC I or MHC II. $CD4^+$ cells only bind to MHC II, whereas $CD8^+$ cells interact with MHC I. Antigen-presenting cells internalize pathogens in the periphery and move to secondary lymphoid organs (such as the thymus) to present these pathogens via MHC to yet immature T cells. This activates the T cells and promotes their proliferation. Somatic recombination allows for every T cell to develop a unique T cell receptor, reacting to a specific antigen (Janeway et al., 2001).

1.3.2.2 B lymphocytes

During adult life, B cells form and maturate from lymphoid progenitor cells in the bone marrow. They migrate to secondary lymphoid organs, such as lymph nodes and the spleen, where they continue their maturation into two main subsets: marginal and follicular zone B cells. After encountering antigens, they are activated to either plasma cells or memory cells. B cells express the membrane-bound B-cell receptor (BCR) which can not only bind to antigens, but also process them, resulting in activation of signalling pathways. Subsequently, plasma cells secrete antibodies (initially especially IgM), the so-called humoral immune response. Memory cells will also secrete antibodies, after the second or another exposure to the same antigen and thereby form an immunological memory (Ratajczak et al., 2018). Plasma and memory cells are mostly tissue-resident instead of circulating in the blood.

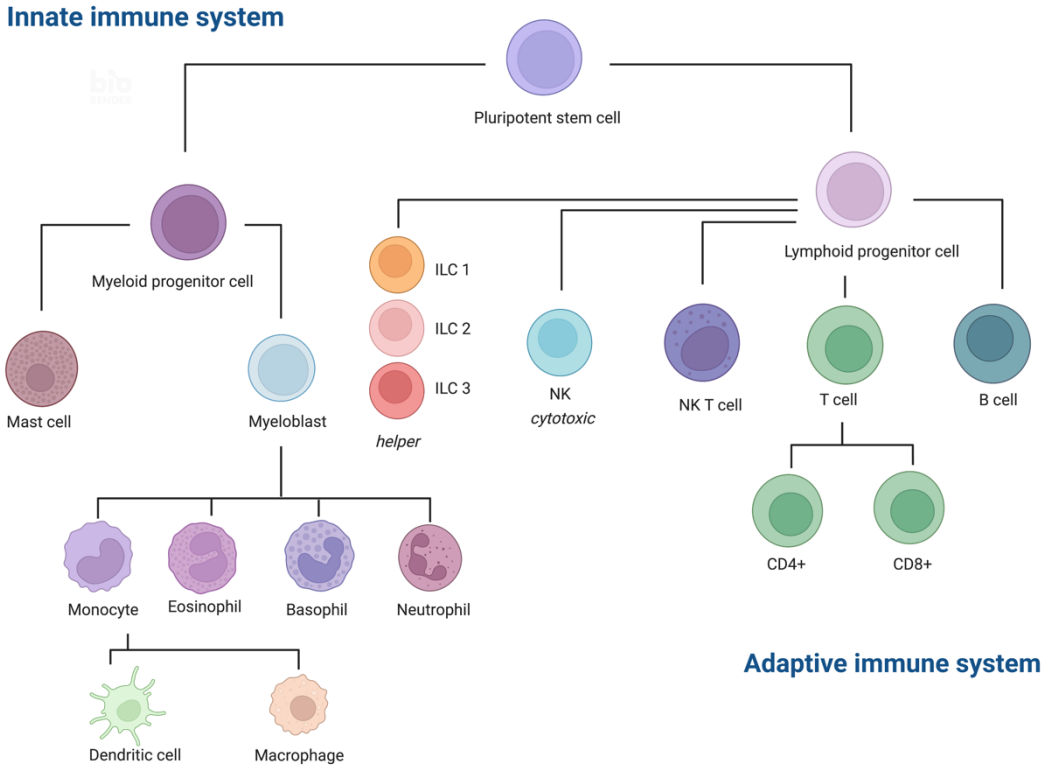

Figure 2: Cell components of the innate and adaptive immune system, modified after Torang *et al.* (Torang et al., 2019), created with BioRender.com

1.3.2.3. Primary lymphoid organs (PLO)

Formation, development and maturation of immune cells take place in primary lymphoid organs including bone marrow, thymus and fetal liver. They are not directly involved in the immune defense but are responsible for the development of immunologically competent B- and T-lymphocytes. The bone marrow is part of the PLO as there leukopoiesis and maturation of B-lymphocytes take place. In contrast, immature T-lymphocytes are also formed in the bone marrow but migrate towards the thymus in order to differentiate.

1.3.2.4. Secondary lymphoid organs (SLO)

Secondary lymphoid organs generally create immune responses and tolerance. They represent spatially defined immune defense compartments, where naive lymphocytes encounter antigens, leading to their differentiation into effector cells. These include

the spleen, lymph nodes, adenoids and tonsils (palatine, pharyngeal and lingual) as well as mucosa associated lymphoid tissue (MALT). The latter describes clusters of lymph follicles within the mucosa and includes Peyer's Patches (PP) or gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), nasal-associated lymphoid tissue (NALT) and vulvovaginal-associated lymphoid tissue (VALT).

After emigrating from the primary lymphoid organs, mature, but naive lymphocytes migrate to the secondary lymphoid organs via blood circulation, where they bind and detect antigens and subsequently develop into mature effector cells. These can leave the SLO in order to approach sites of infection or inflammation, where an immune response is necessary. SLO development depends on a strictly balanced expression of several lymphoid chemokines and cytokines by specific cells, such as lymphoid tissue initiator, inducer and organizer cells (Ruddle & Akirav, 2009; Ulfig, 2019).

1.3.3. Innate lymphoid cells

ILCs are a special subset of lymphocytes that straddle both the innate and adaptive immune system and can be divided into three different groups based on their phenotype and functions, particularly their pattern of cytokine production and transcription factor expression. All groups of ILCs initially derive from a common lymphoid precursor (CLP) cell in the fetal liver and adult bone marrow and then populate their tissue niches early, possibly even during embryogenesis (Yu et al., 2014). This process of homing is mediated by receptors such as α4β7, CCR6, CXCR6 and CCR9. The expression of α4β7 on the surface of all ILC precursors leads to interaction with the adhesion molecule MadCAM-1 on venules of lymphoid tissues (Klose & Artis, 2016; Spits et al., 2013). In contrast to other immune cells, ILCs do not seem to undergo constant renewal from bone marrow precursors but can be replenished from within tissues.

The discovery and investigation of ILCs over the past years has deeply changed the knowledge on the immune system and its regulation. In contrast to B- and T cells, they lack recombinant antigen receptors but can enhance adaptive immunity (Vivier et al., 2018). They do not express known immune cell lineage markers, which makes their characterization complex. ILCs can be seen as the innate equivalents of adaptive immune cells, especially T cells. Each group of ILCs can be considered a counterpart for a certain T cell subset, as they share a similar cytokine profile and depend on the same transcription factors. Further, each pair seems to not only interact, but also amplify the counterpart cell's actions. ILCs can be subdivided into two major lineages: cytotoxic ILCs (NK cells) and helper-like ILCs (Mjösberg et al., 2012).

Recently, it has been suggested that ILC functionality is rather inconsistent and depends on their local microenvironment, thus there appears to be plasticity within the three different subsets, as they have the ability to adjust their phenotypes. This process is depending on the local cytokine milieu they are in. The phenomenon of plasticity seems to be beneficial in creating an even more specific immune response (Colonna, 2018). Due to their localization at mucosal surfaces, ILCs initiate an early, first-line response to pathogens, compared with the adaptive response. ILCs can further interact with microbiota, nutrients, metabolites, neurons and parenchymal cells, and their functions seem to go beyond classic immunological properties as they

also provide tissue and metabolic homeostasis and play a role in the nervous system (Ignacio et al., 2017).

ILCs are mostly tissue-resident cells at mucosal surfaces where they contribute to an appropriate immune response, epithelial barrier integrity and remodelling, repair and regeneration of tissues, especially after damage (Gasteiger et al., 2015; Klose & Artis, 2016; Vivier et al., 2018). However, in the past years, their circulating properties have been of an increasing interest in the context of their migration, for example from peripheral lymph nodes to these tissue barriers (Dutton et al., 2019; Shao et al., 2021).

1.3.3.1 Group 1 ILCs

Group 1 ILCs include the classic NK cell subset and the non-cytotoxic IFN-γ-producing ILC1s, which share many overlapping characteristics. Group 1 ILCs both derive from a common innate lymphoid cell precursor (CILP), though NK cells originate from NK cell precursors and ILC1 from innate lymphoid cell precursors (ILCP). The expression of the natural cytotoxicity receptor (NKp44 in humans, NKp46 in mice) further defines this subgroup (Gronke et al., 2016). Their signature cytokines released upon activation are IFN-γ and TNF-α besides others, such as IL-12. Group 1 ILCs require the T-box transcription factor T-bet and T-box description factor Eomesodermin (Eomes) for their differentiation and maturation. Whereas ILC1s especially require T-bet, the NK subset is dependent on Eomes expression. NK cells have cytotoxic properties as they are able to release cytotoxic vesicles containing granzymes and perforin and can be seen as the equivalent of $CD8^+$ killer cells. In contrast, helper-like ILC1s are considered the innate counterpart of Th1 cells. While ILC1 cells are mostly tissue-resident, NK cells are rather circulating cells (Cortez & Colonna, 2016).

Collectively, they specifically fight viruses and intracellular pathogens, they act in tumor surveillance and inflammation and can lead to activation of Th1 cells. The non-NK (ILC1) subset is either $CD127^+$ or $CD127^-$. The $CD127^-$ subset depends on T-bet and Nfil-3 for development, is independent of IL-15 and can be found in the intestinal epithelium. Possibly some ILC1s develop from the ILC3 subset via IL-12. During this development the expression of the transcription factor retinoic acid receptor-related

orphan receptor gamma T (RORγT) disappears while T-bet increases. T-bet itself promotes IFN-γ production and represses IL-17 (J. Zhang et al., 2018).

1.3.3.2 Group 2 ILCs

Group 2 ILC2s are tissue-resident cells and important components of type 2 immune responses. They are characterized by the production of Th2-associated cytokines. Upon activation with IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), ILC2s secrete IL-5, IL-13 and Amphiregulin (Areg). They require IL-7, retinoic acid receptor-related orphan receptor alpha (RORα) and GATA-3 for their development and differentiation. ILC2s can be further characterized by expression of CD127 (IL-7Rα), ST2 (IL-33R) and IL-17RB (IL-25R) (Tait Wojno & Beamer, 2018; Walker et al., 2013).

ILC2s provide barrier surveillance and epithelial responses. Their cytokine array, especially secretion of Areg, promotes post-infectious tissue repair by inducing proliferation of epithelial cells. This explains the crucial role of ILC2s in wound healing. Additionally, they provide anti-helminth responses as they can recruit eosinophiles via IL-5, provoke mucus production and lead to activation of Th2 cells. Therefore, they play a pivotal role in allergic lung diseases, asthma, atopic diseases and chronic rhinosinusitis, as patients with this type of diseases express a higher number of group 2 ILCs. By expression of IL-17, ILC2s can provide antifungal properties (Walker et al., 2013). Group 2 ILCs have also been shown to play a role in adipocyte differentiation and regulation of adiposity as obese people express a lower number of ILC2s in their adipose tissue (Brestoff et al., 2015; Gronke et al., 2016).

1.3.3.3 Group 3 ILCs

Group 3 ILCs can be divided into ILC3s and lymphoid tissue inducer (LTi) cells and are characterized by the expression of the transcription factor RORγT. They are considered the innate counterpart of Th17 cells but can also regulate a Th17-mediated response.

According to the expression of CCR6, ILC3s can be divided into a CCR6-positive ($CCR6^+$) and CCR6-negative ($CCR6^-$) subset. Upon activation with IL-1β, IL-2 and IL-23, LTi cells are able to produce IL-17, IL-22 and TNFα. Upon activation of group 3 ILCs with IL1-β, IL-2, IL-6, IL-23 or by toll-like receptors (TLR) agonists, they produce IL-17, IL-22, IFN-γ, granulocyte macrophage colony stimulating factor (GM-CSF) and lymphotoxin-α (LT). These effector cytokines resemble those of Th17 cells (Mjösberg & Spits, 2016; Montaldo et al., 2015).

LTi cells mediate the development of secondary lymphoid tissue during embryogenesis, and lymphotoxin additionally promotes lymph node formation in adults. They orchestrate formation of cryptopatches that can be found in the intestinal lamina propria and may act as a stem cell reservoir (Mebius, 2003). LTi cells lack the expression of natural cytotoxicity receptors (NCR), but express the receptor protein c-Kit and chemokine receptor 6 (CCR6).

ILC3s can be classified according to their expression of NCR into a NCR^+ and a NCR^- subset. Both the NCR^+ and NCR^- subset can be activated via IL-1β, IL-2, IL-6 and IL-23. The NCR^+ subset expresses NKp44 and/or NKp46 and upon activation secretes IL-22, Granulocyte macrophage colony stimulating factor (GM-CSF), TNF-α and lymphotoxin. The NCR^- subset produces GM-CSF, LT and IL-17 and therefore can play a role in gut and skin inflammation (Mjösberg & Spits, 2016). In comparison to the other groups of ILCs, type 3 ILCs are less tissue-resident. The functions of group 3 ILCs are diverse. They promote immune response to extracellular pathogens, such as bacterial and fungal infections. They can be found at mucosal sites, especially of the gastrointestinal system, where they promote mucosal defense and barrier integrity and contribute to homeostasis of the intestinal microbiota (IL-22), thus explaining their association with inflammatory bowel disease. In colon inflammation, they up-regulate IL-17A, IL-22, and IFN-γ production (Buonocore et al., 2010). They may also recruit macrophages and play a role in tumor rejection and graft-versus-host-disease (Melo-Gonzalez & Hepworth, 2017).

1.3.3.4 Reservoirs of ILCs

ILCs have been shown to reside in various organs and tissues. They promote diverse functions, depending on the tissue they're found in (Klose & Artis, 2020; Sonnenberg et al., 2013).

In the skin, stimulation by an allergen or inflammation can lead to ILC2 and/or ILC3 response. The ILC2 response can potentially cause further activation of mast cells which subsequently promotes allergic inflammation, while the ILC3 response rather thickens the epidermis. Inflammatory and auto-immune skin diseases are associated with high levels of NCR^{+}ILC3s and ILC2s. For example, an altered number of ILC3s is found in psoriatic skin lesions (Salimi & Ogg, 2014).

In the gastro-intestinal (GI) tract, all three ILC subsets have been observed. Their appearance increases from proximal to distal GI tract. Since the cytokines they produce can affect the commensal bacteria, ILCs can have an impact on their composition and localization (Sonnenberg & Artis, 2012). ILC1s have been shown to contribute to inflammatory responses, they are mostly found in the upper GI tract (Geremia & Arancibia-Cárcamo, 2017). ILC2s promote immunity and protection against parasites, though they are barely found in the gut. ILC3s help with tissue repair though may as well induce colitis via IL-17. IL-23 responsive ILCs have been shown to play a role in Inflammatory Bowel Disease (Geremia & Arancibia-Cárcamo, 2017).

Although being of a relatively small number, ILCs have been shown to play an important role in infectious and allergic lung diseases. While in the murine lung ILC2s comprise the biggest subset, the human lung predominantly hosts ILC3s. Lung-resident ILC2s in both mice and humans can be found in the bronchoalveoloar space. Their depletion has been shown to favour epithelial degeneration and higher risk of influenza infection (Monticelli et al., 2011).

1.3.3.5 ILCs in reproductive organs and pregnancy

Innate immune cells are highly important for reproduction and are a dominant subset at the fetal-maternal interface. NK cells, macrophages and dendritic cells have been

known to play a crucial role for tissue homeostasis and preparation for implantation (Moffett & Colucci, 2014). ILCs have not yet been studied as extensively, though over the past years the investigation of their role in pregnancy has been more and more established. Recent research points to an involvement of ILCs in essential events of pregnancy establishment and maintenance in both mice and humans (Miller et al., 2018).

Uterine NK (uNK) cells seem to be the key players at the fetal-maternal interface. NK cells in the decidua have been characterized extensively as a contribution to an immunosuppressive environment and tissue building in the context of a successful early pregnancy. An early inflammatory stage is necessary for the implantation before later, an immunosuppressive phase prevents fetal rejection (Vacca et al., 2015).

The proportions of uterine ILC1s increase throughout murine gestation, compared with non-pregnant mice. Decidual ILC1s are most abundant mid-gestational and rather scarce in late pregnancy. This is in contrast to the human decidua, where ILC1s are most present during the first trimester. IFN-γ production by stimulated uterine ILCs (uILCs) is increased during pregnancy, to which the ILC1 subset contributes, thus promoting a pro-inflammatory milieu. In contrast to other tissue-resident ILC1s, their development in the uterus is independent of Nfil3 (Doisne et al., 2015).

ILC2s play a minor role in pregnancy. A small proportion of ILC2s has been observed in both the human non-pregnant uterus, specifically the endometrium, and first-trimester decidua. During the third trimester ILC2s appear to be the most abundant subset in the decidua. As ILC2s play a key role in homeostasis, it is likely that they contribute to a homeostatic environment at the feto-maternal junction in late pregnancy rather than to the early-gestational pro-inflammatory milieu. In contrast to this, decidua basalis from women undergoing spontaneous preterm labour contained higher proportions of ILC2s. In mice, the presence of ILC2s and ILC2-like cells was demonstrated in both the non-pregnant and pregnant myometrium with the ILC2-like subset being the most abundant one in the myometrium of pregnant mice, especially from mid-pregnancy throughout term (Doisne et al., 2015; Miller et al., 2018).

ILC3s are the most thoroughly studied subset of ILCs in reproductive organs. Uterine ILC3s have been observed in both murine and human uteri, including the human

endometrium. uILC3s can be subdivided according to their expression of NCR. The NCR^+ILC3 subset is the predominant group in humans, whereas NCR^-ILC3s dominate the murine uterus. During murine pregnancy, ILC3 proportions are predominantly elevated throughout early- and midgestation, but remain rather constant, whereas decidual ILC3s are absent in mid-pregnancy. The development of ILC3s is, similar to uILC1s, not dependent of Nfil3 (Doisne et al., 2015). The human first trimester-decidua contains ILC3s, which feature a unique broad cytokine profile including IFN-γ, IL-13, IL-17A and IL-22. Their expression of GM-CSF and IL-8 contributes to spiral artery remodelling via recruitment of neutrophils during early pregnancy. Further, it has been postulated that uILC3s might contribute to fetal tolerance by reduction of their antigen presentation potential (Einenkel et al., 2020). Impaired uILC3 proportions can be correlated with abnormal placentation and fetal growth restriction, indicating their complex role in decidualization and the early-gestational pro-inflammatory milieu. In this respect, increased numbers of ILC3s were found in the decidua parietalis from women experiencing spontaneous preterm labour (Mendes et al., 2019; C. J. Wang et al., 2020).

LTi cells are rather important in the fetal compartments where they orchestrate formation of secondary lymphoid organs, such as the spleen, mesenteric lymph nodes and Peyer's patches (Cherrier & Eberl, 2012).

1.3.4. Recruitment and composition of immune cells at the fetal-maternal interface

The fetal-maternal interface originates from the site of implantation and consists of the decidua (maternal) and trophoblast (fetal). Immunologically, it can be considered a unique site where the immune system is highly active and challenged. A vivid interplay between decidual stromal cells (DSC), trophoblast, decidual immune cells (DIC), hormones, cytokines and other molecules has been demonstrated. The composition and recruitment of DIC is most likely - at least partially - a result of altered expression of chemokines and chemokine receptors at both the fetal-maternal interface and secondary lymphoid organs. In accordance, the expression of chemokines in DSC, DIC and the trophoblast have been demonstrated, and local chemokine patterns may change throughout gestation (Red-Horse et al., 2001). Farine *et al*. confirmed that peripheral maternal leukocytes (monocytes, granulocytes)

can be activated by decidua-derived chemokines (Farine et al., 2017). Thus, a complex and finely balanced chemokine network contributes to the establishment and maintenance of the fetal-maternal interface (Ramhorst et al., 2016).

Decidual immune cells make up to 50 % of all decidual cells. The composition of decidual leukocytes can be estimated as following: 65-70% uNK cells ($CD56^{bright}CD16^{-}$), 10-20 % macrophages, 15 % T cells and 2-4 % dendritic cells. The balancing of maternal immunotolerance towards the fetus and adequate protection from local infections requires a balanced and dynamic adaptation of these populations (Red-Horse et al., 2001; L. Xu et al., 2021).

Uterine NK (uNK) cells are the predominant leukocyte subpopulation in the decidua (70%), especially during first trimester. Their origin remains controversial, as uNK cells differ from the peripheral NK cells (pNK) phenotypically and functionally: they have less cytotoxic properties. Different chemokines, including CCL3, CX3CL1, CXCL10-12 and adhesion molecules are produced by decidual stromal cells and the trophoblast to promote their migration. The uterine microenvironment, especially altered hormonal levels, affect their migratory ability via up-regulation of chemokines and receptors. uNK cells have an essential impact on trophoblast invasion and play a crucial role in angiogenesis and vascular remodelling of spiral arteries via secretion of VEGF. Their absence leads to termination or impairment of pregnancy (Moffett-King, 2002; Sojka et al., 2019).

Decidual macrophages are the second largest population of lymphocytes at the fetal-maternal interface. They play an essential role in trophoblast invasion, decidualization and remodelling of vessels and tissues (Jena et al., 2019). Monocytes contribute to the removal of cell debris after implantation-derived tissue damage. Their number increases in early pregnancy and remains rather stable until it reaches a peak at term, where they promote the rupture of membranes (Gomez-Lopez et al., 2010; P. Xu et al., 2002). Immature monocytes migrate to the uterine tissues under the influence of trophoblast-derived CCL2, CCL5, M-CSF and CXCL16. Their differentiation *in situ* into mature macrophages recruits and activates further lymphocytes (Imhof & Aurrand-Lions, 2004). Multiple pregnancy complications, including IUGR, infertility, recurrent spontaneous abortions and preeclampsia were associated with dysbalanced macrophage levels (Jena et al., 2019).

T cells are a rather small subset at the fetal-maternal interface and comprise 10-20 % of decidual leukocytes in early pregnancy. However, they are one of the most abundant populations in the decidua (Nancy & Erlebacher, 2014). Different effector T cells, including Th1, Th2, Th17, Tregs and cytolytic T cells have an ambiguous effect on pregnancy. Tregs account for at least half of the T cells around mid-gestation. Progesterone administration in mice was shown to expand Treg populations, indicating the importance of an immunological-endocrinological crosstalk (G. Mao et al., 2010). Tregs express a wide array of chemokine receptors, facilitating migration to the uterus. Hence, Tregs contribute to fetal tolerance, they especially prevent rejection due to paternal antigens. Epigenetic pathways seem to control T cell recruitment to the uterus during murine pregnancy: genes for the expression of chemokines were silenced in pregnancy, indicating an active limitation of T cell access to the uterus (Nancy et al., 2012).

Dendritic cells account for less than 5% of decidual leukocytes and derive from common myeloid progenitors. They provide an immunotolerant milieu towards the fetus and balance the immune response by cross-talking with other immune cells, as they present placental antigens to maternal T cells and increase Treg levels. Especially around implantation, their interaction with NK cells promotes and regulates vascular remodelling. This interaction exists both at the fetal-maternal interface and in secondary lymphoid organs (Tagliani & Erlebacher, 2011). DC migration, especially during the first trimester, is driven by the interactions of CCR7 and ligands such as CCL2, CCL5 and M-CSF. They maturate in a hormone-dependent manner, with progesterone promoting their immunotolerant functions (Du et al., 2014; Li et al., 2011; Wei et al., 2021).

Mast cells are a common subpopulation of cells in the uterus and during pregnancy. They reside as precursors *in situ* and maturate upon pregnancy stimuli or migrate towards the uterus as mature cells. Estradiol and progesterone have been shown to impact their migration in humans and mice by up-regulation of the chemokine receptors CCR3, CCR4 and CCR5. Estradiol and progesterone promote mast cell maturation and degranulation, which has been shown to correlate with angiogenesis during pregnancy. Signals from the embryo can stimulate the secretion of histamine releasing-factor which further triggers degranulation (Cocchiara et al., 1996; Jensen et al., 2010).

B cells play a minor role at the fetal-maternal interface, though they are significantly increased upon intrauterine infection. Their small proportion remains stable throughout the menstrual cycle and pregnancy. However, at term and during infections their numbers slightly increase in response to chemotactic stimuli from the fetal membranes, including CXCL13 (Nhan-Chang et al., 2008).

1.4. Chemotaxis, chemokinesis and chemokines

Chemotaxis describes the process of directional cell migration towards an extracellular chemical gradient, thus playing a role in inflammation, embryogenesis and cancer. Chemokinesis, in contrast, describes the process of undirected random locomotion in response to substances in the environment (Becker E L, 1977; Bignold, 1988).

Chemokines are small (6-14 kD), mostly pro-inflammatory peptides that can bind to specific receptors on cell surfaces. They are secreted by a wide array of cell types, such as T lymphocytes, monocytes and neutrophils (Reikvam et al., 2013). More than 50 chemokines have been described in humans to date (Turner et al., 2014). Based on their gene expression, they have rather homeostatic or inflammatory properties (Zlotnik & Yoshie, 2012). The receptors for chemokines belong to the G-protein coupled receptor superfamily of seven-transmembrane domain receptors. Upon activation of these, leukocytes can be arrested and migrate in response to chemotactic signals, for example to sites of infection. Several chemokines interact with multiple receptors, while others have specific binding partners (Proudfoot, 2002).

Chemokines are divided into 4 subclasses based on their amino-terminal cysteine motif, designated CC, C, CXC and CX3C chemokines. (Bacon et al., 2002; Zlotnik & Yoshie, 2012). The nomenclature of chemokines is based on the receptors they bind. A number indicates the order of their discovery, and R is added for receptor. CC chemokines bind to CCR chemokine receptors and mediate recruitment of lymphocytes, monocytes and eosinophils. These include RANTES (CCL5), MIP-1, MIP-1α (CCL3) and MIP-1ß (CCL4). CXC chemokines bind to CXCR chemokine receptors and recruit neutrophils (Basheer et al., 2013; Sodhi et al., 2004).

Chemokines are involved in many physiological and pathological events. They contribute to cellular proliferation, apoptosis, angiogenesis, hematopoiesis, pro- and anti-tumor activity and most importantly inflammation (Balkwill, 2004; Mackay, 2001; Rossi & Zlotnik, 2000; Zlotnik & Yoshie, 2012). Thus, they are essential in the process of inflammation, they provide host defense, immune surveillance and tissue repair. They may also mediate pathological immune responses as in autoimmune conditions or chronic inflammatory diseases (Godessart & Kunkel, 2001; Karin & Wildbaum, 2015).

Chemokines are further involved in coordinating the interaction between decidual tissues, maternal immune cells and the trophoblast at the fetal-maternal interface. Both the primary trophoblast and decidual stromal cells express different chemokine receptors and secrete chemokine ligands for recruitment of further immune cells. In addition, some pregnancy complications have been associated with abnormally expressed chemokines or chemokine receptors (De La Torre et al., 2007; Kheshtchin et al., 2010; Lockwood et al., 2006; Szarka et al., 2010).

1.4.1. Expression of chemokines and their receptors during pregnancy

Chemokines are well-known regulators of immune cell trafficking. They are common players in the pathogenesis of inflammation, thus they are abundantly expressed in the early phase of pregnancy. They play essential roles during the establishment and development of the fetal-maternal interface in pregnancy as they regulate the access of leukocytes to the decidua and contribute to tissue repair (Red-Horse et al., 2004). Chemokines seem to be expressed in the decidua in a tightly controlled manner, as the composition of local immune cells is very regulated. They are expressed in glandular and luminal epithelium, decidual leukocytes and decidualized endometrial stroma, but also in the maternal vasculature. Chemokine receptors are present on the blastocyst, the human trophoblast and decidual leukocytes. Hence, communication exists between these different components, and a unique and very complex chemokine milieu is established (Hannan et al., 2006). Red-Horse *et al.* identified several chemokine receptor-chemokine ligand pairs that are involved in leukocyte recruitment to the maternal-fetal interface. The most abundant pairs were the ones regulating NK cell, monocyte and T cell recruitment, as these cells make up

the majority: CCR1/HCC-1, CCR2/MCP-1, CCR5/MIP-1alpha, CCR7/SLC, CX3CR1/fractalkine, and CXCR3/ITAC (Red-Horse et al., 2001).

A wide array of chemokine receptors in the primary trophoblast and human DSC were assessed by Du *et al.* CXCR4, CXCR6 were highly and CCR1, CCR3, CCR5, CCR8, CCR9, CXCR1, CXCR4, CXCR6, XCR1 and CX3CR1 moderately expressed in the trophoblast. DSC expressed high levels of CCR2, CCR5 and CCR10 and moderate levels of CCR1, CCR3, CCR4, CCR6, CCR8-9, CXCR1 CXCR4, CXCR6, XCR1 and CX3CR1 (Du et al., 2014).

Besides the recruitment of uterine leukocytes, chemokines are also of interest as they mediate extravasation of leukocytes: SLC (secondary lymphoid-tissue chemokine) examplarily regulates their migration through secondary lymphoid organs. In conclusion, chemokines are not only important for migration to the uterus, but also for emigration, as the leukocyte composition changes throughout gestation. Chemokines have regulating properties on the invasion of the trophoblast as they are involved in its targeting to maternal vessels. The trophoblast invasion is a tightly controlled process: it sets the stage for pregnancy, and malfunctioning of this process is associated with pregnancy failures (Ball et al., 2006; Hustin et al., 1990; Kaufmann et al., 2003; Yamada et al., 2003).

The paracrine and autocrine effect of chemokines on the maternal-fetal interface were demonstrated by Wu *et al.* First trimester human trophoblast-secreted CXCL12 promotes its own proliferation in an autocrine manner, interacts with $CXCR4^+$ DSCs in a paracrine manner and recruits $CXCR4^+$ NK cells to the decidua (Wu et al., 2004). An extensive crosstalk between cytokines, chemokines and the trophoblast has been shown. The pro-inflammatory cytokines TNF-α and IL-1β up-regulate CCL2 expression, which excessively augments macrophage recruitment. This is associated with impaired invasion, and elevated macrophage levels were associated with preeclampsia (Dominguez et al., 2003; Lockwood et al., 2006). CCR4 is a predominantly Th2 associated receptor. Its expression on T cells mediates their recruitment to the fetal-maternal interface, as its ligand TARC (CCL17) is secreted from the trophoblast and endometrial cells. It is also expressed on the invading cytotrophoblast (Tsuda et al., 2002). CCR6 is expressed by the majority of $CD4^+FOXP3^+$ Tregs and Treg migration is enhanced *in vitro* by CCR6 (Acosta-

Rodriguez et al., 2007). The effect of CCR6 and its ligand CCL20 on Treg migration to the feto-placental environment was proposed by Zhang *et al*. The effect of this receptor/ligand pair was found to be important in reproduction, as decidua samples of women experiencing recurrent unexplained abortions contain much lower levels of CCR6 and CCL20 (X. X. Zhang et al., 2015).

Both CCR10, CCR3 and their ligand CCL28 are co-expressed in human DSC from first trimester. Their expression is up-regulated upon exposure to pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-17A. Increased levels of CCL28 can lead to inflammation and DSC apoptosis. In this regard, DSC from spontaneous abortions contained higher levels of CCR3/CCR10/CCL28 compared with successful pregnancy, indicating the manifold effects of chemokines and the need of a well-established equilibrium (Sun et al., 2013).

1.5. Cytokines

Cytokines are small protein molecules, secreted by various cell types in order to regulate the interaction and communication with other cells. Even though they are mainly leukocyte-produced, they can also be secreted by stromal cells. Cytokines can either act on nearby cells (paracrine function), on further distant cells (endocrine function) or even on themselves (autocrine function). They bind to specific receptors on the surface of their target cells, according to which they can be further classified. It must be noted though, that instead of having direct effects on cells, cytokines have rather regulating and modifying properties on immune responses.

An extensive cytokine network exists, where several immune cells coordinate their redundant effects as they can secrete multiple cytokines but are also susceptible to various cytokine stimuli. Cytokines are very potent due to their high affinity to receptors (Fitzgerald et al., 2001). Even though cytokines can mediate both anti- or pro-inflammatory reactions, they play a key role in inflammation-derived immune responses and infections. They are often released in a cascade, at worst leading to a cytokine storm, for example during acute inflammatory diseases, such as COVID-19 (Hojyo et al., 2020). In a similar manner, dysbalanced cytokine responses are

involved in pathological conditions, such as chronic inflammation or pain (J. M. Zhang & An, 2007).

Even though cytokines can be secreted by many different cell types, T cells and macrophages are the predominant source. T helper cells are classified as Th1 and Th2 cells according to their pattern of cytokine production. This paradigm was proposed in 1986 by Mosmann *et al*., leading to a revolution in immunology (Mosmann et al., 1986). Th1 cells characteristically produce pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2, IL-12, IL-18 and promote cell-mediated innate immunity and phagocyte-dependent inflammation. They are also involved in organ-specific autoimmune diseases and allograft rejections. In contrast Th2 cells are defined by production of rather anti-inflammatory cytokines, including IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, and are involved in providing humoral immunity against extracellular pathogens (Charlton & Lafferty, 1995; Kwak-Kim et al., 2003; Singh et al., 1999). Whether activated T helper cells differentiate into a Th1 or Th2 based subset depends on their surrounding microenvironment when encountering antigens. Although innate lymphoid cells express an analogous cytokine milieu, whether they follow the Th1/Th2 paradigm is yet to be elucidated.

1.5.1. Cytokines in female reproductive tract and pregnancy

The female reproductive tract (FRT), including vagina, cervix, uterus and ovaries, requires an adequate immunological response at all times as it is constantly exposed to potential pathogens. Thus, epithelial cells of the FRT must consistently ensure an intact mucosal barrier and a sufficient immune response must be guaranteed at all times (Fahey et al., 2005). During pregnancy, many local and peripheral changes in the composition of immune cells have been reported. The phenomenon of a predominant Th2 type immune response while suppression of the Th1 type was initially considered to be critical for the establishment and maintenance of healthy pregnancy (Calleja-Agius & Brincat, 2008).

Early pregnancy is a complex immunological period. On the one hand, it resembles the tolerance of an allograft, and the abundance of anti-inflammatory molecules protect the conceptus. In this respect, the Th2-type cytokines IL-4 and IL-10 were

identified as protective components (Chatterjee et al., 2014). On the other hand, the process of implantation requires a pro-inflammatory milieu. The invasion of the blastocyst disrupts the endothelial lining and necessitates prompt tissue repair. However, an excessive inflammatory response would jeopardize tolerance of the allograft and could induce fetal rejection.

Many reports have demonstrated the pregnancy-supportive effect of Th2 type response (Raghupathy, 2001). The Th2 type dominance in pregnancy is based on enhanced migration and differentiation, especially at the fetal-maternal interface. Th2 cytokines are even produced by the trophoblast itself, a mechanism to promote protection. The adverse effect of Th1 cytokines on pregnancy outcome has also been observed, as they play a critical role in spontaneous abortions, preterm labour and preeclampsia. Administration of TNF-α, IFN-γ and IL-2 causes miscarriage in mice (Chaouat et al., 1990). Nevertheless, IFN-γ has been shown to promote spiral artery remodelling, indicating its essential role in pregnancy. Thus, it is rather about the balance of Th1 instead of its entire suppression. Tregs are known to contribute to this balance. They possess immunoregulating properties as they induce tolerance, especially of paternal antigens. Further tolerance mechanisms include inhibition of NK cell cytotoxicity and DC maturation, diminution of $CD4^+$ and $CD8^+$ cytokine secretion and decrease of B cell-derived immunoglobulin production. They have a regulatory effect on the composition of T effector cells and have been shown to be essential for successful pregnancy outcome as their induction of immunotolerance is a critical process during implantation. CCR2, CCR4, CCR5 and CCR6 are known to induce Treg migration, which is driven by CCL2, CCL4, CCL17, CCL20, CCL22 (Guerin et al., 2009).

In contrast are Th17 cells, a known producer of IL-17 and inductor of inflammation. Their involvement in autoimmunity and transplant rejection has been reported (Heidt et al., 2010). An imbalance of Th17 and Treg has been characterized as a pathophysiological feature of preeclampsia (Laresgoiti-Servitje, 2013). Hence, the Th1/Th2 paradigm from 1986 has nowadays rather evolved into a Th1/Th2/Th17/Treg paradigm (Peck & Mellins, 2009). This was specifically demonstrated in spontaneous abortions: whereas pregnancy loss was associated with both a Th1 and Th2 predominance, increased levels of Th17 and lower proportions of Tregs were also observed (Sereshki et al., 2014; W. Wang et al.,

2020). Both progesterone and estradiol have an impact on T cell proportions. They promote activation of Tregs and Th2 response, while suppressing the pro-inflammatory pathway via reduction of IL-6 and TNF-α (Hall & Klein, 2017). This confirms the endocrinological-immunological interplay during gestation. A successful pregnancy thus depends on the finely balanced equilibrium of all subsets with a Th2 shifted tendency.

1.6. Cell adhesion and migration

Coordinated cell movements are a fundamental mechanism in the organization of multi-cellular organisms. Cell migration is a crucial part of immune responses, wound healing and tissue repair. In regard of leukocytes, it includes their activation, rolling, attachment and adhesion to the vascular endothelium, transendothelial migration and the targeted movement towards the tissue of destination, for example via chemotactic gradients (Trepat et al., 2012).

Cell attachment is regulated by the formation of cell adhesion complexes, of which integrins are an essential component. Integrins are heterodimeric surface receptor glycoproteins, they consist of an extracellular domain and an intracellular cytoplasmic tail. Two subunits can be distinguished: the α-chain and the β-chain are tightly bound to each other. According to the α-β-constellation, more than 20 different integrins can be classified (Bachmann et al., 2019; Barczyk et al., 2010). Integrins have anchoring properties as they mediate the mechanical connection between cells and the extracellular matrix (ECM). Different components of the ECM, such as fibronectin, collagen, laminin or vitronectin can act as ECM binding partners for integrins via recognition and binding to their Arg-Gly-Asp (RGD) motif. They mediate intracellular signalling by activation of signalling pathways and are of importance in different physiological and pathological conditions.

On the one hand, particular diseases are associated with defects in integrins (Has et al., 2012). On the other hand, many targeted therapies include selective blockade of integrins. Drugs as Tirofiban (αIIb3), Natalizumab (α4) or Vedolizumab (α4β7) have been clinically approved for many years in the fields of acute coronary syndrome, multiple sclerosis and Crohn's disease, respectively (Bachmann et al., 2019; Brandstadter & Sand, 2017; Scribano, 2018).

Integrins have been investigated in pregnancy, particularly for their relevance during implantation. Interestingly, αvβ3 and α4β3 expression in the endometrium is temporary limited to the secretory phase, leading to the hypothesis that their appearance defines the endometrial receptivity during the window of implantation. Here, they may be important for the interaction between the endometrium and trophoblast during nidation and early pregnancy, as their absence correlates with impaired fertility (Lessey et al., 1994).

2. Aim of the work

Innate lymphoid cells (ILCs) are newly described innate immune cells present at the fetal-maternal interface and their roles for establishment and maintenance of pregnancy have been proposed. Yet, knowledge about their migratory capacity during gestation is scarce and remains to be assessed.

The present work addresses the following aims:

- to study the dynamics of the expression of relevant cell homing markers in ILCs in different lymphoid and reproductive organs during murine pregnancy *in vivo*,

- to evaluate the role of pregnancy-related hormones on the expression of these receptors and the consequent migratory capacity of the cells *in vitro*,

- to assess the effect of pregnancy-relevant chemotactic ligands on ILC migration in pregnant and non-pregnant mice *ex vivo*.

3. Methods

3.1. Pregnancy mouse model

BALB/c ($H2^d$) females and C57Bl/6 males were bred in our Central Service and Research Facility for Animals (ZSFV). The animals were kept co-housed in a 12 h light/dark cycle at standardized room temperatures (20 ± 2°C) and air humidity (60 ± 20%). Free access to food and acidulated water (pH = 2,5) was guaranteed at all times. In advance to all experiments, for all mice an acclimatization period of at least 14 days was allowed. Eight- to twelve-week-old BALB/c female mice were paired with C57BL/6J males. Mice were checked for vaginal plug every morning. Observation of a plug was declared as day 0 of pregnancy, and the female was separated from the male. Mice were euthanized either at day 7, 14 or 18 post plug (dpp) in order to display all phases of pregnancy; spleen, thymus, paraaortic and inguinal lymph nodes as well as Peyer's patches were collected. The prepared organs were then stored in buffer on ice until further preparation. Non-pregnant Balb/c female mice were sacrificed as a control group and further used for *in vitro* experiments. Animal experiments were carried out according to institutional guidelines approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLF-MV; 7221.3-1-068/13 to DM). The experiments were conducted in conformity with the European Communities Council Directive 86/609/EEC.

3.2. Isolation of cells from murine organs

3.2.1. Preparation of lymphatic organs

The lymphatic organs (spleen, thymus, lymph nodes and Peyer's patches) were mashed with the back of a syringe plunger through a moistened 40 µm cell strainer and collected in a 50 mL Falcon tube. They were centrifuged for 10 minutes at 300 ×*g*. Following, the erythrocytes in the splenic cell suspension were lysed using 1 mL (1:10) lysis. After five minutes of incubation at room temperature, the lysis was stopped by adding 3 mL FBS in PBS. The cells were then filtered through a moistened cell strainer again. After another centrifugation (300 ×*g*) the supernatant was aspirated and the cell pellet was resuspended in PBS in order to obtain a single-cell suspension. Non-splenic cells from thymus, Peyer's patches (PP) and lymph

nodes (LN) were not lysed. The single cell suspension was counted under a light microscope using a Neubauer cell chamber. Depending on the type of organ, one to three million cells were distributed into FACS tubes and further analysed.

3.2.2. Preparation of bone marrow

Murine femurs were placed into a petri-dish and cut open on both ends using a single-use scalpel. The bone marrow was extracted by inserting a fine cannula into the hollow bone and rinsing them with FACS buffer several times. This solution was centrifuged for 5 minutes at 300 ×*g*, the supernatant was aspirated, and the cells were lysed as mentioned above. Following, they were washed again to obtain a single cell suspension and counted.

3.2.3. Preparation of uterus

The uterus was mechanically chopped in a petri-dish with a single-use scalpel. 0,5 % collagenase was added to the tissue and incubated for one hour at 37 °C in order to digest the tissue. The mashed cell suspension was filtered through a cell strainer and washed as mentioned above. Following, the obtained uterine cells were treated by density gradient centrifugation to separate lymphocytes and remove erythrocytes and cell detritus. 5 mL Lympholyte were placed into a 15 mL Falcon and 4 mL of cell suspension were gently layered on top. After 20 minutes of centrifugation at 1 250 ×*g* (brakes off), the white blood cell layer was carefully aspirated, washed and further treated.

3.2.4. Counting of cells

The cell count was obtained as the number of suspended cells in a specific volume using a Neubauer cell chamber. 10 µL of diluted (1:100/1:50) cell suspension was put in the cell chamber and counted manually. This number was multiplied with the dilution factor and 10 000 to determine the amount of cells in 1 mL of cell suspension.

3.3. Principles of flow cytometry (FACS)

Flow cytometry is a laboratory method to analyse different cells quantitatively and qualitatively according to their size, shape and contents. All Fluorescence Activating Cell Sorting (FACS) analyses are based on the reaction between cell antigens and fluorochrome-linked antibodies. According to their expression of surface markers or intracellular proteins, different cell populations can be distinguished and further analysed. The specific combination of markers allows a rapid multi-parametric analysis.

The flow cytometer uses a fluidics system to transport the cell suspension to an interrogation site, where a laser pulse is applied and used as a light source to produce scattered and fluorescent light signals. Hydrodynamic focusing allows the suspension to be measured cell by cell. Two different directions of the light are distinguished and provide information about the cell. Forward scatter light (FSC) indicates the relative cell size, whereas the side scatter light is applied at a 90° angle and determines the cell granularity (McKinnon, 2018).

In addition to light scatter, the laser pulse excites the antibody-conjugated fluorochrome and emits light energy, which is bundled by a lens, detected, multiplied and converted into an electrical signal. The fluorochromes can be chosen according to their excitation and emission wavelength. Overlapping of the emission spectrum can be compensated by using specific software. BD FACSDiva was used in all experiments. A typical parameter for further analysis is the assessment of the Mean Fluorescence Intensity (MFI). The MFI can be used to obtain the density of a receptor amongst a cell population. The density is equivalent to the fluorescent signal.

3.4. Staining of cells for FACS analysis

3.4.1. Extracellular staining of cells for FACS analysis

Single cell suspensions were obtained as described in chapter 3.2. and transferred into FACS tubes.

All single cell suspensions were then washed with 1 mL PBS and resuspended in either 100 µl Fixable Viability Dye (FVD) eFluor 520 (1:1000 diluted in PBS) for the (positive) samples or in 100 µL of PBS for the negative control. Hereby, dead cells are permanently stained and can be further excluded from the analysis. After 30 minutes of incubation at 4 °C in the dark cells were washed with 1 mL of FACS buffer and then pre-incubated on ice for 5 minutes with either 25 µL of CD16/32 mAb Fc block (1:100 diluted in FACS buffer) for the (positive) samples and 50 µL of plain FACS buffer for the negative control. This prevents unspecific binding of antibodies to Fc-receptors. 25 µL of specific antibody cocktail were added to the positive probes and incubated for 30 minutes at 4°C in the dark. Afterwards, the stained suspension was washed with 1 mL of FACS buffer to remove free antibodies. The cells were then resuspended in 150 µL of FACS buffer and analysed by flow cytometry.

3.4.2. Intracellular staining of cells for FACS analysis

For intracellular staining the cells were fixated and membranes permeabilized to allow staining for structures that are not exposed on the cell surface. The application of Fixation Viability dye, blocking and extracellular staining was performed as mentioned above. After extracellular staining the cells were resuspended in 50 µL of BD Fixation/Permeabilization solution while vortexing thoroughly, incubated for 20 minutes at 4 °C in the dark and then washed with 1 mL of BD Permeabilization Buffer (1:10 dilution in ddH20). After a following incubation on ice (5 minutes), the antibody for the intranuclear staining (RORγT or promyelocytic leukemia zinc finger (PLZF)) was diluted in Permeabilization Buffer (1:10 or 1:100, respectively) and added to the cell pellet. After 30 minutes of incubation, it was washed again in BD Permeabilization Buffer and resuspended in 250 µL of FACS buffer.

A table of the utilized antibodies is displayed in 4.3.4.

3.4.3. Counting Beads

For experiments addressing the migratory capabilities, 20 µL BD Counting Beads were added after extra- and/or intracellular staining to the sample, and the following equation was used to determine concentration of cell populations:

$$\frac{\#\text{ events in cell region}}{\#\text{ events in bead region}} \times \frac{\#\text{ beads/test}}{\text{test volume}} \times dilution\ factor\ = concentration\ of\ cell\ population$$

3.5. Cell culture for hormonal stimulation

3.5.1. Hormonal stimulation time-curve

Single cell suspensions from murine spleen and/or lymph nodes were obtained as previously mentioned in chapter 3.2. The cell culture media consisted of RPMI 1640, 10 % fetale bovine serum (FBS, depleted), Penicillin/Streptomycin (1:100) and ß-Mercapotethanol (1: 286 000). A 24-well plate was prepared with different dilutions of pregnancy hormones. Progesterone (5 ng/mL and 50 ng/mL) and estradiol (5 pg/mL and 100 pg/mL) were diluted in complete medium. 1 million cells were distributed into each well, filled up to 500 µL with either progesterone solution, estradiol solution or medium only for negative samples (Figure 2). The cells were incubated for either 2, 6, 12 or 24 hours at 37 °C and 5 % CO_2. Following the respective period of culture, the wells were rinsed with PBS and the cells were transferred into FACS tubes. After centrifugation (5 minutes, 300 ×*g*), the supernatant was aspirated and the cells were prepared and stained according to the protocol above.

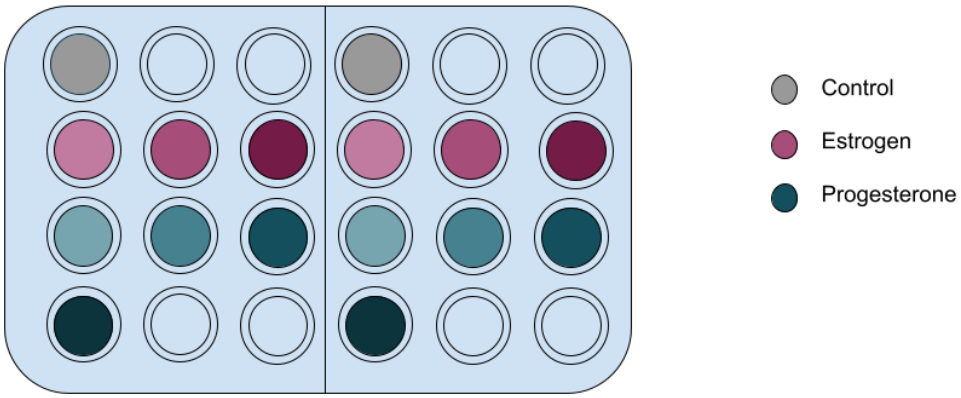

Figure 3: The setting of cell culture-plate for stimulation with different concentrations of progesterone and estradiol (experiment 5)

3.5.2. 48 hour hormonal stimulation of splenocytes

Single cell suspensions from murine splenocytes were obtained as described above (chapter 3.2.). 2 million cells were distributed into each well of a 24-well plate and stimulated with different concentrations of progesterone (50 ng/mL, 500 ng/mL, 5000 ng/mL and 50 µg/mL) and estradiol (5 pg/mL, 100 pg/mL and 100 ng/mL), or bare medium for negative control. Each well contained 1 mL of fluid. The cells were then incubated at 37 ° C and 5% CO_2 for 48 hours (Figure 3).

Following the 48 h stimulation, each well was rinsed with PBS and the well contents were transferred into FACS tubes. After centrifugation (5 minutes, 300 ×*g*), the supernatant was aspirated and the cells were handled and stained according to the protocol above (chapter 3.4.).

3.5.3. Transwell Migration Assay

Migration assays were performed to study the migratory response of lymphocytes towards chemoattractants. Here, the chemoattracting capacities of CCL4, CCL10 and CCL28 were investigated.

Transwell migration inserts (as seen in Figure 4) were carefully placed in each well of a 24-well-plate and thoroughly filled with 500 µL of single cell suspensions, obtained from murine spleen or lymph nodes and prepared as above. Each well contained 500 µL of complete cell culture medium (see above) and different concentrations of the chemoattractant ligands to assess the migratory response of the cells. CCL4 (100 ng/mL and 1000 ng/mL), CCL20 (25 ng/mL and 250 ng/mL) and CCL28 (5 µg/mL) were compared with complete medium only (negative) in different set-ups. After 4 hours of incubation (37°C, 5% CO_2), the cells that had migrated through the membrane into the well were collected from each well, rinsed with PBS and transferred to a FACS tube and washed and stained as previously described.

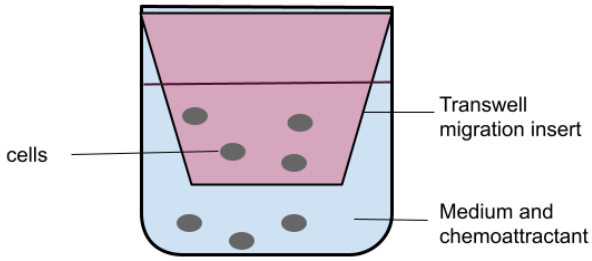

Figure 4: Schematic representation of a transwell-migration assay: inserts are installed into wells and cells are placed onto the semi-permeable inserts. Cell migration towards different chemoattractans can be assessed and compared by analysing the medium in the bottom of each well.

3.6. Statistics

Data were exported to and saved in a Microsoft Excel table (Microsoft Office 2010, Microsoft Corporation; Redmond, USA) and analysed using GraphPad Prism (Version 5.1, GraphPad Inc., La Jolla, USA).

Data from *in vivo* mouse experiments were analysed by one-way analysis of variance (one way-ANOVA), followed by a Dunnett multi-comparison post-test. *In vitro* mouse data were analysed with a paired *t*-test and one-way ANOVA, followed by a Tukey multi-comparison post-test. Here, mean and SEM were calculated and displayed in graphs. Significances are indicated with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Outliers were calculated using an online outlier calculator (Outlier Calculator MiniWebTool). An outlier was defined as a number that is more than 1.5 times the length of the box away from either the lower or upper quartiles.

4. Material

4.1. Laboratory equipment

Device	Brand
Analytical balance MC1 Analytic AC 210P	Sartorius AG (Göttingen, Germany)
Aspiration system DITABIS HLC AZ 02/04	DITABIS - Digital Biomedical Imaging Systems AG (Pforzheim, Germany)
Automatic ice maker RF 0399 A	Manitowoc Deutschland GmbH (Herborn, Germany)
Autoclave VX-150	Systec GmbH Labor-Systemtechnik (Wettenberg, Germany)
Calculator (KF01605)	Q-CONNECT (Gent, Belgium)
Centrifuges:	
5810, 5810 R	Eppendorf (Hamburg, Germany)
VWR Mini Star	VWR (Radnor, PA, USA)
CO_2 incubator MCO-18 AIC	Sanyo (Moriguchi, Japan)
Cryo storage box	neoLab (Heidelberg, Germany)
Digital roller shaker - SRT6D	Bibby Scientific Limited (Stone, Staffordshire, ST15 OSA, UK)
Erlenmeyer flasks	
250 ml, 300 ml, 500 ml	DURAN Group GmbH (Wertheim/Main, Germany) Jenaer Glass GDR

Flow cytometer BD FACSCanto™	BD Biosciences, (Franklin Lakes, NJ, USA)
Freezers:	
4°C laboratory fridge medline LKv 3910	Liebherr-International Germany GmbH (Biberach an der Riß, Germany)
-20°C MDF-U333	Sanyo (Moriguchi, Japan)
-80°C MDF-U53V	
-80°C freezer Kryotec KLS 6086-2	Kryotec-Kryosafe GmbH (Hamburg, Germany)
Fume hood Scala	Waldner Laboreinrichtungen GmbH & Co. KG (Wangen, Germany)
Glass beakers:	
250 mL, 1000 mL	VWR (Radnor, PA, USA)
3000 mL	Vitlab GmbH (Grossostheim, Germany)
Graduated cylinders:	
100 mL	BRAND GMBH + CO KG (Wertheim, Germany)
10 mL, 25 mL, 50 mL, 500 mL, 1000 mL, 2000 mL	Isolab Laborgeräte GmbH (Wertheim, Germany)
Incubation Bath 1003	GFL (Burgwedel, Germany)
Laboratory glass bottles:	
50 mL, 100 mL, 250 mL, 500 mL, 1000 mL, 2000 mL	DURAN Group GmbH (Wertheim/Main, Germany)
Microscopes:	
Light microscope Nikon TMS	Nikon Instruments (Düsseldorf, Germany)

Hoechst 33258 IF Microscope	Invitrogen (Carlsbad, CA, USA)
Neubauer hemocytometer	LO-Laboroptik Ltd. (Lancing, UK)
Personal Computer Elite Desk 800 GI USDT	Hewlett Packard Inc. (Palo Alto, CA, USA)
Pipettes:	
0,1 - 2,5 µl, 0,5 - 10µl, 2 - 20 µl, 20 - 200µl, 100 - 1000µl, 0,5 - 5 mL Multi channel pipette 30 - 300 µL	Eppendorf (Hamburg, Germany)
Pipette controller accu-jet pro	BRAND GmbH (Wertheim, Germany)
Racks:	
neoRack Test Tube Racks 4x4	neoLab (Heidelberg, Germany)
neoRack 4x4, 7x7, 9x9	Thermo Scientific Nalgene Labware (Waltham, MA, USA)
Safety cabinets:	
s@feflow 1.2	Euroclone (Pero, Italy)
HERAsafe KS9	Thermo Electron Corporation, (Waltham, MA, USA)
Table rack for disposable bags	Sarstedt (Nümbrecht, Germany)
Timer SKT338N	Carl Roth (Karlsruhe, Germany)
Tweezers	neoLab (Heidelberg, Germany)

Vortex Mixer:	
VWR™ Analog	VWR (Radnor, PA, USA)
neoLab 7-2020	neoLab, (Heidelberg, Germany)

4.2. Laboratory disposables

Equipment	**Brand**
Cannula	
Cell culture plate Cellstar TC 24-suspension well plate	Cellstar greiner bio one, (Frickenhausen, Germany)
Cell Strainer 40 µm Nylon	BD Biosciences (Franklin Lakes, NJ, USA)
EASYstrainer 40 µm	Greiner Bio-One (Frickenhausen, Germany)
Disposable bags for table rack	Sarstedt (Nümbrecht, Germany)
Petri dish:	
Disposable petri dish Nunclon surface	Nunc A/S (Roskilde, Denmark)
Petri dish	Greiner bio-one (Frickenhausen, Germany)
Feather disposable scalpel with plastic handle	FEATHER Safety Razor Co. (Osaka, Japan)
Gloves: Peha-soft nitrile	Paul Hartmann AG (Heidenheim, Germany)
Indicator tape for steam sterilisation	Carl Roth (Karlsruhe, Germany)
Parafilm „M“ Laboratory Film	Bemis (Neenah, WI, USA)

Pipette tips:	
10 µL SurPhob	Biozym Scientific GmbH (Hessisch Oldendorf, Germany)
200 µL, 1000 µL	Sarstedt (Nümbrecht, Germany)
5000 µL	Eppendorf (Hamburg, Germany)
Serological pipettes:	Sarstedt (Nümbrecht, Germany)
1 mL, 5 mL, 10 mL, 25 mL	
Single-use towels Zick-Zack	Heemann OHG (Falkensee, Germany)
Sterile syringe filters 0.45 µm 16898 2008-11	Carl Roth (Karlsruhe, Germany)
Syringe BD Discardit II 10 mL	BD (Franklin Lakes, NJ, USA)
Table rack for disposable bags	Sarstedt (Nümbrecht, Germany)
Tape Scotch Crystal Clear	3M Deutschland GmbH (Neuss, Germany)
TC-Inserts, 24 well, PET, pore 5 µm, translucent,	Sarstedt (Nümbrecht, Germany)
Tubes for flow cytometry 5 mL, 75x12 mm, PS	Sarstedt (Nümbrecht, Germany)
Tubes for centrifugation	
15 mL, 120 x 17 mm, PP	Sarstedt (Nümbrecht, Germany)
50 mL, 114 x 28 mm, PP	BD (Franklin Lakes, NJ, USA)
Reaction tubes with Safe-lid	
0,5 mL, 1,5 mL, 2,0 mL	Sarstedt (Nümbrecht, Germany)

4.3. Reagents and chemicals

4.3.1. Stimulants for cell culture

Stimulant	Brand
CCL4/IL-1β (200 µg/mL)	BioLegend (San Diego, CA, USA)
CCL20/MIP-3α (200 µg/mL)	BioLegend (San Diego, CA, USA)
CCL28/MEC (200 µg/mL)	BioLegend (San Diego, CA, USA)
17β-Estradiol 30 mM	Merck/Biochrom (Berlin, Germany)
Progesterone 10 mM	Merck/Biochrom (Berlin, Germany)

4.3.2. Biochemical reagents

Chemical product	Brand
β-Mercaptoethanol	Carl Roth (Karlsruhe, Germany)
BD Cell Viability Kit	BD Biosciences (Franklin Lakes, NJ, USA)
BD Fixation and Permeabilization Solution	BD Biosciences (Franklin Lakes, NJ, USA)
BD Perm/Wash buffer I	BD Biosciences (Franklin Lakes, NJ, USA)
Bovine Serum Albumine (BSA)	Sigma Aldrich, (St. Louis, MO,USA)
Collagenase 0,5 %	Merck Chemicals (Darmstadt, Germany)
Ethanol (denatured), 96%	Pharmacy of the University of Greifswald (Greifswald, Germany)
Ethanol (undenatured)	TH. GEYER (Renningen, Germany)
Fetal Bovine Serum (FBS)	Biochrom AG (Berlin, Germany)
Fixable Viability Dye FVD 520, FVD 780	ThermoFisher Scientific/ eBioscience, (Waltham, MA, USA)
Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen, ThermoFisher Scientific (Waltham, MA, USA)
Hand disinfection sensiva	Schülke GmbH (Norderstedt, Germany)

LymphoLyte	Cedarlane/tebu-bio (Burlington, Canada)
Methanol	J. T. Baker (Deventer, Netherlands)
Penicillin/Streptomycin	Gibco, ThermoFisher (Waltham, MA, USA)
Phosphate-buffered Saline (PBS), sterile filtered, liquid, w/o Ca^{2+} w/o Mg^{2+} PBS (dry) w/o Ca^{2+} w/o Mg^{2+}	Biochrom AG (Berlin, Germany)
RPMI1640 medium w L-Glutamine w 2,0 g/l NaHCO3	PAN-Biotech (Wimborne, UK)
Sodium azide (NaN3)	Carl Roth (Karlsruhe, Germany)
Sodium hydroxide (NaOH)	Carl Roth (Karlsruhe, Germany)

4.3.3. Buffers

Buffer	Composition
FACS buffer	10 g BSA, 1g NaN3, 9,55 g PBS in 1000 mL dd H20
10 x PBS buffer	9,55 g PBS in 1000 mL dd H20
10 x Lysis buffer	44,5 g NH4CL, 5,0 g KHCO3, 0,15 g EDTA in 500 mL dd H20
Stopping solution	9 mL PBS buffer + 1 mL FBS

4.3.4. Antibodies

Molecule	Label	Clone	Concentration	Company
CD3e	FITC	145-2C11	1/400	BD Biosciences (Franklin Lakes, NJ, USA)
CD4	FITC	RM4-4	1/400	BD Biosciences (Franklin Lakes, NJ, USA)
CD8a	FITC	53-6.7	1/200	BD Biosciences (Franklin Lakes, NJ, USA)
CD11b	FITC	M1/70	1/200	BD Biosciences (Franklin Lakes, NJ, USA)
Ter119	FITC	TER-119	1/200	BD Biosciences (Franklin Lakes, NJ, USA)
CD19	FITC	6D5	1/400	BioLegend (San Diego, CA, USA)
CD127	APC-eFluor780	A7R34	1/25	eBioScience/ ThermoFisher Scientific (Waltham, MA, USA)
α4β7	APC	DATK32	1/100	Miltenyi Biotec (Bergisch Gladbach, Germany)
CCR4	PE-Cy7	2G12	1/25	BioLegend (San Diego, CA, USA)
CCR10	PE	248918	1/25	R&D (Minneapolis, MN, USA)
CCR6	PerCP-Cy5.5	29-2L17	1/200	BioLegend (San Diego, CA, USA)
CD45	PerCP-Cy5.5	30-F11	1/400	BD (Franklin Lakes, NJ, USA)
RORγT *	APC	REA278	1/10	Miltenyi Biotec (Bergisch Gladbach, Germany)

PLZF *	PerCP-Cy5.5	9E12	1/100	BioLegend (San Diego, CA, USA)
CD 16/32 mAb Fc Block				BD Pharmingen (Heidelberg, Germany)

*intranuclear antibodies

4.4. Software

Program	Use	Brand
BD FACS DIVA Software v6.1.3	Flow cytometry	BD Biosciences (Franklin Lakes, NJ, USA)
FlowJo	Processing of FACS data	BD Biosciences (Franklin Lakes, NJ, USA)
GraphPad Prism 5.01	Statistics	GraphPad Software Inc. (San Diego, CA, USA)
Microsoft Excel 2010	Data processing	Microsoft Corporation (Redmond, WA, USA)
Microsoft Power Point 2010	Image processing	Microsoft Corporation (Redmond, WA, USA)
Microsoft Word 2010	Text processing	Microsoft Corporation (Redmond, WA, USA)

5. Results

5.1. Pregnant uteri contain higher numbers of CCR4$^+$ and CCR10$^+$ ILC3s than non-pregnant uteri

In order to identify uterine ILCs and their migratory potential during pregnancy, ILCs were characterized in pregnant (7 dpp) and non-pregnant uteri. A significant increase of the percentage of ILC3s within ILCs in pregnant uteri from 7 dpp could be observed compared to non-pregnant uteri (from 21.43 ± 5.231 to 43.65 ± 6.528, respectively, p= 0.0285). Further, the expression of CCR4$^+$ ILC3s and CCR10$^+$ ILC3s in pregnant uteri was elevated (from 0.2801 ± 0.1391 to 1.890 ± 1.160; p=0.0369) and from 1.133 ± 0.6292 to 4.607 ± 1.736, p=0.0335, respectively), compared to the non-pregnant control group (Figure 5).

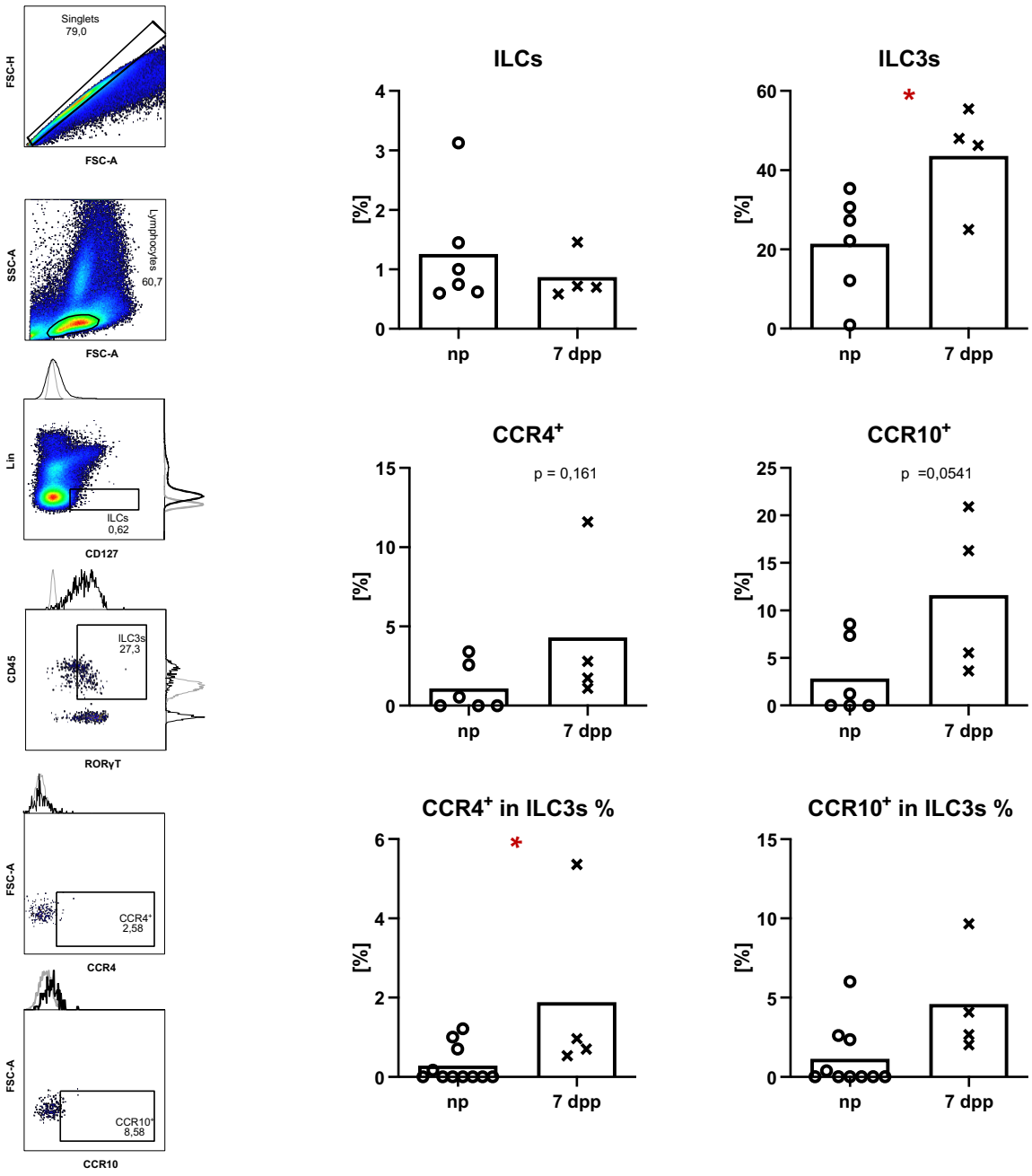

Figure 5: Day 7 (7 dpp) uteri are enriched with CCR4⁺ ILC3s compared to non-pregnant uteri

Proportions of ILCs, ILC3s and their expression of CCR4 and CCR10 were analysed in uterine tissue from either non-pregnant or pregnant (day 7 of pregnancy/7 dpp) murine uteri via flow cytometry. Plots show the gating strategy used in this experiment. Data are shown as mean ± SEM and were analysed by *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n=4 (min.)

5.2. *In vitro* treatment with CCL28 promotes migration of CCR4$^+$ ILC3s from uterus-draining lymph nodes in pregnant mice

In order to gain insight into the mechanisms of ILC3 recruitment during pregnancy, the chemoattracting capacity of two relevant chemokines for the fetomaternal interface (CCL4 and CCL28) were tested on lymphocytes derived from uterus-draining lymph nodes. While no differences between pregnant and non-pregnant mice under treatment with CCL4 were observed, a higher proportion of CCR4$^+$ ILC3s from pregnant mice migrated in response to CCL28 than from non-pregnant mice (0.9737 ± 0.1678 vs. 1.923 ± 0.5345, p= 0.0456). No significant differences were observed in CCR10$^+$ ILC3s (Figure 6). The role of CCL20, another important pregnancy-associated chemokine, was not tested due to the limited numbers of lymphocytes that can be isolated from uterus-draining lymph nodes.

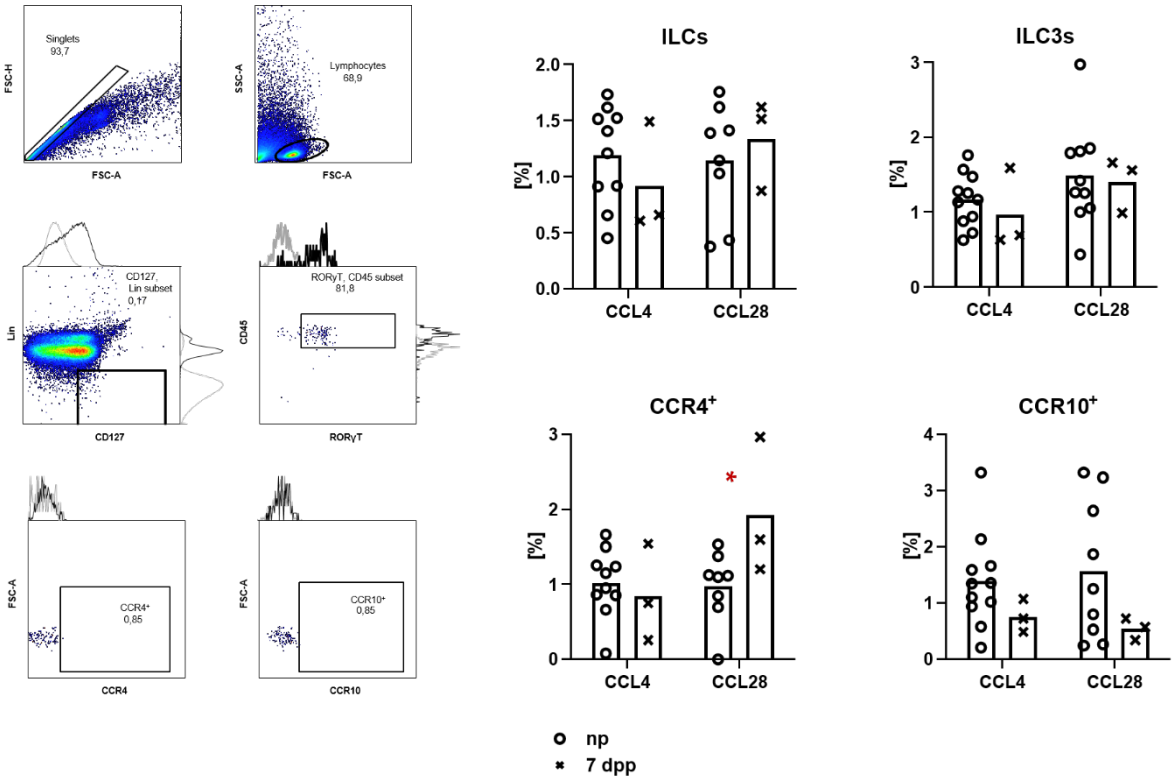

Figure 6: CCL28 promotes migration of CCR4$^+$ ILC3s from uterus-draining lymph nodes of pregnant mice *in vitro*

Migration of murine lymphocytes from paraaortal and inguinal lymph nodes (ILCs, ILC3s) and expression of CCR 4 and CCR 10 was compared in non-pregnant vs. pregnant (7 dpp) mice in response to two chemoattractants (CCL4, 1000 ng/mL) and CCL28 (5 µg/mL). Plots show the gating strategy used in this experiment. Data are shown as mean ± SEM and were analysed by t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=8.

5.3. Migratory behaviour of NCR$^+$ ILC3s from splenocytes is augmented upon chemoattraction with CCL4 and CCL20 *in vitro*

To elucidate a possible role of pregnancy-associated chemokines in the recruitment of ILCs from distant compartments to the murine uterus, splenocytes were treated with CCL4, CCL20 or CCL28 and their migratory capacity was assessed. After 48 h, the percentage of NCR$^+$ ILC3s within total ILCs that have migrated was significantly increased in response to 1000 ng/mL CCL4 (0.2994 ± 0.07867 vs. 0.9586 ± 0.06966, p=0.0453) as well as in response to 250 ng/mL CCL20 (0.2994 ± 0.07867 vs. 0.8665 ± 0.1213, p=0.0143). There were no significant changes in the migratory capacity of other ILC subsets or upon chemoattraction with CCL28 (Figure 7).

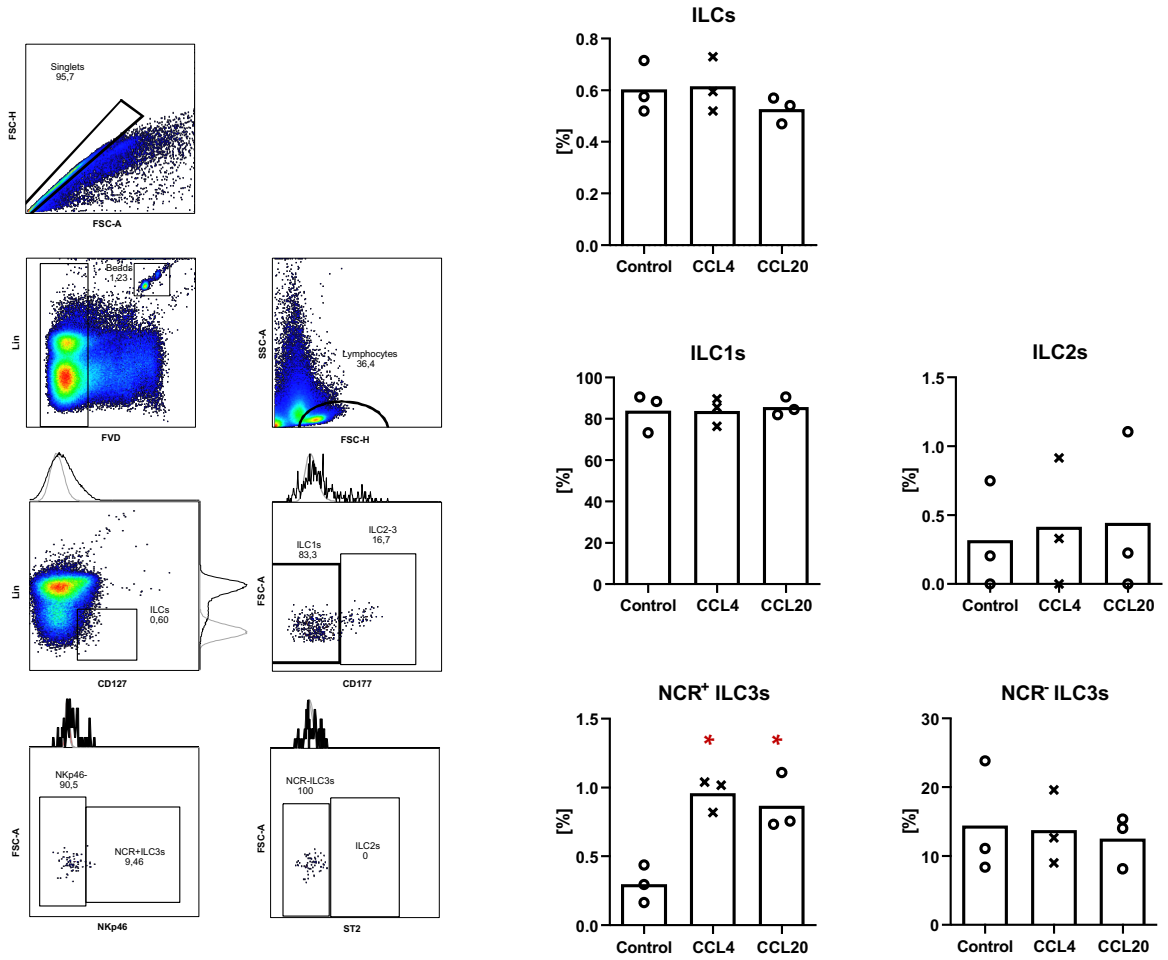

Figure 7: CCL4 and CCL20 enhance migration of NCR^{+}ILC3s from spleen of non-pregnant mice *in vitro.*

The migration of murine splenocytes towards different chemokine gradients were assessed in a migration assay. Two different dilutions of each CCL4 (100 ng/mL vs. 1000 ng/mL) and CCL20 (25 ng/mL vs. 250 ng/mL), and one concentration of CCL28 (5 µg/mL) were utilized in order to analyse the distribution of ILC subsets and the total numbers of lymphocytes, ILC1, ILC2, NCR^{+} ILC3 and NCR^{-} ILC3. Plots show the herein-used gating strategy. Data are shown as mean ± SEM and were analysed by one way-ANOVA, followed by a Tukey multi-comparison post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=3.

5.3.1. The highest migration index occurs after 60 minutes

To assess the dynamics of migratory behaviour upon treatment with chemoattractants, lymphocytes were analysed after 30, 60 and 120 minutes in the presence of progesterone.

The migration index of lymphocytes in response to CCL20 (250 ng/mL) was significantly elevated after 60 minutes as compared to 30 minutes (0.8952 ± 0.05272 vs. 1.391 ± 0.2560, p = 0.0468). ILCs showed a similar, yet not significant migration pattern (Figure 8).

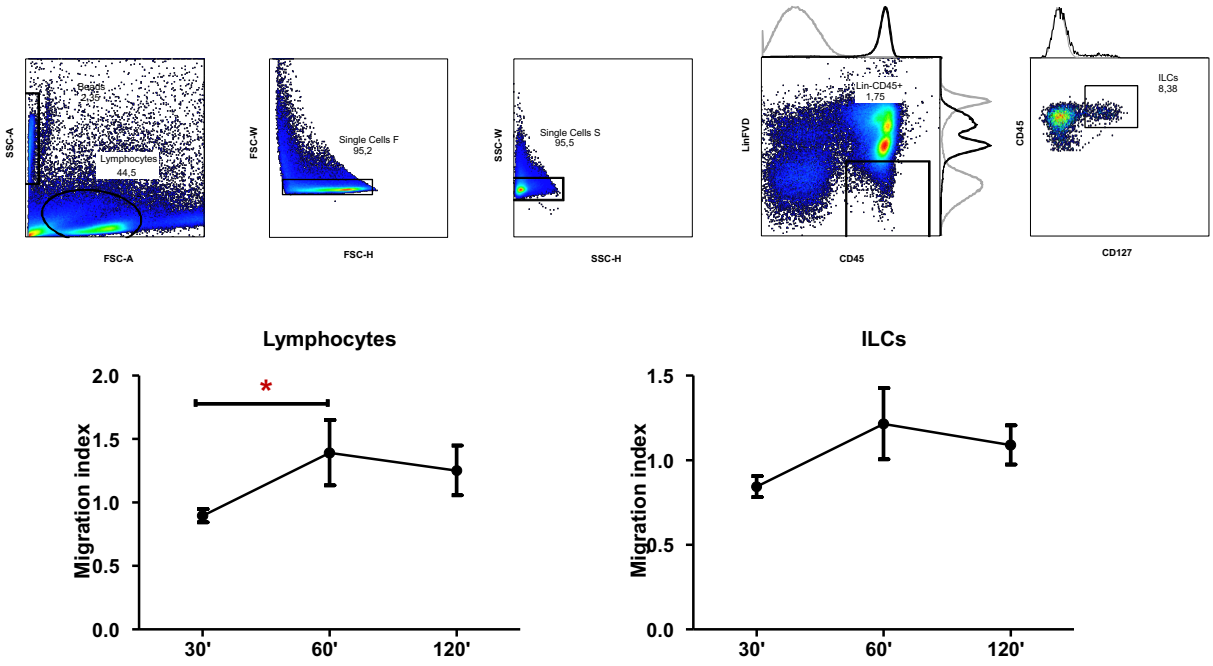

Figure 8: The maximal migratory effect in response to CCL20 was observed after 60 minutes.

Murine splenocytes from non-pregnant mice were stimulated with progesterone and their migration in response to CCL20 (250 ng/mL) was assessed after 30, 60 or 120 minutes. An index was assessed by relating migrated lymphocytes or ILCs/µL towards CCL20 in comparison with the un-stimulated control group. Data are shown as mean ± SEM and were analysed by one-way ANOVA, followed by a Tukey multi-comparison post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

5.4. Expression of CCR4, CCR10 and α4β7 in ILCs during murine pregnancy *in vivo*

To better understand the differential migratory capacity of ILCs during pregnancy, the expression of homing receptors (CCR4, CCR6, CCR10) was analysed in different lymphoid organs in non-pregnant and pregnant mice at different points in pregnancy. The expression of CCR4 in ILCs was significantly higher at 7 dpp compared to non-pregnant mice in murine spleen (-0,6941 ± 2,533 vs. 21,05 ± 7,068; $p < 0,05$), lymph nodes (8,734 ±2,276 vs. 54,11 ± 15,87, $p < 0,05$) and Peyer's Patches (35,20 ± 9,731 vs. 82,97 ± 16,56; $p < 0,05$). In contrast, no significant differences were found in ILCs from thymus and bone marrow. Similar to CCR4, the expression of CCR10 was also found to be significantly higher at 7 dpp compared to non-pregnant mice in ILCs from spleen (95.41 ± 7,532 vs. 192.30 ± 45.91; $p < 0.05$) and lymph nodes (MFI: 107.6 ±17.88 vs. 458.5 ±139.3; $p < 0.05$; %: 15.02 ±3.15 vs. 49.76 ± 13.33; $p < 0.05$) However, this augmentation of CCR10 was not found to be significant in thymus, Peyer's patches or bone marrow. Further, no significant increase of CCR6 was detected in any organs evaluated.

Besides chemokine receptors, the expression of the integrin α4β7 was investigated. A significant decline of α4β7 expression at 7 dpp compared to non-pregnant mice was detected in uterus-draining lymph nodes. α4β7 expression in ILCs from lymph nodes was decreased at 7 dpp. Although, no significant differences were found in the percentage of α4β7-expressing ILCs, the expression levels of α4β7 in this group of cells were found to be decreased at 14 dpp and 18 dpp (data not shown).

In addition to that, the percentage of α4β7-expressing ILCs was increased at 14 dpp and 18 dpp (data not shown) (np vs. 7 dpp: 25.70 ± 9.618 vs. -20.82 ± 11.25; $p < 0.05$; np vs 14 dpp: 728.1 ± 45.34 vs. 510.6 ± 17.14; $p < 0.05$; np vs. 18 dpp: 728.1 ± 45.34 vs. 510.6 ± 17.14; $p < 0.001$, respectively) (Figure 9).

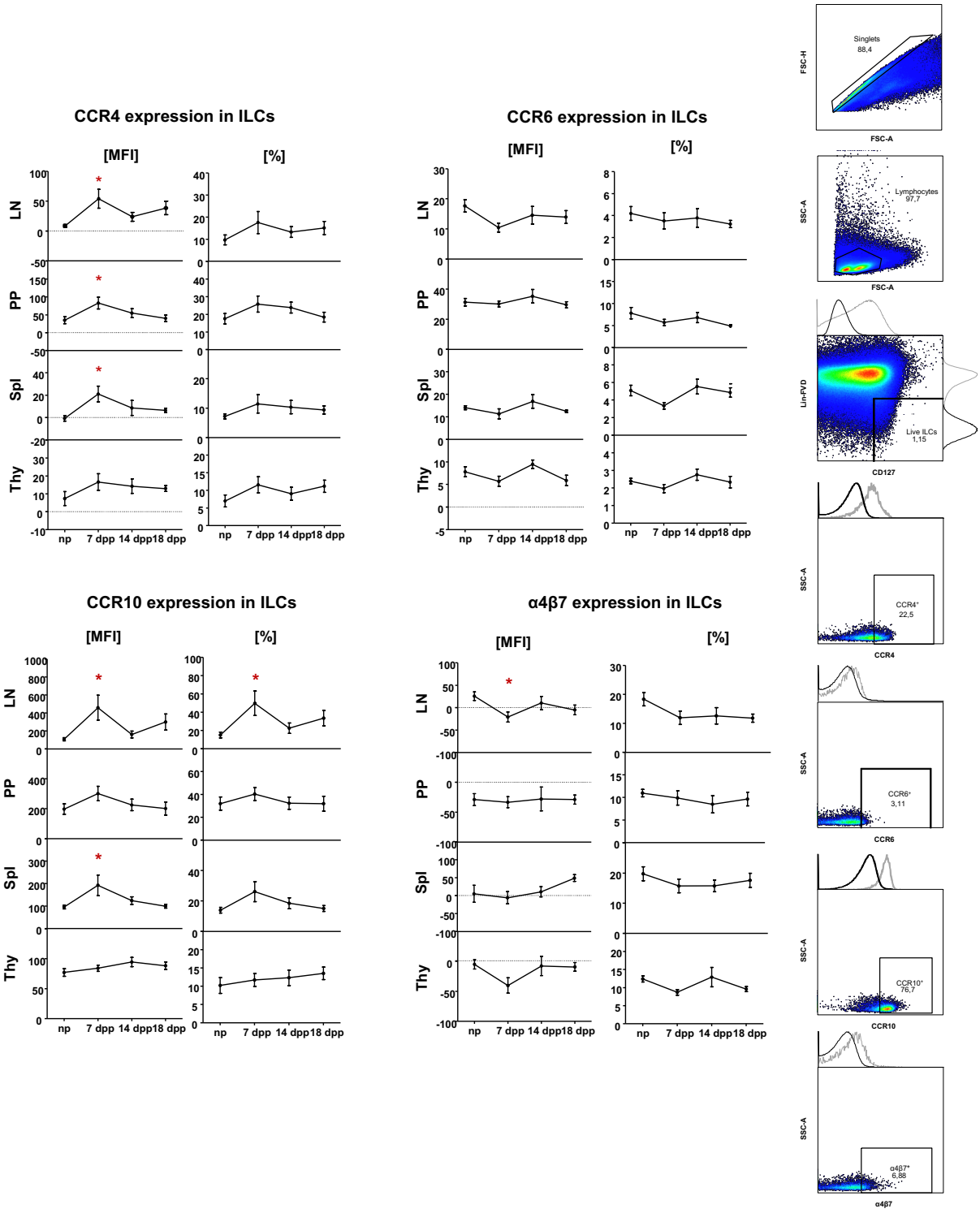

Figure 9: Expression of chemokine receptors in murine lymphatic organs varies during different times of pregnancy.

Expression of CCR4, CCR6, CCR10 and α4β7 were determined by flow cytometry in murine ILCs from spleen (SPL), thymus (Thy), para-aortal and inguinal lymph nodes (LN) and Peyer's Patches (PP).

MFI and percentage (%) of cells with a positive expression of CCR4, CCR6, CCR10 and α4β7 were analysed. Plots show the gating strategy used in this experiment. Data are shown as mean ± SEM and were analysed by one-way ANOVA, followed by a Dunnett multi-comparison post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n>5

5.5. Adaptations in the ILC bone marrow department during different stages of murine pregnancy

To investigate whether the bone marrow could be a potential source of ILCs in pregnancy, ILC precursors and the expression of homing receptors (CCR4, CCR10) was analysed in pregnant and non-pregnant mice. The percentage of ILCs in murine bone marrow was significantly decreased at all times of pregnancy. The decrease at 7 dpp (0.4929 ± 0.05727 vs. 0.2843 ± 0.03123, $p < 0.01$) and 14 dpp (0.4929 ± 0.05727 vs. 0.2850 ±0.0442; $p < 0.01$) was very significant while the reduction at 18 dpp was found to be extremely significant (0.4929 ± 0.05727 vs. 0.1682 ± 0.02357; $p < 0.001$), compared with non-pregnant control group, respectively. In contrast, no differences were found in ILC precursors and their expression of homing receptors (CCR4, CCR10) (figure 10).

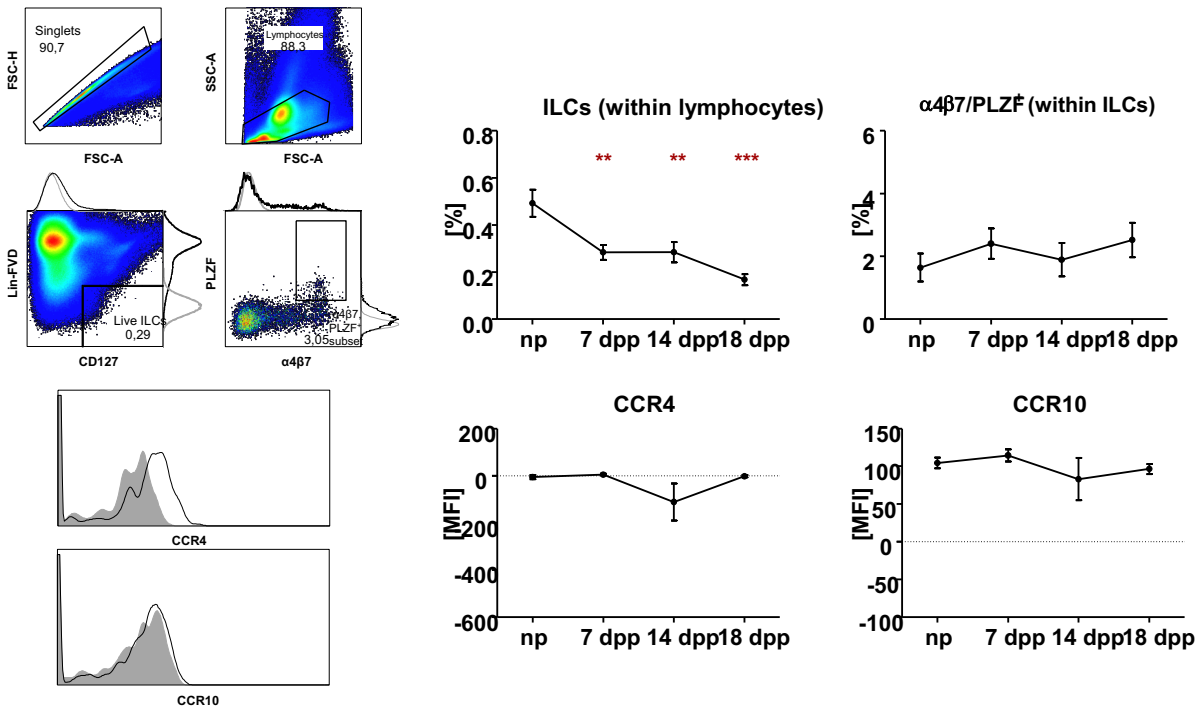

Figure 10: Proportions of ILCs in bone marrow are decreased across all stages of pregnancy.

In murine bone marrow percentage of ILCs and their precursors were determined as the α4β7, PLZF-positive subset and expression of CCR4 and CCR10 were analysed. Plots show the gating strategy used in used in this experiment. Data are shown as mean ± SEM and were analysed by one-way ANOVA, followed by a Dunnett multi-comparison post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n>5$.

5.6. Effect of progesterone on chemokine receptor expression *in vitro* over time

To gain insight into the possible mechanisms regulating the homing receptor expression in ILCs during pregnancy, splenocytes from non-pregnant mice were treated with female hormones that increase during pregnancy and possess immunomodulatory properties. CCR4, CCR6, CCR10 and $\alpha_4\beta_7$ were analysed after 2, 6, 12 and 24 hours of treatment with progesterone and estradiol.

The presence (%) and expression (MFI) of CCR10 in ILCs were significantly lower after 6 hours of incubation with 50 ng/mL of progesterone compared to the unstimulated control group (10.493 ± 4.56 vs. 9.15875 ±4.648, (p=0.0076); 140.0437 ± 33.776 vs. 115.6687 ± 28.4156, (p=0.0243), respectively, n=4). $\alpha_4\beta_7$ expression in ILCs was significantly increased after 6 hours of incubation with progesterone (50 ng/mL) in comparison to control group (66.3375 ± 13.7888 vs. 92.73125 ± 13.5167; p = 0.0022). There were no significant changes upon treatment with estradiol (Figure 11).

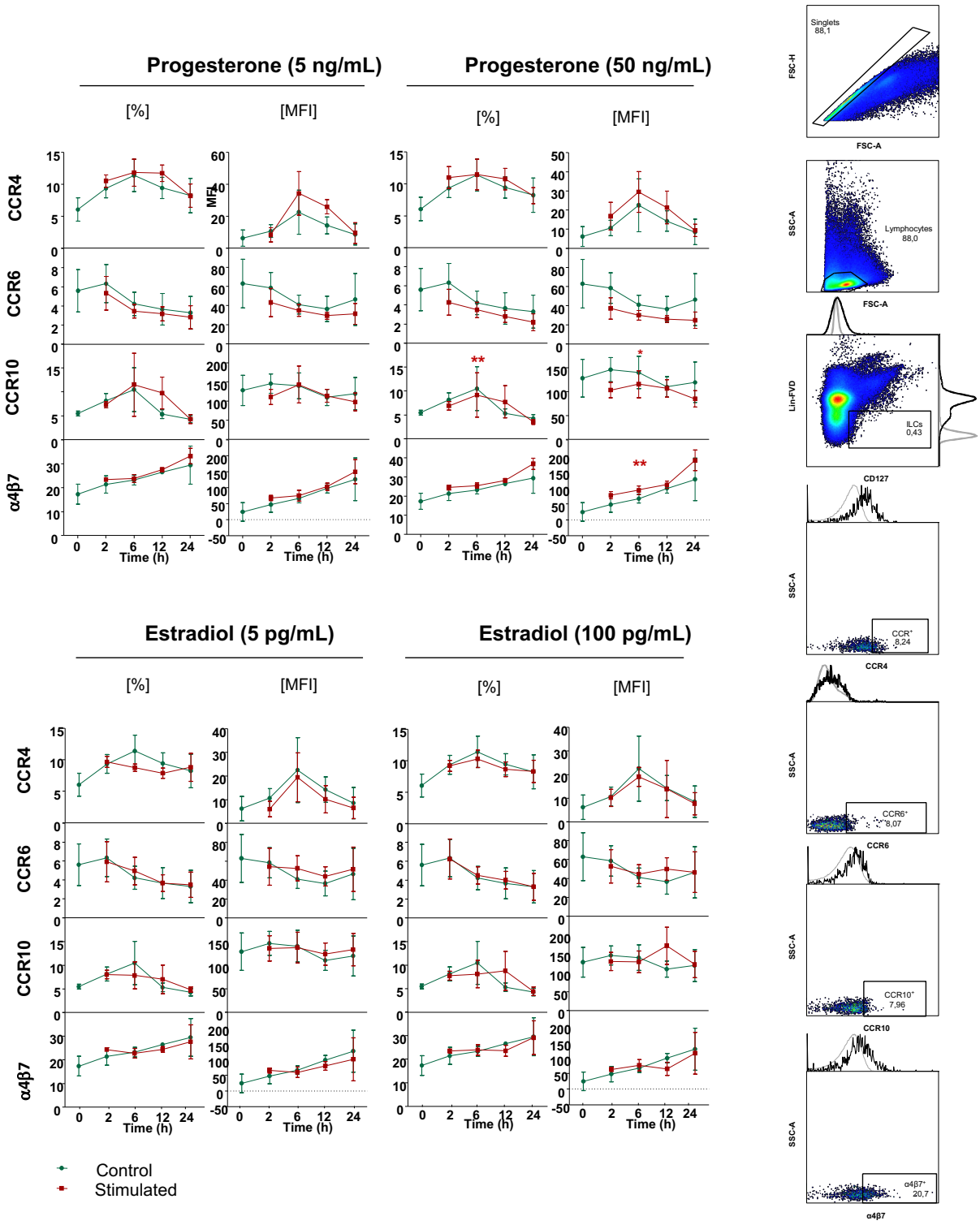

Figure 11: Pregnancy-related hormones influence homing receptor expression most probably after 6 hours of incubation

Murine splenocytes were incubated with pregnancy hormones for two, six, twelve or 24 hours and compared to control. Homing receptors CCR4, CCR6, CCR10 and α4β7 were analysed as percentages and MFI. Estradiol was tested in concentrations of 5 pg/ml and 100 pg/ml versus control. Progesterone was tested in concentrations of 5 ng/ml and 50 ng/ml. Data are shown as mean ± SEM and were analysed by *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=4

5.7. Concentration-dependent effect of progesterone on homing receptor expression *in vitro*

As estradiol and progesterone are produced locally, the effects that these exert on uterine cells may be higher than in the periphery. In order to analyse the effect of a wider spectrum of concentration of female pregnancy hormones on regulation of homing receptor distribution in ILCs, splenocytes from non-pregnant mice were stimulated with different concentrations of these hormones.

Proportions (%) and expression (MFI) of CCR4 were found to be significantly higher in cells stimulated with 5000 ng/mL of progesterone in comparison to the unstimulated control group (6.501 ± 1.354 vs. 16.80 ± 4.34 %, $p < 0.01$; and -10.69 ±8.271 vs. 32.70 ± 25.08; $p < 0.05$, respectively).

Upon stimulation with 50 µg/mL of progesterone the presence (%) and expression (MFI) of $\alpha_4\beta_7$ of were significantly lower compared to the control group (28.72 ± 6.911 vs. 14.8 ± 3.371, $p < 0.05$; and 100.50 ± 64.28 vs. -11.26 ± 36.40, $p < 0.05$).

There were no significant changes observed after treatment with lower concentrations of progesterone neither upon stimulation with estradiol (Figure 12).

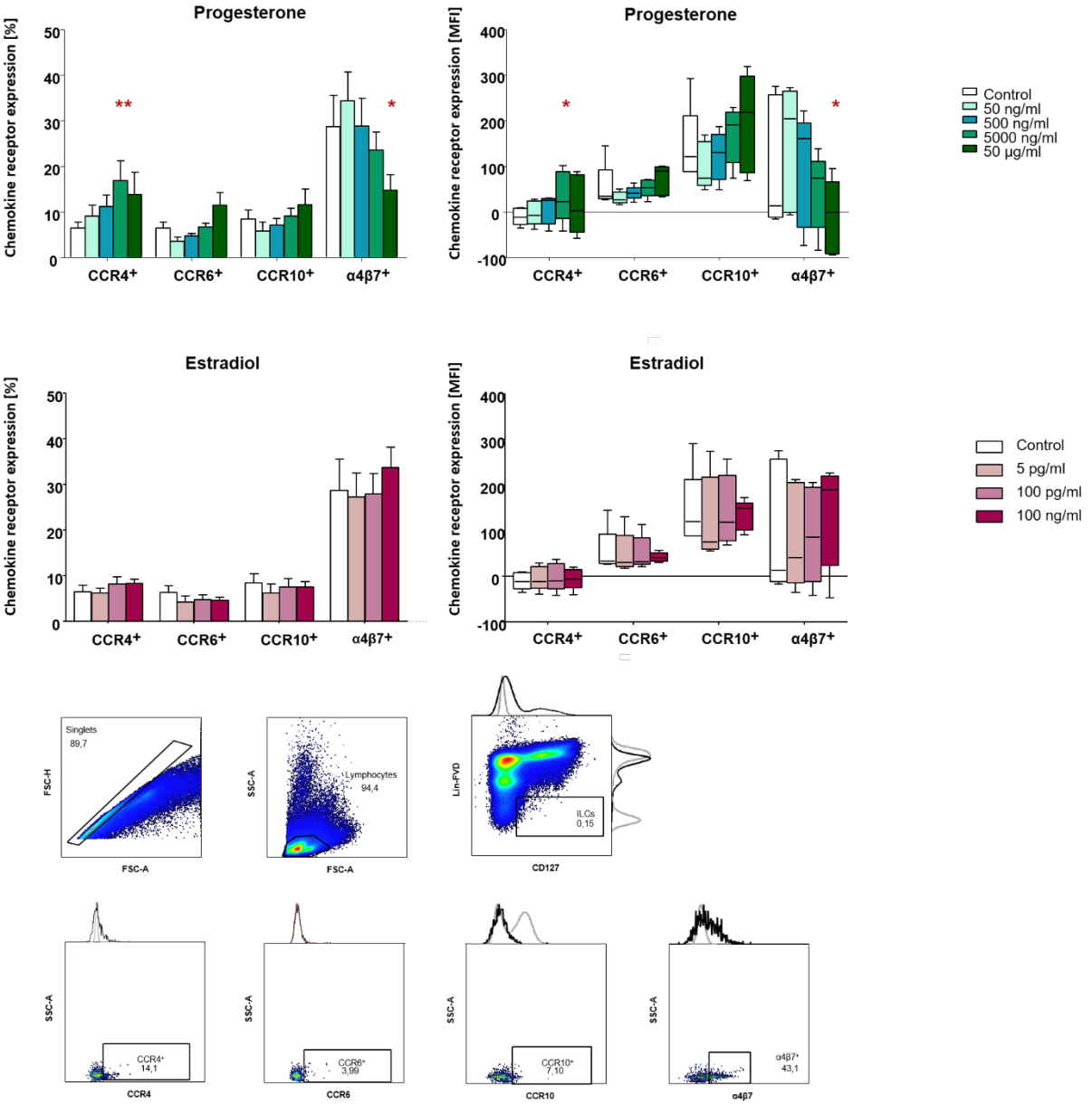

Figure 12: Stimulation of murine splenocytes with different concentrations of pregnancy-related hormones has an impact of homing receptor expression

Murine splenocytes from non-pregnant mice were stimulated with different concentrations of pregnancy-related hormones. Distribution of homing receptors CCR4, CCR6, CCR10 and α4β7 were analysed as MFI and percentage of cells with positive expression by flow cytometry. Progesterone was tested in concentrations of 50 ng/ml, 500 ng/ml, 5000 ng/ml and 50 µg/ml. Estradiol was analysed in concentrations of 5 pg/ml, 100 pg/ml and 100 ng/ml. Plots show the gating strategy. Data are shown as mean ± SEM and were analysed by one-way ANOVA, followed by a Dunnett multi-comparison post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n>5.

6. Discussion

During pregnancy, the female body undergoes a significant amount of changes and adaptations in order to not only tolerate, but also protect the conceptus and enrich its growth and differentiation, while adequately protecting it from infections. In the early stages of reproductive immunology research, Medawar *et al.* proposed a suppression of the maternal immune system so that the host - the maternal body – would not reject the antigenically foreign graft - the fetus (Medawar, 1953). Now, our understanding of the immune adaptation to pregnancy has changed. We assume that instead of the formerly believed maternal immunosuppression, it is rather an immunological modification and an active recognition of the fetus during gestation. Providing both tolerance towards the fetus and defense against pathogens can be considered the main goal, but also the most complex challenge in pregnancy. An imbalance of the maternal-fetal immune regulation can lead to pregnancy complications including preeclampsia, preterm-birth, intrauterine growth restriction or fetal loss (Orsi, 2008).

Understanding the huge variety and complexity of the underlying mechanisms that lead to these changes is one of the major goals of reproductive research. A deeper investigation into the female immune system during different stages of pregnancy will most likely help with the understanding, prevention and treatment of these diseases.

The fetal-maternal interface is a unique site in the human body where a significant number of particular interactions between the maternal immune system and fetal cells take place. It is composed of fetal trophoblast cells, decidual stromal cells and several different decidual immune cells, and an extensive interplay of these different cell types, hormones, cytokines and other signalling molecules has been shown to take place (Chang et al., 2020). Moreover, despite of the formerly believed sterile upper reproductive tract, a specific microbiome has been discovered by the presence of bacterial, viral and even fungal DNA by several recent studies (Einenkel et al., 2019). This underlines the importance of immunological investigation of the female reproductive tract. A correct establishment of this fetal-maternal junction is critical for successful and healthy pregnancy. It has been shown that the first trimester of pregnancy, including implantation and placentation, is defined by a local pro-inflammatory milieu and cytokine profile (Mor et al., 2011). As invasion of the

trophoblast involves disruption of the endometrial endothelial lining, an inflammatory environment is most likely required in order to repair tissues and remove debris. This pro-inflammatory state typically correlates with maternal indisposition, as the maternal body is adapting to changes. The second and beginning of third trimester provides rapid fetal growth and development by providing an immuno-tolerant and anti-inflammatory milieu, thus preventing rejection of the conceptus. The process of parturition involves another influx of inflammatory cells, which among others, populate the myometrium and contribute to expulsion of the fetus and rejection of the placenta (Förger & Villiger, 2020).

6.1. Expression of ILC subtypes in pregnancy

Innate immune cells contribute to tissue remodelling in each menstrual cycle and play an important role in initiation and maintenance of pregnancy as they orchestrate trophoblast invasion, vascular/spiral artery remodelling and prevent fetal rejection. Further, they provide protection from infections of the decidua and amniotic components. ILCs, as an important subgroup, have also been shown to be present in the human and murine uterus, though their distinct role in reproduction remains unclear and is a focus of on-going research. The composition and distribution of uterine ILC subsets changes with the on-going pregnancy (Doisne et al., 2015; Miller et al., 2018).

We were able to observe that uterine ILC3s were more present in the pregnant uterus from day 7 of pregnancy, equivalent to the first trimester, than in virgin uteri from non-pregnant mice. Furthermore, the expression of CCR4 and CCR10 in ILC3s were also increased (Figure 5), indicating that ILC3s at this stage of pregnancy possibly express more homing receptors in order to populate the pregnant uterus. This is in concordance with the previous findings of ILCs being less tissue-resident than initially anticipated (Huang et al., 2018).

ILC3s make up the strongest subset of all uterine ILCs and are mostly composed of the NCR^+ subset (Vacca et al., 2019). The highest proportions of ILC3s in murine uteri are present in early and mid-gestation (Li et al., 2017), similar to the above-mentioned findings. In humans, proportions of ILC3s peak in early pregnancy and around parturition. Through secretion of pro-inflammatory cytokines, such as IL-8, IL-

17, IL-22, TNF-alpha and granulocyte macrophage colony stimulating factor (GM-CSF), they are proposed to contribute to the creation of a pro-inflammatory environment and recruitment of further immune cells (Croxatto et al., 2016). On the other hand, a reduced antigen presentation by ILC3s has been described in early pregnancy, thus possibly promoting tolerance of the fetus (Einenkel et al., 2022). Overall, this facilitates the process of decidualization, trophoblast invasion and placentation, suggesting a pivotal role for ILC3s in early pregnancy.

Besides being important for establishment and maintenance of healthy pregnancy, ILCs are also involved in dysfunctional pregnancies, such as recurrent spontaneous abortions, preterm birth, preeclampsia, morbidly adherent placentas and intrauterine growth restriction (Barnie et al., 2015; Fu et al., 2017; Pang et al., 2019). Especially ILC2s seem to be involved in pregnancy complications. Xu *et al.* reported increased levels of decidual ILC2 and ILC3s in preterm labor (Y. Xu et al., 2018). It remains unclear though, whether this is a cause or a result of preterm birth. Considering that an inflammatory environment can promote preterm birth, the altered lymphocyte composition could correlate with an underlying placental inflammation. Reproductive failure has been associated with dysbalanced proportions of certain immune cells. According to Kamoi *et al.*, women experiencing idiopathic recurrent pregnancy loss show higher levels of NCR^+ ILC3s (Kamoi et al., 2015). This is in alignment with reports from Pang *et al.* who observed increased proportions in recurrent spontaneous abortions in mice (Pang et al., 2019).

In addition, ILC3s seem to promote upregulation of certain adhesion molecules on decidual stromal cells, thus particularly contributing to the influx of immune cells, most of all pro-inflammatory subsets in early pregnancy (Vacca et al., 2018). The murine pregnant uterus is enriched with adhesion molecules, suggesting that enhanced cell trafficking takes place in early pregnancy (Bowen & Hunt, 1999).

Decidual leukocytes are estimated to be rather recruited from the periphery instead of being self-renewed *in situ*, hence mechanisms of recruitment have been of special interest over the past years. Peripheral immune cells are equipped with a broad spectrum of homing receptors that are possibly down-regulated after having reached their tissue of destination (Hanna et al., 2003). It has been discussed whether mature ILCs are also recruited from the periphery or differentiate *in situ* (Kim et al., 2016). By

looking into homing structures and analysis of their homing molecules and receptors, this project aimed to gain insights into mechanisms of their recruitment.

6.2. Effect of pregnancy on migration of ILCs

As seen in Figure 7, enhanced migration of NCR^+ ILC3s in response to CCL4 and CCL20 was observed *in vitro.* Complementing the aforementioned findings of higher ILC3 proportions in early pregnancy (Figure 5), this could indicate their increased migratory capacity during the gestational period, possibly due to increased expression of chemokine receptors and/or augmented susceptibility to chemoattractants. In addition to this, ILCs from pregnant mice showed modified migratory behaviour. Early pregnancy (7 dpp) $CCR4^+$ ILCs migrated in an enhanced manner in response to the chemoattractant CCL28 (Figure 6).

The increase of leukocyte migration in pregnancy is not an unknown phenomenon. Already in 1980 Hawes *et al*. demonstrated the enhanced random migration of monocytes from pregnant women in second and third trimester (Hawes et al., 1980). Interestingly, migration of neutrophils was increased by the plasma of preeclamptic women, indicating that an adequate balance of enhanced leukocyte migration is required in order to provide a supportive, finely adjusted slightly inflammatory milieu in pregnancy (Walsh, 2009).

CCL28 has indeed been shown to play a role in early pregnancy. Choi *et al*. demonstrated its role in placentation in pigs and its abundance at the porcine fetal-maternal interface (Choi et al., 2016). CCL28 is associated with the chemokine receptors CCR10 and CCR3. Interestingly, Sun *et al*. demonstrated the role of the CCL28/CCR10/CCR3 axis in abortions. Decidual tissue from spontaneous abortions contained higher levels of CCL28/CCR3/CCR10 than decidua from healthy pregnancy. It was observed that inflammation-induced up-regulation of CCL28 induces apoptosis of decidual stromal cells, thereby promoting abortion (Sun et al., 2013). This confirms the need of a very finely adjusted chemotactic environment, as it appears to be necessary for successful pregnancy, but an overexpression could jeopardize healthy pregnancy outcome.

CCR10 is a chemokine receptor that is abundantly found in ILCs in the skin. Yang *et al.* demonstrated the expression of CCR10 in ILCs from skin-draining lymph nodes, where they are provided with skin homing abilities. $CCR10^+$ ILCs contribute to immunological homeostasis in the skin as they regulate T cell recruitment, (especially Tregs) and inflammatory conditions (Y. Mao et al., 2020; J. Yang et al., 2016).

It must be noted, that despite the effect of CCL28 on ILC migration, this effect was mostly seen in CCR4-expressing rather than CCR10-expressing ILCs.

The observed enhanced migratory capacity of ILCs during gestation could be a result of higher expression of homing receptors in pregnancy, thus contributing to enhanced recruitment and presence of ILCs in the pregnant uterus, especially in early pregnancy. In order to investigate this hypothesis, the homing potential of ILCs from miscellaneous murine lymphoid organs was assessed in multiple experiments.

6.3. Chemokine receptor expression

The *in vivo* analysis revealed that both CCR4 and CCR10 in ILCs were significantly elevated in early pregnancy. While the CCR4 expression was shown to be increased in ILCs from uterus-draining lymph nodes, spleen and Peyer's patches at day 7 of pregnancy, CCR10 expression was found to be up-regulated in ILCs from uterus-draining lymph nodes and spleen (Figure 9) In addition to this, significantly higher proportions of $CCR4^+$ and $CCR10^+$ ILC3s were found in first-trimester pregnant mice (Figure 5). Taken together, these results point to a pregnancy-driven amplification of homing receptor-dependent cell migration.

CCR4 has been found to be an important chemokine receptor expressed on decidual T cells (predominantly Th2 helper cells) and macrophages as well as the invading extravillous trophoblast and decidual stromal cells (Du et al., 2014; Red-Horse et al., 2001). The recruitment of these aforementioned $CCR4^+$ T lymphocytes into the decidua is critically mediated by thymus and activation chemokine TARC (CCL17), which is expressed by the trophoblast and endometrial glands. Accordingly, Tsuda *et al.* observed a higher expression of CCR4 in early pregnancy decidua compared with peripheral blood. Decreased proportions of decidual $CCR4^+$T cells in cases of miscarriages indicate its critical role in the maintenance of pregnancy (Tsuda et al.,

2002). Aligning with the previously described investigation of CCR4 in ILC migratory behaviour, D'Ambrosio *et al*. reported its role in the recruitment of ILCs to the lung (2001) and into inflamed tissues (D'Ambrosio et al., 2003; Weston et al., 2019). Salimi *et al*. reported the presence of CCR4 in skin-homing ILC2s (Salimi et al., 2013). Altogether, a critical role of CCR4 in the trafficking of T lymphocytes and ILCs is undeniable, however the interplay of CCR4 and ILCs in respect of the fetal-maternal interface has not yet been sufficiently investigated and the role of CCR4 in ILC recruitment into the decidua in pregnancy remains to be elucidated.

6.4. Effect of chemokine ligands on *in vitro* ILC migration

In order to gain insights into the possible role of CCL4 and CCL20 on the recruitment of ILCs to the fetomaternal interface, their chemotactive role on ILCs, derived from uterus-draining lymph nodes, was assessed. Indeed, CCL4 and CCL20 were both able to enhance migration of NCR^+ILC3s *in vitro* (Figure 7). CCL4 is one of the many counter-ligands of CCR4 and along with other ligands, promotes migration and adhesion of the trophoblast (Hannan et al., 2006; Jovanović et al., 2010). Interestingly, endometrium that had been pre-treated ("injured") with a biopsy, showed increased levels of CCL4 and in turn favoured a positive pregnancy outcome, most probably due to the recruitment of DC and macrophages (Gnainsky et al., 2010).

Besides CCL4, CCL20 also increased the migratory capacity of ILC3s *in vitro* and is a ligand that strongly interacts with $CCR6^+$ cells in both humans and mice (Schutyser et al., 2003). CCR6 is a homing receptor on immature DC, B cells, $CD4^+$/$CD8^+$ T cells, NKT cells and ILCs, and is highly expressed on Tregs (Greaves et al., 1997; Ito et al., 2011; Kleinewietfeld et al., 2005; Krzysiek et al., 2000), whereas CCL20 (or MIP-3α or LARC) is found in different epithelial cells, thymus, gastro-intestinal tract, peripheral blood lymphocytes, and secondary lymphoid organs (Charbonnier et al., 1999; Ito et al., 2011; Iwasaki & Kelsall, 2000; Nakayama et al., 2001). The CCL20/CCR6 axis is critical for leukocyte arrest and for the recruitment of antigen-presenting cells to sites of antigen-entry, hence explaining the abundance in lymphoid organs (Varona et al., 2001). Most circulating ILC2s have been shown to express CCR6 in order to migrate into certain epithelial tissues (J. Yang et al., 2016).

CCR6 signalling mediates the recruitment of innate immune cells to the gut, especially to sites of inflammation, explaining the important role of CCR6/CCL20 and ILCs in inflammatory bowel disease (IBD) (Basheer et al., 2013; Skovdahl et al., 2015). Interestingly, CCR6 has been generally found to correlate with disease severity in several autoimmune diseases besides IBD, such as multiple sclerosis, rheumatoid arthritis or psoriasis (Meitei et al., 2021). Accordingly, Takaki-Kuhawara *et al*. found increased levels of $CCR6^+$ ILC3s in inflamed joints in rheumatoid arthritis mouse model (Takaki-Kuwahara et al., 2019). This demonstrates the clear role of CCR6 in inflammatory responses.

Due to the effect of Treg recruitment, the CCL20/CCR6 axis is of interest in the regards of pregnancy. Park *et al.* observed the upregulation of the CCL20/CCR6 axis in porcine endometrium in early pregnancy. A down-regulation of the CCL20/CCR6 axis was found in women experiencing unexplained recurrent miscarriages, as Treg migration was significantly impacted (X. X. Zhang et al., 2015). On the other hand, according to Hamill *et al*. increased levels of CCL20 in amniotic fluid were associated with fetal prematurity (Hamill et al., 2008). Despite the mentioned results, CCR6 expression in ILCs throughout pregnancy remained unchanged. This suggests a stable basal expression of CCR6 in ILCs from lymphoid organs during pregnancy, which is in accordance with a rather low expression of its ligand CCL20 in the absence of strong inflammatory stimuli or molecules (Ito et al., 2011).

In conclusion, even though the CCL20/CCR6 axis contributes to immunotolerance via Treg induction, overexpression is associated with inflammatory and autoimmune conditions. Hence, a finely balanced equilibrium is necessary to provide a pregnancy-supportive microenvironment at the fetal-maternal interface.

6.5. Effect of pregnancy on bone marrow ILC expression

Primary lymphoid organs have been shown to undergo a pregnancy-induced involution (Muzzio et al., 2014; Yokota et al., 2008). The concomitant reduced lymphocyte egression is believed to impact further lymphocyte maturation and distribution during pregnancy. ILC proportions in the murine bone marrow were significantly decreased during all stages of pregnancy (Figure 10). As levels of estrogen are reciprocally related to the number of bone marrow cells, rising estradiol

levels during pregnancy have been proposed to cause this depletion (Yokota et al., 2008). Gestational changes of sex steroid hormone levels can be accounted for this decrease and contribute to the induction of a more tolerant immune milieu (Medina et al., 1993; Medina & Kincade, 1994). However, two possible mechanisms could explain the decrease of ILC proportions in bone marrow during pregnancy. On the one hand, an overall increased recruitment of ILCs from the bone marrow could account for the reduced remaining proportions of ILCs. On the other hand, no pregnancy-related changes in the expression of chemokine receptors were observed. In this concern, the depletion of bone marrow ILCs is likely to be a consequence of an overall reduced lymphopoiesis in pregnancy.

6.6. α4β7 expression on ILCs and pregnancy

α4β7-expressing ILCs from uterus-draining lymph nodes were observed to be significantly reduced at day 7 of pregnancy, and an even stronger decrease of α4β7 expression (MFI) was found at 14 dpp and 18 dpp (data not shown). The integrin α4β7 acts as an adhesion molecule and is therefore critical for leukocyte trafficking into tissues (Berlin et al., 1993). α4β7 is expressed on ILC progenitors where it is involved in cell-cell interactions and migration (Possot et al., 2011; Yu et al., 2014). Major binding partners for α4β7 are expressed on endothelial cells of different peripheral tissues and include predominantly MAdCAM-1, but also VCAM-1 and fibronectin (Strauch et al., 1994; Y. Yang et al., 1998).

Integrins are present during the menstrual cycle and in pregnancy throughout the endometrium, decidua and extravillous trophoblast (Merviel et al., 2001). A switch of the integrin pattern has been observed at the beginning and throughout murine pregnancy, indicating the relevance of cell recruitment but also changes of leukocyte composition at the fetal-maternal interface during pregnancy (Mangale & Reddy, 2007). MAdCAM-1 is expressed by maternal uterine vessels during placentation and its importance for leukocyte trafficking to the uterus was suggested by Salmi et al (Salmi et al., 2001). Thus, the interplay of α4β7-expressing immune cells and MAdCAM-1 seems to be important for the establishment and maintenance of a pregnancy-supportive immune milieu at the fetal-maternal interface. This was confirmed by experiments in mice in which MAdCAM-1 was impaired due to

monoclonal antibodies, which resulted in the impaired recruitment of monocytes and uNK cells, dysfunctional vascular remodelling and a hypocellular decidua, possibly due to reduced access of $\alpha4\beta7^{+}$ monocytes to the decidua and depletion of uterine NK cells (Fernekorn et al., 2004). Interestingly, pregnancy complications, such as IUGR and preeclampsia were associated with the absence or impairment of particular integrins and an impaired integrin expression has been shown to be a marker for poor endometrial receptivity (Chen et al., 2016; Dorostghoal et al., 2017; Zhou et al., 1997; Zygmunt et al., 1997).

α4β7 is also known as a gut homing integrin with altered expression in mucosal inflammation (Meenan et al., 1997). Even though it has been shown by Fernekorn *et al.*, that an impairment of the α4β7-MAdCAM-1 signalling can lead to cellular dysfunctionalities, recent data regarding the monoclonal antibody Vedolizumab has shown otherwise. Vedolizumab has been recently developed as a targeted therapy strategy for treatment of inflammatory bowel disease (IBD). Terjung *et al.* investigated the safety of vedolizumab for female IBD patients during pregnancy, and there were no concerns regarding adverse effects on pregnancy outcome (Terjung et al., 2020). These findings lead to the conclusion that α4β7 is dispensable for homing to the uterus during pregnancy. This stands in contrast to the above-mentioned critical role of α4β7 in pregnancy. However, in the aforementioned study, alterations at the cellular level were not further assessed.

The down-regulation of α4β7 in ILCs from lymph nodes could be interpreted as a mechanism to facilitate lymphocyte migration. Interestingly, the down-regulation of integrins in germinal centres of lymph nodes has been reported already and is supposedly critical in order to allow cell motility (Castanos-Velez et al., 1995). The lack of adhesion promotes enhanced migration and recruitment to peripheral tissues, for example the uterus. Hence, α4β7 downregulation goes along with the increased chemokine receptor expression, both facilitating ILC recruitment to the fetal-maternal interface. The alterations of both chemokine receptor and integrin expression were most significant in ILCs from the uterus-draining lymph nodes, indicating their potential role as a main reservoir for uterine ILCs. Accordingly, Yang *et al.* proposed that ILCs are programmed in the lymph nodes in order to acquire specific homing properties, in this case to the uterus (J. Yang et al., 2016). In general, gestation has been shown to induce mobilization of lymphocytes. Secondary lymphoid organs,

such as lymph nodes and spleen have been shown to undergo hypertrophy during pregnancy, emphasizing their importance in gestational immunomodulations (Bilinski et al., 2008). This could also be attributable to the increased perfusion of the uterine area in pregnancy (Thaler et al., 1990).

6.7. Pregnancy hormones and their effect on the immune system

It has been shown that enhanced migration of leukocytes takes place in pregnancy and that this might be associated with increased migratory ability and altered expression of chemokine receptors and corresponding chemoattracting ligands (Farine et al., 2017; Red-Horse et al., 2001). The significantly increased levels of chemokine receptors found in early pregnancy (Figure 9) pointed to the need of elucidation of the underlying mechanisms.

Hormonal changes play an important role in pregnancy as a complex endocrinological-immune crosstalk is necessary to guarantee tolerance of the fetal allograft. Pregnancy hormones, such as progesterone (P4), estradiol (E2) and human chorionic gonadotropin (β-hCG), are key players in immunomodulation. In this context they might also regulate the expression of chemokine receptors and ligands and might have an impact on cell migration (He et al., 2007; Park & Yang, 2011; Vassiliadou et al., 1999).

Female sex hormones have been shown to play a role in gender differences of autoimmune diseases and immune responses (Voskuhl, 2009; Whitacre, 2001). More than 80 % of patients suffering from Systemic Lupus Erythematodes (SLE), sclerodermia, autoimmune diseases of the thyroid or rheumatoid arthritis (RA) are female (Beeson, 1994). It is well established that different autoimmune diseases (MS, RA) show decreased disease activity during pregnancy and that this is possibly due to endocrinological changes. Typically, disease activity is most decreased in third trimester, where levels of both estradiol and progesterone are the highest, whereas post-partum flare-ups correlate with low hormonal levels, especially when breastfeeding (Nelson & Ostenson, 1997; Voskuhl, 2009; Vukusic & Confavreux, 2006).

Progesterone can act directly or indirectly via Progesterone-Induced-Blocking-Factor (PIBF) on the immune system and has immunomodulatory effects on many different leukocyte subpopulations in the decidua, including T cells, B cells, NK cells, dendritic cells, monocytes and even mesenchymal stem cells. It promotes a Th2-dominated cytokine profile, which has been shown to favor successful pregnancy (Miyaura & Iwata, 2002; Piccinni et al., 1995; Szekeres-Bartho & Schindler, 2019). Progesterone features anti-proliferative and anti-inflammatory properties. It inhibits the antigen-presenting capacities of DC and macrophages and suppresses NK cell cytotoxicity while increasing proportions of systemic and uterine Tregs and regulatory B cells (Mao et al., 2010; Muzzio et al., 2014). Progesterone has further been shown to promote T cell migration into lymphoid tissues while causing transient suppression of T cell activation (Kashiwagi et al., 2022). In conclusion, progesterone modifies different immune cells towards a tolerogenic and pregnancy-supportive state.

6.8. Pregnancy hormones and ILCs

There is only very limited data on steroid hormone receptor expression and the impact of pregnancy hormones on ILCs. A narrow expression pattern of steroid hormone receptors on ILCs has been reported (Blanquart, Laffont, et al., 2021), though a recent study was able to detect progesterone receptors (PR) and estrogen receptors (ER-α) in human circulating ILCs. Their increase in late pregnancy was likely associated with higher levels of progesterone and estradiol during the last trimester (Zhao et al., 2022).

Bartemes *et al.* demonstrated the expression of ER-α on uterine ILC2s, though ILC2s from other origins, such as the lung were negative for ER-α and show no effect of hormonal stimulation (Bartemes et al., 2018). Interestingly, the androgen receptor (AR) seems to play a key role in the regulation of lung ILC2. ILC2 are more abundant in female lymph nodes and lung than in male. This is most probably attributable to the inhibitory effect of testosterone on ILC2s and protects male mice from allergic lung inflammation (Blanquart, Mandonnet, et al., 2021). There is conflicting evidence on the effect of estrogens *in vivo*, potentially due to different forms of administration, concentrations and effector organs. Nilsson *et al*. in 1994 and Hao *et al.* in 2008 observed an inhibitory effect of estradiol on NK cell cytotoxicity whereas Hrushesky

et al. reported the opposite (Hao et al., 2007; Hrushesky et al., 1988; Nilsson & Carlsten, 1994). ILC3s were found to express barely any steroid hormone receptors. NK cells express ER-α whereas PR is only found to some degree (Blanquart, Laffont, et al., 2021).

6.9. Dose-dependent effects of progesterone

Taking into account the regulatory role of sex steroid hormones, progesterone and estradiol could alter the expression of homing receptors as observed *in vivo* in early pregnancy (Figure 9). As shown in experiment 5, short-time stimulation of the murine cells with low doses of progesterone (5 ng/mL, 50 ng/mL) did not significantly alter homing receptor expression. A decrease of CCR10 expression and increase of α4β7 expression in ILCs from murine spleen was found, indicating rather a decrease in their migratory ability. In contrast, when using considerably higher concentrations (5 µg/mL, 50 µg/mL) and longer stimulation times, the opposite effect was observed. Upon stimulation with 5 µg/mL of progesterone, a significant increase of CCR4 expression in ILCs was observed, with CCR6 and CCR10 showing a similar, though non-significant pattern of expression. Stimulation with 50 µg/mL progesterone led to a significant decrease of α4β7 (Figure 12). Interestingly, these results resemble the alterations of homing receptors in early pregnancy *in vivo* (Figure 9). The treatment with estradiol on the other hand had no significant effects on the expression in any concentration.

To date, there are only very few reports on the effect of pregnancy hormones on leukocyte trafficking and the expression of chemokine receptors. Belot *et al.* observed a decrease of CXCR4 expression on the mast cell cell line HMC-1 and reduced cell migration in response to CXCL12 upon treatment with progesterone (1 µM) (Belot et al., 2007). In a similar manner, Vassaliadiou *et al.* reported a suppression of CXCR4 and CCR5 expression on T cells by progesterone, whereas there were no changes seen in these receptors in monocytes (Vassiliadou et al., 1999). Sentman *et al.* demonstrated the up-regulation of CXCL10 and CXCL11, both chemoattractants of uterine NK cells, in response to treatment with progesterone (10^{-9}, 10^{-8} M) and estradiol (10^{-9} M) (Sentman et al., 2004). An interesting effect of progesterone was reported by Ramhorst *et al*.: while an up-regulation of CCL5

(RANTES) in endometrial $CD4^+/CD8^+$ T lymphocytes by progesterone (10^{-5} M) was observed, the opposite effect was found in peripheral $CD4^+/CD8^+$ lymphocytes (Ramhorst et al., 2006).

As seen in experiment 4 *in vivo,* homing receptor alterations did significantly occur at day 7 of pregnancy, hence similar effects of treatment with pregnancy hormones on the expression of these receptors were anticipated. The concentrations used in experiment 5 did not show the expected alteration, while higher levels yielded very similar results as the *in vivo* experiment. Hence, progesterone presents its *in vitro*-potential to alter the expression of homing receptors, especially as a dose-dependent gradual increase of the expression was observed (Figure 12). Even though such high concentrations of progesterone are considered supraphysiological, it should be taken into consideration that local levels at the fetal-maternal interface are much higher than in peripheral blood. Wiest demonstrated that placental levels of progesterone are by far the highest, as compared with plasma, myometrium, amniotic fluid and fetal membrane levels (Wiest, 1967). The amount of progesterone in term human placenta is over 13-fold higher with an average of 237 µg/100 g tissue, compared with peripheral plasma levels at term of 17,4 µg/100 mL. This is not surprising, as the placenta is the main source of progesterone in mid- and late pregnancy. In mice, progesterone levels were assessed as 58 ng/mL in mid/late pregnancy (Chung et al., 2012).

Belot *et al.* observed a similar phenomenon: supraphysiological doses of progesterone were necessary to yield their results. It was suggested that this was due to the low number of PR in mast cells, which could similarly account for ILCs (Belot et al., 2007). It should also be considered that ILCs in general behave differently in response to treatment with progesterone than other other lymphocytes. To date, there is no similar literature about the effect of pregnancy hormones on the expression of chemokine receptors in ILCs. Even though Blanquart *et al.* did not come across abundant expression of PR on ILCs, it is likely that PR expression changes throughout pregnancy. Just recently, Zhao *et al.* were the first ones to demonstrate both the existence of PR on human circulating ILCs as well as the significant increase of PR in ILC2s from late-pregnant women (Blanquart, Laffont, et al., 2021; Zhao et al., 2022). Subsequently, ILCs likely change their susceptibility to

progesterone exposure. The further investigation of PR expression in the course of pregnancy could therefore be an interesting focus for further research.

Further factors besides pregnancy hormones may account for the elevated expression of homing receptors in early pregnancy *in vivo*. The complex interactions that cells experience *in vivo* are difficult to replicate *in vitro*. The effect of costimulatory cells, such as stromal cells, has not been assessed. Furthermore, splenic cells, as they were partially used here, are accurately organized in follicles, whereas in mucosa a very different pattern of cell arrangement is expected.

It should be also taken into account that the ILCs in the latter experiments were of splenic origin to guarantee a significant number of cells. Murine uteri and uterus-draining lymph nodes, though preferred as source of ILCs for this experiment, yield number of cells that limit the realization of meaningful experiments. As Bartemes *et al.* reported, ILCs from other tissues than the uterus do not express estrogen receptors and thus did not react in response to treatment with this hormone (Bartemes et al., 2018). In a similar manner, the effect of progesterone on ILCs from splenocytes could be impacted due to impaired receptor expression. On the other hand, it was postulated that progesterone can also act via glucocorticoid receptors. Furthermore, effects of hormones might also occur indirectly due to interaction with stromal cells, fibroblasts or other soluble factors such as cytokines (Henderson et al., 2003; Lei et al., 2012). In order to further evaluate PR expression and the role of progesterone on ILCs in pregnancy, a knockout mouse model for PR and/or treatment with progesterone antagonists, such as mifepriston (RU486), could be promising options.

6.10. Estradiol and its effect on chemokine receptor expression in ILCs

In several studies significant effects of estradiol on the migratory ability and chemokine receptor signalling in different leukocyte subsets were observed.

Janis *et al.* demonstrated the inhibition of CCR2 and CXCR3 expression on murine monocytes as well as attenuated chemotaxis towards the corresponding chemokine ligand MCP-1 by treatment with estradiol. Accordingly, a decrease of MCP-1 in endometrial stromal cells upon stimulation with estradiol was observed (Arici et al.,

1999; Janis et al., 2004). These findings suggest an important role of estrogens in down-regulated immune surveillance, particularly in female reproductive tissues, thereby facilitating both estrogen-dependent tumor growth and the induction of an immunotolerant state in pregnancy (Boudot et al., 2011).

However, the treatment with estradiol did not present any significant alterations in homing receptor expression of ILCs in any experimental condition tested. Compared with the progesterone concentrations used, administered estradiol levels were rather low. There is contradictory data regarding serum estradiol levels in pregnant mice. According to Barkley *et al.*, murine plasma levels were quantified as ca. 20 pg/mL in early pregnancy and reached levels of ca. 50 pg/mL in late pregnancy (Barkley et al., 1979). Slightly different, but similar ranges were obtained by Chung *et al*. They determined a broader spectrum of hormone levels throughout murine pregnancy with mid-pregnancy serum concentrations of 7.24 pg/mL and a peak of 101.8 pg/mL in late pregnancy (Chung et al., 2012). Zhang, Fishman *et al.* on the other hand detected much higher levels and a different gradient. An early-pregnancy peak of 2000 pg/mL was followed by a decrease towards 1000 pg/mL, then reaching late-pregnancy levels of 1500 pg/mL (L. Zhang et al., 1999). In accordance, we tested a broad spectrum of estradiol levels of 5 pg/mL, 100 pg/mL and 100 ng/mL. None of these concentrations were able to alter homing receptor expression in ILCs, so a lack of a detectable effect on ILC chemokine receptor distribution can be postulated.

6.11. Chemokine signalling as a potential therapeutical target

In summary, chemokines and their receptors play a regulatory role in several pregnancy-associated processes, and dysbalanced or abnormally expressed chemokine levels have been associated with adverse pregnancy outcomes. Lopez *et al.* suggested that the decreased disease activity of MS during pregnancy correlates with down-regulation of CXCR3, a chemokine receptor that is directly involved in MS pathogenesis. Its down-regulation suppresses the Th1 response and thus supports pregnancy (Gomez-Lopez et al., 2010).

In contrast, up-regulation of CCL2, CCL5 and CXCL10 seems to be involved in the pathogenesis of preeclampsia (Li et al., 2011; Szarka et al., 2010). Augmented levels of CCL2 and CCL8 were found to be associated with preterm labour, as increased

chemokine levels lead to an early influx of immune cells and result in pre-term onset of labour (Farine et al., 2017; Hamilton et al., 2013).

Other pathological conditions in which increased chemokine signalling plays a role, have been successfully treated by targeted blockade of the involved receptors or ligands. An anti-CCR2 antibody was favorably tested in prevention and treatment of multiple sclerosis, rheumatoid arthritis and other inflammatory diseases. In a recent work, Tu *et al.* proposed a beneficial role of the CCR2/CCL2 pathway inhibition in the treatment of cancer (Tu et al., 2020). The CCR5-inhibiting antibody Maraviroc has been successfully established as an emerging antiretroviral drug in HIV treatment (Latinovic et al., 2011). The abundance of CCR4 on T cells has been taken advantage of in the development of the anti-CCR4 monoclonal antibody Mogamulizumab, which has been clinically approved in the treatment of T cell neoplasias, such as adult T cell leukemia or peripheral T cell lymphoma (Ueda, 2015). Furthermore, AMD3100 as a CXCR4 inhibitor has been used in the treatment of malignant hematopoietic diseases for over 10 years, as it contributes to stem cell mobilization from the bone marrow (De Clercq, 2009).

Therefore, targeted inhibition of chemokine signalling could be a promising approach in prevention and treatment of inflammation-derived pregnancy complications in the future.

6.12. Critical appraisal of the work

6.12.1. Methodological limitations

Due to the small proportions of ILCs within lymphocytes, their analysis is challenging and difficulties in identification of the subsets were faced. ILCs can be identified according to their surface markers and their cytokine array, hence flow cytometry is the most reliable method for ILC analysis (Tait Wojno & Beamer, 2018). Nevertheless, the use of only one method limits the validity of the results. The expression of chemokine receptors and α4β7 integrin could have been further investigated via PCR or Western blot, though in contrast to flow cytometry, purified ILCs in relatively high numbers would have been necessary.

Immunofluorescence analysis of uterine ILCs in frozen and embedded uteri was planned and performed in order to compare the distribution in non-pregnant and pregnant uteri. Even though formerly established protocols were used and adjusted to staining for ILCs, the presence of such low numbers of positive stained cells were poorly distinguishable from unspecific, background staining. Further animal models (rat, pig) or human uteri may serve as a better alternative due to the increased organ size and cell numbers.

In the reported experiments, different murine lymphoid organs were analysed for number and properties of ILCs. Lymphoid organs seem to be important reservoirs of ILCs and other innate immune cells but the investigation of ILC distribution within the uterus would be more significant in regard to homing. The methods for analysing ILCs in uterine tissues were only improved in the on-going studies, hence the initial focus was put on the lymphoid organs. A more accurate examination of chemokine receptors in uterine ILCs during different stages of pregnancy will be a valuable approach in future experiments. Nevertheless, the small size of uteri in non-pregnant and early pregnant mice will most probably always aggravate the number of assessed ILCs.

It must be considered that *in vitro* results have limited meaningfulness as they neglect the complexity of tissues *in vivo*. Interactions of cells *in vivo* depend on several immunological and endocrine interactions between cells, vessels, glands and extracellular matrix that cannot be reproduced in cell culture.

For many of the experiments, a higher sample number would have improved the experiment's significance. Unfortunately, the number of lymphocytes that can be isolated from certain organs, such as lymph nodes and Peyer's patches, is very limited and can be as low as 1.5 million cells from pooled inguinal and para-aortal lymph nodes or 750.000 cells from Peyer's Patches. Differences in the number of replicates for each subgroup can be explained as a result of difficulties in reliably determining the pregnancy of a mouse. The appearance of a vaginal copulation plug was used as a pregnancy sign and declared day 0 of pregnancy, though technically it only indicates that mating has occurred. Subsequently, some mice eventually did not turn out as pregnant and had to be sacrificed as non-pregnant samples. Additionally,

the assessment of the third phase of pregnancy group was challenging, as 18 dpp is very close to term and some mice had littered before they could be sacrificed.

It must be noted that in terms of chemotaxis, the differentiation from chemokinesis can be relevant and necessary. Chemotaxis and chemokinesis are both important chemotropic responses that have been shown to often go along. Their co-existence and relevance for cell migration has been investigated in different cell types, such as spermatozoa, polymorphonuclear leukocytes, mesenchymal stem cells, malignant mesothelioma cells and even neurons (Behar et al., 1996; Fräki et al., 1983; Ralt et al., 1994; Yoon et al., 2016). While chemotaxis describes the directed cell movement towards or away from a concentration gradient, chemokinesis describes the random movement of cells in the presence of a chemotropic substance without a gradient.

Practically, the assessment of chemotropic responses thus depends on the presence of a chemoattractant gradient. As depicted in Figure 4, for a chemotaxis migration assay, the bottom well department contains one or more chemoattractans, whereas the upper department contains medium and cells only. For assessment of chemokinesis, both compartments share the same composition.

It must be considered that experiments in this work only addressed the process of chemotaxis, as every measured cell migration was related to a chemical gradient. Even though it is generally important to distinguish the cell movement from random migration, the enhanced presence of chemoattractants at the feto-maternal interface mimics a chemical gradient, similar to the one created in a well. As an essential part of this work aims to evaluate a possible role of uterine derived chemoattractants in the recruitment of ILCs to the feto-maternal interface, the focus during the experiments was put on chemotaxis.

6.12.2. Mouse model and its limitations and animal-human translation

It is important to highlight that every mice model has limited validity as compared to experiments with human probes. Murine experiments can be very helpful to gain information on underlying mechanisms, though it is important to understand that their value has its limits. An important advantage is the option to remove and analyse different organs during pregnancy, a standardized setting can be guaranteed, experiments can be repeated multiple times and results are reproducible.

Murine and human pregnancy differ in several aspects. The mouse pregnancy has a very short duration in comparison to humans, and due to litters with multiple embryos, multiple implantation sites are present. Mice lack β-hCG and part of their organogenesis takes place postnatally. Major differences in placenta structure limit the utility of a mouse model for the understanding of placentation (Carter, 2020).

Especially during pregnancy, human probe sampling is either very difficult or even impossible, both ethnically and technically, so murine studies are a very important component of reproductive research. Many parameters of the immune system and their effect on pregnancy have been assessed in rodents, and a selection of them can be potentially further investigated in human pregnancy, if possible. Nonetheless, the results of mouse model experiments should be interpreted carefully, as they cannot be simply translated onto humans. Humanized immune system mouse models could be an approach in order to generate valuable data with greater relevance for human diseases. Here, immunodeficient mice are reconstituted with human immune cells and thus mimic more valuable conditions. This strategy combines the favourable advantages of mouse models with more beneficial data in regard to the investigation of the human immune system (Allen et al., 2019).

6.12.3. Outlook

For further investigation, the distinction of all groups of ILCs in all experiments is a useful approach and could have delivered more valuable data. As each group has different characteristics and functions, their role in pregnancy is also different and variable. Especially in the assessment of different lymphoid organs for ILC homing receptor expression, a breakdown into subgroups can upgrade the results.

In vivo studies of migration are difficult and to date, only few systems to track ILC migration are available. A promising approach is the *in situ*-labelling of cells: lymphocytes can be marked via fluorescent dyes and subsequently injected into rodents. Different dyes are available for visualization of migration and proliferation including CFDA-SE (CFSE), PKH26, Hoechst 33342 or calcein, though CFSE is the most commonly used due to its highly reliable characteristics and low cost (Becker et al., 2004; Parish, 1999). The labeled cells can then be tracked and analysed in the

tissues where they accumulated. In a similar manner, Dutton *et al.* distinguished migratory from tissue-resident ILCs in peripheral lymph nodes in transgenic photoconvertible mice (Dutton et al., 2019) Such methods or similar techniques could be a promising approach for future studies to ameliorate the investigation of ILC migration during pregnancy in mice, though due to the lack of unique specific markers, the systems to track ILCs are technically challenging to date (Kim et al., 2016).

To start screening the function of ILCs in the context of their migration to uterine - derived chemoattractants, flow cytometry is a valid tool to identify relevant ILC populations in a complex cell composition. However, the low numbers of ILCs present in most tissues strongly limit the experimental approach. Based on the data gained from the experiments that were performed in this thesis, further experimental approaches can focus on behaviour of type 3 ILCs. For instance, after sorting of ILC type 3 cells, a broader spectrum of experiments could be performed without the need of further characterization by flow cytometry. This includes the assessment of chemotaxis and chemokinesis via specific assays. Different chambers including µ-Slide, Boyden, Zigmond or Dunn chambers are available to perform an even more exact analysis of cell migration via life cell imaging or immunofluorescence microscopy (Zengel et al., 2011).

Gene knock-out mice are mouse models in which particular genes of interest are inactivated or disrupted, allowing reliable *in vivo* analyses of biological mechanisms and gene functions. The selective knock-out of certain homing receptors, such as CCR4, CCR6, CCR10 and α4β7 and the subsequent effect on murine pregnancy would be a useful tool to deliver valuable information on the importance and process of ILC migration for pregnancy. As mentioned above, the effect of progesterone on ILCs and homing receptors could be assessed via blocking of progesterone receptors or the administration of progesterone antagonists such as RU 486.

7. Summary

Pregnancy involves adaptations of the cellular composition *in utero* to establish a functioning fetal-maternal interface. Different subsets of leukocytes populate the endometrium and contribute to tolerance of the fetal allograft while protecting it from potentially threatening infections or rejection. Innate lymphoid cells are recently discovered immune cells that, besides the gut, lung and skin, possess immunoregulatory functions in the female reproductive tract, especially during gestation. Although present at the fetal-maternal interface, the dynamics of ILC migration during pregnancy remains poorly investigated. The involvement of homing receptors in ILC migration to the uterus was the main subject of the present work.

First, the expression of homing receptors on ILCs from miscellaneous organs was assessed across the course of murine pregnancy *in vivo* by means of flow cytometry. Then, their migratory capacity towards pregnancy-relevant chemokines was investigated *in vitro*. The impact of pregnancy related hormones on the migration and homing of ILCs was then analysed *in vitro* via migration assays.

The results confirm altered proportions of ILCs *in utero* and the altered expression of homing receptors in ILCs in pregnancy. Different murine lymphoid organs showed augmented expression of chemokine receptors and decreased levels of homing integrin α4β7 in the first trimester, suggesting enhanced migration patterns of ILCs during early pregnancy. Subsequently, migration assays were used to demonstrate the role of different chemokine ligands in enhancing ILC migration.

Eventually, the alterations in homing receptor expression were correlated with female pregnancy hormones. Progesterone treatment caused similar effects on homing receptor expression in ILCs as observed during early gestation. These results represent the first study evaluating the effect of sex steroid hormones on ILC chemokine receptor distribution.

Taken together, our results indicate the involvement of pregnancy-relevant chemokines, including CCL4, CCL20 and CCL28, in the recruitment of ILCs to the uterus during pregnancy. The data highlight an endocrinological-immune crosstalk in the regulation of ILC homing to the female reproductive tract. Gestation alters chemokine receptor expression in order to regulate the access of immune cell

subsets to the fetal-maternal interface. An adequate regulation is highly needed, as a lack or abundance of different subgroups could result in pregnancy complications, including fetal loss, pre-eclampsia or pre-term birth. Thus, the role of ILC chemotaxis to the pregnant uterus and its regulation are of interest in the understanding, prevention and treatment of the clinically relevant obstetric diseases.

Figures

Abbreviations

°C	degrees Celsius
µL	microliter
APC	antigen-presenting cells; allophycocyanin (fluorochrome)
BSA	bovine serum albumin
β-hCG	beta- human chorionic gonadotropin
CCL	chemokine ligand
CD	cluster of differentiation
CILP	common innate lymphoid progenitor
CLP	common lymphoid progenitor
C(X)CR	chemokine receptor
DC	dendritic cell
DIC	decidual immune cells
dpp	days post plug
DSC	decidual stromal cells
FACS	fluorescence-activated cell sorting (synonym: flow cytometry)
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate (fluorochrome)
FOXP3	Forkhead-Box-Protein P3
FSH	follicle stimulating hormone
FRT	female reproductive tract
GM-CSF	granulocyte macrophage-colony stimulating factor

H	hour
IBD	inflammatory bowel disease
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
IUGR	intra-uterine growth restriction
kD	kiloDalton
LH	luteinizing hormone
LN	lymph node(s)
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MHC	major histocompatibility complex
MS	multiple sclerosis
NCR	natural cytotoxicity receptor
PE	phycoerythrin (fluorochrome)
PE-Cy7	phycoerythrin-cyanine7 (fluorochrome)
PerCP	peridinin-chlorophyll- protein complex (fluorochrome)
PLO	primary lymphoid organs
PLZF	promyelocytic leukemia zinc finger
PP	Peyer's patches
RA	rheumatoid arthritis
RORα	Retinoic-acid-receptor-related orphan nuclear receptor alpha
RORγT	Retinoic-acid-receptor-related orphan nuclear receptor gamma T

rpm	rounds per minute
s	seconds
SLO	secondary lymphoid organs
T-bet	T-box expressed in T cells
TGF-β	transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegeben Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Greifswald, den 10.05.2023 _____

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