UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Geburtshilfe, Kinder- und Jugendmedizin
Klinik für Geburtshilfe und Pränatalmedizin

Prof. Dr. med. Kurt Hecher

Fetal origin of immune diseases: Maternal microchimerism during gestation and its modulation upon a prenatal stress challenge

Dissertation

zur Erlangung des Doktorgrades Dr. rer. biol. hum.
an der Medizinischen Fakultät der Universität Hamburg

vorgelegt von

Ina Annelies Stelzer
aus Meppen

Hamburg 2016

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 08.09.2016

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, die Vorsitzende: Prof. Dr. Petra Arck

Prüfungsausschuss, zweiter Gutachter: Prof. Dr. Udo Markert

Prüfungsausschuss, dritter Gutachter: Prof. Dr. Bettina Toth

Für Oma Kühn und Tante Annelies

Table of Contents

Table of Contents……iv

1 Introduction 1
1.1 Developmental origins of health and disease 1
1.2 Prenatal stress as a challenge to maternal immune adaptation to pregnancy 1
1.3 Maternal prenatal stress and DOHaD: potential mediators and mechanisms 2
1.4 Maternal microchimerism 4
1.5 Ontogeny of the fetal immune system 10
1.6 Fetal bone marrow as hematopoietic niche during late gestation 11
1.7 Objectives of this thesis 13
2 Material and Methods 14
2.1 Material 14
2.2 Methods 19
2.2.1 Mice and timed pregnancy 19
2.2.2 Tissue isolation and generation of single cell suspensions 20
2.2.3 Flow cytometry 21
2.2.4 Histological analysis of the placenta 23
2.2.5 Experimental design: Prenatal stress challenge 24
2.2.6 Fluorescence-activated cell sorting 24
2.2.7 *Ex vivo* co-culture 25
2.2.8 Statistical analyses 28
2.2.9 Study approval 28
3 Results 29
3.1 Combined expression of congenic CD45.2 and major histocompatibility complex I H-2D^{b} allow for advanced identification of gestational maternal microchimerism by flow cytometry 29
3.2 Maternal microchimeric cells are present in various fetal tissues during late gestation 30
3.3 Maternal microchimeric T cells in the fetal bone marrow express cell adhesion molecule ligands 34
3.4 The feto-maternal interface expresses cell adhesion molecules in the cellular linings of labyrinthine blood spaces 34
3.5 A mid-gestational stress challenge hampers maternal pregnancy-associated weight increase but does not affect late gestational fetal weight and placental histomorphometry 36
3.6 A mid-gestational maternal stress challenge affects frequency of maternal microchimerism in gd 18.5 fetal bone marrow 37
3.7 Maternal microchimeric cells promote differentiation of fetal hematopoietic stem and progenitor cells of the fetal bone marrow *in vitro* 40
3.8 A mid-gestational stress challenge results in a reduced frequency of late-gestational fetal innate immune cells in the bone marrow 42

4 Discussion 43
5 Abstract 60
6 Zusammenfassung 61
7 Abbreviations 62
8 References 64
9 Acknowledgements 77
10 Publications 79
11 Affidavit 800

1 Introduction

1.1 Developmental origins of health and disease

Epidemiological data indicate a strong increase in the incidence of chronic non-communicable diseases, including cancer, diabetes, cardiovascular, and allergic diseases, as well as auto-immune diseases, in the last century of the previous millennium, now accounting for more than 60% of deaths worldwide (WHO, 2011, Bach, 2002, Lundback et al., 2016). Environmental conditions, in particular a Western lifestyle, which is accompanied by urbanization, migration, improved hygiene, nutritional changes and a sedentary activity level, are assumed to underlie these developments rather than improved diagnosis or mere genetic predisposition (Gluckman and Hanson, 2004, Hanson and Gluckman, 2014, Okada et al., 2010).

It is recognized that early life environmental influences during the perinatal period could have long-term consequences for the risk of developing chronic diseases in postnatal life (Hanson and Gluckman, 2014), a concept summarized as “fetal programming” or the “developmental origins of health and disease” (DOHaD). Unraveling the underlying mechanisms that link adverse early life circumstances with a higher susceptibility to chronic immune diseases in later life has implications for understanding causes of the increasingly emerging onset of immune diseases, and, ultimately, for developing potential preventive measures and treatment options.

1.2 Prenatal stress as a challenge to maternal adaptation to pregnancy

Pregnancy is an exceptional immunological circumstance. The semi-allogeneic fetus expresses paternal and thus, from the maternal point of view, foreign antigens which require to be tolerated to avoid rejection by the immune system while the fetus needs to be nourished at the same time. The maternal immune system actively responds to fetal antigen and is capable to adapt both local and systemic immune responses to pregnancy (Arck and Hecher, 2013). This adaptation can be challenged with consequences for growth and development of the fetus. Failure or insufficiency in immune adaptation can result in fetal loss, or pregnancy complications such as pre-eclampsia and pre-term birth, which are often accompanied by poor fetal development. These complications can manifest in low birth weight (Hamilton et al., 2015), which in turn is a risk factor for developing chronic diseases like coronary heart disease, stroke, type 2 diabetes mellitus, adiposity, the metabolic syndrome, and osteoporosis later in life (Barker, 2006, Gluckman et al., 2008, Smith and Ryckman, 2015). Exogenous challenges to the maternal immune adaptation to pregnancy include medication such as use of acetaminophen (Thiele et al., 2015), infectious viral diseases such as Influenza A virus infection (Gabriel and Arck, 2014, Jamieson et al., 2009, Van Kerkhove et al., 2011), possibly Vitamin D deficiency (Brannon, 2012, Fried et al., 2016), as well as overnutrition (Frias et al., 2011, Lumeng and Saltiel, 2011).

Additionally, a maternal stress burden during pregnancy has been proposed to disturb immune adaptation and thereby account for pregnancy complications (Arck et al., 2008, Nepomnaschy et al., 2004, Nepomnaschy et al., 2006) and poor fetal development. In humans, maternal stress has been

correlated with a decreased gestational length and birth weight (Wadhwa, 2005), or intra-uterine growth (Precht et al., 2007), an effect mirrored in mouse models of prenatal stress (Solano et al., 2015). Epidemiological data indicate a link of a prenatal stress exposure with a child's physiology and predisposition to postnatal diseases, such as mood disorders, depression or schizophrenia (Kinney et al., 2010, Kinsella and Monk, 2009), autism (Walder et al., 2014), cardiovascular diseases (Cottrell and Seckl, 2009, Leduc et al., 2010), cancer (Bermejo et al., 2007), and autoimmune type 1 diabetes (Virk et al., 2010). This may be mediated by an activated stress response, which is accompanied by increased levels of glucocorticoid hormones, adding to the physiological pregnancy-associated hypercortisolism. This response not only exerts its effects on metabolic and adrenergic circuits but also influences the maternal immune system through immune-endocrine interactions (Glaser and Kiecolt-Glaser, 2005).

Negative repercussions of prenatal stress on the developing fetal immune system may program the offspring's immunity and impact the risk to develop chronic immune diseases later in life (Veru et al., 2015, Veru et al., 2014). Indeed, in human birth cohorts prenatal stress exposure was associated with the later development of allergic diseases like asthma, eczema, and atopic dermatitis (Andersson et al., 2016a, Fang et al., 2011, Pincus et al., 2010, Sausenthaler et al., 2009, von Hertzen, 2002, Wright, 2007). Evidence in humans shows that maternal anxiety during late pregnancy was associated with asthma in children at 7 years of age (Cookson et al., 2009). Similarly, the likelihood to develop asthma and eczema at 14 years of age was significantly increased in Australian children of mothers who had experienced adverse life events during the second half of gestation irrespective of maternal genetic predisposition (Hartwig et al., 2014). Similarly, maternal stress perception during pregnancy was associated with wheezing in children in Mexico (Rosa et al., 2016), USA (Lee et al., 2016), and with wheezing, asthma, eczema or allergic rhinitis in Italian children (de Marco et al., 2012).

Stress-induced persistent effects on immunity have been linked with the onset of immune diseases in experimental settings. In adult offspring, airway hyperresponsiveness in mice (Pincus-Knackstedt et al., 2006) and leukocyte infiltration in rats (Nogueira et al., 1999) in response to allergen sensitization was increased after a prenatal stress exposure. This indicates an increased vulnerability to airway inflammation, linking the epidemiological observations of a higher risk to postnatally develop immune diseases when conditioned during early life.

1.3 Maternal prenatal stress and DOHaD: potential mediators and mechanisms

Underlying mechanisms and mediators that directly link maternal conditions with fetal development and long-term health consequences remain a matter of intense research (Hanson and Gluckman, 2014). An impaired functional integrity of the feto-maternal interface, hormone level alterations, and epigenetic modifications have been proposed, as described in the following.

Maternal prenatal stress perception alters the maternal immune adaptation at the murine feto-maternal interface, including an increased secretion of pro-inflammatory cytokines (Blois et al., 2004), an enhanced migration of inflammatory cells into the feto-maternal interface (Blois et al., 2005), decreased levels of regulatory T (Treg) cells, Galectin-1 (Blois et al., 2007, Blois et al., 2004), as well as $CD8^+CD122^+$ suppressor T cells, concomitant with an increased cytotoxic CD8 T cell activity

(Solano et al., 2015). Progesterone is crucial for pregnancy initiation and essentially involved in maintenance of pregnancy-protective immune response regulation (Arck et al., 2008, Szekeres-Bartho et al., 2005). Stress exposure decreased levels of serum progesterone in mammalian pregnancies (Arck et al., 2008, Joachim et al., 2003, Solano et al., 2015).

These stress-related alterations point towards a decreased tolerogenic and rather inflammatory maternal state at the feto-maternal interface, a condition emerging concomitantly with an excess secretion and availability of glucocorticoids through activation of the maternal hypothalamic-pituitary-adrenocortical (HPA) axis in response to stress. Although glucocorticoids contribute to fetal development in a gestational age- and concentration-specific manner (Fowden and Forhead, 2015, Rog-Zielinska et al., 2014), an excess of fetal glucocorticoid exposure due to their excessive transplacental transfer has been shown to link maternal stress responses to an impaired fetal growth in human and murine pregnancies (Seckl and Meaney, 2004, Wyrwoll et al., 2009). Reduced activity of the enzyme 11β-hydroxysteroid dehydrogenase type 2 which converts maternal glucocorticoids to their inactive form in the placenta is shown to be involved in an increased prenatal stress signaling to the fetus (Seckl and Holmes, 2007, Solano et al., 2015). Increased glucocorticoid levels act on the developing endocrine systems, alter the expression of several growth factors and their hormone receptors, and result in programming of e.g. the HPA axis responsiveness towards stress signaling in rodent and non-human primate offspring (Fowden and Forhead, 2015, Jellyman et al., 2015, Kofman, 2002, Maccari et al., 2003, Moisiadis and Matthews, 2014, Welberg and Seckl, 2001). An increased passage of glucocorticoids into the fetus might also influence the fetal immune system directly by binding to glucocorticoid-sensitive hematopoietic fetal cells that express glucocorticoid and mineralocorticoid receptors, thereby potentially affecting their immediate immune function (Coutinho and Chapman, 2011, Speirs et al., 2004).

Additionally, epigenetic modifications have been suggested as key mediators of long-term programming. Epigenetic mechanisms provide the molecular evidence of the impact adverse developmental conditions can have on priming subsequent disease (Hanson and Gluckman, 2014, Nistala et al., 2011, Whitelaw and Whitelaw, 2006). These include DNA methylation, histone modification, and non-coding RNA processes (Meaney and Ferguson-Smith, 2010). A link between epigenetic modifications in the offspring and previous pathological metabolic conditions during human pregnancy has been reported (Lillycrop, 2011). While, inevitably, the established epigenetic correlations between pre- and postnatal conditions require confirmation through cause effect relationships (Saffery, 2014), studies aiming to elucidate long-term effects of prenatal exposure to excess glucocorticoids in guinea pigs, mice and humans indicate e.g. CpG residue methylation and thus gene silencing in key regulatory genes, altered expression of DNA methyltransferases, and differential binding of promoter response elements (Crudo et al., 2012, Moisiadis and Matthews, 2014, Wyrwoll et al., 2007, Yehuda et al., 2014).

Apart from stress hormones and their interactions with immune, endocrine, and gene-regulatory circuits, another potential mechanism accounting for the fetal origin of a child's immune disease development could be of cellular origin: maternal microchimerism.

1.4 Maternal microchimerism

Microchimerism is defined as the presence of a small amount of genetically distinct cells in an individual. Bi-directional transfer of nucleated cells occurs between mother and child during pregnancy resulting in maternal and fetal microchimerism (Nelson, 2012).

Maternal microchimerism (MMc) is defined as the persistent presence of maternal cells in the organs of the fetus and offspring. MMc cells are acquired during pregnancy and lactation and persist until adulthood. The variety of MMc cell phenotypes, the widespread location of MMc cells in the offspring's organism, and its detection in the context of human auto-immune diseases have raised interest in its short- and long-term functional role for the offspring. To date, the importance of MMc in humans has been discussed in two rather contradictory settings: beneficial effects of MMc have been described e.g. in tolerance induction in allograft transplantation and a re-inforcement of maternal reproductive fitness; while conversely, MMc can have disadvantageous effects such as in priming of the child's immune response against allo-antigens, possibly pre-disposing to auto-immunity.

1.4.1 Advantages for the child's health - Induction of tolerance toward non-inherited maternal antigens

Microchimerism-induced, non-inherited maternal antigen (NIMA)-specific tolerance is evident in allograft transplantation, as recipients transplanted with maternal tissue experienced a decreased acute rejection rate, graft failure after 6 months, and graft-versus-host disease as well as markedly improved long-term (5 and 10 year) allograft survival in comparison to those transplanted with paternal tissue (Burlingham et al., 1998, Ichinohe et al., 2005, Joo et al., 2013, Nijagal et al., 2012, van Rood et al., 2002). Further functional consequences are recognized in transfusion-dependent individuals that are broadly exposed to foreign human leukocyte antigen (HLA) alleles. These patients were found to selectively lack antibodies with specificity to NIMA, but frequently carried antibodies against non-inherited paternal antigens (NIPA) HLA haplotypes (Claas et al., 1988).

Thus, exposure to genetically foreign maternal tissue beginning in utero primes tolerance to immunologically discordant NIMA in the offspring (Dutta and Burlingham, 2011, Eikmans et al., 2014, Kinder et al., 2015b). This tolerance is mediated by the suppression of cytotoxic T cell responses (Akiyama et al., 2011, Bonilla et al., 2006) and the expansion of immune suppressive Tregs with NIMA specificity, the latter establishing during human and murine pregnancy (Mold et al., 2008, Molitor-Dart et al., 2007) and persisting until mouse adulthood (Kinder et al., 2015a).

The advantageous effects of prenatal NIMA exposure with regard to transplantation outcome in humans have been confirmed by studies in a mouse model that employs major histocompatibility complex (MHC) class I H-$2^{b/b}$ offspring that was exposed to non-inherited maternal MHC H-2^{d} allo-antigens via MMc cell trafficking. This NIMA-exposed offspring accepts fully allogeneic (H-$2^{d/d}$) heart grafts (Andrassy et al., 2003, Dutta and Burlingham, 2011, Molitor-Dart et al., 2007), showed extended survival of semi-allogeneic skin allografts (Andrassy et al., 2003, Zhang and Miller, 1993), and reduced graft-versus-host disease in bone marrow transplantation (Matsuoka et al., 2006).

1.4.2 Disadvantages for the child's health - A predisposing role in auto-immunity?

The engraftment of semi-allogeneic MMc cells in the offspring's organs may in contrast to tolerance induction sensitize the developing offspring's immune system and may influence the offspring's predisposition to auto-immune diseases (Leveque and Khosrotehrani, 2011). Higher prevalence or frequencies of MMc were associated with various human auto-immune diseases, such as type I diabetes, neonatal lupus syndrome, scleroderma, juvenile myopathies and dermatomyositis (Artlett et al., 2001, Khosrotehrani et al., 2006, Lambert et al., 2004, Nelson et al., 2007, Reed et al., 2004, Stevens et al., 2003, Vanzyl et al., 2010, Ye et al., 2014b). In human type 1 diabetes, for example, MMc cells are present at an increased frequency in intact β cell islets of juvenile patients (Nelson et al., 2007, Vanzyl et al., 2010, Ye et al., 2014a). Mouse models of MMc in auto-immunity (Lopez-Guisa et al., 2011, Roy et al., 2011) or allo-reactivity (Leveque et al., 2014) provide hints that MMc cells could be an integral part of auto-immune pathogenesis. However, MMc cells do not appear to be the effectors mediating the inflammatory process and direct evidence of their role in the onset and progression of different auto-immune responses remains elusive (Stevens, 2016, Ye et al., 2014a).

1.4.3 Techniques of MMc cell detection

Research into the clinical relevance of human MMc (Jeanty et al., 2014, Nelson, 2012) is essentially complemented by data from animal models. In humans, MMc cells are distinguished from offspring's cells using the HLA haplotype alleles, which differ between mother and child. The HLA haplotype is identified by different polymerase chain reaction (PCR)-based methodologies or fluorescence-activated cell sorting (Mold et al., 2008). Alternatively, sex chromosome-based fluorescence in situ hybridization (FISH) has been used to identify single maternal cells in offspring's tissues (Eikmans et al., 2014). Immunofluorescence- and PCR-based approaches can be combined to verify the genetic identity of single microchimeric cells (Kroneis et al., 2010).

In contrast, mouse models offer a broader spectrum of markers and methods that permit the detection of a low amount of MMc cells among a high quantity of offspring's cells. During the last 20 years, various models have been designed to generate detectable genetic differences between mother and fetus. Allogeneic transfer of blastocysts into pseudo-pregnant foster dams was employed, that differed from the blastocysts with regard to their expression of maternal-specific markers which can be detected in the fetus. These include the H-2 genes, which comprise the MHC class I homologous to HLA in humans (Shimamura et al., 1994), their I-E genes, comprising MHC class II (Shimamura et al., 1994), or reporter transgenes such as LacZ (Marleau et al., 2003, Piotrowski and Croy, 1996). Following a similar approach, other models employed the adoptive transfer of cells, e.g. of human origin or radioactively labeled (Chen et al., 2008, Wienecke et al., 2012), into the pregnant female. Despite enabling the detection of MMc cells in the fetus, these experimental approaches significantly intervene with pregnancy, potentially affecting the appearance of MMc *per se*. New mating strategies to overcome this limitation employ mating of mice with differing H-2 genes (Kinder et al., 2015a), different leukocyte marker CD45 subtypes (CD45.1, CD45.2) (Nijagal et al., 2011, Roy et al., 2011), or mice that differ in the expression of transgenic markers (e.g. enhanced green fluorescent protein (eGFP)) (Dutta and Burlingham, 2009, Dutta and Burlingham, 2011, Lopez-Guisa et al., 2011, Vernochet et al., 2005, Vernochet et al., 2007, Zhou et al., 2000).

The experimental model selected must be accompanied by an appropriate detection method. The detection of MMc cells in a fetus or neonate is limited by the amount of cells that can be obtained from an organ or tissue at a certain early stage of life. The detection methods most frequently used in the context of MMc are microscopy-based methodologies, flow cytometry, and PCR. Microscopy-based methodologies include fluorescence microscopy or immunohistochemistry, which provide information on the MMc cell morphology and tissue localization. For example, CD1 fetuses obtained from blastocyst transfer into LacZ-transgenic dams showed positive signals following X-Gal staining (Piotrowski and Croy, 1996). Compared with PCR-based methods or flow cytometry, microscopy-based methodologies provide mostly semi-quantitative information, which relies on the selected sections, visual fields or areas analyzed. Quantitative PCR (qPCR) can be employed to genetically detect and quantify the occurrence of MMc cells in the organs of the offspring. To date, established protocols allow discrimination of the H-2 haplotype and the presence of the eGFP gene (Dutta and Burlingham, 2009, Dutta and Burlingham, 2011, Kinder et al., 2015a). In contrast, a genetic approach to identifying the CD45 subtype is not yet available, as CD45.1 and CD45.2 differ in only 12 nucleotides (Zebedee et al., 1991). The limitation of a DNA-based method is, however, that target detection, although quantified as genomic equivalents per fetal cells, does not allow phenotypical or functional characterization of MMc. This limitation can be overcome by flow cytometry, which detects cell surface antigens. Flow cytometry allows not only the identification, but also the characterization of the cellular identity of MMc cells, which is fundamental to understanding their possible role in fetal organs during gestation. EGFP and CD45 have both been recognized to be reliable markers in initial MMc detection by flow cytometry. A comparative sensitivity analysis of our group revealed equal detection limits for CD45 and eGFP (Thiele et al., 2014). The fluorescence of eGFP requires no cofactors for its detection, while CD45.1 and CD45.2 can be reliably distinguished using specific fluorochrome-conjugated antibodies.

To date, an experimental model has not been described that relies on naturally occurring genetic variation between mouse strains to avoid experimental interference with pregnancy progression and concomitantly allows to detecting and at the same time phenotyping MMc cells with high specificity.

1.4.4 Transplacental route of MMc cell migration

Although microchimerism is assumed to originate from transplacental trafficking, the mechanisms by which cells cross the feto-maternal interface in the mouse and in humans are unclear. During the establishment of blood circulation in the murine placenta at mid-gestation, maternal cells progressively invade the labyrinthine zone starting at gestational day (gd) 13 to reach its maximum between gd 17 and 19 (Vernochet et al., 2007). These cells have been observed to spread across the zone of materno-fetal blood exchange, which could facilitate the traffic through the placenta (Unno et al., 2010, Vernochet et al., 2007) in a process of transplacental migration (Figure 1).

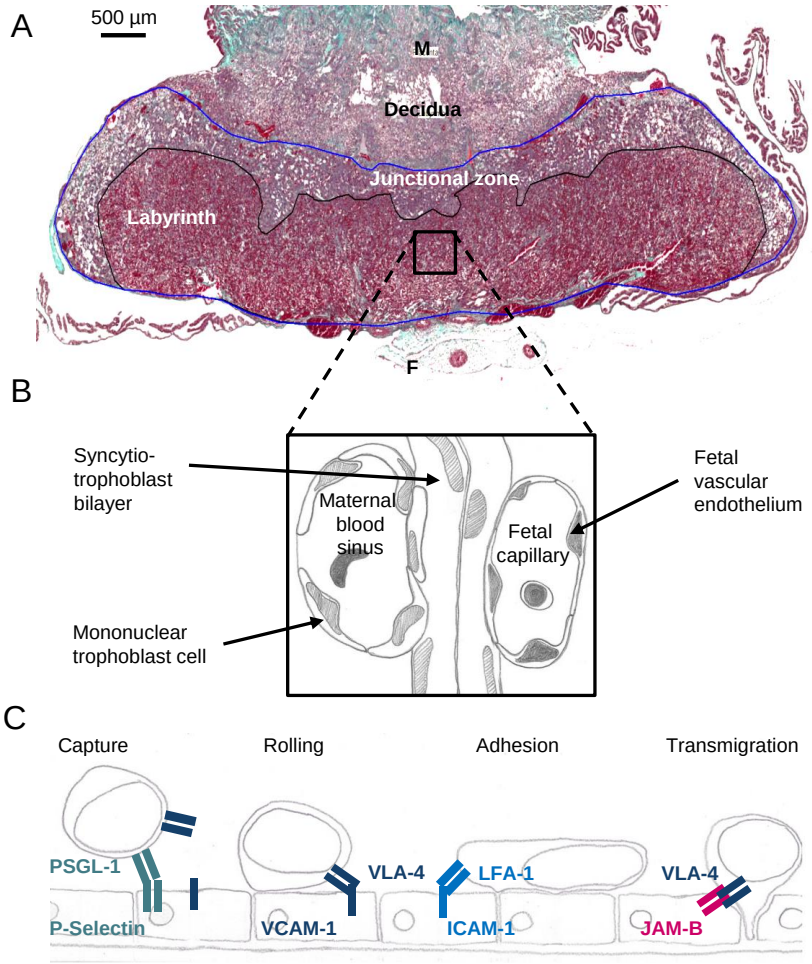

Figure 1: *Transplacental migration across the trophoblast-endothelial barrier.* In order to reach the fetal circulation, maternal cells at the feto-maternal interface (A) in maternal blood sinuses have to cross the mononuclear trophoblast cell layer, two layers of multinucleated syncytiotrophoblast, and, after traveling the labyrinthine stroma, migrate through the basal membrane and fetal endothelial cell layer (Rossant and Cross, 2001) in the blood spaces of the placental labyrinth (B) in a process of transendothelial migration. (C) Labyrinthine cell layers are hypothesized to express the necessary molecular machinery, including molecules for cell capture (Selectins), rolling (vascular cell adhesion molecule (VCAM)-1), adhesion (intercellular adhesion molecule (ICAM)-1), and transmigration (e.g. Junctional adhesion molecule (JAM)-B). The reciprocal expression of cell adhesion molecule ligands such as P-Selectin glycoprotein ligand (PSGL)-1, very late antigen (VLA)-4, and lymphocyte function-associated antigen (LFA)-1 on the MMc cells is a prerequisite to allow for placental transfer. M: maternal side; F: fetal side.

It has been hypothesized that the process of cell adhesion and transmigration of leukocytes in the placenta is similar to that seen at the blood–brain barrier (Dawe et al., 2007). Whilst in humans the expression of cellular adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 that could mediate cell traffic has been addressed by some studies (Cartwright and Balarajah, 2005, Dye et al., 2001, Xiao et al., 1997), in mice it has been less frequently investigated. For early capture, a unique E-selectin gene expression pattern has been attributed to the trophoblast (Milstone et al., 2000), which also expresses P-selectin (Fernekorn et al., 2007). During rolling, VCAM-1 slows down velocity of cells in the blood stream, preparing for ICAM-1-mediated firm adhesion. Explicit evidence of expression in the murine labyrinth for both VCAM-1 and ICAM-1 is lacking, still, the human evidence as well as their involvement in leukocyte recruitment in the murine decidua basalis (Blois et al., 2005, Kruse et al., 2002, Solano et al., 2011, Tometten et al., 2006), suggests them to be candidate molecules participating in MMc migration mechanisms.

Junctional adhesion molecules may play a role in facilitating integrin-mediated MMc cell transendothelial migration (Looman et al., 2007, Prados et al., 2011, Rutland et al., 2007). Among the superfamily of junctional adhesion molecules (JAMs), JAM-B is expressed in early murine gestation (Su et al., 2012). Interestingly, JAM-B is expressed in human placenta (Aurrand-Lions et al., 2002, Johnson-Leger et al., 2002) and its ligand, the integrin complex Very late antigen (VLA)-4, has been shown to be necessary for transplacental migration of surrogate MMc cells (Chen et al., 2008). This makes JAM-B a candidate protein which could be involved in MMc transmigrational processes, while its expression remains to be histologically determined in the mid-to late gestational mouse placenta.

Overall, to date, it remains hypothetical and thus requires investigation whether MMc migration could occur via the transplacental route.

1.4.5 Tissue distribution, kinetics and phenotype of pre- and postnatal MMc cells

As observed in humans, murine MMc cells can be detected in multiple fetal, neonatal, and adult organs, such as peripheral blood, lymphoid organs, but also non-lymphoid tissues (Stelzer et al., 2015).

There is compelling evidence that in mice MMc originates as early as mid-gestation upon the establishment of the blood circulation in the hemochorial placenta (Marleau et al., 2003, Rossant and Cross, 2001). From gd 12.5, MMc cells are found in the fetal thymus, although only in immune-deficient offspring, and in peripheral blood. On gd 13.5 MMc can also be detected in the liver, which is the major hematopoietic organ at that gestational age (Vernochet et al., 2005). In parallel with the rapid build-up of the prenatal immune system, MMc appears to become a widespread phenomenon in late murine pregnancy. Although Marleau et al. (2003) did not find any MMc cells present in whole fetal tissues until gd 15.5, on gd 16.5, they observed abundant MMc in bone marrow and less prominently in the liver. Increased levels of radioactivity accumulated in gd 19 fetuses compared with gd 15 upon adoptive transfer of radioactive cells into the maternal circulation (Wienecke et al., 2012). In contrast, the frequency of MMc cells among fetal blood cells decreased from gd 13.5 – 14.5 to late gestation and neonatal life (Nijagal et al., 2011). This relative decrease could be explained by increased fetal blood cellularity due to the progressing hematopoiesis or by the homing of MMc cells into the fetal organs. Indeed, in late gestation, MMc cells were more prevalently found in the liver, bone marrow, and spleen, followed by the thymus and also the lung (Piotrowski and Croy, 1996, Unno et al., 2010, Zhou et al., 2000).

In neonatal life, MMc has a similar distribution to that observed during late gestation. Still, in the early postnatal period, a modulation of the MMc level is apparent (Su et al., 2008). This may reflect the dynamic transition of the prenatal immune system, which might be more tolerogenic toward MMc, to the adult immunogenic program (Burt, 2013, Dakic et al., 2004), which could be accompanied by the partial deletion of semi-allogeneic MMc cells. The hematopoietic organs containing maternal cells were shown to continually increase until young adulthood (Vernochet et al., 2005). In young adults, maternal cells are commonly found in the bone marrow, thymus (Vernochet et al., 2005), and in blood (Araki et al., 2010) and lymph nodes (Dutta and Burlingham, 2009). Even up to 12 weeks of age, MMc is present in the bone marrow and at variable levels in the spleen of the offspring (Marleau et al., 2003). In non-lymphoid organs such as the brain, heart, skin, small intestine and kidney, MMc is more

evident in early postnatal life, when it is also more frequently analyzed in these organs (Leveque et al., 2014, Su et al., 2008). Still, in neonatal and young offspring (postnatal day (pnd) 0 – 45), the level of MMc in the brain correlated with the level of MMc found in lymphoid organs (Kaplan and Land, 2005) and the heart, liver, lung, brain, small intestine, and kidney remained sites of MMc in young adults (Dutta and Burlingham, 2009). This indicates a widespread peripheral distribution of MMc that is not restricted to the lymphoid compartment. At 6 – 8 weeks of age, though, the frequency of MMc per organ was decreased compared with pnd 1 offspring (Dutta and Burlingham, 2009), further supporting the association between the persistence of MMc and the maturation of the offspring's immune system. Importantly, during prenatal and early postnatal life, MMc is present in the liver, bone marrow, thymus, and later in the spleen (Godin et al., 1995), at a time at which these organs are prominent sites of immune ontogeny.

In order to address a potential role of MMc in the progeny it is necessary to consider the phenotype of MMc cells. Evidence for the transfer of MMc via transplacental migration from the maternal circulation (Vernochet et al., 2007) implies that MMc cells belong to the maternal blood pool, and hence most likely to the hematopoietic lineage. It is noteworthy that the MMc cell phenotype differs among the host organs, suggesting that MMc cells are either of heterogeneous nature and/or have the capacity to differentiate locally. As early as gd 12.5 – 15.5, MMc cells from hematopoietic cell lineages can be found in fetal lymphoid organs (Nijagal et al., 2011, Vernochet et al., 2005), where they can also be observed later in gestation and in postnatal life (Stelzer et al., 2015).

Adding to the significance of MMc establishment and persistence in fetal organs, a non-hematopoietic mesenchymal stem cell fraction also showed a weak signal for maternal DNA after *in vitro* expansion of adult mice bone marrow (Dutta and Burlingham, 2010). Thus, a MMc stem cell pool could be the source of maternal cells residing in non-hematopoietic tissue, such as the MMc thymic epithelial cells (Dutta and Burlingham, 2009), renal proximal tubular and endothelial cells (Lopez-Guisa et al., 2011), and cardiomyocytes, endothelial, and smooth muscle cells, contained in the Lineage $(Lin)^{neg}c\text{-}Kit^{neg}$cardiac compartment (Dutta and Burlingham, 2009, Dutta and Burlingham, 2010).

In summary, the widespread distribution of MMc in lymphoid and non-lymphoid organs, but especially their immune phenotype, suggests that MMc cells might participate in the development of the offspring's immune system. Although several studies have shown the presence of MMc in fetal organs, a comparative investigation of MMc frequency in various fetal tissues and the concomitant elucidation of its phenotype during late murine gestation remains elusive.

1.4.6 Factors modulating MMc

To date, highly variable prevalence and levels of murine MMc were reported among different experimental set-ups. Differences may be enhanced by the diverse detection approaches, which render the data from separate studies difficult to contrast. Interestingly, a large variability in the frequency of MMc is also observed in different cohorts of human studies (reviewed by Nelson (2012)) and in rhesus monkeys (Bakkour et al., 2014), supporting the idea that a disparity among individuals is inherent to MMc.

Histo(in)compatibility: The observed disparity may in fact be due to human materno-fetal histocompatibility, which has been shown to intrinsically influence the level of MMc (Berry et al., 2004). In mice, too, the engraftment of MMc cells in lymphoid organs was facilitated to a greater extent in syngeneic offspring compared with allogeneic and outbred offspring (Lopez-Guisa et al., 2011, Marleau et al., 2003, Piotrowski and Croy, 1996, Vernochet et al., 2005). In a different study, allogenicity and H-2 heterozygosity decreased MMc in the brain and in pooled lymphoid organs (Kaplan and Land, 2005).

Immune deficiency: In immune-deficient offspring, the level of MMc was higher in both lymphoid and non-lymphoid organs compared with immune-competent offspring (Piotrowski and Croy, 1996, Zhou et al., 2000). Thus, the lower the offspring's immune response against maternal antigens, the higher the level of MMc. This supports the concept that the offspring's immune system can react against maternal semi-alloantigens and confine the passage, engraftment, and survival of MMc. In line with this, in human infants suffering from severe combined immunodeficiency (SCID), maternal cell engraftment is commonly recognized (Liu et al., 2016, Touzot et al., 2012). This nurtures the idea that MMc cells might be capable of substituting for functional immune deficits in the offspring.

Fetal injury, intervention, and inflammation: The occurrence of MMc is also subject to exogenous modulation. Indeed, intrauterine non-specific tissue injury appears to enhance the transfer of MMc cells or their survival in the fetal organism. In a case of in utero intervention, maternal fetal-specific T cells accumulated in the uterus and its draining lymph nodes (Wegorzewska et al., 2014) seeming to have sensed the ongoing fetal inflammation. Maternal cell trafficking was increased in in utero sham-injected fetuses compared with unmanipulated offspring (Nijagal et al., 2011), an observation also made in human prenatal surgery (Saadai et al., 2012). MMc was also increased upon pertussis toxin-induced asymptomatic systemic inflammation in pregnant dams (Wienecke et al., 2012). The mechanisms regulating such dynamic changes in MMc are, however, unclear.

Overall, together with the observations of its multiple phenotypes, these observations support the existence of mechanisms that control the selective transfer and persistence of MMc cells. However, besides the induction of fetal maternal-specific Tregs to generate fetal tolerance towards maternal antigens, the physiological meaning of MMc for the host offspring remains unknown. Environmental challenges such as prenatal stress could affect the MMc cell pool that might mirror the maternal immune status not only under healthy, but also under adverse conditions. MMc cells might act as a materno-fetal messenger executing prenatal immune cross-talk from mother to child with an impact on pillars of the innate and adaptive immune system, e.g. by influencing immune development in the liver, spleen, and bone marrow.

1.5 Ontogeny of the fetal immune system

The development of the fetal immune system presents as a sequential trafficking of fetal hematopoietic stem cells (HSC) through hematopoietic sites (Figure 2) and starts with the generation of hematopoietic progenitors in the first weeks of gestation in the human para-aortic splanchnopleura and yolk sac (Mikkola and Orkin, 2006) and in early midgestation (starting at gd 10.5) in mice in the aorto-gonado-mesonephros region (Bertrand et al., 2010, Cumano and Godin, 2007), placenta and

yolk sac (Mikkola and Orkin, 2006, Zovein et al., 2010). Early fetal HSC then migrate into the fetal liver (from 7 weeks of gestation in humans (Holsapple et al., 2003) and gd 12.5 in mice (Kieusseian et al., 2012, Kumaravelu et al., 2002), which remains the main hematopoietic site in the human embryonic and early fetal phase (first and second trimester) (Palmer, 2011) and during mid-gestation in mice (Mikkola and Orkin, 2006). In the murine fetal liver, next to erythroid-myeloid progenitors (EMPs), which transiently give rise to cells of the erythroid and myeloid lineages before definitive HSC-derived hematopoiesis assumes responsibility (Gomez Perdiguero et al., 2015), fetal HSC expand and differentiate into mature HSC (Ema and Nakauchi, 2000, Kieusseian et al., 2012). Seeding of the fetal thymus with fetal liver-derived T cell precursors occurs in waves starting during mid-gestation (Ramond et al., 2014, Solano et al., 2011). Colonization of the fetal bone marrow from gd 15.5 onwards subsequently founds the beginning of definitive hematopoiesis in this compartment at and after gd 16.5, thus during late gestation (Delassus and Cumano, 1996). It is at this time that the first quiescent adult-like HSC are identified (Coskun et al., 2014), while it takes until weaning for HSC proliferation to cease to adult levels (Bowie et al., 2006). In contrast, in human embryos, seeding of the bone marrow by circulating stem cells derived from the fetal liver is initiated already during the first trimester and the bone marrow becomes the main site of hematopoiesis as early as during the second half of the second trimester (Palmer, 2011).

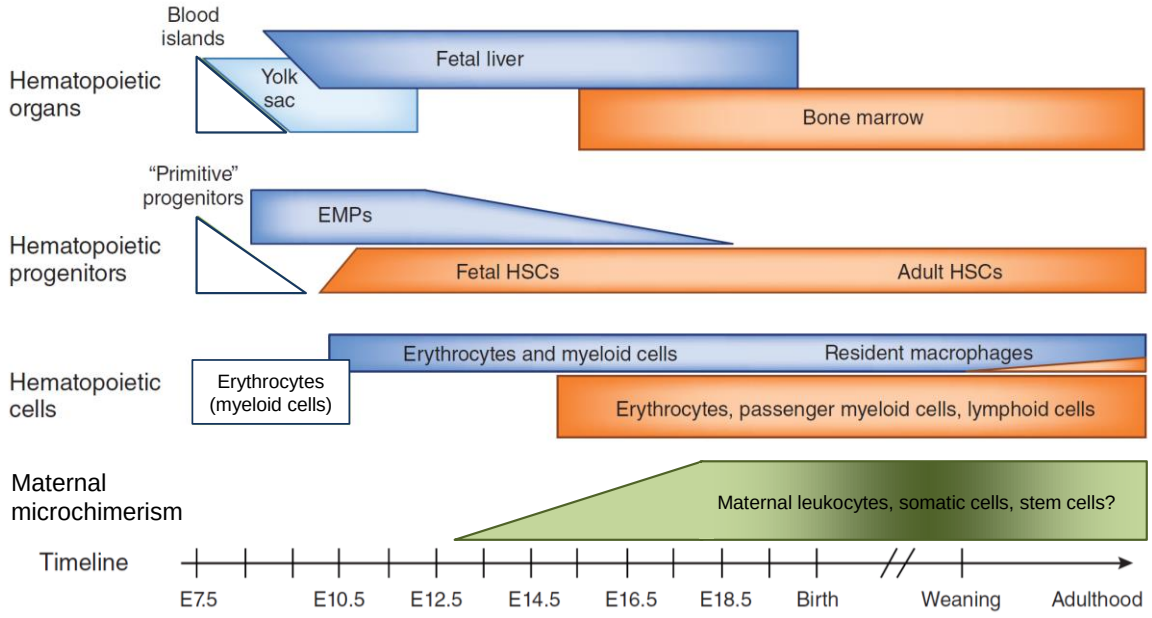

Figure 2: *Murine fetal immune ontogeny is accompanied by the emergence of maternal microchimerism*. The bone marrow as the definitive site of hematopoiesis around birth and during postnatal life emerges during late gestation from progressive shifts of hematopoietic sites and progenitor cells during prenatal development. MMc is first described on gd 13.5 and is increasingly detected in various fetal organs until late gestation. Lactational enrichment could lead to a peripartal peak of MMc prevalence, with persistence of leukocytes, somatic cells and possibly hematopoietic and mesenchymal stem cells until adulthood in women and 6-12 week old young adult mice (Stelzer et al., 2015). EMP: erythroid-myeloid progenitor (adapted by permission from Macmillan Publishers Ltd: Nat Immunol (Perdiguero and Geissmann, 2016)).

1.6 Fetal bone marrow as hematopoietic niche during late gestation

The establishment of the bone marrow as the main site of hematopoiesis during late murine gestation is accompanied by the development of a micro-environment necessary for maintenance of HSC self-renewal potential and differentiation. Bone vascularization and the emergence of a calcified matrix

allow human and murine HSC to interact with mesenchyme-derived stromal cells (Coskun et al., 2014, Holsapple et al., 2003), which maintain the prerequisite conditions for HSC survival and homing (Mazo et al., 2011). Stromal cells such as fibroblasts, osteoblasts, osteoclasts and adipocytes represent the specific niche to which HSC lodge. This niche expresses chemo-attractant signals such as CXC chemokine ligand (CXCL) 12 or stem cell factor (SCF) to attract circulating fetal HSC (Christensen et al., 2004). Binding to their respective receptors CXC chemokine receptor (CXCR) 4 and c-Kit on HSC not only ensures chemotaxis (Nagasawa et al., 1996) but also activates adhesion molecules expressed by HSC such as P-Selectin glycoprotein ligand (PSGL)-1 and integrin complexes such as lymphocyte function-associated antigen (LFA)-1 and VLA-4 to mediate interactions of these adhesion molecules with their respective receptors on the BM microvasculature and stroma e.g. P-Selectin, ICAM-1, and VCAM-1 (Ciriza et al., 2013, Mazo et al., 2011). At the same time, interactions of CXCL12 with CXCR4, VCAM-1 with VLA-4, and SCF with c-Kit ensure long-term stem cell retention by adhesive contacts between stromal cells and HSC which require proteolytic cleavage should HSC mobilization from the niche become necessary.

Owing to the long-term self-renewal potential of HSC, the cascade of hematopoiesis is capable of replenishing all blood cell components throughout an individual's life. Although fetal and adult human HSC are recognized to give rise to distinct progenitor cells, at least with respect to T cells (Mold et al., 2010), the basic process of hematopoiesis itself is assumed to be the same in fetal and adult age.

The mid-to-late gestational period (gd 12.5 – birth) of maturation of HSC in the fetal liver and colonization of the bone marrow by HSC could be a window of vulnerability to challenges such as adverse maternal conditions during pregnancy. This period corresponds to the late first and second trimester human fetal immune ontogeny. It seems plausible that programming of the offspring's peri- and postnatal immunity would be mediated by effects on persistent entities, such as HSCs, rather than on short-lived terminally differentiated cells. Affected precursors with long-term potential would then give rise to differentially primed hematopoietic progenitors in postnatal life.

Overall, while available studies provide evidence for the impact of a prenatal stress challenge on long-term immunity, less is known about immediate stress effects on the cells of the developing immune system of the fetus. These effects could lie at the basis of persistent alterations in the functioning of the offspring's immune response.

1.7 Objectives of this thesis

This thesis intends to investigate maternal microchimerism (MMc) during late murine gestation and its modulation upon a prenatal maternal stress challenge. It is aimed to test the hypothesis that MMc is a mechanism of materno-fetal communication involved in fetal immune development with, if challenged, possible consequences for the child's peri- and postnatal immunity.

In order to address this hypothesis, it is aimed to

1. Advance the detection of MMc cells in fetal tissues by establishment of a mouse mating model that allows identification of MMc by flow cytometry.

2. Detect frequency and phenotype of MMc cells during late gestation in fetal tissues.

3. Investigate adhesion molecule and adhesion molecule ligand expression as prerequisite for the route of transplacental migration of MMc cells.

4. Investigate the modulation of MMc cell frequency and phenotype by a prenatal maternal stress challenge.

5. Investigate a functional role of MMc cells in the fetal bone marrow.

6. Investigate the impact of a midgestational maternal stress challenge on immune cell populations in the fetal bone marrow during late gestation.

2 Material and Methods

2.1 Material

Table 1: *Chemicals*

Chemicals

	Company	Catalog number
Bluing Reagent for hematoxylin stained IHC sections	Ventana	760-2037
Bovine serum albumin (BSA)	Sigma-Aldrich	A7906-100 g
Cell Conditioning Solution, CC1	Ventana	950-124
CellTracker™ fluorescent probe Blue (CTB) CMAC (7-amino-4-chloromethylcoumarin)	Life Technologies	C2110
Collagenase IV	Roche	11088866001
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	4540-500 ml
DNase I	Sigma	D5307
Ethanol (99%), denatured	Th. Geyer	22.945.000
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	03690-100 ml
Eukitt medium	O. Kindler	
Fetal calf serum (FCS)	Gibco	10082147
Formaldehyde solution	Sigma-Aldrich	F1635-500ml
Giemsa's azur eosine-methylene blue solution	Merck	1.092.040.500
Goldner 1 (azophloxine solution)	Sigma	P2395-25G; F8129-25G
Goldner 2 (phosphotungstic acid)	Merck	1.005.320.100
Goldner 3 (light-green SF solution)	Merck	1.159.410.025
Hematoxylin	Ventana	760-2021
L-Glutamine 200mM (100x)	Gibco	25030-024
May Grünwald's eosine-methylene blue solution modified	Merck	1.014.240.500
Normal rat serum (NRS)	Jackson Immuno Research	012-000-120
Orange G solution	Roth	0318.1
Penicillin (10.000 units)/ Streptomycin (10 mg/ml)	Sigma-Aldrich	P4333-100ml
Potassium dihydrogen phosphate ("Weisesche Pufferlösung")	Provided inhouse	
Proteinase K	Roche	760-2018

Red blood cell (RBC) lysis buffer	eBioscience	00-4333
Tissue Tek	Sakura	REF1408
Trypsin (0.25%)/EDTA	Gibco	25200-056
Trypan Blue Stain (0.4%)	Gibco	15250-061
ultraView Universal DAB Detection Kit	Ventana	760-500
Weigert's iron hematoxylin	Waldeck	2E-032; 2E-052
Xylol replacement medium (Xylolersatzmedium (XEM)) HS200	DiaTec	1-019614-1

Table 2: *Plastic and other materials*

Material	Company	Catalog number
Cell strainer (40 µm nylon)	Falcon	352340
Cell culture microplate, 96 well, F-Bottom, µClear, Chimney well, Black, Sterile	Greiner bio-one655090	655090
Microvette 500 K3E; 100 K3E	Sarstedt	20.1341; 20.1278
Polystyrene, round-bottom FACS tube (5 ml)	Falcon	352052
Rodent repellent device	Conrad Electronics, Germany	P7901
Syringe needle 27G x ½'' 0,4x12mm	Braun	4665406
Ultrasound rodent repellent device	Weitech, Belgium	WK0220-E

Table 3: *Media and buffers*

Medium	Components	Company	Catalog number
complete αMEM (c-αMEM)	α Minimum Essential Medium (MEM)	Gibco	22571-020
	20% FCS		
	1% Penicillin/Streptomycin		
complete RPMI (cRPMI)	Roswell Park Memorial Institute (RPMI) 1640	Gibco	21875-034
	10% FCS		
	1% Penicillin/Streptomycin		
	1% L-Glutamine		

FACS buffer	Dulbecco's Phosphate-Buffered Saline (PBS) 0.5% BSA (optional) 2mM EDTA	Gibco	14190-094
IHC buffer	Reaction Buffer (Tris based buffer solution (pH 7.6 ± 0.2))	Ventana	950-300

Table 4: *Mice and cell lines*

Mouse strain	Gender	Company	UKE-internal strain identifier
C57BL/6	female	Charles River	5050
CByJ.SJL(B6)-Ptprca/J	male	Jackson Laboratories, Stock no. 006584	3363

Cell line	Description	Original publication
OP9	mouse stromal bone marrow cells derived from (C57BL/6 x C3H)F2 - op/op	Nakano et al., 1994

Table 5: *Antibodies*

Flow cytometry

Antigen	Conjugated fluorochrome	Clone	Dilution	Company	Catalog number
Basic panel: MMc detection					
CD45.2	APC-Cy7	104	1:100	Biolegend	109824
CD45.1	FITC	A20	1:400	Biolegend	110706
H-2Db	PE	KH95	1:50	Biolegend	111508
H-2Dd	Alexa Fluor 647	34-2-12	1:200	Biolegend	110612
+ Panel 1: MMc cell phenotype - immune cell lineages					
CD3e	PE-Cy7	145-2C11	1:200	Biolegend	100320
CD19	PerCP-Cy5.5	6D5	1:200	Biolegend	115534
CD11c	Alexa Fluor® 700	N418	1:200	Biolegend	117320
CD11b	BV 605	M1/70	1:200	Biolegend	101237
CD335 (NKp46)	eFluor® 450	29A1.4	1:100	eBioscience	48-3351
Fixable viability dye	eFluor® 506	na	1:1000	eBioscience	65-0866

+ Panel 2: MMc cell phenotype - hematopoietic stem cells

Lineage cocktail	Alexa Fluor® 700	CD3 (17A2), Ly-6G/C (RB6-8C5), CD11b (M1/70), CD45R(B220) (RA3-6B2), TER-119 (Ter-119)	1:25	Biolegend	133313
Sca-1	PE-Cy7	D7	1:100	Biolegend	108114
c-Kit (CD117)	BV605	ACK2	1:100	Biolegend	135121
Fixable viability dye	eFluor® 506	na	1:1000	eBioscience	65-0866

+ Panel 3: MMc cell phenotype - cell adhesion molecule ligands

CD3	PE eFluor®610	145-2C11	1:100	eBioscience	61-0031-80
CD45R(B220)	Alexa Fluor 700	RA3-6B2	1:100	eBioscience	56-0452-80
CD11b	Horizon V500	M1/70	1:200	BD Horizon	562128
CD162 (PSGL-1)	BV421	2PH1	1:500	BD Horizon	562805
CD11a (LFA-1)	PerCP eFluor®710	M17/4	1:300	eBioscience	46-0111
CD49d (VLA-4)	PE-Cy7	R1-2	1:300	Biolegend	103617
Zombie yellow™ fixable viability kit	BV570	na	1:500	Biolegend	423103

+ Panel 4: MMc cells and fetal HSPC - fluorescence-activated sorting and post-culture analysis

Lineage detection cocktail	Biotinylated	CD5, CD11b, CD45R(B220), Anti-7-4, Anti-Ly6-G/C, TER-119	1:10	Miltenyi Biotec	130-092-613
CD3	BV421	145-2C11	1:100	Biolegend	100335
Sca-1 (Ly-6A/E)	BV711	D7	1:100	Biolegend	108131
c-Kit (CD117)	BV605	ACK2	1:100	Biolegend	135121
Biotin	Streptavidin-BV650	na	1:200	Biolegend	405231
7-AAD viability staining	PerCP-Cy5.5	na	1:300	Biolegend	420404

Fc-receptor blocking, isotype control and compensation beads

	Conjugated fluorochrome	Clone	Dilution	Company	Catalog number
TruStain fcX (anti-mouse CD16/32)	na	93	1:200	Biolegend	101320
CompBeads (anti-rat/anti-hamster Ig κ and negative control compensation particles)	na	na	na	Becton Dickinson	552845
CompBeads (anti-mouse Ig κ and negative control compensation particles)	na	na	na	Becton Dickinson	552843
Isotype control Mouse IgG2b, κ	PE	MPC-11	1:20	Becton Dickinson	559529

Immunohistochemistry

	Conjugated fluorochrome	Clone	Dilution	Company	Catalog number
primary					
Rabbit polyclonal anti-mouse JAM-B	na	na	1:400	abcam	ab139645
Goat polyclonal anti-mouse P-Selectin	na	na	1:500	Santa Cruz	sc-6943
Goat polyclonal anti-mouse VCAM-1	na	na	1:200	R&D systems	AF643
secondary					
Anti-rabbit horse reddish peroxidase (HRP)-conjugate	na	na	undiluted	Histofine	414142F
Anti-goat universal immuno-peroxidase polymer	na	na	undiluted	Histofine	414161F

Table 6: *Equipment and software*

Equipment

	Company
Flow cytometer LSR Fortessa	Becton Dickinson
Flow sorter Aria Fusion	Becton Dickinson
Zeiss ApoTome	Zeiss
Slide scanner Mirax Midi	Zeiss
CO_2 Incubator HeraCell 150!	Thermo Scientific
Benchmark XT	Ventana Medical Systems, Inc.
NanoZoomer 2.0 HT	Hamamatsu Photonics K.K.
Microtome SM2010R	Leica
Cytospin 2	Shandon

Software	**Company**
FACSDiva	Becton Dickinson
Pannoramic Viewer	3DHISTECH
FlowJo Version 9.8 for Mac	TreeStar, Inc.
SPSS Version 20	SPSS, Inc.
Office Windows 2010	Microsoft Inc.
Nano Zoomer Digital Pathology Image	Hamamatsu Photonics K.K.
Prism	GraphPad Software, Inc.

2.2 Methods

2.2.1 Mice and timed pregnancy

Six – eight week-old female C57BL/6J (H-2D^{b}; CD45.2^{+}) and male CByJ.SJL(B6)-Ptprc^{a}/J (H-2D^{d}; CD45.1^{+}) were upon arrival in the animal facility of University Medical Center Hamburg-Eppendorf, allowed to rest for 7 days prior to timed mating (Table 4). Mating was started between 01:00 and 03:00 p.m. for five consecutive days and the presence of a vaginal plug was checked between 07:00 and 09:00 a.m. every morning. The day of an apparent plug was designated as gd 0.5. Pregnancy was confirmed by a 1.5 – 2 g body weight increase relative to gd 0.5 on gd 8.5 and 10.5. Mice were

maintained in the animal facility of University Medical Center Hamburg-Eppendorf with a 12-hour light/12-hour dark cycle. Food and water was provided ad libitum.

2.2.2 Tissue isolation and generation of single cell suspensions

Fetal tissues

Dams were sacrificed on gd 18.5 by cervical dislocation after anesthesia with CO_2. Pregnancy outcome (number of implantations, aborted fetuses, and individual fetal weight) was recorded. Fetuses were sacrificed by immediate decapitation after opening of the uterus and fetal peripheral blood mononuclear cells (PBMC) were collected in an EDTA-treated microvette (up to 100µl per fetus). Each fetus was kept on ice in a PBS-filled well of a 24-well plate on ice. Placentae were fixed in 4% formaldehyde solution in PBS for 24h and transferred to PBS until embedding in paraffin for subsequent immunohistochemistry. Individual fetal tissues (thymus, spleen, lung, liver, hind limbs (femur and tibia)) were harvested using a binocular microscope into cRPMI medium. In case of subsequent culture of fetal bone marrow cells, hind limbs were processed in c-αMEM medium.

Fetal tissues were processed on ice to generate single cell suspensions as follows: Thymi and spleens (pooled per litter) were grinded through a cell strainer (40 µm) into a 50 ml Falcon tube using the rubber edge of a syringe cap, while flushing with 30 ml of cRPMI. Pooled unperfused lung tissue of one litter was cut into small pieces using scissors and enzymatically digested with Collagenase IV (working concentration: 2 mg/ml) and DNase (30U) for 15 min at 37°C at 700-800 rpm in a thermomixer. The cell suspension was passed through a 40µm cell strainer into a 50 ml Falcon tube by flushing with 30 ml PBS supplemented with 2 mM EDTA to stop enzyme activity. Liver tissue per fetus was grinded through a cell strainer (40 µm) on a 3.5 cm Petri dish, while flushing with 5 ml cRPMI. The liver cell suspension was re-filtered through a fresh cell strainer into a 50 ml Falcon tube by flushing with 30 ml of cRPMI. To isolate the bone marrow from both femurs and tibiae of each fetus (fetal bone marrow (BM)), bones were placed onto a 3.5 cm Petri dish with grinding surface, generated by scratching a net into the dish using a syringe needle. Using the plastic edge of a syringe cap, bones were minced to generate a bone marrow cell suspension, which was diluted in 5 ml cRPMI and filtered through a cell strainer (40 µm) into a 50 ml Falcon tube by flushing with 30 ml of cRPMI. Cells in suspension of all processed tissues were pelleted by centrifugation (8 min, 4°C, 1500 rpm). Erythrocyte lysis was required for liver and spleen samples. To this end, cell pellets were resuspended in 2 ml of RBC lysis buffer and incubated for 5 min on ice. Reaction was stopped with 30 ml PBS and cells were re-pelleted by centrifugation (8 min, 4°C, 1500 rpm). To generate single cell suspensions of fetal PBMC, samples were pooled from one litter, diluted in 5 ml of RBC lysis buffer and incubated for 5 min on ice to eliminate erythrocytes. Reaction was stopped with 30 ml PBS and cells pelleted by centrifugation (8 min, 4°C, 1500 rpm). Cell pellets of all tissues were re-suspended in cRPMI and cell counts were manually determined in a Neubauer hemocytometer using Trypan blue solution to exclude dead and dying cells.

Adult tissues

Maternal PBMC were harvested by retroorbital puncture prior to killing in an EDTA-treated microvette (500 – 1000 µl). To eliminate erythrocytes, samples were diluted with 5 ml of RBC lysis buffer and incubated for 5 min on ice. Reaction was stopped with 30 ml PBS and cells pelleted by centrifugation

(8 min, 4°C, 1500 rpm). Maternal and paternal spleen and bone marrow were collected in PBS and used as control tissues during establishment and for quality control of the MMc detection technique. Spleen tissue was grinded through a cell strainer (40 µm) on a 3.5 cm Petri dish while flushing with 5 ml PBS. The cell suspension was re-filtered through a fresh cell strainer into a 50 ml Falcon tube by flushing with 30 ml PBS. Adult femur and tibia were exposed from the hind limbs and cut with a scalpel at each end to flush the bone marrow cavity using a syringe and needle with 5 ml PBS. Bone marrow cells were homogenized by resuspension in PBS. Cells in suspension were pelleted by centrifugation (8 min, 4°C, 1500 rpm) and resuspended in PBS to determine the cell count in a hemocytometer.

2.2.3 Flow cytometry

To identify MMc cells among the bulk offspring's cells in the different fetal organs, extracellular antigens were stained with fluorochrome-conjugated antibodies. $1x10^6$ cells in 50 µl FACS buffer in FACS tubes were initially incubated with NRS (1:100) and TruStain fcX (anti-mouse CD16/32) for 15 min at 4°C on ice to block unspecific FcγRII/III binding. Antibodies directed against CD45.2, CD45.1, H-2D^b, and H-2D^d were used to detect CD45.1^{neg}CD45.2$^{+/+}$ H-2D^{dneg}H-2D$^{b/b}$ MMc cells. To analyze the cell phenotype, cells were additionally stained for CD3e (T cells), CD45R (B220) (B cells), CD19 (B cells), CD11b (myeloid cells), Gr-1 (granulocytes), CD11c (dendritic cells), CD335 (NKp46) (Panel 1, Table 5), or a lineage cocktail, Sca-1, and c-Kit (CD117), identifying HSCs (Panel 2, Table 5). To analyze cell adhesion molecule ligand expression, cells were stained for CD162 (PSGL-1), CD49d (VLA-4), and CD11a (LFA-1) (Panel 3, Table 5). Undiluted antibodies were added in pre-determined dilutions (ranging from 1:50 – 1:400, Table 5) to the staining tube without washing after Fc blocking and incubated for 30 min at 4°C on ice in the dark. Samples were washed twice in 600 µl PBS by centrifugation (8 min, 4°C, 1500 rpm) to remove unbound antibody and soluble proteins. Supernatant was discarded and cell viability staining was performed using either fixable viability dye or Zombie Yellow™ fixable viability kit for 30 min at 4°C on ice in the dark. Ultimately, cells were washed in 600 µl FACS buffer and re-suspended in 300 µl FACS buffer. At least $0.2x10^6$ events in the forward (FSC) vs. sideward (SSC) scatter leukocyte gate were acquired using a LSR Fortessa flow cytometer. Data were analyzed using FlowJo software. Doublet cells and dead cells were excluded from the analysis. Fluorescence minus one (FMO) controls, which contain all antibodies of the panel except one, were added to set gating boundaries of cell populations positive for the respective missing antibody (Figure 3 A). During establishment, an IgG2b, κ isotype control for PE H-2D^b was included to confirm specificity of MMc staining (Figure 3 B).

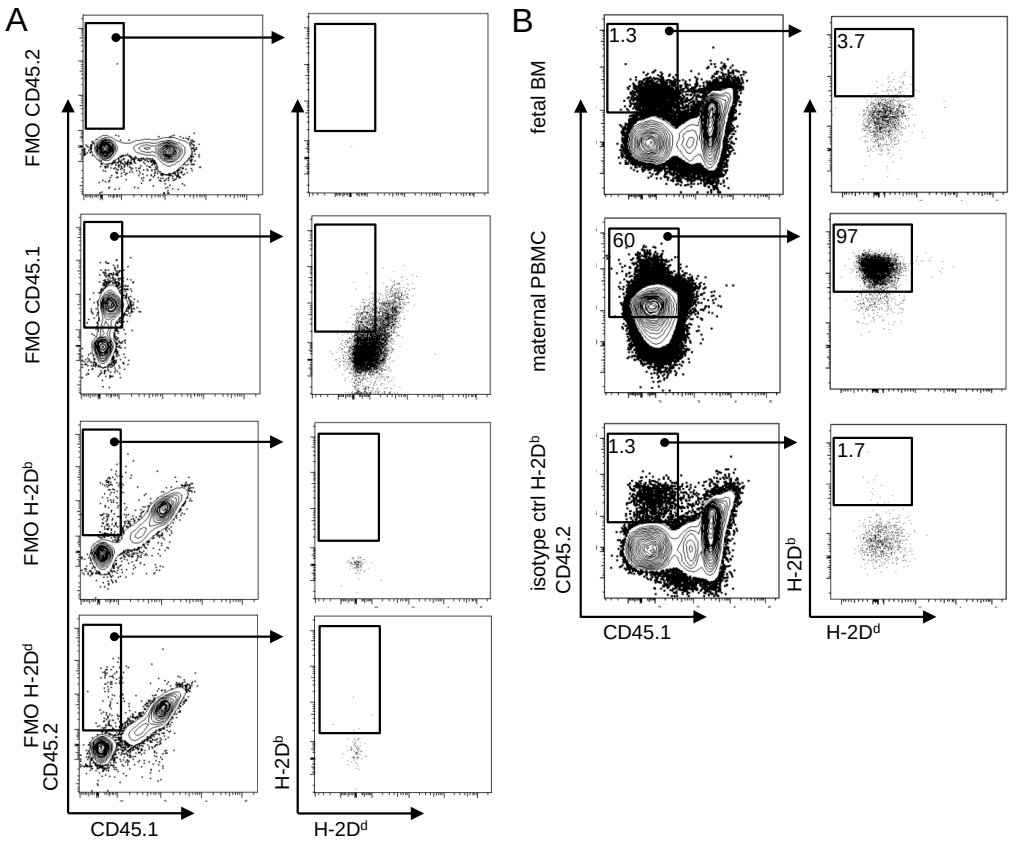

Figure 3: *Representative dot plots of control stainings of the maternal microchimeric (MMc) cell gating strategy.* (A) Fetal bone marrow (BM) (gd 18.5) fluorescence minus one (FMO) controls of the MMc detection markers. (B) Fetal BM and maternal peripheral mononuclear cells (PBMC) stained for the MMc detection markers including PE H-2D^b (clone: KH95) and fetal BM isotype-control with PE IgG2b, κ isotype antibody (clone: MCP-11) staining. Numbers represent cell frequencies of parent gate.

Compensation of spectral overlap

Compensation of spectral overlap between the fluorochromes employed in the respective FACS staining panels was set up prior to experimental analyses using compensation beads. For each antibody used in the panel, single stained samples were prepared by adding the antibody to one drop of anti-mouse/rat/hamster Ig Kappa (κ) beads diluted in 100 µl FACS buffer, corresponding to the host in which the antibody had been generated. Antibody volumes were determined empirically and ranged from 0.1 to 1.25 µl in 100 µl. A single stained cell viability sample was generated using an aliquot of 1x10^6 experimental fetal BM cells, of which half were killed prior to staining at 65°C for 1 min. Antibody-to-bead coupling was performed by incubating for 20 min at room temperature (RT) in the dark, followed by wash in 750 µl PBS by centrifugation (5 min, RT, 1500 rpm), and re-suspension in FACS buffer. In addition to unstained Ig κ beads as control, unstained cells served as controls for the auto-fluorescence of the experimental samples. Samples were acquired in the LSR Fortessa in the Compensation Setup mode of FACSDiva software. The automatically calculated compensation values were re-evaluated in the FlowJo software by carrying out a post-analysis compensation using FMO controls.

2.2.4 Histological analysis of the placenta

Paraffin-embedded placental tissues (gd 13.5 and 18.5) were cut at the mid-sagittal plane into histological sections of 4 mm thickness using a microtome. Prior to histochemical treatments, sections were deparaffined using xylol replacement medium (2x 5 min) and rinsing twice in 100% ethanol (1st rinse 3 min, 2nd rinse 5 min). Sections were then rinsed in distilled water and dehydrated twice in 96% ethanol (2x 5 min).

Histomorphological assessment

Masson-Goldner trichrome staining of the histological sections was employed to visualize the morphologically different areas of placental tissue. For Masson-Goldner trichrome staining, tissue sections were stained with Weigert's iron hematoxylin, Goldner 1, Goldner 2, orange G solution, and Goldner 3 solution in a stepwise manner. Finally, sections were dehydrated and mounted using Eukitt medium. Images were acquired using a slide scanner. Areas of junctional zone (JZ) and labyrinth (L) zone were quantified using the software Pannoramic Viewer and an L/JZ ratio was calculated. The number of blood spaces was counted in two visual fields (each 0.05 mm^2) in the peripheral region of the labyrinth adjacent to the chorionic plate.

Immunohistochemistry

Automated slide staining was performed with Benchmark XT. Tissue specimen of mouse abdominal aorta, lung and intestine were used as positive controls. A parallel staining devoid of the primary antibody served as negative control.

JAM-B staining: Prior to staining, heat-mediated antigen retrieval was performed with Cell Conditioning Solution (pH 8.5) according to the manufacturer's instruction. Slides were incubated with rabbit polyclonal anti-mouse JAM-B antibody (Table 5) in IHC buffer for 32 min at RT. Bound αJAM-B was detected with anti-rabbit horse reddish peroxidase (HRP)-conjugated secondary antibody (Table 5) according to the manufacturer's instructions after quenching of endogenous peroxidase activity by hydrogen peroxide (H_2O_2).

P-Selectin staining: Prior to staining, antigen retrieval was performed using Proteinase K according to the manufacturer's instruction for 8 min. Slides were incubated with goat polyclonal anti-mouse P-Selectin antibody (Table 5) in IHC buffer for 32 min at RT. Bound αP-Selectin was detected by anti-goat Universal Immuno-peroxidase Polymer (Table 5) according to the manufacturer's instructions after quenching of endogenous peroxidase activity by hydrogen peroxide (H_2O_2).

VCAM-1 staining: Prior to staining, heat-mediated antigen retrieval was performed with Cell Conditioning Solution (pH 8.5) according to the manufacturer's instruction. Slides were incubated with goat polyclonal anti-mouse VCAM-1 antibody (Table 5) in IHC buffer for 32 min at RT. Bound αVCAM-1 was detected by anti-goat Universal Immuno-peroxidase Polymer (Table 5) according to the manufacturer's instructions after quenching of endogenous peroxidase activity by hydrogen peroxide (H_2O_2).

The product of peroxidase activity was visualized using the ultraView Universal DAB Detection Kit according to the manufacturer's instructions. Sections were counterstained with Hematoxylin and

Bluing Reagent and mounted with Tissue Tek. Slides were scanned using NanoZoomer 2.0 HT and analyzed using the software Nano-Zoomer Digital Pathology Image.

2.2.5 Experimental design: Prenatal stress challenge

To challenge the pregnancy by a maternal stress perception, C57BL/6J dams were exposed to sound stress on gd 10.5, 12.5, and 14.5. Single-housed dams were relocated from their home into stress cages during the stress periods (24h, 09:00 – 09:00 a.m.) with ad libitum access to food and water. Sound sources were two rodent repellent devices placed inside of the stress cage, one emitting at 300 Hz in intervals of 15 s and the other continuously emitting ultrasound waves. Unstressed, single-housed dams in their home cages served as controls. Mothers of both groups were intraperitoneally injected with 200 µl sterile PBS at 9.00 a.m. from gd 8.5 – 12.5. Maternal body weight was recorded every morning starting on gd 8.5 and continuing throughout the stress period (gd 10.5 – 15.5), as well as on gd 16.5 and experimental day gd 18.5.

2.2.6 Fluorescence-activated cell sorting

To sort MMc cells and fetal hematopoietic stem and progenitor cells (HSPC) from the bulk offspring's cells, fetal BM cells of one litter were harvested as described in section "Fetal tissue isolation and generation of single cell suspensions", pooled and stained with fluorochrome-conjugated antibodies against extracellular antigens under sterile conditions. Up to $2x10^6$ cells in 50µl were initially stained with NRS (1:100) and TruStain (CD16/32) for 15 min at 4°C on ice to block unspecific Fc binding. Antibody and buffer volumes were adjusted according to the bone marrow cell count present in the tube. Antibodies directed against CD45.2, CD45.1, H-$2D^b$, H-$2D^d$ were used to detect MMc cells. A biotinylated lineage cell detection cocktail, as well as Sca-1 and c-Kit (CD117) were used to identify fetal $Lin^{neg}Sca\text{-}1^+c\text{-}Kit^+$ HSPC (Panel 4, Table 5). Undiluted antibodies were added to the staining tube without washing after Fc blocking and incubated for 30 min at 4°C on ice in the dark. Samples were diluted in FACS buffer and cells washed and pelleted twice in 600ml PBS by centrifugation (8 min, 4°C, 1500 rpm) to remove unbound antibody. Supernatant was discarded and the cell pellet was incubated with fluorochrome-conjugated streptavidin for 15 min at 4°C on ice in the dark. Cells were washed as described before, re-filtered through a cell strainer (40 µm), and re-suspended in FACS buffer containing 2 mM EDTA to avoid cell clotting during sorting. Cell viability staining was performed by addition of 7-Aminoactinomycin D (7-AAD, Table 5) to the sample tube 15 min before sorting using an Aria Fusion flow sorter. Maternal PBMC served as positive staining control. Cells were sorted into sterile FACS tubes holding 2 ml PBS supplemented with 2 mM EDTA, that were pre-coated overnight with FCS, to enhance cell viability after sorting. Nozzle size 70 and 4-way purity sorting was used. Purity of the sorted populations was determined after sorting by flow cytometry (Figure 4). The yield of sorted cells is depicted in Table 7.

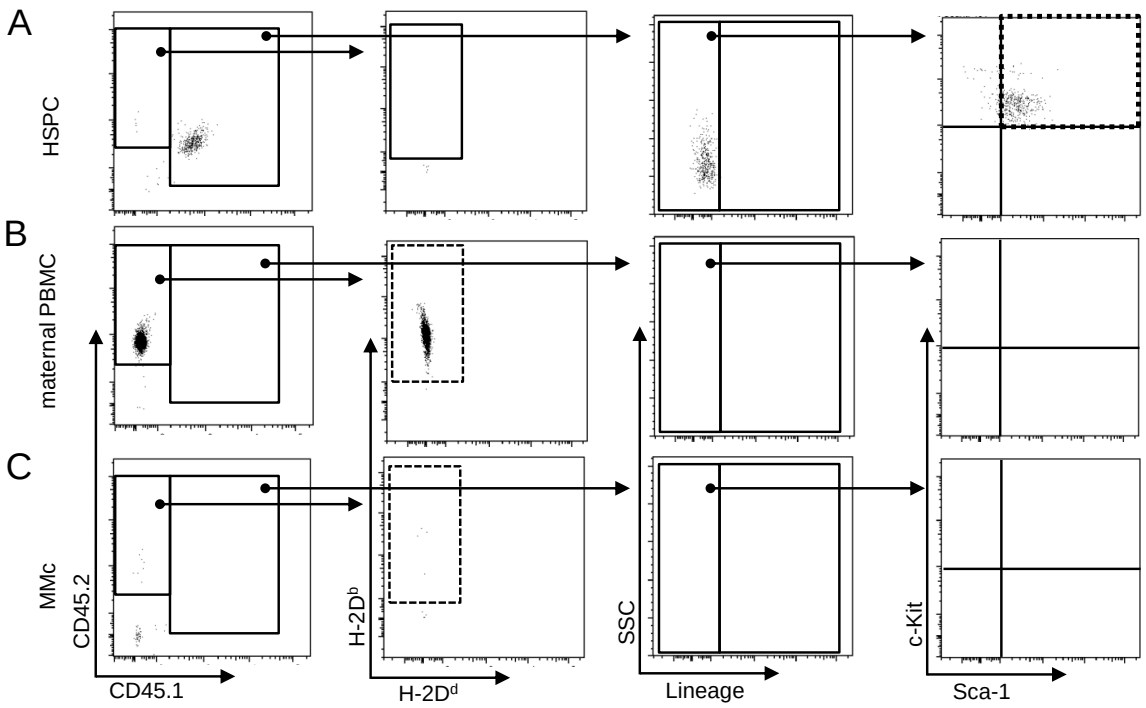

Figure 4: *Representative dot plots of sorted cell populations on culture day 0.* Sorted fetal hematopoietic stem and progenitor cells (HSPC) (A), maternal peripheral blood mononuclear (PBMC) $CD3^+$ T cells (B), and maternal microchimeric (MMc) cells (C) at day of initiation of culture (d0) were analyzed for purity after sorting. Dotted line represents fetal HSPC sorting gate. Stippled line represents MMc cell and maternal PBMC sorting gates.

Table 7: *Cell yield after fluorescence-activated cell sorting.* Maternal microchimeric (MMc) cells and fetal hematopoietic stem and progenitor cells (HSPC) were sorted from fetal bone marrow (BM) cells pooled per litter on gd 18.5.

fetal BM cell count	litter 1	2	3	4	5	6	7	8	9	10	**Mean**	± SD	± SEM
harvested total cells x10^6	7.0	8.85	13.4	16.7	12.0	15.45	8.6	15.8	17.7	20.05	**13.3**	4.6	1.6
sorted MMc cells	412	440	246	596	227	492	156	812	476	230	**418.6**	203.5	72
sorted fetal HSPC	1055	1567	549	1012	778	620	1330	2529	440	496	**1150.9**	633.1	223.8

2.2.7 *Ex vivo* co-culture

In vitro maintenance and pre-seeding of OP9 feeder layer

To co-culture sorted MMc cells and fetal HSPC, commercially available OP9 cells served as feeder layer. OP9 cells (Nakano et al., 1994) were kept in c-αMEM medium under optimal cell culture conditions of 37°C, 5% CO_2. At 80-90% confluence cells were either split 1:2 or cryo-preserved in αMEM (1% Penicillin/Streptomycin, 30% FCS, 10% DMSO) in aliquots of 2x10^5 cells/ml in liquid nitrogen. To avoid differentiation when culturing without irradiation, cells were kept in culture for

maximally 7 consecutive days. For co-culture, one aliquot of OP9 cells was grown to confluence in 3 ml c-αMEM medium on a 3.5 cm Petri dish for 4 days. 48h prior to addition of sorted MMc cells and fetal HSPC, OP9 cells were harvested by incubating with 500 µl 0.25% trypsin/EDTA for 3 min at 37°C after washing the dish with PBS. Reaction was stopped with c-αMEM medium and cells were washed, counted and seeded onto a 96-well microplate (5000 cells/well in 200 µl c-αMEM).

Fluorescent cell tracking

To detect MMc cells during and after the culture period, cells were stained with fluorescent dye Cell Tracker Blue (CTB) prior to plating. Sorted cells in collection tubes were centrifuged for (10 min, 21°C, 1500 rpm) and the pellet was subsequently washed again with PBS to remove protein from the solution. Up to 1x10^6 pelleted cells were quickly re-suspended in 1 ml 20µM CTB in protein-free PBS at 37°C, shortly vortexed to ensure homogenous cell labeling, and incubated for 20 min at 37°C, 5% CO_2. Reaction was stopped with 2 ml of pre-warmed c-αMEM and cells were washed twice in c-αMEM by centrifugation (10 min, 21°C, 1500 rpm) before plating. Sorted maternal $CD3^+$ T cells served as positive control. Both maternal PBMC T cells and MMc cells were analyzed under the ApoTome microscope using ultraviolet (UV) light (**Figure 5**).

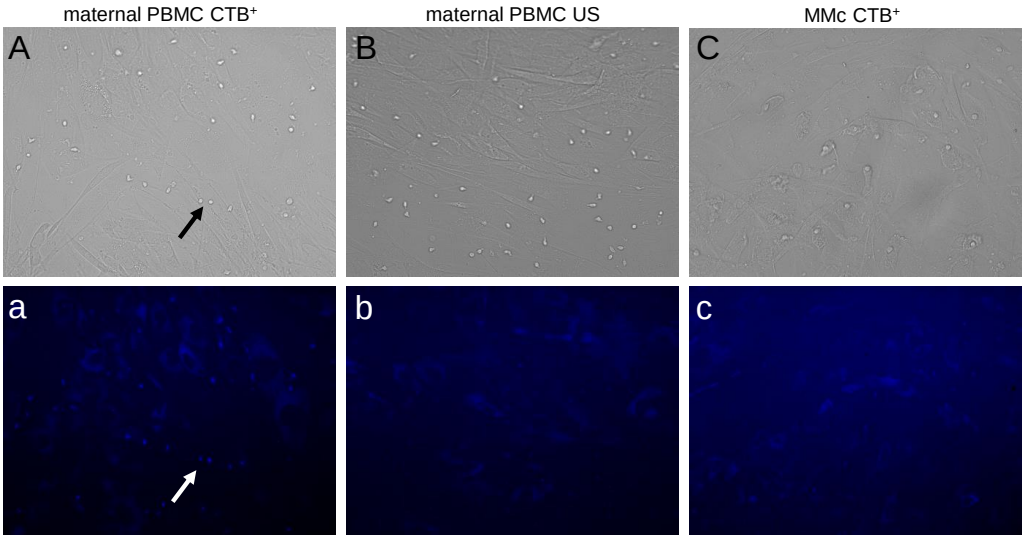

Figure 5: *Fluorescence tracking of sorted cells.* Representative depiction of sorted maternal peripheral blood mononuclear (PBMC) $CD3^+$ T cells (10^4/well), stained with Cell Tracker Blue (CTB) (A (bright field), a (UV laser)), or unstained (B, b), and cultured for 120h supported by OP9 stromal feeder cells. Cells presented with a stable morphology for the entire culture period (C, c) The number of maternal microchimeric (MMc) cells that could be sorted from fetuses pooled from one litter was too low to reliably detect 400 fluorescently labelled MMc cells, cultured alone on OP9 cells for 120h, by fluorescence microscopy. Arrows indicate an example of a CTB-stained T cell. Magnification: 20x.

Co-Culture

Sorted MMc cells and fetal HSPC were washed in c-αMEM medium after sorting and cultured in a ratio of 1 MMc to 10 fetal HSPC cells. Since cell yield after sorting varied between litters (Table 7), 220-350 fetal HSPC with corresponding numbers of MMc cells were initially seeded in adjusted total culture medium volumes, designated as culture day 0. For example, 35 MMc cells and 350 fetal HSPC were suspended in 100 µl c-αMEM and added to 100 µl of feeder layer-conditioned medium. Fetal HSPC cells and MMc cells cultured alone served as control conditions. Cells were kept at 37°C, 5%

CO_2 for 120h (5 days) without changing the medium. On culture day 5, microscopic co-culture appearance was photographied using ApoTome microscopy before harvesting cells (Figure 6). Culture supernatant was collected into FACS tubes, centrifuged to pellet cells, and stored in aliquots of 25 µl at -20°C for later analysis of secreted factors. Subsequently, adherent cells were harvested by trypsinization (20 µl 0.25% trypsin/EDTA for 3 min at 37°C) into the same FACS tube to combine adherent and suspension cells of each well. Cells were stained for flow cytometric analysis using the same panel as employed on culture day 0 (staining procedure as described in section "Fluorescence-activated cell sorting"; Panel 4, Table 5) to analyze fetal HSPC proliferation and differentiation and detect MMc (Figure 7). The fraction of $CD45^+$ cells was sorted from all harvested cells for further analyses.

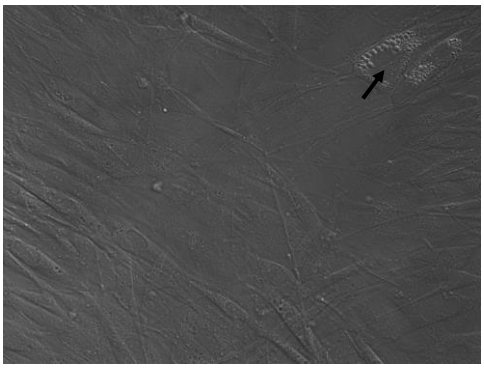

Figure 6: *OP9 stromal bone marrow cells in culture.* Representative depiction of the stromal feeder cells without added experimental cells after 120 h of culture in a 96 well plate. A continuous layer is apparent after 48h of initial seeding and remains stable during the culture period with characteristic occasional adipocytic appearance (arrow).

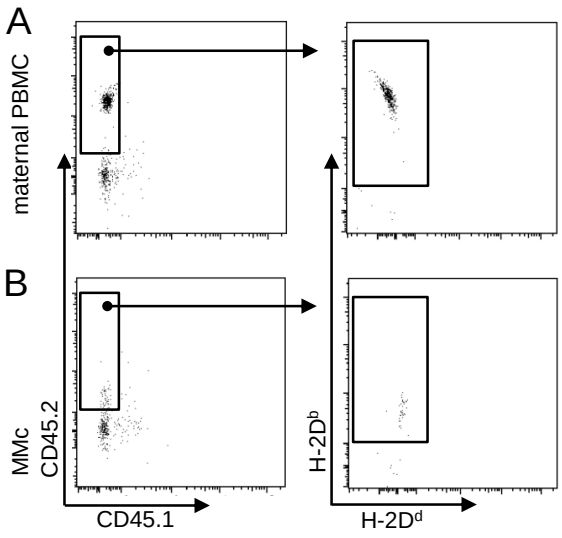

Figure 7: *Representative dot plots of cultured cell populations on culture day 5.* After 120h of culture (d5), 10^4 maternal peripheral blood mononuclear (PBMC) T cells (A) and 400 maternal microchimeric (MMc) cells (B), cultured alone as control condition and negative for cell viability staining using 7-AAD, can be recovered by flow cytometric detection, indicating the survival of both cell populations during the culture period.

Cytospin

Sorted $CD45^+$ cells on culture day 5 were centrifuged for 5 min at 1500rpm, resuspended in 200 µl PBS and transferred into a cytospin carrier. Cells were spun onto a glass slide in a cytospin centrifuge (800 rpm, 8 min) and stained with Pappenheim (Giemsa-May-Grünwald) staining for subsequent morphological assessment. To this end, slides were immersed in 100% May-Grünwald solution for 4 min, passed to 50% May-Grünwald solution for 3 min, and washed in distilled water. Subsequently,

slides were immersed in Potassium dihydrogen phosphate buffer solution (2% Giemsa) for 7 min, washed in distilled water and air-dried. Slides were digitally scanned and analyzed for hematopoietic cell morphology.

2.2.8 Statistical analyses

All statistics such as descriptive and explorative data analyses were performed using SPSS Version 20 (SPSS, Inc.). For group comparisons, the nonparametric Mann-Whitney-U test for independent data sets was employed. Chi-Square-Test with Yates' correction tested the expected vs. observed categorical frequencies between groups. For paired group comparisons, the Wilcoxon matched pairs signed-rank-test was used. Outlier analysis was carried out by excluding values above or below a Z-value of ±1.96. Level of significance was set to $p \leq 0.05$.

2.2.9 Study approval

Animal care and all experimental procedures were performed according to institutional guidelines and conform to requirements of the German Animal Welfare Act. Ethical approvals were obtained from the State Authority of Hamburg (Germany, approval numbers G067/10, ORG_615, ORG_702, ORG_764, ORG_795).

3 Results

3.1 Combined expression of congenic CD45.2 and major histocompatibility complex I H-2D^{b} allow for advanced identification of gestational maternal microchimerism by flow cytometry

This thesis employs a novel approach to advance the detection of naturally occurring hematopoietic maternal microchimeric cells among bulk haploidentical fetal cells by flow cytometry. To this end, an allogeneic mating model in which female C57BL/6J (H-2D^{b}; CD45.2^{+}) were mated to male CByJ.SJL(B6)-Ptprc^{a}/J (H-2D^{d}; CD45.1^{+}) was employed. This combination results in offspring heterozygous for both the congenic marker CD45 as well as the MHC class I haplotype (CD45.2/1; H-2D$^{b/d}$). MMc cells can be distinguished from fetal cells by their homozygous expression of H-2D^{b} and CD45.2^{+} (Figure 8 A, B).

During establishment of this technique for detection of rare microchimeric cells, the staining combination using the four extracellular markers – CD45.1, CD45.2, MHC class I haplotypes H-2D^{b} and H-2D^{d} – was validated using maternal (H-2D^{b}; CD45.2^{+}) and paternal (H-2D^{d}; CD45.1^{+}) (Figure 8 C) tissues as well as artificially created microchimerism of 5% maternal cells (H-2D^{b}; CD45.2^{+}) added to bulk fetal cells of gd 18.5 bone marrow (CD45.2/1; H-2D$^{b/d}$) (Figure 8 D). Observing the expected staining outcomes allowed to identifying naturally occurring MMc cells in fetal bone marrow (Figure 8 E), as determined by the frequency of CD45.2$^{+/+}$H-2D$^{b/b}$ maternal cells among CD45.2/1; H-2D$^{b/d}$ fetal cells. Affirmed by fluorescence minus one controls (Figure 3), the use of H-2D as a second marker for detecting MMc cells improved the specificity of the detection as it permitted to exclude false positive CD45.2^{+} cells from the MMc population (Figure 8 E, right column).

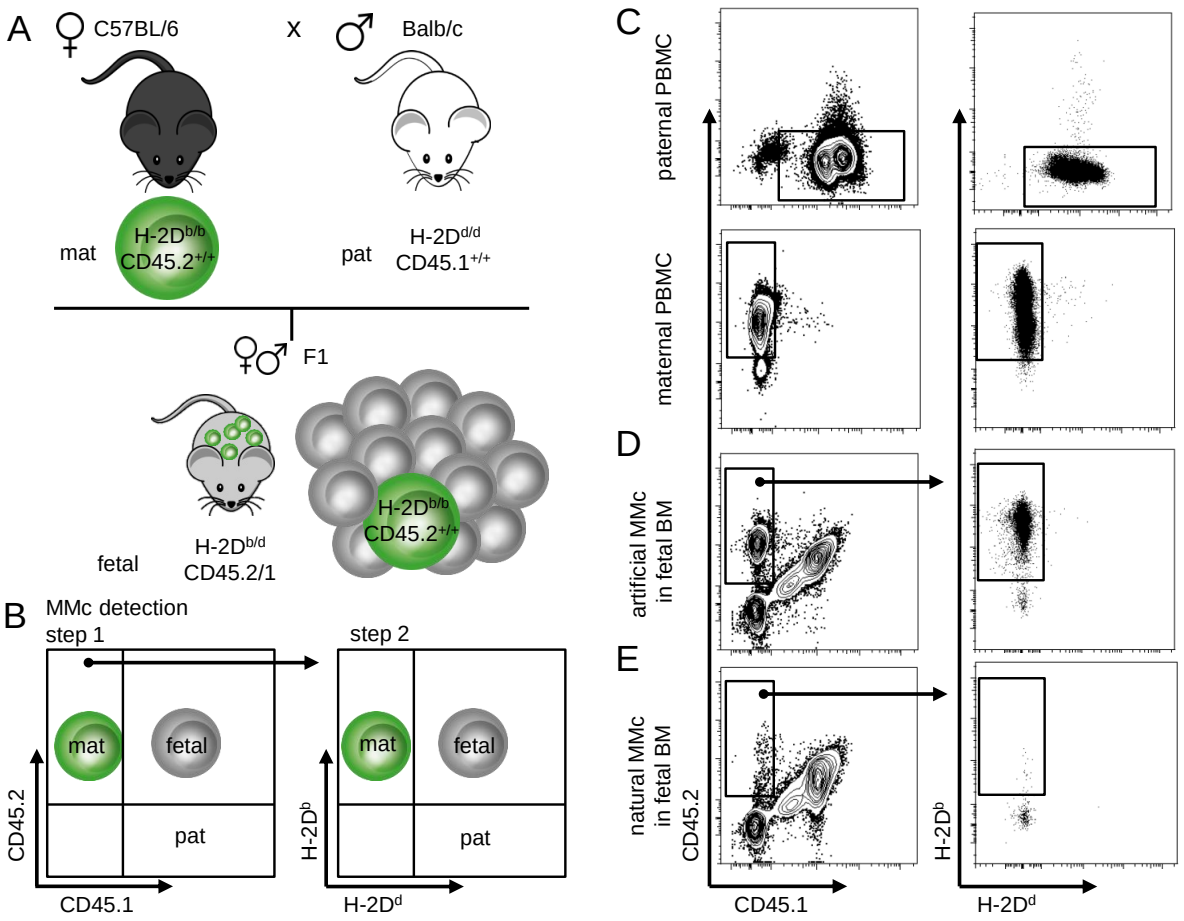

Figure 8: *Technical approach for detection of maternal microchimerism (MMc) among fetal cells in offspring's organs by flow cytometry*. (A) Allogeneic mating strategy and (B) gating approach to identify CD45.2$^{+/+}$H-2D$^{b/b}$ MMc cells among heterozygous fetal cells (CD45.2/1 H-2D$^{b/d}$) in two detection steps based on the congenic markers CD45.1/2 and major histocompatibility complex class I (MHC) haplotypes H-2D^{b} and H-2D^{d}. (C) Representative staining of maternal and paternal peripheral blood mononuclear cells (PBMC). After doublet exclusion and cell viability gating, among the living cells in the fetal bone marrow (BM) on gd 18.5, artificially generated (fetal BM spiked with 5% maternal PBMC) (D) and naturally occurring MMc (E) can be identified. mat: maternal, pat: paternal.

3.2 Maternal microchimeric cells are present in various fetal tissues during late gestation

The possible role of MMc in materno-fetal immune cross talk was investigated by detecting MMc cells in various fetal organs. Bone marrow, liver, spleen, lung, peripheral blood, and thymus were harvested from heterozygous CD45.2/1 H-2D$^{b/d}$ fetuses on gd 18.5. While MMc cells were found in fetal organs such as liver, spleen, lung, and in the peripheral blood, they were scarce in the fetal thymus (Figure 9). MMc cells were particularly frequent in the bone marrow compared to the other tissues, revealing it as a major site of MMc during late gestation.

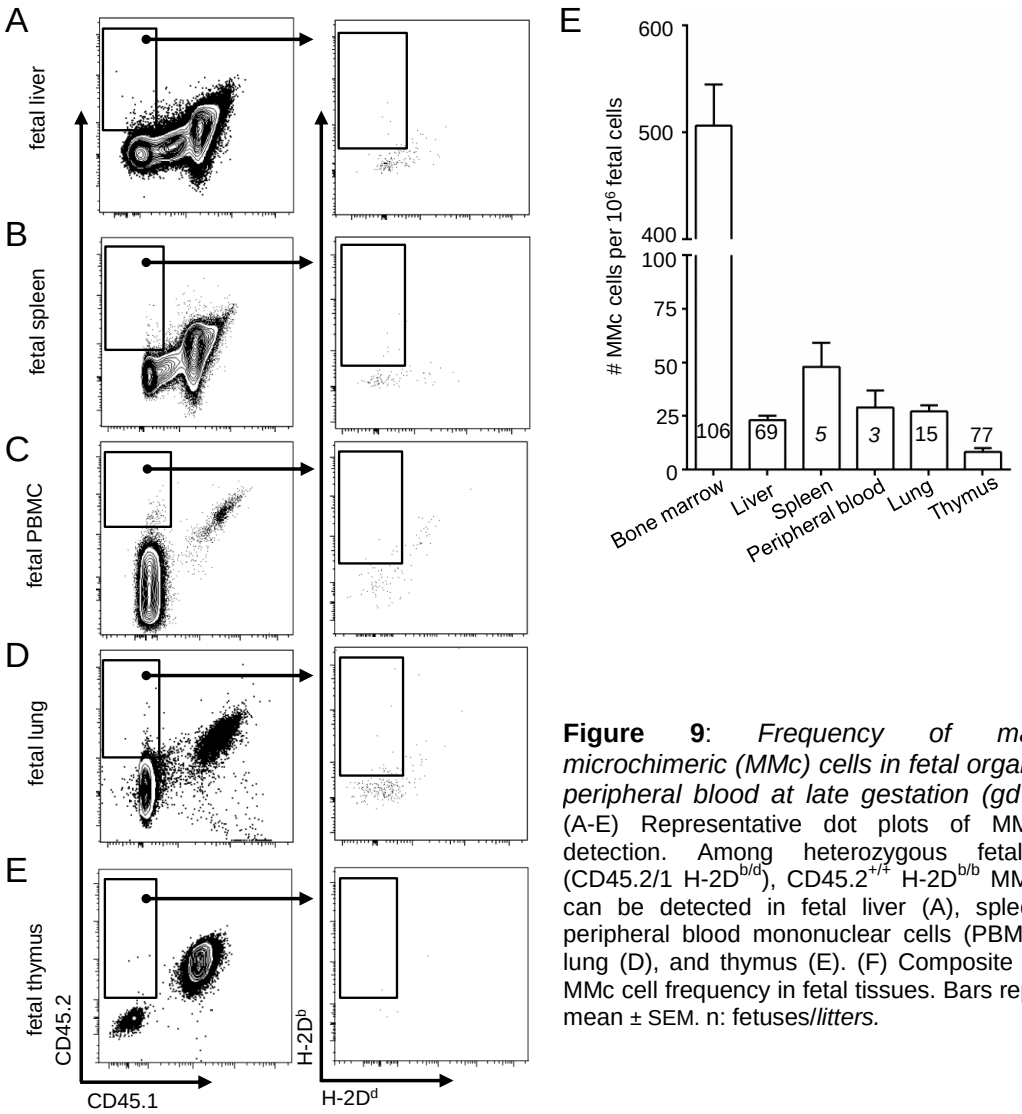

Figure 9: *Frequency of maternal microchimeric (MMc) cells in fetal organs and peripheral blood at late gestation (gd 18.5).* (A-E) Representative dot plots of MMc cell detection. Among heterozygous fetal cells (CD45.2/1 H-2D$^{b/d}$), CD45.2$^{+/+}$ H-2D$^{b/b}$ MMc cells can be detected in fetal liver (A), spleen (B), peripheral blood mononuclear cells (PBMC) (C), lung (D), and thymus (E). (F) Composite data of MMc cell frequency in fetal tissues. Bars represent mean ± SEM. n: fetuses/*litters.*

In addition to the mere detection of MMc cells, the employed flow cytometric approach allowed to analyze the phenotypical identity of MMc cells using the cell-lineage defining surface markers such as NKp46 to identify NK cells, CD3 (T cells), CD19 (B cells), CD11c (dendritic cells), and CD11b (myeloid cells), as well as Sca-1 and c-Kit to identify fetal HSPC. Pilot data of this phenotypical analysis showed T cells to be a prominent subset among the MMc cell population in the primary hematopoietic organs during late gestation, the bone marrow, liver, and spleen, followed by B cells, CD11b^{+} and CD11c^{+} cells (Figure 10).

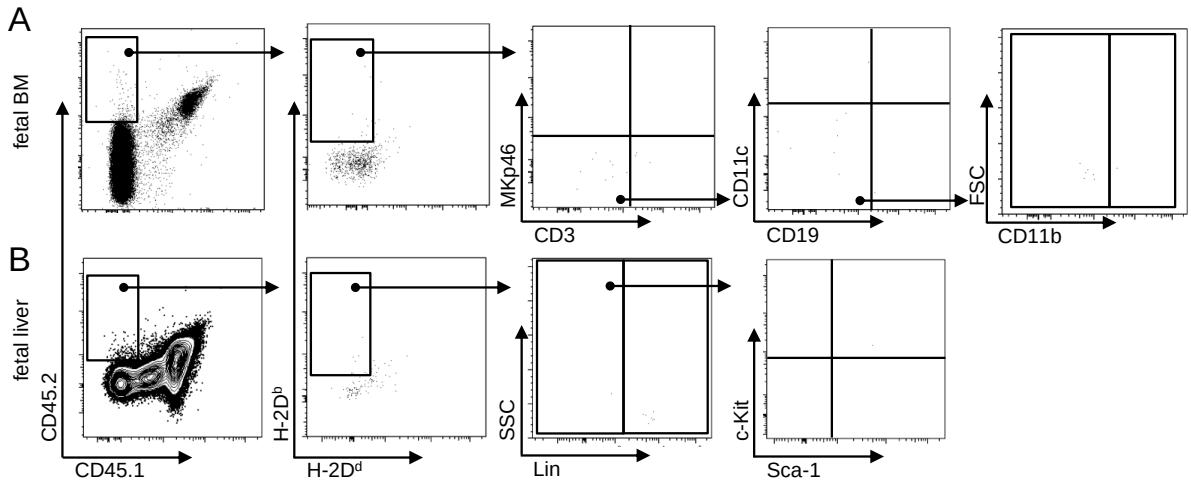

Figure 10: *Representative dot plots of hematopoietic cell phenotypes identified among the maternal microchimeric (MMc) cell population.* (A) In the fetal bone marrow (BM), $CD3^{+}NKp46^{neg}$ T cells, $CD19^{+}$ B cells, $CD11c^{+}$ dendritic cells and $CD11b^{+}$ myeloid cells can be identified. (B) In the fetal liver, extremely rarely MMc cells with a phenotype of Lineage $(Lin)^{neg}Sca\text{-}1^{+}c\text{-}Kit^{+}$ hematopoietic stem and progenitor cells were detected.

Therefore, only CD3, B220, and CD11b were targeted in subsequent analyses to assess frequency of the major leukocyte populations T cells, B cells, and myeloid cells, respectively, among MMc cells in the fetal liver and the MMc cell-rich bone marrow.

In a more detailed analysis, the frequencies of T, B, and myeloid cells among MMc cells in fetal liver and bone marrow were compared to the putative source of MMc, the maternal peripheral blood, to investigate a possible selective transfer of cells from mother to child. While the composition of MMc cells in the fetal liver (Figure 11 A) closely resembled that in the maternal peripheral blood (Figure 11 B), in the fetal bone marrow on gd 18.5, the majority of MMc cells were $CD3^{+}$ T cells (>75%), while B and myeloid MMc cells were less frequently present than in the peripheral maternal blood. This indicates an accumulation of MMc T cells in the fetal bone marrow (Figure 11 C).

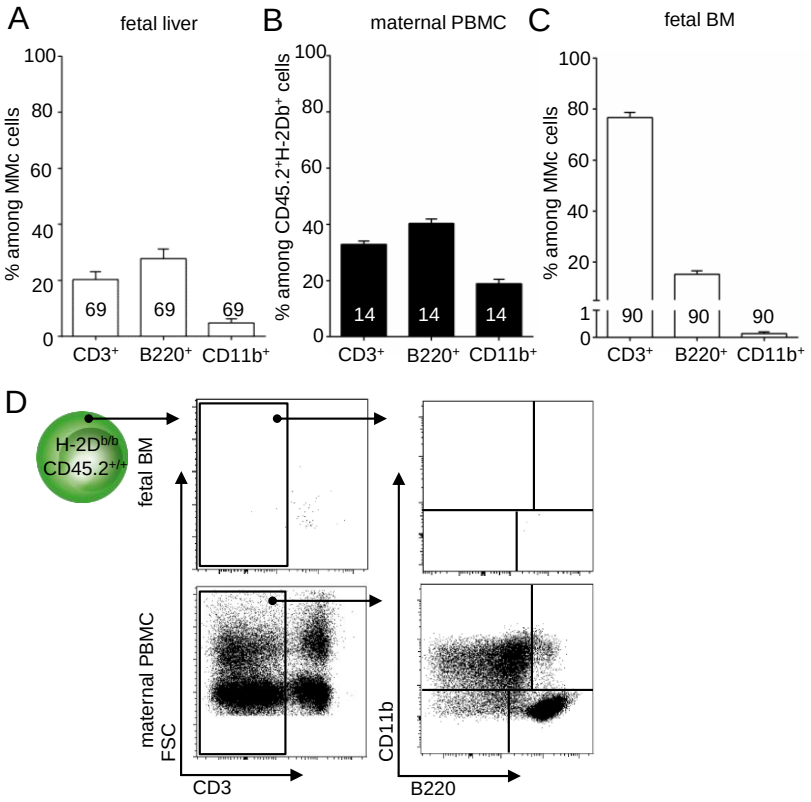

Figure 11: *Phenotypical composition of the maternal microchimeric (MMc) cell population in fetal bone marrow (BM) on gd 18.5 differs from maternal peripheral blood cells (PBMC).* Composite percentage of $CD3^+$ T cells, $B220^+CD11b^{neg}$ B cells and $CD11b^+B220^{neg}$ myeloid cells among $CD45.2^{+/+}H\text{-}2D^{b/b}$ MMc cells found in fetal liver (A) and fetal BM (C). (B) Corresponding percentages of $CD3^+$ T cells, $B220^+CD11b^{neg}$ B cells and $CD11b^+B220^{neg}$ myeloid cells among maternal PBMC. (D) Representative dot plots of immune cell staining of MMc cells in the fetal BM and in maternal PBMC. Bars represent mean ± SEM and n fetuses (A, C) and mothers (B).

In summary, MMc cells of hematopoietic origin were identified in differing frequencies among various immune and non-immune tissues in fetuses close before birth. Among these cells, T, B, and myeloid cells were present. The phenotypical composition of the MMc population appeared organ-specific and resembled the maternal peripheral blood frequencies in case of the fetal liver, but not the fetal bone marrow, where MMc T cells seemed to selectively accumulate. To further investigate this observation, and because the fetal bone marrow generally contained the highest frequency of MMc cells, subsequent MMc cell analyses were based on the fetal bone marrow.

3.3 Maternal microchimeric T cells in the fetal bone marrow express cell adhesion molecule ligands

The presence of MMc cells in the offspring raises questions on the mechanisms of migration these cells use to reach the fetal organs. In order to investigate the migration capacity of MMc T cells, it was hypothesized that these cells express cell adhesion molecule ligands (CAML), which would enable them to bind to cell adhesion molecules (CAM) expressed in the blood sinus linings of the placenta and to cross the trophoblast-endothelial barrier, which separates the maternal from the fetal circulation. As expected, the CAMLs VLA-4, PSGL-1, and LFA-1 were expressed by virtually all T cells present in the maternal peripheral blood (>90%) (Figure 12 B). Surprisingly, MMc T cells present in the fetal bone marrow almost completely lacked expression of LFA-1, but expressed both VLA-4 and PSGL-1 at a lower level than maternal PBMC T cells (Figure 12 A). This data shows that MMc cells express molecular ligands that could allow them to migrate across the trophoblast-endothelial barrier. However, these observations do not allow conclusions on whether the specific CAML signature observed in MMc T cells in the fetal BM is necessary to cross the placenta or is established subsequently during home to the fetal bone marrow.

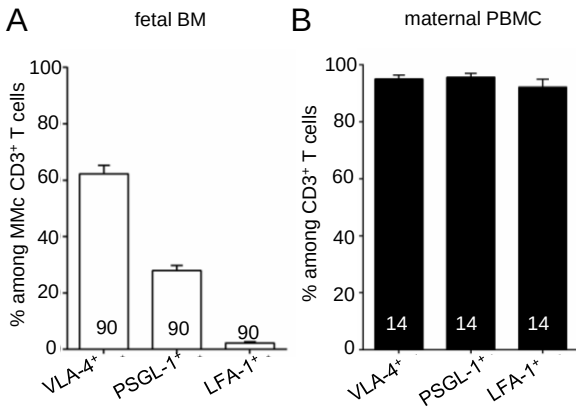

Figure 12: *Cell adhesion molecule ligand expression (CAML) by maternal microchimeric (MMc) and maternal peripheral blood mononuclear (PBMC) CD3$^+$ T cells*. MMc CD3$^+$ T cells in the fetal bone marrow on gd 18.5 express CAML Very late antigen (VLA)-4 and P-Selectin glycoprotein ligand (PSGL)-1, while the majority lacks Lymphocyte function-associated antigen (LFA)-1 expression (A). In contrast, CD3$^+$ T cells among maternal peripheral blood leukocytes express virtually all three CAML (>90%) (B). Bars represent the mean ± SEM and n fetuses (A) and mothers (B).

3.4 The feto-maternal interface expresses cell adhesion molecules in the cellular linings of labyrinthine blood spaces

To allow for trans-placental migration of CAML-expressing MMc cells, we further hypothesized that the corresponding cell adhesion molecules are expressed in the placental labyrinth. Using immunohistochemistry, we confirmed the labyrinthine expression of P-Selectin and VCAM-1 during gestation, which have the potential to bind to PSGL-1 and VLA-4 expressed on MMc cells, respectively (Figure 13 A-D). P-Selectin protein expression was localized to thin cell layers in the complete labyrinth, while VCAM-1 expression appeared more clustered in larger, presumably

trophoblast cells across the labyrinth zone. JAM-B, involved in trans-endothelial migration processes, was not found to be expressed in the cell layers of the labyrinth on gd 18.5 (Figure 13 e-f), suggesting that a different protein must enable trans-trophoblast/-endothelial passage of MMc cells. Still, JAM-B was specifically and prominently expressed in the spongiotrophoblast cells of the junctional zone (Figure 13 E-F). This previously unknown presence of JAM-B in the mouse placenta prompted us to investigate its spatio-temporal expression in more detail, revealing a mouse-strain specific increase in spongiotrophoblast JAM-B expression over gestation. Further, JAM-B expression was responsive to modulation by progesterone (Stelzer et al. 2016).

In summary, expression of P-Selectin and VCAM-1 in the labyrinth could facilitate trans-placental passage of MMc cells. MMc cells in turn express VLA-4 and PSGL-1 to adhere to the linings of the labyrinthine blood spaces, the first step necessary for trans-endothelial migration.

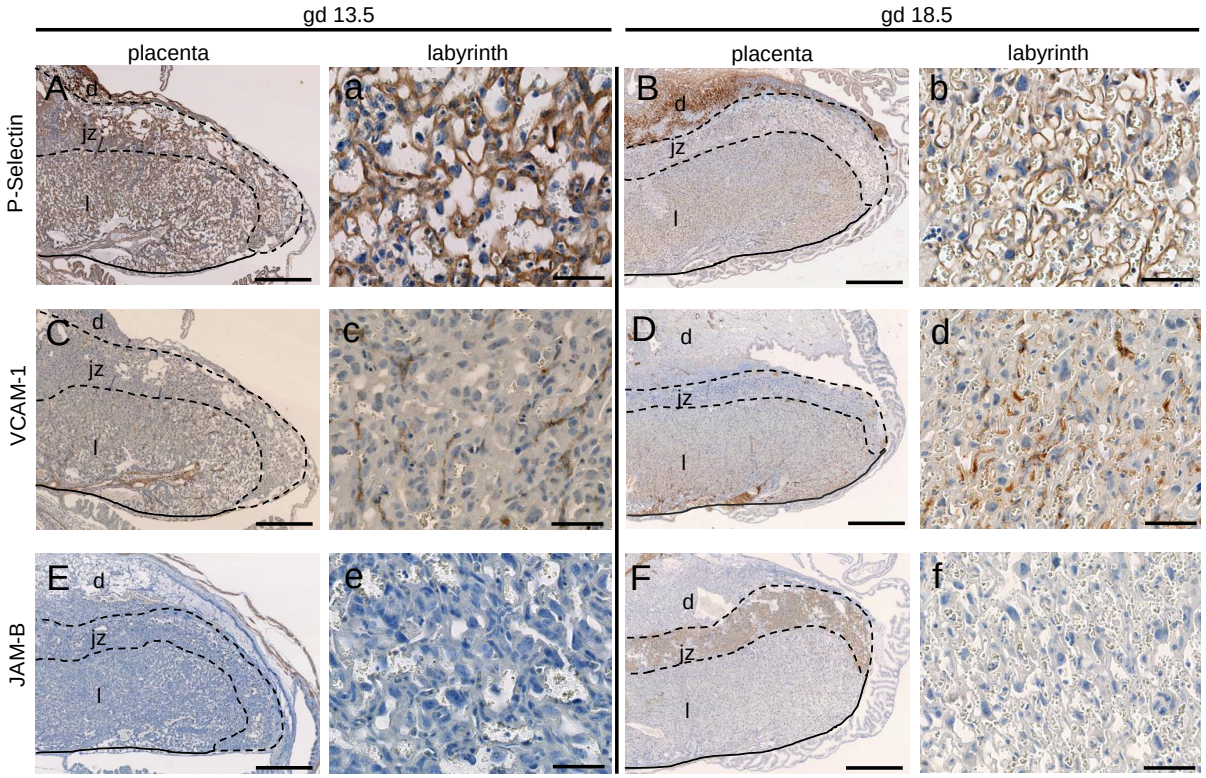

Figure 13: *Cell adhesion molecule expression in the placental labyrinth on gd 13.5 and 18.5.* The feto-maternal interface of in Balb/c-mated C57BL6 females expresses P-selectin (A, B) and VCAM-1 (C, D) protein, including the labyrinth (a - d). JAM-B protein is absent in the labyrinth (e, f), but shows spongiotrophoblast-lineage specific expression in the junctional zone, which increases from gd 13.5 to 18.5 (E, F). d: decidua, jz: junctional zone, l: labyrinth. Bars: A-F = 500 µm, a-f = 50 µm.

3.5 A mid-gestational stress challenge hampers maternal pregnancy-associated weight increase but does not affect late gestational fetal weight and placental histomorphometry

To eventually test the vulnerability of MMc to exogenous influences during pregnancy, an experimental model of maternal stress perception was employed to challenge the maternal immune adaptation to pregnancy. To this end, dams were exposed to an acute mid-gestational sound stress recurring on gd 10.5, 12.5, and 14.5 for 24h each (Figure 14 A). In a first step, fetal parameters and placental condition were tested to assess stress effects on the overall reproductive outcome. Stress exposure resulted in a dampened pregnancy-related maternal weight increase compared to unstressed dams, as apparent in the mornings after stress exposure. Stressed dams had a significantly reduced weight on gd 13.5, after the second stress treatment period. This effect was present as a trend also after the first and third stress treatment but was compensated for on gd 12.5, 14.5 and during late gestation (gd 16.5 and 18.5) (Figure 14 B). Effects of the stress challenge on pregnancy outcome parameters, such as fetal weight, abortion rate, and number of implantations, as well as placental histomorphometry were assessed on gd 18.5. Fetal weight, an indicator of prenatal development, was not different between stressed and unstressed pregnancies (Figure 14 C). Also abortion rates and number of implantations per litter were comparable between stressed and unstressed pregnancies (Figure 14 D, E). Further, the ratio between placental labyrinth and junctional zone area (L/JZ ratio), a measure of overall placental development, and the number of blood spaces per visual field, a measure of labyrinthine vascularization, was not different between stressed and control pregnancies. This indicates that the overall placental condition after a mid-gestational stress challenge does not remain affected at late gestation (Figure 14 F, G).

While overall pregnancy outcome is unaffected on gd 18.5, the employed stress model results in increased maternal glucocorticoid hormone level after stress challenges on gd 11.5 and 13.5, which is accompanied by a decreased fetal weight on gd 15.5 in comparison to unchallenged pregnancies (unpublished data of our lab, Clara Perani). This indicates disturbing effects of the stress challenge on maternal immune adaptation to pregnancy and fetal development. This makes it a suitable model to study subclinical psychogenic stress perception during pregnancy, resembling the situation in human females.

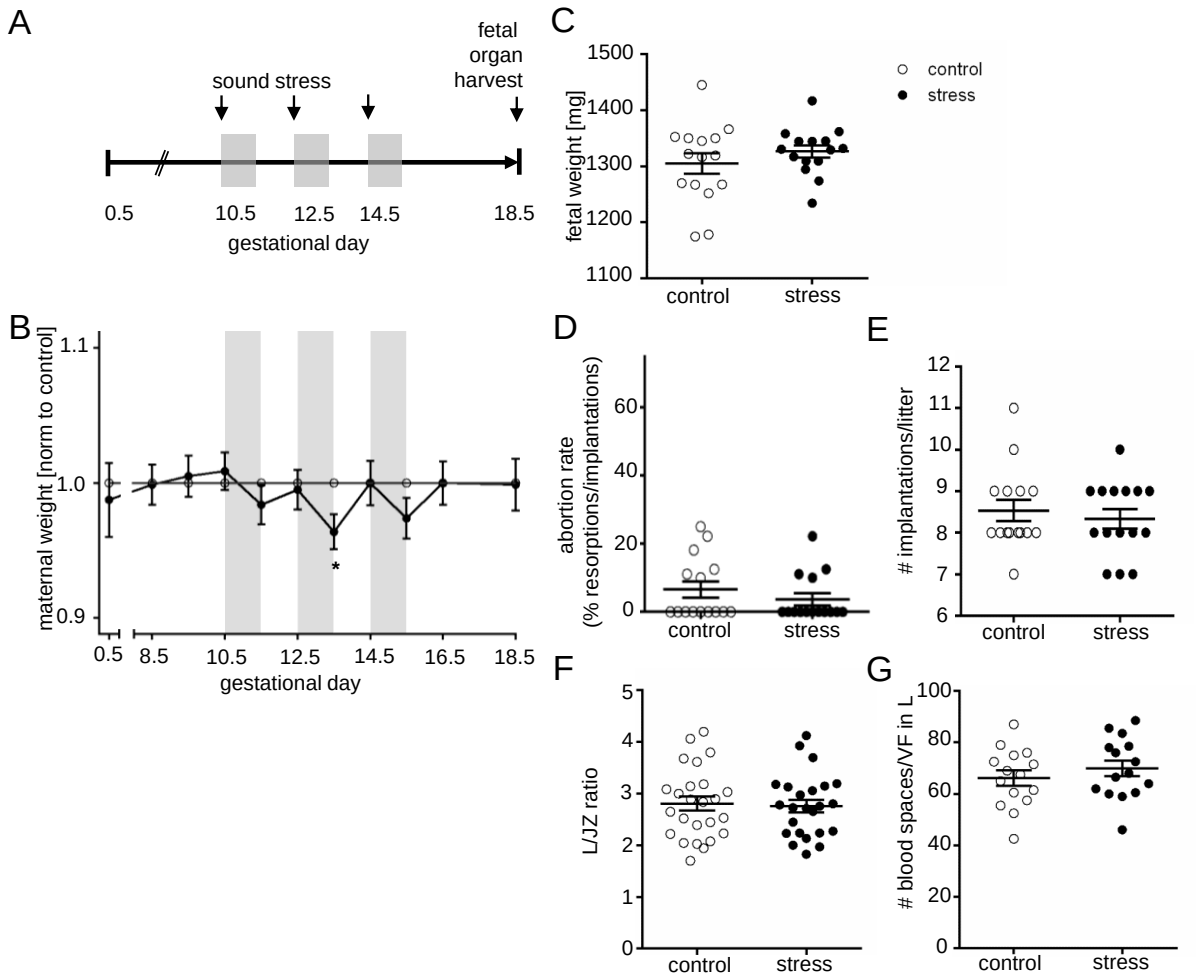

Figure 14: *Experimental design and overall reproductive outcome in pregnancies exposed to a mid-gestational stress challenge vs. control.* (A) Schematical depiction of the employed stress model. Balb/c-mated C57BL/6 females were exposed to sound stress for three periods of each 24h during mid-gestation (gd 10.5, 12.5, 14.5). Fetal organs were harvested on gd 18.5. (B) Maternal weight development is impaired throughout stress treatment as apparent in the decreased weight in the mornings after each stress period in comparison to the control group, an effect which is compensated until late pregnancy. On gd 18.5, mean fetal weight per litter (C), abortion rate (D), and litter size (E) were not different between stressed and control pregnancies. Two measures for placental development, the ratio between placental labyrinth and junctional zone area (L/JZ ratio) (F) and the number of blood spaces per visual field (G), did not differ between stressed and control pregnancies. Lines and data points represent the mean ± SEM. (B) n= 8 mothers per group. *: p≤0.05 as assessed by Mann-Whitney-U-test.

3.6 A mid-gestational maternal stress challenge affects frequency of maternal microchimerism in gd 18.5 fetal bone marrow

We hypothesized that MMc could be a mechanism for fetal programming. To test if MMc is vulnerable to a prenatal maternal stress perception, frequency and phenotype of maternal microchimeric cells in the fetal bone marrow was analyzed after a mid-gestational maternal stress challenge. A strong trend towards reduced numbers of MMc cells ($CD45.2^{+/+}H\text{-}2D^{b/b}$) per one million fetal bone marrow cells was observed on gd 18.5 in fetuses from stressed mothers compared to control fetuses in unstressed mothers (p=0.07 by Mann-Whitney-U-test). Significantly fewer fetuses in the stressed group presented with MMc quantities at the 75^{th} percentile of the control group (* p≤0.05 assessed by Chi-Square-Test followed by Yates's correction) (Figure 15 A). The lower number of MMc cells was due to a shift towards reduction in the MMc $CD3^+$ T cells in the fetal bone marrow, while numbers of

$B220^{+}CD3^{neg}CD11b^{neg}$ B cells and $CD11b^{+}CD3^{neg}B220^{neg}$ myeloid cells remained unaffected (Figure 15 B). To test whether this trend towards a decrease in T cell number was accompanied by stress-induced alterations of their migration capacity, MMc T cell expression of cell adhesion molecule ligands VLA-4, PSGL-1 and LFA-1 was investigated. MMc T cells mainly expressed VLA-4 and stressed fetuses showed a trend towards lower CAML expression in comparison to control fetuses (Figure 15 C). More specifically, most of the MMc T cells showed the specific phenotype of VLA-4^{+}, but PSGL-1^{neg} and LFA-1^{neg} which was slightly reduced upon stress (Figure 15 D). No other CAML-expression subgroups were affected by stress. We also tested if a maternal stress challenge would affect these same populations in the maternal peripheral blood, the presumable source of MMc. We found that neither the number of $CD45.2^{+/+}H\text{-}2D^{b/b}$ cells in all living leukocytes nor the number of T, B or $CD11b^{+}CD3^{neg}B220^{neg}$ myeloid cells was altered upon stress (Figure 15 E, F). The number of $CD3^{+}$ T cells expressing either VLA-4, PSGL-1 or LFA-1 was also unaffected by a mid-gestational stress challenge (Figure 15 G). VLA-4^{+}, but PSGL-1^{neg} and LFA-1^{neg} T cells were found to be an extremely rare population in the maternal peripheral blood (Figure 15 H).

In summary, a mid-gestational maternal stress challenge does not affect the size of immune cell populations in the maternal peripheral blood in late gestation and does not impact overall pregnancy outcome. However, stress challenge to maternal adaptation to pregnancy results in a lower proportion of fetuses with high numbers of MMc in their bone marrow and in a trend towards a reduced MMc T cell population at late gestation, supporting the hypothesis of MMc as a mechanism for materno-fetal immune cross talk which can be exogenously modulated.

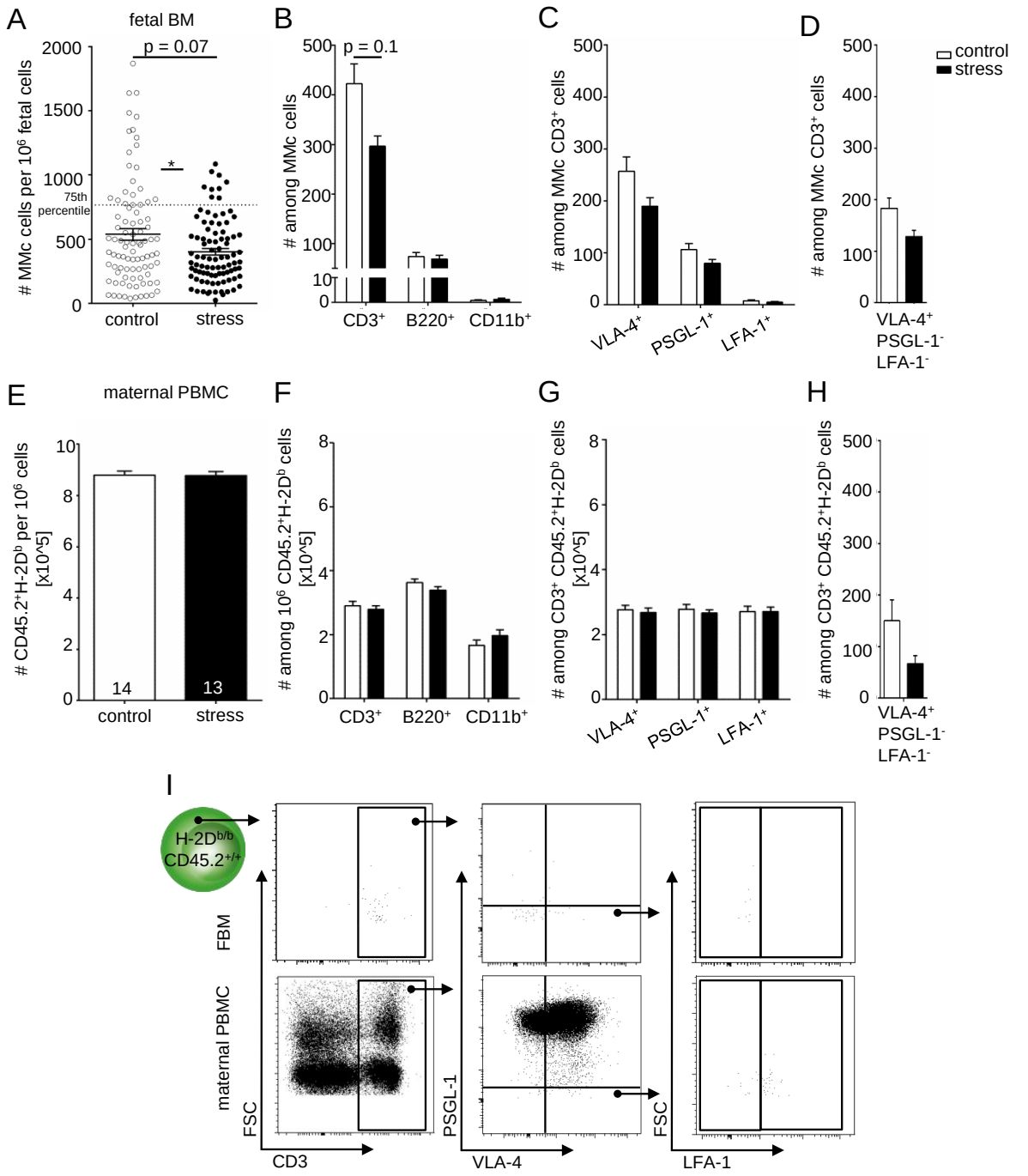

Figure 15: *A mid-gestational stress challenge modulates maternal microchimerism (MMc) in the fetal bone marrow (BM) at late gestation*. The proportion of fetuses with a high number of CD45.2$^{+/+}$H-2D$^{b/b}$ MMc cells (above 75th percentile) is significantly decreased in fetal BM on gd 18.5, while the overall frequency among all fetuses shows a strong trend towards a reduced number of MMc cells in stressed compared to control fetuses (p=0.07) (A). This could be due to a decrease in MMc T cells (B), mostly VLA-4^{+} (C), and specifically PSGL-1^{neg} and LFA-1^{neg} (D). Corresponding late gestational leukocyte populations in the maternal peripheral blood (PBMC) (E-F) are not affected by a mid-gestational stress challenge. The population of VLA-4^{+}PSGL-1^{neg}LFA-1^{neg} CD3^{+} T cells, prominent among MMc cells (D), is a very rare population among all CD45.2$^{+/+}$H-2D$^{b/b}$ maternal PBMC (H). (I) Representative dot plots of MMc and maternal PBMC T cell cell adhesion molecule ligand (CAML) expression. Bars represent mean ± SEM. * p≤0.05 as assessed by Chi-Square-test. Comparison of group means by Mann-Whitney-U-test. n (A-D): 90.

3.7 Maternal microchimeric cells promote differentiation of fetal hematopoietic stem and progenitor cells of the fetal bone marrow *in vitro*

The accumulation of MMc cells in the fetal bone marrow during late gestation and its vulnerability to a mid-gestational maternal stress challenge provoked the question of their functional role in this fetal tissue. We hypothesized that MMc immune cells influence fetal hematopoietic stem and progenitor cell (HSPC) survival and differentiation shortly before birth. To overcome limitations regarding the quantity of MMc cells *in vivo* and to evaluate the effect of MMc cells on fetal HSPC in a controlled environment, MMc cells were isolated from the fetal bone marrow by fluorescence-activated cell sorting and co-cultured with simultaneously isolated fetal HSPC ($Lin^{neg}Sca\text{-}1^{+}c\text{-}Kit^{+}$) *in vitro*. After 120h of HSPC culture in the presence or absence of MMc cells in FCS-containing, growth factor-free medium, an increase in the number of living and $CD45^{+}$ cells per well was apparent microscopically and by flow cytometric analysis (Figure 16 A, C, D), demonstrating the proliferation of fetal HSPC. The fold-increase of $CD45^{+}$ cells was significantly higher when fetal HSPC were cultured in the presence of MMc cells, compared to HSPC cultured alone (Figure 16 C, D). The frequency of dead cells was not different between culture conditions (data not shown).

To test whether the increased proliferation was associated with fetal HSPC differentiation, the presence of fetal HSPC-derived lineage-committed progenitor cells ($CD45^{+}Lin^{+}$) was assessed. Presence of $CD45^{+}Lin^{+}$ cells in the wells after seeding $CD45^{+}Lin^{neg}$ fetal HSPC demonstrated the differentiation of fetal HSPC, which was quantified as fold increase of $CD45^{+}Lin^{+}$ cells relative to the number of initially seeded fetal HSPC (Figure 16 F). The higher fold-increase in $CD45^{+}$ cells in those wells in which fetal HSPC were cultured in the presence of MMc cells was due to an increase in $CD45^{+}Lin^{+}$ cells as compared to when fetal HSPC were cultured alone (Figure 16 D, F). The increased number of $CD45^{+}Lin^{+}$ cells in MMc-fetal HSPC co-cultures was not due to proliferation of the MMc cells themselves, since single-cultured MMc cells were not expanding (Figure 7). Complementarily, while the number of $CD45^{+}Lin^{neg}$ cells was increased as well after five days of culture (Figure 16 E), there was no difference in fold-expansion of $CD45^{+}Lin^{neg}$ between fetal HSPC cultured in presence or absence of MMc. This fold-increase of cells was not due to the expansion of fetal HSPC in the culture wells (data not shown).

In order to further characterize the lineage commitment of $CD45^{+}$ cells that differentiated from fetal HSPC cultured in the presence or absence of MMc, these cells were cytospun after sorting at 120 h of culture and analyzed histomorphologically. The majority of cells were committed to the myeloid lineage after five days of culture, giving rise to monocytes and monocytic precursors, while cells of the lymphoid lineage and erythrocyte progenitors were only rarely found. In contrast to fetal HSPC cultured alone, the $CD45^{+}$ population arising from HSPC co-cultured with MMc comprised higher numbers of common myeloid progenitors as well as terminally committed colony-forming unit monocytes and mature monocytes, while granulocyte precursors were rare (Figure 16 G).

In summary, this data strongly suggests a functional role of MMc in promoting fetal hematopoietic stem and progenitor cell differentiation into $CD45^{+}$ lineage-committed immune cells shortly before birth. Additionally, MMc cells skew stem and progenitor cell differentiation towards the myeloid monocytic lineage, suggesting a direct influence on myeloid hematopoiesis and thus, the development of the fetal immune system.

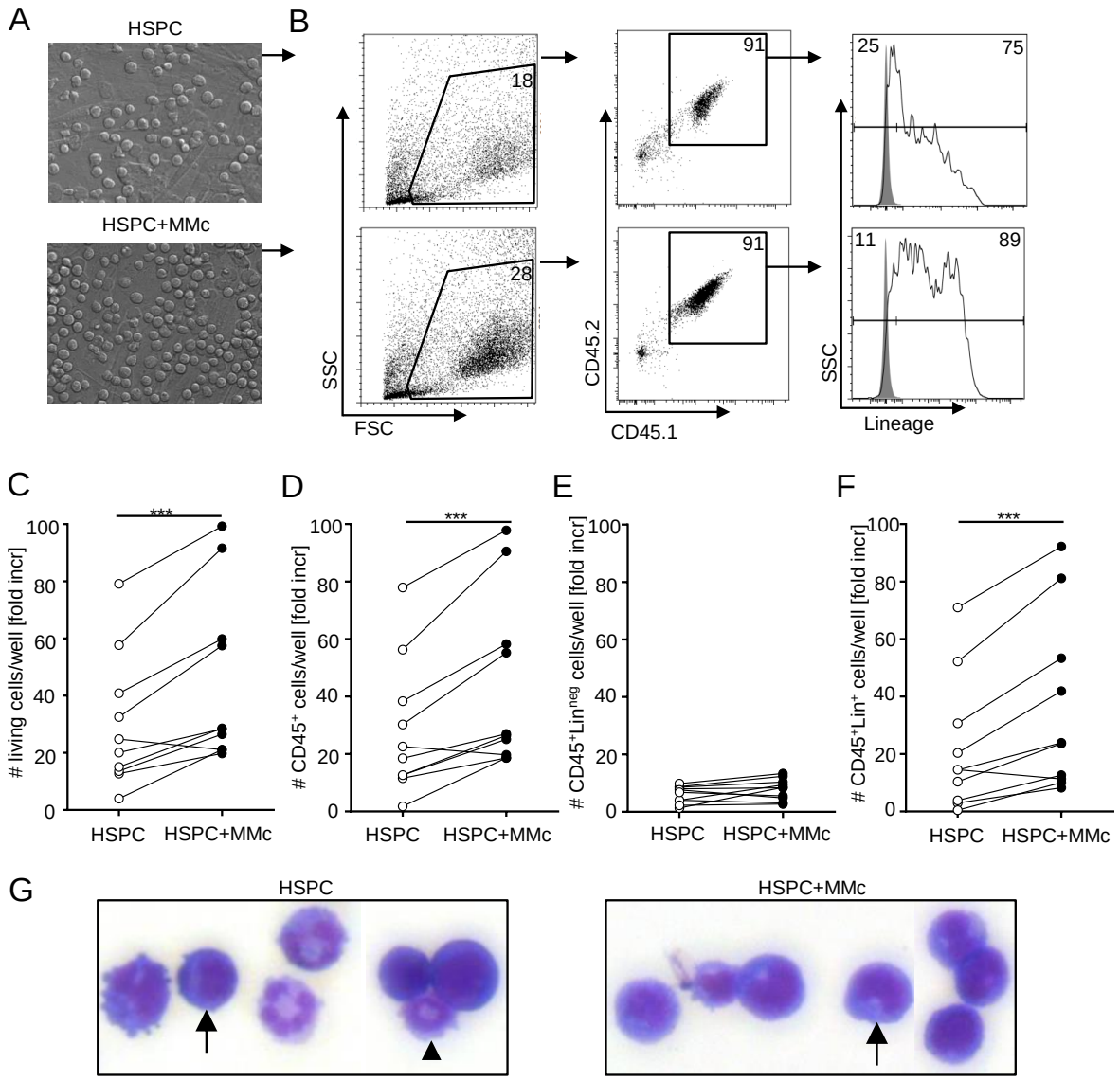

Figure 16: *Maternal microchimeric (MMc) cells promote the differentiation of fetal hematopoietic stem and progenitor cells (HSPC) into Lineage (Lin)$^+$ leukocytes in vitro.* (A) Representative microscopy of fetal HSPC-derived hematopoietic cells after 120h of (co-)culture (magnification: 20x). (B) Gating strategy and representative dot plots of HSPC cultured alone (upper panel) or in the presence of MMc cells (lower panel). Forward (FSC) vs. sideward (SSC) scatter indicates the employed leukocyte gate. Living (7-AAD^{neg}) cells of the leukocyte gate express the fetal genotype CD45.2/1. CD45$^+$ cells stain positive for markers of the committed major immune cell lineages (Lin$^+$), indicating differentiation of HSPC. Grey histogram bars represent fluorescence minus one control for lineage antibody staining. Numbers represent cell frequencies of parent gate. (C-F) Accompanying composite data showing the fold increase of the number of living (7-AAD^{neg}) (C) cells in the leukocyte gate, the number of CD45$^+$ (D), CD45$^+$Lin^{neg} (E), and CD45$^+$Lin$^+$ (F) per well, depicted as fold increase relative to the number of initially seeded HSPC. (G) Morphological assessment of HSPC-derived lineage-committed CD45$^+$ immune cells shows that the presence of MMc cells favors myeloid differentiation towards monocytes/macrophages over granulocyte differentiation. Arrows indicate representative examples of cells with pro-monocytic/macrophage morphology which are more abundant when derived from fetal HSPC in presence of MMc compared to when HSPC are cultured alone. Arrowhead indicates an example of a pre-granulocytic cell. (C-F) n=10 litters. ***: p≤0.001 as assessed by Wilcoxon matched pairs signed rank test.

3.8 A mid-gestational stress challenge results in a reduced frequency of late-gestational fetal innate immune cells in the bone marrow

In addition to the effect of MMc on fetal HSPC that bear long-term potential for self-renewal, we were interested in the short-term consequences of a mid-gestational stress challenge on the development of the fetal immune system. The number of $CD45^+$ leukocytes in the fetal bone marrow was reduced on gd 18.5 in fetuses whose mothers experienced stress during mid-gestation (Figure 17 A), while the overall number of living cells per fetus was not different between groups (data not shown). This overall decrease in bone marrow leukocytes was mainly due to a significant decrease in $CD11b^+$ myeloid cells in stressed fetuses (Figure 17 B). T and B cells were generally much less frequent and remained unaffected by stress challenge. Although innate $CD11b^+$-expressing cells comprise a major immune cell population in the bone marrow, expressed by all cells committed to the myeloid lineage, we cannot exclude that the stress-induced reduction in $CD45^+$ cells is also due to decreases in other hematopoietic cells, such as the erythroid lineage.

In summary, a mid-gestational stress challenge results in a reduced frequency of innate $CD11b^+$ myeloid cells in the fetal bone marrow during late gestation. This data, in combination with our observation of stress-reduced quantities of MMc cells in the fetal bone marrow, which might have a role in stimulating fetal HSPC differentiation towards the myeloid lineage, reveal an immediate stress effect on the development of the fetal innate immune system, with a potential direct involvement of MMc cells.

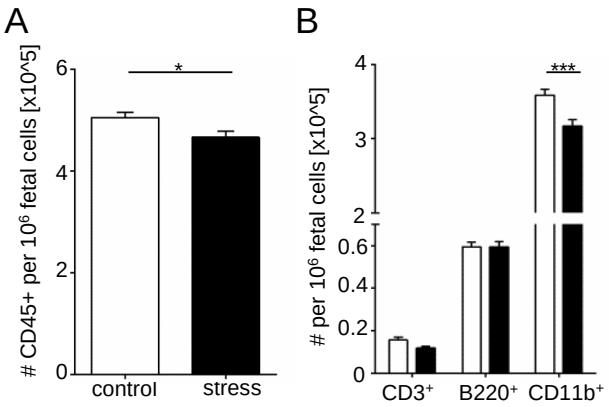

Figure 17: *A mid-gestational stress challenge affects fetal bone marrow (BM) immune ontogeny at late gestation*. Stress decreases the number of fetal $CD45^+$ cells (A), which is due to a reduction in $CD11b^+$ myeloid cells (B). This observation accompanies the trend towards a frequency reduction of maternal microchimeric (MMc) cells in the fetal BM, which promote the differentiation of fetal hematopoietic stem and progenitor cells (HSPC) towards maturation of the $CD11b^+$ monocyte/macrophage lineage. Bars represent mean ± SEM and n fetuses. *: $p \leq 0.05$; ***: $p \leq 0.001$ as assessed by Mann-Whitney-U-test.

4 Discussion

This thesis aimed to investigate maternal microchimerism during late murine gestation and its modulation upon a prenatal maternal stress challenge in order to test the hypothesis of a role of MMc as a mechanism of materno-fetal communication involved in fetal immune development with, if challenged, possible consequences for the child's peri- and postnatal immunity.

Advancing the flow cytometric detection of murine maternal microchimeric cells by usage of two independent cell surface markers (congenic CD45 as well as the MHC class I H-2D haplotype) allowed to enumerating the frequency of MMc cells in various fetal organs during late gestation. The fetal bone marrow was shown to be a major site of MMc. Exploiting the possibility to also phenotypically characterize the MMc population by this technique indicated the heterogeneic presence of the major immune cell lineages in fetal organs. The majority of cells in the fetal bone marrow were determined to be $CD3^+$ T cells. These cells showed expression of cell adhesion molecule ligands VLA-4 and PSGL-1, involved in migration and homing, while they were surprisingly lacking expression of LFA-1. To test their hypothetical transplacental route of migration, the presence of cell adhesion molecule expression in the placental labyrinth, where the trophoblast-endothelial barrier separates fetal from maternal circulation, was assessed. VCAM-1 and P-Selectin, the respective counter-receptors of VLA-4 and PSGL-1, were found to be expressed throughout late mid- to late gestation, providing prerequisites for materno-fetal cell transfer.

To test if prenatal MMc is relevant for immune communication from mother to fetus during pregnancy, the susceptibility of MMc to challenges of the maternal adaptation to pregnancy was tested. Dams were exposed to psychogenic noise stress during mid-gestation, resulting in a strong trend towards a decreased frequency of MMc in the bone marrow in late gestation, which was specifically due to reduced numbers of MMc T cells.

To address a functional role of MMc cells in the fetal BM, a co-culture system was established to test the influence of MMc cells on fetal HSPC homeostasis. MMc cells promoted the differentiation of fetal HSPC as shown by increased levels of $CD45^+Lin^+$ cells when compared to fetal HSPC cultured alone, indicating a role of MMc in promoting fetal hematopoiesis shortly before birth. Additionally, the presence of MMc cells seemed to favor HSPC differentiation and progenitor cell maturation towards monocytes and macrophages over granulocytic differentiation.

This observation was complemented by a significantly decreased frequency of fetal $CD11b^+$ myeloid cells in the BM in fetuses growing in mid-gestationally stressed mothers in comparison to control fetuses. This could be partially mediated by the decreased presence of hematopoiesis-promoting MMc cells in the fetal BM.

Our observations support the initial hypothesis of a direct role of MMc for the offspring's perinatal immune development and its modulation upon a challenge to maternal adaptation to pregnancy.

4.1 Technical advancement of MMc detection

Previous work of our group explored the sensitivity of a molecular biology approach, qPCR, and a cellular approach, flow cytometry, to detect cellular microchimerism. To that purpose, mixed cells suspensions containing reciprocal frequencies of wild type cells and surrogate target cells positive for eGFP or CD45.2, respectively, were analyzed, revealing that both techniques possess the sensitivity and limit of detection to reliably detect microchimeric cells and thus, are suitable for experimental approaches (Thiele et al., 2014). While most published evidence relies on qPCR techniques to identify the frequency of MMc in various solid tissues with high accuracy (Dutta and Burlingham, 2009, Kinder et al., 2015a, Mold et al., 2008), an experimental approach that aims to address the phenotype of MMc cells required a cellular technique, such as flow cytometry. Accordingly, combining the parental expression of the congenic markers CD45.1 and CD45.2 to identify homozygous congenic CD45.2 maternal cells among offspring's heterozygous cells (CD45.2/1) has been employed before to detect and phenotype microchimerism in fetal and neonatal blood (Nijagal et al., 2011). However, flow cytometric detection has intrinsic technical limitations, which need to be controlled carefully to minimize the false positive error rate such as cell auto-fluorescence, unspecific antibody binding and spectral overlap in the fluorescent signal emission.

Here, we sought to introduce the H-2 complex, which defines the MHC class I in mice and is homologous to HLA in human, as an additional independent target to enhance the accuracy of detection of very rare MMc cells among a bulk of fetal haploidentical cells. To this end, we here took advantage of the natural polymorphism between mouse inbred strains with regard to their H-2D gene, which is H-2D^{b} in the C57BL/6 strain and H-2D^{d} in Balb/c mice. As published by our group, including H-2D as additional congenic marker improved the specificity of the identification of MMc cells. False positive signals in the CD45.2^{+} gate were markedly reduced after gating for homozygous H-2D^{b} positivity in the second detection step, thereby enriching for the population of MMc cells among the fetal cells heterozygous for both markers (Solano, 2014). Concomitantly, high forward/sideward scatter thresholds and doublet exclusion during acquisition, as well as viability staining, fluorescence minus one (FMO) and isotype antibody control stainings minimized the inclusion of false positive signals and validated our approach.

Clearly, this approach fundamentally relies on expression of leukocyte-specific CD45, precluding the detection of MMc cells other than of immune lineage origin. It does not allow to experimentally depleting MMc due to the absence of a unique maternal marker for cell-specific targeting by e.g. antibody-mediated depletion (Kinder et al., 2015a). However, the development of this approach takes advantage of the natural genomic variation between mouse strains, thereby avoiding the use of transgenically modified animals or models employing artificially generated microchimerism, as has been done previously using eGFP (Dutta and Burlingham, 2009), luciferase (Su et al., 2008), or neoR (Kaplan and Land, 2005), upon adoptive transfer of labeled, transgenic leukocytes into the mother (Wienecke et al., 2012), by using antigen-specific T cell mouse models (Leveque et al., 2014, Roy et al., 2011) or adoptively transferring wild-type blastocysts into LacZ transgene-tagged mothers (Marleau et al., 2003, Piotrowski and Croy, 1996). All of these approaches can be considered as interventions that may have immunogenic properties, which could interfere with our endeavor to reveal the transfer of a delicate amount of immune cells between mother and fetus. Furthermore, in contrast

to other models that rely on wild-type and heterozygote mutant offspring, among which only in half of the fetuses maternal cells can be identified (Arvola et al., 2000, Dutta and Burlingham, 2009, Kaplan and Land, 2005, Su et al., 2008, Zhou et al., 2000), this model does not require genotyping prior to MMc cell detection, since all offspring is genetically identical, rendering *ex vivo* analyses of freshly isolated cellular material readily feasible. Lastly, the allogeneic mating combination results in semi-allogeneic fetuses, resembling human allogeneic pregnancies, in which maternal microchimeric are recognized by the fetus as NIMA-expressing cells. This is of interest when considering differences in MMc levels between syn-, allogeneic, and outbred offspring, where the frequency is highest in syngeneic offspring, proposing an inherent regulation of the level of MMc by the fetal host (Vernochet et al., 2005, Vernochet et al., 2007).

Interestingly, we observed the level of MHC class I expression to be lower among the detected MMc cells in comparison to maternal PBMC cells (Figure 8 C, E). Similar observations are made in tumor cells, which use alterations of MHC class I cell surface expression as an escape route to avoid immune rejection (Haworth et al., 2015). It is tempting to speculate that a downregulation could be a mechanism of MMc cells to avoid rejection by the fetal immune system. This would allow evasion of deletion by cytotoxic T lymphocytes that respond to foreign antigen while retaining MHC class I on the surface avoids fetal recognition by NK cells that are activated when MHC class I surface expression is absent.

Staining intact cells for flow-cytometry not only enabled to precisely track MMc cells but also to analyze this pool of cells for their phenotype based on expression of cell-lineage defining markers. The potential of subsequent fluorescence activated cell sorting was exploited for *in vitro* functional analyses of MMc in this study. Even further advancing this approach towards single-cell based approaches will facilitate the pursuit of in-depth phenotypic, gene expression and functional analyses of microchimeric cells.

4.2 Transplacental passage

We here show the complementary expression of cell adhesion molecules VCAM-1 and P-Selectin at the feto-maternal interface and their ligands VLA-4 and PSGL-1 on MMc cells, providing evidence of prerequisites for the hypothesis of MMc transplacental migration.

The specific expression of P-Selectin and VCAM-1 in the placental murine labyrinth during mid- and late gestation adds to the published knowledge about their expression at the feto-maternal interface, as prominently shown for the decidua in earlier pregnancy (Kruse et al., 2002, Solano et al., 2011). Cell adhesion molecules are necessary to recruit immune cells to the feto-maternal interface (Blois et al., 2005, Nancy et al., 2012) and their expression is responsive to challenges, e.g. decidual P-Selectin expression at mid-gestation was upregulated provoked by a maternal stress perception, and/or in abortion-prone mouse models (Fernekorn et al., 2007, Tometten et al., 2006). VCAM-1 expression follows a kinetic over the normal course of pregnancy, being highest in decidual blood vessels during mid-gestation (Kruse et al., 2002).

We hypothesized that a mid-gestational stress challenge would lead to alterations in the expression of P-Selectin and VCAM-1. However, in the labyrinth, we found the P-Selectin protein expression intensity to be equal between mid-gestational stress-challenged and control placentae on gd 18.5.

Also, the number of VCAM-1$^+$ blood space linings per total count of blood spaces in the labyrinth was not altered in mid-gestational stress-challenged in comparison to control placentae (data not shown). A mid-gestational maternal stress challenge does thus not persist in an altered cell adhesion molecule expression at the late-gestational feto-maternal interface, while this does not preclude modulations to the CAM machinery that might have occurred earlier during the stress perception period itself or shortly after with consequences for the potential of MMc cells to transmigrate.

In contrast, JAM-B, which we observed to be prominently expressed in the junctional zone on gd 18.5, was susceptible to maternal perceived stress. While the percentage of JZ area positive for JAM-B expression was not different in stressed versus control placenta, surprisingly, the protein expression intensity was significantly decreased in mid-gestational stress-challenged in comparison to control placentae (data not shown), indicating that JAM-B expression is susceptible to modulation by a stress challenge. This adds to our observations of an increase of the percentage of JAM-B expressing area in the junctional zone as well as its expression intensity during mid-to late gestation (Stelzer et al., 2016). Next to its role in adhesion and transendothelial migration, JAM-B is functionally involved in stabilization of inter-cellular contacts, stem cell homeostasis, pro-angiogenesis and vascular lumen formation, processes necessary for a proper development of the placenta. In case of a stress challenge, one could speculate that placental functional integrity might be weakened upon a reduced JAM-B expression. Besides that, progesterone, which is essential for pregnancy establishment and maintenance, had been shown to regulate JAM-B expression during implantation (Su et al., 2012). We showed progesterone to be a negative regulator of JAM-B expression, as apparent in increased protein expression intensities in mid-gestational progesterone receptor-deficient placentae (Stelzer et al., 2016). The possible role of JAM-B as trophoblast-lineage specific protein in supporting placental integrity remains to be investigated.

Since we could not observe JAM-B expression in the labyrinthine intercellular contacts to enable transendothelial migration, other candidate proteins remain to be investigated, among them platelet endothelial cell adhesion molecule (PECAM)-1 (Looman et al., 2007, Prados et al., 2011), vascular-endothelial (VE)-cadherin, and β-catenin (Rutland et al., 2007), which are expressed by endothelial cells at the murine feto-maternal interface.

Additionally, it is possible that the selective passage of MMc cells is driven by gradients of chemo-attractants existing across fetal and maternal compartments. In human pregnancies, the vascular endothelial growth factor (VEGF)-A, which is shown to have chemo-attractant properties next to its angiogenic function (Lash et al., 2003), forms a gradient that increases in concentration from the maternal blood plasma to the human umbilical cord blood. In a cross-species study using rat and human cells, VEGF-A has been shown to promote surrogate microchimeric cell migration via a mechanism that involves VEGF receptor (R)-1, and the integrins α4β1 (VLA-4), α2β1, and α5β1 (Chen et al., 2008). It remains unknown whether this mechanism is also of relevance in mice. The only available study on MMc cell trafficking in mice revealed that the chemokine ligand (CCL) 3, a chemo-attractant highly expressed by the fetal trophoblast during late gestation, is not essential for MMc cell migration (Unno et al., 2010).

4.3 MMc is present in the fetal BM, but scarce in the thymus

The technique to detect MMc cells described above enabled to thoroughly characterize the presence of MMc across various fetal tissues during late gestation. Surprisingly, MMc appeared to be rather rare in the fetal thymus, and not all fetuses were positive for thymic MMc. Published evidence has described the wide-spread presence of MMc in fetal bone marrow, liver, thymus, spleen, peripheral blood, and lung in immune-competent offspring during late gestation using semi-quantitative techniques such as ß-galactosidase-reactivity (Marleau et al., 2003, Piotrowski and Croy, 1996) or detection of GFP^+ cell count by fluorescence microscopy (Unno et al., 2010), as well as overall levels of radioactivity that accumulated in the fetus (Wienecke et al., 2012). Detection of a maternal transgene by PCR revealed the frequency of GFP^+ samples among all organ samples pooled from gd 15.5 until birth (Vernochet et al., 2005) or, by flow cytometry, the number of GFP^+ cells per organ in 32 gd 12 – 18 fetuses (Zhou et al., 2000). In a small representative sample group, $CD45.2^+$ MMc cells were identified among the fetal peripheral blood from mid- to late gestation by flow cytometry (Nijagal et al., 2011).

Here, we enrich these observations with quantitative evidence derived from our MMc detection model and for the first time provide directly comparable MMc quantities between the different fetal organs isolated from gd 18.5 fetuses. In the context of fetal immune development, the observation of the fetal bone marrow being a prominent site of MMc parallels the preceding migration of hematopoietic activity from fetal liver to bone marrow from gd 15.5 onwards and the manifestation of the fetal BM as a major hematopoietic organ in this gestational period. Accordingly, MMc in the fetal liver is quantitatively lower in comparison to the fetal BM on gd 18.5, while it is evident in this organ as early as mid-gestational gd 13.5 (Vernochet et al., 2005), at a time in which the liver assumes key hematopoietic function.

Since the thymus is a primary lymphoid organ during fetal development responsible for differentiation and selection of T cells, the rare presence of MMc cells in this organ was unexpected with regard to our hypothesis of a role of MMc in fetal immune ontogeny. The thymus is colonized by hematopoietic progenitors in waves during gestation, while the first wave spans gd 11.5 – 15.5, and the second wave occurs after gd 16.5 (Misslitz et al., 2006, Shortman and Wu, 1996). Fetal thymopoiesis is dynamically transitioning, initially originating from T cell lineage–restricted progenitor cells, while the second wave shortly before birth no longer gives rise to embryonic T cells and includes less-differentiated progenitors with high proliferate capacity (Ramond et al., 2014). In immune-deficient offspring, the earliest murine MMc has been detected on gd 12.5 in the region of the developing fetal thymus (Piotrowski and Croy, 1996) and MMc has been shown to persist in the thymus postnatally (Kaplan and Land, 2005, Vernochet et al., 2005) and even until adulthood (Leveque et al., 2014). By then, it has been reported to be present among non-hematopoietic thymic epithelial cells (Dutta and Burlingham, 2009) in immune-competent offspring. In human newborns, MMc was highly present in the thymus (Srivatsa et al., 2003), supporting the thymus being one of the targets for MMc migration. In our analyses, the observed relatively small MMc cell count in the thymus in comparison to the fetal BM and its lower frequency among all samples analyzed suggests a rather transient than resident role in this fetal organ, suggesting that MMc cells play a rather minor role in the thymic stromal environment to support T cell development at this gestational age.

Among the various fetal organs, it was aimed to investigate the presence of the major hematopoietic lineages among the MMc population. We surprisingly extremely rarely found $Lin^{neg}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells among the MMc cell pool in the fetal liver, hinting towards the migration of stem and progenitor cells with long-term potential. Evidence from human studies indicates that MMc could be replenished during adult life from microchimeric HSC residing in the offspring's tissues since pregnancy (Hall et al., 1995, Jonsson et al., 2008, Sunku Cuddapah et al., 2010). In mice, maternal DNA was present among $Lin^{neg}c\text{-}Kit^{+}$ HSC in the bone marrow and heart of 6 – 8-week-old mice (Dutta and Burlingham, 2009, Dutta and Burlingham, 2010). These intriguing observations certainly require further experimental proofs.

Among the committed lineages, we found minor percentages of $CD11c^{+}$ dendritic cells and NK cells in the fetal bone marrow, liver and spleen. This indicates the establishment of antigen-presenting cell (APC) MMc during gestation, matching the detection of maternal antigens among $CD11c^{+}$ and MHC class II^{+} cell populations in the fetal peripheral blood and spleen of young adult mice, suggesting that a portion of persisting MMc cells could belong to the APC compartment (Dutta and Burlingham, 2009, Dutta and Burlingham, 2011, Nijagal et al., 2011). Also MMc NK cells were found before in fetal, neonatal and adult lymphoid organs and peripheral blood (Nijagal et al., 2011, Vernochet et al., 2005).

We found myeloid MMc to be rare in the late gestational fetal BM, while it was more frequent in the fetal liver. Isolated from adult bone marrow and spleen, maternal-specific DNA was identified *ex vivo* and *in vitro* among $CD11b^{+}$ myeloid cells (Dutta and Burlingham, 2009, Dutta and Burlingham, 2010, Dutta and Burlingham, 2011). Even in a non-lymphoid tissue, the heart, maternal DNA has been shown to be present among cardiac macrophages scattered across the tissue (Dutta and Burlingham, 2009).

Further, B cells were the main immune cell type among MMc in the fetal liver. B cells were found in fetal, neonatal and adult lymphoid organs and peripheral blood (Nijagal et al., 2011, Vernochet et al., 2005). Interestingly, in offspring genetically deficient in B cells, MMc cells were a source of IgG-secreting cells (Arvola et al., 2000), providing evidence for the capacity of MMc cells to substitute functionally for offspring's immune deficiencies.

Overall, the presence of different immune cells among the MMc population in the fetus and adult suggests a variety of functions that these cells could adopt in the various tissues they home to and reside in. Our data complement the earlier notion of a potential selectivity of MMc cell transfer, showing that the phenotype of the MMc cells in the fetal circulation differed from the maternal peripheral blood composition (Nijagal et al., 2011). While we observe the fetal liver MMc cell composition to resemble its source, possibly due to the placental blood flow draining into the fetal liver, the MMc population composition in the fetal bone marrow strongly diverged from the one in the maternal peripheral blood, especially with regard to the high frequency of T cells. Mechanisms guiding the targeted homing of different MMc cell types remain unknown.

4.4 MMc T cells in the offspring

Furthermore, we here report the pre-dominant existence of MMc T cells among MMc cells in the fetal bone marrow during late gestation. Several previous studies focused on MMc T cells (Leveque et al., 2014, Roy et al., 2011, Vernochet et al., 2005, Wan et al., 2002) owing to their relative longevity,

capacity to specifically recognize non-self-antigens, and the central role they play in adaptive immune responses. In mice, MMc T cells were found in fetal and neonatal lymphoid organs (Leveque et al., 2014, Nijagal et al., 2011, Vernochet et al., 2005) and endured until adult life, when both $CD4^+$and $CD8^+$ T cells could be identified (Dutta and Burlingham, 2009, Dutta and Burlingham, 2011). Interestingly, MMc $CD4^+$ T cells were also found in non-lymphoid organs, such as the skin and small intestine of neonates and also in the small intestine of adult animals (Leveque et al., 2014). In humans, maternal MMc T cells have been identified as early as in fetal organs of the 2^{nd} trimester (Jonsson et al., 2008) and in cord blood of male neonates (Hall et al., 1995). Their life-long persistence is evident in peripheral blood of infants, adolescents (Kowalzick et al., 2005, Reed et al., 2004), and adult women (Loubiere et al., 2006).

Whilst the functionality of these cells was not directly assessed in the murine models mentioned above, MMc T cells significantly influenced the offspring's immunity, triggering either tolerance to foreign antigens (Fujii and Yamaguchi, 1992, Wan et al., 2002) or the loss of tolerance and subsequent inflammation when MMc cells were highly allo-reactive toward the offspring's antigens (Leveque et al., 2014, Roy et al., 2011). In addition to allo-antigen presentation, MMc may contribute to the protection of the organism from foreign challenges during early life. For example, in a mouse model of in utero hematopoietic cell transplantation (IUHCTx) the engraftment was limited by MMc T cells (Nijagal et al., 2011). In humans suffering from severe combined immunodeficiency (SCID), maternal T cells engraft in the fetus and are capable of expansion in the infant (Liu et al., 2016, Touzot et al., 2012).

In summary, MMc T cells can fulfil specific functions in the offspring, underlining their potential meaning for immune development when residing in the fetal bone marrow.

4.5 MMc T cells in the fetal bone marrow

MMc cell migration takes place in parallel with the egress of fetal HSC from the fetal liver and their circulation and homing to the fetal bone marrow to there establish the definitive hematopoietic activity. Fetal liver HSCs circulate and migrate into the fetal bone marrow in response to stromal cell-derived factor (SDF)-1α which binds to its receptor CXCR4 (Christensen et al., 2004). This interaction remains important for maintenance of the HSC in the bone marrow niche, a specialized microenvironment supporting HSC survival and function. This niche is found in the perivascular region of sinusoids and arterioles and contains osteoblasts, osteoclasts, endothelial cells, adipocytes, and mesenchymal/stromal cells, which control HSC through secretion of not only SDF-1α (Ding and Morrison, 2013), but also stem cell factor (SCF) (Ding et al., 2012), as well as angiopoietin-1 (Riether et al., 2015). Additionally, cell-to-cell contact between VCAM-1 and its binding integrin VLA-4 is a key signal for niche retention of HSC (Mendez-Ferrer et al., 2010). VCAM-1 is constitutively expressed in the adult bone marrow microenvironment by endothelial (Zhao et al., 2012) and mesenchymal cells (Chow et al., 2013). Notably, VCAM-1/VLA-4 interaction is re-inforced by CXCR4 engagement (Hidalgo et al., 2001, Petty et al., 2009). In the fetal bone marrow, VCAM-1 expression starts around gd 16.5 and after gd 18.5, the area of VCAM-1 expression spreads throughout the hematopoietic compartment (Tada et al., 2006).

The majority of MMc cells homing to the fetal bone marrow were T cells, which expressed mainly VLA-4 enabling them to bind to VCAM-1, suggesting similar mechanisms of homing to and retention in the bone marrow may apply as for HSCs. In reductionist studies, α4 integrins (VLA-4 is assembled from α4 and β1 integrins) have been shown to be involved in interactions of immune cell precursors with the bone marrow stromal cells in the peripartal period (Arroyo et al., 1996). Also, the bone marrow microenvironment provides an appropriate support for extrathymic T cell development in infant mice (Dejbakhsh-Jones et al., 1995), suggesting that the fetal bone marrow may display structural and functional features of a lymphoid organ appreciated by immune cells, especially T cells.

MMc T cells also expressed PSGL-1, enabling them to bind to P-Selectin expressed in the bone marrow vasculature and stroma (Zhao et al., 2012), although on a lower level as compared to the maternal peripheral blood. Its expression level was more comparable to the expression of CAML observed in the maternal uterus draining lymph nodes (data not shown), suggesting that BM-homed T cells might adapt their integrin surface localization to the local secondary lymphoid organ-like environment provided by the bone marrow.

We found the MMc T cells in the fetal bone marrow to show a specific phenotype of VLA-expression, while PSGL-1 was expressed to a lower extent and LFA-1 was almost completely absent. LFA-1 not only functions as an adhesion molecule but is at the same time involved in the dendritic cell-T cell immunological synapse for promoting early T cell activation. Binding is stimulated at very low antigen concentrations and is initially weak until a TCR-signal enhances the cell contact leading to T cell proliferation in response to antigen presentation (Bachmann et al., 1997, Li et al., 2009, Mueller et al., 2004). The suspicious absence which might be due to downregulation or surface dislocation of LFA-1 on MMc T cells might render these T cell less responsive towards antigen presentation. This could be a mechanism to avoiding recognition of semi-allogeneic fetal antigens presented by fetal APCs thereby preventing activation and ensuring their survival in the host. Additionally, this phenotype underlines a function of these cells which might be different from being effectors in an (auto-)immune response.

T cells with this phenotype were extremely rare among the maternal peripheral blood cells. At this point of the study, there is no conclusion possible whether this specific T cell adhesion molecule signature is necessary to migrate transplacentally or whether it is acquired during homing to and required for residency in the fetal bone marrow. A proportion of fetal T cells present in the fetal bone marrow expressed the same phenotype (data not shown), suggesting it might be specific to bone marrow T cells. A next experimental approach would need to interfere with MMc migration by antibody-mediated blocking of VLA-4 using a Natalizumab analogon, injected into the mother from gd 12.5 onwards. The expected observation of a depletion of MMc in the fetus would then support the hypothesis of the essential requirement of VLA-4 for transplacental MMc migration.

In the adult stem cell niche, cells of the innate and adaptive immune system are known to contribute to regulation of HSPC during homeostasis, inflammation and infection. Various mature immune cells including T and B cells, plasma cells, NKT cells, dendritic cells, neutrophils, macrophages, and myeloid-derived suppressor cells are described to reside in the bone marrow (Zhao et al., 2012, Mercier et al., 2012). In addition to $CD3^+$ T cells, we here report the migration of adult maternal microchimeric $CD11b^+$ myeloid cells and $B220^+$ B cells into the fetal bone marrow. At least in

the adult, the bone marrow serves as a reservoir for mature myeloid cells and supports naïve B as well as antibody-secreting plasma cells (Mercier et al., 2012), which home back to the bone marrow after peripheral antigen-exposure and there maintain their longevity in specialized microenvironments, thus ensuring long-term immunity (Zhao et al., 2012). As for the MMc T cells, expression of cell adhesion molecules on the microchimeric myeloid and B cells might enable their lodging into their respective niches.

4.6 MMc cells promote fetal hematopoiesis *in vitro*

We here observe maternal microchimeric cells to promote hematopoiesis derived from late gestational fetal HSPC *in vitro*. As shown previously in morphological studies during late gestation, MMc cells presented to be scattered across the bone marrow, localizing next to hematopoietic cavities (Piotrowski and Croy, 1996), which in turn express VCAM-1 during late gestation (Tada et al., 2006). The process of hematopoiesis is hierarchically regulated, as depicted in Figure 18.

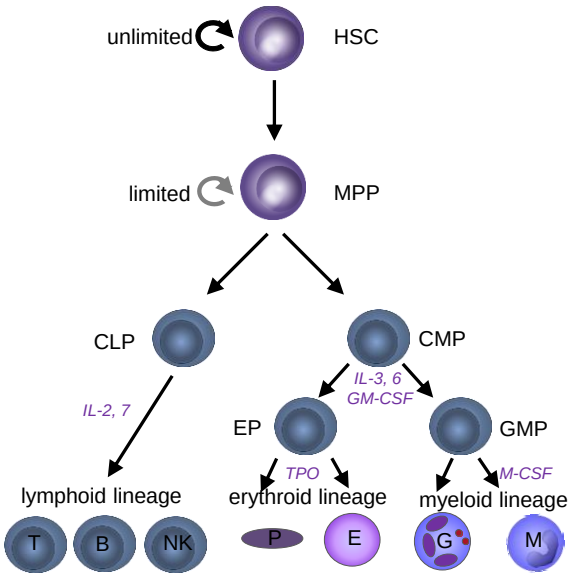

Figure 18: *Hierarchical structure of hematopoiesis*. Rarely dividing hematopoietic stem cells (HSC) with unlimited self-renewal capacity, defined as Lin^{neg} (negative for surface marker expression of the committed myeloid or lymphoid lineages) Sca-1^{+}c-Kit^{+}, give rise to multipotent progenitors (MPP) which have limited or lost long-term self-renewal potential. MPPs differentiate into lineage-restricted oligopotent progenitors consisting of common lymphoid precursors (CLP) and common myeloid precursors (CMP), the latter in turn giving rise to erythroid precursors (EPs), and granulocyte/macrophage precursors (GMP). In response to specific factors of the hematopoietic niche (Interleukin (IL)- 2 and -7 for lymphoid lineage, IL-3, -6, and granulocyte macrophage colony-stimulating factor (GM-CSF) for the myeloid lineage, macrophage colony-stimulating factor (M-CSF) for monocyte, and thrombopoietin (TPO) for megakaryocyte differentiation), these progenitors proliferate intensely and produce all mature blood cell types of the erythroid, myeloid and lymphoid lineages, such as megakaryocyte progenitors of platelets (P) and erythrocytes (E); monocytes/macrophages (M) and granulocytes (G), as well as pro-T, pro-B, pro-dendritic and Natural Killer (NK) cells. Lineage-restricted progenitors undergo additional maturation, selection, and specification to become mature effector cells, capable of migrating into peripheral and secondary lymphoid tissues (modified from (Riether et al., 2015)).

This role in increasing hematopoietic cell numbers suggests MMc cells to equipping the fetal immune system for the anti- and pathogenic challenges during birth and in the early neonatal life. In the adult, during high pathogenic load such as infections, innate immune cells need to be rapidly

replenished from BM HSPC, when immune effector cells are heavily consumed at inflammatory sites. In the bone marrow, the cellular composition of the microenvironment as well as the cytokine milieu change fundamentally to meet the requirements for this so-called demand-adapted hematopoiesis (Takizawa et al., 2012). MMc could be a mechanism to stimulate the maturation of the fetal innate immune capacity shortly before birth, since HSPC differentiation was skewed towards maturation of pro-monocytes and mature macrophages, thus myelopoiesis, in the presence of MMc cells.

While data of a direct role of fetal immune cells in prenatal hematopoiesis are elusive, such relations are recognized for regulation of adult stem cells by cells of the immune system. Macrophages in the adult murine BM secrete soluble factors that, besides being involved in erythropoiesis, indirectly regulate hematopoiesis by stimulating mesenchymal cells to produce HSC retention factors (Chow et al., 2013, Ludin et al., 2012). Bone marrow resident B cells secrete considerable amounts of IL-6 and Transforming growth factor (TGF)-β, contributing to a myelopoietic balance (Tokoyoda et al., 2004).

The T cells among the MMc cells migrating into the fetal bone marrow reside next to a population of fetal T cells, which in the present study make up less than 0.1% of all cells present in the fetal bone marrow on gd 18.5. The adult mouse bone marrow contains 1% – 5% $CD3^+$ T cells among the mononuclear cells, including both $CD4^+$ and $CD8^+$ T cells (Zhao et al., 2012). A significant proportion of $CD4^+$ T cells are $CD4^+CD25^+$ Treg cells, which have been described to confer immune-privilege to the BM HSC niche (Fujisaki et al., 2011). These cells are rather suppressive to colony formation and myeloid differentiation of HSPC (Urbieta et al., 2010). In contrast, $CD4^+$ T cells with a memory phenotype have been shown to reside in the adult bone marrow in close association with VCAM-1 expressing stromal cells (Tokoyoda et al., 2009), and it is these cells, and not $CD8^+$ T cells, that secrete hematopoiesis-promoting cytokines such as IL-3 and Granulocyte macrophage colony-stimulating factor (GM-CSF) (Monteiro et al., 2005, Sharara et al., 1997). Another cytokine, interferon (IFN)-gamma, secreted by activated T cells, exerts its effects on hematopoiesis rather indirectly: mesenchymal cells are stimulated to secrete IL-6, which in turn induces the proliferation of multipotent progenitors and myeloid differentiation (de Bruin et al., 2012, Schurch et al., 2014). Lastly, CD40L, prominently expressed by T cells, has been determined to promote myelopoiesis from human $CD34^+$ progenitors isolated from cord blood *in vitro*. This, again, is indirectly mediated via upregulation of SCF and thrombopoietin production by stromal cells (Solanilla et al., 2000).

It is rather speculative to translate the involvement of adult (human) T cells in adult hematopoiesis to the fetal situation. Fetal and adult hematopoietic progenitors are distinct populations (Perdiguero and Geissmann, 2016) that give rise to immune effector cells specific to the developmental needs of the organism (Burt, 2013). It can be assumed that these cells in consequence also react to different cues for homeostasis and differentiation. Still, some evidence suggests the involvement of $CD3^+$ T cells in HSPC regulation during the fetal period:

Fetal $CD3^+$TER-119^{neg} cells, isolated from the fetal liver Lin^+ fraction on gd 15.5, have been shown to stimulate the expansion of fetal liver HSC ($Lin^{neg}Sca\text{-}1^+c\text{-}Kit^+$). This was due to their production of different growth factors, among them angiopoietin-like protein 2 and Insulin like growth factor (IGF-2), the latter binding to and increasing the *in vivo* long-term repopulation potential of fetal HSC transplanted into irradiated mice (Zhang and Lodish, 2004). Further analyses confirmed the significance of IGF-2 in promoting the expansion of long-term-HSC from the mouse adult bone marrow

ex vivo (Zhang and Lodish, 2005). Interestingly, these effects on the *in vivo* repopulation capacity of gd 15.5 fetal HSC were observed after a three day co-culture period at a ratio of 1 HSC to 1 $CD3^{+}TER\text{-}119^{neg}$ (Zhang and Lodish, 2004). In our setting on gd 18.5, an even lower ratio of 10 HSPC to 1 MMc cell was sufficient to reveal a hematopoiesis-promoting effect after 5 days of co-culture. This indicates that HSPC maintenance and proliferation is strongly influenced by mid-gestational fetal liver $CD3^{+}$ and late-gestational fetal BM MMc cells, both very rare populations among all cells in the hematopoietic organs at the respective gestational age.

During experimental establishment of the fetal HSPC culture, initially, primary fetal $CD45^{neg}$ BM stromal cells were taken as feeder layer to cultivate fetal HSPC. Pilot data revealed the same results with regard to HSPC differentiation as presented in this thesis, however, heterogeneity of the feeder layer resulting in fluctuating proliferation rates was inherent to the primary cell stock. To overcome this influencing factor we employed the commercially available cell line OP9, which is derived from newborn bone marrow stromal cells (Nakano et al., 1994). This ensured reproducibility of co-culture results and excluded the possibility that the allologous stroma would nebulize the effect of the small quantities of co-cultured MMc cells. In addition, OP9 cells are devoid of Macrophage colony-stimulating factor (M-CSF) expression due to a mutation in its gene (op/op) (Yoshida et al., 1990). As determined in knock-out studies, M-CSF is a major factor responsible for embryonic macrophage development from monoblasts and pro-monocytes (Pollard, 2009). The absence of stromal M-CSF implies to allocate responsibility for the enhanced differentiation of pro-monocytes and mature macrophages in fetal HSPC-MMc cell co-culture directly to the MMc cells, at least with regard to the potential secretion of this specific hematopoietic cytokine.

In summary, at least in the adult, the bone marrow has been described to function as secondary lymphoid organ which harbors mature, resident immune cells which, in concert with the stromal niche, are involved in regulation of hematopoiesis. During the establishment of the fetal bone marrow as the definite site of hematopoiesis during late gestation, MMc cells could serve to support this process. It remains to be investigated whether MMc secrete hematopoiesis-promoting factors that specifically induce myeloid lineage differentiation thereby equipping the fetus with appropriate amounts of innate cells during the perinatal period. Future analyses will aim to identifying IL-3, IL-6, GM-CSF, and M-CSF in co-culture supernatants.

4.7 Prenatal stress challenge and the modulation of MMc

We here tested the influence of a maternal stress challenge during pregnancy on the frequency and phenotype of prenatal MMc in the fetal bone marrow and observed a strong tendency towards a decreased frequency, which was due to a reduction in MMc T cell numbers. MMc presents with an inherent disparity in frequencies between individuals due to modulating factors such as materno-fetal histo(in)compatibility in humans (Berry et al., 2004) and mice (Kaplan and Land, 2005, Lopez-Guisa et al., 2011, Marleau et al., 2003, Piotrowski and Croy, 1996, Vernochet et al., 2005, Nijagal et al., 2011, Wegorzewska et al., 2014, Wienecke et al., 2012), variations in fetal immunocompetence (Piotrowski and Croy, 1996, Zhou et al., 2000), and states of fetal injury and inflammation (Nijagal et al., 2011, Saadai et al., 2012, Wegorzewska et al., 2014, Wienecke et al., 2012). Maternal stress perception has been associated with a pro-inflammatory environment at the materno-fetal interface, leading us to

hypothesize this could be mirrored in a pro-inflammatory MMc footprint that migrates into the fetus. In previous studies of states of fetal injury and inflammation, such as intrauterine non-specific tissue injury (Nijagal et al., 2011, Wegorzewska et al., 2014), human prenatal surgery (Saadai et al., 2012), or pertussis toxin-induced asymptomatic systemic inflammation in pregnant dams (Wienecke et al., 2012) enhanced MMc migration was shown. In contrast, we here observe a trend towards reduced numbers of MMc cells in the bone marrow.

The observed trend could be due to a decreased overall migration, decreased homing to this specific fetal tissue, egress from the bone marrow after initial homing, or a reduced expansion or survival. Of note, since we did not distinguish fetal samples by gender, we cannot rule out a possible sex difference in MMc frequency between male and female fetuses. The reduction we observe here is due to the significantly reduced proportion of fetuses with a high frequency of MMc cells upon a stress challenge in comparison to the control group. Morphological assessments of fetal BM MMc cells revealed their potential selective proliferation, as demonstrated by an increased quantity and estimated diameter from gd 16.5 until gd 18.5 – 19.5 in unchallenged fetuses (Marleau et al., 2003, Piotrowski and Croy, 1996) which could argue for a disturbed expansion or survival upon a prenatal stress challenge. Other than that, (1) their re-distribution into different tissues to patrol the periphery after sensing a stressed fetal environment, (2) their reduced local expansion or (3) an overall reduced level of MMc migration could be of reason. In all cases, lower numbers of fetal BM-resident MMc cells could result in a less efficient stimulation of myeloid hematopoiesis in stress-challenged pregnancies.

A re-distribution of MMc T cells (1) into fetal peripheral or other lymphoid tissues seems plausible on the one hand when considering evidence for the presence of MMc hematopoietic cells in addition to lymphoid organs in non-lymphoid organs such as lung and heart of both mouse (Piotrowski and Croy, 1996, Unno et al., 2010) and human fetuses (Jonsson et al., 2008) and human fetal kidney, adrenal gland, pancreas, brain, ovary, and testis (Jonsson et al., 2008). This implies that they could participate in peripheral immune monitoring. Earlier reports strongly suggest a role in the surveillance of the offspring's developing system under adverse conditions. MMc immune cells have been shown to protect the offspring's organism from foreign challenges and compensate for immature immune function, such as the MMc T cell-mediated limitation of in utero transplant engraftment in mice (Nijagal et al., 2011) and MMc T cell engraftment in human infants suffering from SCID (Liu et al., 2016, Touzot et al., 2012). In line with this, IL-2-deficient offspring acquired IL-2-expressing cells in the thymus and spleen, probably via materno-fetal transmigration (Wrenshall et al., 2007).

On the other hand, the peripheral presence of MMc cells has been suggested to play a role in the pathogenesis of auto-immune diseases (Lopez-Guisa et al., 2011). Their semi-allogenicity may provoke an offspring's immune response in the long term, or, dependent on the MMc cell phenotype, they may themselves be reactive toward the offspring's antigen, thereby initiating inflammation and predisposing to auto-immunity (Leveque and Khosrotehrani, 2011). In mice, a model of juvenile type I diabetes showed that MMc T cells with an ovalbumin (OVA)-specific T cell receptor in offspring that expressed OVA in the pancreatic β cells triggered peri-insulitis in the young adult offspring (Roy et al., 2011). In a similar transgenic approach, highly allo-reactive MMc T cells induced inflammation in the small intestines of 6 - 8-week-old offspring (Leveque et al., 2014). It has been suggested that antigen-specific MMc T cells may educate the offspring's immune cells to elicit an immune response against

self-antigens through a shift of antigen-presenting cells (APC) toward an immunogenic phenotype (Roy et al., 2011) and skew immunity toward auto-reactivity by suppressing the fetal regulatory compartment (Leveque et al., 2014).

A reduced local expansion (2) or their overall reduced level of migration (3) into the fetus upon stress would result in a reduced exposure of the fetus to tolerance-inducing benefits of MMc.

Semi-allogeneic MMc cells express NIMA, which are foreign to the offspring's immune system and shape the offspring's immune ontogeny with consequences for its postnatal life. During late gestation, $CD8^+$ T cell tolerance toward NIMA was shown to be achieved in the thymus (Akiyama et al., 2011). In the periphery, gestational NIMA exposure induces the activation and expansion of specific $CD4^+CD25^+FoxP3^+$ Treg cells in young adults (Akiyama et al., 2011, Matsuoka et al., 2006, Molitor-Dart et al., 2007). Here, Tregs possessed a NIMA-specific suppressive function (Dutta and Burlingham, 2009, Matsuoka et al., 2006, Molitor-Dart et al., 2007), which correlated with the level of MMc found in immune and non-lymphoid tissues of the animals analyzed (Dutta and Burlingham, 2009). Further, MMc induces the suppression of murine cytotoxic T cell responses (Akiyama et al., 2011, Bonilla et al., 2006). Parallel oberservations were made in human fetuses, where maternal alloantigen exposure induced the maturation of allo-antigen-specific fetal Tregs (Mold et al., 2008).

MMc introduces antigens to the fetus, which can result in long-lasting tolerance towards these antigens. MMc may not only display maternal alloantigens to the fetus. It has been suggested that MMc cells could also expose it to foreign material such as food and bacterial antigens in preparation for postnatal immune challenges (Mold et al., 2008). In a process referred to as de novo allo-antigen acquisition, professional APCs of the offspring could internalize and re-present maternal antigen at the offspring's periphery (Bracamonte-Baran and Burlingham, 2014, Dutta and Burlingham, 2009, Dutta and Burlingham, 2011). Indeed, there is evidence that such a mechanism could play a role in the case of asthma, where it has recently been suggested that the presence of MMc might confer protection against development of the allergic disease (Thompson et al., 2013). This may be due to the migration of allergen-specific MMc T cells that might introduce the offspring to this specific allergen, a mechanism proposed from studies in mice (Fujii and Yamaguchi, 1992, Wan et al., 2002).

In conclusion, the modulated MMc cell frequency in the fetal bone marrow can be assumed to be only one of the alterations in the MMc footprint that occurs upon a maternal stress challenge. MMc cells originating in a stressed maternal environment might be skewed from exerting beneficial functions such as the published observations of immune monitoring and tolerance induction, or, as we report here for the first time, promotion of hematopoiesis, towards rather detrimental signal transduction, representing a "stressed" phenotype. To further advance our understanding of a role of MMc for the offspring's immune system, a deeper characterization of the naturally occurring MMc T cell phenotype and its modulation upon a stress challenge could provide hints for functional involvements. Elucidating an effector memory (either CD4 or CD8) or innate-like (γδ T cell) phenotype could indicate a potential role in the pathogenesis of auto-immunity, e.g. in case of the expression of MHC class II molecules necessary for antigen presentation to fetal APCs, B and T cells.

4.8 Prenatal stress and consequences for the offspring's immune function

Adverse maternal conditions during stress perception are accompanied by changes in the immune function of the offspring. In human cord blood samples, prenatal stress resulted in altered pro-inflammatory cytokine profiles, such as higher levels of interleukin (IL)-1, 4, 5, 6 (Andersson et al., 2016b, O'Connor et al., 2013) and 8 (Andersson et al., 2016b, Wright et al., 2010), higher Immunoglobulin (Ig)E antibody levels (Lin et al., 2004, Peters et al., 2012) and inhibited adaptive immunity in response to mitogen stimulation (O'Connor et al., 2013). This cytokine profile indicates a bias towards a T helper (Th) 2-skewed immune response, which is also observed in mice (Iwakabe et al., 1998, Pincus-Knackstedt et al., 2006) and persists in adolescent (Veru et al., 2015) and adult prenatally stressed humans (Entringer et al., 2008). Experimental studies aiming to elucidate persistent stress-induced alterations in more detail gained inconsistent results regarding IL-6, IL-2, and Interferon (IFN)-γ secretion, probably due to differences in species, stress type, duration and time point between studies (Coe et al., 2002, Collier et al., 2011, Iwakabe et al., 1998, Pincus-Knackstedt et al., 2006, Vanbesien-Mailliot et al., 2007). Still, secretion of the Th1 cytokine Tumor necrosis factor (TNF)-α was decreased in young monkeys (Coe et al., 2002) and murine offspring (Collier et al., 2011, Pincus-Knackstedt et al., 2006) of stressed mothers.

Adult adaptive and innate immune responses were shown to be affected when the offspring was stressed during the period of maturation of HSC in the fetal liver and colonization of the bone marrow by HSC, corresponding to mid-to-late gestation (gd 12.5 – birth) in mice (Veru et al., 2014). Adult rat T cell populations were found to be decreased with regard to $CD4^+$ T cells (Gotz et al., 2007) or modulated in case of $CD8^+$ T cells frequencies (Llorente et al., 2002, Vanbesien-Mailliot et al., 2007). A weakened Th1 type immunity in response to challenges such as skin allograft transplantation or a model of induced arthritis was apparent in prenatally stressed rats (Gorczynski, 1992, Sobrian et al., 1997). Pokeweed mitogen-induced proliferation of B cells was decreased in prenatally stressed adult and adolescent rats (Gotz and Stefanski, 2007, Gotz et al., 2007, Kay et al., 1998).

The collection of all these snapshot examples of immune shifts in adult prenatally stressed offspring substantiate the influence early environmental conditions have on the outcome of an individual's immunity.

The prenatal stress challenge employed in this study resulted in a reduced overall number of $CD45^+$ leukocytes in the fetal bone marrow during late gestation. Reduced numbers of whole leukocyte counts were shown to persist until adulthood of prenatally stressed adult offspring in rat and swine (Couret et al., 2009, Gotz and Stefanski, 2007). The decrease we observed here was due to a decrease in innate $CD11b^+$ myeloid cell frequency. As is the case in adult mice, $CD11b^+$ cells, including granulocytes, monocytes and mature macrophages, play a key role in the innate response to pathogens. They constitute the primary host defense in the mouse embryo. Their reduced number in the fetal bone marrow in response to stress might be due to a decrease in hematopoietic output, possibly in part due to lower levels of myelopoiesis-promoting MMc cells or a disturbed functionality of MMc cells derived from stressed pregnancies. Alternatively, $CD11b^+$ cells could have been migrated into the periphery of the stressed fetus. Published experimental evidence shows that innate immune cell subsets are able to react towards stressors e.g. social stress. Adult innate immune cells were mobilized by stress and a reduction in bone marrow glucocorticoid-insensitive $CD11b^+$ cell frequencies

was reported due to their re-distribution into the circulation and spleen (Engler et al., 2004, Engler et al., 2005). Similarly, macrophages were specifically recruited from the bone marrow to the perivascular spaces in the brain, accompanied by increased circulating monocyte levels, and were involved in anxiety-like behavior in adult socially stressed mice (Wohleb et al., 2013). Also functionally, adult monocytes were primed by stressors like social defeat, resulting in an increased pro-inflammatory cytokine production (Bailey et al., 2009, Wohleb et al., 2011) and glucocorticoid insensitivity (Stark et al., 2001). Similarly, prenatal stressors, e.g. endotoxin-mediated pro-inflammatory conditions, were observed to result in a reduced innate response in the adult and senescent rat offspring (Hodyl et al., 2007). More specifically, macrophage activity in offspring of murine dams stressed during late gestation was hampered with regards to phagocytosis and spreading (Fonseca et al., 2005, Palermo Neto et al., 2001), indicating the susceptibility of the myeloid lineage to programming during the prenatal period. Thus, reduced frequencies of $CD11b^+$ cells in the fetal bone marrow after a midgestational stress challenge might be due to their mobilization into the periphery and could be accompanied by functional alterations.

Apart from the long-term consequences on innate immunity, the acute reduction of monocytic cells might threat fetal development. Evidence for the importance of macrophages during fetal development shows their involvement in ductal branching, neuronal networking, angiogenesis, lung development as well as bone morphogenesis (Jones and Ricardo, 2013, Pollard, 2009). Decreased numbers or an impaired function of myeloid cells might thus not only hamper proper immune responses in case of perinatal infections but could program fetal lung organogenesis (Jones et al., 2013) and bone marrow niche formation with consequences for hematopoietic capacity (Begg et al., 1993, Pollard, 2009).

Ultimately, cells of the myeloid lineage are short-lived cells. Long-lasting consequences on the offspring's immunity would be expected to be primed in fetal HSPC that possess long-term potential presumably via epigenetic mechanisms. Different methylation patterns have been observed between fetal liver and young postnatal HSCs (Beerman et al., 2013), suggesting a potential susceptibility to challenges during this fetal-to-adult transition which is ongoing during murine late gestation. In the context of stress perception, glucocorticoid effects on HSC differentiation need to be considered. The methylation of genes involved in glucocorticoid receptor signaling pathways is altered when HSC commit to common myeloid and megakaryocyte-erythrocyte progenitors (Bartholdy et al., 2014) suggesting the responsiveness of hematopoietic progenitors to glucocorticoid signals. Further, glucocorticoids might play a role in the perinatal bone marrow niche, as suggested by their promotion of osteoblast proliferation *in vitro* and increased levels of CXCL12 expression on osteoblasts (Kollet et al., 2013). CXCL12 is an important factor in HSC chemotaxis and quiescence, again indicating that glucocorticoids could indirectly influencing hematopoietic capacity and alter the developing environment fetal HSC home to and will reside in throughout life. A potential involvement of MMc cells in providing epigenetic cues towards the dynamic regulation of HSC in the perinatal period remains to be investigated.

4.9 Scientific significance and outlook

The data presented in this thesis provides support for a role of MMc cells as materno-fetal messengers during pregnancy, which are functionally involved in fetal immune development and susceptible to modulation by a maternal stress challenge (Figure 19). These observations add knowledge to our understanding of MMc being a beneficial signal for the fetus, while at the same time implying the strong potential of detrimental consequences for the fetal recipient via direct cell-to-cell signaling, in case of adverse maternal pregnancy conditions.

An immediate question deriving from this data is whether a maternal stress-induced reduced fetal myelopoiesis and the concomitant lower frequency of available innate immune cells results in higher susceptibility towards perinatal infection. In fact, neonatal mice, like newborn humans, are intrinsically susceptible to infection. This has been shown to be due to a suppressive population of $CD71^+$ erythroid cells which at the expense of high infection susceptibility allow for commensal microbial colonization in neonatal mice by suppression of aberrant immune activation (Elahi et al., 2013). As an example for the developing neonatal immune function in contrast to the adult one, it has been reported that the neonatal myelopoietic response during induced murine sepsis is less efficient in comparison to adult myelopoiesis (Cuenca et al., 2015). It is tempting to speculate that persisting MMc cells, whose presence is enforced by lactational transfer after birth (Arvola et al., 2000, Dutta and Burlingham, 2009, Dutta and Burlingham, 2010), could participate in demand-adapted hematopoiesis and promote myelopoiesis centrally in the BM which possesses primary hematopoietic function to counter-balance peripheral immune suppression during the peripartal period.

While we here report comprehensive *ex vivo* and *in vitro* data derived from our mouse model of detection of naturally occurring MMc, our results require to be complemented by reductionist approaches. Therefore, a next approach will investigate fetal immune development in a mouse model devoid of MMc. To this end, blastocysts derived from $CD45.1^+$ BALB/c ($H\text{-}2D^d$)-mated $CD45.2^+$ C57BL/6 ($H\text{-}2D^b$) females will be transferred into RAG-$2^{del/del}$ (C57BL6 background) pseudo-pregnant female mice, which lack T and B lymphocytes due to a deletion of the RAG-2 protein coding region. Blastocysts transferred into wildtype C57BL6 female mice will serve as controls. Based on our current results, we expect the absence of lymphoid MMc to be accompanied by reductions in hematopoietic capacity. Subsequently, the susceptibility towards immediate perinatal infection and a long-term impact on fetal immunity e.g. upon an allergic airway sensitization, will be investigated hypothesizing that a lack of MMc results in an increased vulnerability to infectious challenges and immune disease development.

We here elucidate a hematopoietic function of MMc cells for fetal immune development besides tolerance induction at gd 18.5 in murine gestation. MMc cells could have a similar role in humans, since, with regard to immune development of the fetus, this gestational age corresponds to the late first and second trimester in human embryos (Veru et al., 2014), at which time human MMc has already been detected (Jonsson et al., 2008). The physiological significance of MMc and its susceptibility to modulation described in this thesis together with the life-long persistence of these cells in the offspring strongly suggests novel trans-generational immune regulatory pathways engrained within development and reproduction. Indeed, persisting MMc has been shown to enhance reproductive fitness of female offspring in subsequent pregnancies (Kinder et al., 2015a). While limited

access to human tissue material restricts tracking of MMc cells in the infant, adolescent and adult human in tissues other than the blood, we envision that future endeavors in mouse model research will allow the functional characteristics of MMc to be thoroughly investigated in the context of the offspring's physiology and pathology, e.g. auto-immunity, by comprehensive analyses of the MMc cell transcriptome using e.g. single-cell RNA sequencing.

In the context of the concept of developmental origins of health and disease, vertical transfer of cells from mother to child can be considered a part of the mechanisms connecting the maternal and fetal (immune) systems during pregnancy with possible long-term implications for the offspring's postnatal health.

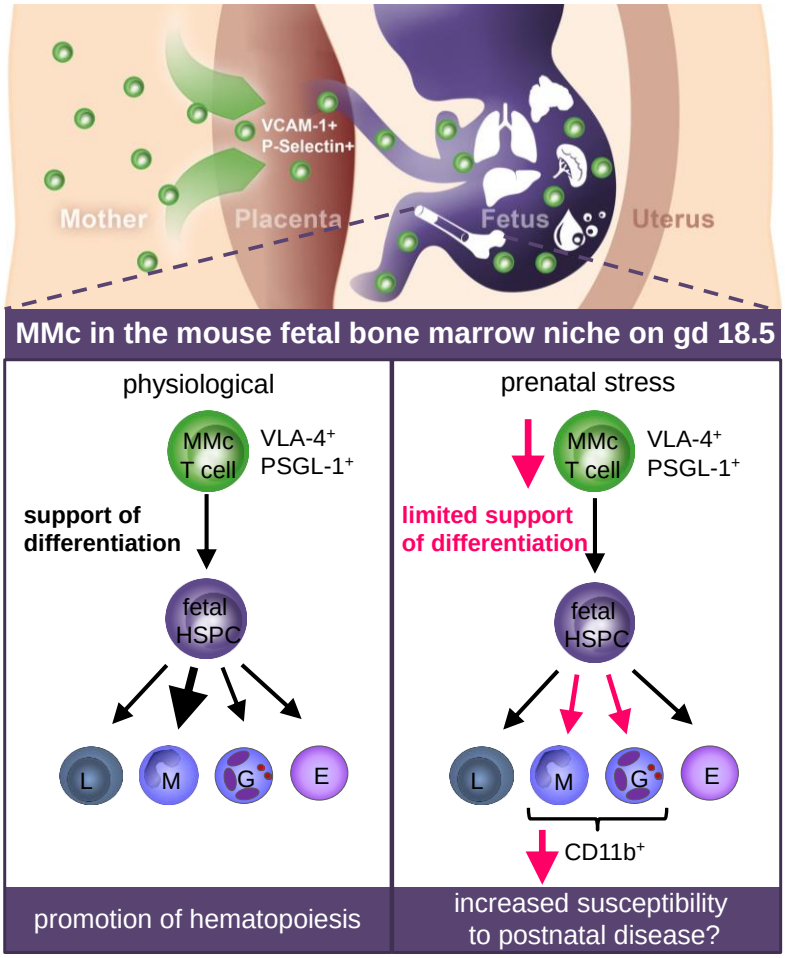

Figure 19: *Graphical summary*. *Maternal microchimerism (MMc) as a mechanism of materno-fetal immune communication during pregnancy.* Maternal cells are detected in various fetal tissues during late murine gestation (gestational day (gd) 18.5). The fetal bone marrow is a main site of MMc. Among the MMc population, $CD3^+$ T cells are the most abundant subset, expressing the cell adhesion molecule ligands VLA-4 and PSGL-1, while LFA-1 expression is largely absent. This might enable them to cross the placental trophoblast-endothelial barrier which in turn expresses VCAM-1 and P-Selectin. During normal development, MMc cells have a functional role in supporting differentiation of fetal hematopoietic stem and progenitor cells (HSPC) *in vitro* and favor the differentiation of lineage-committed immune cells toward myeloid monocytes/macrophages (M). This could be mediated by the secretion of hematopoietic factors by MMc T cells. In contrast, upon a mid-gestational stress challenge, a strong trend towards a reduced number of MMc cells is observed, which is due to a reduction of $CD3^+$ T cells and might be accompanied by a limited promotion of hematopoiesis. This, in turn, could partially mediate the observed decrease in fetal $CD11b^+$ myeloid cells in the fetal bone marrow via several potential mechanisms, such as a reduced secretion of hematopoietic factors, impaired MMc cell proliferation, reduced migration into or egress from the bone marrow, or a stress-altered MMc cell phenotype. Overall, MMc cells directly influence immune development by promotion of hematopoiesis prior to birth, hereby shaping the offspring's immune system for its perinatal life with, if challenged, potential consequences for its susceptibility to diseases later in life.

5 Abstract

Early life conditions and the onset of (immune) diseases later in life have been shown to be linked in epidemiological and experimental studies, a concept termed developmental origins of health and disease. A healthy prenatal development of the fetus is critically dependent on its nourishing and immunologically tolerant mother. Challenges to maternal immune adaptation to pregnancy, such as a prenatal stress perception, have been linked with an increased risk to develop diseases in the postnatal life of the child, including allergies and asthma. We here propose maternal microchimerism (MMc) as a potential mediating mechanism. MMc describes the presence of a small amount of maternal cells in fetal tissues, which establishes during pregnancy and has been shown to persist until both human and murine adulthood. We aimed to investigate MMc during late murine gestation and its modulation upon a prenatal maternal stress challenge in order to test the hypothesis that MMc is a mechanism of materno-fetal communication involved in fetal immune development with, if challenged, possible consequences for the child's peri- and postnatal immunity.

To this end, an allogeneic mouse model was established to detect MMc cells, identified as CD45.1^{neg}/CD45.2^{+} and H-2D^{dneg}/H-2D^{b}, in offspring derived from CD45.1^{+}BALB/c(H-2D^{d})-mated CD45.2^{+}C57BL/6(H-2D^{b}) females by flow cytometry on gestational day (gd) 18.5. Pregnant females were challenged by sound stress exposure on gd 10.5, 12.5 and 14.5.

Among the various fetal organs in which MMc was detected, the fetal bone marrow (BM) was a main site of MMc (<1000 MMc/10^{6} fetal cells). Among this MMc population, CD3^{+} T cells are the most abundant immune cell subset, expressing the cell adhesion molecule ligands Very late antigen (VLA)-4 und P-Selectin glycoprotein ligand (PSGL)-1, while Lymphocyte function-associated antigen (LFA)-1 expression is largely absent. The adhesion molecule expression might enable them to cross the placental trophoblast-endothelial barrier which in turn expresses the corresponding cell adhesion molecules VCAM-1 and P-Selectin in the placental labyrinth during mid- to late gestation (gd 13.5 and 18.5). In contrast, CD3^{+} T cells among maternal peripheral blood leukocytes, the presumptive source of MMc, expressed virtually all three CAML, whilst their overall frequency among all blood leukocytes was significantly lower compared to the one among MMc cells, suggesting a preferential migration of MMc T cells into the fetal bone marrow. A prenatal stress challenge resulted in a strong trend towards a reduced number of MMc cells in the fetal bone marrow on gd 18.5, specifically due to a reduction in CD3^{+} MMc T cells. To test their functional role in this hematopoietic organ, co-cultures of MMc cells with fetal hematopoietic stem and progenitor cells (HSPC) showed their support of stem cell differentiation *in vitro*, while they favored the maturation of lineage-committed immune cells toward myeloid monocytes/macrophages over granulocyte differentiation. Strikingly, a mid-gestational stress challenge mediated a decreased frequency of fetal CD11b^{+} myeloid cells in the fetal bone marrow on gd 18.5.

In conclusion, hematopoietic MMc cells are distributed across fetal organs during late gestation and have a functional role in directly supporting immune development prior to birth by promoting hematopoiesis in the fetal BM, hereby playing a role in shaping the offspring's immune system for its perinatal life. Adverse conditions, e.g. maternal stress perception, have immediate consequences for fetal innate immune ontogeny, possibly mediated by a limited support of HSPC differentiation by a smaller or functionally impaired MMc population, thus a modulated materno-fetal MMc signal. This could have a long-term impact on the child's susceptibility to develop diseases later in life.

6 Zusammenfassung

Das Konzept des „Entwicklungsursprungs von Gesundheit und Krankheit“ umfasst Beobachtungen aus epidemiologischen und experimentellen Studien, die Umweltbedingungen im pränatalen Lebensalter und das Auftreten von (Immun)erkrankungen im späteren Leben miteinander in Verbindung bringen. Eine gesunde vorgeburtliche Entwicklung des Fötus ist entscheidend angewiesen auf Nährstoffzufuhr durch die aus immunologischer Sicht gegenüber ihrem Kind tolerante Mutter. Störungen der maternalen immunologischen Anpassung an die Schwangerschaft, wie etwa eine pränatale Stressbelastung der Mutter, stehen in Zusammenhang mit einem erhöhten Risiko des Kindes im Laufe seines Lebens Krankheiten wie z.B. Allergien und Asthma zu entwickeln. Mütterlicher Mikrochimärismus (MMc) könnte ein potentieller vermittelnder Mechanismus sein. MMc beschreibt die Anwesenheit einer geringen Anzahl mütterlicher Zellen in fötalen Geweben, ein Phänomen, das sich während der Schwangerschaft etabliert und bis ins menschliche und murine Erwachsenenalter erhalten bleibt. In der vorliegenden Arbeit geht es darum, MMc während der späten murinen Schwangerschaft und seine Modulation durch eine mütterliche Stressbelastung zu untersuchen. Es gilt der Hypothese nachzugehen, inwiefern MMc ein Mechanismus der maternal-fötalen Kommunikation sein kann, der in die fötale Immunentwicklung involviert ist und, wenn gestört, möglicherweise Auswirkungen auf die peri- und postnatale Immunkapazität des Kindes hat.

Zu diesem Zweck wurde ein allogenes Mausmodell entwickelt, um mütterliche Zellen, identifiziert als CD45.1^{neg}/CD45.2^{+} und H-2D^{dneg}/H-2D^{b}, in Nachwuchs aus CD45.1^{+}BALB/c(H-2D^{d})-verpaarten CD45.2^{+}C57BL/6(H-2D^{b}) Weibchen mit Hilfe von Durchflusszytometrie an Gestationstag (gd) 18.5 zu detektieren. Tragende Weibchen wurden an gd 10.5, 12.5 und 14.5 einer Stressbelastung durch Lärm ausgesetzt.

Unter den verschiedenen fötalen Geweben, in denen MMc Zellen detektiert werden konnten, wurde das Knochenmark als Hauptort für MMc ermittelt (<1000 MMc/10^{6} fötaler Zellen). Innerhalb dieser MMc Population sind CD3^{+} T Zellen die größte Immunzellpopulation. Diese Zellen exprimieren die Zelladhäsionsmolekülliganden Very late antigen (VLA)-4 und P-Selectin glycoprotein ligand (PSGL)-1, die es den Zellen ermöglichen können, über die Trophoblast-Endothel-Barriere im plazentalen Labyrinth zu migrieren, die ihrerseits die zugehörigen Zelladhäsionsmoleküle Vascular cell adhesion molecule (VCAM)-1 und P-Selectin in der mittleren bis späten Gestationsperiode (gd 13.5 und 18.5) exprimiert. Eine pränatale Stressbelastung der Mutter resultiert in einem starken Trend zu einer verminderten Anzahl von MMc Zellen im fötalen Knochenmark an gd 18.5, und hier im speziellen von CD3^{+} MMc T Zellen. Um einer funktionellen Rolle der MMc Zellen im Knochenmark nachzugehen, wurden MMc Zellen mit fötalen hematopoietischen Stamm- und Vorläuferzellen co-kultiviert. Es konnte gezeigt werden, dass MMc Zellen die Stammzelldifferenzierung unterstützen und die Reifung von „lineage-committed“ Immunzellen in Richtung von Monozyten und Makrophagen begünstigen. Interessanterweise ließ sich im fötalen Knochenmark an gd 18.5 eine stress-vermittelte verminderte Frequenz von fötalen CD11b^{+} myeloiden Zellen beobachten.

In dieser Arbeit konnte die Präsenz von hematopoietischen MMc Zellen in fötalen Organen in der späten murinen Schwangerschaft gezeigt werden. Speziell im Knochenmark übernehmen diese Zellen eine direkte Funktion für die Immunentwicklung kurz vor der Geburt, indem sie die fötale Hematopoiese unterstützen. Sie sind somit an der Prägung des Immunsystems des Nachwuchses für das perinatale Leben beteiligt. Ungünstige Umweltbedingungen, wie eine pränatale Stressbelastung der Mutter, haben unmittelbare Konsequenzen auf die fötale Entwicklung des angeborenen Immunsystems. Möglicherweise hängt dies mit einer eingeschränkten Unterstützung der Stammzelldifferenzierung durch eine kleinere oder funktionell eingeschränkte MMc Zellpopulation, also ein moduliertes maternal-fötales MMc Signal, zusammen. Dies könnte mit einer langfristigen Auswirkung auf die Anfälligkeit des Kindes einhergehen, in späteren Lebensphasen Erkrankungen zu entwickeln.

7 Abbreviations

(q)PCR	(quantitative) Polymerase chain reaction
7-AAD	7-Aminoactinomycin D
µm	Micrometer
µl	Microliter
APC	Allophycocyanin
APC	Antigen-presenting cell
B	B cell
BM	Bone marrow
BSA	Bovine serum albumin
BV	Brilliant Violet
CAM	Cell adhesion molecule
CAML	Cell adhesion molecule ligand
CD	Cluster of differentiation
CLP	Common lymphoid precursor
CMP	Common myeloid precursor
CpG	5'—Cytosine—phosphate—Guanine—3'
CTB	Cell tracker blue
CXCL	Chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
D	Decidua
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Erythrocyte
EDTA	Ethylenediaminetetraacetic acid
eGFP	enhanced Green fluorescent protein
EMP	Erythroid-myeloid progenitor
EP	Erythroid precursor
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one control
FSC	Forward scatter
G	Granulocyte
gd	Gestational day
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Granulocyte/macrophage precursor
GR	Glucocorticoid receptor
hCG	human chorionic gonadotropin
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenocortical axis
HRP	Horse reddish peroxidase
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IGF-2	Insulin-like growth factor-2
IHC	Immunohistochemistry
IL	Interleukin

JAM-B	Junctional Adhesion Molecule-B
JZ	placental junctional zone
KIR	Killer-cell immunoglobulin-like
L	placental labyrinth
LFA-1	Lymphocyte function-associated antigen-1
Lin	Lineage antibody cocktail
LSK	$Lineage^{neg}Sca\text{-}1^{+}c\text{-}Kit^{+}$ hematopoietic stem cell
M	Monocyte/macrophage
M-CSF	Macrophage colony-stimulating factor
MEM	Minimum essential medium
MHC	Major histocompatibility complex
ml	Milliliter
mM	Millimolar
MMc	maternal microchimerism
MMc cell	maternal microchimeric cell
MPP	Multipotent progenitor
MR	Mineralocorticoid receptor
na	not applicable
NIMA	Non-inherited maternal antigen
NIPA	Non-inherited paternal antigen
NK	Natural Killer cell
NRS	Normal rat serum
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
P	Platelet
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein
pnd	Postnatal day
PSGL-1	P-selectin glycoprotein ligand-1
RBC	Red blood cell
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEM	Standard error of the mean
SCF	Stem cell factor
SSC	Sideward scatter
T	T cell
Th	T helper cell
TPO	Thrombopoietin
TNF	Tumor necrosis factor
Treg	regulatory T cell
UV	Ultraviolet light
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4 (α4β1 integrin)
XEM	Xylol replacement medium

8 References

AKIYAMA, Y., CAUCHETEUX, S. M., VERNOCHET, C., IWAMOTO, Y., TANAKA, K., KANELLOPOULOS-LANGEVIN, C. & BENICHOU, G. 2011. Transplantation tolerance to a single noninherited MHC class I maternal alloantigen studied in a TCR-transgenic mouse model. *J Immunol,* 186**,** 1442-9.

ANDERSSON, N. W., HANSEN, M. V., LARSEN, A. D., HOUGAARD, K. S., KOLSTAD, H. A. & SCHLUNSSEN, V. 2016a. Prenatal maternal stress and atopic diseases in the child: a systematic review of observational human studies. *Allergy,* 71**,** 15-26.

ANDERSSON, N. W., LI, Q., MILLS, C. W., LY, J., NOMURA, Y. & CHEN, J. 2016b. Influence of prenatal maternal stress on umbilical cord blood cytokine levels. *Arch Womens Ment Health*.

ANDRASSY, J., KUSAKA, S., JANKOWSKA-GAN, E., TORREALBA, J. R., HAYNES, L. D., MARTHALER, B. R., TAM, R. C., ILLIGENS, B. M., ANOSOVA, N., BENICHOU, G. & BURLINGHAM, W. J. 2003. Tolerance to noninherited maternal MHC antigens in mice. *J Immunol,* 171**,** 5554-61.

ARAKI, M., HIRAYAMA, M., AZUMA, E., KUMAMOTO, T., IWAMOTO, S., TOYODA, H., ITO, M., AMANO, K. & KOMADA, Y. 2010. Prediction of reactivity to noninherited maternal antigen in MHC-mismatched, minor histocompatibility antigen-matched stem cell transplantation in a mouse model. *J Immunol,* 185**,** 7739-45.

ARCK, P. C. & HECHER, K. 2013. Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nat Med,* 19**,** 548-56.

ARCK, P. C., RUCKE, M., ROSE, M., SZEKERES-BARTHO, J., DOUGLAS, A. J., PRITSCH, M., BLOIS, S. M., PINCUS, M. K., BARENSTRAUCH, N., DUDENHAUSEN, J. W., NAKAMURA, K., SHEPS, S. & KLAPP, B. F. 2008. Early risk factors for miscarriage: a prospective cohort study in pregnant women. *Reprod Biomed Online,* 17**,** 101-13.

ARROYO, A. G., YANG, J. T., RAYBURN, H. & HYNES, R. O. 1996. Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. *Cell,* 85**,** 997-1008.

ARTLETT, C. M., MILLER, F. W., RIDER, L. G. & CHILDHOOD MYOSITIS HETEROGENEITY COLLABORATIVE STUDY, G. 2001. Persistent maternally derived peripheral microchimerism is associated with the juvenile idiopathic inflammatory myopathies. *Rheumatology (Oxford),* 40**,** 1279-84.

ARVOLA, M., GUSTAFSSON, E., SVENSSON, L., JANSSON, L., HOLMDAHL, R., HEYMAN, B., OKABE, M. & MATTSSON, R. 2000. Immunoglobulin-secreting cells of maternal origin can be detected in B cell-deficient mice. *Biol Reprod,* 63**,** 1817-24.

AURRAND-LIONS, M., JOHNSON-LEGER, C., LAMAGNA, C., OZAKI, H., KITA, T. & IMHOF, B. A. 2002. Junctional adhesion molecules and interendothelial junctions. *Cells Tissues Organs,* 172**,** 152-60.

BACH, J. F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med,* 347**,** 911-20.

BACHMANN, M. F., MCKALL-FAIENZA, K., SCHMITS, R., BOUCHARD, D., BEACH, J., SPEISER, D. E., MAK, T. W. & OHASHI, P. S. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity,* 7**,** 549-57.

BAILEY, M. T., KINSEY, S. G., PADGETT, D. A., SHERIDAN, J. F. & LEBLEBICIOGLU, B. 2009. Social stress enhances IL-1beta and TNF-alpha production by Porphyromonas gingivalis lipopolysaccharide-stimulated CD11b+ cells. *Physiol Behav,* 98**,** 351-8.

BAKKOUR, S., BAKER, C. A., TARANTAL, A. F., WEN, L., BUSCH, M. P., LEE, T. H. & MCCUNE, J. M. 2014. Analysis of maternal microchimerism in rhesus monkeys (Macaca mulatta) using real-time quantitative PCR amplification of MHC polymorphisms. *Chimerism,* 5**,** 6-15.

BARKER, D. J. 2006. Adult consequences of fetal growth restriction. *Clin Obstet Gynecol,* 49**,** 270-83.

BARTHOLDY, B., CHRISTOPEIT, M., WILL, B., MO, Y., BARREYRO, L., YU, Y., BHAGAT, T. D., OKOYE-OKAFOR, U. C., TODOROVA, T. I., GREALLY, J. M., LEVINE, R. L., MELNICK, A., VERMA, A. & STEIDL, U. 2014. HSC commitment-associated epigenetic signature is prognostic in acute myeloid leukemia. *J Clin Invest,* 124**,** 1158-67.

BEERMAN, I., BOCK, C., GARRISON, B. S., SMITH, Z. D., GU, H., MEISSNER, A. & ROSSI, D. J. 2013. Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging. *Cell Stem Cell,* 12**,** 413-25.

BEGG, S. K., RADLEY, J. M., POLLARD, J. W., CHISHOLM, O. T., STANLEY, E. R. & BERTONCELLO, I. 1993. Delayed hematopoietic development in osteopetrotic (op/op) mice. *J Exp Med,* 177**,** 237-42.

BERMEJO, J. L., SUNDQUIST, J. & HEMMINKI, K. 2007. Risk of cancer among the offspring of women who experienced parental death during pregnancy. *Cancer Epidemiol Biomarkers Prev,* 16**,** 2204-6.

BERRY, S. M., HASSAN, S. S., RUSSELL, E., KUKURUGA, D., LAND, S. & KAPLAN, J. 2004. Association of maternal histocompatibility at class II HLA loci with maternal microchimerism in the fetus. *Pediatr Res,* 56**,** 73-8.

BERTRAND, J. Y., CHI, N. C., SANTOSO, B., TENG, S., STAINIER, D. Y. & TRAVER, D. 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature,* 464**,** 108-11.

BLOIS, S., TOMETTEN, M., KANDIL, J., HAGEN, E., KLAPP, B. F., MARGNI, R. A. & ARCK, P. C. 2005. Intercellular adhesion molecule-1/LFA-1 cross talk is a proximate mediator capable of disrupting immune integration and tolerance mechanism at the feto-maternal interface in murine pregnancies. *J Immunol,* 174**,** 1820-9.

BLOIS, S. M., ILARREGUI, J. M., TOMETTEN, M., GARCIA, M., ORSAL, A. S., CORDO-RUSSO, R., TOSCANO, M. A., BIANCO, G. A., KOBELT, P., HANDJISKI, B., TIRADO, I., MARKERT, U. R., KLAPP, B. F., POIRIER, F., SZEKERES-BARTHO, J., RABINOVICH, G. A. & ARCK, P. C. 2007. A pivotal role for galectin-1 in fetomaternal tolerance. *Nat Med,* 13**,** 1450-7.

BLOIS, S. M., JOACHIM, R., KANDIL, J., MARGNI, R., TOMETTEN, M., KLAPP, B. F. & ARCK, P. C. 2004. Depletion of CD8+ cells abolishes the pregnancy protective effect of progesterone substitution with dydrogesterone in mice by altering the Th1/Th2 cytokine profile. *J Immunol,* 172**,** 5893-9.

BONILLA, W. V., GEUKING, M. B., AICHELE, P., LUDEWIG, B., HENGARTNER, H. & ZINKERNAGEL, R. M. 2006. Microchimerism maintains deletion of the donor cell-specific CD8+ T cell repertoire. *J Clin Invest,* 116**,** 156-62.

BOWIE, M. B., MCKNIGHT, K. D., KENT, D. G., MCCAFFREY, L., HOODLESS, P. A. & EAVES, C. J. 2006. Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. *J Clin Invest,* 116**,** 2808-16.

BRACAMONTE-BARAN, W. & BURLINGHAM, W. 2014. Non-inherited Maternal Antigens, Pregnancy, and Allotolerance. *Biomed J*.

BRANNON, P. M. 2012. Vitamin D and adverse pregnancy outcomes: beyond bone health and growth. *Proc Nutr Soc,* 71**,** 205-12.

BURLINGHAM, W. J., GRAILER, A. P., HEISEY, D. M., CLAAS, F. H., NORMAN, D., MOHANAKUMAR, T., BRENNAN, D. C., DE FIJTER, H., VAN GELDER, T., PIRSCH, J. D., SOLLINGER, H. W. & BEAN, M. A. 1998. The effect of tolerance to noninherited maternal HLA antigens on the survival of renal transplants from sibling donors. *N Engl J Med,* 339**,** 1657-64.

BURT, T. D. 2013. Fetal regulatory T cells and peripheral immune tolerance in utero: implications for development and disease. *Am J Reprod Immunol,* 69**,** 346-58.

CARTWRIGHT, J. E. & BALARAJAH, G. 2005. Trophoblast interactions with endothelial cells are increased by interleukin-1beta and tumour necrosis factor alpha and involve vascular cell adhesion molecule-1 and alpha4beta1. *Exp Cell Res,* 304**,** 328-36.

CHEN, C. P., LEE, M. Y., HUANG, J. P., APLIN, J. D., WU, Y. H., HU, C. S., CHEN, P. C., LI, H., HWANG, S. M., LIU, S. H. & YANG, Y. C. 2008. Trafficking of multipotent mesenchymal stromal cells from maternal circulation through the placenta involves vascular endothelial growth factor receptor-1 and integrins. *Stem Cells,* 26**,** 550-61.

CHOW, A., HUGGINS, M., AHMED, J., HASHIMOTO, D., LUCAS, D., KUNISAKI, Y., PINHO, S., LEBOEUF, M., NOIZAT, C., VAN ROOIJEN, N., TANAKA, M., ZHAO, Z. J., BERGMAN, A., MERAD, M. & FRENETTE, P. S. 2013. CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress. *Nat Med,* 19**,** 429-36.

CHRISTENSEN, J. L., WRIGHT, D. E., WAGERS, A. J. & WEISSMAN, I. L. 2004. Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol,* 2**,** E75.

CIRIZA, J., THOMPSON, H., PETROSIAN, R., MANILAY, J. O. & GARCIA-OJEDA, M. E. 2013. The migration of hematopoietic progenitors from the fetal liver to the fetal bone marrow: lessons learned and possible clinical applications. *Exp Hematol,* 41**,** 411-23.

CLAAS, F. H., GIJBELS, Y., VAN DER VELDEN-DE MUNCK, J. & VAN ROOD, J. J. 1988. Induction of B cell unresponsiveness to noninherited maternal HLA antigens during fetal life. *Science,* 241**,** 1815-7.

COE, C. L., KRAMER, M., KIRSCHBAUM, C., NETTER, P. & FUCHS, E. 2002. Prenatal stress diminishes the cytokine response of leukocytes to endotoxin stimulation in juvenile rhesus monkeys. *J Clin Endocrinol Metab,* 87**,** 675-81.

COLLIER, C. T., WILLIAMS, P. N., CARROLL, J. A., WELSH, T. H., JR. & LAURENZ, J. C. 2011. Effect of maternal restraint stress during gestation on temporal lipopolysaccharide-induced neuroendocrine and immune responses of progeny. *Domest Anim Endocrinol,* 40**,** 40-50.

COOKSON, H., GRANELL, R., JOINSON, C., BEN-SHLOMO, Y. & HENDERSON, A. J. 2009. Mothers' anxiety during pregnancy is associated with asthma in their children. *J Allergy Clin Immunol,* 123**,** 847-53 e11.

COSKUN, S., CHAO, H., VASAVADA, H., HEYDARI, K., GONZALES, N., ZHOU, X., DE CROMBRUGGHE, B. & HIRSCHI, K. K. 2014. Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. *Cell Rep,* 9**,** 581-90.

COTTRELL, E. C. & SECKL, J. R. 2009. Prenatal stress, glucocorticoids and the programming of adult disease. *Front Behav Neurosci,* 3**,** 19.

COURET, D., JAMIN, A., KUNTZ-SIMON, G., PRUNIER, A. & MERLOT, E. 2009. Maternal stress during late gestation has moderate but long-lasting effects on the immune system of the piglets. *Vet Immunol Immunopathol,* 131**,** 17-24.

COUTINHO, A. E. & CHAPMAN, K. E. 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol,* 335**,** 2-13.

CRUDO, A., PETROPOULOS, S., MOISIADIS, V. G., IQBAL, M., KOSTAKI, A., MACHNES, Z., SZYF, M. & MATTHEWS, S. G. 2012. Prenatal synthetic glucocorticoid treatment changes DNA methylation states in male organ systems: multigenerational effects. *Endocrinology,* 153**,** 3269-83.

CUENCA, A. G., CUENCA, A. L., GENTILE, L. F., EFRON, P. A., ISLAM, S., MOLDAWER, L. L., KAYS, D. W. & LARSON, S. D. 2015. Delayed emergency myelopoiesis following polymicrobial sepsis in neonates. *Innate Immun,* 21**,** 386-91.

CUMANO, A. & GODIN, I. 2007. Ontogeny of the hematopoietic system. *Annu Rev Immunol,* 25**,** 745-85.

DAKIC, A., SHAO, Q. X., D'AMICO, A., O'KEEFFE, M., CHEN, W. F., SHORTMAN, K. & WU, L. 2004. Development of the dendritic cell system during mouse ontogeny. *J Immunol,* 172**,** 1018-27.

DAWE, G. S., TAN, X. W. & XIAO, Z. C. 2007. Cell migration from baby to mother. *Cell Adh Migr,* 1**,** 19-27.

DE BRUIN, A. M., LIBREGTS, S. F., VALKHOF, M., BOON, L., TOUW, I. P. & NOLTE, M. A. 2012. IFNgamma induces monopoiesis and inhibits neutrophil development during inflammation. *Blood,* 119**,** 1543-54.

DE MARCO, R., PESCE, G., GIRARDI, P., MARCHETTI, P., RAVA, M., RICCI, P. & MARCON, A. 2012. Foetal exposure to maternal stressful events increases the risk of having asthma and atopic diseases in childhood. *Pediatr Allergy Immunol,* 23**,** 724-9.

DEJBAKHSH-JONES, S., JERABEK, L., WEISSMAN, I. L. & STROBER, S. 1995. Extrathymic maturation of alpha beta T cells from hemopoietic stem cells. *J Immunol,* 155**,** 3338-44.

DELASSUS, S. & CUMANO, A. 1996. Circulation of hematopoietic progenitors in the mouse embryo. *Immunity,* 4**,** 97-106.

DING, L. & MORRISON, S. J. 2013. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature,* 495**,** 231-5.

DING, L., SAUNDERS, T. L., ENIKOLOPOV, G. & MORRISON, S. J. 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature,* 481**,** 457-62.

DUTTA, P. & BURLINGHAM, W. J. 2009. Tolerance to noninherited maternal antigens in mice and humans. *Curr Opin Organ Transplant,* 14**,** 439-47.

DUTTA, P. & BURLINGHAM, W. J. 2010. Stem cell microchimerism and tolerance to non-inherited maternal antigens. *Chimerism,* 1**,** 2-10.

DUTTA, P. & BURLINGHAM, W. J. 2011. Correlation between post transplant maternal microchimerism and tolerance across MHC barriers in mice. *Chimerism,* 2**,** 78-83.

DYE, J. F., JABLENSKA, R., DONNELLY, J. L., LAWRENCE, L., LEACH, L., CLARK, P. & FIRTH, J. A. 2001. Phenotype of the endothelium in the human term placenta. *Placenta,* 22**,** 32-43.

EIKMANS, M., VAN HALTEREN, A. G., VAN BESIEN, K., VAN ROOD, J. J., DRABBELS, J. J. & CLAAS, F. H. 2014. Naturally acquired microchimerism: Implications for transplantation outcome and novel methodologies for detection. *Chimerism,* 5.

ELAHI, S., ERTELT, J. M., KINDER, J. M., JIANG, T. T., ZHANG, X., XIN, L., CHATURVEDI, V., STRONG, B. S., QUALLS, J. E., STEINBRECHER, K. A., KALFA, T. A., SHAABAN, A. F. &

WAY, S. S. 2013. Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. *Nature,* 504**,** 158-62.
EMA, H. & NAKAUCHI, H. 2000. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood,* 95**,** 2284-8.
ENGLER, H., BAILEY, M. T., ENGLER, A. & SHERIDAN, J. F. 2004. Effects of repeated social stress on leukocyte distribution in bone marrow, peripheral blood and spleen. *J Neuroimmunol,* 148**,** 106-15.
ENGLER, H., ENGLER, A., BAILEY, M. T. & SHERIDAN, J. F. 2005. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. *J Neuroimmunol,* 163**,** 110-9.
ENTRINGER, S., KUMSTA, R., NELSON, E. L., HELLHAMMER, D. H., WADHWA, P. D. & WUST, S. 2008. Influence of prenatal psychosocial stress on cytokine production in adult women. *Dev Psychobiol,* 50**,** 579-87.
FANG, F., HOGLUND, C. O., ARCK, P., LUNDHOLM, C., LANGSTROM, N., LICHTENSTEIN, P., LEKANDER, M. & ALMQVIST, C. 2011. Maternal bereavement and childhood asthma-analyses in two large samples of Swedish children. *PLoS One,* 6**,** e27202.
FERNEKORN, U., BUTCHER, E. C., BEHRENDS, J., KARSTEN, C. M., ROBKE, A., SCHULZE, T. J., KIRCHNER, H. & KRUSE, A. 2007. Selectin, platelet plays a critical role in granulocyte access to the pregnant mouse uterus under physiological and pathological conditions. *Biol Reprod,* 76**,** 645-53.
FONSECA, E. S., SAKAI, M., CARVALHO-FREITAS, M. I. & PALERMO NETO, J. 2005. Naloxone treatment prevents prenatal stress effects on peritoneal macrophage activity in mice offspring. *Neuroendocrinology,* 81**,** 322-8.
FOWDEN, A. L. & FORHEAD, A. J. 2015. Glucocorticoids as regulatory signals during intrauterine development. *Exp Physiol,* 100**,** 1477-87.
FRIAS, A. E., MORGAN, T. K., EVANS, A. E., RASANEN, J., OH, K. Y., THORNBURG, K. L. & GROVE, K. L. 2011. Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. *Endocrinology,* 152**,** 2456-64.
FRIED, D. A., RHYU, J., ODATO, K., BLUNT, H., KARAGAS, M. R. & GILBERT-DIAMOND, D. 2016. Maternal and cord blood vitamin D status and childhood infection and allergic disease: a systematic review. *Nutr Rev,* 74**,** 387-410.
FUJII, Y. & YAMAGUCHI, N. 1992. Maternal T cells of immunized pregnant mice induce immune suppression in their offspring. *Immunology,* 77**,** 171-6.
FUJISAKI, J., WU, J., CARLSON, A. L., SILBERSTEIN, L., PUTHETI, P., LAROCCA, R., GAO, W., SAITO, T. I., LO CELSO, C., TSUYUZAKI, H., SATO, T., COTE, D., SYKES, M., STROM, T. B., SCADDEN, D. T. & LIN, C. P. 2011. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature,* 474**,** 216-9.
GABRIEL, G. & ARCK, P. C. 2014. Sex, immunity and influenza. *J Infect Dis,* 209 Suppl 3**,** S93-9.
GLASER, R. & KIECOLT-GLASER, J. K. 2005. Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol,* 5**,** 243-51.
GLUCKMAN, P. D. & HANSON, M. A. 2004. The developmental origins of the metabolic syndrome. *Trends Endocrinol Metab,* 15**,** 183-7.
GLUCKMAN, P. D., HANSON, M. A., COOPER, C. & THORNBURG, K. L. 2008. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med,* 359**,** 61-73.
GODIN, I., DIETERLEN-LIEVRE, F. & CUMANO, A. 1995. Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc Natl Acad Sci U S A,* 92**,** 773-7.
GOMEZ PERDIGUERO, E., KLAPPROTH, K., SCHULZ, C., BUSCH, K., AZZONI, E., CROZET, L., GARNER, H., TROUILLET, C., DE BRUIJN, M. F., GEISSMANN, F. & RODEWALD, H. R. 2015. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature,* 518**,** 547-51.
GORCZYNSKI, R. M. 1992. Conditioned stress responses by pregnant and/or lactating mice reduce immune responses of their offspring after weaning. *Brain Behav Immun,* 6**,** 87-95.
GOTZ, A. A. & STEFANSKI, V. 2007. Psychosocial maternal stress during pregnancy affects serum corticosterone, blood immune parameters and anxiety behaviour in adult male rat offspring. *Physiol Behav,* 90**,** 108-15.
GOTZ, A. A., WITTLINGER, S. & STEFANSKI, V. 2007. Maternal social stress during pregnancy alters immune function and immune cell numbers in adult male Long-Evans rat offspring during stressful life-events. *J Neuroimmunol,* 185**,** 95-102.

HALL, J. M., LINGENFELTER, P., ADAMS, S. L., LASSER, D., HANSEN, J. A. & BEAN, M. A. 1995. Detection of maternal cells in human umbilical cord blood using fluorescence in situ hybridization. *Blood,* 86**,** 2829-32.

HAMILTON, B. E., MARTIN, J. A., OSTERMAN, M. J., CURTIN, S. C. & MATTHEWS, T. J. 2015. Births: Final Data for 2014. *Natl Vital Stat Rep,* 64**,** 1-64.

HANSON, M. A. & GLUCKMAN, P. D. 2014. Early developmental conditioning of later health and disease: physiology or pathophysiology? *Physiol Rev,* 94**,** 1027-76.

HARTWIG, I. R., SLY, P. D., SCHMIDT, L. A., VAN LIESHOUT, R. J., BIENENSTOCK, J., HOLT, P. G. & ARCK, P. C. 2014. Prenatal adverse life events increase the risk for atopic diseases in children, which is enhanced in the absence of a maternal atopic predisposition. *J Allergy Clin Immunol,* 134**,** 160-9.

HAWORTH, K. B., LEDDON, J. L., CHEN, C. Y., HORWITZ, E. M., MACKALL, C. L. & CRIPE, T. P. 2015. Going back to class I: MHC and immunotherapies for childhood cancer. *Pediatr Blood Cancer,* 62**,** 571-6.

HIDALGO, A., SANZ-RODRIGUEZ, F., RODRIGUEZ-FERNANDEZ, J. L., ALBELLA, B., BLAYA, C., WRIGHT, N., CABANAS, C., PROSPER, F., GUTIERREZ-RAMOS, J. C. & TEIXIDO, J. 2001. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-dependent adhesion to fibronectin and VCAM-1 on bone marrow hematopoietic progenitor cells. *Exp Hematol,* 29**,** 345-55.

HODYL, N. A., KRIVANEK, K. M., LAWRENCE, E., CLIFTON, V. L. & HODGSON, D. M. 2007. Prenatal exposure to a pro-inflammatory stimulus causes delays in the development of the innate immune response to LPS in the offspring. *J Neuroimmunol,* 190**,** 61-71.

HOLSAPPLE, M. P., WEST, L. J. & LANDRETH, K. S. 2003. Species comparison of anatomical and functional immune system development. *Birth Defects Res B Dev Reprod Toxicol,* 68**,** 321-34.

ICHINOHE, T., TESHIMA, T., MATSUOKA, K., MARUYA, E. & SAJI, H. 2005. Fetal-maternal microchimerism: impact on hematopoietic stem cell transplantation. *Curr Opin Immunol,* 17**,** 546-52.

IWAKABE, K., SHIMADA, M., OHTA, A., YAHATA, T., OHMI, Y., HABU, S. & NISHIMURA, T. 1998. The restraint stress drives a shift in Th1/Th2 balance toward Th2-dominant immunity in mice. *Immunol Lett,* 62**,** 39-43.

JAMIESON, D. J., HONEIN, M. A., RASMUSSEN, S. A., WILLIAMS, J. L., SWERDLOW, D. L., BIGGERSTAFF, M. S., LINDSTROM, S., LOUIE, J. K., CHRIST, C. M., BOHM, S. R., FONSECA, V. P., RITGER, K. A., KUHLES, D. J., EGGERS, P., BRUCE, H., DAVIDSON, H. A., LUTTERLOH, E., HARRIS, M. L., BURKE, C., COCOROS, N., FINELLI, L., MACFARLANE, K. F., SHU, B., OLSEN, S. J. & NOVEL INFLUENZA, A. P. W. G. 2009. H1N1 2009 influenza virus infection during pregnancy in the USA. *Lancet,* 374**,** 451-8.

JEANTY, C., DERDERIAN, S. C. & MACKENZIE, T. C. 2014. Maternal-fetal cellular trafficking: clinical implications and consequences. *Curr Opin Pediatr,* 26**,** 377-82.

JELLYMAN, J. K., VALENZUELA, O. A. & FOWDEN, A. L. 2015. HORSE SPECIES SYMPOSIUM: Glucocorticoid programming of hypothalamic-pituitary-adrenal axis and metabolic function: Animal studies from mouse to horse. *J Anim Sci,* 93**,** 3245-60.

JOACHIM, R., ZENCLUSSEN, A. C., POLGAR, B., DOUGLAS, A. J., FEST, S., KNACKSTEDT, M., KLAPP, B. F. & ARCK, P. C. 2003. The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. *Steroids,* 68**,** 931-40.

JOHNSON-LEGER, C. A., AURRAND-LIONS, M., BELTRAMINELLI, N., FASEL, N. & IMHOF, B. A. 2002. Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. *Blood,* 100**,** 2479-86.

JONES, C. V. & RICARDO, S. D. 2013. Macrophages and CSF-1: implications for development and beyond. *Organogenesis,* 9**,** 249-60.

JONES, C. V., WILLIAMS, T. M., WALKER, K. A., DICKINSON, H., SAKKAL, S., RUMBALLE, B. A., LITTLE, M. H., JENKIN, G. & RICARDO, S. D. 2013. M2 macrophage polarisation is associated with alveolar formation during postnatal lung development. *Respir Res,* 14**,** 41.

JONSSON, A. M., UZUNEL, M., GOTHERSTROM, C., PAPADOGIANNAKIS, N. & WESTGREN, M. 2008. Maternal microchimerism in human fetal tissues. *Am J Obstet Gynecol,* 198**,** 325 e1-6.

JOO, S. Y., SONG, E. Y., SHIN, Y., HA, J., KIM, S. J. & PARK, M. H. 2013. Beneficial effects of pretransplantation microchimerism on rejection-free survival in HLA-haploidentical family donor renal transplantation. *Transplantation,* 95**,** 1375-82.

KAPLAN, J. & LAND, S. 2005. Influence of maternal-fetal histocompatibility and MHC zygosity on maternal microchimerism. *J Immunol,* 174**,** 7123-8.

KAY, G., TARCIC, N., POLTYREV, T. & WEINSTOCK, M. 1998. Prenatal stress depresses immune function in rats. *Physiol Behav,* 63**,** 397-402.

KHOSROTEHRANI, K., GUEGAN, S., FRAITAG, S., OSTER, M., DE PROST, Y., BODEMER, C. & ARACTINGI, S. 2006. Presence of chimeric maternally derived keratinocytes in cutaneous inflammatory diseases of children: the example of pityriasis lichenoides. *J Invest Dermatol,* 126**,** 345-8.

KIEUSSEIAN, A., BRUNET DE LA GRANGE, P., BURLEN-DEFRANOUX, O., GODIN, I. & CUMANO, A. 2012. Immature hematopoietic stem cells undergo maturation in the fetal liver. *Development,* 139**,** 3521-30.

KINDER, J. M., JIANG, T. T., ERTELT, J. M., XIN, L., STRONG, B. S., SHAABAN, A. F. & WAY, S. S. 2015a. Cross-Generational Reproductive Fitness Enforced by Microchimeric Maternal Cells. *Cell,* 162**,** 505-15.

KINDER, J. M., JIANG, T. T., ERTELT, J. M., XIN, L., STRONG, B. S., SHAABAN, A. F. & WAY, S. S. 2015b. Tolerance to noninherited maternal antigens, reproductive microchimerism and regulatory T cell memory: 60 years after 'Evidence for actively acquired tolerance to Rh antigens'. *Chimerism,* 6**,** 8-20.

KINNEY, D. K., HINTZ, K., SHEARER, E. M., BARCH, D. H., RIFFIN, C., WHITLEY, K. & BUTLER, R. 2010. A unifying hypothesis of schizophrenia: abnormal immune system development may help explain roles of prenatal hazards, post-pubertal onset, stress, genes, climate, infections, and brain dysfunction. *Med Hypotheses,* 74**,** 555-63.

KINSELLA, M. T. & MONK, C. 2009. Impact of maternal stress, depression and anxiety on fetal neurobehavioral development. *Clin Obstet Gynecol,* 52**,** 425-40.

KOFMAN, O. 2002. The role of prenatal stress in the etiology of developmental behavioural disorders. *Neurosci Biobehav Rev,* 26**,** 457-70.

KOLLET, O., VAGIMA, Y., D'UVA, G., GOLAN, K., CANAANI, J., ITKIN, T., GUR-COHEN, S., KALINKOVICH, A., CAGLIO, G., MEDAGLIA, C., LUDIN, A., LAPID, K., SHEZEN, E., NEUFELD-COHEN, A., VAROL, D., CHEN, A. & LAPIDOT, T. 2013. Physiologic corticosterone oscillations regulate murine hematopoietic stem/progenitor cell proliferation and CXCL12 expression by bone marrow stromal progenitors. *Leukemia,* 27**,** 2006-15.

KOWALZICK, L., ARTLETT, C. M., THOSS, K., BAUM, H. P., ZIEGLER, H., MISCHKE, D., BLUM, R., PONNIGHAUS, J. M. & QUIETZSCH, J. 2005. Chronic graft-versus-host-disease-like dermopathy in a child with CD4+ cell microchimerism. *Dermatology,* 210**,** 68-71.

KRONEIS, T., GUTSTEIN-ABO, L., KOFLER, K., HARTMANN, M., HARTMANN, P., ALUNNI-FABBRONI, M., WALCHER, W., DOHR, G., PETEK, E., GUETTA, E. & SEDLMAYR, P. 2010. Automatic retrieval of single microchimeric cells and verification of identity by on-chip multiplex PCR. *J Cell Mol Med,* 14**,** 954-69.

KRUSE, A., MARTENS, N., FERNEKORN, U., HALLMANN, R. & BUTCHER, E. C. 2002. Alterations in the expression of homing-associated molecules at the maternal/fetal interface during the course of pregnancy. *Biol Reprod,* 66**,** 333-45.

KUMARAVELU, P., HOOK, L., MORRISON, A. M., URE, J., ZHAO, S., ZUYEV, S., ANSELL, J. & MEDVINSKY, A. 2002. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development,* 129**,** 4891-9.

LAMBERT, N. C., ERICKSON, T. D., YAN, Z., PANG, J. M., GUTHRIE, K. A., FURST, D. E. & NELSON, J. L. 2004. Quantification of maternal microchimerism by HLA-specific real-time polymerase chain reaction: studies of healthy women and women with scleroderma. *Arthritis Rheum,* 50**,** 906-14.

LASH, G. E., WARREN, A. Y., UNDERWOOD, S. & BAKER, P. N. 2003. Vascular endothelial growth factor is a chemoattractant for trophoblast cells. *Placenta,* 24**,** 549-56.

LEDUC, L., LEVY, E., BOUITY-VOUBOU, M. & DELVIN, E. 2010. Fetal programming of atherosclerosis: possible role of the mitochondria. *Eur J Obstet Gynecol Reprod Biol,* 149**,** 127-30.

LEE, A., MATHILDA CHIU, Y. H., ROSA, M. J., JARA, C., WRIGHT, R. O., COULL, B. A. & WRIGHT, R. J. 2016. Prenatal and postnatal stress and asthma in children: Temporal- and sex-specific associations. *J Allergy Clin Immunol*.

LEVEQUE, L., HODGSON, S., PEYTON, S., KOYAMA, M., MACDONALD, K. P. & KHOSROTEHRANI, K. 2014. Selective organ specific inflammation in offspring harbouring microchimerism from strongly alloreactive mothers. *J Autoimmun,* 50**,** 51-8.

LEVEQUE, L. & KHOSROTEHRANI, K. 2011. Can maternal microchimeric cells influence the fetal response toward self antigens? *Chimerism,* 2**,** 71-77.

LI, D., MOLLDREM, J. J. & MA, Q. 2009. LFA-1 regulates CD8+ T cell activation via T cell receptor-mediated and LFA-1-mediated Erk1/2 signal pathways. *J Biol Chem,* 284**,** 21001-10.

LILLYCROP, K. A. 2011. Effect of maternal diet on the epigenome: implications for human metabolic disease. *Proc Nutr Soc,* 70**,** 64-72.

LIN, Y. C., WEN, H. J., LEE, Y. L. & GUO, Y. L. 2004. Are maternal psychosocial factors associated with cord immunoglobulin E in addition to family atopic history and mother immunoglobulin E? *Clin Exp Allergy,* 34**,** 548-54.

LIU, C., DUFFY, B., BEDNARSKI, J. J., CALHOUN, C., LAY, L., RUNDBLAD, B., PAYTON, J. E. & MOHANAKUMAR, T. 2016. Maternal T-Cell Engraftment Interferes With Human Leukocyte Antigen Typing in Severe Combined Immunodeficiency. *Am J Clin Pathol,* 145**,** 251-7.

LLORENTE, E., BRITO, M. L., MACHADO, P. & GONZALEZ, M. C. 2002. Effect of prenatal stress on the hormonal response to acute and chronic stress and on immune parameters in the offspring. *J Physiol Biochem,* 58**,** 143-9.

LOOMAN, C., SUN, T., YU, Y., ZIEBA, A., AHGREN, A., FEINSTEIN, R., FORSBERG, H., HELLBERG, C., HELDIN, C. H., ZHANG, X. Q., FORSBERG-NILSSON, K., KHOO, N., FUNDELE, R. & HEUCHEL, R. 2007. An activating mutation in the PDGF receptor-beta causes abnormal morphology in the mouse placenta. *Int J Dev Biol,* 51**,** 361-70.

LOPEZ-GUISA, J. M., HOWSMON, R., MUNRO, A., BLAIR, K. M., FISHER, E., HERMES, H., ZAGER, R. & STEVENS, A. M. 2011. Chimeric maternal cells in offspring do not respond to renal injury, inflammatory or repair signals. *Chimerism,* 2**,** 42-49.

LOUBIERE, L. S., LAMBERT, N. C., FLINN, L. J., ERICKSON, T. D., YAN, Z., GUTHRIE, K. A., VICKERS, K. T. & NELSON, J. L. 2006. Maternal microchimerism in healthy adults in lymphocytes, monocyte/macrophages and NK cells. *Lab Invest,* 86**,** 1185-92.

LUDIN, A., ITKIN, T., GUR-COHEN, S., MILDNER, A., SHEZEN, E., GOLAN, K., KOLLET, O., KALINKOVICH, A., PORAT, Z., D'UVA, G., SCHAJNOVITZ, A., VORONOV, E., BRENNER, D. A., APTE, R. N., JUNG, S. & LAPIDOT, T. 2012. Monocytes-macrophages that express alpha-smooth muscle actin preserve primitive hematopoietic cells in the bone marrow. *Nat Immunol,* 13**,** 1072-82.

LUMENG, C. N. & SALTIEL, A. R. 2011. Inflammatory links between obesity and metabolic disease. *J Clin Invest,* 121**,** 2111-7.

LUNDBACK, B., BACKMAN, H., LOTVALL, J. & RONMARK, E. 2016. Is asthma prevalence still increasing? *Expert Rev Respir Med,* 10**,** 39-51.

MACCARI, S., DARNAUDERY, M., MORLEY-FLETCHER, S., ZUENA, A. R., CINQUE, C. & VAN REETH, O. 2003. Prenatal stress and long-term consequences: implications of glucocorticoid hormones. *Neurosci Biobehav Rev,* 27**,** 119-27.

MARLEAU, A. M., GREENWOOD, J. D., WEI, Q., SINGH, B. & CROY, B. A. 2003. Chimerism of murine fetal bone marrow by maternal cells occurs in late gestation and persists into adulthood. *Lab Invest,* 83**,** 673-81.

MATSUOKA, K., ICHINOHE, T., HASHIMOTO, D., ASAKURA, S., TANIMOTO, M. & TESHIMA, T. 2006. Fetal tolerance to maternal antigens improves the outcome of allogeneic bone marrow transplantation by a CD4+ CD25+ T-cell-dependent mechanism. *Blood,* 107**,** 404-9.

MAZO, I. B., MASSBERG, S. & VON ANDRIAN, U. H. 2011. Hematopoietic stem and progenitor cell trafficking. *Trends Immunol,* 32**,** 493-503.

MEANEY, M. J. & FERGUSON-SMITH, A. C. 2010. Epigenetic regulation of the neural transcriptome: the meaning of the marks. *Nat Neurosci,* 13**,** 1313-8.

MENDEZ-FERRER, S., MICHURINA, T. V., FERRARO, F., MAZLOOM, A. R., MACARTHUR, B. D., LIRA, S. A., SCADDEN, D. T., MA'AYAN, A., ENIKOLOPOV, G. N. & FRENETTE, P. S. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature,* 466**,** 829-34.

MERCIER, F. E., RAGU, C. & SCADDEN, D. T. 2012. The bone marrow at the crossroads of blood and immunity. *Nat Rev Immunol,* 12**,** 49-60.

MIKKOLA, H. K. & ORKIN, S. H. 2006. The journey of developing hematopoietic stem cells. *Development,* 133**,** 3733-44.

MILSTONE, D. S., REDLINE, R. W., O'DONNELL, P. E., DAVIS, V. M. & STAVRAKIS, G. 2000. E-selectin expression and function in a unique placental trophoblast population at the fetal-maternal interface: regulation by a trophoblast-restricted transcriptional mechanism conserved between humans and mice. *Dev Dyn,* 219**,** 63-76.

MISSLITZ, A., BERNHARDT, G. & FORSTER, R. 2006. Trafficking on serpentines: molecular insight on how maturating T cells find their winding paths in the thymus. *Immunol Rev,* 209**,** 115-28.

MOISIADIS, V. G. & MATTHEWS, S. G. 2014. Glucocorticoids and fetal programming part 2: Mechanisms. *Nat Rev Endocrinol,* 10**,** 403-11.

MOLD, J. E., MICHAELSSON, J., BURT, T. D., MUENCH, M. O., BECKERMAN, K. P., BUSCH, M. P., LEE, T. H., NIXON, D. F. & MCCUNE, J. M. 2008. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science,* 322**,** 1562-5.

MOLD, J. E., VENKATASUBRAHMANYAM, S., BURT, T. D., MICHAELSSON, J., RIVERA, J. M., GALKINA, S. A., WEINBERG, K., STODDART, C. A. & MCCUNE, J. M. 2010. Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. *Science,* 330**,** 1695-9.

MOLITOR-DART, M. L., ANDRASSY, J., KWUN, J., KAYAOGLU, H. A., ROENNEBURG, D. A., HAYNES, L. D., TORREALBA, J. R., BOBADILLA, J. L., SOLLINGER, H. W., KNECHTLE, S. J. & BURLINGHAM, W. J. 2007. Developmental exposure to noninherited maternal antigens induces CD4+ T regulatory cells: relevance to mechanism of heart allograft tolerance. *J Immunol,* 179**,** 6749-61.

MONTEIRO, J. P., BENJAMIN, A., COSTA, E. S., BARCINSKI, M. A. & BONOMO, A. 2005. Normal hematopoiesis is maintained by activated bone marrow CD4+ T cells. *Blood,* 105**,** 1484-91.

MUELLER, K. L., DANIELS, M. A., FELTHAUSER, A., KAO, C., JAMESON, S. C. & SHIMIZU, Y. 2004. Cutting edge: LFA-1 integrin-dependent T cell adhesion is regulated by both ag specificity and sensitivity. *J Immunol,* 173**,** 2222-6.

NAGASAWA, T., HIROTA, S., TACHIBANA, K., TAKAKURA, N., NISHIKAWA, S., KITAMURA, Y., YOSHIDA, N., KIKUTANI, H. & KISHIMOTO, T. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature,* 382**,** 635-8.

NAKANO, T., KODAMA, H. & HONJO, T. 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science,* 265**,** 1098-101.

NANCY, P., TAGLIANI, E., TAY, C. S., ASP, P., LEVY, D. E. & ERLEBACHER, A. 2012. Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science,* 336**,** 1317-21.

NELSON, J. L. 2012. The otherness of self: microchimerism in health and disease. *Trends Immunol,* 33**,** 421-7.

NELSON, J. L., GILLESPIE, K. M., LAMBERT, N. C., STEVENS, A. M., LOUBIERE, L. S., RUTLEDGE, J. C., LEISENRING, W. M., ERICKSON, T. D., YAN, Z., MULLARKEY, M. E., BOESPFLUG, N. D., BINGLEY, P. J. & GALE, E. A. 2007. Maternal microchimerism in peripheral blood in type 1 diabetes and pancreatic islet beta cell microchimerism. *Proc Natl Acad Sci U S A,* 104**,** 1637-42.

NEPOMNASCHY, P. A., WELCH, K., MCCONNELL, D., STRASSMANN, B. I. & ENGLAND, B. G. 2004. Stress and female reproductive function: a study of daily variations in cortisol, gonadotrophins, and gonadal steroids in a rural Mayan population. *Am J Hum Biol,* 16**,** 523-32.

NEPOMNASCHY, P. A., WELCH, K. B., MCCONNELL, D. S., LOW, B. S., STRASSMANN, B. I. & ENGLAND, B. G. 2006. Cortisol levels and very early pregnancy loss in humans. *Proc Natl Acad Sci U S A,* 103**,** 3938-42.

NIJAGAL, A., FLECK, S., HILLS, N. K., FENG, S., TANG, Q., KANG, S. M., ROSENTHAL, P. & MACKENZIE, T. C. 2012. Decreased risk of graft failure with maternal liver transplantation in patients with biliary atresia. *Am J Transplant,* 12**,** 409-19.

NIJAGAL, A., WEGORZEWSKA, M., JARVIS, E., LE, T., TANG, Q. & MACKENZIE, T. C. 2011. Maternal T cells limit engraftment after in utero hematopoietic cell transplantation in mice. *J Clin Invest,* 121**,** 582-92.

NISTALA, R., HAYDEN, M. R., DEMARCO, V. G., HENRIKSEN, E. J., LACKLAND, D. T. & SOWERS, J. R. 2011. Prenatal Programming and Epigenetics in the Genesis of the Cardiorenal Syndrome. *Cardiorenal Med,* 1**,** 243-254.

NOGUEIRA, P. J., FERREIRA, H. H., ANTUNES, E. & TEIXEIRA, N. A. 1999. Chronic mild prenatal stress exacerbates the allergen-induced airway inflammation in rats. *Mediators Inflamm,* 8**,** 119-22.

O'CONNOR, T. G., WINTER, M. A., HUNN, J., CARNAHAN, J., PRESSMAN, E. K., GLOVER, V., ROBERTSON-BLACKMORE, E., MOYNIHAN, J. A., LEE, F. E. & CASERTA, M. T. 2013. Prenatal maternal anxiety predicts reduced adaptive immunity in infants. *Brain Behav Immun,* 32**,** 21-8.

OKADA, H., KUHN, C., FEILLET, H. & BACH, J. F. 2010. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clin Exp Immunol,* 160**,** 1-9.

PALERMO NETO, J., MASSOCO, C. O. & FAVARE, R. C. 2001. Effects of maternal stress on anxiety levels, macrophage activity, and Ehrlich tumor growth. *Neurotoxicol Teratol,* 23**,** 497-507.

PALMER, A. C. 2011. Nutritionally mediated programming of the developing immune system. *Adv Nutr,* 2**,** 377-95.

PERDIGUERO, E. G. & GEISSMANN, F. 2016. The development and maintenance of resident macrophages. *Nat Immunol,* 17**,** 2-8.

PETERS, J. L., COHEN, S., STAUDENMAYER, J., HOSEN, J., PLATTS-MILLS, T. A. & WRIGHT, R. J. 2012. Prenatal negative life events increases cord blood IgE: interactions with dust mite allergen and maternal atopy. *Allergy,* 67**,** 545-51.

PETTY, J. M., LENOX, C. C., WEISS, D. J., POYNTER, M. E. & SURATT, B. T. 2009. Crosstalk between CXCR4/stromal derived factor-1 and VLA-4/VCAM-1 pathways regulates neutrophil retention in the bone marrow. *J Immunol,* 182**,** 604-12.

PINCUS-KNACKSTEDT, M. K., JOACHIM, R. A., BLOIS, S. M., DOUGLAS, A. J., ORSAL, A. S., KLAPP, B. F., WAHN, U., HAMELMANN, E. & ARCK, P. C. 2006. Prenatal stress enhances susceptibility of murine adult offspring toward airway inflammation. *J Immunol,* 177**,** 8484-92.

PINCUS, M., KEIL, T., RUCKE, M., BRUENAHL, C., MAGDORF, K., KLAPP, B. F., DOUGLAS, A. J., PAUS, R., WAHN, U. & ARCK, P. 2010. Fetal origin of atopic dermatitis. *J Allergy Clin Immunol,* 125**,** 273-5 e1-4.

PIOTROWSKI, P. & CROY, B. A. 1996. Maternal cells are widely distributed in murine fetuses in utero. *Biol Reprod,* 54**,** 1103-10.

POLLARD, J. W. 2009. Trophic macrophages in development and disease. *Nat Rev Immunol,* 9**,** 259-70.

PRADOS, M. B., SOLANO, M. E., FRIEBE, A., BLOIS, S., ARCK, P. & MIRANDA, S. 2011. Stress increases VCAM-1 expression at the fetomaternal interface in an abortion-prone mouse model. *J Reprod Immunol,* 89**,** 207-11.

PRECHT, D. H., ANDERSEN, P. K. & OLSEN, J. 2007. Severe life events and impaired fetal growth: a nation-wide study with complete follow-up. *Acta Obstet Gynecol Scand,* 86**,** 266-75.

RAMOND, C., BERTHAULT, C., BURLEN-DEFRANOUX, O., DE SOUSA, A. P., GUY-GRAND, D., VIEIRA, P., PEREIRA, P. & CUMANO, A. 2014. Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. *Nat Immunol,* 15**,** 27-35.

REED, A. M., MCNALLAN, K., WETTSTEIN, P., VEHE, R. & OBER, C. 2004. Does HLA-dependent chimerism underlie the pathogenesis of juvenile dermatomyositis? *J Immunol,* 172**,** 5041-6.

RIETHER, C., SCHURCH, C. M. & OCHSENBEIN, A. F. 2015. Regulation of hematopoietic and leukemic stem cells by the immune system. *Cell Death Differ,* 22**,** 187-98.

ROG-ZIELINSKA, E. A., RICHARDSON, R. V., DENVIR, M. A. & CHAPMAN, K. E. 2014. Glucocorticoids and foetal heart maturation; implications for prematurity and foetal programming. *J Mol Endocrinol,* 52**,** R125-35.

ROSA, M. J., JUST, A. C., TAMAYO, Y. O. M., SCHNAAS, L., SVENSSON, K., WRIGHT, R. O., TELLEZ ROJO, M. M. & WRIGHT, R. J. 2016. Prenatal and postnatal stress and wheeze in Mexican children: Sex-specific differences. *Ann Allergy Asthma Immunol,* 116**,** 306-312 e1.

ROSSANT, J. & CROSS, J. C. 2001. Placental development: lessons from mouse mutants. *Nat Rev Genet,* 2**,** 538-48.

ROY, E., LEDUC, M., GUEGAN, S., RACHDI, L., KLUGER, N., SCHARFMANN, R., ARACTINGI, S. & KHOSROTEHRANI, K. 2011. Specific maternal microchimeric T cells targeting fetal antigens in beta cells predispose to auto-immune diabetes in the child. *J Autoimmun,* 36**,** 253-62.

RUTLAND, C. S., LATUNDE-DADA, A. O., THORPE, A., PLANT, R., LANGLEY-EVANS, S. & LEACH, L. 2007. Effect of gestational nutrition on vascular integrity in the murine placenta. *Placenta,* 28**,** 734-42.

SAADAI, P., LEE, T. H., BAUTISTA, G., GONZALES, K. D., NIJAGAL, A., BUSCH, M. P., KIM, C. J., ROMERO, R., LEE, H., HIROSE, S., RAND, L., MINIATI, D., FARMER, D. L. & MACKENZIE, T. C. 2012. Alterations in maternal-fetal cellular trafficking after fetal surgery. *J Pediatr Surg,* 47**,** 1089-94.

SAFFERY, R. 2014. Epigenetic change as the major mediator of fetal programming in humans: Are we there yet? *Ann Nutr Metab,* 64**,** 203-7.

SAUSENTHALER, S., RZEHAK, P., CHEN, C. M., ARCK, P., BOCKELBRINK, A., SCHAFER, T., SCHAAF, B., BORTE, M., HERBARTH, O., KRAMER, U., VON BERG, A., WICHMANN, H. E., HEINRICH, J. & GROUP, L. S. 2009. Stress-related maternal factors during pregnancy in relation to childhood eczema: results from the LISA Study. *J Investig Allergol Clin Immunol,* 19**,** 481-7.

SCHURCH, C. M., RIETHER, C. & OCHSENBEIN, A. F. 2014. Cytotoxic CD8+ T cells stimulate hematopoietic progenitors by promoting cytokine release from bone marrow mesenchymal stromal cells. *Cell Stem Cell,* 14**,** 460-72.

SECKL, J. R. & HOLMES, M. C. 2007. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. *Nat Clin Pract Endocrinol Metab,* 3**,** 479-88.

SECKL, J. R. & MEANEY, M. J. 2004. Glucocorticoid programming. *Ann N Y Acad Sci,* 1032**,** 63-84.

SHARARA, L. I., ANDERSSON, A., GUY-GRAND, D., FISCHER, A. & DISANTO, J. P. 1997. Deregulated TCR alpha beta T cell population provokes extramedullary hematopoiesis in mice deficient in the common gamma chain. *Eur J Immunol,* 27**,** 990-8.

SHIMAMURA, M., OHTA, S., SUZUKI, R. & YAMAZAKI, K. 1994. Transmission of maternal blood cells to the fetus during pregnancy: detection in mouse neonatal spleen by immunofluorescence flow cytometry and polymerase chain reaction. *Blood,* 83**,** 926-30.

SHORTMAN, K. & WU, L. 1996. Early T lymphocyte progenitors. *Annu Rev Immunol,* 14**,** 29-47.

SMITH, C. J. & RYCKMAN, K. K. 2015. Epigenetic and developmental influences on the risk of obesity, diabetes, and metabolic syndrome. *Diabetes Metab Syndr Obes,* 8**,** 295-302.

SOBRIAN, S. K., VAUGHN, V. T., ASHE, W. K., MARKOVIC, B., DJURIC, V. & JANKOVIC, B. D. 1997. Gestational exposure to loud noise alters the development and postnatal responsiveness of humoral and cellular components of the immune system in offspring. *Environ Res,* 73**,** 227-41.

SOLANILLA, A., DECHANET, J., EL ANDALOUSSI, A., DUPOUY, M., GODARD, F., CHABROL, J., CHARBORD, P., REIFFERS, J., NURDEN, A. T., WEKSLER, B., MOREAU, J. F. & RIPOCHE, J. 2000. CD40-ligand stimulates myelopoiesis by regulating flt3-ligand and thrombopoietin production in bone marrow stromal cells. *Blood,* 95**,** 3758-64.

SOLANO, M. E., JAGO, C., PINCUS, M. K. & ARCK, P. C. 2011. Highway to health; or How prenatal factors determine disease risks in the later life of the offspring. *J Reprod Immunol,* 90**,** 3-8.

SOLANO, M. E., KOWAL, M. K., O'ROURKE, G. E., HORST, A. K., MODEST, K., PLOSCH, T., BARIKBIN, R., REMUS, C. C., BERGER, R. G., JAGO, C., HO, H., SASS, G., PARKER, V. J., LYDON, J. P., DEMAYO, F. J., HECHER, K., KARIMI, K. & ARCK, P. C. 2015. Progesterone and HMOX-1 promote fetal growth by CD8+ T cell modulation. *J Clin Invest,* 125**,** 1726-38.

SOLANO, M. E., THIELE, K., STELZER, I. A., MITTRÜCKER, H-W., ARCK, P. C. 2014. Advancing the detection of maternal hematopoietic microchimeric cells in fetal immune organs in mice by flow cytometry. *Chimerism,* accepted for publication.

SPEIRS, H. J., SECKL, J. R. & BROWN, R. W. 2004. Ontogeny of glucocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type-1 gene expression identifies potential critical periods of glucocorticoid susceptibility during development. *J Endocrinol,* 181**,** 105-16.

SRIVATSA, B., SRIVATSA, S., JOHNSON, K. L. & BIANCHI, D. W. 2003. Maternal cell microchimerism in newborn tissues. *J Pediatr,* 142**,** 31-5.

STARK, J. L., AVITSUR, R., PADGETT, D. A., CAMPBELL, K. A., BECK, F. M. & SHERIDAN, J. F. 2001. Social stress induces glucocorticoid resistance in macrophages. *Am J Physiol Regul Integr Comp Physiol,* 280**,** R1799-805.

STELZER, I. A., MORI, M., DEMAYO, F., LYDON, J., ARCK, P. C. & SOLANO, M. E. 2016. Differential mouse-strain specific expression of Junctional Adhesion Molecule (JAM)-B in placental structures. *Cell Adh Migr,* 10**,** 2-17.

STELZER, I. A., THIELE, K. & SOLANO, M. E. 2015. Maternal microchimerism: lessons learned from murine models. *J Reprod Immunol,* 108**,** 12-25.

STEVENS, A. M. 2016. Maternal microchimerism in health and disease. *Best Pract Res Clin Obstet Gynaecol,* 31**,** 121-30.

STEVENS, A. M., HERMES, H. M., RUTLEDGE, J. C., BUYON, J. P. & NELSON, J. L. 2003. Myocardial-tissue-specific phenotype of maternal microchimerism in neonatal lupus congenital heart block. *Lancet,* 362**,** 1617-23.

SU, E. C., JOHNSON, K. L., TIGHIOUART, H. & BIANCHI, D. W. 2008. Murine maternal cell microchimerism: analysis using real-time PCR and in vivo imaging. *Biol Reprod,* 78**,** 883-7.

SU, R. W., JIA, B., NI, H., LEI, W., YUE, S. L., FENG, X. H., DENG, W. B., LIU, J. L., ZHAO, Z. A., WANG, T. S. & YANG, Z. M. 2012. Junctional adhesion molecule 2 mediates the interaction between hatched blastocyst and luminal epithelium: induction by progesterone and LIF. *PLoS One,* 7**,** e34325.

SUNKU CUDDAPAH, C. S., GADI, V. K., DE LAVAL DE LACOSTE, B., GUTHRIE, K. A. & NELSON, J. L. 2010. Maternal and fetal microchimerism in granulocytes. *Chimerism,* 1**,** 11-14.

SZEKERES-BARTHO, J., POLGAR, B., KOZMA, N., MIKO, E., PAR, G., SZEREDAY, L., BARAKONYI, A., PALKOVICS, T., PAPP, O. & VARGA, P. 2005. Progesterone-dependent immunomodulation. *Chem Immunol Allergy,* 89**,** 118-25.

TADA, T., WIDAYATI, D. T. & FUKUTA, K. 2006. Morphological study of the transition of haematopoietic sites in the developing mouse during the peri-natal period. *Anat Histol Embryol,* 35**,** 235-40.

TAKIZAWA, H., BOETTCHER, S. & MANZ, M. G. 2012. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood,* 119**,** 2991-3002.

THIELE, K., HOLZMANN, C., SOLANO, M. E., ZAHNER, G. & ARCK, P. C. 2014. Comparative sensitivity analyses of quantitative polymerase chain reaction and flow cytometry in detecting cellular microchimerism in murine tissues. *J Immunol Methods,* 406**,** 74-82.

THIELE, K., SOLANO, M. E., HUBER, S., FLAVELL, R. A., KESSLER, T., BARIKBIN, R., JUNG, R., KARIMI, K., TIEGS, G. & ARCK, P. C. 2015. Prenatal acetaminophen affects maternal immune and endocrine adaptation to pregnancy, induces placental damage, and impairs fetal development in mice. *Am J Pathol,* 185**,** 2805-18.

THOMPSON, E. E., MYERS, R. A., DU, G., AYDELOTTE, T. M., TISLER, C. J., STERN, D. A., EVANS, M. D., GRAVES, P. E., JACKSON, D. J., MARTINEZ, F. D., GERN, J. E., WRIGHT, A. L., LEMANSKE, R. F. & OBER, C. 2013. Maternal microchimerism protects against the development of asthma. *J Allergy Clin Immunol,* 132**,** 39-44.

TOKOYODA, K., EGAWA, T., SUGIYAMA, T., CHOI, B. I. & NAGASAWA, T. 2004. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity,* 20**,** 707-18.

TOKOYODA, K., ZEHENTMEIER, S., HEGAZY, A. N., ALBRECHT, I., GRUN, J. R., LOHNING, M. & RADBRUCH, A. 2009. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity,* 30**,** 721-30.

TOMETTEN, M., BLOIS, S., KUHLMEI, A., STRETZ, A., KLAPP, B. F. & ARCK, P. C. 2006. Nerve growth factor translates stress response and subsequent murine abortion via adhesion molecule-dependent pathways. *Biol Reprod,* 74**,** 674-83.

TOUZOT, F., DAL-CORTIVO, L., VERKARRE, V., LIM, A., CRUCIS-ARMENGAUD, A., MOSHOUS, D., HERITIER, S., FRANGE, P., KALTENBACH, S., BLANCHE, S., PICARD, C., HACEIN-BEY-ABINA, S., CAVAZZANA-CALVO, M. & FISCHER, A. 2012. Massive expansion of maternal T cells in response to EBV infection in a patient with SCID-Xl. *Blood,* 120**,** 1957-9.

UNNO, A., SUZUKI, K., KITOH, K. & TAKASHIMA, Y. 2010. Fetally derived CCL3 is not essential for the migration of maternal cells across the blood-placental barrier in the mouse. *J Vet Med Sci,* 72**,** 1399-403.

URBIETA, M., BARAO, I., JONES, M., JURECIC, R., PANOSKALTSIS-MORTARI, A., BLAZAR, B. R., MURPHY, W. J. & LEVY, R. B. 2010. Hematopoietic progenitor cell regulation by CD4+CD25+ T cells. *Blood,* 115**,** 4934-43.

VAN KERKHOVE, M. D., VANDEMAELE, K. A., SHINDE, V., JARAMILLO-GUTIERREZ, G., KOUKOUNARI, A., DONNELLY, C. A., CARLINO, L. O., OWEN, R., PATERSON, B., PELLETIER, L., VACHON, J., GONZALEZ, C., HONGJIE, Y., ZIJIAN, F., CHUANG, S. K., AU, A., BUDA, S., KRAUSE, G., HAAS, W., BONMARIN, I., TANIGUICHI, K., NAKAJIMA, K., SHOBAYASHI, T., TAKAYAMA, Y., SUNAGAWA, T., HERAUD, J. M., ORELLE, A., PALACIOS, E., VAN DER SANDE, M. A., WIELDERS, C. C., HUNT, D., CUTTER, J., LEE, V. J., THOMAS, J., SANTA-OLALLA, P., SIERRA-MOROS, M. J., HANSHAOWORAKUL, W., UNGCHUSAK, K., PEBODY, R., JAIN, S., MOUNTS, A. W. & INFECTION, W. H. O. W. G. F. R. F. F. S. H. N. P. 2011. Risk factors for severe outcomes following 2009 influenza A (H1N1) infection: a global pooled analysis. *PLoS Med,* 8**,** e1001053.

VAN ROOD, J. J., LOBERIZA, F. R., JR., ZHANG, M. J., OUDSHOORN, M., CLAAS, F., CAIRO, M. S., CHAMPLIN, R. E., GALE, R. P., RINGDEN, O., HOWS, J. M. & HOROWITZ, M. H. 2002. Effect of tolerance to noninherited maternal antigens on the occurrence of graft-versus-host disease after bone marrow transplantation from a parent or an HLA-haploidentical sibling. *Blood,* 99**,** 1572-7.

VANBESIEN-MAILLIOT, C. C., WOLOWCZUK, I., MAIRESSE, J., VILTART, O., DELACRE, M., KHALIFE, J., CHARTIER-HARLIN, M. C. & MACCARI, S. 2007. Prenatal stress has pro-inflammatory consequences on the immune system in adult rats. *Psychoneuroendocrinology,* 32**,** 114-24.

VANZYL, B., PLANAS, R., YE, Y., FOULIS, A., DE KRIJGER, R. R., VIVES-PI, M. & GILLESPIE, K. M. 2010. Why are levels of maternal microchimerism higher in type 1 diabetes pancreas? *Chimerism,* 1**,** 45-50.

VERNOCHET, C., CAUCHETEUX, S. M., GENDRON, M. C., WANTYGHEM, J. & KANELLOPOULOS-LANGEVIN, C. 2005. Affinity-dependent alterations of mouse B cell development by noninherited maternal antigen. *Biol Reprod,* 72**,** 460-9.

VERNOCHET, C., CAUCHETEUX, S. M. & KANELLOPOULOS-LANGEVIN, C. 2007. Bi-directional cell trafficking between mother and fetus in mouse placenta. *Placenta,* 28**,** 639-49.

VERU, F., DANCAUSE, K., LAPLANTE, D. P., KING, S. & LUHESHI, G. 2015. Prenatal maternal stress predicts reductions in CD4+ lymphocytes, increases in innate-derived cytokines, and a Th2 shift in adolescents: Project Ice Storm. *Physiol Behav,* 144**,** 137-45.

VERU, F., LAPLANTE, D. P., LUHESHI, G. & KING, S. 2014. Prenatal maternal stress exposure and immune function in the offspring. *Stress,* 17**,** 133-48.

VIRK, J., LI, J., VESTERGAARD, M., OBEL, C., LU, M. & OLSEN, J. 2010. Early life disease programming during the preconception and prenatal period: making the link between stressful life events and type-1 diabetes. *PLoS One,* 5**,** e11523.

VON HERTZEN, L. C. 2002. Maternal stress and T-cell differentiation of the developing immune system: possible implications for the development of asthma and atopy. *J Allergy Clin Immunol,* 109**,** 923-8.

WADHWA, P. D. 2005. Psychoneuroendocrine processes in human pregnancy influence fetal development and health. *Psychoneuroendocrinology,* 30**,** 724-43.

WALDER, D. J., LAPLANTE, D. P., SOUSA-PIRES, A., VERU, F., BRUNET, A. & KING, S. 2014. Prenatal maternal stress predicts autism traits in 6(1/2) year-old children: Project Ice Storm. *Psychiatry Res,* 219**,** 353-60.

WAN, W., SHIMIZU, S., IKAWA, H., SUGIYAMA, K. & YAMAGUCHI, N. 2002. Maternal cell traffic bounds for immune modulation: tracking maternal H-2 alleles in spleens of baby mice by DNA fingerprinting. *Immunology,* 107**,** 261-7.

WEGORZEWSKA, M., NIJAGAL, A., WONG, C. M., LE, T., LESCANO, N., TANG, Q. & MACKENZIE, T. C. 2014. Fetal intervention increases maternal T cell awareness of the foreign conceptus and can lead to immune-mediated fetal demise. *J Immunol,* 192**,** 1938-45.

WELBERG, L. A. & SECKL, J. R. 2001. Prenatal stress, glucocorticoids and the programming of the brain. *J Neuroendocrinol,* 13**,** 113-28.

WHITELAW, N. C. & WHITELAW, E. 2006. How lifetimes shape epigenotype within and across generations. *Hum Mol Genet,* 15 Spec No 2**,** R131-7.

WHO 2011. World Health Organisation. Global Status Report on Non-Communicable Diseases 2010. *Geneva: World Health Organisation,* 2011.

WIENECKE, J., HEBEL, K., HEGEL, K. J., PIERAU, M., BRUNE, T., REINHOLD, D., PETHE, A. & BRUNNER-WEINZIERL, M. C. 2012. Pro-inflammatory effector Th cells transmigrate through anti-inflammatory environments into the murine fetus. *Placenta,* 33**,** 39-46.

WOHLEB, E. S., HANKE, M. L., CORONA, A. W., POWELL, N. D., STINER, L. M., BAILEY, M. T., NELSON, R. J., GODBOUT, J. P. & SHERIDAN, J. F. 2011. beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. *J Neurosci,* 31**,** 6277-88.

WOHLEB, E. S., POWELL, N. D., GODBOUT, J. P. & SHERIDAN, J. F. 2013. Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. *J Neurosci,* 33**,** 13820-33.

WRENSHALL, L. E., STEVENS, E. T., SMITH, D. R. & MILLER, J. D. 2007. Maternal microchimerism leads to the presence of interleukin-2 in interleukin-2 knock out mice: implications for the role of interleukin-2 in thymic function. *Cell Immunol,* 245**,** 80-90.

WRIGHT, R. J. 2007. Prenatal maternal stress and early caregiving experiences: implications for childhood asthma risk. *Paediatr Perinat Epidemiol,* 21 Suppl 3**,** 8-14.

WRIGHT, R. J., VISNESS, C. M., CALATRONI, A., GRAYSON, M. H., GOLD, D. R., SANDEL, M. T., LEE-PARRITZ, A., WOOD, R. A., KATTAN, M., BLOOMBERG, G. R., BURGER, M., TOGIAS, A., WITTER, F. R., SPERLING, R. S., SADOVSKY, Y. & GERN, J. E. 2010. Prenatal maternal stress and cord blood innate and adaptive cytokine responses in an inner-city cohort. *Am J Respir Crit Care Med,* 182**,** 25-33.

WYRWOLL, C. S., MARK, P. J. & WADDELL, B. J. 2007. Developmental programming of renal glucocorticoid sensitivity and the renin-angiotensin system. *Hypertension,* 50**,** 579-84.

WYRWOLL, C. S., SECKL, J. R. & HOLMES, M. C. 2009. Altered placental function of 11beta-hydroxysteroid dehydrogenase 2 knockout mice. *Endocrinology,* 150**,** 1287-93.

XIAO, J., GARCIA-LLORET, M., WINKLER-LOWEN, B., MILLER, R., SIMPSON, K. & GUILBERT, L. J. 1997. ICAM-1-mediated adhesion of peripheral blood monocytes to the maternal surface of placental syncytiotrophoblasts: implications for placental villitis. *Am J Pathol,* 150**,** 1845-60.

YE, J., VIVES-PI, M. & GILLESPIE, K. M. 2014a. Maternal microchimerism: Friend or foe in type 1 diabetes? *Chimerism,* 5.

YE, J., VIVES-PI, M. & GILLESPIE, K. M. 2014b. Maternal microchimerism: increased in the insulin positive compartment of type 1 diabetes pancreas but not in infiltrating immune cells or replicating islet cells. *PLoS One,* 9**,** e86985.

YEHUDA, R., DASKALAKIS, N. P., LEHRNER, A., DESARNAUD, F., BADER, H. N., MAKOTKINE, I., FLORY, J. D., BIERER, L. M. & MEANEY, M. J. 2014. Influences of maternal and paternal PTSD on epigenetic regulation of the glucocorticoid receptor gene in Holocaust survivor offspring. *Am J Psychiatry,* 171**,** 872-80.

YOSHIDA, H., HAYASHI, S., KUNISADA, T., OGAWA, M., NISHIKAWA, S., OKAMURA, H., SUDO, T., SHULTZ, L. D. & NISHIKAWA, S. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature,* 345**,** 442-4.

ZEBEDEE, S. L., BARRITT, D. S. & RASCHKE, W. C. 1991. Comparison of mouse Ly5a and Ly5b leucocyte common antigen alleles. *Dev Immunol,* 1**,** 243-54.

ZHANG, C. C. & LODISH, H. F. 2004. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood,* 103**,** 2513-21.

ZHANG, C. C. & LODISH, H. F. 2005. Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. *Blood,* 105**,** 4314-20.

ZHANG, L. & MILLER, R. G. 1993. The correlation of prolonged survival of maternal skin grafts with the presence of naturally transferred maternal T cells. *Transplantation,* 56**,** 918-21.

ZHAO, E., XU, H., WANG, L., KRYCZEK, I., WU, K., HU, Y., WANG, G. & ZOU, W. 2012. Bone marrow and the control of immunity. *Cell Mol Immunol,* 9**,** 11-9.

ZHOU, L., YOSHIMURA, Y., HUANG, Y., SUZUKI, R., YOKOYAMA, M., OKABE, M. & SHIMAMURA, M. 2000. Two independent pathways of maternal cell transmission to offspring: through placenta during pregnancy and by breast-feeding after birth. *Immunology,* 101**,** 570-80.

ZOVEIN, A. C., TURLO, K. A., PONEC, R. M., LYNCH, M. R., CHEN, K. C., HOFMANN, J. J., COX, T. C., GASSON, J. C. & IRUELA-ARISPE, M. L. 2010. Vascular remodeling of the vitelline artery initiates extravascular emergence of hematopoietic clusters. *Blood,* 116**,** 3435-44.

9 Acknowledgements

First and foremost, I would like to thank my Doktormutter Petra Arck, without whom this work would not have been possible. I thank her for being so extremely open to me approaching her in the first place and putting trust in me from the very beginning and ever after. Her unwavering and demanding, motivating and honest support and scientific mentoring kept me on track throughout this project and made me grow not only as a scientist but also as a person. I am deeply grateful to my supervisor Emilia Solano, who constantly guided me through these graduate studies with all her straightforward patience and resources allocated to let the project and me develop. Thank you for all the scientific discussions and invested time which encouraged me to try to bring out the best of me, pushing me forward. Employing the scientific and personal skills that I learned from both of you will without doubt lead me safely through my future work and personal life.

Writing of this thesis has initially been made possible thanks to the positive vote of the committee of the Promotionsförderung at the Cusanuswerk-Bischöfliche Studienförderung, especially consultant Dr. Manuel Ganser. Granting a PhD scholarship allowed me to walk down a path of tremendous professional and personal development, certainly supported by the Cusanuswerk Graduiertentagungen.

I would like to thank Professors Eva Tolosa and Gisa Tiegs for being part of my thesis committee and spending time and effort into giving advice to let the project progress. Our discussions helped me to widen my angle of looking at and interpreting data.

I thank Professor Udo Markert for outrightly agreeing to be the second reviewer of this thesis and part of the evaluating committee at the defense. Thank you so much, not only for traveling all the way from Jena to Hamburg for this occasion but also for letting me join the exciting organization of the ISIR-ESRI congress.

I am most grateful for being part of the Arck group and to all current and former members: Alexandra, Dimitra, Géraldine, Hendrik, Isabel, Janina, Katherina, Kathrin, Lesley, Martin, Mayumi, René, and Thomas. Ein großes Danke to Detlev Riller who contributed exceptional graphic art to our pinkish MMc poster, the colorful chapter and this green-purple thesis. Special thanks to my earliest PhD student companion Greta, “office ladies” Clara and Kristin (who was co-developing graphical summary, discussing, reading, and as a matter of course supporting this thesis), and lab heroes Agnes and Christopher: all of you, with all your helpfulness and humor, rather feel like friends than just co-workers.

Also, I would like to thank all the people outside our group, who helped in realizing this thesis, such as ever so supportive and flexible Susanne Roscher, Julia Spötter, Melanie Lachmann, and Regine Thiele of the FACS Core Facility. Nora Kersten (Institute for Immunology) helped to sort at the most odd day times and Kristin Hartmann (Mouse Pathology Core Facility) managed the most straightforward immunohistochemistry support. Anja Fischer and Anne Willing (ZMNH) adopted me during the very first weeks of my PhD in Hamburg in their lab and became such wonderful friends afterwards. Last but not least, Ioanna Triviai (Department of Stem Cell Transplantation) is to be

especially acknowledged for her excitement about our project and the sharing of all her hematopoietic knowledge to support our stem cell culture experiments.

I would like to thank Beatrice Lugger (NaWik Karlsruhe) for being my mentor in managing the next steps in my professional life. With all her awareness of the scientific community and our modern work life, she is the neutral career-experienced adult person that I unknowingly missed for a long time and who, with her strong manner of giving Ina-tailored advice, helped me to zoom out of my box and re-orientate.

Lastly, I thank all my beloved closest persons for letting me dissolve in my PhD project during the last years while constantly making sure reassembly is still possible, foremost my Mom and Dad (P.), Carla, Phil, and Lars, and auntie Kerstin. I thank dearest Johanna for her ever so critical “Brückentier” support and invaluable friendship. It was the most supportive combination to live in parallel PhD worlds with Natalie and Sandra and to spend energizing and neutralizing times together in Hamburg, Berlin, and Basel. I thank Julia for making me aware of my winged backpack, and Alexander and Dorothee for taking care of cooking, talking, running, and all the other everyday things a joyful life needs.

10 Publications

Research articles

Ina Annelies Stelzer, Mayumi Mori, Francesco DeMayo, John Lydon, Petra Clara Arck, Maria Emilia Solano “Differential mouse-strain specific expression of Junctional Adhesion Molecule (JAM)-B in placental structures” *Cell Adhesion & Migration*, 2016 DOI: 10.1080/19336918.2015.1118605

Maria Emilia Solano, Kristin Thiele, **Ina Annelies Stelzer**, Hans-Willi Mittrücker, Petra Clara Arck. “Advancing the detection of maternal haematopoietic microchimeric cells in fetal immune organs in mice by flow cytometry” *Chimerism*. 2014 Oct 30:1-4. DOI:10.4161/19381956.2014.959827.

Review articles

Ina Annelies Stelzer, Kristin Thiele, Maria Emilia Solano. “Maternal microchimerism: Lessons learned from murine models”. *Journal of Reproductive Immunology*. 2015 Jan 8. DOI: 0.1016/j.jri.2014.12.007.

Book chapter

Ina Annelies Stelzer, Petra Clara Arck. “Immunity and the endocrine system” in: *Encyclopedia of Immunobiology*, Editor: Michael Ratcliffe. Elsevier, 2016. DOI: 10.1016/B978-0-12-374279-7.19001-0

11 Affidavit

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....

Hamburg im Juli 2016 Ina Stelzer