Consequences of Antenatal Corticosteroid Treatment on the Developing Immune System

Dissertation

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ABSTRACT

Women at risk of preterm birth are routinely given antenatal corticosteroids (ACS) to accelerate fetal lung maturation of the child and consequently reduce respiratory distress syndrome and neonatal mortality. This treatment is widely used and prescribed without any restrictions since there are no known immediate side-effects. Long term side-effects of this treatment include changes of the hypothalamic-pituitary-adrenal-axis (HPA-axis) in children and - in multiple courses of corticosteroid treatment to animals- impaired brain growth. Despite the pronounced effects of glucocorticoids (GC) on developing T cells, only few studies have addressed the effect of antenatal steroid treatment on the immune system of the offspring.

In this thesis, I established a mouse model to investigate short- and long-term consequences of ACS treatment on the immune system. I found that immature thymocytes of neonates whose mothers received steroids underwent massive apoptosis. This effect was transient, but triggered an accelerated transition from immature to mature compartments to counteract the loss of cells. I also found that not all Vγ chains of the unconventional TCRγδ cells are equally sensitive to betamethasone, and changes in their distribution persisted into adulthood. ACS treatment to animals prone to develop spontaneous autoimmunity resulted in marked changes in T helper cells and acceleration of disease symptoms. Finally, I found that human T cell precursors are also sensitive to low doses of betamethasone relevant in ACS treatment. Analysis of human cord blood samples from preterm infants that received steroids revealed a correlation between the frequency of T helper cells and the time elapsed after steroid treatment.

In summary, I could show transient and persistent changes on different T cell types in both mouse and human after antenatal steroid treatment. It is conceivable that overexposure of glucocorticoids at the end pregnancy may influence the immune system towards higher autoreactivity, leading to an increased risk for the development of autoimmune and allergic diseases.

ZUSAMMENFASSUNG

Im klinischen Bereich werden antenatale Glucocorticoide bei drohender Frühgeburt verabreicht um die Lungenreifung des Fötus zu fördern und somit die Häufigkeit eines Atemnotsyndroms und die dadurch verursachte Mortalität der Frühgeborenen zu verringern. Die Therapie wird routinemäßig in der Klinik angewendet, da bislang keine kurzfristigen Nebenwirkungen bekannt sind. Langfristige Nebenwirkungen umfassen Veränderungen in der Hypothalmus-Pituitary-Adrenal Achse (HPA-Achse) und - im Falle von mehrfach angewendeten Therapie-Regimen in Tierversuchen - ein beeinträchtigtes Hirnwachstum. Trotz ausgeprägter Wirkung von Steroiden auf in der Entwickelung befindlichen Thymozyten, sind bisher kaum Studien zum Effekt der antenatalen Steroidtherapie auf das Immunsystem durchgeführt worden.

In der vorgelegten Arbeit habe ich ein Tiermodell etabliert mit dem ich kurz- und langfristige Effekte der antenatalen Steroidtherapie auf das Immunsystem der Nachkommen untersuchen konnte. Ich konnte nachweisen, dass unreife Thymozyten von behandelten Neonaten massive Apoptoseraten aufwiesen. Dieser Effekt war vorübergehend, führte jedoch zu einem beschleunigten Übergang von unreifen zu reifen Zellkompartimenten um den durch Apoptose verursachten Zellverlust auszugleichen. Weiterhin konnte ich nachweisen, dass nicht alle Vγ Ketten der unkonventionellen TCR$\gamma\delta$ Zellen gleich sensitiv auf die Behandlung mit Betamethasone reagierten. Die durch die Steroidtherapie verursachten Veränderungen in der Verteilung der Ketten blieben bis ins Erwachsenenalter bestehen. Die Anwendung der Steroidtherapie bei Tieren, die spontane Autoimmunität entwickelten führte zu merklichen Veränderungen der T Helfer Zellen sowie einer Beschleunigung der Krankheitssymptome beim systemischen Lupus erythematodes. Schließlich konnte ich in *in vitro* Experimenten nachweisen, dass unreife humane T Zellen ebenfalls sensitiv auf geringe Dosen von Betamethason reagieren. Die Analyse von humanen Nabelschnurblutproben von Frühgeborenen ergab einen Zusammenhang zwischen der Frequenz der vorhandenen T Helfer Zellen und der seit der Steroidtherapie verstrichenen Zeit.

Insgesamt konnten schwerwiegende kurz- und langfristige Effekte der Steroidtherapie auf unterschiedliche T Zell Populationen in der Maus und im Menschen nachgewiesen werden. Es erscheint möglich, dass die antenatale Steroidtherapie das Immunsystem hin zu verstärkter Autoreaktivität prägt, und somit die Entwicklungen von Autoimmunerkrankungen und Asthma begünstigt.

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1 INTRODUCTION

1.1 The Immune System

The immune system is a complex network of cells, tissues, and organs protecting the body from infectious organisms and other invaders. To function properly, the immune system must detect a wide variety of agents, from viruses to parasitic worms, and likewise discriminate them from the organism's own healthy tissue. During an immune response, the immune system attacks foreign substances and infected cells to maintain health. In vertebrates, two arms of the immune system, known as the innate and the adaptive immune system, complement each other (Table 1.1).

The innate immune system poses the first line of defense against agents that have invaded the body, responding rapidly but unspecifically. Contrary to this, the adaptive immune response is slower, but highly specific, mediated by T- and B-cells expressing receptors recognizing distinct epitopes of a foreign antigen. Between these two arms, some recently described cell types turned up featuring characteristics of both classical arms of the immune systems. These cells are considered to be a bridge between adaptive and innate immunity and are often crucial at mucosal interfaces. They are especially important during the first stages of life when the adaptive immune system is not fully developed (Delves and Roitt, 2000; Playfair and Chain, 2010; Murphy et al., 2012).

Table 1.1: Cellular components of the immune system and their assignation to either innate or adaptive immunity.

Innate Immunity (rapid response)		Adaptive immunity (slow response)
Dendritic cell (DC)	TCR$\gamma\delta$ cell	T cell (CD4+ and CD8+)
Mast cell	Natural killer T cell	B cell
Macrophage		
Granulocytes (Neutrophil, Basophil, Eosinophil)		
Natural killer cell		

1.2 Properties of T cells

T cells mature in the thymus and characteristically express a distinct T cell receptor (TCR). Approximately 30.000 identical TCR molecules are expressed on the surface of a single T cell (Murphy et al., 2012). The T cell receptor is made of an amino-terminal variable region which

shows homology to an immunoglobulin V domain and a constant region with homology to an immunoglobulin C domain (Figure 1.1 a). The variable domain is further divided into different gene segments: the variable (V)-, diversity (D)- and joining (J)-region which undergo specific rearrangements during thymic development (Chien et al., 1984). In case of the α chain, a V_α gene segment rearranges to a J_α gene segment to create a functional V-region exon. Further transcription and splicing of the combined VJ_α exon to the C_α leads to the generation the T cell receptor α-chain protein. The variable domain of the β-chain is encoded in three gene segments: V_β, D_β and J_β. Rearrangement of these gene segments establishes a functional VDJ_β V-region exon that is joined to C_β, producing the T cell receptor β chain. The α and β chains pair soon after their synthesis to yield the $\alpha\beta$-T cell receptor heterodimer (Murphy et al., 2012) (Figure 1.1 b). A small proportion of T cells do not express the $\alpha\beta$ TCR, but a TCR$\gamma\delta$ receptor.

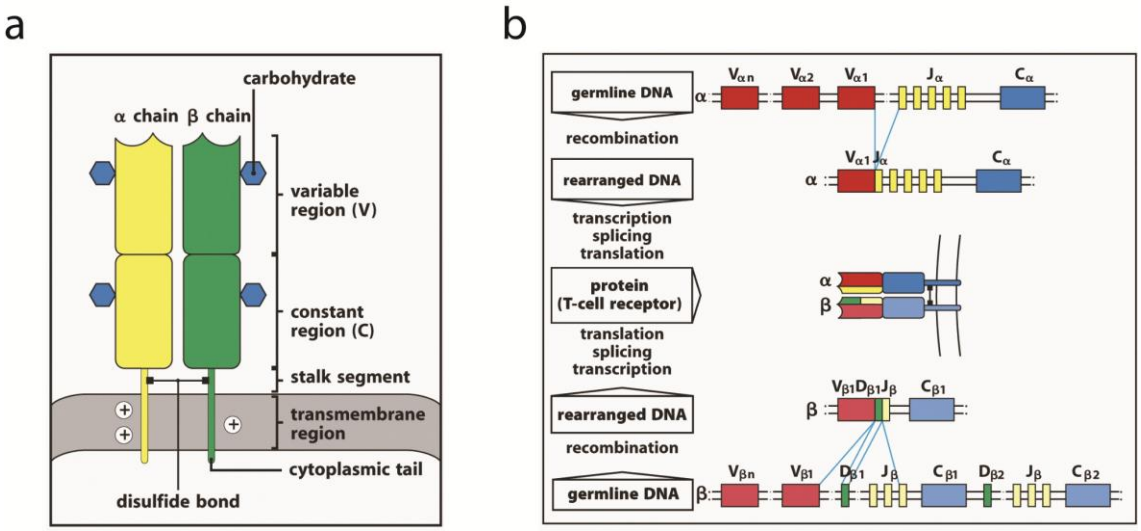

Figure 1.1: Structure and rearrangement of the T cell receptor. (**a**) Characteristic heterodimeric structure of the T cell receptor, composed of two transmembrane glycoprotein chains, the α and β chain each displaying a constant (C) and variable (V) region. (**b**) Development of the T cell receptor by somatic recombination of TCRα and TCRβ gene segments. (Murphy et al., 2012)

T cells fall into two major classes which have different effector functions and are determined by the expression of the cell-surface proteins CD4 and CD8. During antigen recognition, CD4 or CD8 associates with the T cell receptor and binds to invariant sites on the MHC portion of the composite peptide:MHC ligand. This binding is required for the T cell to make an effective response, and so CD4 and CD8 are called co-receptors. The co-receptors recognize different MHC molecules. CD8 is expressed by cytotoxic T cells and recognizes MCH class I molecules, while CD4 is found on T helper cells and recognizes MHC class II complexes.

1.3 Development and selection of T cells

The thymus, as the organ where T cells develop and mature, plays a key role in the development of an effective immune system including self-tolerance (Le Douarin et al., 1984; Sakaguchi, 2003). The thymus is anatomically located in the anterior superior mediastinum of the thoracic cavity, in front of the heart and behind the sternum. It is a soft, roughly triangular organ composed of two identical lobes which are divided into many lobules. The outer region is the cortex with a high cell content; the inner region is the medulla. Lymphoid precursors originating from the bone marrow migrate into the thymus. The colonization of the thymus by lymphoid progenitor cells in mouse occurs in two waves (Misslitz et al., 2006; Ramond et al., 2014), peaking between embryonic days 11 and 13 and between embryonic days 18 and 21 (Misslitz et al., 2006).

The maturation of progenitor cells in the thymus can easily be tracked by the expression of the T cell receptor and the co-receptors CD4 and CD8 or lack thereof. The most immature thymocytes do not express the TCR and also lack CD4 and CD8 and are referred to as double-negative cells (DN). On the basis of the cell surface expression of the markers CD25 and CD44, these DN progenitors can be organized into four subsets according to the following sequence of maturation: CD44+CD25- (DN1), CD44+CD25+ (DN2), CD44-CD25+ (DN3), andCD44-CD25- (DN4), until they reach the CD4+ CD8+ double-positive (DP) stage (Rodewald and Fehling, 1998). DN cells can give rise to two different T cells lineages which can be differentiated by the expression of their TCR chains. A small proportion of cells (about 3%) expresses the $\gamma\delta$-chains, while the majority express the conventional $\alpha\beta$ chains (Yates, 2014). The differentiation of the two receptor types occurs during the DN2 and DN3 stages, when genes for the TCR β, γ and δ chains rearrange (Capone et al., 1998; Livak et al., 1999). Successful gene rearrangement leads to the expression of a TCR$\gamma\delta$ or a surrogate TCR complex (the pre-TCR) which is composed of a TCRβ chain and an invariant preTα chain on the cell surface. For cells committed to the $\alpha\beta$ lineage, bearing the pre-TCR, this process is known as β-selection. Following this, the cells proliferate and progress to the DP stage when they start to rearrange their final α-chain locus. Once the TCRα is rearranged, DP thymocytes migrate through the cortex. Most thymocytes in the cortex of the adult thymus are highly motile (Witt et al., 2005; Bousso et al., 2002), pausing to interact through their TCR with peptide–MHC complexes that are expressed by stromal cells, such as cTECs and dendritic cells in the cortex. Thymocytes failing to produce a TCR capable of recognizing self-MHC-peptides die by apoptosis (“death by neglect”). Although T cells with a non-functional TCR$\alpha\beta$ can undergo repeated gene arrangement, about 90% of all generated cells die by neglect (Petrie et al., 1993; Kyewski and Klein, 2006). Cells capable of

binding peptide:MHC complexes with moderate affinity receive a survival signal (positive selection) and migrate to the medulla. The thymocytes surviving this selection process down-regulate the expression of either CD4 or CD8 co-receptors yielding single-positive (SP) T helper (CD4+CD8-) or cytotoxic (CD4-CD8+) T cells (Singer et al., 2008). The SP T cells additionally upregulate the chemokine receptor CCR7, causing migration from the cortex to the medulla. Medullary thymic epithelial cells express tissue-specific self-antigens. Thymocytes that respond to these cells by binding, therefore having TCR that recognizes self-MHC-peptides with high affinity and marking them as potentially autoreative, are deleted. An estimated 5% or less (Egerton et al., 1990) cells survive the selection process and exit the thymus to populate the peripheral compartments (Figure 1.2).

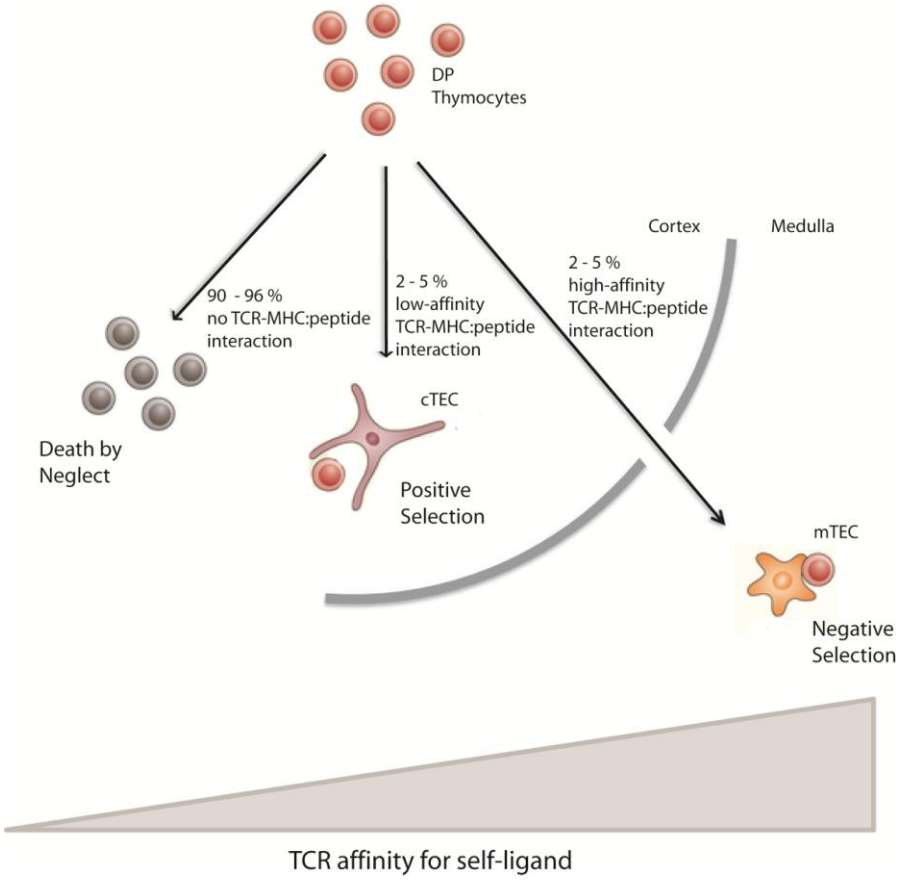

Figure 1.2: Simplified schematic representation of T cell selection events in the thymus. During their CD4+CD8+ DP stage, thymocytes undergo selection processes and lineage commitment, resulting either in their differentiation into CD4+ or CD8+ single positive cells (positive selection) or deletion (negative selection). The intensity of TCR signals - mainly affected by the avidity between TCR and MHC:self-peptide - determines their fate.

Additionally to the thymic selection, TCR$\alpha\beta$ cells undergo further specific selection processes by exposition to super-antigens (SAg). The SAg binds MHC molecules outside the peptide

binding groove and thus is able to bind to T cells independent of their antigen-specificity but specific of the Vβ chain employed. SAg can be of endogenous, bacterial or viral origin. Most inbred mouse strains permanently integrated mammary tumor retroviruses (Mtv) (Lee and Eicher, 1990) which encode functional SAg with particular Vβ preferences. Expression of the Mtv within the thymus leads to the deletion of SAg reactive T cells (Holt et al., 2013). C57BL/6 mice of the H-2b haplotype are described to feature the Mtv-8, Mtv-9 and Mtv-17 provirus (Peterson et al., 1985; Holt et al., 2013) which lead to deletion or increase of certain Vβ cells (Woodland et al., 1991; Lee et al., 2011; Braun et al., 1995). These processes lead to a specific Vβ repertoire, distinct for individual mouse strains. Previous animal and human genetic studies have shown that a biased TCR Vβ repertoire is a characteristic feature of some autoimmune diseases (Tzifi et al., 2013), however this issue remains controversial.

1.4 Different types of T cells

1.4.1 A second T cell receptor lineage - TCRγδ cells

Beside the T cells expressing the TCRαβ homodimer, a small T cell population expresses a γδ T cell receptor. γδ T cells are conserved throughout evolution and across species (Prinz et al., 2013). TCRγδ represent a numerically small (approximately 5%) T cell population in peripheral blood and lymphoid tissues but are more abundant in mucosal tissue like the gastrointestinal tract, lung and skin (Kabelitz et al., 2013). In mice, γδ T cells comprise the predominant population at certain epithelial or mucosal sites - in the epidermis and epithelia of intestine, reproductive organs, and tongue - where they can make up to 50% of the T cells (Kabelitz et al., 1999). The usage of a γ and δ chain sets them apart from the classical CD4+ helper T cells and CD8+ cytotoxic T cells that are defined by TCRαβ receptor. The γδ lymphocytes arise from a common thymocyte progenitor for both receptor types during development in the thymus (Born et al., 2010). While this T cell branching point is obviously influenced by the nature of the rearranged β, γ and δ TCR genes, also TCR signal strength, as well as epigenetic phenomena, cytokines and transcription factors can influence the developmental outcome (Taghon and Rothenberg, 2008). It has been proposed that strong TCR signaling promotes a TCRγδ fate while weaker signaling drives αβ-committed double positive cells (Haks et al., 2005). Likewise, Notch signaling has been shown to be involved in regulating the TCR αβ/γδ lineage decision. In the mouse, several lines of evidence suggest that TCRαβ T cells require higher levels of Notch activation compared to TCRγδ T cells. Reducing Notch1 gene dosage in vivo favored γδ T cell

development over αβ-lineage cells (Washburn et al., 1997). Instead, γδ T cells seem more dependent on jagged ligands (Jiang et al., 1998) which are considered to induce lower Notch activation in comparison with DL ligands (Bruckner et al., 2000).

As with the αβ lineage, the amino-terminal V-region of γδ T cells is formed by rearrangement of V-segments with J- or D- and J- segments. Contrary to TCRαβ cells, there are only a few variable genes available to construct Vγ/Vδ proteins. Fetal thymic murine γδ T cell development progresses in successive waves that associate with variable Vγ segment usage (Carding and Egan, 2002). The cells in each wave assemble the same Vγ and Vδ regions, but each wave uses a different set of V, D and J gene segments. γδ T cell subsets develop in the following order: Vγ5 cells after embryonic day 12 (E12) to approximately E17, Vγ6 cells from E14 to around birth, and finally Vγ1 and Vγ4 cells from E16 and E18 onward (Hayday and Gibbons, 2008). Only Vγ7 migrating to the gut are thought to develop extrathymically (Rocha et al., 1994) (Figure 1.3). Why certain V, D and J gene segments are selected for rearrangement at particular times during embryonic development is still poorly understood, though the usage of the Vγ/Vδ genes appears to be predetermined by the localization. γδ T cells with distinct Vγ/Vδ usage are present in the skin and mucosal tissues such as the intestine, the lung, or the reproductive tract (Bonneville et al., 2010; Hayday, 2000).

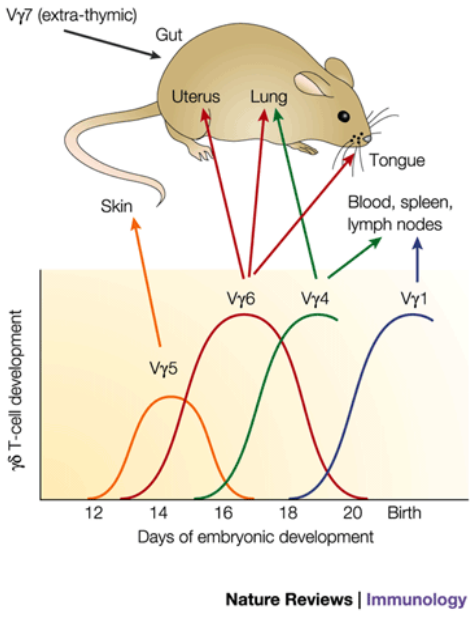

Figure 1.3: Development of murine TCRγδ cells. T cells bearing the γ and δ chain develop during distinct embryonic stages between days E12 and birth. Indicated also the tissues these cells preferentially migrate to (from Carding and Egan, 2002).

At the functional level, γδ T cells and conventional αβ T cells share many features, like the production of pro-inflammatory cytokines such as TNF-α and interferon-γ (IFN-γ) or cytotoxic effector activity (Bonneville et al., 2010; Hayday, 2000). Murine γδ T cells can also function as an early source of 'innate' cytokine interleukin-17 (IL-17) production (O'Brien et al., 2009). Interestingly, the effector function of γδ T cells is associated with the type of Vγ or Vδ region expressed. While the proinflammatory cytokine IL-17 is mainly produced by the Vγ4 subset, Vγ1 cells produce IFN-γ (Blanco et al., 2014). In contrast to recognition of antigens by αβ T cells, γδ T cells do not require antigen processing but recognize certain bacterial, lipid, and tumor antigens directly without any presentation by major histocompatibility complex (MHC) molecules (Shin et al., 2005; Murphy et al., 2012).

Recent reports showed that in contrast to TCRαβ cells, IL-17-production by TCRγδ cells does not need TCR engagement but can merely be triggered by the cytokines IL-23 and IL-1 which are produced by pathogen-activated dendritic cells (Sutton et al., 2009) or by the direct triggering of Toll-like receptor2 and dectin-1 by pathogens (Martin et al., 2009). This feature highlights the innate function of γδ T-17 cells that act like other innate immune cells by directly responding to pathogens or to cytokines produced by cells in infected tissue and, as a result, producing cytokines that amplify the function of other innate or adaptive immune cells.

Although the role of γδ T cells in the mammalian adaptive immune system is still not fully understood, an early divergence of the two classes of T cell receptors and their conservation through evolution suggests distinct functions. The specific location and abundance of TCRγδ cells implicates a role in epithelial/mucosal immunity (Jaffar et al., 2009; Jameson et al., 2003).

1.4.2 Agonist selected unconventional T cells display regulatory functions

Thymic selection depends on the affinity of T-cell receptor-peptide major histocompatibility complex glycoprotein (TCR-pMHC) interactions. In conventional TCRαβ+ T cell development, death by neglect eliminates weak or absent TCR-pMHC interactions while medium affinity interactions are positively selected. T cell precursors that interact with high affinity for self-peptides are generally deleted. However, some self-reactive thymocytes mature into unconventional T cells through an alternative selection process defined as agonist selection (Baldwin et al., 2004; Stritesky et al., 2012). Agonist-selected unconventional T cell subsets are thought to have a regulatory role in immune system and are classified into three main cell types: regulatory T (Treg) cells, invariant natural killer T (iNKT) cells, and TCRαβ+ CD8αα+ intestinal intraepithelial lymphocytes (IELs) (Hsieh et al., 2012; Kronenberg and Gapin, 2002;

Lambolez et al., 2007). The usefulness of lymphocytes with reactivity to self-antigens is twofold: They can both promote immunological tolerance and enhance immunity to foreign antigens (Stritesky et al., 2012).

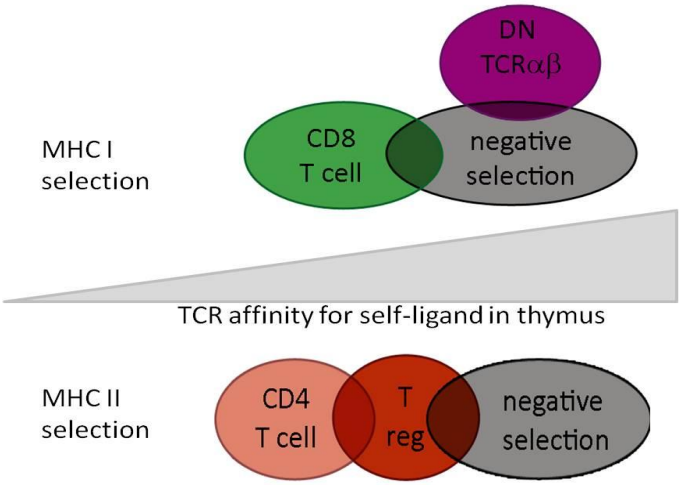

Figure 1.4: T cell selection. Overlap in TCR repertoire for positive, negative, and agonist selection of tTregs and DN TCRαβ IELs in the thymus (adapted from Kurd and Robey, 2014).

1.4.2.1 Regulatory T cells prevent autoimmunity

Regulatory T (Treg) cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. They are by now widely regarded as the primary mediators of peripheral tolerance. At the same time, they reduce beneficial responses in certain circumstances such as limiting anti-tumor immunity. Regulatory T cells are marked by constitutive expression of CD25 and the transcription factor FoxP3, a member of the forkhead/winged-helix family of transcription factors and a master regulator of Treg development and function. Another critical molecule for the development and function is interleukin 2 (IL-2). A component of the high-affinity IL-2 receptor (IL-2R) is CD25, which can be used for the detection of Tregs (Sakaguchi et al., 2008).

Tregs are either generated in the thymus (thymically-derived Tregs; tTregs) or induced in the periphery from classical CD4+ T cells (peripherally-induced Tregs; pTreg). Foxp3+ thymocytes are detectable from late DP stage to the CD4+ or CD8+ single positive stage, constituting ~5% of mature CD4+ and less than 1% of CD8+ thymocytes in the thymus (Fontenot et al., 2005). Thymically derived Tregs differentiate from thymocytes that express T cell receptors with an increased affinity for self-peptide-MHC complexes (Lim et al., 2006; Maggi et al., 2005). They reside at the threshold between positive and negative selection, but are at the same time not

negatively selected and resistant to thymic deletion (Lim et al., 2006). The Treg repertoire is highly diverse with a wide range of antigen specificities but marked reactivity to self antigens, and some degree of overlap with the repertoire of conventional T cells (Hsieh et al., 2006; Fazilleau et al., 2007). A hallmark of tTregs is that - unlike the majority of thymus-produced naive T cells - they are already functionally mature and "antigen-primed" in the thymus, before encountering antigen in the periphery (Sakaguchi et al., 1982; Itoh et al., 1999). Upon antigen exposure, Tregs are activated and exert suppression of effector T cells.

1.4.2.2 Unconventional Intraepithelial lymphocytes - a component of mucosal immunity

In addition to conventional TCR$\alpha\beta$+ or TCR$\gamma\delta$+ resident effector cells expressing either the co-receptor CD4+ or CD8$\alpha\beta$+, the intestinal epithelium harbors unique and abundant innate-like lymphocytes. These unconventional intraepithelial lymphocytes (IEL) are made up of a prominent population of TCR$\alpha\beta$-CD4-CD8$\alpha\beta$- and TCR$\gamma\delta$+CD4-CD8$\alpha\beta$- (Cheroutre et al., 2011). The co-receptor CD4- and CD8$\alpha\beta$- double-negative (DN or co-receptor negative) TCR$\alpha\beta$+ intraepithelial T cells are no circulating T lymphocytes (Guy-Grand et al., 2013) that can represent up to one fourth of the total T cell pool (Fang et al., 2010) and about one third of the TCR$\alpha\beta$+ cells in the intestinal epithelium (Mayans et al., 2014). DN TCR$\alpha\beta$+ intraepithelial T cells were historically called CD8$\alpha\alpha$+TCR$\alpha\beta$+ T cells since they express an $\alpha\alpha$ homodimeric form of the CD8 (Guy-Grand et al., 1991), but unlike CD4 and CD8$\alpha\beta$, the CD8$\alpha\alpha$ homodimer does not function as a TCR co-receptor (Cheroutre and Lambolez, 2008).

DN IELs exhibit unusual features compared to conventional T cells, including phenotype, TCR repertoire, and thymic selection pathway (Cheroutre et al., 2011; Lambolez et al., 2007; Pobezinsky et al., 2012). They harbor numerous potentially autoreactive T cells, as evidenced by the presence of cells expressing forbidden Vβs reactive with autologous, retroviral-encoded superantigens (Guy-Grand et al., 1992; Rocha et al., 1991). Conventional T cells are positively selected by weak interactions with self-peptides. By contrast, DN IELs express strongly self-reactive TCRs but - like regulatory T cells - escape negative selection (Pobezinsky et al., 2012) by "agonist selection". TCR$\alpha\beta$+ IELs have an oligoclonal TCR repertoire enriched for self-reactive clones (Regnault et al., 1994; Rocha et al., 1991) but are not destructive.

Although there is no complete understanding of the individual capabilities of each IEL subset, similar gene expression programs suggest overlapping functions that include homeostatic crosstalk with intestinal epithelial cells and microbiota (Li et al., 2011; Shui et al., 2012) or promoting repair and regeneration of the epithelium by secreting various growth factors, and

killing intestinal bacteria (Boismenu and Havran, 1994; Ismail et al., 2011). IL-17 and IL-22 enhance basic innate barrier defenses at mucosal surfaces, such as antimicrobial peptide production and neutrophil recruitment; both events that occur rapidly and precede adaptive phase immunity. At the intestinal mucosal surface IEL cells are important sources of IL-17 and IL-22 during early phases of infection (Rubino et al., 2012).

1.5 Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones that are produced by the adrenal glands after cytokine stimulation of the hypothalamus-pituitary-adrenal (HPA) axis. GCs bind to the glucocorticoid receptor (GR) that is present in almost every vertebrate animal cell. They are involved in the regulation of a variety of biological processes, including immune responses, metabolism, cell growth and proliferation, as well as development and reproduction. They are part of the feedback mechanism of the immune system that reduces inflammation and are used in medicine to treat a wide spectrum of diseases such as allergies, asthma, autoimmune diseases, and sepsis. All natural steroid hormones share a common chemical structure (Figure 1.5). Corticosterone (Figure 1.5 a, corticosterone) is the dominant glucocorticoid in mice and rats, while cortisol (or hydrocortisone) is predominant in humans. A number of synthetic steroids engaging the GR with different agonistic power have been produced (Nahar et al., 2007). Among them Betamethasone (Figure 1.5 b) shares the steroidal multiring structure of natural glucocorticoids, but an added fluorine atom increases drug potency by slowing metabolism and also increasing the affinity of the synthetic steroid for the receptor (Tannock and Hill, 1992).

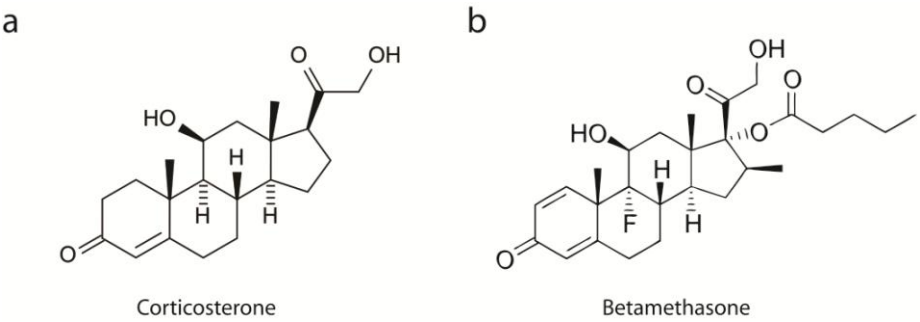

Figure 1.5: Typical steroidal structure. Common multiring structure of steroids of (**a**) corticosterone, a natural steroid which is predominant in mouse and rat and (**b**) of betamethasone, a synthetic corticosteroid which potency is enhanced by an added fluorine atom.

GCs mediate their effects by binding to the glucocorticoid receptor (GR). In its resting state, the intracellular GR protein exists in the cytoplasm (Herrlich, 2001). Ligand binding induces a conformational change in the receptor, which results in the translocation to the nucleus where it then binds to glucocorticoid responsive elements (GREs) and acts as a modulator of transcription, either activating or repressing gene transcription (Greenstein et al., 2002). The modulation of protein expression by this genomic pathway is assumed to take at least 30-60 min while the overall effect takes hours or days (Stahn et al., 2007).

A large fraction of endogenous GCs are bound to proteins (such as albumin) when transported through the blood. Accordingly, only a low amount of GCs is free and available to mediate biological functions (Ashwell et al., 2000). In contrast, the potency of synthetic GC relies on their ability to be carrier-free and available (Kadmiel and Cidlowski, 2013).

1.5.1 Effects of glucocorticoids on the immune system

Glucocorticoids have inhibitory effects on a broad range of immune responses mediated by both the innate and adaptive immune system. Because of their inhibitory effects on both arms of the immune system, glucocorticoids are remarkably effective in managing acute inflammation and autoimmune disorders (Chatham and Kimberly, 2001). There are diverse mechanisms by which steroids exert their functions.

First, GCs prevent or suppress the full inflammatory reaction by inhibiting early inflammatory events such as edema, fibrin deposition, capillary dilatation and migration of leukocytes into the area (Kufe et al., 2003). GCs lead to termination of inflammation by enhancing the clearance of foreign antigens, toxins, microorganisms, and dead cells by supporting opsonisation and by stimulating macrophage phagocytic ability and antigen uptake (Cox, 1995; Liu et al., 1999; Piemonti et al., 1999; van der Goes et al., 2000). They act further by suppressing or stimulating a large number of pro-inflammatory or anti-inflammatory mediators respectively.

The effects of steroids on B- and T-cells are also diverse. Glucocorticoids are known to moderately influence the different phases of B cell activation, survival, proliferation, and differentiation (Kadmiel and Cidlowski, 2013). The effect of glucocorticoids on T cells is specific to the subtype of T cells. Activated and immature T lymphocytes are especially susceptible to glucocorticoid-induced apoptosis (Schwartzman and Cidlowski, 1994; Cohen and Duke, 1984), and several studies suggest a role for glucocorticoids in thymocyte development (Ashwell et al., 2000; van den Brandt et al., 2007). Indeed, epithelial cells in the mouse thymus synthesize steroid hormones, with a production peak at birth and decreasing steadily until adult levels at

approximately four weeks of age (Vacchio et al., 1994; Taves et al., 2014), coinciding with the peak of T cell production during embryonic and postnatal development. It has been postulated that signaling provided upon GR engagement sets the threshold between positive and negative selection (Tolosa et al., 1998).

In addition to GC, TCR engagement results in cell death (activation-induced apoptosis) of immature thymocytes. Simultaneous exposure to both signals, circumstances that take place during thymocyte development and selection processes, antagonizes cell death signals (Zacharchuk et al., 1990; Vacchio et al., 1994). Thymocytes with intermediate binding avidity are able to antagonize apoptosis, while cells above or below a certain threshold cannot, leading to GC-induced (death by neglect) or activation-induces apoptosis (negative selection) (Vacchio et al., 1999; Stephens et al., 2003) (Figure 1.6 a). Reduced glucocorticoid signaling shifts the selection window towards lower TCR affinities, allowing cells with T cell receptors which would normally die by neglect to be positively selected (Ashwell et al., 2000) (Figure 1.6 b). This results in an altered T cell repertoire with less autoreactive cells (Tolosa et al., 1998; Lu et al., 2000).

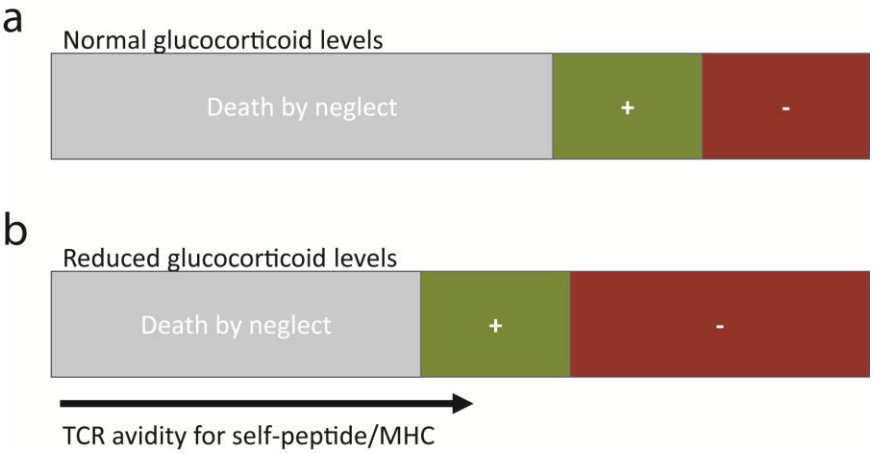

Figure 1.6: Mutual antagonism model of thymocyte selection. (**a**) Activation-induced and glucocorticoid-induced apoptosis are mutually antagonistic. Cells with an intermediate affinity of TCR-peptide-MHC complexes are rescued and positively selected (*green area*). Cells below or above this threshold enter apoptosis; either by glucocorticoid-induced (death by neglect, *grey area*) or activation-induced (negative selection, *red area*) apoptosis. (**b**) Reduced glucocorticoid signaling shifts the selection window towards lower TCR affinities. Cells with low affinity between TCR and self-peptide/MHC which would normally die by neglect are positively selected and those which would normally be positively selected to undergo negative selection (Adapted and modified from Ashwell et al., 2000).

1.5.2 Antenatal Corticosteroid Treatment

Pulmonary surfactant is a surface-active lipoprotein complex formed by alveolar cells. The proteins and lipids that make up the surfactant have both hydrophilic and hydrophobic

regions. The main lipid component of surfactant reduces surface tension and enables the alveoli to unfold. This is particularly important around the time of birth, readying the fetus' lungs to breathe independently after delivery. Surfactant is detectable in low amounts in the amniotic fluid starting at 24 weeks of gestation (Taeusch et al., 2005). Despite this, the lung is not functionally mature until the 34th or 35th week of gestation (Hess, 2012) and babies born before this time point are at high risk of developing respiratory problems. Preterm birth rates range between 5% and 7% in industrial countries, and are estimated to be substantially higher in developing countries (Lawn et al., 2006).

In 1972, administration of antenatal GCs was shown to accelerate neonatal lung maturation (Liggins and Howie, 1972), triggering a very rapid development of the offspring's lungs. GCs activate gene expression of a number of surfactant proteins in the lung, thus reducing the surface tension and enabling the alveoli to unfold. The use of synthetic GCs led to a decrease in the incidence of respiratory distress syndrome (RDS) and preterm mortality (Mendelson and Boggaram, 1991) and is by now considered a standard prophylactic treatment of pregnant women at risk of preterm delivery during gestational weeks 24 to 34. After 34 weeks' gestation the use of ACS is still effective but the reduction in RDS and neonatal death is not significant (Miracle et al., 2008). The recommended ACS treatment consists of two doses of 12 mg betamethasone administered intramuscularly 24 h apart (Miracle et al., 2008). It could be shown though that the use of ACS reduces neonatal death even when infants are born less than 24 h after the first dose has been given (Roberts and Dalziel, 2006). Babies born more than 48 hours, but less than seven days after the administration of steroids, show the greatest benefit (McEvoy et al., 2000). Data establishing the clinical benefit beyond seven days after antenatal corticosteroid therapy are insufficient and recent reports estimate that more than 50% of women receiving ACS remain pregnant 7 to 14 days longer, meaning that more than half of women receive this treatment needlessly and without benefit for the offspring at birth (Asztalos et al., 2013). Beside the undisputed benefits for neonates, excessive exposure to either natural or synthetic GCs may have adverse effects on the offspring in the context of development and growth of several organs (heart, brain, kidney) (Uno et al., 1990; Zhang et al., 2010). More surprisingly, despite well documented effects of steroids on developing immune cells, very few efforts have been made to study the effects of ACS on the immune system. Some studies now establish a link between ACS treatment with the development of asthma early in life (Pole et al., 2010) or diabetes (Greene et al., 2013), hinting that there may be long lasting consequences of antenatal glucocorticoids on the immune system of the offspring.

1.6 Aims

Steroids, in particular betamethasone, are routinely administered to pregnant women at risk of preterm birth to promote lung maturation in the offspring. Until now, few studies have addressed the effect of ACS on the immune system of the offspring, although corticosteroids are known to induce death of developing thymocytes.

Low amounts of steroids influence the selection processes of immature thymocytes, leading to more negative selection. Alterations in the selection process induces changes in the peripheral T cell repertoire and affects the adaptive immune response (Mittelstadt et al., 2012; van den Brandt et al., 2007; Lu et al., 2000; Tolosa et al., 1998; Tolosa and Ashwell, 1999). The effect of excessive steroid signaling around birth - a crucial time for T cell development and thymic export - is not known. Following the mutual antagonism model of thymocyte selection, we hypothesize that increasing GC levels shift the selection window towards higher TCR avidity, resulting in a T cell repertoire with increased levels of autoreative T cells.

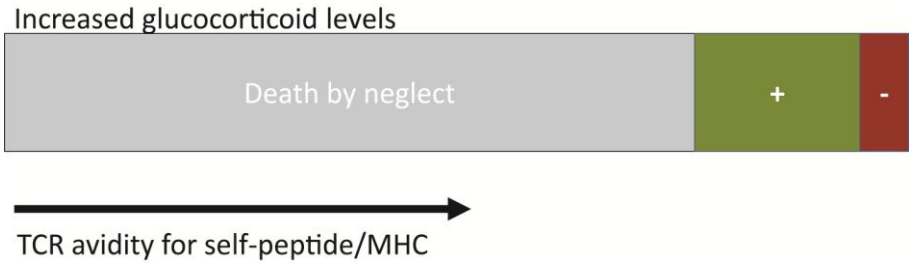

Figure 1.7: Hypothesized selection process with increased glucocorticoid levels. Increased levels of glucocorticoid around birth may shift the selection towards higher TCR affinities. Positive selection of cells which would normally be negatively selected results in a repertoire harboring more autoreactive T cells, thus increasing the potential of autoimmunity.

Consequently, antenatal steroid treatment may impair the normal development of the offspring's immune system and increase the risk for autoreactivity and poor host defense in later life.

To investigate the effects of steroids on the immune system of the offspring, the aim of this project is:

1. To evaluate short-term consequences of ACS treatment.
    - Perform MRI based calculations of thymic size,
    - Assess the frequency of cell death and the effect on different T cell subsets by flow-cytometry

2. To investigate long-term consequences of antenatal steroid treatment on the immune system of the offspring.
    - Analyzing the frequency of the main T cell subsets and the Vγ TCRγδ T cell repertoire by flow cytometry in antenatally treated adult mice
3. To assess the effects of ACS on the development of autoimmune diseases. For this, the development of cellular changes and progression of clinical symptoms in two animal models of spontaneous autoimmune disease are monitored:
    - Colitis in the IL-10 KO
    - Lupus-like disease in the MRL lpr/lpr model

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Reagents and solutions

Material	Company
Betamethasone	**Sigma**
Bouin's solution	**Sigma**
FACS Clean Solution	**BD Bioscience**
Dulbecco's Phosphate Buffered Saline (PBS), 1x	**Gibco**
EDTA (0.5M)	**AppliChem**
Ethanol, ≥99,8%	**Roth**
Eosin	**Chroma**
FACS Flow Sheath Fluid	**BD Biosciences**
Hematoxylin	**Chroma**
L-Glutamine, 200 mM	**Gibco**
Live/Dead dye (Pacific Orange succinimidyl ester)	**Invitrogen**
Lysing Solution	**BD Bioscience**
MultiHance	**Bracco**
Penicillin/Streptomycin, 100x	**PAA**
Biocoll Separating Solution	**BIOCHROM AG**
FACS Rinse Solution	**BD Bioscience**
RPMI 1640	**Gibco**
Serum, fetal bovine	**Biochrom AG**
Trypan blue solution, 0.4%	**Sigma Aldrich**
Xylol	**National diagnostics**

2.1.2 Composition of media

Buffer	Compounds
FACS buffer	0.1% BSA 0.02% NaN_3 In 1x PBS
Fc blocking solution	0.5 µl Fc block 1 µl NRS In 1x PBS/0.2% BSA
Standard RPMI medium	10% FCS (heat inactivated) 1% Penicillin/Streptomycin 2 mM L-Glutamine In RPMI
Digestion medium (lung)	30 U DNase 2 mg/ml Collagenase IV In PBS
Digestion medium (colon)	30 U DNase 100 mg/ml Collagenase IV In RPMI

2.1.3 Antibodies against human surface markers for Flow Cytometry

Specificity	Fluorochrome	Clone	Marker for	Company
CD3	PerCP-Cy5.5	OKT3	Lymphocytic cell subsets	**eBioscience**
CD4	AF488	RPA-T4	T helper cells	**BioLegend**
CD8	V510	RPA-T8	cytotoxic T cells	**BD Bioscience**
CD8	PECy7	SK1	cytotoxic T cells	**BioLegend**
CD14	Pacific Blue	HCD14	Monocytes and Macrophages	**BioLegend**
CD16	APC-Cy7	3G8	NK cells, Monocytes, Macrophages	**BioLegend**
CD19	PE-Cy7	HIB19	B cells	**BioLegend**
CD45	BV510	HI30	Hematopoietic cells	**BD Bioscience**
TCR$\gamma\delta$	PE	11F2	TCR$\gamma\delta$ cells	**BD Bioscience**

2.1.4 Antibodies against mouse surface markers for Flow Cytometry

Specificity	Fluorochrome	Clone	Host	Staining for	Company
CD3	eFluor450	500A2	Golden Syrian Hamster	T cells	**eBioscience**
CD3	BV510	17A2	Rat	T cells	**BioLegend**
CD4	APC-eFluor780	RM4-5	Rat	T helper lymphocytes	**eBioscience**
CD4	BV421	GK1.5	Rat	T helper lymphocytes	**BioLegend**
CD8	PECy7	53-6.7	Rat	Cytotoxic T cells	**eBioscience**
CD24	BV421	M1/69	Rat	Heat stable antigen, maturation marker	**BioLegend**
CD25	PE	3C7	Rat	IL-2 receptor alpha chain; Treg marker, activation marker	**BioLegend**
CD44	APC	IM7	Rat	Cell surface glycoprotein; maturation marker	**eBioscience**
CD44	PECy7	IM7 H23:N23	Rat	Cell surface glycoprotein; maturation marker	**BioLegend**
45R/B220	FITC	RA3-6B2	Rat	B cells, activated T cells, T cells of the lpr/lpr mouse strain	**BioLegend**
CD69	BV421	H1.2F3	Armenian Hamster	„Very early activation antigen“, Activation marker	**BioLegend**
TCR β chain	PerCPCy5.5	H57-597	Armenian Hamster	TCRβ chain marker	**BioLegend**
TCRγδ chain	FITC	eBioGL3	Armenian Hamster	TCRγδ cell marker	**eBioscience**
TCR Vγ1.1/Cr4	PE	2.11	Armenian hamster	TCR Vγ1 cell marker	**BioLegend**
TCR Vγ2	APC	UC3-10A6	Armenian Hamster	TCR Vγ4 cell marker	**BioLegend**
CD304/Nrp1	APC	3E12	Rat	Neuropilin, thymic Treg marker	**BioLegend**
GARP	PE	F011-5	Rat	„Glycoprotein A Repetitions Predominant“ Activation marker in Tregs	**BioLegend**
			Apoptosis markers		
Annexin V	FITC				**BD Bioscience**
			Fc Block		
CD16/CD32	unconjugated	2.4G2			**BioXCell**

2.1.5 Kits

	Company
Mouse anti-dsDNA ELISA Kit	**Shibayagi Co., Ltd**
FITC BrdU Flow Kit	**BD Bioscience**

2.1.6 Recombinant Proteins

CR2-Fc	J. Thurman, University of Colorado, USA

2.1.7 General Equipment

Equipment	Model/Type	Company
Autoclave	**Varioclave**	**H+P Labortechnik**
Centrifuges	**5810R**	**Eppendorf**
ELISA plate reader	**Wallac Victor 1420**	**PerkinElmar**
Flow cytometer	**FACS Canto II**	**BD Bioscience**
Freezers -20 °C		**Liebherr and Kryotec**
Freezer -80 °C	**905**	**Thermo Scientific**
Fridges		**Liebherr**
Microscope	**Standard20**	**Zeiss**
Neubauer cell chamber		**Marienfeld**
pH meter		**Mettler**
Pipettes		**Eppendorf, Gilson**
Pipette boy		**Eppendorf**
Racks		**Roth**
Slide scanner	**Mirax Midi**	**Zeiss**
Standard power pack P25	**Bio105 LVD**	**Biomed Analytic**
Sterile bank		**Heraeus**
Table centrifuge	**5424R**	**Eppendorf**
Thermoshaker with heated lid	**2.0 ml**	**Biometra**
Water bath		**GFL**
Vortex	**Gene 2**	**Scientific Industries**

2.1.8 Consumables

Consumable	Company
96-well plate, U-bottom	**Greiner**
Cell strainer, 70 µm	**BD Falcon**
Embedding cassettes	**Carl Roth**
Eppendorf tubes	**Eppendorf**
FACS tubes	**Sarstedt**
Falcon tubes	**BD Falcon**
Gloves, nitrile and latex	**Supermax Glove**
Multistix 10 SG	**SIEMENS**
Needles	**B. Braun**
Petri dish	**nunc**
Pipette tips	**Eppendorf, Sarstedt**
Serological pipettes	**BD Falcon**
Surgical blade	**B. Braun**
Syringes	**B. Braun**
TruCount tubes	**BD Bioscience**

2.1.9 Software

Software	Company
BD FACSDiva 8.0	**BD Bioscience**
FlowJo vX.0.7	**Tree Star, Inc.**
Prism 5.01	**GraphPad Software, Inc.**
TBase	**4D SAS**
OsiriX	**Pixmeo**
Panoramic Viewer software	**3DHISTECH Ltd.**
K-Pacs	**IMAGE Information System Ltd**

2.1.10 Donors

Donors	Source	Ethical approval
Cord blood	Prof. P. Arck/Prof. K. Hecher	PV3694
Thymic tissue	Prof. Ali Dodge-Khatami	PV3459

2.1.11 Mouse strains

Strains	
C57Bl/6J	UKE, Hamburg
BALB/c-CD45.1	Charles River, Sulzbach, Germany
IL-10 KO	UKE, Hamburg, through Dr. Gelderblom
MRL lpr/lpr	Charles River, Sulzbach, Germany

2.2 Methods

2.2.1 Human samples

Cord blood

Umbilical cord blood samples were obtained from the maternity clinic (Prof. Dr. Kurt Hecher), in collaboration with the Laboratory for Experimental Feto-Maternal Medicine and under supervision of Prof. Petra Arck (Department of Obstetrics and Fetal Medicine)

Thymus samples

Thymus tissue was provided by Prof. Ali Dodge-Khatami (Department of Paediatric Cardiac Surgery) from children undergoing cardiac surgery. The sample collection was approved by the local ethics committee (study code: PV3459; Immunregulatorische Mechanismen in Autoimmunerkrankungen: Studien zur Phänotypisierung und Funktion von regulatorischen und Effektor-Vorläuferzellen; Applicant: Eva Tolosa)

2.2.2 Animal experimentation

2.2.2.1 Ethical approval

Animal experiments were conducted according to the German Animal Protection Law care and the guidelines of our institution.

	Ethical approval
„Auswirkungen von Betamethason-Applikation auf die Entwicklung des Immunsystems sowie als mögliche Ursache von Autoimmunerkrankungen“	G12/122
„Auswirkungen von Betamethason-Applikation auf die Entwicklung des Immunsystems sowie als mögliche Ursache von chronisch-entzündlichen Darmerkrankungen“	G31/13
„Auswirkungen pränataler Betamethason-Applikation auf die Entwicklung des Immunsystems sowie der Genese von Autoimmunerkrankungen in einem systemischen Lupus Modell“	G13/119
„Auswirkungen von Steroid-Applikation auf Glucocorticoid-Rezeptor-Expression einzelner Immunpopulationen sowie auf Thymus-Epithelzellen in vitro“	ORG 674

2.2.2.2 Housing of mice

C57Bl/6J, BALB/c-CD45.1 and MRL lpr/lpr mice were housed in individually ventilated cages of the S1-barrier of the animal facility in N27 at the University Medical Center Hamburg-Eppendorf. The IL-10 KO mice were housed in open cages in the S2-barriere of the same facility.

2.2.2.3 Timed pregnancies

For mating, two female mice were paired with individual males. Females were checked the morning for the presence of a vaginal plug, and plugged females were separated from males. The day of the plug detection was considered embryonic day (E) E0.5Betamethasone (Sigma-Aldrich, Germany) ranging from 0.01 to 0.1 mg or PBS as control were administered on day E18.5 by intra peritoneal (i.p.) injection of the pregnant female (Figure 2.1). Bromodeoxyuridine (BrdU, 1 mg) was administered on day E18.5 by i.p. injection.

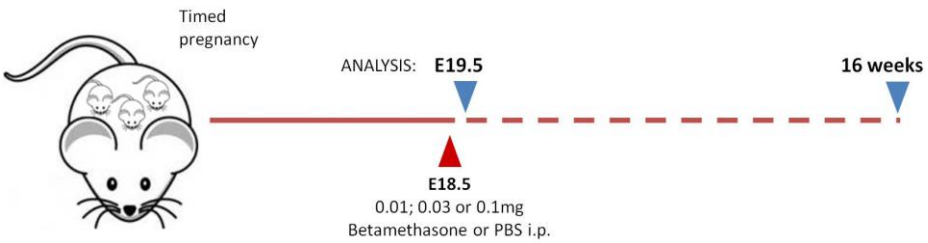

Figure 2.1: Mouse model for antenatal steroid treatment (ACS). Male and female mice were paired and the detection of vaginal plug was considered E0.5. Betamethasone or PBS was administered on E18.5 by i.p. injection. Analysis of the offspring was conducted at different time points (only the first and last time points analyzed are depicted).

2.2.2.4 IL-10 KO mice – Colitis model

Interleukin-10 (IL-10) is an important regulatory cytokine that suppresses effector functions of macrophages/monocytes, T helper 1 (Th1) cells, and natural killer cells (for review, see Howard and O'Garra, 1992). Mice homozygous for the IL-10 gene deletion do not produce IL-10 and spontaneously develop chronic inflammatory bowel disease (IBD) (Kuhn et al., 1993; Berg et al., 1996), with an incidence of 100% at 3 months of age (Berg et al., 1996). Clinical signs of inflammation are diarrhea, perianal ulceration, intestinal bleeding, and occasionally rectal prolapse (Kuhn et al., 1993; Berg et al., 1996).

Offspring of betamethasone- or sham-treated mothers were screened for disease symptoms by colonoscopy. Scoring was based on 5 parameters:

- Stool consistence
- Vascularity
- Granularity of mucosal surface
- Translucency of colon

For each of the parameters, a 3-point-scale was used with 0=no signs of disease and 3=severe signs of disease. All parameters added up to a cumulative score raging from 0 to 15 points. Additionally, mice were weighted weekly to screen for weight loss induced by diarrhea. Mice were sacrificed at the age of 14 weeks. Organs were collected and used for FACS analysis of immune cell populations.

2.2.2.5 MRL/MpJ-Fas^{lpr} - Systemic lupus erythematosus

Mice homozygous for the lymphoproliferation (lpr) spontaneous mutation on the MRL/Mpj background - MRL lpr/lpr mice - develop systemic autoimmunity resembling human systemic lupus erythematosus (Andrews et al., 1978). Hallmarks of the disease include massive lymphadenopathy - which is associated with proliferation of aberrant T cells, and production of anti-dsDNA autoantibodies, and glomerulonephritis due to immune complex deposition in the kidney. Disease incidence is 100% with an earlier onset and progression of SLE symptoms in female mice.

Female offspring were screened weekly for disease symptoms starting at the age of 8 weeks. Scoring was based on three parameters:

- Skin Lesion Scoring
- Lymphadenopathy Scoring
- Urine protein content scoring

Additionally, mice were weighted weekly. At 12 and 16 weeks of age, blood was collected and serum stored for analysis of anti-dsDNA Antibodies. To follow the deposit of immune complexes in the kidney, in vivo MRI was conducted at the ages of 12 and 16 weeks. Mice were sacrificed either at 12 or 16 weeks of age and organs were collected and used for FACS analysis of immune cell populations.

2.2.2.6 Organ and blood collection

Pups were sacrificed by decapitation on days E19.5 to E21.5. Adult mice were sedated by CO_2/O_2 inhalation and sacrificed by cervical dislocation.

Blood was collected directly from the heart into tubes containing EDTA and used for analysis of peripheral blood cells by FACS or for analysis of serum. To separate the serum, blood was centrifuged at 8000 rpm for 20 mins at 4 °C. The serum was collected and frozen at -80 °C. Thymus, lung, spleen, colon and lymph nodes were harvested and processed immediately (see next paragraph). Kidneys were fixed in Bouin´s solution for 24 h and stored in EtOH (70%) at 4° C.

2.2.3 Cell culture

2.2.3.1 Isolation of primary cells

Thymus and lymph nodes

Thymi or lymph nodes were placed in a small petri dish containing 2 ml 1x PBS. Single cell suspensions were prepared by mechanical disruption using a syringe embol. During this process, cells were released into the medium and they were subsequently transferred into a falcon tube. After centrifugation (5 mins, 1500 rpm) the supernatant was discarded and the cell pellet was resuspended in 1 ml PBS before counting.

Spleen

Spleens were processed as above, and the cell pellet was further resuspended in 1 ml distilled H_2O for 20 seconds to lyse erythrocytes. Lysis was stopped by addition of 10 ml 1x PBS. Samples were centrifuged as described before. The supernatant was discarded and the cell pellet was resuspended in 1 ml PBS before counting.

Lung

Lung tissue was placed in a small petri dish containing 2 ml 1x PBS. Tissue was cut into small pieces and digested in 1 ml of PBS containing 30 U DNase and 2 mg/ml Collagenase IV. Tissue was incubated for 2 h at 37 °C in a rotating platform. Samples were additionally vortexed every 15 mins. The recovered cell suspension was passed through a cell strainer into a falcon tube and washed with PBS containing 2 mM EDTA. After centrifugation (5 mins, 1500 pm) the cell pellet was resuspended in 1 ml Lysis buffer and incubated for 5 mins at room temperature. Lysis was stopped by addition of 10 ml PBS containing 2 mM EDTA. The cell pellet was subsequently washed and resuspended in 1 ml PBS before counting.

Colon

Colonic tissue was placed in a petri dish containing 2 ml 1x PBS with 1% FBS. Colon was cut open and washed twice. Tissue was placed in a tube containing RPMI medium and 1mM EDTA and incubated for 30 mins at 37 °C under constant shaking. The intraepithelial lymphocytes (IEL) were released into the medium. Remaining tissue was washed twice with 1x PBS containing 1% FBS while the medium containing the IELs was stored on ice. To extract lamina propria lymphocytes (LPL), colonic tissue was cut into small pieces and digested in RPMI containing 30 U DNase and 100 mg/ml Collagenase IV for 45 mins at 37 °C under constant shaking in a water bath. Remaining tissue was processed through a cell strainer (70 µm) and washed twice with 1x PBS with 1% FBS. Cell suspension was combined with the previously isolated and stored cells. The resulting cell suspension was washed and resuspended in 40% Biocoll before layering onto a 90% Biocoll solution. The Biocoll gradient was centrifuged (20 mins, 1500 rpm without braking) at room temperature. Lymphocytes were isolated from the interphase and washed with 1x PBS and 1% FBS. The supernatant was discarded and cells were resuspended in 1 ml PBS before counting.

2.2.3.2 Cell count

Isolated cells were centrifuged and resuspended in a defined volume of medium. 10 µl cell suspension was diluted 100-fold in Trypan blue, which stains dead cells. A Neubauer counting chamber was used for counting the cells under the microscope.

2.2.3.3 In vitro apoptosis assay

Normal human thymic tissue was obtained from children undergoing corrective cardiac surgery after informed consent from the parents. Thymic tissue was mechanically disrupted in full RPMI (containing 10% Fetal bovine serum, 1% Penicillin/Streptomycin and 2 mM L-Glutamine) until a single cell suspension was obtained. Fresh thymocytes ($5x10^5$) were seeded into 48-well plates and betamethasone was added at a final concentration ranging from 1 nM to 1 µM. Cells were incubated overnight at 37°C and cell death was subsequently analyzed by flow cytometry. Mouse thymocytes from C57Bl/6J mice were prepared accordingly.

2.2.4 Methods in immunology

2.2.4.1 Enzyme-linked-immunosorbent-assay (ELISA)

The enzyme-linked-immunosorbent-assay (ELISA) can be used to detect the presence of a protein in a sample. The sandwich ELISA is based on the binding of antigen to an immobilized antibody, which can be subsequently quantified using a second antibody directed against a different epitope of the antigen and a detection method. For the detection of anti dsDNA antibodies in MRL lpr/lpr mice, serum was measured using the mouse anti-dsDNA ELISA kit according to the manufacturer's protocol (Shibayagi Co., Ltd). dsDNA coated 96 well-plate was washed with washing buffer. For quantification, a serial dilution of the standard was prepared and serum samples were diluted. Wells were filled with 100 µl standard or mouse sera and incubated for 2 h at room temperature on a plate shaker. Wells were washed and 100 µl of HRP-labeled anti-mouse IgG antibody solution added before 2 h incubation at room temperature on a shaker. Wells were washed and 100 µl chromogenic substrate was added to the wells and incubated for 20 mins before the reaction was stopped by addition of 100 µl reaction stopper. Absorbance of each well was measured using a plate reader at 450 nm.

2.2.4.2 Flow Cytometry

Flow cytometry is a laser-based technique used for analysis and isolation of fluorescently labeled cells. After staining the cells, flow cytometric analysis was performed on a BD FACS Canto II flow cytometer using the FACS Diva software (BD Biosciences) for data collection. Analysis of data was performed with FlowJo software (Tree Star, Inc.).

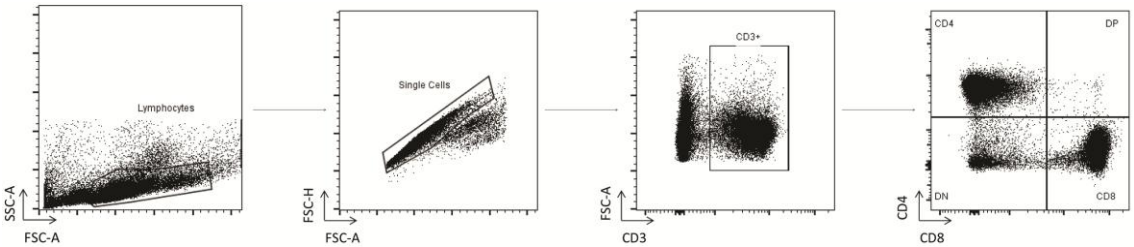

Figure 2.2: Gating strategy for T cell subsets in spleen. The first gate is set according to the size and granularity of the cells using FSC-A/SSC-A (A=area). Comparison of FSC-A and FSC-H (H=height) is used to discriminate between doublets and single cells. Fluorochrome labeled antibodies can be used to distinguish between specific cell types, here gating first on CD3 positive cells and then using CD4 and CD8 antibodies to discriminate the subsets of double negative (DN), double positive (DP) and CD4 and CD8 single positive cells.

2.2.4.3 Staining of surface and intracellular markers for Flow Cytometry

For cell surface FACS analysis, unspecific binding was first prevented by incubating cells with Fc-blocking agent (anti CD16/32) and normal rat serum for 5 mins on ice. After incubation with Fc-block, approximately $1x10^6$ cells were stained with different fluorochrome-coupled surfaced antibodies diluted in FACS buffer and incubated for 20 mins at RT. In case of whole blood, erythrocytes were lysed by adding 1 ml 1x Lysing Solution (BD) for 10 mins at RT in the dark followed by a washing step.

For the assessment of cell death, Pacific Orange succinimidyl ester (Invitrogen) and annexin V FITC (BD Biosciences) were used according to the manufacturer's instructions for analysis of dead and apoptotic cells, respectively. Annexin V detects phosphatidylserine and phosphatidylethanolamine on the cell surface, an event found in apoptotic and dead cells, but not on healthy living cells. Pacific Orange succinimidyl ester can cross the cell membranes of dead cells and react with free amines in the cytoplasm. Live cells exclude these dyes since their cell membranes are intact and the free dye is washed away after staining. After staining, cells were washed by adding 1 ml of PBS and centrifuged at 1500 rpm for 5 mins. The supernatant was discarded and cells were resuspended in 200 µl FACS buffer and analyzed by FACS.

Using BD TruCount Tubes containing fluorescent beads, the absolute number of cells in the sample can be determined by comparing cellular events to bead events. Thymocyte proliferation was analyzed by measuring BrdU incorporation using anti-BrdU antibodies (BrdU Flow kit (BD)).

For intracellular staining, cells were first stained for cell surface markers as described above. Cells were incubated with 1 ml fresh prepared 1x Fixation Buffer (R&D) for 30 mins at 4 °C in the dark and washed twice with 1x Permeabilisation/Wash Buffer (PermBuffer; R&D). Subsequently the antibody directed against the transcription factor FoxP3 was added and

incubated for 30 min at 4 °C in the dark. After washing with Permeabilisation/Wash Buffer, the cells were resuspended in 200 µl FACS buffer and analyzed by FACS.

2.2.5 Methods of protein analysis or Biochemistry methods

2.2.5.1 Mass spectrometry

Mass spectrometry (MS) is an analytical chemistry technique for identification of the amount and type of chemicals present in a sample.

Betamethasone and cortisol-d_4 (CIL, USA) were used as reference and internal standards, respectively. Newborn calf serum (Biochem, Germany) was used as blank serum sample. Serum samples were spiked with 500 pg Cortisol-d_4, diluted and liquid-liquid-extracted. The combined extracts were dried under nitrogen at 50 °C, reconstituted and injected onto the HPLC- C18-column (Thermo Fisher Scientific, Germany). The chromatographic separation was performed on the LC-system 1200 series (Agilent Technologies, USA). Tandem mass spectrometry was carried out on a Q-Trap 5500 mass spectrometer (AB Sciex, USA) equipped with an ESI interface. The instrument was operated in the positive ionization mode at +5500 V and a temperature of 550 °C. Nitrogen was employed as nebulising gas. Specific multiple reaction monitoring (MRM) transitions were optimized for betamethasone and for cortisol. For validation, calibration samples prepared with 30 µL of calf serum spiked with different concentrations of betamethasone. Detection limit was 0.3 pg/µL (signal-to-noise ratio greater than 3) and the calibration curve showed linear relationship (R^2 = 99%).

Mass spectrometry analysis for the detection of betamethasone in serum samples of newborn mice was performed by Aniko Krumbholz, at the Institute of Doping Analysis and Sports Biochemistry, Kreischa, Germany.

2.2.6 Histology

Fetuses were fixed in Bouin's solution and then embedded in paraffin. Transversal 4 µm thick sections were cut from the neck area of the fetus and stained for haematoxylin and eosin (H&E). Haematoxylin staining results in a blue color of cell nuclei while counterstaining with an aqueous or alcoholic solution of eosin Y colors other, eosinophilic structures in various shades of red, pink and orange.

Paraffin was removed from the slides by incubating twice for 10 mins in xylol, followed by subsequent incubation in different dilutions of Ethanol (100%, 96%, 90%, 80% and 70%) for 2

mins each. Sections were then washed in PBS for 5 mins. For H&E staining, sections were placed in Hematoxylin for 15 mins. Slides were transferred and rinsed for 15 mins with water and then stained for 60 sec with Eosin. Three incubation steps of 2 mins in 100% ethanol were followed by two further 5 minute incubation steps in xylol.
Slides were scanned using a slide scanner. The Panoramic Viewer software was used to prepare the pictographs.

2.2.7 Magnetic resonance imaging (MRI)

Radiology is a medical field that uses imaging to diagnose and treat disease seen within the body. Magnetic resonance imaging (MRI) uses strong magnetic fields to align atomic nuclei within body tissues, followed by the use of a radio signal to disturb the axis of rotation of these nuclei. The radio frequency signal generated can be observed as the nuclei return to their baseline states. The radio signals are collected by coils placed near the area of interest. MRI scans give the best soft tissue contrast of all the imaging modalities.
All MRI-based methods were performed by the Diagnostic and Interventional Radiology Department (UKE) on a dedicated small animal MR scanner at 7.0 Tesla (ClinScan, Bruker BioSpin, Germany).

2.2.7.1 Thymic volumetry

Fetuses were harvested on gestation day 19.5, fixed in Bouin´s solution for 24 h and stored in EtOH (70%) at 4° C. For contrast enhancement, fetuses were placed in a 4% gadolinium-based contrast agent (Multihance, Bracco, Germany) for 48 h, and subsequently embedded in 1% agarose for imaging. Volumetric analysis of the fetal thymus was performed using open source image analysis software (OsiriX, Pixmeo). Thymic tissue in successive MRI sequences was outlined and marked as region of interest (ROI). The volume of the thymus was calculated from the determined ROIs.

2.2.7.2 Size of axillary and renal lymph nodes

Mice were placed in an animal bed equipped with circulating warm water to regulate the body temperature. Animals were anaesthetized using 2% isoflurane (Forene® 100% V/V, Abbott GmbH & Co. KG, Germany), keeping the breathing rate between 30 - 40 breaths per minute. A T2–weighted turbo–spin–echo sequence (TR 1760 ms, TE 49 ms, FoV 40 mm, voxel size 0.156 x

0.156 x 0.6 mm³, slice thickness 0.6 mm, number of signal acquisitions (NSA) 4, time 6:10 min) was performed for evaluation of lymph node size. Analysis of lymph node was performed using open image analysis software (K-Pacs, IMAGE Information System Ltd.), measuring the widest distance of the organ.

2.2.8 Statistical Analysis

Statistical differences were determined by Student's *t* test or one-way ANOVA using the GraphPad Prism 5.01 software. If one-way ANOVA showed significant differences within the group means, Dunnett's test was performed as a posthoc test. Significance is displayed as (*) if $p<0.05$, (**) if $p < 0.01$, and (***) if $p < 0.001$.

3 RESULTS

3.1 Immediate effects of antenatal steroid treatment

3.1.1 Antenatal betamethasone can be detected in the offspring

To address the immediate effects of antenatal steroids on the offspring, we first had to determine if the steroid reaches the offspring's body. For this, we injected the pregnant female mice with different amounts of betamethasone or with PBS on pregnancy day E18.5 and analyzed the serum of the pups by mass spectrometry 24 h after treatment. The range of concentrations used in these experiments varied between 0.01 and 0.1 mg. It has been shown previously that concentrations in the range of 0.1 mg elicited a positive effect on lung maturation of the unborn pups (Christensen et al., 1997) and also represented an equivalent body surface area dosage compared to human treatment (Stewart et al., 1998).

Betamethasone could be readily detected in a dose-dependent manner in the serum of the pups of betamethasone-treated mothers, but not of mock-treated mothers, 24 h after application (Figure 3.1). The presence of the antenatally applied synthetic steroid in the pups is transient, as analysis at 48 h after application revealed that betamethasone was only detected in one of five pups whose mothers had been treated with the maximum dose of betamethasone.

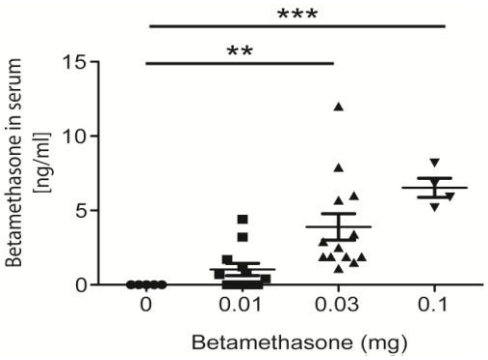

Figure 3.1: Betamethasone can be detected in the pups' peripheral blood. Betamethasone (0.01, 0.03 or 0.1 mg) was injected i.p. to a pregnant female mouse on day E18.5. Levels of betamethasone in the pup 24 h after injection, analyzed by mass spectrometry. Values are expressed as mean ± SEM (*n* = 4-13)

3.1.2 Antenatal betamethasone results in reduced size of the offspring's thymus

Steroids are long known to induce death of immature thymocytes (Cohen and Duke, 1984). Since the synthetic steroids could be detected in the offspring's body, we wanted to know whether antenatal application had an effect on the thymus of the offspring. Using MRI and flow cytometry, we assessed thymus volume, absolute cell numbers and cellular composition in the offspring. MRI analysis of the pups revealed that even minimal doses (0.01 mg) of

betamethasone resulted in a significant reduction in thymic volume. The degree of reduction was dependent on the dose of betamethasone administered to the mother and reached a maximum 30% of the total volume (Figure 3.2 a and b). Of note, the reduced thymus volume was unrelated to the size of the pup (data not shown). In addition, single-cell suspensions from isolated thymi were prepared and absolute numbers were counted by flow cytometry, using BD TruCount tubes. The number of viable thymocytes was massively reduced in the offspring of mothers treated with 0.1 mg betamethasone (Figure 3.2 c).

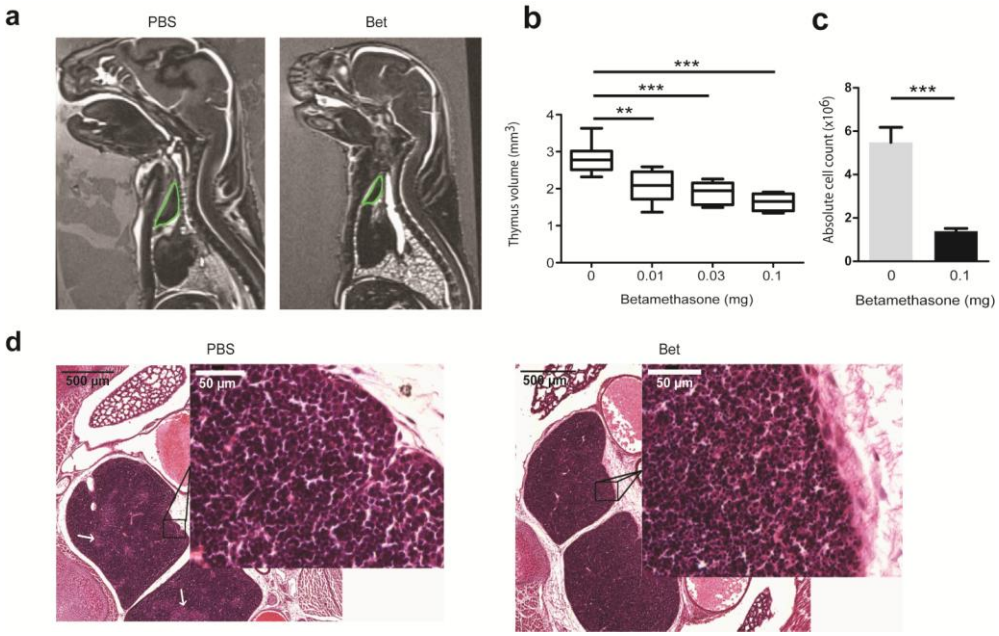

Figure 3.2: Antenatal glucocorticoids cause a reduction in the thymic volume of the offspring. Betamethasone (0.01, 0.03 or 0.1 mg) was injected i.p. to a pregnant female mouse on day E18.5. (**a**) Representative MRI pictures displaying the thymus of the offspring whose mothers were treated with PBS (*left*) or with 0.1 mg Betamethasone (*right*). (**b**) MRI quantification of the thymic volume of the fetuses 24 h after betamethasone application. The *boxes* represent the 25th and 75th percentiles of the values, with the *bars* showing the minimum and maximum values (*n*=4-8). Statistical analyses were performed by one-way ANOVA and Dunnett post hoc test. (**c**) Absolute cell count of viable thymocytes from pups of PBS- and betamethasone-treated mothers. Values are expressed as mean ± SEM (*n*=8-9). Statistical analysis was performed using Student's *t* test. (**d**) Representative H&E staining of paraffin sections displaying the thymus of the offspring whose mothers were treated with PBS (*left*) or with 0.1 mg Betamethasone (*right*). In the PBS-treated animal, the white *arrows* indicate the thymic medulla, which is not visible in the betamethasone-treated animal. The *inset* shows a magnification of the thymic cortex. The *scale* is indicated in the figure.

Histological examination of the cellular composition and structure of the thymus showed the expected two distinct areas in the pups from control-treated mothers: The densely packed cortex and the more sparse cellular distribution in the medulla. In contrast, in the thymi of pups from betamethasone-treated mothers, these two areas could not be differentiated. The

massive loss of immature thymocytes resulted in a sparse cellular distribution in the cortex, making it indistinguishable from the medulla (Figure 3.2 d). Taken together, these findings show that betamethasone leads to a marked reduction of thymic volume and cellularity even at the lowest dosage applied.

3.1.3 Betamethasone treatment induces death of developing thymocytes

To investigate the effect of betamethasone on a cellular level, we assessed the range of apoptosis in thymic cells from the offspring of betamethasone- or sham-treated mothers by flow cytometry using the panel depicted below (Figure 3.3 a).

In viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. When entering the intermediate stages of apoptosis, PS is exposed to the external cellular environment by translocation to the outer leaflet of the membrane where it can be detected by annexin V conjugates. We used Pacific Orange succinimidyl ester to discriminate dead cells. The dye can cross cell membranes of dead cells and reacts with free amines in the cytoplasm while live cells exclude these dyes due to intact cell membranes and the unbound dye is washed away after staining. Early apoptotic cells (annexin-V positive only) can be distinguished from late apoptotic/necrotic cells (annexin-V and dye positive)(Wlodkowic et al., 2011). More than 90% of the thymocytes were alive in the thymus of sham-treated mothers 24 h after injection (determined as negative for both Annexin V and Dead Cell Stain). With rising doses of betamethasone applied to the mothers, the amount of living cells decreased significantly (Figure 3.3 b and c). In the group treated with the highest dose of betamethasone (0.1 mg), the frequency of live cells was 33% reduced compared to the control group (Figure 3.3 c). A subsequent increase in apoptotic and necrotic cells was equally evident.

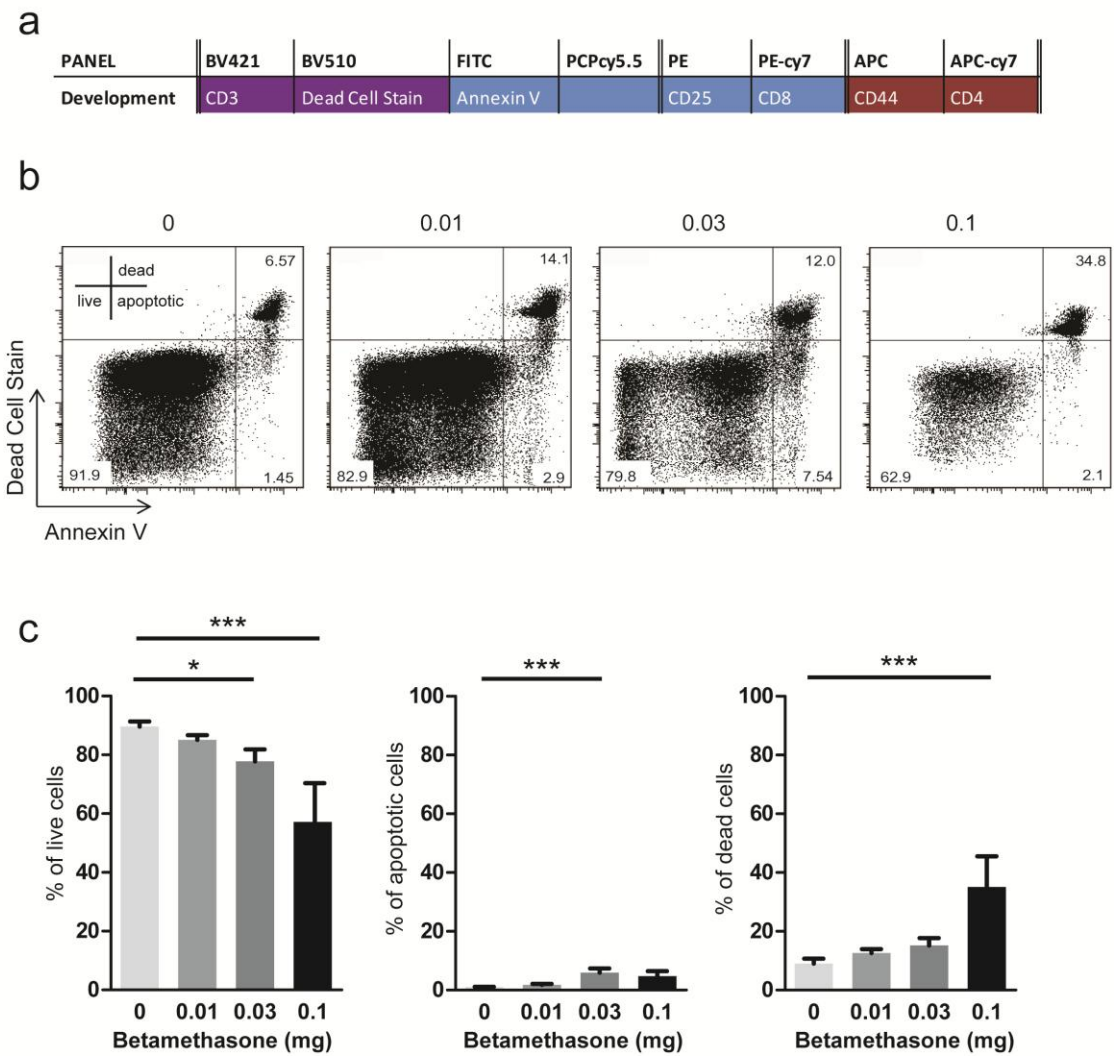

Figure 3.3: Antenatal betamethasone induces thymocyte death. Betamethasone (0.01, 0.03 or 0.1 mg) was injected i.p. to a pregnant female (E18.5). Cell death was assessed 24 h after injection by flow cytometry (Annexin V and Pacific Orange succinimidyl ester) on single cell suspensions from the offspring's thymi. (**a**) Staining panel used for FACS analysis. (**b**) Representative dot plot analysis of cell death for each applied dose and the control group. (**c**) Percentage of live, apoptotic and dead thymocytes. Values are expressed as mean ± SEM (*n*=7-26). Statistical analyses were performed by one-way ANOVA and Dunnett post hoc test.

Using the cellular markers CD4 and CD8, we found that the difference in thymic volume was mainly due to a massive reduction in the number and frequency of CD4+ CD8+ double positive (DP) immature thymocytes (Figure 3.4 a, b and c). The relative frequency of CD4+ and CD8+ single positive (SP) mature thymocytes remained comparable, and so did the ratio between these mature cell types. Concomitant to the reduction in the DP thymocytes, an increase in the frequency of the early precursor CD4- CD8- (DN) compartment was observed (Figure 3.4 b). Moreover, we found an inverse correlation between the percentage of living DP

cells and the concentration of betamethasone detected in the sera of the offspring (Figure 3.4 d), indicating a direct effect of this steroid on the developing thymic cells.

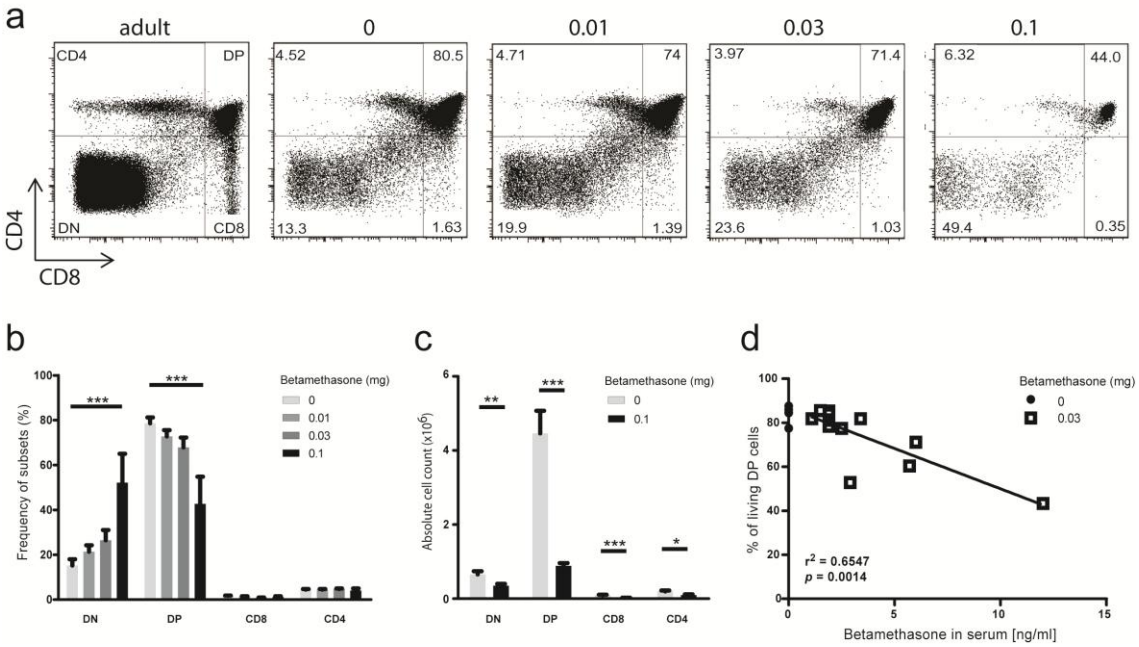

Figure 3.4: Double-positive thymocytes are most sensitive to antenatal steroid treatment. Cell suspensions of the offspring's thymi were prepared as before and stained for CD4 and CD8 in addition to Annexin V and Pacific Orange succinimidyl ester. (**a**) Representative CD4/CD8 dot plots for each condition after gating on living cells. A dot plot corresponding to an 8-week-old mouse is shown for comparison (*left panel*). (**b**) Frequency of immature double-negative (*DN*), double-positive (*DP*), and mature CD4 and CD8 single-positive thymocytes for each condition. Values are expressed as mean ± SEM (*n*=7-26). Statistical analyses were performed by one-way ANOVA and Dunnett post hoc test. (**c**) Absolute cell count of immature DN, DP, and mature CD4 and CD8 single-positive thymocytes from pups of PBS- and betamethasone-treated mothers. Values are expressed as mean ± SEM (*n*=8-9). Student's *t* test was used for statistical analysis. (**d**) Frequency of living DP thymocytes correlated with the concentration of betamethasone in the serum of the offspring 24 h after ACS of the mother (*n*=5-13).

3.1.4 Antenatal betamethasone treatment reduces proliferation of immature thymocytes and accelerated transition in the CD4-CD8- DN compartment

In addition to thymocyte apoptosis, the dramatic loss of thymocytes observed after antenatal betamethasone treatment could also be due to reduced proliferation. To address this point, we injected BrdU - a thymidine analog - to pregnant females together with 0.1 mg betamethasone or PBS. BrdU incorporation, indicating cell proliferation, was indeed massively reduced in all immature thymocyte subsets after antenatal betamethasone treatment (Figure 3.5 a and b). The amount of proliferating DN and DP thymocytes in treated animals is five-fold reduced compared to the sham-treated offspring.

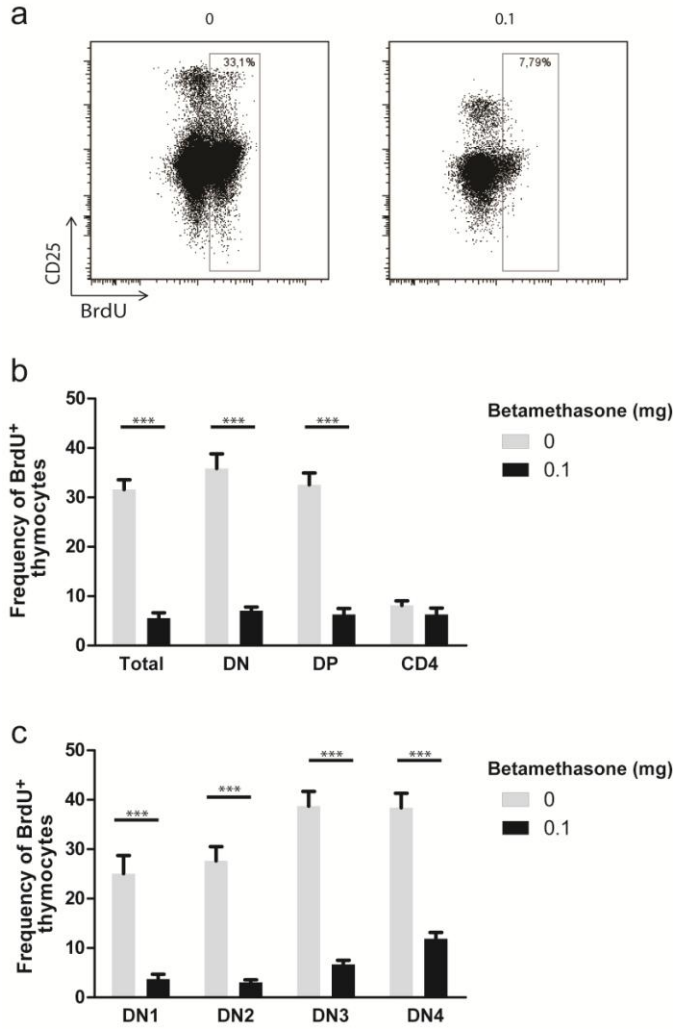

Figure 3.5: Antenatal steroid treatment results in reduced thymocyte proliferation. Pregnant mice were injected with 0.1 mg betamethasone or PBS and 1 mg BrdU i.p. at E18.5. Thymocyte proliferation was measured by BrdU incorporation 24 h after treatment. (**a**) Representative CD25/BrdU dot plots of total thymocytes from a thymus of the offspring of a PBS- and a betamethasone-treated mother. (**b**) Frequencies of proliferating thymocytes in total live cells, immature double-negative (*DN*), double-positive (*DP*), and mature CD4 SP cells. (**c**) Frequency of BrdU-positive immature thymocytes in each of the DN1-4 stages. Values are expressed as mean ± SEM (*n*=8-9). Statistical analyses were performed by Student's *t* test.

Within the immature CD4- CD8- DN thymocytes, proliferation is decreased in all DN subsets after betamethasone application, the DN4 being the least affected (Figure 3.5 c). Consequently, we found the frequency of the DN4 subset overrepresented in the treated animals (40% increase), while the DN2 and DN3 subsets were reduced to a 20% of the values found in control animals (Figure 3.6 b and c). Analogous changes, albeit at a lesser extent, were observed in the offspring of mothers treated with lower doses of betamethasone (Figure 3.6 c). Of note, the absolute cell count for the DN3 and DN4 was still reduced compared to control animals (Figure 3.6 d). These findings indicate that antenatal betamethasone has a

profound effect on proliferation of immature thymocytes, and suggest that the massive loss of DP cells forces the transition of the immature cells to DN4 in order to quickly replenish the DP compartment.

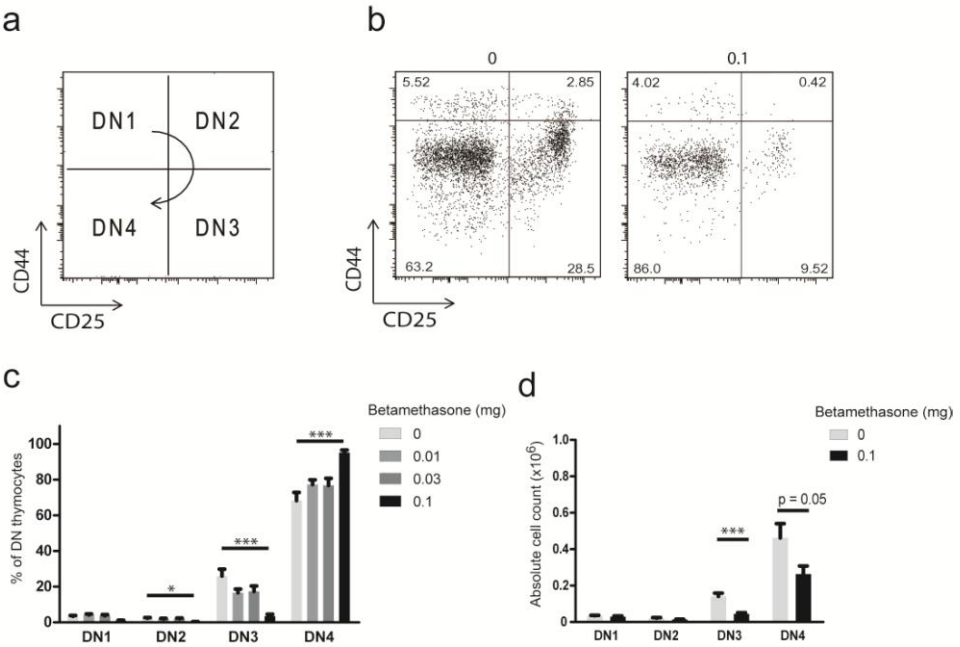

Figure 3.6: Changes in the most immature thymocyte compartments after antenatal steroid treatment. Cell suspensions of the offspring's thymi were prepared as before and stained for CD44 and CD25 in addition to CD4 and CD8, Annexin V, and Pacific Orange succinimidyl ester. CD4-CD8- DN thymocytes were selected from the living cells for further analysis. (**a**) Gating strategy for the analysis of the early developmental stages of DN thymocytes. (**b**) Representative CD25/CD44 dot plots of thymocytes from a thymus from pups of a PBS- and a betamethasone-treated mother. (**c**) Frequency of thymocytes in each of the DN1-4 stages from animals under the different treatment regimes. Values are expressed as mean ± SEM (*n*=7-26). Statistical analyses were performed by one-way ANOVA and Dunnett post hoc test. (**d**) Absolute cell count of immature thymocytes in the DN1-4 stages from pups of PBS- and betamethasone-treated mothers. Values are expressed as mean ± SEM (*n*=8-9). Student's *t* test was used for statistical analysis.

3.1.5 Thymic reconstitution of the offspring after ACS

DN1 and DN2 thymocytes undergo massive proliferation, and thus it would be expected that the thymic cellularity and volume are recovered after a short period of time. To address the duration of the observed changes, we assessed death of thymocytes in the different developmental stages at several time points after antenatal betamethasone administration. At the first time point (day 1 after injection, 1dpi), the pups were born. The other animals were analyzed 1 day post-partum (pp1, equivalent to 2 dpi), and 2 days post-partum (pp2, equivalent to 3dpi). Importantly, there was no difference in offspring mortality, time of delivery or in the sex distribution of the offspring between the treated mothers and the

controls (data not shown). As expected, we observed the dramatic increase in cell death 24 h after betamethasone injection to the mother, however, at the later time points (2dpi and 3dpi), there were no significant differences in cell death in treated animals compared to control animals (Figure 3.7 a). Thus, massive proliferation of the early DN stages compensated quickly for betamethasone-induced cell loss. However, careful analysis of the cell subsets revealed that two and three days post injection there was still a dramatic reduction in the relative frequency of DP cells. Two days post injection, DP cells accounted for barely over 50% of the total thymocytes, and three days after injection the proportion of DP increased to 65%, still lower than the more than 80% observed in the offspring from sham-treated mothers (Figure 3.7 b). Again, a compensatory acceleration in the maturation of the DN subsets could be observed until d3 post injection, with higher percentages of cells in the DN4 stage (Figure 3.7 c). Thus, the effect of antenatal steroid treatment is still noticeable three days post injection, around birth, a moment which is especially delicate for the selection events taking place in the thymus.

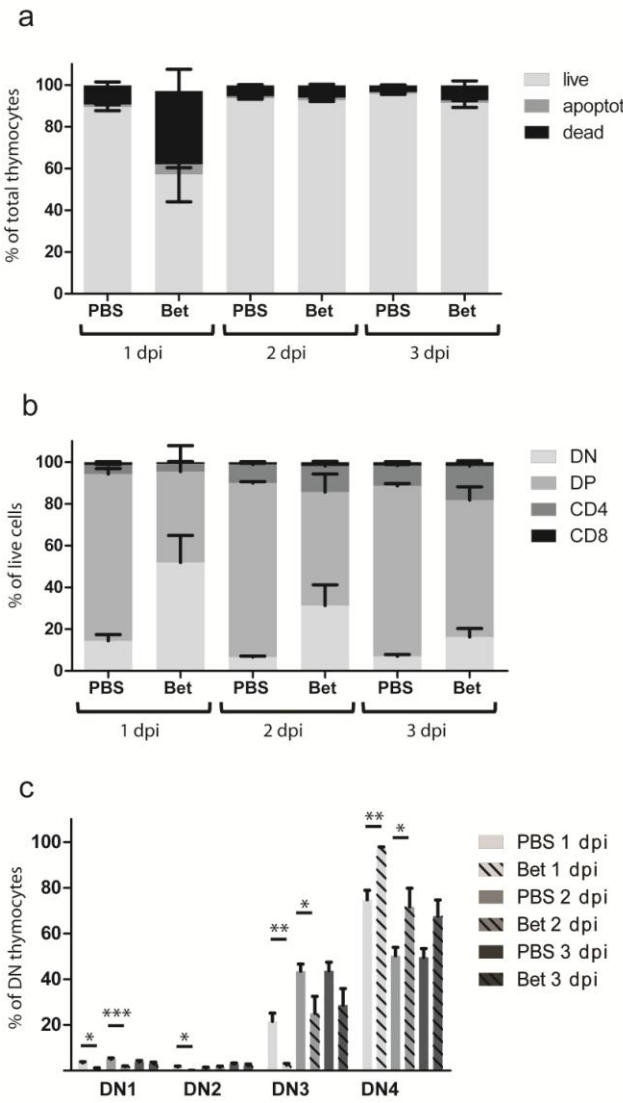

Figure 3.7: Reconstitution of the offspring's thymus after antenatal steroid treatment of the pregnant mother. Pregnant mice were given 0.1 mg betamethasone or PBS i.p. at E18.5. Thymic tissue from the offspring was prepared and stained 24 h (1 dpi), 48 h (2 dpi), or 72 h (3 dpi) after injection. Cell isolation and staining was performed as indicated in Fig. 3.6. (**a**) Percentage of live, apoptotic, and dead thymocytes at different time points after injection. (**b**) Frequency of DN, DP, CD4 SP and CD8 SP thymocytes within live thymocytes. (**c**) Frequency of DN thymocytes in the early developmental stages DN1 to DN4. In all graphs, values are expressed as mean ± SEM (*n*=7-22). Statistical analyses were performed by Student's *t* test.

3.1.6 Betamethasone treatment results in increased frequencies of regulatory T cells

In addition to the conventional single positive (SP) CD4 or CD8 cells, we analyzed the effect of antenatal betamethasone specifically on regulatory T cells (Treg) 24 h after treatment. To address this, single-cell suspensions of thymic tissue were stained for CD25 in addition to CD4 and CD8 (Figure 3.3 a), and Tregs were identified as CD4+CD8-CD25+ cells (Figure 3.8 a). We could show that offspring of betamethasone-treated mothers had a slightly increased

frequency of mature CD4+CD8-CD25+ Treg compared to the sham-treated pups (Figure 3.8 c). The increase was, however, not significant.

Considering that Treg development in the thymus undergoes the DP stage, we assessed the effect of betamethasone in the Treg precursors. The frequency of these cells, identified as CD4+CD8+CD25+ (see Figure 3.8 a) showed a more than two fold increase in viable Treg precursors after antenatal betamethasone treatment. These results indicate that regulatory T cells and their precursors may be less sensitive to corticosteroids than their conventional CD4 counterparts.

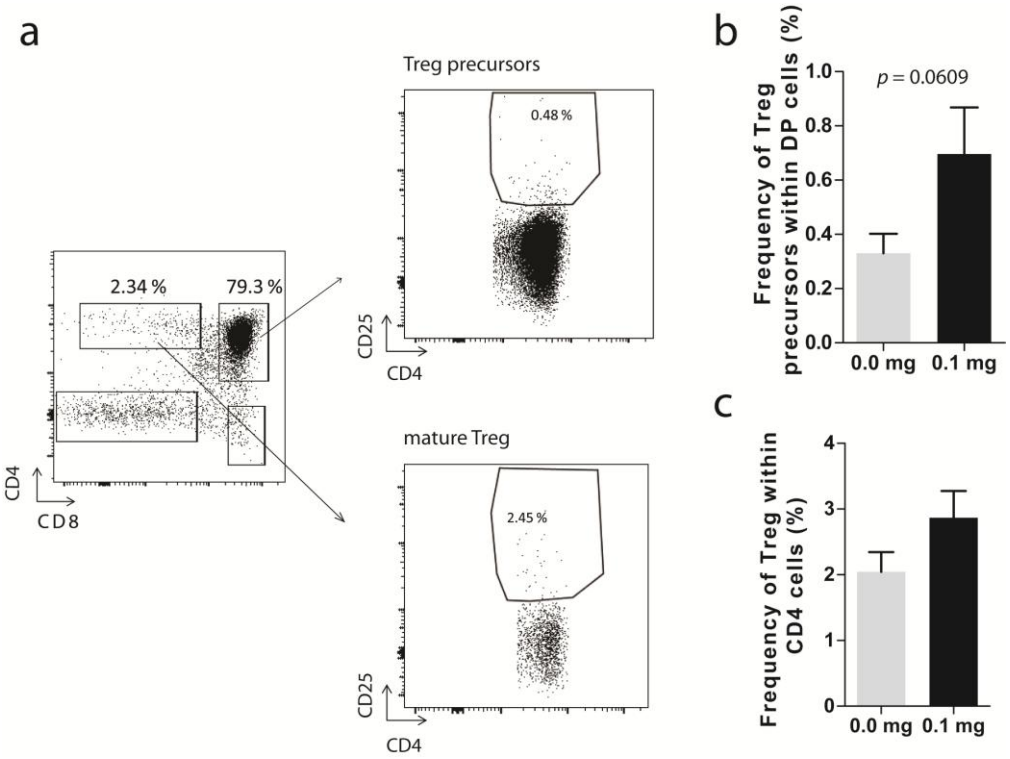

Figure 3.8: Antenatal steroid treatment increases the frequency of regulatory T cells and their precursors. Cell suspensions of the offspring's thymi were prepared as before and stained for CD25 in addition to CD4 and CD8. Treg thymocytes were selected from CD4 compartment and Treg precursors from the DP cells. (**a**) Gating strategy for the analysis of regulatory T cells and their precursors. Frequency of T regulatory precursors (**b**) and regulatory T cells (**c**) from animals under the different treatment regimes. Values are expressed as mean ± SEM (*n*=6-7). Student's *t* test was used for statistical analysis.

3.1.7 TCRγδ T cells subsets bearing variable γ chains are differentially sensitive to antenatal betamethasone treatment

TCRγδ cells also develop in the thymus, although they follow a different path of differentiation and do not go through the highly GC-sensitive DP stage. Between stages DN2 and DN3, these cells rearrange a T cell receptor consisting of a TCR-γ and a TCR-δ chain. To investigate the

effect of antenatal corticosteroids on thymic TCRγδ cells 24 h after application, we used a combination of anti-CD3 and anti-TCRγδ antibodies to gate on TCRγδ cells (Figure 3.9 b). The relative frequency of TCRγδ cells was slightly but not significantly increased (Figure 3.9 c) in offspring of betamethasone-treated mothers while the absolute number of TCRγδ cells was significantly reduced (Figure 3.9 d). The reduction of absolute numbers indicates that TCRγδ cells - like conventional TCRαβ T cells - are sensitive to steroids.

The population of TCRγδ cells could be further divided according to their maturation status (Figure 3.9 b, *lower panel*) and their distinct usage of variable segments of the γ-chain (Figure 3.9 b, *upper panel*). CD24 - or heat stable antigen - is generally expressed in immature thymocytes, while CD44 expression increases as T cells become activated or progress to the memory stage. Thus, immature TCRγδ cells express CD24 and low levels of CD44, while mature TCRγδ cells lose the expression of CD24 (Figure 3.9 b, *lower panel*). We found no differences in the relative frequencies of immature and mature TCRγδ cells in the offspring of betamethasone- and sham-treated mothers (Figure 3.9 e).

Analyzing the usage of different Vγ-chains using Vγ1 and Vγ4-antibodies, we found a small but significant increase in TCRγδ cells expressing the variable region Vγ1 in ACS-treated offspring. Regarding the Vγ4-chain, we observed a 10%-decrease in the use of this Vγ chain in offspring of betamethasone- compared to sham-treated mothers (Figure 3.9 f).

Taken together, the results indicate that TCRγδ cells - and especially those bearing the Vγ4 chain - may be less sensitive to steroids compared to conventional T cells.

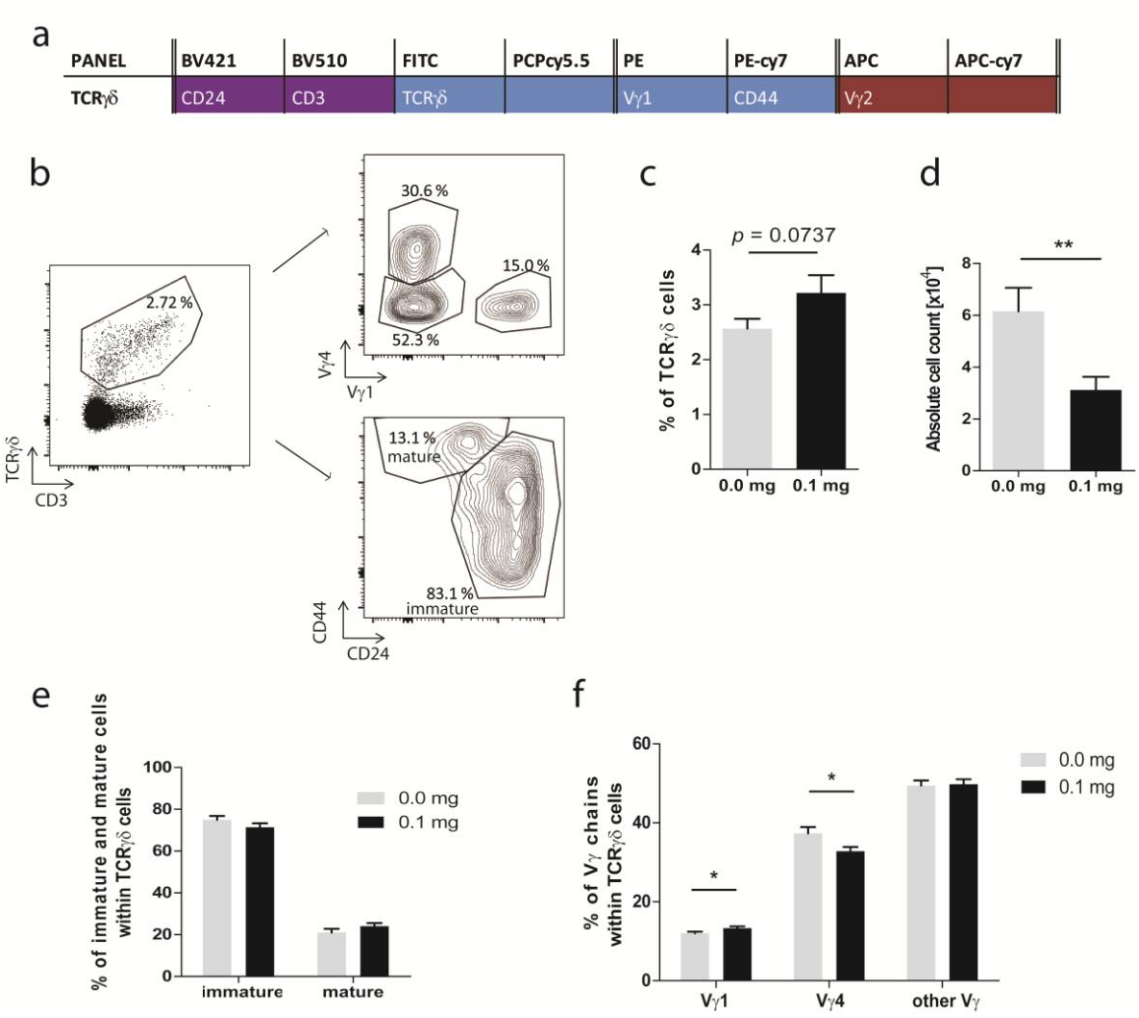

Figure 3.9: Vγ chains are differentially sensitive to steroid treatment. Cell suspensions of the offspring's thymi were prepared as before and stained for Vγ1, Vγ4, CD24 and CD44 in addition to CD3 and TCRγδ. TCRγδ thymocytes were selected from single cell compartment for further analysis. (**a**) Staining panel and (**b**) gating strategy for the analysis of developmental stages of TCRγδ thymocytes (*lower panel*) and the presence of different Vγ chains (*lower panel*). (**c**) Frequency of TCRγδ cells from animals under the different treatment regimes. (**d**) Absolute cell count of TCRγδ cells from animals under the different treatment regimes. (**e**) Frequency of maturation stages of TCRγδ cells from animals whose mothers were treated with betamethasone or PBS. (**f**) Frequency of the usage of Vγ chains within TCRγδ cells from a thymus of the offspring of a PBS- and a betamethasone-treated mother. In all graphs, values are expressed as mean ± SEM (*n*=11-20). Student's *t* test was used for statistical analysis.

3.2 Persistent effects of antenatal steroid treatment

After establishing the drastic consequences of antenatal betamethasone treatment at organ and cell levels in our mouse model, we asked if all these effects were transient around birth or whether they persisted into adulthood. To address this point, we analyzed spleen cells from adult offspring from betamethasone- or sham-treated mothers.

3.2.1 No effects on the distribution of cytotoxic and helper T cells

We first assessed if the classical CD4+ T-helper and the CD8+ T cytotoxic lymphocytes were present at the normal frequencies at ages ranging from 4 to 7 weeks. We found a ratio 1:2 (CD8 to CD4 cells) in both PBS- and betamethasone-treated offspring at all times and concluded that all subsets had recovered similarly from antenatal betamethasone (Figure 3.10). Of note, splenocytes from offspring of betamethasone- or sham-treated mothers were fully functional as determined by cytokine production profiles and proliferation to a variety of stimuli (data not shown).

These results indicate that the effect of antenatal steroid treatment on major populations of conventional T cells is transient and does not have an obvious consequence for T cell function.

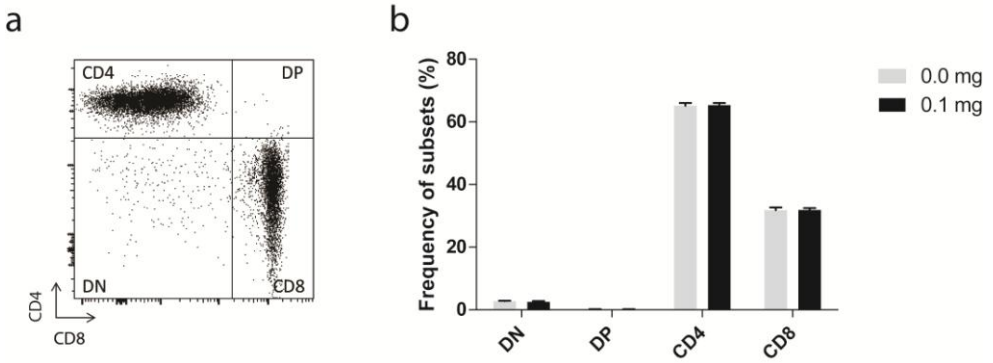

Figure 3.10: Complete reconstitution of the offspring's peripheral T cell compartment in adult offspring. Cell suspensions of the offspring's spleen were prepared and stained for CD4 and CD8. (**a**) Representative CD4/CD8 dot plot of a 7-weeks-old mouse. (**b**) Frequency of immature double-negative (*DN*), double-positive (*DP*), and mature CD4 and CD8 single-positive splenocytes from adult offspring antenatally treated with betamethasone or PBS. Values are expressed as mean ± SEM (*n*=11-12). Statistical analyses were performed by Student's *t* test.

3.2.2 Antenatal betamethasone increases the frequency of activated Treg

In addition to the conventional CD4 or CD8 cells, we analyzed the effect of antenatal steroid treatment on regulatory T cells in adult offspring (4-7 weeks of age) of betamethasone- or sham-treated mothers. Like in thymocytes, CD25 was used for the identification of regulatory T cells (Figure 3.11 b). Offspring of betamethasone- and sham-treated mothers showed no differences in the frequency of Treg (Figure 3.11 c). Glycoprotein A Repetitions Predominant (GARP) is a type I membrane glycoprotein expressed on activated regulatory T cells. It serves as a receptor for latent TGF-β and is suggested to play a role in controlling suppressor function of Tregs. We found the frequency of GARP-expressing regulatory T cells was 1.5-fold elevated

in betamethasone-treated offspring compared to untreated control offspring (Figure 3.11 d) though with a high inter-individual variance.

We used neuropilin 1 (Nrp-1) to discriminate between thymus-derived and peripherally induced Tregs. Nrp-1 (or CD304) is a transmembrane protein expressed by thymic Tregs, but not by Tregs induced in the periphery from conventional CD4 T cells (Weiss et al., 2012). The frequency of these two Treg subsets remained unchanged in treated adult offspring, with a slight tendency towards a higher frequency of peripherally derived Tregs in betamethasone treated animals (Figure 3.11 e).

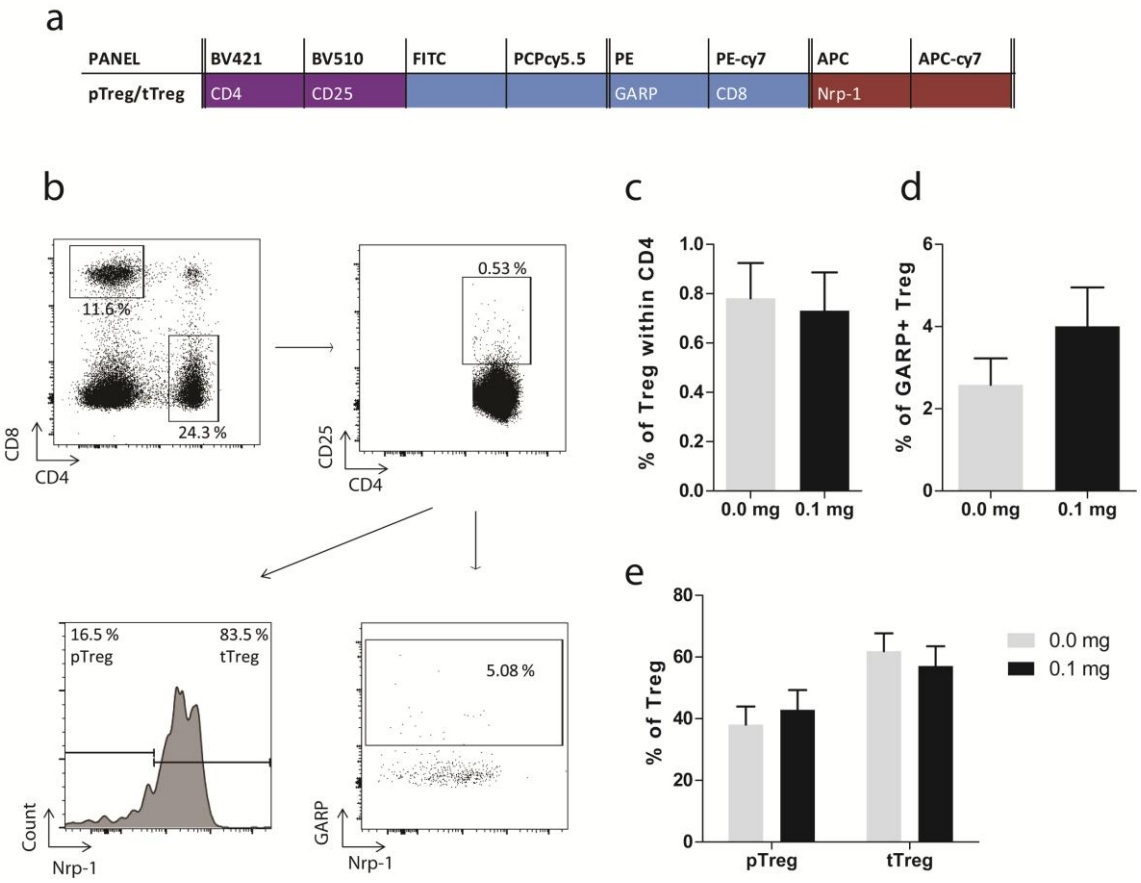

Figure 3.11: Antenatal betamethasone induces a decrease of thymically derived Tregs in the periphery and increases activated regulatory T cells. Analysis of the peripheral immune system in the spleen was performed at the age of 4-7 weeks. Single cell suspensions from splenic tissue were prepared and stained for CD4, CD8, CD25, GARP and Nrp-1. (**a**) Staining panel and (**b**) gating strategy for the analysis of regulatory T cells (CD4+CD25+), their activation status (GARP) and further discrimination between thymic Treg (Nrp-1 positive) and peripherally induced Treg (Nrp-1 negative). Percentage of regulatory T cells (**c**) and of activated Treg (**d**). (**e**) Frequency of thymically derived (tTreg) and peripherally generated regulatory T cells (pTreg) in betamethasone- and PBS-treated offspring. In all graphs, values are expressed as mean ± SEM (*n*=7-11). Student's *t* test was used for statistical analysis.

3.2.3 Betamethasone treatment induces persistent changes in Vγ chain distribution (Vγ repertoire) in different organs

During embryonic development, distinct subsets of TCRγδ cells develop and leave the thymus in waves (Figure 1.3, introduction). These subsets of TCRγδ cells can be distinguished by the TCRγ chain that is employed during receptor rearrangements (Figure 3.12 b). Given the specific time point of antenatal steroid application, we wanted to know if the repertoire of Vγ chains is intact in the offspring of betamethasone-treated mothers.

When looking at the thymus of 4 to 7 weeks old animals, we found that neither the frequency of pan TCRγδ cells nor the Vγ chain segments employed was affected by antenatal steroid treatment (Figure 3.12 c and d). In contrast, a significant increase in the frequency of Vγ4 T cells could be seen in the spleen of antenatally treated animals. The frequency of Vγ1-bearing gamma delta T cells was also increased, while a compensatory decrease was found in TCRγδ cells bearing other Vγ chains (Figure 3.12 e). The situation in TCRγδ cells isolated from lung tissue was even more pronounced with a 44% overrepresentation of Vγ1 bearing TCRγδ cells and a 30% decrease in Vγ4 (Figure 3.12 g). No substantial differences were detected in blood cells (Figure 3.12 f).

These results show that antenatal betamethasone has a long lasting effect on the usage of variable segments of the γ-chain, and suggest that either selection events in the thymus or the V, D and J gene rearrangements could be affected by steroids.

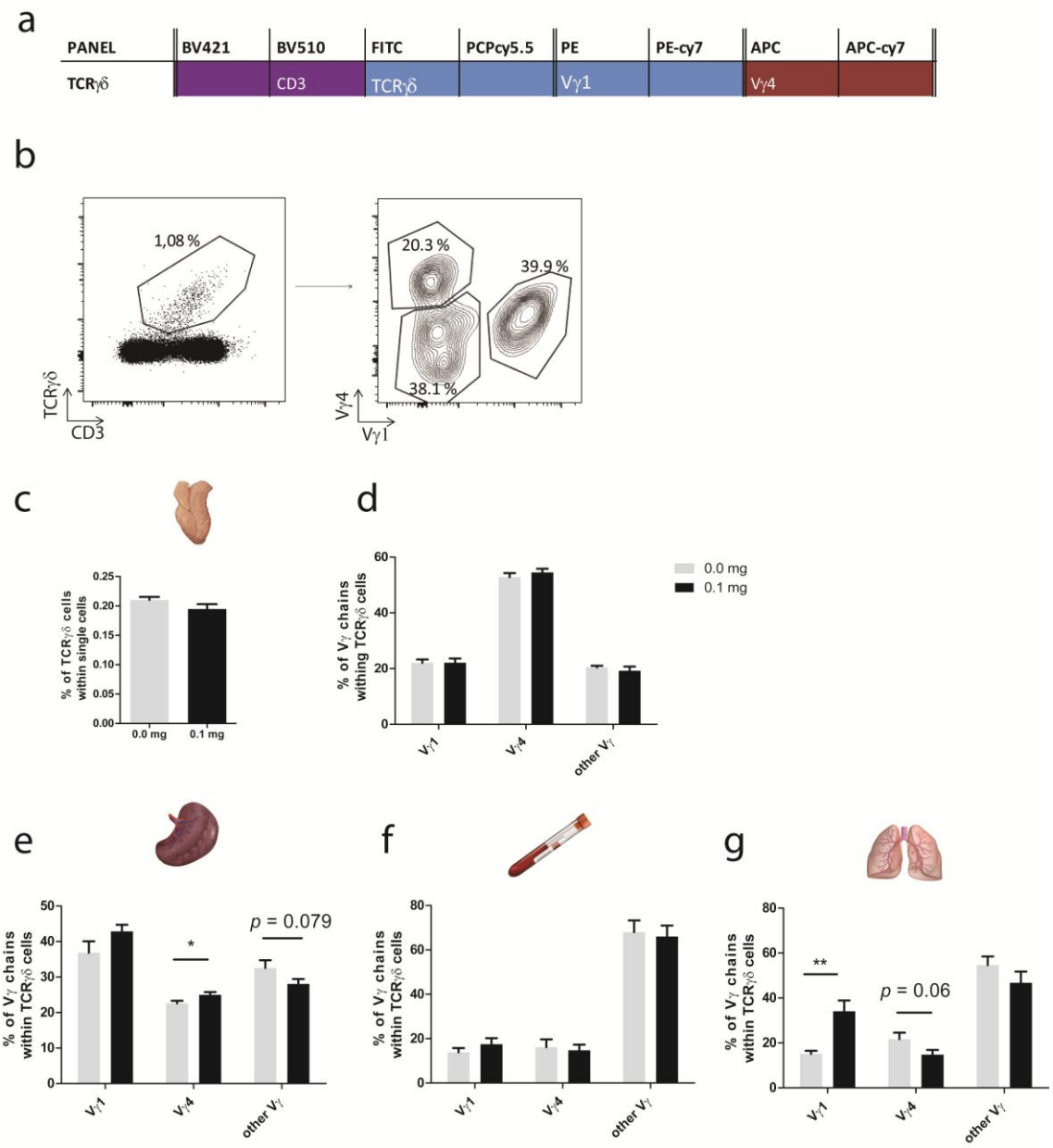

Figure 3.12: Antenatal corticosteroid treatment results in changes in Vγ distribution in the periphery. Analysis of the immune system in the thymus, spleen, blood and lung was performed at the age of 4-7 weeks. Single cell suspensions from thymic, splenic and lung tissue were prepared and stained for CD3, TCRγδ, Vγ1 and Vγ4. Whole blood was stained directly for the same markers. (**a**) Staining panel and (**b**) gating strategy for the analysis of TCRγδ cells and the major Vγ subsets. (**c**) Percentage of TCRγδ and (**d**) the major Vγ subsets in the thymus. Frequency of Vγ chain usage in (**e**) spleen, (**f**) peripheral blood and (**g**) lung. In all graphs, values are expressed as mean ± SEM (*n*=11-19). Student's *t* test was used for statistical analysis.

3.3 Effect of ACS on the development of autoimmunity

Upon finding short-term and persistent effects of antenatal betamethasone treatment on the immune system of the offspring, we wondered whether the changes evoked could lead to

consequences for the immune function, and put the offspring at risk for autoimmune diseases. To evaluate these possibilities, we investigated two different mouse models for spontaneous autoimmune diseases: the MRL lpr/lpr model of systemic lupus erythematosus and the IL-10 KO mice, which develop spontaneous colitis.

3.3.1 Antenatal betamethasone changes the composition of intestinal intraepithelial lymphocytes and leaves mice more susceptible to develop colitis

Interleukin-10 (IL-10) is an important regulatory cytokine that suppresses effector functions of macrophages/monocytes, T helper 1 (Th1) cells, and natural killer cells. Mice homozygous for the IL-10 gene deletion spontaneously develop chronic inflammatory bowel disease (IBD) (Kuhn et al., 1993; Berg et al., 1996). To address the effects of antenatal steroids on the development of colitis, we applied betamethasone or PBS to pregnant IL-10 KO mice on E18.5. The offspring were reared and weaned normally.

The mean colitis score was similar in betamethasone- and PBS-treated offspring remaining at background levels but with a high inter-individual variability (Figure 3.13 a). Only about 8% of the sham treated and 25% of ACS-treated animals developed relevant disease symptoms (colitis score >5) at the age of 14 weeks. The incidence of colitis was three-fold higher in the group of animals treated antenatally with betamethasone (Figure 3.13 b), but remained generally low, hinting that high hygiene standards in the animal facility preclude the development of colitis.

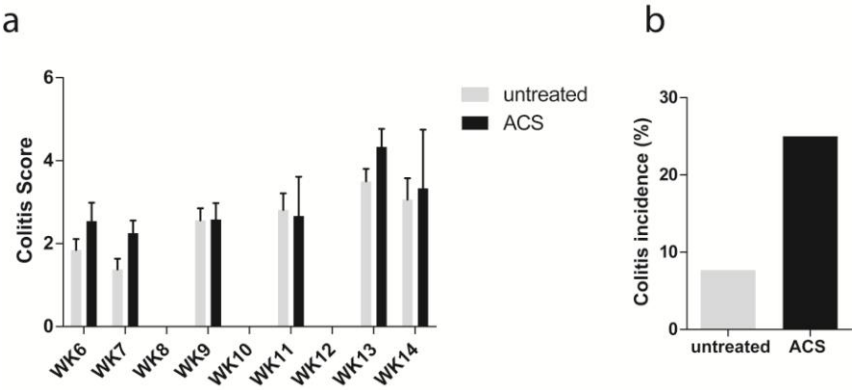

Figure 3.13: Colitis progression and incidence in offspring treated antenatally with corticosteroids or PBS. Offspring of treated and untreated mothers were regularly screened for onset of colitis symptoms. (**a**) Colitis scores as determined by endoscopy. Values are expressed as mean ± SEM (*n*=6-13). (**b**) Colitis incidence in the offspring from ACS-treated and -untreated mothers. Mice were considered sick if the colitis score was 5 or higher. Values are expressed as frequency of sick animals in the group.

To ascertain if there was a subclinical manifestation of colitis, we sacrificed the offspring at 14 weeks of age to analyze the composition of immune cells in the colon.

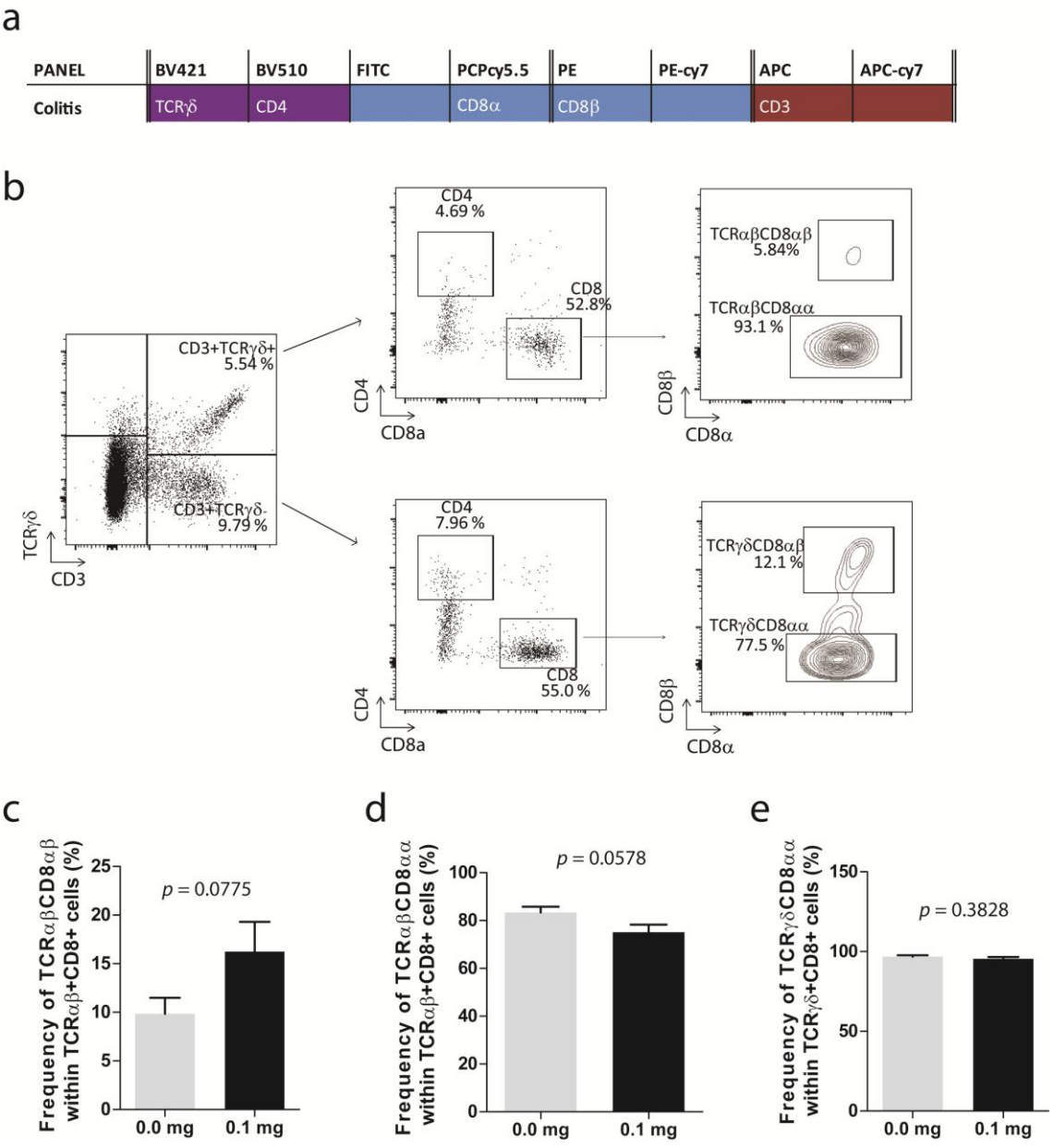

Figure 3.14: ACS induces a reduction in unconventional TCRαβCD8αα intraepithelial lymphocytes (IEL). Analysis of the peripheral immune system in the colon of treated and untreated offspring was performed at the age of 14 weeks. Single cell suspensions from colonic tissue was prepared and stained for CD3, TCRγδ, CD4, CD8α and CD8β. (**a**) Staining panel and (**b**) gating strategy for the analysis of intraepithelial lymphocytes. Frequency of (**c**) conventional TCRαβ+CD8αβ IELs, (**d**) unconventional TCRαβ+CD8αα IELs and (**e**) unconventional TCRγδ+CD8αα IELs. In all graphs, values are expressed as mean ± SEM (*n*=7-8). Student's *t* test was used for statistical analysis.

Intestinal intraepithelial lymphocytes (IELs) include conventional CD4 and CD8αβ T cells, as well as a significant population of unconventional T cell types - TCRγδ cells and T cell receptor

TCR$\alpha\beta$ co-receptor-negative CD8$\alpha\alpha$ T cells. Using the markers CD3 and TCR$\gamma\delta$, we distinguished between lymphocytic cells displaying the $\alpha\beta$ or the $\gamma\delta$ T cell receptor chains. Gating on CD8 cells, we could further differentiate between cells employing a normal CD8$\alpha\beta$ co-receptor and cells being co-receptor negative, instead expressing two CD8α-chains on their surface (Figure 3.14 b) marking them as CD8$\alpha\alpha$.

In the intestinum, a substantial portion of the T cell population is made up of three different cell types: the conventional TCR$\alpha\beta$CD8$\alpha\beta$, the unconventional TCR$\alpha\beta$CD8$\alpha\alpha$ and the unconventional TCR$\gamma\delta$CD8$\alpha\alpha$ cells. The frequency of conventional TCR$\alpha\beta$CD8$\alpha\beta$ IELs was 1.5-fold increased (Figure 3.14 c) in mice antenatally treated with betamethasone while the unconventional TCR$\alpha\beta$CD8$\alpha\alpha$ subset was decreased by 8% (Figure 3.14 d). Though the differences were not significant for either TCR$\alpha\beta$ subset, the results indicate that selection of TCR$\alpha\beta$ bearing IELs could be affected by antenatal steroids. The frequencies of TCR$\gamma\delta$-bearing cells remained undisturbed by ACS treatment (Figure 3.14 e).

3.3.2 Effects of antenatal betamethasone in a mouse model of systemic lupus erythematosus

The second autoimmune model used in our study was the MRL lpr/lpr mouse model, where the mice spontaneously develop symptoms reminiscent of the human systemic lupus erythematosus. Mice of the MRL background are prone to autoreactivity (Theofilopoulos and Dixon, 1982). The lpr (fas) mutation accelerates the phenotypic changes by preventing apoptosis of activated autoreactive cells (Watanabe-Fukunaga et al., 1992).

3.3.2.1 Antenatal betamethasone accelerates onset and progression of lupus-like symptoms

Female offspring of betamethasone- or PBS-treated mothers were scored weekly for the development of disease symptoms, beginning at the age of eight weeks. Scoring parameters included clinical features like the development of lymphadenopathy and skin lesions as well as the protein content measured in the urine of each mouse. Signs of disease increased longitudinally in both groups of mice observed. The disease progression showed an acceleration of symptoms in betamethasone-treated mice compared to untreated offspring (Figure 3.15 a). Offspring that had received antenatal steroids showed significantly higher disease symptoms at the ages of 10 and 14 weeks, but not consistently throughout the scoring

period. Beginning at week 13, the disease symptoms were consistently higher in betamethasone-treated offspring (Figure 3.15 a).

The onset of disease was similar in both treatment-groups. All betamethasone-treated offspring were affected by disease at the age of 13 weeks, while 20% of PBS-treated offspring remained healthy until this time-point and developed lupus only two weeks later (Figure 3.15 b).

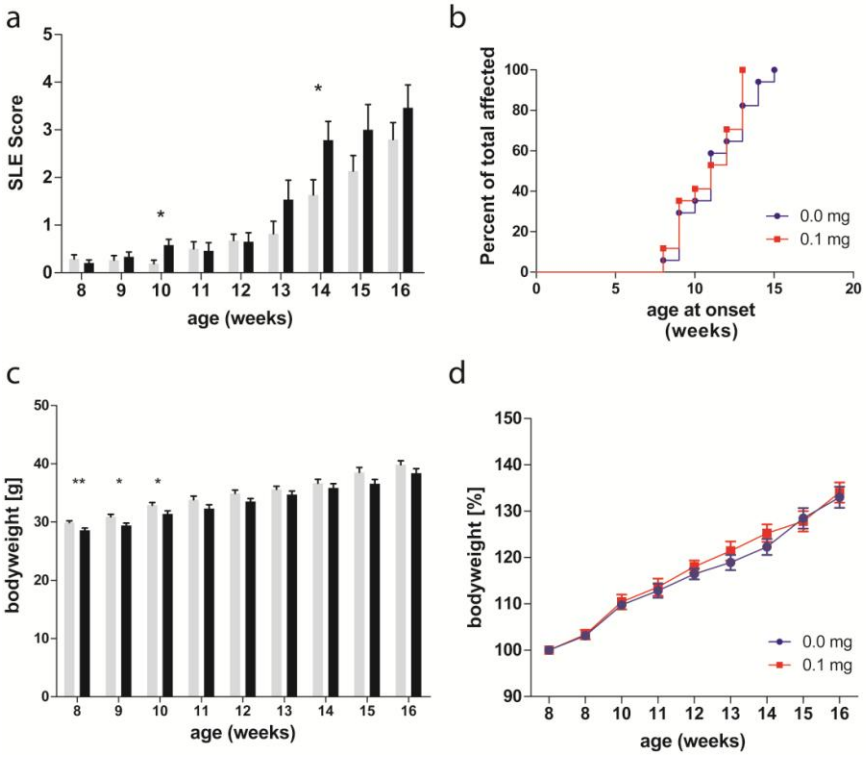

Figure 3.15: Observation of clinical symptoms of lupus-like symptoms in offspring antenatally treated with corticosteroids or PBS. Offspring of treated and untreated mothers were regularly screened for onset of lupus. (**a**) Lupus progress as determined by visual scoring parameters. Values are expressed as mean ± SEM (*n*=21-26 for 8-12 weeks of age and *n*=11-14 for 13-16 weeks of age). (**b**) Onset of lupus symptoms in the offspring from ACS-treated and -untreated mothers. Mice were considered sick when reaching a score of 0.5 or higher in two consecutive scoring weeks. Values are expressed as percent of affected animals. Increase in (**c**) absolute and (**d**) relative bodyweight during the scoring phase. Values are expressed as mean ± SEM (*n*=21-26 for 8-12 weeks of age and *n*=11-14 for 13-16 weeks of age). Student's *t* test was used for statistical analysis.

Additionally to the scoring parameters, the bodyweight was analyzed weekly. During disease progression the bodyweight increases with disease progression due to edema upon loss of renal function and exacerbated lymphadenopathy. Throughout the whole scoring phase, the weight of antenatally treated offspring remained lower although significant differences were only observed early in disease (Figure 3.15 c). The relative increase in bodyweight was similar in both treatment groups (Figure 3.15 d).

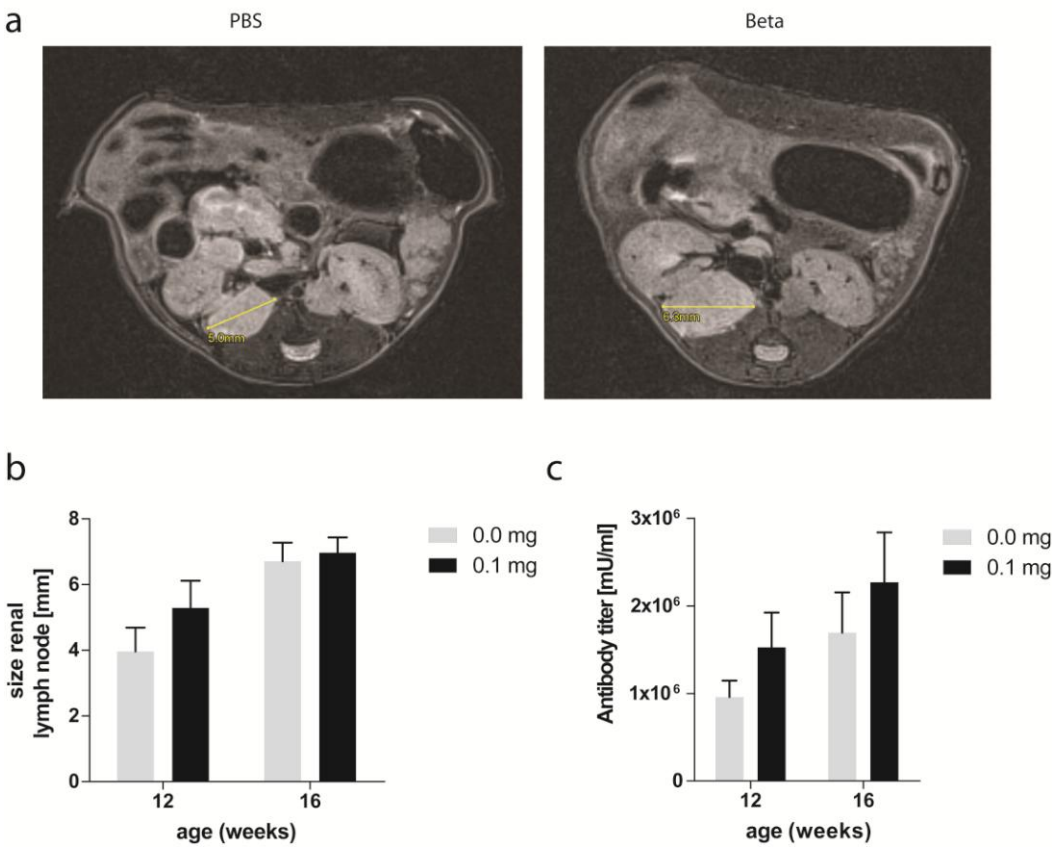

Figure 3.16: Antenatal glucocorticoids cause increased lymph node size and tendencies towards higher anti-dsDNA antibodies in the offspring. (**a**) Representative MRI pictures displaying the renal lymph node of the offspring whose mothers were treated with PBS (*left*) or with 0.1 mg Betamethasone (*right*). (**b**) MRI quantification of the renal lymph node size of the offspring at 12 weeks of age. Values are expressed as mean ± SEM (*n* = 21-26 for 4-6). (**c**) Anti-dsDNA antibody titer in serum of offspring treated antenatally with PBS or Betamethasone as obtained by ELISA. Values are expressed as mean ± SEM (*n* = 5-10).

MRI analysis of renal lymph node size in the offspring revealed that the application of betamethasone resulted in a 25% increase at 12 weeks of age (Figure 3.16 a and b). By the end of the scoring phase, this difference was not obvious any longer (Figure 3.16 b). Of note, similar results were observed for the size of axillary lymph nodes (data not shown). In addition, we found higher titers of anti-dsDNA antibodies in the antenatally treated offspring at both time points assessed (Figure 3.16 c).

Taken together, the findings show antenatal steroid treatment leads to a faster disease progression of systemic lupus erythematosus as indicated by disease progression scoring, lymph node size and anti-dsDNA antibody titers.

3.3.2.2 Betamethasone induces persistent changes in the Vγ repertoire

Similar to the observation in B6 mice (Figure 3.12), we could also find a skewed Vγ repertoire in TCRγδ cells in animals with the MRL background. The cells isolated from spleen of MRL mice showed a Vγ repertoire that was similarly affected after antenatal betamethasone treatment as observed in wild type mice (data not shown). Additionally, we analyzed the Vγ repertoire in axillary lymph nodes and found profound changes in the relative frequencies of Vγ chains. The frequency of Vγ1-bearing gamma delta T cells was increased by 20% while a pronounced decrease of approximately 50% was found in TCRγδ cells bearing other Vγ chains (Figure 3.17).

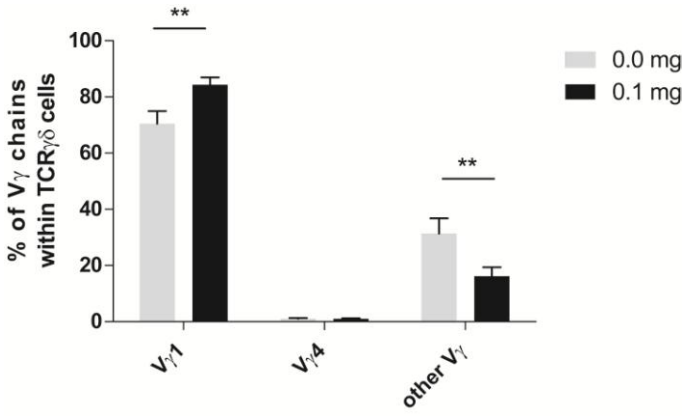

Figure 3.17: Antenatal corticosteroid treatment results in changes in Vγ distribution in axillary lymph nodes. Analysis of the Vγ repertoire was performed at 12-16 weeks. Lymph node cells were stained for CD3, TCRγδ, Vγ1 and Vγ4 and analyzed by flow cytometry. Values are expressed as mean ± SEM (*n*=15-17). Student's *t* test was used for statistical analysis.

3.3.2.3 Betamethasone induces prominent changes in the CD4 T cell compartment

Peripheral T lymphocytes in the MRL lpr/lpr strain undergo drastic phenotypic changes: at age 8 weeks, most T cells of the TCRαβ subset display the CD45 isoform B220, which is normally absent in T cells, and they do not express the co-receptors CD4 or CD8 (double negative or DN cells). The relative frequency of this unique population increases with the age of the mouse, and it's thought to be a result of the absence of the fas gene in the lpr strain (Morse et al., 1982).

Analysis of classical and aberrant TCRαβ cells revealed no differences in the frequencies of either cell types in mice antenatally treated with PBS or betamethasone (Figure 3.18 c and d). The frequency of mature conventional cytotoxic and helper T cells was significantly higher (Figure 3.18 d) in lymph nodes but not in spleen of 16 week old betamethasone-treated animals, with a relative increase of 20% for CD4 T cells and a compensatory decrease in CD8 T

cells (Figure 3.18 d). The frequency of regulatory T cells in 16 week old mice was slightly lower in betamethasone-treated offspring. The relative frequency of regulatory T cells increased in PBS- and betamethasone-treated offspring between the age of 12 and 16 weeks. In the untreated animals, this increase in Treg is significant (Figure 3.18 e).

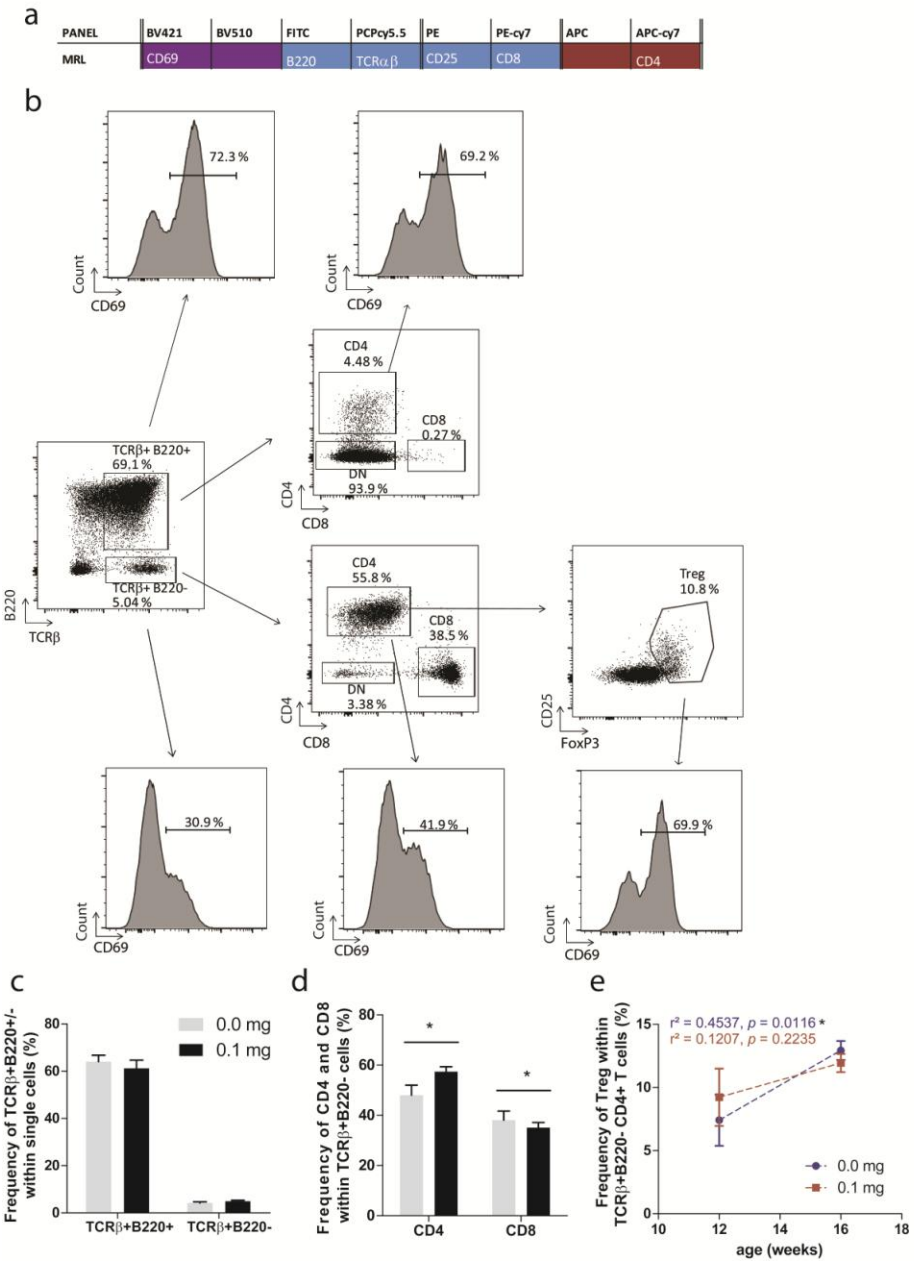

Figure 3.18: ACS induces an increase in the relative frequency of CD4 T helper and regulatory T cells. Lymph node or spleen cells from PBS- or betamethasone-treated offspring were analyzed for the expression of CD4, CD8, CD25, CD69, B220 and TCRαβ. (**a**) Staining panel and (**b**) gating strategy for the analysis of lymphocytes. (**c**) Frequency of conventional TCRβ+B220- and aberrant TCRβ+B220+ T cells. (**d**) Frequency of T helper and cytotoxic T cells in axillary lymph nodes. Analysis was performed at 16 weeks of age. Values are expressed as mean ± SEM (*n*=11-14). Student's *t* test was used for statistical analysis. (**e**) Linear changes in the frequencies of regulatory T cells over time in spleen. Values are expressed as mean ± SEM (*n*=5-9). Linear regression was used for statistical analysis of the deviation from zero.

CD69, a classical marker for early activation of T and B cells, was expressed at lower levels in conventional CD4 cells of betamethasone-treated mice at 16 weeks of age (Figure 3.19 a, b and c) in peripheral organs. Regulatory T cells also showed a reduction in CD69 at 16 weeks (data not shown).

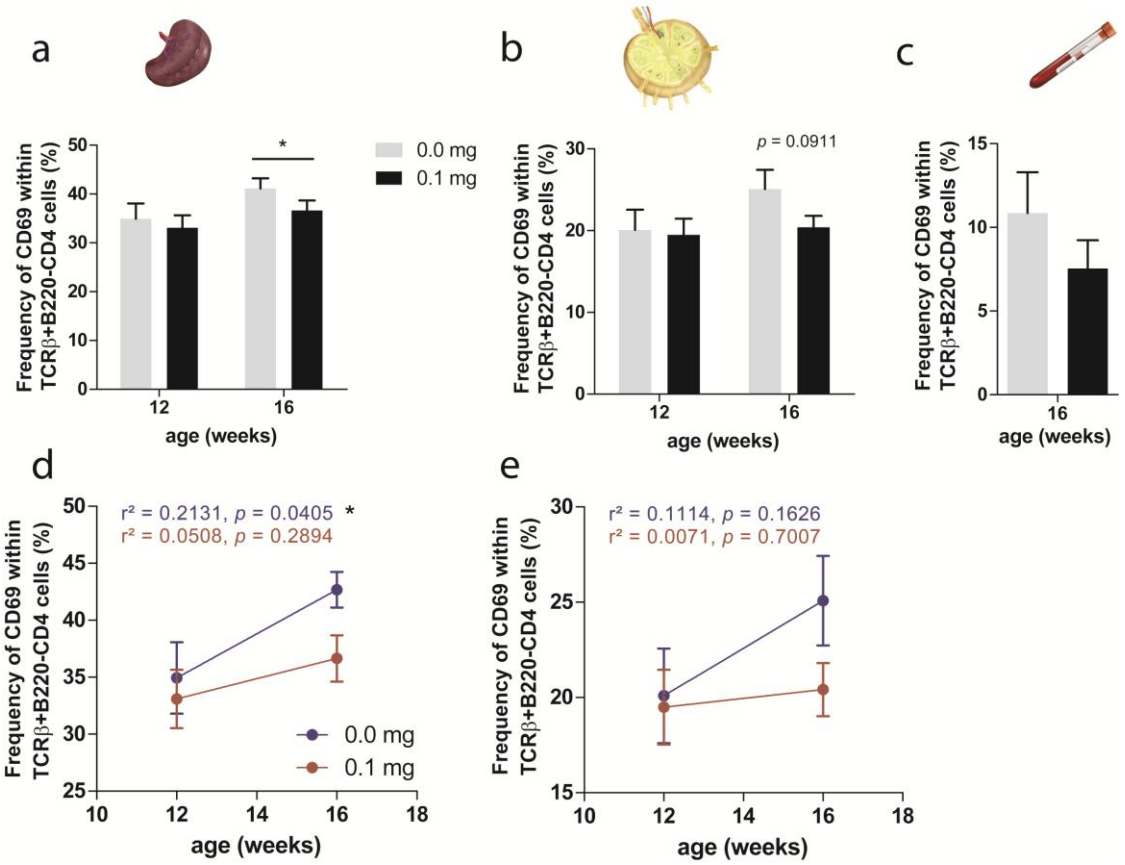

Figure 3.19: Antenatal betamethasone results in lower CD69 expression on T helper cells. Surface expression of CD69 on T helper cells was analyzed in spleen (**a**) and axillary lymph node (**b**) by flow cytometry at the age of 12 and 16 weeks and in (**c**) peripheral blood at the age of 16 weeks. In all graphs, values are expressed as mean ± SEM (*n*=6-14). Student's *t* test was used for statistical analysis. Changes in the frequencies of CD69 expression on T helper cells in (**d**) spleen and (**e**) lymph node over time. Values are expressed as mean ± SEM (*n*=11-14). Linear regression values are shown in the graph.

Between 12 and 16 weeks of age, expression of CD69 in CD4 cells increased in both treatment-groups. This increase was, however, less prominent in glucocorticoid-treated offspring compared to untreated animals in both spleen and lymph node (Figure 3.19 d and e).

3.4 In vitro effect of betamethasone on human and mouse thymocytes

There are obvious differences in the development of the immune system between mice and humans. Thus, the question remains if steroids have an effect on human T cell development. We used two approaches to tackle this question: First, we assessed *in vitro* the effect of betamethasone on human thymocytes, and second, we analyzed T cells in the cord blood of children whose mothers received steroids before their birth.

To assess the sensitivity of human thymocytes to betamethasone, we incubated single-cell suspensions from freshly isolated mouse and human thymocytes with betamethasone. We used annexin V and Pacific Orange succinimidyl ester to discriminate between live, apoptotic and necrotic cells and used the lineage markers CD3, CD4 and CD8 to identify the cellular subsets DN, DP, SP CD4 and CD8. Similar to mouse thymocytes (Figure 3.20 a), human thymic cells enter apoptosis readily after incubation with betamethasone for 18 h (Figure 3.20 b). Already at low concentrations of betamethasone (3-6 nM for mouse and 6-12 nM for human thymocytes respectively) the frequency of live cells decreases drastically. Assuming a blood volume of approximately 1.5 ml of a mouse and a betamethasone dose of 0.1 mg used in ACS treatment in our model, a peak concentration of 0.06 mg betamethasone per ml blood directly after injection can be reached. This concentration is equivalent to approximately 170 µM, a molarity which is more than 3000-fold over the highest concentration used in the in vitro experiments. Even considering that only a fraction of the injected steroids reach the fetus, the range of betamethasone concentration that showed an effect on thymocyte death in the *in vitro* experiments is physiologically relevant in the context of antenatal steroid treatment.

Again, similar to mouse thymocytes and to the in vivo treatment in mice (Figure 3.4), we observed a drastic reduction in the proportion of DP thymocytes in the human thymocyte cultures (lower panels). Concomitantly, the frequencies of the immature DN cells, as well as of mature CD4+ and CD8+ SP thymocytes increased proportionally, while in the murine system we observed a very dominant increase of DN thymocytes and no obvious changes in the mature SP subsets. Altogether, this data show that the human thymocytes are susceptible to the effects of steroids administered.

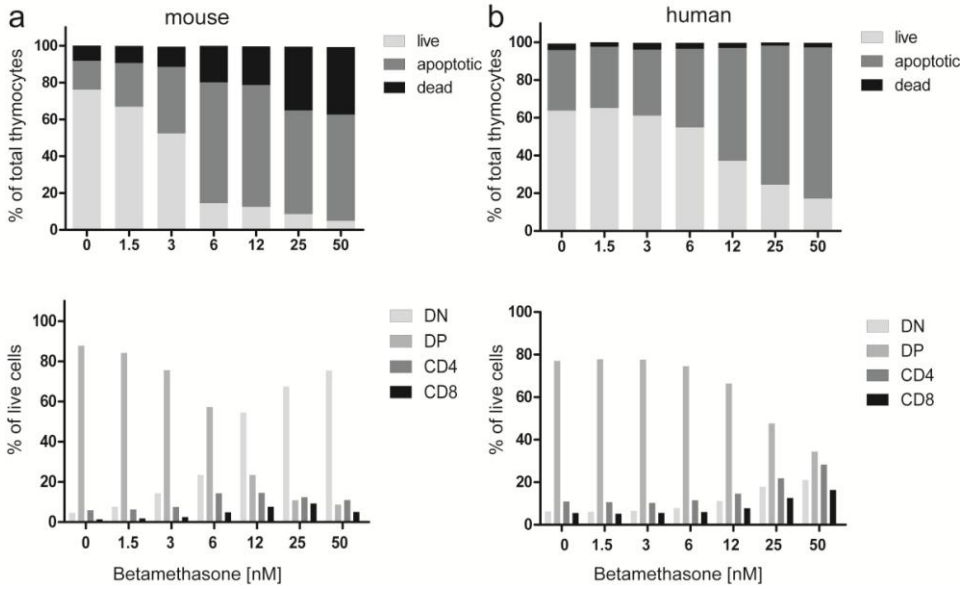

Figure 3.20: Low doses of betamethasone induce apoptosis and death of thymocytes. Mouse (**a**) or human (**b**) thymocytes were isolated from fresh tissue and cultured in the presence (1.5 to 50 nM) or absence of betamethasone for 18 h and subsequently stained for cell surface markers, Cell Dead Stain and Annexin V. The *top panels* display the percentage of live, apoptotic, and dead cells in total thymocytes. The *lower panels* indicate the frequency of DN, DP, and CD4 and CD8 SP thymocytes within the live cell gate. One representative experiment is shown in each case.

3.5 Effect of antenatal corticosteroids on human immune cells

3.5.1 Effects of antenatal betamethasone on CD4 cell population in cord blood

In our mouse model, antenatal steroid treatment results in short- and long-term consequences for the offspring's immune system. Similarly, there are clear indications that human thymocytes are sensitive to steroids in *in vitro* assays. To assess immediate effects of antenatal corticosteroid treatment in humans, we analyzed cord blood samples of preterm children.

Table 3.1: List of human cord blood donors. Human cord blood of children born prematurely was obtained at the University Medical Center Hamburg-Eppendorf. Table includes data on antenatal steroid treatment (and elapsed time past since the treatment) and gestational age.

Cord blood sample	Antenatal Steroid treatment (ACS)	Gestational age at birth (weeks)	Time between ACS and birth (days)
1	No	33	
2	No	34	
3	No	34	
4	No	35	
5	No	35	
6	No	35	
7	No	36	
8	No	36	
9	Yes	32	0
10	Yes	32	2
11	Yes	33	2
12	Yes	34	8
13	Yes	35	29
14	Yes	36	42
15	Yes	36	92

We stained the cord blood samples from a small cohort of children whose mothers had been treated (n = 7) or not (n = 8) with betamethasone during their pregnancy (Table 3.1). Lineage markers (CD3, CD14, CD16, CD19 and CD56) were used to assess the relative frequencies of the leukocytes (monocytes, neutrophils) and lymphocytes (T- and B-cells, NK cells). Within the T cells, we further specified conventional T helper and cytotoxic T cells as well as unconventional TCR$\gamma\delta$, mucosal associated invariant T cells (MAIT) and regulatory T cells. In addition, we assessed in all T lymphocyte subsets their activation status with the expression of activation markers CD25 and CD69, and their effector phenotype (naïve, central and effector memory, and effector cells) using the markers CD45RA, CD27 and CCR7. In this very small cohort we could not observe differences in the frequency of the cell populations analyzed (Table 3.2 and Figure 3.21 c).

Table 3.2: Frequencies of different immune populations in a human cohort using cord blood. Study cohort was divided into treated and untreated children.

	out of CD45+ cells					out of CD3+ cells				out of CD4+ cells						out of CD8+ cells				
Case #	% of Monocytes	% of Neutrophils	% of CD3	% of CD19	% of CD56	% of CD4	% of CD8	% of TCR$\gamma\delta$	% of MAIT	% of Treg	% of CD25^{int}	% of naive	% of Central Memory (CM)	% of Effector Memory (EM)	% of TEMRA	% of CD25^{int}	% of naive	% of CM	% of EM	% of TEMRA
1	8.91	44.8	32.9	2.44	9.23	70	23.4	2.53	1.34	5.47	3.57	85.1	10	3.25	1.65	0.077	92	3.32	1.93	2.75
2	8.53	30.4	44.1	4.92	5.91	64.9	21.3	1.43	1.2	9.88	3.32	87	5.71	3.32	3.99	0.051	92.7	1.17	1.31	4.86
3	9.92	48.2	21.6	5.08	11.6	73.8	23.4	0.47	1.17	7.96	5.03	63.3	29.2	6.77	0.75	0.09	85.9	7.52	2.64	3.97
4	11.3	43.4	20.5	8.64	12.8	66.2	24.2	0.917	1.04	9.87	4.92	74.8	14.6	6.09	4.46	0.13	86.7	4.02	3.21	6.07
5	3.16	33.7	37.4	4.38	10.9	71.3	13.3	1.59	0.83	8.81	2.85	67.8	25.6	4.62	1.99	2.48	83.6	7.14	5.5	3.77
6	8.97	14.6	24.7	13.9	0.57	68.2	28.1	2.35	0.49	6.71	2.59	74.8	20	3.89	1.36	0.04	82.9	6.59	4.85	5.67
7	8.97	29.2	32.3	8.08	6.58	74.8	19.9	1.03	0.38	6.88	0.81	63	32.6	3.37	1.03	0.04	71.8	19.2	6.44	2.58
8	8.91	39.1	33.6	9.95	2.4	80.6	11.7	1.48	0.56	8.92	2.37	66.3	26.3	5.75	1.6	0.12	71.7	13.8	9.74	4.71
Mean (SEM)	8,58 (0,83)	35,4 (3.84)	30.8 (2.87)	7.17 (1.3)	7.5 (1.56)	71.23 (1.8)	20.6 (1.97)	1.47 (0.24)	0.87 (0.13)	8.06 (0.56)	3.18 (0.49)	72.7 (3.3)	20.5 (3.39)	4.63 (0.49)	2.10 (0.48)	0.38 (0.3)	83.4 (2.83)	7.84 (2.09)	4.45 (0.98)	4.29 (0.45)
9	10.9	32.9	37.7	4.62	11.9	67.8	22.3	1.55	1.65	9.78	5.36	78.8	8.33	6.77	6.07	0.09	88	1.38	1.57	9.02
10	8.17	40.2	37	9.11	1.51	82.6	16	0.45	0.30	7.88	2.83	69.6	23.9	4.78	1.71	0	86.5	5.84	2.6	5.1
11	10	43.4	16.3	3.11	17.8	53.2	40	1.12	1.32	5.84	5.29	82	9.35	5.69	2.93	0.16	89.2	2.86	1.38	6.51
12	11.6	39	28.4	5.11	8.11	63.6	21.6	0.79	1.06	5.51	1.94	62.4	22.8	12.7	2.13	0.11	74.8	12.2	7.08	5.89
13	2.56	9.14	69.4	7.39	8.79	70.3	24.2	0.48	0.39	9.03	1.39	77.9	15.9	3.83	2.3	0.04	92.8	4.96	0.93	1.27
14	6.64	46.4	28.7	3.83	9.85	68.6	18.7	1.73	0.64	9.98	3.81	81.4	6.71	6.33	5.54	0.27	88.3	1.51	1.99	8.16
15	8.13	29.8	31.7	8.64	8.67	72.8	25.8	1.3	0.28	9.04	2.1	71.6	22.2	4.84	1.41	0.18	85.5	5.96	3.9	4.64
Mean (SEM)	8,28 (1.15)	34.41 (4.73)	35.6 (6.24)	5.9 (0.9)	9.5 (1.8)	68.41 (3.37)	24.09 (2.92)	1.06 (0.19)	0.80 (0.2)	8.15 (0.69)	3.24 (0.61)	74.8 (2.7)	15.6 (2.83)	6.42 (1.11)	3.15 (0.71)	0.12 (0.03)	86,4 (2.13)	4.95 (1.41)	2.78 (0.81)	5.79 (0.96)

Careful analysis of T helper cells in combination with children's gestational age revealed some minor effects of betamethasone-treatment. In this, we found that the frequency of CD4 cells increased throughout gestation in children who were not treated with antenatal steroids (Figure 3.21 d, *blue*). Children that received ACS treatment lacked this increase in the frequency of T helper cells (Figure 3.21 d, *red*). To investigate whether this was a direct effect of the received steroids, we matched the frequency of CD4 cells in the cord blood samples to the time between antenatal steroid treatment and the sample collection. A slight increase in the frequency of T helper cells correlated with the time passed since steroid treatment (Figure 3.21 e), indicating that the frequency of T helper cells recovered with time.

Taken together, the results indicate that the application of antenatal steroids could potentially lead to changes in the immune system also in human. It should be noted that the study cohort used in this thesis was very small and very heterogeneous in terms of gestational age and the time elapsed since the application of steroids so effects of the treatment may remain hidden. Further studies are needed to draw conclusions on this topic.

a

PANEL	BV421	BV510	FITC	PCPcy5.5	PE	PE-cy7	APC	APC-cy7
Subsets	CD14	CD45	CD4	CD3	TCRγδ	CD19	CD56	CD16
Teff	CD45RA	CD8	CD4	CD3	TCRγδ	CD28	CCR7	CD27
Treg	CD45RA	CD8	HLA-DR	CD127	CD25	CD39	CD4	CD31
MAIT	CD161	CD8α	Vα24Jα18	CCR6	CD3	CD56	Vα7.2	CD4
TCRγδ	CD8α	CD4	Vδ2	CD3	TCRγδ	CD45RA	CD161	CD27

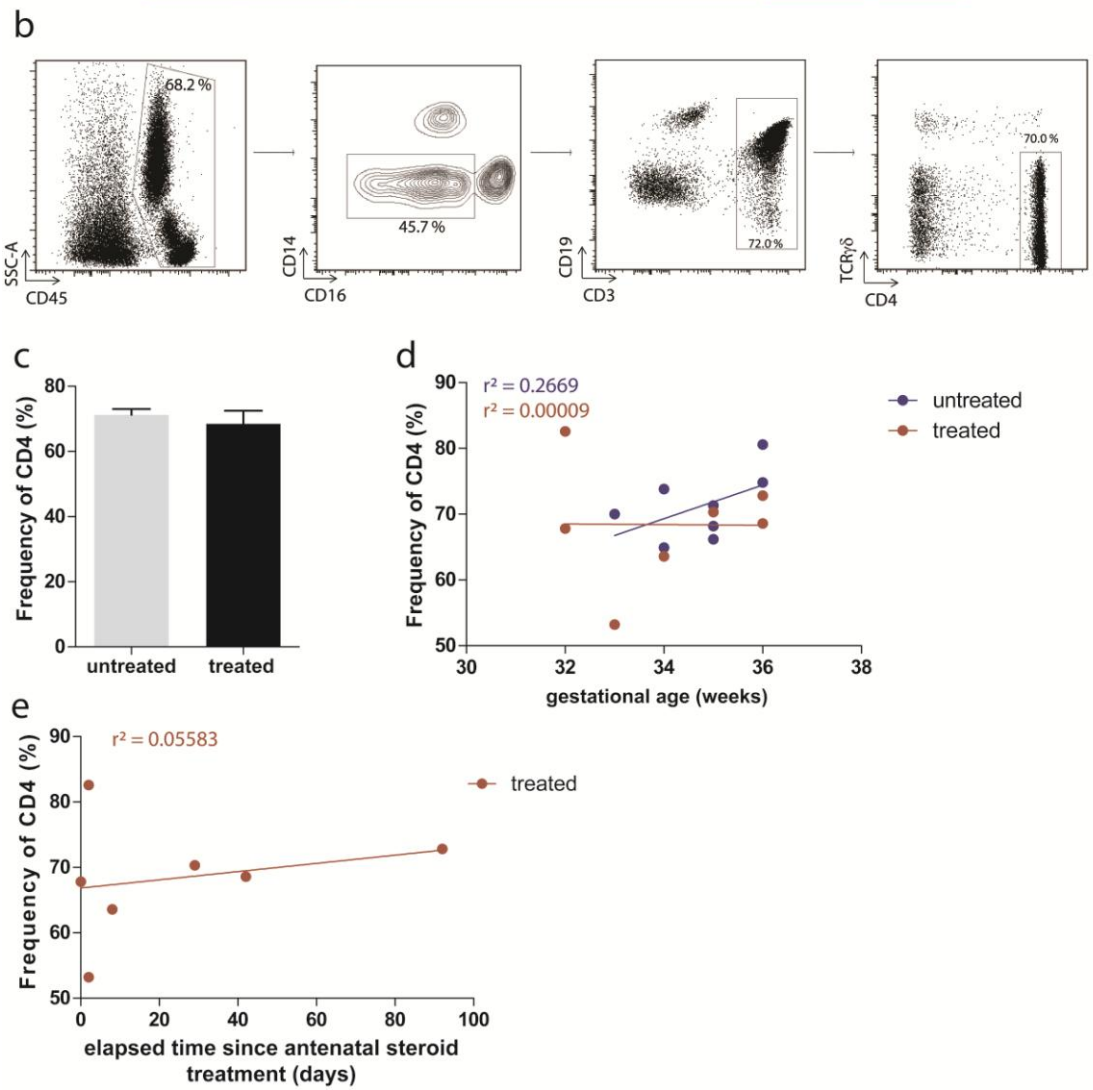

Figure 3.21: ACS treatment inhibits the gestational age-dependent increase of the frequency of T helper cells. Cord blood samples from preterm children were collected and stained for the main immune cell populations. (**a**) Staining panels used for the identification of different immune populations and (**b**) gating strategy employed for the analysis of T helper cells in the subset staining panel. (**c**) Frequency of conventional T helper cells in betamethasone treated and untreated preterm children. Values are expressed as mean ± SEM (*n*=7-8). Student's *t* test was used for statistical analysis. (**d**) Frequency of T helper cells correlated with the gestational age of the children (*n*=7-8 for each group). (**e**) Frequency of T helper cells in correlation with elapsed time since antenatal steroid treatment (*n* = 7).

4 DISCUSSION

In developed countries, seven to 10% of pregnant women are treated with antenatal glucocorticoids due to suspected pre-term delivery. The therapy significantly reduces neonatal morbidity and mortality in infants born before 34 weeks of gestation (McKinlay et al., 2012; Roberts and Dalziel, 2006), while negative secondary effects at short term seem to be minor compared to the benefits. Reported long term effects of antenatal steroid exposure in animal models include altered cardiometabolic functions (Benediktsson et al., 1993; Sugden et al., 2001), neuropathology (Antonow-Schlorke et al., 2003; Huang et al., 2001), as well as a changed glucose-insulin homeostasis and metabolism (Lindsay et al., 1996; Nyirenda et al., 1998). There is much less data on humans, but children exposed to antenatal steroid treatment show increased cortisol reactivity to acute psychosocial stress (Alexander et al., 2012).

Maternal glucocorticosteroid levels rise during pregnancy and peak before or during birth (Jung et al., 2011). Even though systemic fetal glucocorticoid levels are much lower compared to maternal levels, the placenta only represents a partial barrier for these hormones. Maternal active endogenous steroids (cortisol and corticosterone) are rapidly converted to inactive 11-keto forms by a NAD-dependent 11β-dehydrogenase (11β-HSD-2) (Cottrell et al., 2012), however, synthetic glucocorticoids are poor substrates for this enzyme, are not easily metabolized and therefore cross the feto-placental barrier (Khulan and Drake, 2012). In addition to direct effects of excess glucocorticoids, these molecules are also involved in “developmental programming”, a process by which a stimulus or insult during fetal or infant development elicits long-term changes in tissue structure or function that can persist into the next generation (Seckl, 2004; Crudo et al., 2013; Seckl and Holmes, 2007). Throughout this developmental programming, the environment encountered in fetal and neonatal life may exert a profound influence on physiological functions and on risk for disease in adult life. Animal studies clearly demonstrate that there is a direct association between nutrient imbalance in fetal life and disease in adulthood, including hypertension, diabetes, obesity and renal disease (Langley-Evans, 2006). Similar studies reported alterations such as impaired fetal growth or development, or pathophysiological outcomes later in life (Gluckman et al., 2008; Pincus-Knackstedt et al., 2006; Solano et al., 2011). Given their potent actions during development, excess glucocorticoids likely represent a common pathway by which environmental conditions are signaled from the mother to the fetus, triggering changes in offspring growth and persistently affecting tissue and organ function (Cottrell and Seckl, 2009).

Developmental programming effects also include epigenetic changes in target chromatin, affecting tissue-specific expression of the GR and glucocorticoid signaling. The effects of intrauterine exposure to antenatal steroids (or other events such as nutritional imbalance, hormone exposure or endocrine disruptions) can be transmitted to subsequent generations, without further exposure of the F1 generation (Drake et al., 2005). This suggests that exposure must induce either stable chromosomal alterations or involve epigenetic modification that is maintained through germ cell maturation (Jirtle and Skinner, 2007).

Despite known modulating effects of glucocorticoids on immune cells, few efforts have been made to study the effects of antenatal steroid treatment in the context of the immune system. An isolated report describes the lack of a thymic shadow in human newborns whose mothers received dexamethasone before delivery, a setback that subsided in some of the infants several weeks later (Michie et al., 1998). In the study, the authors did not detect any changes in immune cell counts in the first four weeks of life, but phenotype and function of these cells were not investigated. Some recent studies establish a link between ACS treatment and the development of asthma (Pole et al., 2010), and the development of diabetes (Greene et al., 2013) early in life. These studies are the first results hinting that there may be long lasting consequences of antenatal glucocorticoids on the immune system of the offspring.

Using an animal model for antenatal steroid application, we have shown that betamethasone remains in the bloodstream of the pups for at least 24 hours. This is consistent with reports from cord blood analysis where the synthetic steroid could be detected for at least 20 hours after administration (Ballard et al., 1975). Of note, the concentration detected in human cord blood serum is higher compared to the amounts detected in our mouse model. Effects on size and cellular composition of the thymus are clearly visible with a reduction in thymic volume that is mainly due to a dramatic loss of double positive thymocytes. The loss of double positive thymocytes is probably compensated with an accelerated transition from early double negative (DN2 and DN3) stages to the DN4 stage, the step before DP, in an attempt to replenish the depleted double positive compartment. At the same time, regulatory T cells and T cells bearing a gamma delta T cell receptor show a compensatory increase in frequency, hinting that these cells are less sensitive to steroid treatment compared to conventional T cells. This finding could be explained by a lower expression of the glucocorticoid receptor in these cells (our unpublished observations). Interestingly - and in contrast to the TCR$\alpha\beta$ cells - the maturation stages of the TCR$\gamma\delta$ cells are not differentially sensitive to betamethasone application, suggesting that changes within the TCR$\gamma\delta$ cells not related to their maturation

status. Instead, changes were observed in TCR$\gamma\delta$ chain usage. The frequencies of the Vγ1 and Vγ4 subsets in ACS-treated offspring differed from those of untreated offspring shortly after birth. Fetal thymic murine $\gamma\delta$ T cell development progresses in successive waves that associate with variable Vγ segment usage (Carding and Egan, 2002). The cells within each wave assemble the same Vγ and Vδ regions, but for each wave a different set of V, D and J gene segments is employed. Vγ1 and Vγ4 cells develop from E16 and E18 onward (Hayday and Gibbons, 2008). Our betamethasone injection protocol on gestational day 18.5, explains the most prominent changes in these subsets. We found a higher frequency of Vγ1 cells in betamethasone-treated animals. A recent report showed that Vγ1 cells promote Treg generation by CCR5 ligand production (Blink et al., 2014), which may offer further explanation to the increase in regulatory T cells seen shortly after ACS. Also, thymic epithelial cells and thymocytes express TGF-β, a highly conserved cytokine which is released during apoptosis (Chen et al., 2001). Due to the increased rates of apoptosis after antenatal steroid treatment, the thymic microenvironment is altered towards a milieu with increased levels of TGF-β which may further enhance Treg generation (Karagiannidis et al., 2004).

The effects of the antenatal betamethasone on cellular composition and thymic volume are noticeable for at least three days after administration, in the postnatal period, a time of highly active thymocyte development and thymocyte selection events. Several studies suggest a role for glucocorticoids in thymocyte development (Ashwell et al., 2000; van den Brandt et al., 2007). Indeed, epithelial cells in the mouse thymus synthesize steroid hormones, with a production peak at birth and decreasing steadily until adult levels at approximately 4 weeks of age (the inverse of glucocorticoid production by the adrenal cortex) (Vacchio et al., 1994; Taves et al., 2014). It has been postulated that signaling provided upon glucocorticoid receptor engagement sets the threshold between positive and negative selection, events that take place at the double positive stage of development, and alterations in this process lead to changes in the T cell repertoire that affect the adaptive immune response (Lu et al., 2000; Mittelstadt et al., 2012; Tolosa and Ashwell, 1999; Tolosa et al., 1998; van den Brandt et al., 2007). The initial thymic function during fetal and early post-natal life is crucial, and disruption of the early thymic microenvironment could result in long-term consequences including altered cell-mediated immunity (Gui et al., 2012). Thus, it seemed conceivable that overexposure of glucocorticoids at the end of pregnancy may shift a fragile balance of signals during the process of thymocyte selection.

Together with thymic output, homeostatic regulation of the peripheral niche is responsible for the maintenance of constant cell numbers. Therefore, it is not surprising that the absolute amounts and relative frequencies of the main immune cell subsets are unchanged in adult offspring of ACS-treated mothers. Still, within these main subsets, changes induced by ACS treatment remain visible and consequences can become significant in disease. In this study, we report persistent changes in the frequencies of Vγ segment usage in peripheral organs such as spleen and lung of healthy adult offspring of ACS-treated mothers. As seen in the analysis immediately after birth, the subsets most affected are the Vγ1- and Vγ4-bearing TCRγδ cells. It has to be noted again that the ACS treatment used in this study was routinely applied on gestational day 18.5. We did not investigate changes in the immune system induced by treatment administration at a different gestational day. In recent years, several reports have shown that TCRγδ cells can inhibit or suppress the maturation and/or activation of immune cells around them, leading to beneficial or potentially pathological consequences (Wesch et al., 2014). Interestingly, the Vγ1 and Vγ4 subsets in mouse often appear to have opposing functional roles; while cells of one subset exacerbate disease pathology, cells within the other subset diminish it (Hahn et al., 2004; O'Brien et al., 2000; Welte et al., 2008; Huber et al., 2000; Cui et al., 2009; Lahn et al., 1999; Gao et al., 2003). In the context of our findings of an increased frequency of Vγ1 cells in the lung, it is important to note that this particular subset has been marked as pathogenic and proinflammatory in allergic airway hyperresponsivness (AHR) (Hahn et al., 2004; Lahn et al., 1999). It is of course tempting to speculate that a dysregulation in the Vγ chain repertoire may be cause of the reported association of ACS treatment and the development of asthma early in life, a hypothesis which could be investigated in further studies using a well established mouse model for asthma. While the development of TCRγδ cells has been shown to progress in waves in mouse, how human gamma delta T cells are generated is not clear (McVay et al., 1998), making any translational conclusion to be regarded with caution. Contrary to mouse TCRγδ development, rearranged TCRDV-DC genes are expressed outside of the thymus in the liver and primitive gut in human (McVay et al., 1998), hinting that extra-thymic development of TCRγδ cells plays an important role in humans. At the same time, the patterns of TCRDVR transcripts and receptor structure change in the tissues examined during ontogeny, which may reflect the trafficking of different DV1 populations to and from distinct anatomical sites at different times during development (McVay et al., 1998), possibly mimicking the waves of development of TCRγδ cells seen in mouse.

Ligand mediated positive and negative selection through TCR$\gamma\delta$ remain poorly understood (Turchinovich and Pennington, 2011) but changes in the frequencies of several other cell types analyzed in this study also indicate that thymocyte selection events are shifted after antenatal steroid therapy. Similar to the TCR$\gamma\delta$ repertoire described above, the Vβ repertoire of TCR$\alpha\beta$ cells was likewise persistently altered in offspring whose mothers received antenatal steroids (our unpublished observations). Previous animal and human genetic studies have shown that a biased TCR Vβ repertoire is a characteristic feature of some autoimmune diseases (Tzifi et al., 2013), however this issue remains controversial. Autoimmune diseases arise from abnormal and exacerbated immune responses of the body against substances and tissues normally present in the body. The broad field of autoimmune disorders can be divided into local (e.g. Chron's disease, Colitis, Type 1 Diabetes) and systemic (e.g. systemic lupus erythematosus or rheumatoid arthritis) diseases. The trigger of autoimmune diseases is as of yet unclear. In many cases they result from multiple interactions of genes and environmental factors (Theofilopoulos and Kono, 1999; Ermann and Fathman, 2001. Figure 4.1 a). An increasing prevalence of many autoimmune diseases has been reported in industrial countries in the last fifty years (Bach, 2002. Figure 4.1 b), a period that is much too short to account for genetic evolutionary changes. Considering the shift in the repertoire of the immune system reported in this study, it seems possible that antenatal steroid treatment - which has become a regular clinical treatment since 1972 - may be one factor among many leading to the rising prevalence of autoimmune diseases.

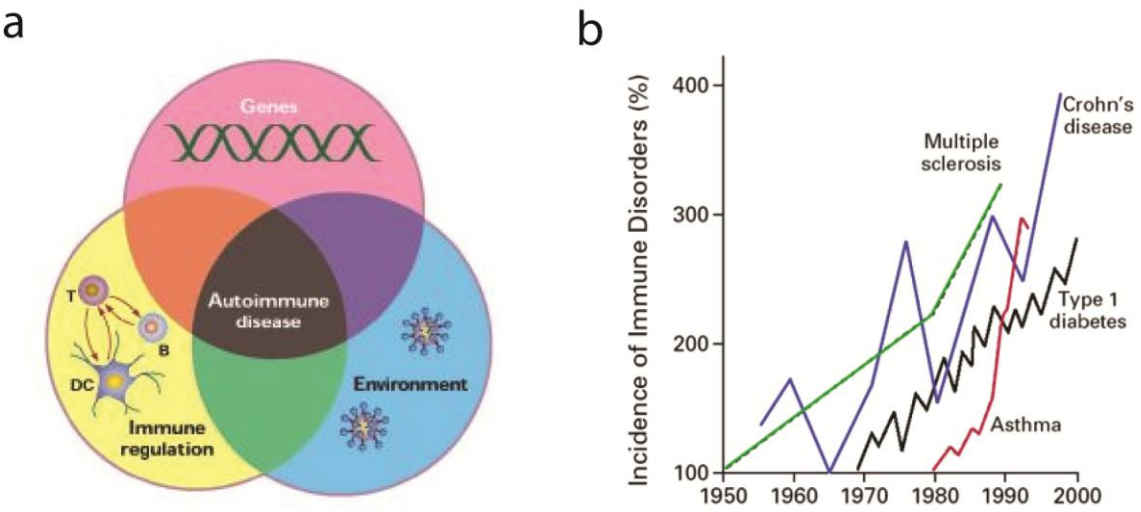

Figure 4.1: Autoimmune disease. a) Interplay of genetics, environment and immune regulation that can act as a trigger for autoimmune diseases (Ermann and Fathman, 2001). **b)** Rising incidence of autoimmune diseases in western societies (Bach, 2002).

We used two autoimmune mouse models in this study: a model for colitis (local autoimmune disease) and a model for systemic lupus erythematosus (systemic disease). Due to the lack of the immune regulatory cytokine IL-10, the IL-10 KO mice spontaneously develop a chronic colitis (Scheinin et al., 2003). IL-10 inhibits the activity of Th1 cells, NK cells, and macrophages, which are required for optimal pathogen clearance but also contribute to tissue damage. Many effects of IL-10 on T cell and NK cell function are indirect, being mediated by an effect of IL-10 on monocyte-macrophages (Couper et al., 2008). However, the IL-10 KO mouse model is dependent on the housing conditions. Thus, if maintained under conventional conditions, mice develop enterocolitis, but they only develop a limited form of colitis when kept in specific-pathogen-free environments. Germfree IL-10-deficient mice show no evidence of colitis or immune system activation (Sellon et al., 1998). Our housing facility is very clean and several research groups working with IL-10 KO mice report difficulties with this model (personal communications S. Huber, G. Tiegs). It was therefore not surprising that only few animals observed in this study developed colitis while most animals remained healthy. Despite this, the incidence of disease was higher in the group of animals treated antenatally with steroids. The decrease of unconventional IEL (TCR$\alpha\beta$CD8$\alpha\alpha$ T cells) in the gut reported in this study may be a reason. While these agonist-selected cells display a self-reactive potential, they are not destructive to healthy self-tissue and play a protective role in induced models of colitis (Poussier et al., 2002). Whether this is true for mouse models of spontaneous colitis like the IL-10 KO mice is unclear. Contrary to the TCR$\alpha\beta$CD8$\alpha\alpha$ T cells, the second set of unconventional co-receptor negative cells, the TCR$\gamma\delta$CD8$\alpha\alpha$ cells, show no such decrease after ACS treatment. Vγ5-bearing TCR$\gamma\delta$ cells, which comprise the majority of the TCR$\gamma\delta$ CD8$\alpha\alpha$ population in the gut, develop extrathymically, which may explain the unchanged frequencies in this cell type.

The second autoimmune model analyzed in this study is the MRL lpr/lpr mouse. This mouse strain develops a clinically similar form of systemic lupus erythematosus spontaneously. Disease incidence in this mouse model is 100%, thus any differences between ACS-treated and untreated offspring would only be visible as earlier/retarded onset of disease or a slower/faster progression. The results provided in this study indicate a slightly accelerated disease progression in betamethasone treated offspring. The onset of disease remained parallel in both treatment groups, with a similar occurrence of first signs of disease. Despite this, all of the offspring who had received antenatal steroids showed disease symptoms at 13 weeks of age while the last sham-treated offspring only presented with signs of disease at 15 weeks of age. Although not significantly different, ACS-treated offsprings tended to have

higher titers of anti-dsDNA antibodies. The presence of anti-dsDNA in serum in SLE patients correlates with greater severity of renal involvement and is considered a marker of disease activity (Tozzoli et al., 2002; ter Borg et al., 1990; Esdaile et al., 1996). In vivo imaging of renal and axillary lymph nodes by MRI revealed that at 12 weeks of age the lymph nodes of betamethasone treated animals were larger than those of control treated offspring. At 16 weeks, the lymph nodes of both groups showed similar sizes, a result that could be explained by anatomical limitations of lymph node growth.

These clinical signs hinted that ACS-treated offspring show stronger symptoms of autoimmune disease. The results could be further strengthened by analysis of the immune system on a cellular level where changes in several different T cell populations suggested a more active disease in offspring treated antenatally with steroids. A higher frequency of CD4 T cells in the ACS-treated offspring was an indication for stronger autoimmune disease as CD4 T cells are viewed as one of the most important players in autoimmunity (Dittel, 2008) and in mediating many aspects of autoimmune inflammation. In contrast, regulatory T cells show the capacity to prevent and down modulate inflammation. In light of this, it was interesting to see that ACS-treated animals had lower frequencies of regulatory T cells. It has been previously reported that regulatory T cells display quantitative and/or qualitative deficiencies in autoimmune diseases like systemic lupus erythematosus (Bonelli et al., 2010). It is especially interesting to note that the imbalance in regulatory T cells is worsening in the later stages of disease when the acceleration of disease symptoms in ACS-treated offsprings becomes more prominent.

One of the earliest cell surface antigens expressed by T cells upon activation is CD69. Once expressed, CD69 acts as a costimulatory molecule for T cell activation and proliferation. It is likely a pleiotropic immune regulator, potentially important in the activation and differentiation of a wide variety of hematopoietic cells (Ziegler et al., 1994). An analysis of this marker revealed an unexpected lower expression on the cell surface of CD4 T cells in spleen, lymph node and peripheral blood of ACS-treated animals. In autoimmune diseases, CD69 seems to have opposing roles as either a brake or an accelerator of disease. Patients with systemic lupus erythematosus show a diminished number of CD69 on peripheral blood CD4 T cells, which is associated with resistance of effector T lymphocytes to suppression by Treg cells (Gonzalez-Amaro et al., 2013). The authors also showed a decreased number of CD69 on regulatory T cells in patients with active SLE. This finding correlates well with the results observed in our study where betamethasone treated animals display lower expression of CD69 on regulatory T cells (data not shown). Taken together, we find higher disease activity in

offsprings whose mother's were treated with a single injection of betamethasone during pregnancy.

In order to translate these findings to the human system, we have to consider obvious differences between the species. Yet, pregnancy is a process that involves many complex physiological changes in the mother which makes *in vitro* experiments difficult. The use of animal models is therefore crucial for the understanding of effects of stressors applied during pregnancy. The immune system of the mouse is well characterized and is in many aspects similar to that of the human body. Despite this conservation significant differences exist between mice and humans in immune system development, activation, and response to challenge, in both the innate and adaptive arms (Mestas and Hughes, 2004). The most significant difference lies in the developmental maturity of the immune system before and after birth, which has been associated with the length of gestation. Animals with short gestation periods (e.g. mice) have relatively immature immune systems with significant immune development occurring postpartum, while the immune system of humans is mostly developed at birth. The period of gestation for the mouse is approximately 20 days compared to 40 weeks in the human. Therefore, each day of gestation for a mouse is approximately equivalent to 2 weeks of gestation for the human (Holladay and Smialowicz, 2000). Despite these differences, there are a lot of similarities in the ontogeny of the human immune system compared to that of mice. The mechanisms of thymus organogenesis are conserved between mouse and human (Farley et al., 2013) and the pattern of differentiation of T cells within the thymus is similar in both species (Holladay and Smialowicz, 2000).

In vitro data generated using human thymocytes show similar sensitivity of these immune cells compared to mice, validating the results acquired in mouse models regarding the apoptotic effects of antenatal steroids. The analysis of human cord blood from preterm babies whose mothers have received ACS treatment compared to those who remained untreated, we found minor differences within the T helper cells. The frequency of CD4+ T cells increased in untreated children with gestational age, while this was not observed in ACS-treated children. In the cord blood samples of ACS-treated children, the frequency of T helper cells increased with the time elapsed since the application of ACS, suggesting a slow recovery of T helper cells after steroid treatment. This data ties well together with the results obtained in our mouse model. The interpretation of this data has to be taken with caution as the human study cohort was very small and heterogeneous, especially with regard to the time point of steroid treatment and the varying duration of time between antenatal steroid treatment and sample collection at birth. A few studies on human cord blood suggest that ACS treatment results in

lower absolute lymphocyte cell counts (Chabra et al., 1998) and decreased proliferation of T cells while the activity of natural killer cells was increased (Kavelaars et al., 1999). On the other hand, exposure to ACS did not reveal significant effects on the concentrations of cytokines (IL-10, TGF-β and NT-3) measured in cord blood (Kumar et al., 2011). Drawing conclusions from human studies will remain challenging as steroids are applied at different time points of gestation, therefore potentially targeting different cell populations developing at that moment. Still, it is important to generate data to study the effect of ACS treatment in humans to complement the data generated in mouse models. More research on this topic is required in order to evaluate the risks and the benefits of antenatal steroid treatment.

4.1 Conclusion and Perspectives

In this study I showed marked effects of steroid application on different T cell types. Effects encompass both transient and persistent changes for the immune system. Because of the loss of thymocytes, short-term effects may include reduced thymic export and, as a consequence, a diminished contribution of the thymus to the peripheral lymphocyte pool. This effect - although transient - may lead to persistent alterations of the immune system such as a bias in the T cell repertoire or a constrained diversity of T cell specificities (Figure 4.2). The use of autoimmune mouse models likewise suggests the changes seen in the immune system after ACS render these offspring more prone to developing autoimmune diseases in general and an accelerated development in particular.

Antenatal betamethasone application is a standard treatment for women presenting with signs of pre-term delivery. Despite known immunomodulatory effects of steroids, few studies have addressed the effect of antenatal steroids on the immune system of the offspring. The use of antenatal steroids is paralleled by the rapidly rising incidence of allergic and auto-immune diseases in developed countries within the last decades (Bach, 2002; Subbarao et al., 2009). Antenatal stress is suggested to be one of the risk factors for the development of asthma (Subbarao et al., 2009; Pole et al., 2010) and there is evidence that antenatal exposure to GC poses a higher risk of developing type 1 diabetes (Greene et al., 2013).

The results of this study indicate the importance to further investigate the influences of antenatal steroid treatment on the developing immune system of the offspring. The undisputed benefits for neonates born before the 34th week of gestation probably outweigh the long term consequences for the immune system. However, in light of the results presented in this study, it still seems crucial for clinicians to assess the threat of preterm delivery and

develop more restrictive guidelines for the necessity of ACS treatment instead of a wide-spread prophylactic use of ACS treatment including women at low risk of imminent preterm birth.

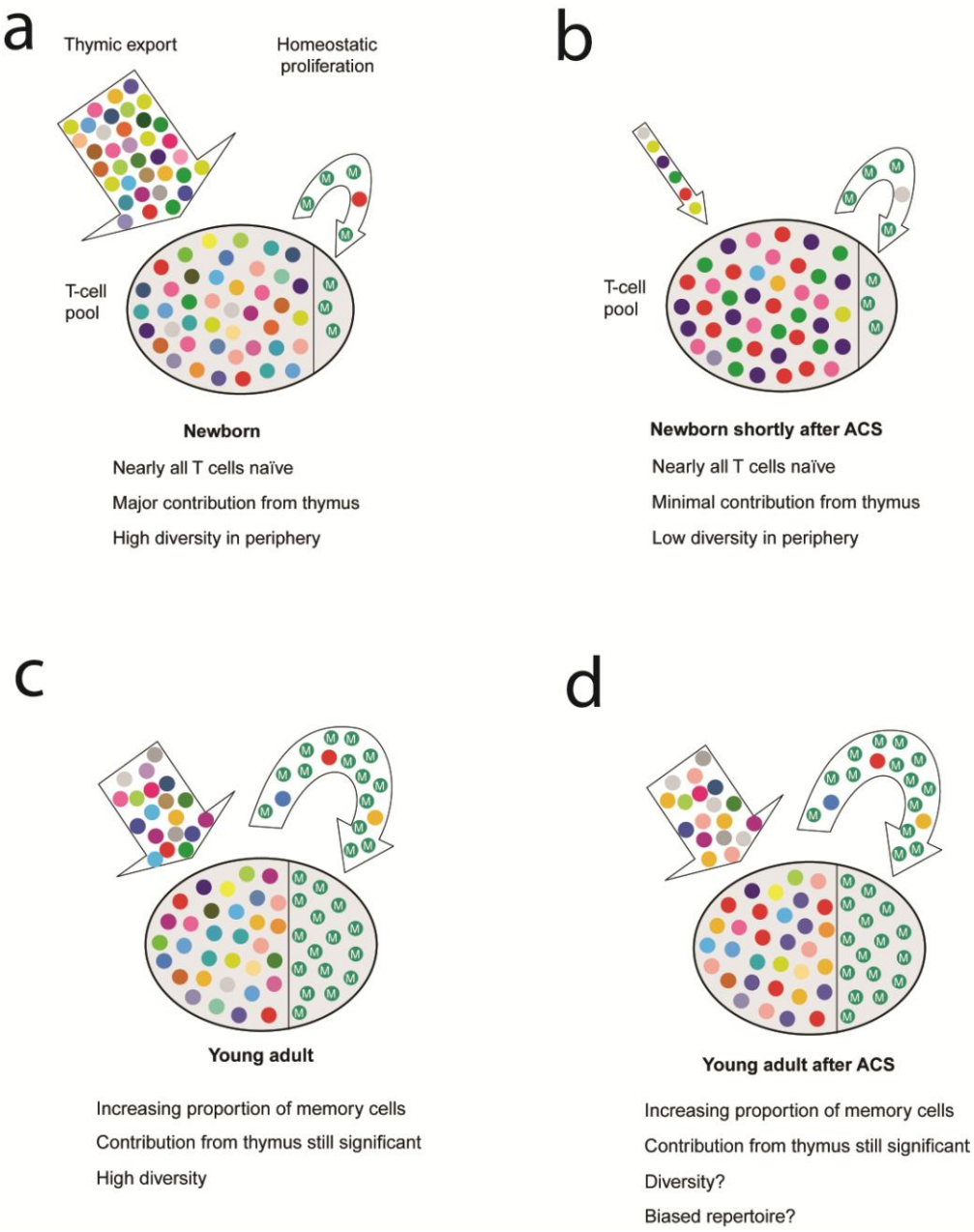

Figure 4.2: Possible influence of antenatal corticosteroid treatment on the immune system of the offspring. The peripheral T cell pool is maintained through both thymic output and proliferation of existing cells. **a)** In newborns, thymic output is the major contributor, leading to a diverse T cell repertoire. **b)** ACS treatment massively reduces thymic output, likely resulting in a lower T cell diversity in the periphery. **c)** In young adults, thymic output is less prominent, while proliferation of existing cells leads to an increasing proportion of memory cells. **d)** In young adults treated antenatally with ACS, T cell diversity may be compromised and therefore bias the repertoire. Adapted from Berzins et al., 2002.

5 REFERENCES

Alexander, N., Rosenlocher, F., Stalder, T., Linke, J., Distler, W., Morgner, J., and Kirschbaum, C. (2012). Impact of antenatal synthetic glucocorticoid exposure on endocrine stress reactivity in term-born children. The Journal of clinical endocrinology and metabolism *97*, 3538-3544.

Andrews, B.S., Eisenberg, R.A., Theofilopoulos, A.N., Izui, S., Wilson, C.B., McConahey, P.J., Murphy, E.D., Roths, J.B., and Dixon, F.J. (1978). Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. The Journal of experimental medicine *148*, 1198-1215.

Antonow-Schlorke, I., Schwab, M., Li, C., and Nathanielsz, P.W. (2003). Glucocorticoid exposure at the dose used clinically alters cytoskeletal proteins and presynaptic terminals in the fetal baboon brain. The Journal of physiology *547*, 117-123.

Ashwell, J.D., Lu, F.W., and Vacchio, M.S. (2000). Glucocorticoids in T cell development and function*. Annual review of immunology *18*, 309-345.

Asztalos, E.V., Murphy, K.E., Willan, A.R., Matthews, S.G., Ohlsson, A., Saigal, S., Armson, B.A., Kelly, E.N., Delisle, M.F., Gafni, A.*, et al.* (2013). Multiple courses of antenatal corticosteroids for preterm birth study: outcomes in children at 5 years of age (MACS-5). JAMA pediatrics *167*, 1102-1110.

Bach, J.F. (2002). The effect of infections on susceptibility to autoimmune and allergic diseases. The New England journal of medicine *347*, 911-920.

Baldwin, T.A., Hogquist, K.A., and Jameson, S.C. (2004). The fourth way? Harnessing aggressive tendencies in the thymus. Journal of immunology *173*, 6515-6520.

Ballard, P.L., Granberg, P., and Ballard, R.A. (1975). Glucocorticoid levels in maternal and cord serum after prenatal betamethasone therapy to prevent respiratory distress syndrome. The Journal of clinical investigation *56*, 1548-1554.

Benediktsson, R., Lindsay, R.S., Noble, J., Seckl, J.R., and Edwards, C.R. (1993). Glucocorticoid exposure in utero: new model for adult hypertension. Lancet *341*, 339-341.

Berg, D.J., Davidson, N., Kuhn, R., Muller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M.W., and Rennick, D. (1996). Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. The Journal of clinical investigation *98*, 1010-1020.

Berzins, S.P., Uldrich, A.P., Sutherland, J.S., Gill, J., Miller, J.F., Godfrey, D.I., and Boyd, R.L. (2002). Thymic regeneration: teaching an old immune system new tricks. Trends in molecular medicine *8*, 469-476.

Blanco, R., Borroto, A., Schamel, W., Pereira, P., and Alarcon, B. (2014). Conformational changes in the T cell receptor differentially determine T cell subset development in mice. Science signaling *7*, ra115.

Blink, S.E., Caldis, M.W., Goings, G.E., Harp, C.T., Malissen, B., Prinz, I., Xu, D., and Miller, S.D. (2014). gammadelta T cell subsets play opposing roles in regulating experimental autoimmune encephalomyelitis. Cellular immunology *290*, 39-51.

Boismenu, R., and Havran, W.L. (1994). Modulation of epithelial cell growth by intraepithelial gamma delta T cells. Science *266*, 1253-1255.

Bonelli, M., Smolen, J.S., and Scheinecker, C. (2010). Treg and lupus. Annals of the rheumatic diseases *69 Suppl 1*, i65-66.

Bonneville, M., O'Brien, R.L., and Born, W.K. (2010). Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. Nature reviews Immunology *10*, 467-478.

Born, W.K., Yin, Z., Hahn, Y.S., Sun, D., and O'Brien, R.L. (2010). Analysis of gamma delta T cell functions in the mouse. Journal of immunology *184*, 4055-4061.

Bousso, P., Bhakta, N.R., Lewis, R.S., and Robey, E. (2002). Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. Science *296*, 1876-1880.

Braun, M.Y., Jouvin-Marche, E., Marche, P.N., MacDonald, H.R., and Acha-Orbea, H. (1995). T cell receptor V beta repertoire in mice lacking endogenous mouse mammary tumor provirus. European journal of immunology *25*, 857-862.

Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. Nature *406*, 411-415.

Capone, M., Hockett, R.D., Jr., and Zlotnik, A. (1998). Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)CD25(+) Pro-T thymocytes. Proceedings of the National Academy of Sciences of the United States of America *95*, 12522-12527.

Carding, S.R., and Egan, P.J. (2002). Gammadelta T cells: functional plasticity and heterogeneity. Nature reviews Immunology *2*, 336-345.

Chabra, S., Cottrill, C., Rayens, M.K., Cross, R., Lipke, D., and Bruce, M. (1998). Lymphocyte subsets in cord blood of preterm infants: effect of antenatal steroids. Biology of the neonate *74*, 200-207.

Chatham, W.W., and Kimberly, R.P. (2001). Treatment of lupus with corticosteroids. Lupus *10*, 140-147.

Chen, W., Frank, M.E., Jin, W., and Wahl, S.M. (2001). TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. Immunity *14*, 715-725.

Cheroutre, H., and Lambolez, F. (2008). Doubting the TCR coreceptor function of CD8alphaalpha. Immunity *28*, 149-159.

Cheroutre, H., Lambolez, F., and Mucida, D. (2011). The light and dark sides of intestinal intraepithelial lymphocytes. Nature reviews Immunology *11*, 445-456.

Chien, Y.H., Gascoigne, N.R., Kavaler, J., Lee, N.E., and Davis, M.M. (1984). Somatic recombination in a murine T-cell receptor gene. Nature *309*, 322-326.

Christensen, H.D., Sienko, A.E., Rayburn, W.F., Gonzalez, C.L., and Coleman, F.H. (1997). A placebo-controlled, blinded comparison between betamethasone and dexamethasone to enhance lung maturation in the fetal mouse. Journal of the Society for Gynecologic Investigation *4*, 130-134.

Cohen, J.J., and Duke, R.C. (1984). Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. Journal of immunology *132*, 38-42.

Cottrell, E.C., Holmes, M.C., Livingstone, D.E., Kenyon, C.J., and Seckl, J.R. (2012). Reconciling the nutritional and glucocorticoid hypotheses of fetal programming. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *26*, 1866-1874.

Cottrell, E.C., and Seckl, J.R. (2009). Prenatal stress, glucocorticoids and the programming of adult disease. Frontiers in behavioral neuroscience *3*, 19.

Couper, K.N., Blount, D.G., and Riley, E.M. (2008). IL-10: the master regulator of immunity to infection. Journal of immunology *180*, 5771-5777.

Cox, G. (1995). Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. Journal of immunology *154*, 4719-4725.

Crudo, A., Suderman, M., Moisiadis, V.G., Petropoulos, S., Kostaki, A., Hallett, M., Szyf, M., and Matthews, S.G. (2013). Glucocorticoid programming of the fetal male hippocampal epigenome. Endocrinology *154*, 1168-1180.

Cui, Y., Shao, H., Lan, C., Nian, H., O'Brien, R.L., Born, W.K., Kaplan, H.J., and Sun, D. (2009). Major role of gamma delta T cells in the generation of IL-17+ uveitogenic T cells. Journal of immunology *183*, 560-567.

Delves, P.J., and Roitt, I.M. (2000). The immune system. First of two parts. The New England journal of medicine *343*, 37-49.

Dittel, B.N. (2008). CD4 T cells: Balancing the coming and going of autoimmune-mediated inflammation in the CNS. Brain, behavior, and immunity *22*, 421-430.

Drake, A.J., Walker, B.R., and Seckl, J.R. (2005). Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. American journal of physiology Regulatory, integrative and comparative physiology *288*, R34-38.

Egerton, M., Scollay, R., and Shortman, K. (1990). Kinetics of mature T-cell development in the thymus. Proceedings of the National Academy of Sciences of the United States of America *87*, 2579-2582.

Ermann, J., and Fathman, C.G. (2001). Autoimmune diseases: genes, bugs and failed regulation. Nature immunology *2*, 759-761.

Esdaile, J.M., Abrahamowicz, M., Joseph, L., MacKenzie, T., Li, Y., and Danoff, D. (1996). Laboratory tests as predictors of disease exacerbations in systemic lupus erythematosus. Why some tests fail. Arthritis and rheumatism *39*, 370-378.

Fang, X., Du, P., Liu, Y., and Tang, J. (2010). Efficient isolation of mouse liver NKT cells by perfusion. PloS one *5*, e10288.

Farley, A.M., Morris, L.X., Vroegindeweij, E., Depreter, M.L., Vaidya, H., Stenhouse, F.H., Tomlinson, S.R., Anderson, R.A., Cupedo, T., Cornelissen, J.J*., et al.* (2013). Dynamics of thymus organogenesis and colonization in early human development. Development *140*, 2015-2026.

Fazilleau, N., Bachelez, H., Gougeon, M.L., and Viguier, M. (2007). Cutting edge: size and diversity of CD4+CD25high Foxp3+ regulatory T cell repertoire in humans: evidence for similarities and partial overlapping with CD4+CD25- T cells. Journal of immunology *179*, 3412-3416.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005). A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nature immunology *6*, 1142-1151.

Gao, Y., Yang, W., Pan, M., Scully, E., Girardi, M., Augenlicht, L.H., Craft, J., and Yin, Z. (2003). Gamma delta T cells provide an early source of interferon gamma in tumor immunity. The Journal of experimental medicine *198*, 433-442.

Gluckman, P.D., Hanson, M.A., Cooper, C., and Thornburg, K.L. (2008). Effect of in utero and early-life conditions on adult health and disease. The New England journal of medicine *359*, 61-73.

Gonzalez-Amaro, R., Cortes, J.R., Sanchez-Madrid, F., and Martin, P. (2013). Is CD69 an effective brake to control inflammatory diseases? Trends in molecular medicine *19*, 625-632.

Greene, N.H., Pedersen, L.H., Liu, S., and Olsen, J. (2013). Prenatal prescription corticosteroids and offspring diabetes: a national cohort study. International journal of epidemiology *42*, 186-193.

Greenstein, S., Ghias, K., Krett, N.L., and Rosen, S.T. (2002). Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies. Clinical cancer research : an official journal of the American Association for Cancer Research *8*, 1681-1694.

Gui, J., Mustachio, L.M., Su, D.M., and Craig, R.W. (2012). Thymus Size and Age-related Thymic Involution: Early Programming, Sexual Dimorphism, Progenitors and Stroma. Aging and disease *3*, 280-290.

Guy-Grand, D., Cerf-Bensussan, N., Malissen, B., Malassis-Seris, M., Briottet, C., and Vassalli, P. (1991). Two gut intraepithelial CD8+ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation. The Journal of experimental medicine *173*, 471-481.

Guy-Grand, D., Vanden Broecke, C., Briottet, C., Malassis-Seris, M., Selz, F., and Vassalli, P. (1992). Different expression of the recombination activity gene RAG-1 in various

populations of thymocytes, peripheral T cells and gut thymus-independent intraepithelial lymphocytes suggests two pathways of T cell receptor rearrangement. European journal of immunology *22*, 505-510.

Guy-Grand, D., Vassalli, P., Eberl, G., Pereira, P., Burlen-Defranoux, O., Lemaitre, F., Di Santo, J.P., Freitas, A.A., Cumano, A., and Bandeira, A. (2013). Origin, trafficking, and intraepithelial fate of gut-tropic T cells. The Journal of experimental medicine *210*, 1839-1854.

Hahn, Y.S., Taube, C., Jin, N., Sharp, L., Wands, J.M., Aydintug, M.K., Lahn, M., Huber, S.A., O'Brien, R.L., Gelfand, E.W.*, et al.* (2004). Different potentials of gamma delta T cell subsets in regulating airway responsiveness: V gamma 1+ cells, but not V gamma 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation. Journal of immunology *172*, 2894-2902.

Haks, M.C., Lefebvre, J.M., Lauritsen, J.P., Carleton, M., Rhodes, M., Miyazaki, T., Kappes, D.J., and Wiest, D.L. (2005). Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. Immunity *22*, 595-606.

Hayday, A., and Gibbons, D. (2008). Brokering the peace: the origin of intestinal T cells. Mucosal immunology *1*, 172-174.

Hayday, A.C. (2000). [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annual review of immunology *18*, 975-1026.

Herrlich, P. (2001). Cross-talk between glucocorticoid receptor and AP-1. Oncogene *20*, 2465-2475.

Hess, D. (2012). Respiratory care : principles and practice, 2nd edn (Sudbury, Mass.: Jones & Bartlett Learning).

Holladay, S.D., and Smialowicz, R.J. (2000). Development of the murine and human immune system: differential effects of immunotoxicants depend on time of exposure. Environmental health perspectives *108 Suppl 3*, 463-473.

Holt, M.P., Shevach, E.M., and Punkosdy, G.A. (2013). Endogenous mouse mammary tumor viruses (mtv): new roles for an old virus in cancer, infection, and immunity. Frontiers in oncology *3*, 287.

Howard, M., and O'Garra, A. (1992). Biological properties of interleukin 10. Immunology today *13*, 198-200.

Hsieh, C.S., Lee, H.M., and Lio, C.W. (2012). Selection of regulatory T cells in the thymus. Nature reviews Immunology *12*, 157-167.

Hsieh, C.S., Zheng, Y., Liang, Y., Fontenot, J.D., and Rudensky, A.Y. (2006). An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. Nature immunology *7*, 401-410.

Huang, W.L., Harper, C.G., Evans, S.F., Newnham, J.P., and Dunlop, S.A. (2001). Repeated prenatal corticosteroid administration delays astrocyte and capillary tight junction maturation in fetal sheep. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience *19*, 487-493.

Huber, S.A., Graveline, D., Newell, M.K., Born, W.K., and O'Brien, R.L. (2000). V gamma 1+ T cells suppress and V gamma 4+ T cells promote susceptibility to coxsackievirus B3-induced myocarditis in mice. Journal of immunology *165*, 4174-4181.

Ismail, A.S., Severson, K.M., Vaishnava, S., Behrendt, C.L., Yu, X., Benjamin, J.L., Ruhn, K.A., Hou, B., DeFranco, A.L., Yarovinsky, F.*, et al.* (2011). Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. Proceedings of the National Academy of Sciences of the United States of America *108*, 8743-8748.

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F., and Sakaguchi, S. (1999). Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and

suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. Journal of immunology *162*, 5317-5326.

Jaffar, Z., Ferrini, M.E., Herritt, L.A., and Roberts, K. (2009). Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. Journal of immunology *182*, 4507-4511.

Jameson, J., Witherden, D., and Havran, W.L. (2003). T-cell effector mechanisms: gammadelta and CD1d-restricted subsets. Current opinion in immunology *15*, 349-353.

Jiang, R., Lan, Y., Chapman, H.D., Shawber, C., Norton, C.R., Serreze, D.V., Weinmaster, G., and Gridley, T. (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. Genes & development *12*, 1046-1057.

Jirtle, R.L., and Skinner, M.K. (2007). Environmental epigenomics and disease susceptibility. Nature reviews Genetics *8*, 253-262.

Jung, C., Ho, J.T., Torpy, D.J., Rogers, A., Doogue, M., Lewis, J.G., Czajko, R.J., and Inder, W.J. (2011). A longitudinal study of plasma and urinary cortisol in pregnancy and postpartum. The Journal of clinical endocrinology and metabolism *96*, 1533-1540.

Kabelitz, D., Peters, C., Wesch, D., and Oberg, H.H. (2013). Regulatory functions of gammadelta T cells. International immunopharmacology *16*, 382-387.

Kabelitz, D., Wesch, D., and Hinz, T. (1999). gamma delta T cells, their T cell receptor usage and role in human diseases. Springer seminars in immunopathology *21*, 55-75.

Kadmiel, M., and Cidlowski, J.A. (2013). Glucocorticoid receptor signaling in health and disease. Trends in pharmacological sciences *34*, 518-530.

Karagiannidis, C., Akdis, M., Holopainen, P., Woolley, N.J., Hense, G., Ruckert, B., Mantel, P.Y., Menz, G., Akdis, C.A., Blaser, K.*, et al.* (2004). Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. The Journal of allergy and clinical immunology *114*, 1425-1433.

Kavelaars, A., van der Pompe, G., Bakker, J.M., van Hasselt, P.M., Cats, B., Visser, G.H., and Heijnen, C.J. (1999). Altered immune function in human newborns after prenatal administration of betamethasone: enhanced natural killer cell activity and decreased T cell proliferation in cord blood. Pediatric research *45*, 306-312.

Khulan, B., and Drake, A.J. (2012). Glucocorticoids as mediators of developmental programming effects. Best practice & research Clinical endocrinology & metabolism *26*, 689-700.

Kronenberg, M., and Gapin, L. (2002). The unconventional lifestyle of NKT cells. Nature reviews Immunology *2*, 557-568.

Kufe, D.W., Holland, J.F., Frei, E., and American Cancer Society. (2003). Cancer medicine 6, 6th edn (Hamilton, Ont. ; Lewiston, NY: BC Decker).

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263-274.

Kumar, P., Venners, S.A., Fu, L., Pearson, C., Ortiz, K., and Wang, X. (2011). Association of antenatal steroid use with cord blood immune biomarkers in preterm births. Early human development *87*, 559-564.

Kurd, N., and Robey, E.A. (2014). Unconventional intraepithelial gut T cells: the TCR says it all. Immunity *41*, 167-168.

Kyewski, B., and Klein, L. (2006). A central role for central tolerance. Annual review of immunology *24*, 571-606.

Lahn, M., Kanehiro, A., Takeda, K., Joetham, A., Schwarze, J., Kohler, G., O'Brien, R., Gelfand, E.W., and Born, W. (1999). Negative regulation of airway responsiveness that is dependent on gammadelta T cells and independent of alphabeta T cells. Nature medicine *5*, 1150-1156.

Lambolez, F., Kronenberg, M., and Cheroutre, H. (2007). Thymic differentiation of TCR alpha beta(+) CD8 alpha alpha(+) IELs. Immunological reviews *215*, 178-188.

Langley-Evans, S.C. (2006). Developmental programming of health and disease. The Proceedings of the Nutrition Society *65*, 97-105.

Lawn, J.E., Cousens, S.N., Darmstadt, G.L., Bhutta, Z.A., Martines, J., Paul, V., Knippenberg, R., Fogstad, H., and Lancet Neonatal Survival Series steering, t. (2006). 1 year after The Lancet Neonatal Survival Series--was the call for action heard? Lancet *367*, 1541-1547.

Le Douarin, N.M., Dieterlen-Lievre, F., and Oliver, P.D. (1984). Ontogeny of primary lymphoid organs and lymphoid stem cells. The American journal of anatomy *170*, 261-299.

Lee, B.K., and Eicher, E.M. (1990). Segregation patterns of endogenous mouse mammary tumor viruses in five recombinant inbred strain sets. Journal of virology *64*, 4568-4572.

Lee, Y.K., Chiu, S., Chew, A., Greenhalgh, D.G., and Cho, K. (2011). Prevalent de novo somatic mutations in superantigen genes of mouse mammary tumor viruses in the genome of C57BL/6J mice and its potential implication in the immune system. BMC immunology *12*, 5.

Li, Y., Innocentin, S., Withers, D.R., Roberts, N.A., Gallagher, A.R., Grigorieva, E.F., Wilhelm, C., and Veldhoen, M. (2011). Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. Cell *147*, 629-640.

Liggins, G.C., and Howie, R.N. (1972). A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics *50*, 515-525.

Lim, H.W., Broxmeyer, H.E., and Kim, C.H. (2006). Regulation of trafficking receptor expression in human forkhead box P3+ regulatory T cells. Journal of immunology *177*, 840-851.

Lindsay, R.S., Lindsay, R.M., Waddell, B.J., and Seckl, J.R. (1996). Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone. Diabetologia *39*, 1299-1305.

Liu, Y., Cousin, J.M., Hughes, J., Van Damme, J., Seckl, J.R., Haslett, C., Dransfield, I., Savill, J., and Rossi, A.G. (1999). Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. Journal of immunology *162*, 3639-3646.

Livak, F., Tourigny, M., Schatz, D.G., and Petrie, H.T. (1999). Characterization of TCR gene rearrangements during adult murine T cell development. Journal of immunology *162*, 2575-2580.

Lu, F.W., Yasutomo, K., Goodman, G.B., McHeyzer-Williams, L.J., McHeyzer-Williams, M.G., Germain, R.N., and Ashwell, J.D. (2000). Thymocyte resistance to glucocorticoids leads to antigen-specific unresponsiveness due to "holes" in the T cell repertoire. Immunity *12*, 183-192.

Maggi, E., Cosmi, L., Liotta, F., Romagnani, P., Romagnani, S., and Annunziato, F. (2005). Thymic regulatory T cells. Autoimmunity reviews *4*, 579-586.

Martin, B., Hirota, K., Cua, D.J., Stockinger, B., and Veldhoen, M. (2009). Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity *31*, 321-330.

Mayans, S., Stepniak, D., Palida, S.F., Larange, A., Dreux, J., Arlian, B.M., Shinnakasu, R., Kronenberg, M., Cheroutre, H., and Lambolez, F. (2014). alphabetaT cell receptors expressed by CD4(-)CD8alphabeta(-) intraepithelial T cells drive their fate into a unique lineage with unusual MHC reactivities. Immunity *41*, 207-218.

McEvoy, C., Bowling, S., Williamson, K., Collins, J., Tolaymat, L., and Maher, J. (2000). Timing of antenatal corticosteroids and neonatal pulmonary mechanics. American journal of obstetrics and gynecology *183*, 895-899.

McKinlay, C.J., Crowther, C.A., Middleton, P., and Harding, J.E. (2012). Repeat antenatal glucocorticoids for women at risk of preterm birth: a Cochrane Systematic Review. American journal of obstetrics and gynecology *206*, 187-194.

McVay, L.D., Jaswal, S.S., Kennedy, C., Hayday, A., and Carding, S.R. (1998). The generation of human gammadelta T cell repertoires during fetal development. Journal of immunology *160*, 5851-5860.

Mendelson, C.R., and Boggaram, V. (1991). Hormonal control of the surfactant system in fetal lung. Annual review of physiology *53*, 415-440.

Mestas, J., and Hughes, C.C. (2004). Of mice and not men: differences between mouse and human immunology. Journal of immunology *172*, 2731-2738.

Michie, C.A., Hasson, N., and Tulloh, R. (1998). The neonatal thymus and antenatal steroids. Archives of disease in childhood Fetal and neonatal edition *79*, F159.

Miracle, X., Di Renzo, G.C., Stark, A., Fanaroff, A., Carbonell-Estrany, X., Saling, E., and Coordinators Of World Associatin of Perinatal Medicine Prematurity Working, G. (2008). Guideline for the use of antenatal corticosteroids for fetal maturation. Journal of perinatal medicine *36*, 191-196.

Misslitz, A., Bernhardt, G., and Forster, R. (2006). Trafficking on serpentines: molecular insight on how maturating T cells find their winding paths in the thymus. Immunological reviews *209*, 115-128.

Mittelstadt, P.R., Monteiro, J.P., and Ashwell, J.D. (2012). Thymocyte responsiveness to endogenous glucocorticoids is required for immunological fitness. The Journal of clinical investigation *122*, 2384-2394.

Morse, H.C., 3rd, Davidson, W.F., Yetter, R.A., Murphy, E.D., Roths, J.B., and Coffman, R.L. (1982). Abnormalities induced by the mutant gene Ipr: expansion of a unique lymphocyte subset. Journal of immunology *129*, 2612-2615.

Murphy, K., Travers, P., Walport, M., and Janeway, C. (2012). Janeway's immunobiology, 8th edn (New York: Garland Science).

Nahar, L., Sarker, S.D., and Turner, A.B. (2007). A review on synthetic and natural steroid dimers: 1997-2006. Current medicinal chemistry *14*, 1349-1370.

Nyirenda, M.J., Lindsay, R.S., Kenyon, C.J., Burchell, A., and Seckl, J.R. (1998). Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. The Journal of clinical investigation *101*, 2174-2181.

O'Brien, R.L., Roark, C.L., and Born, W.K. (2009). IL-17-producing gammadelta T cells. European journal of immunology *39*, 662-666.

O'Brien, R.L., Yin, X., Huber, S.A., Ikuta, K., and Born, W.K. (2000). Depletion of a gamma delta T cell subset can increase host resistance to a bacterial infection. Journal of immunology *165*, 6472-6479.

Peterson, D.O., Kriz, K.G., Marich, J.E., and Toohey, M.G. (1985). Sequence organization and molecular cloning of mouse mammary tumor virus DNA endogenous to C57BL/6 mice. Journal of virology *54*, 525-531.

Petrie, H.T., Livak, F., Schatz, D.G., Strasser, A., Crispe, I.N., and Shortman, K. (1993). Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. The Journal of experimental medicine *178*, 615-622.

Piemonti, L., Monti, P., Allavena, P., Sironi, M., Soldini, L., Leone, B.E., Socci, C., and Di Carlo, V. (1999). Glucocorticoids affect human dendritic cell differentiation and maturation. Journal of immunology *162*, 6473-6481.

Pincus-Knackstedt, M.K., Joachim, R.A., Blois, S.M., Douglas, A.J., Orsal, A.S., Klapp, B.F., Wahn, U., Hamelmann, E., and Arck, P.C. (2006). Prenatal stress enhances susceptibility of murine adult offspring toward airway inflammation. Journal of immunology *177*, 8484-8492.

Playfair, J., and Chain, B. (2010). Immunology at a glance, 9th edn (Wiley-Blackwell).

Pobezinsky, L.A., Angelov, G.S., Tai, X., Jeurling, S., Van Laethem, F., Feigenbaum, L., Park, J.H., and Singer, A. (2012). Clonal deletion and the fate of autoreactive thymocytes that survive negative selection. Nature immunology *13*, 569-578.

Pole, J.D., Mustard, C.A., To, T., Beyene, J., and Allen, A.C. (2010). Antenatal steroid therapy for fetal lung maturation and the subsequent risk of childhood asthma: a longitudinal analysis. Journal of pregnancy *2010*, 789748.

Poussier, P., Ning, T., Banerjee, D., and Julius, M. (2002). A unique subset of self-specific intraintestinal T cells maintains gut integrity. The Journal of experimental medicine *195*, 1491-1497.

Prinz, I., Silva-Santos, B., and Pennington, D.J. (2013). Functional development of gammadelta T cells. European journal of immunology *43*, 1988-1994.

Ramond, C., Berthault, C., Burlen-Defranoux, O., de Sousa, A.P., Guy-Grand, D., Vieira, P., Pereira, P., and Cumano, A. (2014). Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. Nature immunology *15*, 27-35.

Regnault, A., Cumano, A., Vassalli, P., Guy-Grand, D., and Kourilsky, P. (1994). Oligoclonal repertoire of the CD8 alpha alpha and the CD8 alpha beta TCR-alpha/beta murine intestinal intraepithelial T lymphocytes: evidence for the random emergence of T cells. The Journal of experimental medicine *180*, 1345-1358.

Roberts, D., and Dalziel, S. (2006). Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. The Cochrane database of systematic reviews, CD004454.

Rocha, B., Vassalli, P., and Guy-Grand, D. (1991). The V beta repertoire of mouse gut homodimeric alpha CD8+ intraepithelial T cell receptor alpha/beta + lymphocytes reveals a major extrathymic pathway of T cell differentiation. The Journal of experimental medicine *173*, 483-486.

Rocha, B., Vassalli, P., and Guy-Grand, D. (1994). Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice. The Journal of experimental medicine *180*, 681-686.

Rodewald, H.R., and Fehling, H.J. (1998). Molecular and cellular events in early thymocyte development. Advances in immunology *69*, 1-112.

Rubino, S.J., Geddes, K., and Girardin, S.E. (2012). Innate IL-17 and IL-22 responses to enteric bacterial pathogens. Trends in immunology *33*, 112-118.

Sakaguchi, S. (2003). The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. The Journal of clinical investigation *112*, 1310-1312.

Sakaguchi, S., Takahashi, T., and Nishizuka, Y. (1982). Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. The Journal of experimental medicine *156*, 1577-1586.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. Cell *133*, 775-787.

Scheinin, T., Butler, D.M., Salway, F., Scallon, B., and Feldmann, M. (2003). Validation of the interleukin-10 knockout mouse model of colitis: antitumour necrosis factor-antibodies suppress the progression of colitis. Clinical and experimental immunology *133*, 38-43.

Schwartzman, R.A., and Cidlowski, J.A. (1994). Glucocorticoid-induced apoptosis of lymphoid cells. International archives of allergy and immunology *105*, 347-354.

Seckl, J.R. (2004). Prenatal glucocorticoids and long-term programming. European journal of endocrinology / European Federation of Endocrine Societies *151 Suppl 3*, U49-62.

Seckl, J.R., and Holmes, M.C. (2007). Mechanisms of disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. Nature clinical practice Endocrinology & metabolism *3*, 479-488.

Sellon, R.K., Tonkonogy, S., Schultz, M., Dieleman, L.A., Grenther, W., Balish, E., Rennick, D.M., and Sartor, R.B. (1998). Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infection and immunity *66*, 5224-5231.

Shin, S., El-Diwany, R., Schaffert, S., Adams, E.J., Garcia, K.C., Pereira, P., and Chien, Y.H. (2005). Antigen recognition determinants of gammadelta T cell receptors. Science *308*, 252-255.

Shui, J.W., Larange, A., Kim, G., Vela, J.L., Zahner, S., Cheroutre, H., and Kronenberg, M. (2012). HVEM signalling at mucosal barriers provides host defence against pathogenic bacteria. Nature *488*, 222-225.

Singer, A., Adoro, S., and Park, J.H. (2008). Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nature reviews Immunology *8*, 788-801.

Solano, M.E., Jago, C., Pincus, M.K., and Arck, P.C. (2011). Highway to health; or How prenatal factors determine disease risks in the later life of the offspring. Journal of reproductive immunology *90*, 3-8.

Stahn, C., Lowenberg, M., Hommes, D.W., and Buttgereit, F. (2007). Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. Molecular and cellular endocrinology *275*, 71-78.

Stephens, G.L., Ashwell, J.D., and Ignatowicz, L. (2003). Mutually antagonistic signals regulate selection of the T cell repertoire. International immunology *15*, 623-632.

Stewart, J.D., Sienko, A.E., Gonzalez, C.L., Christensen, H.D., and Rayburn, W.F. (1998). Placebo-controlled comparison between a single dose and a multidose of betamethasone in accelerating lung maturation of mice offspring. American journal of obstetrics and gynecology *179*, 1241-1247.

Stritesky, G.L., Jameson, S.C., and Hogquist, K.A. (2012). Selection of self-reactive T cells in the thymus. Annual review of immunology *30*, 95-114.

Subbarao, P., Mandhane, P.J., and Sears, M.R. (2009). Asthma: epidemiology, etiology and risk factors. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne *181*, E181-190.

Sugden, M.C., Langdown, M.L., Munns, M.J., and Holness, M.J. (2001). Maternal glucocorticoid treatment modulates placental leptin and leptin receptor expression and materno-fetal leptin physiology during late pregnancy, and elicits hypertension associated with hyperleptinaemia in the early-growth-retarded adult offspring. European journal of endocrinology / European Federation of Endocrine Societies *145*, 529-539.

Sutton, C.E., Lalor, S.J., Sweeney, C.M., Brereton, C.F., Lavelle, E.C., and Mills, K.H. (2009). Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity *31*, 331-341.

Taeusch, H.W., Ballard, R.A., and Gleason, C.A. (2005). Avery's diseases of the newborn, 8th edn (Philadelphia: Elsevier Saunders).

Taghon, T., and Rothenberg, E.V. (2008). Molecular mechanisms that control mouse and human TCR-alphabeta and TCR-gammadelta T cell development. Seminars in immunopathology *30*, 383-398.

Tannock, I., and Hill, R.P. (1992). The Basic science of oncology, 2nd edn (New York: McGraw-Hill, Health Professions Division).

Taves, M.D., Plumb, A.W., Sandkam, B.A., Ma, C., Van Der Gugten, J.G., Holmes, D.T., Close, D.A., Abraham, N., and Soma, K.K. (2014). Steroid profiling reveals widespread local regulation of glucocorticoid levels during mouse development. Endocrinology, en20131606.

ter Borg, E.J., Horst, G., Hummel, E.J., Limburg, P.C., and Kallenberg, C.G. (1990). Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus. A long-term, prospective study. Arthritis and rheumatism *33*, 634-643.

Theofilopoulos, A.N., and Dixon, F.J. (1982). Autoimmune diseases: immunopathology and etiopathogenesis. The American journal of pathology *108*, 319-365.

Theofilopoulos, A.N., and Kono, D.H. (1999). The genes of systemic autoimmunity. Proceedings of the Association of American Physicians *111*, 228-240.

Tolosa, E., and Ashwell, J.D. (1999). Thymus-derived glucocorticoids and the regulation of antigen-specific T-cell development. Neuroimmunomodulation *6*, 90-96.

Tolosa, E., King, L.B., and Ashwell, J.D. (1998). Thymocyte glucocorticoid resistance alters positive selection and inhibits autoimmunity and lymphoproliferative disease in MRL-lpr/lpr mice. Immunity *8*, 67-76.

Tozzoli, R., Bizzaro, N., Tonutti, E., Villalta, D., Bassetti, D., Manoni, F., Piazza, A., Pradella, M., Rizzotti, P., and Italian Society of Laboratory Medicine Study Group on the Diagnosis of Autoimmune, D. (2002). Guidelines for the laboratory use of autoantibody tests in the diagnosis and monitoring of autoimmune rheumatic diseases. American journal of clinical pathology *117*, 316-324.

Turchinovich, G., and Pennington, D.J. (2011). T cell receptor signalling in gammadelta cell development: strength isn't everything. Trends in immunology *32*, 567-573.

Tzifi, F., Kanariou, M., Tzanoudaki, M., Mihas, C., Paschali, E., Chrousos, G., and Kanaka-Gantenbein, C. (2013). Flow cytometric analysis of the CD4+ TCR Vbeta repertoire in the peripheral blood of children with type 1 diabetes mellitus, systemic lupus erythematosus and age-matched healthy controls. BMC immunology *14*, 33.

Uno, H., Lohmiller, L., Thieme, C., Kemnitz, J.W., Engle, M.J., Roecker, E.B., and Farrell, P.M. (1990). Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. Brain research Developmental brain research *53*, 157-167.

Vacchio, M.S., Lee, J.Y., and Ashwell, J.D. (1999). Thymus-derived glucocorticoids set the thresholds for thymocyte selection by inhibiting TCR-mediated thymocyte activation. Journal of immunology *163*, 1327-1333.

Vacchio, M.S., Papadopoulos, V., and Ashwell, J.D. (1994). Steroid production in the thymus: implications for thymocyte selection. The Journal of experimental medicine *179*, 1835-1846.

van den Brandt, J., Luhder, F., McPherson, K.G., de Graaf, K.L., Tischner, D., Wiehr, S., Herrmann, T., Weissert, R., Gold, R., and Reichardt, H.M. (2007). Enhanced glucocorticoid receptor signaling in T cells impacts thymocyte apoptosis and adaptive immune responses. The American journal of pathology *170*, 1041-1053.

van der Goes, A., Hoekstra, K., van den Berg, T.K., and Dijkstra, C.D. (2000). Dexamethasone promotes phagocytosis and bacterial killing by human monocytes/macrophages in vitro. Journal of leukocyte biology *67*, 801-807.

Washburn, T., Schweighoffer, E., Gridley, T., Chang, D., Fowlkes, B.J., Cado, D., and Robey, E. (1997). Notch activity influences the alphabeta versus gammadelta T cell lineage decision. Cell *88*, 833-843.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature *356*, 314-317.

Weiss, J.M., Bilate, A.M., Gobert, M., Ding, Y., Curotto de Lafaille, M.A., Parkhurst, C.N., Xiong, H., Dolpady, J., Frey, A.B., Ruocco, M.G.*, et al.* (2012). Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. The Journal of experimental medicine *209*, 1723-1742, S1721.

Welte, T., Lamb, J., Anderson, J.F., Born, W.K., O'Brien, R.L., and Wang, T. (2008). Role of two distinct gammadelta T cell subsets during West Nile virus infection. FEMS immunology and medical microbiology *53*, 275-283.

Wesch, D., Peters, C., and Siegers, G.M. (2014). Human gamma delta T regulatory cells in cancer: fact or fiction? Frontiers in immunology *5*, 598.

Witt, C.M., Raychaudhuri, S., Schaefer, B., Chakraborty, A.K., and Robey, E.A. (2005). Directed migration of positively selected thymocytes visualized in real time. PLoS biology *3*, e160.

Wlodkowic, D., Telford, W., Skommer, J., and Darzynkiewicz, Z. (2011). Apoptosis and beyond: cytometry in studies of programmed cell death. Methods in cell biology *103*, 55-98.

Woodland, D.L., Lund, F.E., Happ, M.P., Blackman, M.A., Palmer, E., and Corley, R.B. (1991). Endogenous superantigen expression is controlled by mouse mammary tumor proviral loci. The Journal of experimental medicine *174*, 1255-1258.

Yates, A.J. (2014). Theories and quantification of thymic selection. Frontiers in immunology *5*, 13.

Zacharchuk, C.M., Mercep, M., Chakraborti, P.K., Simons, S.S., Jr., and Ashwell, J.D. (1990). Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. Journal of immunology *145*, 4037-4045.

Zhang, J., Massmann, G.A., Rose, J.C., and Figueroa, J.P. (2010). Differential effects of clinical doses of antenatal betamethasone on nephron endowment and glomerular filtration rate in adult sheep. Reproductive sciences *17*, 186-195.

Ziegler, S.F., Ramsdell, F., and Alderson, M.R. (1994). The activation antigen CD69. Stem cells *12*, 456-465.

6 ABBREVIATIONS

°C	degree Celsius
%	percent
Ag	Antigen
AHR	airway hyperresponsivness
ANOVA	analysis of variance
ACS	antenatal corticosteroid treatment
APC	Allophycocyanin
Beta	Betamethasone
BSA	bovine serum albumin
BV421	Brilliant Violet421
BV510	Brilliant Violet510
CD	cluster of differentiation molecule
cm	centimetre
DMSO	Dimethylsulfoxid
DN	double negative ($CD4^-CD8^-$ thymocytes)
DP	double positive ($CD4^+CD8^+$ thymocytes)
E	embryonic day
EDTA	Ethylenediaminetetraacetic
ELISA	enzyme-linked immunosorbent assay
et al.	*et alii*; and others
EtOH	ethanol
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	gram
GARP	Glycoprotein A Repetitions Predominant
GC	glucocorticoid
GR	glucocorticoid receptor
h	hour
HPA	hypothalamus-pituitary-adrenal
IEL	intraepithelial lymphocytes
i.p.	intra peritoneal
i.v.	intravenous
l	litre
LPL	lamina propria lymphocytes

M	molar
min	minute(s)
ml	millilitre
mm^2	square millimetre
mM	millimolar
mol	unit of measurement
µl	microlitre
µg	microgram
n	nano
ng	nanogram
nm	nanometre
OD_{400}	optical density at a wavelength of 400 nm
p	pico
PBS	phosphate buffered saline
PE	Phycoerythrin
PE-cy7	Phycoerythrin cyanine 7
pg	pico gram
pH	negative logarithm of the activity of the hydronium iron
PS	phosphatidylserine
pTreg	peripherally induced Treg
RDS	respiratory distress syndrome
RLU	relative luminescence units
ROI	Region of interest
Rpm	round per minute
SAg	Super antigen
SEM	standard error of the mean
sec	second(s)
SP	single positive ($CD4^+$ or $CD8^+$ thymocyte)
SSC	sideward scatter
TCR	T cell receptor
Treg	regulatory T cell
tTreg	thymically derived Treg
U	unit
v	volumen
v/v	volume per volume
w	weight
WT	wildtype
x g	centrifugal force

7 LIST OF FIGURES

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9 APPENDIX

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Posters and Talks

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Sehr geehrte Damen und Herrn,

hiermit bestätige ich, dass die von Frau Ines Diepenbruck mit dem Titel "Consequences of antenatal corticosteroid treatment on the developing immune system" vorgelegte Doktorarbeit in korrektem Englisch geschrieben ist.

Mit freundlichen Grüßen,

Carol Stocking

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, den 10.02.2015

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Ines Diepenbruck