# Unraveling the potential of Human Chorionic Gonadotropin as an approach for the treatment of Multiple Sclerosis using a mouse model

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"The important thing is to not stop questioning.
Curiosity has its own reason for existing."

Albert Einstein

# Abstract

Multiple sclerosis (MS) is one of the most common neuroinflammatory diseases affecting young adults. Based on observations that MS patients recover during pregnancy, it was suggested that pregnancy hormones play a role in disease amelioration. Therefore, the influence of the pregnancy hormone human chorionic gonadotropin (hCG), known to possess multiple immunomodulatory functions, was investigated in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS. Female myelin oligodendrocyte glycoprotein (MOG)-immunized C57BL/6J mice were preventively and therapeutically treated with two different hCG preparations (recombinant hCG [rhCG] or urine-derived hCG [uhCG]). EAE disease severity and different immunological parameters in the CNS, inguinal lymph nodes, and spleen were analyzed during disease onset (day 10), the initial phase (day 20), and the progressive phase (day 35). Treatment with rhCG did not alter disease severity and was only able to reduce Th17 cell frequencies at the progressive EAE phase. uhCG treatment resulted in significantly less pronounced disease signs at day 13, 16, and 20. Markedly, uhCG-treated MOG-immunized animals showed approximately a 50 % lower survival rate when injection of uhCG was accompanied with EAE disease onset. During the progressive phase, a decrease in the pro-inflammatory Th17 cell population and a significant reduction in the B17 frequency was found within the CNS after uhCG treatment. However, lymphocyte populations were not altered after uhCG treatment at disease onset or the initial EAE phase. Although uhCG was able to reduce B17 cell frequencies *ex vivo*, it was suggested that the reduction of EAE signs at the initial disease phase is not mediated through alterations of lymphocytes during this phase *in vivo*. Taken together, uhCG containing various isoforms of hCG and having a purity of 70 %, but not rhCG

containing 99 % of recombinant hCG, was able to reduce EAE severity. This suggests that the reduced EAE severity may be due to other components within the uhCG preparation and not by hCG itself. Thus, it remains unclear which component in the uhCG preparation is able to diminish signs of EAE and which immune cell populations contribute to the diminished EAE severity observed by uhCG. For this, the role of the B17 cell population, which was significantly reduced in the CNS after uhCG administration, as well as other cell populations in the EAE mouse model need further investigation. Finally, further studies are needed to clarify which component or components of the uhCG preparation have resulted in a significantly reduced EAE score and may represent a new potential treatment option for MS.

# Zusammenfassung

Multiple Sklerose (MS) ist eine der häufigsten neuroinflammatorischen Erkrankungen bei jungen Erwachsenen. Basierend auf vorherigen Beobachtungen kann angenommen werden, dass bei MS Patienten eine Verbesserung der Krankheitssymptome während der Schwangerschaft einhergeht. Aus diesem Grund wird ein Einfluss von Schwangerschaftshormonen auf den Krankheitsverlauf während der Schwangerschaft vermutet. Daher wurde in dieser Studie der Einfluss des humanen Choriongonadotropins (hCG), welches immunomodulatorische Wirkungen besitzt, auf den Krankheitsverlauf im experimentellen Mausmodell für MS, der Autoimmunenzephalomyelitis (EAE), untersucht. Weibliche Myelin Oligodendrocyte Glycoprotein (MOG)-immunisierte C57BL/6J Mäuse wurden präventiv und therapeutisch mit zwei unterschiedlichen hCG Präparaten (rekombinantes hCG [rhCG] oder Urin-aufgereinigtes hCG [uhCG]) behandelt. Analysen wurden direkt zu Beginn der Erkrankung (Tag 10), während der Anfangsphase (Tag 20) und während der progressiven Phase (Tag 35) durchgeführt, wobei verschiedene immunologische Parameter im zentralen Nervensystem (ZNS), den inguinalen Lymphknoten und der Milz erhoben wurden. Die Behandlung mit rhCG veränderte den EAE-Krankheitsverlauf nicht und konnte die Th17-Zellhäufigkeit nur in der progressiven EAE-Phase (Tag 35) senken. Die Behandlung mit uhCG nach EAE Induktion führte hingegen zu signifikant weniger ausgeprägten Krankheitssymptomen an Tag 13, 16 und 20. Interessanterweise zeigten uhCG-behandelte MOG-immunisierte Tiere eine um etwa 50 % niedrigere Überlebensrate, wenn die Injektion von uhCG mit Eintreten der ersten Krankheitssymptome einherging. Während der progressiven Phase wurde nach uhCG Behandlung eine Abnahme der proinflammatorischen

Th17-Zellpopulation und eine signifikante Reduktion der B17-Zellpopulation im ZNS festgestellt. Die Lymphozytenpopulationen waren jedoch zu Beginn der Erkrankung und in der Anfangsphase nach uhCG-Behandlung nicht verändert. Obwohl uhCG in der Lage war *ex vivo* die B17-Zellfrequenzen zu reduzieren, kann vermutet werden, dass die Reduktion der Anzeichen einer EAE-Erkrankung in der anfänglichen EAE-Krankheitsphase nicht durch Veränderungen der Lymphozyten während dieser Phase *in vivo* vermittelt wird. Insgesamt konnte nur mit uhCG, bestehend aus verschiedenen hCG Isoformen und einer Reinheit von ungefähr 70 %, jedoch nicht mit rhCG, welches eine Reinheit von 99 % aufweist und die intakte Form von hCG enthält, eine Reduktion der EAE Symptome erreicht werden. Daher kann vermutet werden, dass der verbesserte EAE-Krankheitsverlauf durch andere Komponenten in dem uhCG-Präparat erreicht werden konnte und nicht durch das enthaltene hCG per se. Somit bleibt unklar, welche Komponenten in dem uhCG-Präparat in der Lage sind Anzeichen von EAE zu vermindern und welche Immunzellpopulationen zu den verminderten Anzeichen der EAE-Erkrankung beitragen, die durch uhCG hervorgerufen wurden. Daher muss die Rolle der B17-Zellpopulation, die nach der Verabreichung von uhCG im ZNS signifikant reduziert wurde, sowie anderer Immunzellpopulationen nach uhCG-Behandlung im EAE-Mausmodell weiter untersucht werden. Schließlich sind weitere Studien erforderlich, um zu klären, welche Komponente oder Komponenten der uhCG Präparation zu einem signifikant verringert ausgeprägten Krankheitssymptomen geführt haben und möglicherweise eine neue potenzielle Behandlungsoption für MS darstellen.

# Contents

# 1. Introduction

## 1.1 Autoimmune diseases

Over the last years, the incidence of autoimmune disorders has risen by approximately 19.1 % per year with a prevalence of approximately 12.5 % making autoimmune disorders one of the leading causes of death in women [1]. Additionally, the number of patients suffering from autoimmune diseases increases steadily [1]. For example, about 3.2 of 100,000 people are diagnosed with Multiple sclerosis (MS) per year affecting approximately 64 % of women in the population diagnosed with MS [2–4]. Diagnosing autoimmune disorders in patients is a complex procedure and can be difficult to do due to the broad spectrum of clinical features. The time passing from the beginning of the autoimmune response until the appearance of first symptoms can vary between days, months, or years [5]. Therefore, not only the health care system will be challenged in the future, but also the understanding of the development of autoimmune disorders and successful treatment options are detrimental.

Autoimmunity occurs when the immune system mistakenly attacks host cells and tissues. Consequences of this failure of self-tolerance can lead to autoimmune disorders caused by autoantigens or autoantibodies [6, 7]. To avoid autoimmune reactions, different mechanisms are described to maintain self-tolerance within the body. On the one hand, naïve lymphocytes strongly react to self-antigens, causing

apoptosis or inactivation of the cell, named anergy [8, 9]. On the other hand, self-antigens are constitutively expressed in the body tissue and this can lead to tolerance of self-antigens [8, 9]. The immune system regulates self-tolerance by triggering an adaptive immune response comprised of the antigen-specific lymphocytes: T and B cells [10]. These cells are activated when they are presented with a specific antigen and different co-stimulatory molecules (human leukocyte antigen [HLA]-DR, cluster of differentiation [CD] 80, CD86). The generation of lymphocytes in primary lymphoid organs is one of the main regulation points for self-tolerance and the activation of autoreactive lymphocytes. With regard to T cells, T cells mature in the primary lymphoid organs where they can bind self peptides and therefore become autoreactive [10]. Usually these autoreactive T cells are eliminated, which is called negative selection. Additionally, regulatory T (Treg) cells suppress autoreactive T cells that could have escaped negative selection. However, in autoimmune states, T cells within the thymus recognize self-peptides, mature and become autoreactive, disrupting the negative selection process [8, 10, 11]. Similarly, dysfunctional Treg cells are associated with autoimmunity. T cells can then migrate from primary lymphoid organs and spread to different tissues [8, 10, 11]. These autoreactive T cells can lead to tissue specific or systemic inflammation, causing an imbalance of T effector cells and Treg cells (Figure 1.1) [10, 12]. Examples of tissue specific autoimmune diseases are MS affecting the CNS, diabetes mellitus type 1 affecting the pancreas or Grave's disease affecting the thyroid [13, 14]. Systemic autoimmune diseases, like rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), are characterized by a chronic inflammatory immune response and show a progressive clinical course as well as increased mortality [14]. However, not every autoreactive T cell automatically leads to an autoimmune disease, because autoantigens need to be presented to both T and B cells for their survival and therefore autoimmune reactions are necessary in low doses [12]. These autoreactive T cells can be modulated by Treg cells in the periphery through inhibition [10, 12]. Despite, naïve T cells with low-binding affinity to self-antigens can still escape the self-tolerance mechanisms and lead to an autoimmune reaction [10].

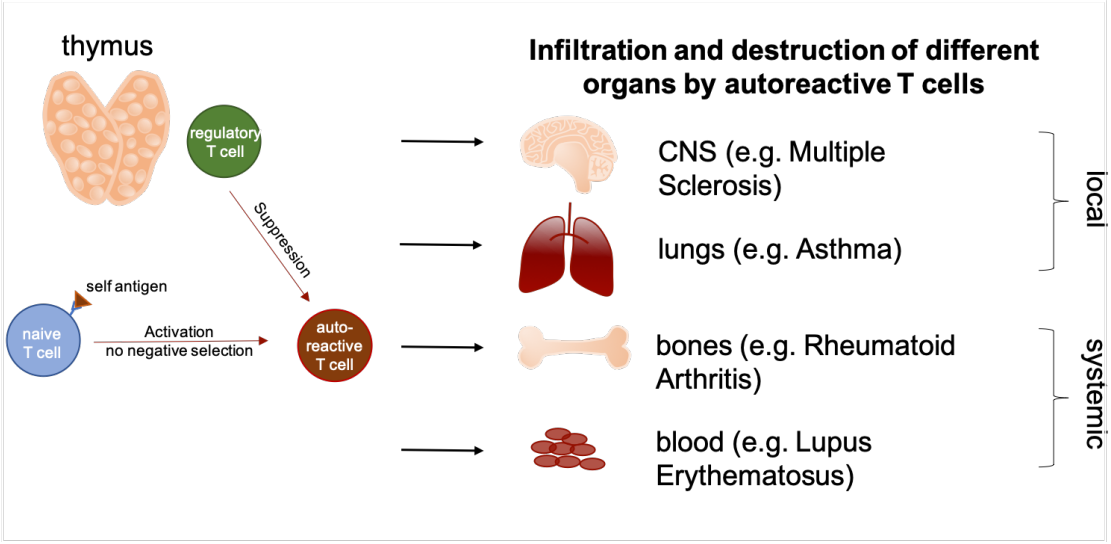


**Figure 1.1: Development of autoimmunity and autoimmune diseases.**
The development of autoimmunity leading to tissue-specific or systemic autoimmune disorders. Within the thymus, T cells are binding self peptides and are not eliminated through the negative selection process. Therefore, autoreactive T cells can escape into the periphery and attack different tissues causing autoimmunity. Figure modified according to Johnsen et al., 2016 [13].

Gender specificity plays a critical role in autoimmune disorders as woman have a stronger immune reactivity compared to men with women being more likely to develop autoimmune diseases [15, 16]. Regarding immune cell responses, there are gender specific alternations of T cell and antibody responses [17]. As an example, female MS patients exhibit increased interferon $\gamma$ (IFN$\gamma$) cytokine levels in response to the proteolipid protein peptide and enhanced T cell reactivity compared to men [17, 18]. Furthermore, sex hormones contribute to the development of autoimmune disorders [19, 20]. Lymphocytes express receptors for different sex hormones like progesterone, androgens, human chorionic gonadotropin (hCG), and estrogens [21–23]. Progesterone, androgens, and hCG obtain immunosuppressive functions, whereas estrogen can activate the immune system due to the estrogen receptor present on activated T cells [24–26]. Bird and colleagues studied the effect of androgenic steroid 19-nortestos-terone on postmenopausal RA patients, which led to an improvement of chronic anaemia [27]. This shows that gender specificity has a strong influence on autoimmune disease development. However, this field of study requires further investigations.

Also, the gene *autoimmune Regulator* (AIRE) has been found to play an important role in the regulation of autoimmunity [28–30]. *AIRE* leads to an expression of

different tissue-specific peptides, which are presented on lymphocytes, being usually negatively selected [28, 29]. The disruption of the *AIRE* gene results in a disturbed negative selection process and autoreactive cells can escape the thymus causing autoimmune polyglandular syndrome 1 [28, 29].

Other factors also contribute to autoimmunity such as genetic variations (cytotoxic T-lymphocyte-associated protein (CTLA)-4 [disruption of the anergy mechanism], HLA-DR, forkhead-box-protein P3 (FoxP3) [reduced Treg function], FAS [disrupted apoptosis of autoreactive cells]), altered major histocompatibility complex (MHC)/peptide recognition, viral or bacterial infections, environmental factors (measle infection, smoking), or endocrine disruptors (dioxins, phthalates or polychlorinated biphenyls) [31–33].

Nonetheless, due to the complex clinical picture of various autoimmune diseases, the main factors which directly contribute to disease pathology are still unknown [34–36].

## 1.2 Multiple sclerosis

MS is one of the most common neuroinflammatory diseases affecting mainly young people between the age of 20 - 40 years, showing a 2:1 women to men ratio [37]. The first descriptions of the disease go back to the $14^{th}$ century, where the progression of MS was described over the course of 24 years in the diary of Augustus Frederick d'Este (1794-1848) [38]. William MacKenzie (1791–1886) reported the first medical description, describing a 23-year-old man having visual problems and paralysis of the limbs with a subsequent appearance of urinary incontinence [39]. Pathologic evidence of the clinical symptoms was reported by Jean-Martin Charcot in 1868. He described the presence of lesions and atrophy within the CNS and named the disease "la sclérose en plaques disséminées" [40].

MS represents a complex immune reaction effectively leading to neuroinflammation in the CNS. Different immune cell populations like autoreactive T cells, B cells, and macrophages contribute to the disease progression (chapter 1.2.4). The triggering factor is still unknown and many different factors have been suggested for disease

development and progression. For MS diagnosis, the cerebral spinal fluid (CSF) is tested for the presence of oligoclonal bands of predominantly immunoglobulin (Ig) G, but also IgM [41]. Nonetheless, the factors contributing to the development of the disease as well as the symptoms, ranging from visual problems, dizziness, fatigue to muscle stiffness are broad making the disease hard to identify and to treat.

### 1.2.1 Clinical course and forms of the disease

In general, MS is characterized by intermittent episodes of neurological dysfunction referred to as relapses. In the first stage of the disease peripheral immune cells attack the CNS leading to episodes of acute inflammation (relapses). During the later phase of the disease, an inflammatory immune response is present within the CNS leading to demyelination causing nerve dysfunction resulting in progressive disability of the patient [42].

The first recognized symptoms are mostly visual problems which are caused by inflammation of the optic nerve as well as sensory symptoms like tingling, numbness, or loss of sensation [43]. Throughout disease progression, other symptoms become more pronounced like clumsiness, dizziness, spasticity, fatigue, mood changes like depression or euphoria, concentration disabilities, urinary incontinence, and muscle stiffness. Over time, MS slowly affects a patients daily routine and ultimately reaches a stage where an autonomous life is not possible [44, 45]. Depending on the progression of MS, patients will become dependent on continuous care. About two-third of MS patients die due to MS symptoms or infections approximately 30 years after disease onset.

The course of MS can vary for every patient depending on the form of MS diagnosed. Four different forms are described in the literature: relapsing-remitting, primary progressive, secondary progressive, and progressive-relapsing (Figure 1.2) [46, 47]. The relapsing-remitting form is the most common form and is characterized by clearly defined relapses followed by partial or complete recovery periods affecting about 85 % of MS patients [48–50]. About 65 % of these patients show a secondary progressive form later in life. The primary progressive form shows

a steady worsening from the beginning of disease onset affecting about 10 % of all patients [46, 47]. In contrast, the secondary progressive type shows a more steady progression and can transform into the progressive-relapsing form [51, 52]. This progressive-relapsing type of MS is characterized by a steady disease progression from disease onset including occasional exacerbation of symptoms (Figure 1.2) [46, 53].

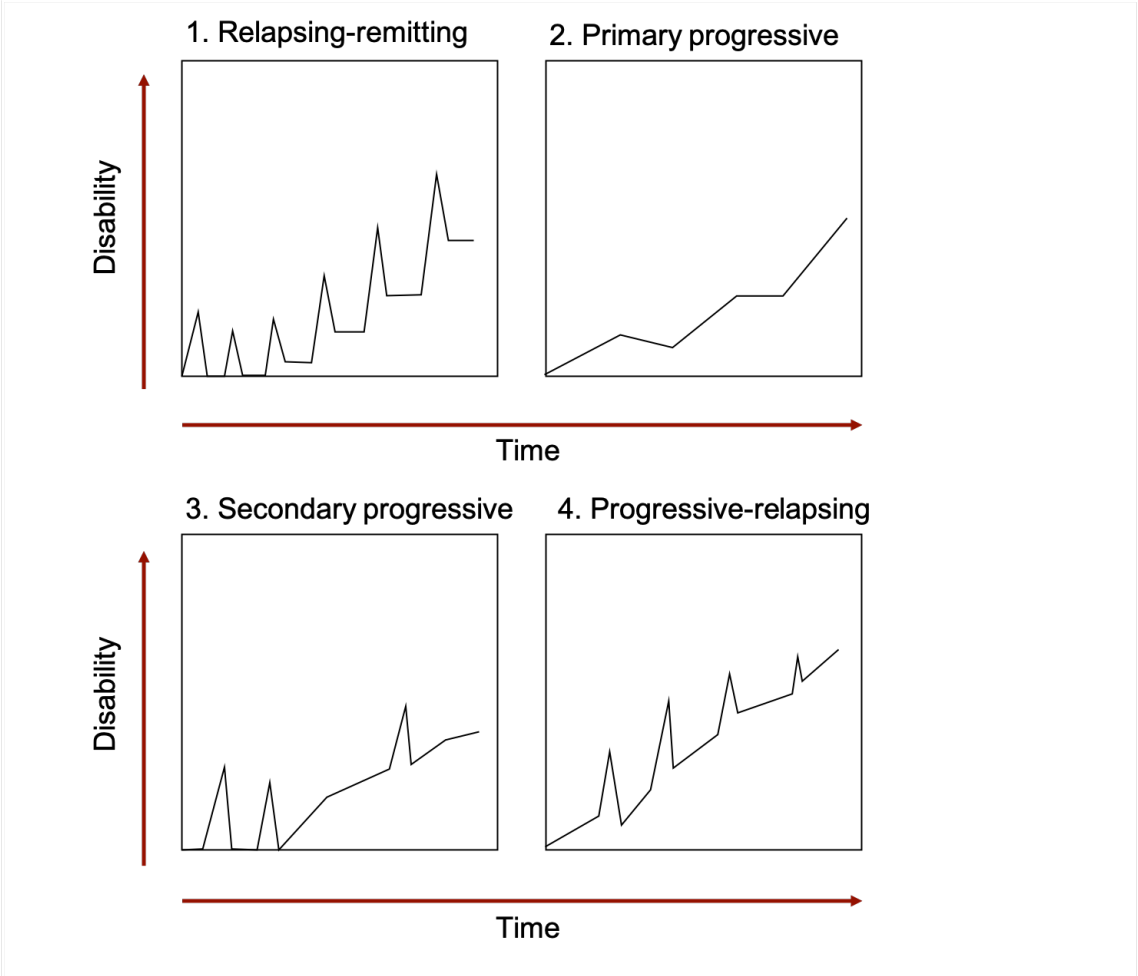


**Figure 1.2: MS is defined by four different types of disease progression.**
1.) The relapsing-remitting form is characterized by relapses with recovery periods, but worsening of disease symptoms long term. 2.) The primary progressive type shows a progressive course and continuous worsening of the disease. 3.) The relapsing-remitting form can convert into the secondary progressive form and is characterized by relapses at the beginning and ongoing worsening of the symptoms later on. 4.) The fourth type is the progressive-relapsing form having relapses but disease symptoms will continuously worsen over time. Figure modified according to Jacobs, 2016 [46, 47].

### 1.2.2 Epidemiology and risk factors

MS is often stated as a disease of the northern countries due to its higher prevalence in this region. Many patients live in North America and Europe, with MS being the most common chronic infection [54]. Worldwide there are about 2.5 million people diagnosed with MS. In 2010, about 120,000 to 140,000 people were affected in Germany [55]. Due to the higher prevalence in northern countries, MS is associated with lower sunlight exposure and thus lower vitamin D levels [56, 57]. Other indicated factors are genetic variations of the HLA gene complex. Additionally, previous infections, like the measles virus, human herpes virus-6 (HHV-6), or the Epstein-Barr virus (EBV) are associated with disease outbreak [56–60]. Other factors like smoking, stress, and vaccines can also increase susceptibility of disease outbreak and progression [59, 61].

### 1.2.3 Histopathology

Disease manifestations of MS are acute demyelinating white matter lesions and the infiltration of various immune cells (B cells, T cells, macrophages, dendritic cells, and monocytes) [62, 63]. During secondary degeneration, gray matter lesions appear within the CNS where neuronal and axonal damage takes place [44, 64]. More specifically, the brain stem, cerebellum, spinal cord, optic nerve, brain ventricles, and also white matter is affected by the disease. These lesions can be identified using functional magnetic resonance imaging (fMRI) scans. In MS patients, nerve cell bodies are typically damaged and 30 to 80 % of axons show demyelination and cellular damage within these lesions [44, 64]. Patients exhibit a disrupted blood-brain barrier (BBB) as well as cortical atrophy. As mentioned earlier, within the CSF oligoclonal bands of IgG and IgM and inflammatory markers (chemokine [C-X-C motif] ligand 13 [CXCL-13], IFN$\gamma$, fetuin-A and osteopontin) can be detected in MS patients [41, 65–67].

### 1.2.4 Inflammatory immune response

The inflammatory process mimicking MS pathology has been widely studied using the experimental autoimmune encephalomyelitis (EAE) model. The CNS is an immunoprivileged organ. These organs are usually protected from the periphery by functional and intact barriers, such as the blood-brain-barrier (BBB). However, different cells as well as molecules can pass the BBB to an extent. During an autoimmune reaction in MS the BBB breaks down and autoreactive myelin-specific T and B cells, as well as macrophages enter the CNS [68]. These autoreactive T cells express integrins such as the very late antigen-4 (VLA-4), which can bind to the vascular cell adhesion molecule (VCAM) expressed by the endothelial cells of the BBB, resulting in an increase of tumor necrosis factor (TNF)$\alpha$ and IFN$\gamma$ [69]. Additionally, the activation of the choroid plexus by IFN$\gamma$ leading to a higher infiltration of immune cells in the CNS has been emphasized as well as an interaction of Th17 cells with the choroid plexus by the chemokine (C-C motif) ligand (CCL)20 and the chemokine receptor (CCR)6 during neuroinflammation [70–72].

After CNS infiltration, autoreactive $CD4^+$ T cells are activated by antigen-presenting cells (APCs) presenting myelin-specific antigens on the MHC complexes. Furthermore, additional T cells, B cells, and macrophages are recruited from the periphery further driving an inflammatory immune response in the CNS [68]. $CD4^+$ and $CD8^+$ T cells target different proteins of the myelin sheath as well as protein structures of oligodendrocytes leading to apoptosis and activation of microglia and plasma cells [68]. During this pro-inflammatory process, different cytokines such as the granulocyte colony-stimulating factor (GM-CSF), interleukin (IL)-17, TNF$\alpha$, IFN$\gamma$, reactive oxygen species (ROS) and nitric oxide synthase (NOS) are secreted [73–76]. This leads to a destruction of the myelin sheath of neurons and oligodendrocytes, axonal damage, neurological dysfunction, and lesions within the CNS [77–79]. Moreover, during neuroinflammation B cells produce autoantibodies causing antibody-dependent cell-mediated cytotoxicity, release of inflammatory cytokines by other leukocytes (T cells, macrophages), phagocytosis of macrophages, and complement activation (Figure 1.3) [80, 81].

This neuroinflammatory immune response shows increased T helper (Th) 1 and Th17 cell responses, which are thought to be the key players. Also, B cells strongly contribute to MS disease progression leading to clinical worsening of symptoms in MS patients [68, 82, 83]. Nonetheless, partial remyelination by oligodendrocytes takes place, resulting in the recovery between relapses in some forms of MS [84]. However, the initial step that leads to disease outbreak still needs to be unraveled.

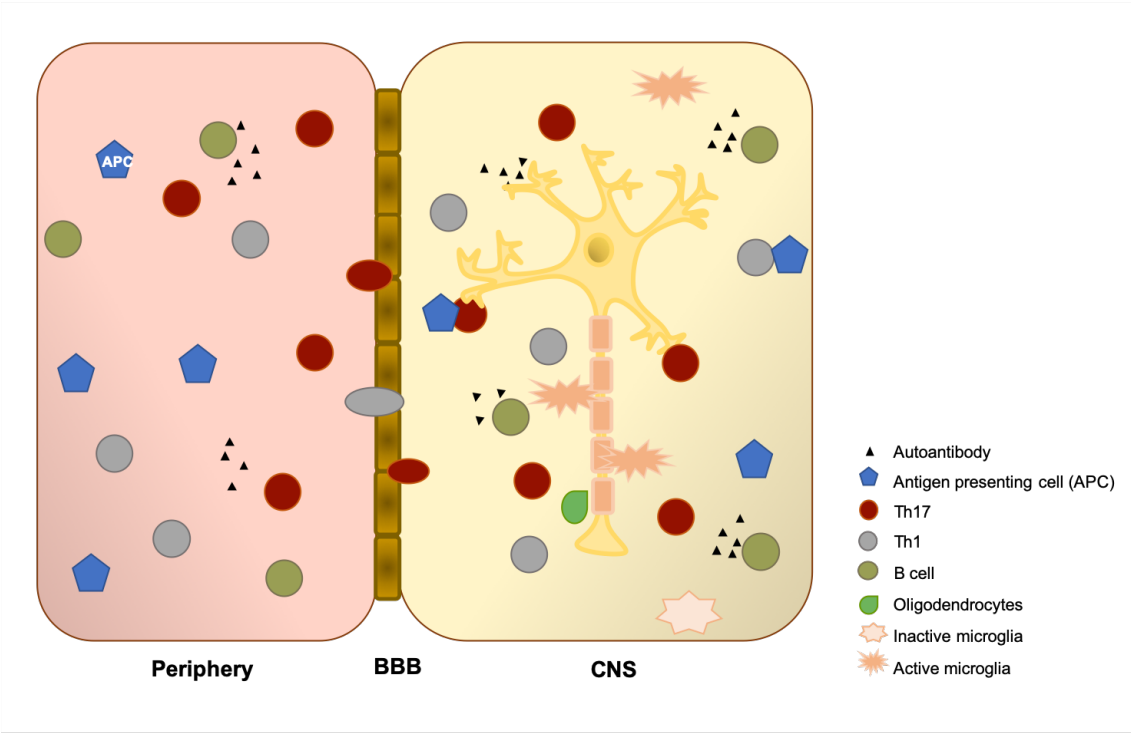


**Figure 1.3: Hypothetical schema of an immune response in MS.**
The complex immune reaction of MS involves different cell subsets driving disease progression. Autoreactive T and B cells enter the CNS promoting an inflammatory immune response mainly driven by Th1 and Th17 cells targeting the myelin sheaths of neurons. This leads to the disruption of the myelin sheaths and of the neuron itself promoting first symptoms of the disease and its progression. Figure modified according to Hemmer et al., 2002 [85].

#### 1.2.4.1 T cells as regulators of neuroinflammation

Belonging to the adaptive immune system, T cells are lymphocytes which originate from the bone marrow and mature in the thymus [86]. Within the thymus, T cells are presented with different antigens, including self antigens. In this process, usually autoreactive T cells cannot escape the selection process and leave the thymus. In secondary lymphoid organs, these naïve T cells will be exposed to antigens presented

by APCs. They will then develop into central memory T cells and further into effector memory T cells and effector T cells. These T cells can be subdivided into $CD4^+$ T helper (Th) cells, $CD8^+$ cytotoxic T cells, and Treg cells [86].

As mentioned earlier (Chapter 1.1) during the development of T cells, different checkpoints have to be passed to ensure successful T cell development (negative selection within the thymus, anergy, or deletion of T cells, induction of tolerance). In MS, these checkpoints are somehow disrupted and lead to a neuroinflammatory state within the CNS promoted by autoreactive T (Th1, Th17) and B cells playing a prominent role [83]. Usually, Th17 cells induce a pro-inflammatory immune response to protect the host from extracellular pathogens by secreting pro-inflammatory cytokines such as IL-17A, IL-17F, IL-21, IL-22, and IL-26, recruiting B cells, neutrophils and other innate immune cells [86]. Th17 cells can be generated through the secretion of IL-1$\beta$, IL-6, transforming growth factor (TGF)$\beta$, IL-21, and IL-23 by other cells. IL-23 is able to stabilize Th17 cells and IL-6 inhibits cells with immunosuppressive capacities (Treg cells) [87, 88]. In MS however, autoreactive Th17 cells attack the myelin sheaths of neurons within the CNS and the Th17 immune response is exaggerated during disease progression [83]. Not only MS, but also RA and SLE are associated with a similar exaggerated Th17 elevation [89, 90].

Th1 cells are important for the progression of MS [91, 92]. Th1 cells produce IFN$\gamma$ and TNF$\alpha$ inducing inflammation. However, IFN$\gamma$ knockout mice suffer from a more severe disease progression in the experimental autoimmune encephalomyelitis (EAE) model leading to the assumption that even though IFN$\gamma$ is a pro-inflammatory cytokine, it may have anti-inflammatory properties or that some degree of inflammation is still needed for combating EAE [93]. A specific Th cell subset, called Th9, is able to produce IL-9 and IL-10 [94]. The cytokine IL-9 has been investigated regarding neuroinflammation conversely driving Th17 development, but also inducing suppressive functions of Treg cells [94]. Adoptive transfer of Th9 cells led to EAE development as well as enhanced EAE disease progression using IL9R deficient mice [94, 95]. This highlights that other cell subsets can strongly contribute to disease progression and that rather a cell network and not isolated cell subsets are responsible for the outcome.

Contrarily, Treg cells are important counter-regulators of inflammation being $CD4^+$ T cells that express CD25, low levels of CD127, and the transcription factor FoxP3 [87, 96]. They can be induced by TGFβ and IL-2 and induce tolerance through IL-10, IL-35, and TGFβ production. The role of Treg cells in the maintenance of self-tolerance was first described in the study investigating cardiac allograft transplants in rats [97]. Also, Treg cells play a pivotal role in pregnancy maintenance and reportedly counter-regulate Th17 cells. The suppression of immune responses can be modified through production of anti-inflammatory cytokines such as IL-10, induced apoptosis of target cells, modification of maturation and function of APCs, or metabolic disruption (Figure 1.4) [98].

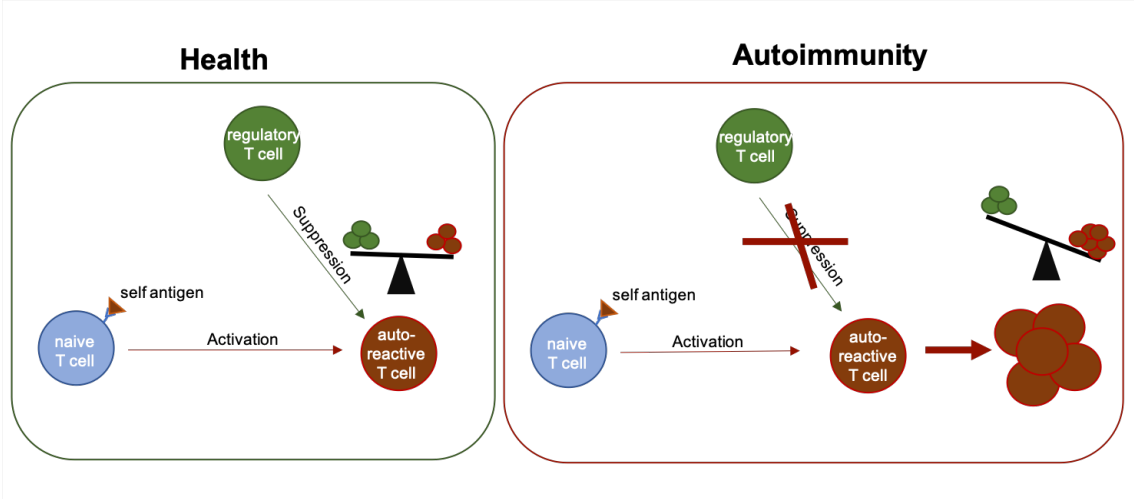


**Figure 1.4: Hypothetical presence of a Th17/Treg imbalance in autoimmunity.**
In healthy individuals autoreactive T cells are suppressed to avoid autoimmune reactions. During autoimmunity this process is disturbed due to impaired Treg cell functioning causing autoimmunity. Figure modified according to Grant et al., 2015 [99].

Studies suggest that there is a disrupted balance of Th17 and Treg cells in several autoimmune disorders like RA, acute coronary syndrome, but also in other immunological disturbed processes like recurrent fetal loss [100, 101]. An impaired suppressive Treg function was also found in the blood of MS patients [102]. However, relapsing-remitting MS patients showed impaired naïve and memory Treg function during the early disease stage, but during the chronic disease stage no differences were found compared to healthy controls [103]. Vigletta and colleagues reported that in MS patients Treg cell frequencies remain unchanged, but these cells possess an impaired functionality [104, 105]. Furthermore, it was shown that Treg

cell transfer partially protects from EAE by IL-10 production and the depletion of Treg cells leads to worsening of the disease [106, 107].

#### 1.2.4.2 B cells and neuroinflammation

Together with T cells, B cells belong to the adaptive immune system and are the only immune cell population able to present antigens and to produce antibodies and cytokines. They originate from hematopoietic stem cells of the bone marrow (B2 cells) or the fetal liver (B1 cells) and modulate the humoral immune response (Figure 1.5). General B cell markers are CD19, CD45R (human), or B220 (murine) [86]. Mature B cells produce IgM and IgD and are called naïve B cells remaining in peripheral lymphatic organs until further activation through their specific antigen. Usually, when an autoantigen is present, an immature B cell can react in four different ways. If there is a multivalent autoantigen, then the cell undergoes apoptosis. If there is a soluble autoantigen, the B cell may undergo anergy. In case there is a non cross-linked autoantigen with low affinity or no reaction against self-determinants, the B cell matures [86]. Once they are activated by antigens, they can differentiate into plasmablast and plasma cells or memory B cells [86]. They further differentiate into effector B cells producing antibodies. These antibodies are specific with a unique antigen-binding site to induce an antigen specific immune reaction [86]. These effector B cells can be divided into type 1 (TNF, IFN$\gamma$, IL-12 producer) and type 2 (IL-2, IL-4, IL-6, TNF producer) B cells, both releasing pro-inflammatory cytokines.

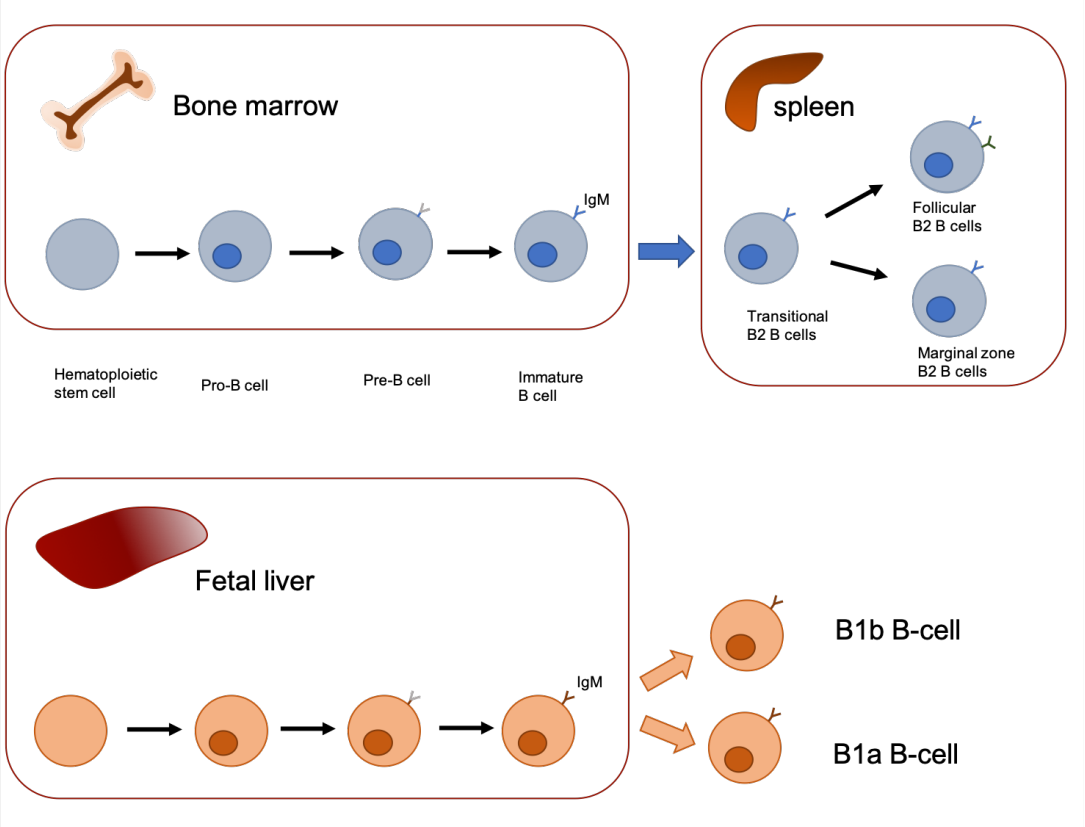


**Figure 1.5: B cell development.**
B cells can either originate from the bone marrow (B2 cells) or from the fetal liver (B1 cells). B2 cells further mature in the spleen and B1 cells in the periphery. Figure modified according to Montecino et al., 2012 [108].

A role of B cells in autoimmune reactions has been emphasized in the last decades. Effector B cells and impaired Breg cell function were shown to have detrimental functions in MS progression [109]. It has been found that specific factors like IL-4 contribute to disease progression by rescuing autoreactive B cells and increasing their survival [110]. However, IL-4 has also been shown to reduce the clinical score in MS mouse models without affecting the neuroinflammatory response [111, 112]. The dysregulation of B cells can fundamentally influence various autoimmune disorders regarding disease development and progression, showing that not only T cells can mediate autoimmunity, but also B cells strongly contribute. Interestingly, different autoimmune disorders like RA and SLE show a disruption in B cell tolerance due to autoreactive B cells [113]. It is already known that human genetic variants that contribute to the systemic autoimmune response modulate B cell receptors as well as other receptors to generate dysfunctional B cells [114]. Autoreactive B cells convert into autoreactive plasma cells producing autoantibodies and these cells activate Th

cells leading to inflammation. Hence, tolerogenic mechanisms seem to be disrupted. Christensen and colleagues focused on toll-like receptor (TLR)7 and TLR9, which are important for autoantibody specificity. TLR7 showed a protective effect in a lupus mouse model, whereas the deletion of TLR9 led to an exacerbation of the disease [115]. In MS, the dysregulation of B cell tolerance is induced by the Th1/Th17/Treg dysbalance [104, 116]. Additionally, even though autoantibodies are necessary for disease progression, an antibody independency of MS disease progression was highlighted by using soluble factors from B cell cultures from MS patients not containing IgM or IgG [117]. The soluble factors in the supernatant were still toxic for neurons and oligodendrocytes *in vitro* [117]. Contrarily, the programmed cell death ligand 1 (PD-L1) expression on B cells also leads to protection against EAE [118].

B cells are proposed to play a more significant role in MS progression than initially thought. New treatments using CD19 and CD20 antibodies in MS patients revealed promising results and paved the way for new therapeutic strategies targeting B cells in MS [119]. Notably, the depletion of both T and B cells by targeting CD52 showed a stronger exacerbation of the disease. This was interpreted as relevant, because B cells recover faster than T cells which suggests a significant contribution of B cells for disease progression [120]. However, this needs to be further investigated.

With MS being a primarily IL-17 driven autoimmune disease and the role of B cells in disease progression being under investigation, a possible connection between B cells and IL-17 may arise. B cells have been shown to produce IL-17 (B17) [121]. This specific B cell subset is not well studied and has not been investigated in MS until now. A study in RA patients highlights that this distinct B17 cell population may also drive disease progression due to its IL-17 production [122]. Bermejo and colleagues studied B17 cells in an infection model with *Trypanosoma cruzi*. They identified a $CD19^{+}$ cell population, apparently plasmablasts, that produces IL-17. Interestingly, these B cells produced more IL-17 than T cells in the initial phase of infection (day 10 and day 19) contributing to the establishment of inflammation [123]. Nevertheless, in this specific infection model, IL-17 also possesses protective functions because of its capacity to recruit neutrophils producing

IL-10 [123]. This study implies that other IL-17 producing cells besides Th17 cells may be involved in MS pathology. It can be hypothesized that B17 cells may promote disease progression during disease onset and Th17 cells may be an important disease enhancer at later stages of the disease. Thus, studies investigating B17 cells are urgently needed to elucidate their participation, particularly in the context of autoimmunity and infections.

Breg cells represent another important counter-regulator suppressing inflammation. Their induction takes place in two different ways. Tolerogenic cells are either directly induced within the bone marrow which is highly TLR regulated, or in the lymphoid organs, where co-stimulatory molecules like CD40/CD40L and MHC, and also Treg cells are mandatory [124, 125]. IL-10 inhibition in mice developing EAE exhibit a more severe disease progression and transferring of IL-10-producing B cells showed a suppression of EAE disease signs [126]. Moreover, IL-35 induces IL-10-producing B cells and is also known to decrease inflammation [127].

## 1.3 Multiple sclerosis mouse models

Mouse model organisms are an important tool for the investigation of clinical disease progression and treatment options. Not only because the mouse genome shows a 85 % homology to the human genome, but mice are also a good model organism to study a variety of diseases, as husbandry and breeding is economic and transgenic mouse models are relatively easy to produce [128]. Many different mouse models are available and each mouse strain utilizes specific advantages for individual questioning and investigations.

Until now, none of the published mouse models is able to mimic the broad spectrum of the MS pathology completely. However, many researchers take advantage of inducing EAE in different animal models to study CNS demyelinating and immunological pathways underlying MS pathology [129, 130]. Many different EAE induction protocols are available. Commonly an injection of the myelin oligodendrocyte glycoprotein peptide (MOG35-59, 92-106), proteolipid protein (PLP139-151, 178-191), or myelin basic protein (MBP84-104) in complete Freud's

adjuvant containing heat-inactivated *Mycobacterium tuberculosis* and injections of pertussis toxin are applied (Figure 1.6) [131, 132].

EAE is characterized by demyelination, inflammation, axonal loss, and gliosis combined with recovery mechanisms such as remyelination and reduction of inflammation [129, 130]. There are specific rodent strains or induction models for studying different mechanisms of MS pathology. For instance, when checking for demylination by T cells and macrophages, a chronic EAE model can be used by inducing EAE in C57BL/6 mice using the MOG peptide (MOG35-55) [133]. Other models are more suitable for the investigation of different mechanisms in disease progression, like inflammation by $CD4^+$ T cells to study inflammation-induced hypoxia-like tissue injury [134, 135]. There are two different forms of EAE, active EAE (induced by MBP, MOG, PLP) and passive EAE (induced through the injection of lymphocytes isolated from animals showing active EAE signs), which also varies with the animal strain used [136, 137]. Using a model to study demyelination evoked by T cells, C57BL/6 mice induced with the MOG peptide show a chronic progressive course of EAE with manifestations of demyelination and axonal damage [138]. However, there is a high variability in disease incidence and course. Different results from different laboratories suggest that both microbiome and husbandary are relevant as well [139]. These animals often show cytotoxic demyelination and further administration of pertussis toxin is needed [140].

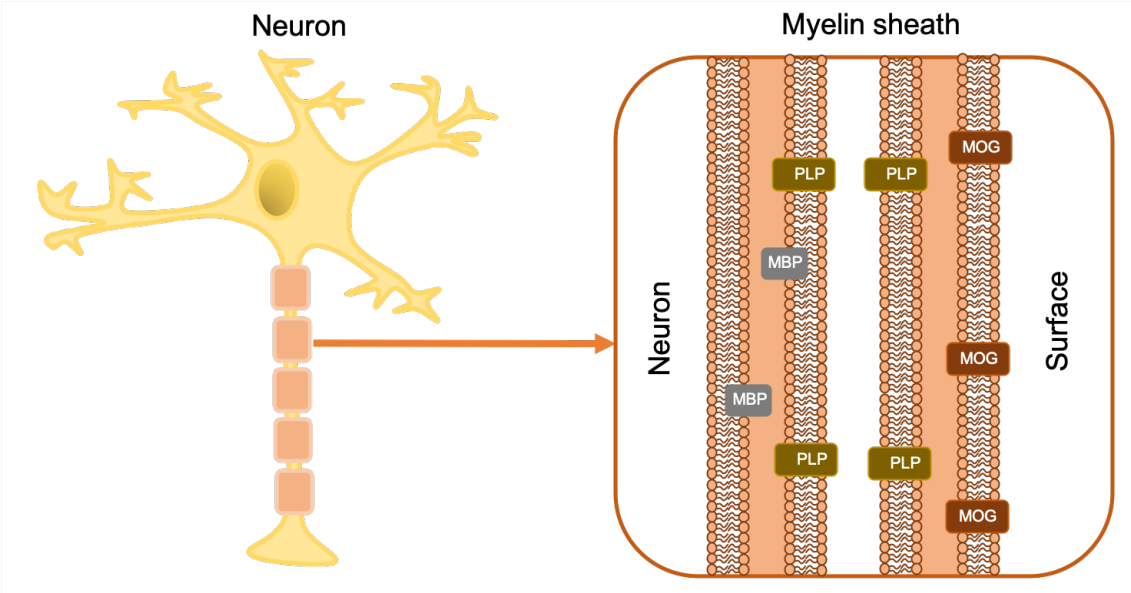


**Figure 1.6: Protein composition of the myelin sheath presenting targets for EAE induction.**
The myelin sheath composed of the different proteins used for successful EAE induction is shown. Each target protein is used to study individual approaches in the EAE animal model to study MS progression. Figure modified according to Hemmer et al., 2002 [85].

## 1.4 Autoimmune diseases and pregnancy

There is a strong interrelation between autoimmunity and pregnancy in both directions. Many women diagnosed with an autoimmune disorder in their reproductive age have the wish to become pregnant even though the effects and consequences for the baby and the mother are uncertain. Other women are diagnosed with an autoimmune disorder once pregnant and often because they suffered a miscarriage. This is quite frequent for the autoimmune polyglandular syndrome [141, 142]. Until recently, physicians often advised women with autoimmune diseases to avoid pregnancy. However, currently with appropriate medical advice and follow up pregnancies are possible [143].

Autoimmunity affects pregnancy establishment, development, maintenance, and outcome, and pregnancy itself also has an effect on several autoimmune disorders. Patients diagnosed with MS or SLE often suffer from infertility or recurrent pregnancy loss, while other autoimmune disorders are attenuated with a milder disease progression during pregnancy [144–146]. On the cellular level, an increased Th1 and Th17 response can be observed in MS, whereas pregnancy is largely associated with a more pronounced Th2 response [82]. Successful pregnancy requires

the induction of Treg cells and adequate regulation of Th17 cells [82, 147, 148]. Having these different immune modulatory mechanisms of autoimmunity and pregnancy in mind, there is no doubt that pregnancy-driven immune modulations will interfere with the immune responses present in patients suffering from autoimmunity.

Depending on the type of autoimmune disease, a shift towards a Th2 immune response during pregnancy can lead to a remission of the disease which has been reported for Grave's disease, MS, and RA [149–151]. It should be noted, Grave's disease and MS have increased flare rates after pregnancy [149, 150, 152]. Alternatively, studies investigating SLE and pregnancy have reported that there are increased flares during pregnancy due to its Th2 mediated immune response. These flares increase as pregnancy progresses [153, 154]. Using a mouse model to study the influence of pregnancy on SLE, an absence of skin lesions and increased IL-10 and IFN$\gamma$ expression levels was observed [155, 156]. Also, increased nephritis and no suppressive effect from Treg cells was present [155, 156]. Multiple studies have revealed that estrogens, progesterone as well as testosterone are capable of modulating autoimmunity, resulting in declined relapse rates and also contributing to repair mechanisms in the CNS [157–159]. Moreover, males diagnosed with MS display often a more rapid disease course leading to disability compared to women due to low estrogen and progesterone levels, indicating that these hormones may have an important influence in males [160].

### 1.4.1 Pregnancy hormones

Hormones are produced by endocrine glands and are important mediators of information needing small amounts for action [161]. They can function far from their origin binding to specific receptors, but can also act as autocrine mediators [161]. During pregnancy, estrogens, progesterone, and human chorionic gonadotropin (hCG) play a pivotal role to maintain pregnancy [162]. During gestation, hormone levels change in secretion, concentrations and metabolism (Figure 1.7) [163]. Progesterone is mainly produced by the *corpus luteum* in the second half of the

menstrual cycle and in large amounts throughout pregnancy increasing towards the end of pregnancy [164]. However, during pregnancy the production of progesterone declines in the first trimester and the trophoblasts of the placenta overtake the production [164].

Estrogens are produced by the follicles of the ovaries during the menstrual cycle and by the synthiotrophoblasts of the placenta during pregnancy where it also increases strongly throughout pregnancy [165]. Estrogen is also produced in testicles, the adrenal glands, fat, liver, the breasts, and the brain. Small amounts of progesterone and estrogens are also produced by the adrenal cortex in men and women throughout life [165]. Estrogens support myometrial growth, promote the uterine blood flow, and stimulate breast growth [166].

hCG hormone levels peak within the first trimester, being produced by the syncytiotrophoblasts of the placenta stimulating the *corpus luteum* for progesterone production. Furthermore, hCG supports pregnancy by facilitation of trophoblast invasion right away after successful conception [167–169]. Specifically, regular hCG is produced by syncytiotrophoblasts and villous cytotrophoblasts to modulate the immune response and to support immunomodulation, angiogenesis, and trophoblast invasion. Hyperglycosylated hCG is produced by extravillous cytotrophoblasts (EVCTs) and syncytiotrophoblasts promoting EVCT and trophoblast invasion, angiogenesis, and supporting immunomodulation [170, 171].

Progesterone is necessary for successful decidualization and together with estrogens prepares the uterus for implantation [172]. hCG facilitates trophoblast invasion, angiogenesis, and the nourishment of the fetus [173, 174]. Pregnancy hormones are able to modulate different immune cell subsets such as T cells, B cells, and dendritic cells to allow fetal tolerance induction and are therefore critically involved in normal pregnancy progression [175–177].

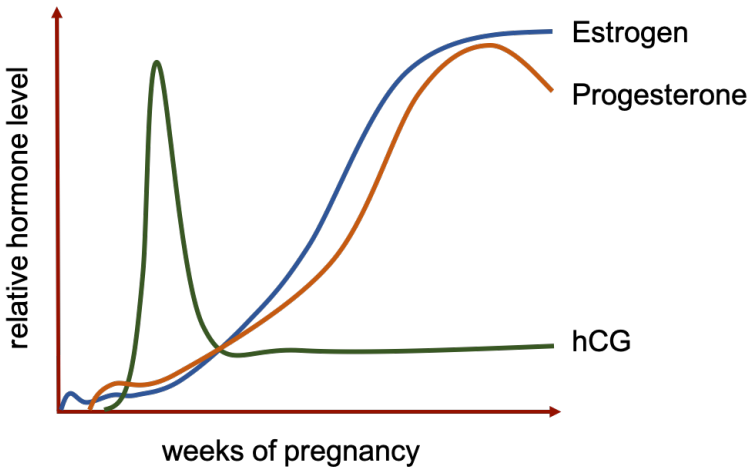


**Figure 1.7: Relative hormone levels of estrogens, progesterone, and hCG during pregnancy.**
The relative hormone levels of estrogens, progesterone, and hCG throughout pregnancy are shown. Estrogen levels increase throughout pregnancy as well as progesterone levels. However, right before labor sets in, the progesterone levels drop. hCG levels peak during the first trimester and maintains in a steady-state throughout the pregnancy. Figure modified according to Lu et al., 2018 [163].

Pregnancy hormones have also been found to be involved in the regulation of autoimmune diseases and several studies already addressed their potential as treatment options [150, 178]. Nonetheless, most studies focused on the role of estrogens and progesterone and further studies are needed in regards to hCG.

### 1.4.2 Progesterone and estrogens

The steroid hormones, estrogen and progesterone, affect various immune cell subsets (NK cells, T cells, B cells, Dendritic cells) and therefore have a broad range of action. Using an RA mouse model, progesterone treatment induced a Th2 response reduced the Th1 response and caused an induction of Treg cells leading to remission of the disease [179]. The preventive subcutaneous injection of a progesterone pellet in an EAE mouse model exhibited a protection against axonal damage [180]. Progesterone reduces disease severity in mice developing EAE and increases IL-10 cytokine levels [181]. Also, therapeutical treatment with progesterone after EAE onset resulted in a remission of EAE disease signs, induced recovery mechanisms within the CNS, and promoted an increase in IL-10 cytokine production [181]. More precisely,

progesterone was able to increase maturation and proliferation of oligodendrocyte progenitor cells and promoted myelin protein synthesis [182].

Due to their steady increase throughout pregnancy, estrogens can strongly affect the progression of a pregnant women's autoimmune disease. In MS, animal studies showed that estrogen protects from EAE through the inhibition of Th1 and Th17 cells, which has been found to be estrogen receptor dependent, using estrogen receptor knock-out mice and a preventive treatment with applied estradiol (E2) pellets [183]. An increase of Treg cell frequencies, an elevation of IL-10 cytokine levels, and a decrease of IFN$\gamma$ cytokine levels produced by lymphocytes isolated from E2-treated animals developing EAE compared to control animals was further observed [183–185]. The application of estrogen pellets in the EAE mouse model highlights a protective effect by increasing Breg cell frequencies and limiting the recruitment of pro-inflammatory cells from the periphery protecting against EAE [186]. IL-17 and TNF$\alpha$ were reduced in pregnant mice having EAE compared to non-pregnant mice exhibiting EAE progression suggesting that pregnancy itself has beneficial effects on EAE progression [187]. Furthermore, treatment with estriol mediates a shift towards the Th1 response and has already elicited protective effects in MS and RA. Jansson and colleagues described a delayed disease onset after estrogen application in an EAE mouse model and a suppression of collagen-induced arthritis [188]. Also, estriol increased the Th2 response in an EAE mouse model [157]. Furthermore, the treatment of human T cells isolated from MS patients presented an inhibited nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF$\kappa$B) activity which is necessary for the inflammation cascade [157, 189].

Due to these promising results, the first trial with estradiol treating patients diagnosed with relapsing-remitting MS was performed in 2002 [190]. Here, lesions in the brain were reduced and analysis of peripheral blood mononuclear cells (PBMCs) showed an increase of IL-10 and IL-5 and a decrease of TNF$\alpha$ cytokine levels after 12 month estradiol treatment [190]. A clinical trial phase II followed, but a clinical phase III has not been performed yet [191]. Estrogen treatment in female RA patients after menopause induced anti-inflammatory effects [192]. In regard to menopause, there is a hormone replacement therapy (HRT) available to lower

the symptoms. HRT is usually a combination of estradiol and progesterone and specific individual effects evoked by each hormone cannot be separately elaborated. HRT was suggested to increase the life quality, which was reported by MS patients included in a survey after HRT therapy [193].

Estrogens can also have negative effects [16]. In SLE, disease severity worsens and flares increase. Estradiol treatment leads to increased IFN$\gamma$ cytokine levels and systemic inflammation [194]. Contrarily, the lack of the progesterone receptor in a SLE mouse model leads to an increase of IgG autoantibody levels and indicates a protective effect of progesterone [195]. It has been found that there is an increased expression of the estrogen receptor-$\alpha$ (ER$\alpha$) and not of the ER$\beta$ in PBMCs of SLE patients and this might result in an exacerbation of this disease [196, 197]. Studying osteoporosis in postmenopausal women undergoing estrogen replacement therapy diagnosed with SLE, it has been shown that estrogens did indicate a positive effect on bone mass [198].

### 1.4.3 human Chorionic Gonadotropin

hCG is currently used in *in vitro* fertilization (IVF) cycles for final oocyte maturation using two different hCG preparations: urine-derived hCG (uhCG) or recombinant hCG (rhCG) [199]. Both of them show equal effects, however, the rhCG preparation is better tolerated by patients [200]. hCG is a primate specific heterodimeric glycoprotein produced by the syncytiotrophoblasts of the placenta [169]. It consists of two different subunits, the $\alpha$-subunit encoded on chromosome 6 and the $\beta$-subunit encoded on chromosome 19 [201, 202]. Interestingly, the luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and follicle-stimulating hormone (FSH) share the structural identical $\alpha$-subunit, whereas the $\beta$-subunit is unique for each hormone [201].

There are five different forms of hCG: regular hCG, $\beta$-hCG, hyperglycosylated $\beta$-hCG, hyperglycosylated hCG, and pituitary hCG [203, 204]. While hCG is mostly referred to as a pregnancy hormone, some forms are not exclusively pregnancy related and can be produced by other cell types. For example, $\beta$-hCG is produced

in large amounts by choriocarcinoma cells or non-trophoblastic neoplasms and is therefore used as a marker in tumor diagnostics. Pituitary hCG is produced by the pituitary gland in small amounts throughout life and increases during menopause [204, 205]. Even though its natural function is to sustain *corpus luteum*-mediated progesterone secretion, hCG is also able to modulate different immune cell subsets of the innate and the adaptive immune system supporting fetal survival. In detail, hCG binds to the luteinizing hormone/choriogonadotropin (LH/CG) receptor and thereby induces naïve T cells to differentiate into Treg cells which is mandatory for fetal tolerance induction [25, 206]. Additionally, hCG can modulate cells indirectly through cytokine and growth factor release secreted by the target cell [173, 207]. Most immune cells express hormone receptors as the LH/CG receptor like macrophages, DCs, T cells, and B cells. However, hCG can also act on cell types that do not possess the LH/CG receptor. Here, hCG binds to the mannose receptor which has been identified in natural killer (NK) cells as hCG probably induces their proliferation [208]. Follicular fluid macrophages show increased vascular endothelial growth factor (VEGF) in reaction to hCG which is important for vessel formation during pregnancy [209]. Also, an increased M1 to M2 polarization of macrophages is present [210]. Macrophages are further able to degrade hCG through transient vacuole formation in monocytes in case of too high hCG concentrations [211, 212]. DCs show a decreased expression of maturation markers such as MHC-II, CD80, and HLA-DR after hCG treatment [213, 214]. However, the data obtained for DCs is still inconsistent. Yoshimura and colleagues describe an activation of myeloid and plasmacytic DCs by the upregulation of CD40 and CD80 [215]. Regarding conventional T cells and B cells hCG has a rather suppressing effect [216–218]. Pioneer work from our group demonstrated that during pregnancy, hCG induces Treg cells in the periphery and is able to attract Treg cells to the feto-maternal interface supporting fetal tolerance [147, 206]. The release of hCG by trophoblasts is able to modulate $CD4^+$ $FoxP3^-$ T cells into $CD4^+$ $FoxP3^+$ Treg cells being able to inhibit responder T cells [219]. Other studies underlined the influence of hCG in inducing Treg cells [220, 221]. Interestingly, hCG can modulate B cells towards Breg cells which support fetal survival [217, 218]. hCG is able to enhance the secretion

of IL-10 which is lost when the LH/CG receptor is blocked [218]. Here, both Treg and Breg cells show elevated IL-10 expression levels [218, 222].

Due to its suppressive immunomodulatory properties, hCG may be also effective in autoimmunity or acceptance of allograft transplants due its ability to convert T cells into Treg cells. It has been suggested that hCG may be a successful modulator in some autoimmune disorders (RA, diabetes, Sjögren syndrome) [223–225]. It has been shown that hCG can prevent autoimmune diabetes by reducing $CD4^+$ and $CD8^+$ cell populations and increasing Treg cell populations in NOD mice [224]. Using a rat model to study rheumatoid arthritis, hCG treatment resulted in decreased arthritis as well as reduced TNF$\alpha$, IL-1$\beta$ and IL-6 cytokine levels as well as nitric oxide and iNOS protein levels [223]. Concerning MS, little is known about a potential effect of hCG on MS progression. Caspary and colleagues claimed no effect of hCG on EAE disease progression in guinea pigs, which was published in 1977 [226]. The treatment with hCG at day 7 and 11 led to decreased perivascular inflammation, but slightly enhanced disease score [226]. Later on, Han and colleagues investigated the influence of hCG in the EAE mouse model and showed an increased disease severity and mortality after hCG application [227]. The MMP-9 activity was increased in the CNS and plasma and also IL-4 and IL-6 cytokine levels were increased in hCG-treated mice developing EAE [227].

## 1.5 Aim of the study

hCG has already been proven to possess immunomodulatory properties and to induce immune tolerance in pregnancy. However, evidence is lacking about its possible immunomodulatory effects in autoimmune diseases. MS patients experience an improvement of their symptoms during pregnancy which may be interpreted as hCG being immunomodulatory for MS outcome. The main aim of this study was to investigate whether hCG exerts protective effects in MS by modulating the systemic and/or local immune response. For this, two different hCG preparations were applied preventively and therapeutically using the EAE mouse model to study

their efficiency. Moreover, we sought to identify the underlying mechanisms of hCG action at the cellular level. More precisely, the following questions were addressed in this study:

**1.** Does preventive treatment with hCG alter the disease score and the weight of MOG-immunized C57BL/6J female mice developing EAE?

**2.** Are there differences in EAE disease progression after hCG treatment depending on the hCG preparation used?

**3.** Does preventive treatment with hCG affect demyelination and cellular infiltration in the CNS?

**4.** Do hCG-treated animals developing EAE show modified lymphocyte populations after preventive hCG treatment?

**5.** Does therapeutic treatment with hCG affect EAE disease progression?

# 2. Material and Methods

## 2.1 Material

### 2.1.1 Equipment

| Equipment | Company |
|---|---|
| Attune NxT flow cytometer | ThermoFisher, Germany |
| Attune NxT autosampler | ThermoFisher, Germany |
| Autoclave Laboklav 80-V | SHP Steriltechnik AG, Germany |
| Centrifuge 5810R | Eppendorf, Germany |
| Centrifuge 5417R | Eppendorf, Germany |
| Centrifuge "mini spin" | Eppendorf, Germany |
| Chemiluminescence plate reader SYNG/A609 | Synoptics, USA |
| Clean bench Nu-437-400E | IntegraBiosciences, Germany |
| Drying cabinett | Memmert, Germany |
| FACSCalibur TM | BD Bioscience, Germany |
| Freezer -80°C | Sanyo/Thermo Fischer, Germany |
| Freezer -20°C | Kirsch/Liebherr, Germany |
| Hood Airflow-control EN14175 | Bense, Germany |
| Ice machine ZBE70-35 | Ziegra Eismaschinen, Germany |
| Incubator Hera cell 240 | Thermo Scientific, Germany |
| MACS separator | Miltenyi Biotec, Germany |
| Magnetic stirrer RH Basic 2 IKAM | Windhaus-Labortechnik, Germany |
| Mastercycler "Matercycler personal" | Eppendorf, Germany |
| Microplate reader Synergy HT | BioTek Instruments, USA |
| Microscope Zeiss Axio Hall 100 | Zeiss, Germany |
| Microscope Zeiss Axiovert 40C | Zeiss, Germany |
| Microtome Microm HM 355S | Thermo Scientific, Germany |
| Paraffin embedding machine EC 350-1 | Microm, Germany |

| Equipment | Company |
|---|---|
| Paraffin cooling station EC 350-2 | Microm, Germany |
| pH meter Microprocessor pH211 | NeolabMigge Laborbedarf, Germany |
| Refrigerator FKEX300, KTO 1810-20 | Rexel and Unielektro, Germany |
| Scale Sartorius CPA 225 D | Sartorius AG, Germany |
| Scale 440-2IN | Kern, Germany |
| Scale Sartorius LA 620P | Sartorius AG, Germany |
| Shaker Labnet Rocher 25 | Labnet, USA |
| Ultra Turrax T8 | Neolab, Germany |
| Thermocycler "iQ5 Multicolor" | Biorad Laboratories, Germany |
| Real-Time PCR Setection System" | Biorad Laboratories, Germany |
| Vortexer IKA MS 3 basic | Ika Works Ink., USA |
| Vortexer Vortex Mixer MS3 basic | Laborfachhandel Gaudig, Germany |
| Water bath HAAKE SWB25 | ThermoFisher, Germany |
| Water treatment plant Elix10 | Millipore, Germany |
| Water bath for histology | GFL, Germany |

| Further Equipment | Company |
|---|---|
| Aluminium foil | Roth, Germany |
| Autoclave bags | Labsolute, Germany |
| Bouffant | Medline, France |
| Beaker | Simax, Czech Republic |
| CPE overshoes | TransatlanticClinic, Germany |
| Coverslip 24x60 mm | Menzel Gläser, Germany |
| Canules 26G/30G | Braun, Germany |
| Cuvettes | NeolabMigge Laborbedarf, Germany |
| Dissection equipment | Fine Science Tools, Germany |
| Embedding cassettes | Roth, Germany |
| Filter 0.2 µm pore size | Whatman, Germany |
| Flask 100 ml/500 ml | Schott Duran, Germany |
| Glass syringe 1 ml/5 ml Luer-Lock Tip | Poulten & Graf GmbH, Germany |
| Gloves | Ansell/Meditrade, Germany |
| Ice bucket | NeolabMigge Laborbedarf, Germany |
| MACS MS, LD column | Miltenyi Biotec, Germany |
| MACS separator | Miltenyi Biotec, Germany |
| Multichannel pipette 300 µl | Brand, Germany |
| Neubauer counting chamber | VWR, Germany |
| Operating mask | 3M healthcare, Canada |
| Parafilm M | Roth, Germany |
| Pasteur pipette 3 ml | Sarstedt, Germany |
| Pipettes 10/100/1000 µl volume | Eppendorf, Germany |
| Pipette tips 10/100/1000 µl | Sarstedt/Eppendorf, Germany |
| Pipette filter tips 10/100/1000 µl | Sarstedt/Eppendorf, Germany |
| Scalpell | Feather, Japan |
| Sieve 100 µm, 70 µm | Corning, USA |
| Slide 76x26 mm | Menzel Gläser, Germany |

| Further Equipment | Company |
|---|---|
| Syringe 1 ml Omnifix-F | Braun, Germany |
| Syringe 30 ml | Braun, Germany |
| Three-way valve Discofix | Braun, Germany |
| Reaction tube holder | NeolabMigge Laborbedarf, Germany |
| Reaction tube 1.5 ml, 2 ml | Eppendorf, Germany |
| Reaction tube 15 ml, 50 ml | Greiner Bio-One, Germany |
| RNAse free reaction tube 1.5 ml, 2 ml | Eppendorf, Germany |
| Round bottom tubes | Corning, USA |
| 6 well plate | Sarstedt, Germany |
| 24 well plate | Sarstedt, Germany |
| 96 well plate | Sarstedt, Germany |
| 96 well plate (round bottom) | Corning, USA |

### 2.1.2 Chemicals and compounds

| Reagent | Company |
|---|---|
| Aqua ad injectabilia AMPUWA | Fresenius Kabi, Germany |
| Attune 1x shutdown solution | ThermoFisher, Germany |
| Attune 1x wash solution | ThermoFisher, Germany |
| Attune 1x focussing fluid | ThermoFisher, Germany |
| Bovine Serum Albumin (BSA) | Sigma, Germany |
| Brefeldin A 1000x | Biolegend, Germany |
| Chloroform | Sigma, Germany |
| Complete Freud's adjuvant (CFA) | Sigma, Germany |
| dNTP | Promega, Germany |
| DNAse | Stratagene, Germany |
| EDTA | Merck, Germany |
| Eosin | Sigma, Germany |
| Ethanol | Fischer, Germany |
| FACS Clean | BD Bioscience, Germany |
| FACS Flow | BD Bioscience, Germany |
| FACS Rinse | BD Bioscience, Germany |
| FBS | Biochrom, Germany |
| Fixation/permeabilization concentrate | ThermoFisher, Germany |
| Fluorescein | Fermentas, Germany |
| HCl | Roth, Germany |

| **Reagent** | **Company** |
|---|---|
| Hematoxylin | Sigma, Germany |
| Heparin | Zentralapotheke MD, Germany |
| $H_2SO_4$ | Sigma, Germany |
| $H_2O_2$ | Zentralapotheke Magdeburg, Germany |
| Ionomyin | ThermoFisher, Germany |
| Immersion oil 518F | Zeiss, Germany |
| Isopropyl | Roth, Germany |
| KCl | Merck, Germany |
| Ketamin-HCl | Pfizer, USA |
| $KH_2PO_4$ | Merck, Germany |
| Luxol fast blue solution | Sigma, Germany |
| Lympholyte M$^{TM}$ | Cederlane, Canada |
| Myelin oligodendrocyte glycoprotein (MOG) peptide 35-59 | JPT, Germany |
| M-MLV reverse transcriptase Reaction buffer 5x | Promega, Germany |
| *Mycobacterium tuberculosis* H37 Ra | DIFCO, USA |
| NaCl | Roth, Germany |
| $Na_2HPO_4$ | Merck, Germany |
| NaOH | Sigma, Germany |
| Paraplast (Paraffin) | Roth, Germany |
| paraformaldehyde (PFA) | Roth, Germany |
| PBS | PAN Biotech, Germany |
| Recombinant hCG (Ovitrelle) | Merck, Germany |
| Percoll | GE Healthcare, Germany |
| PMA | Sigma, Germany |
| Penicillin/streptomycin | ThermoFisher, Germany |
| Permebilization diluent | ThermoFisher, Germany |
| Pertussin toxin | Sigma, Germany |
| Reverse Transcriptase | Promega, Germany |
| RNAse inhibitor | Promega, Germany |
| PMA | Sigma, Germany |
| RPMI 1640 | Life Technologies, Germany |
| Rompun (Xylazinhydrochloride) | Bayer, Germany |
| Roti-Histokitt | Roth, Germany |
| Sucrose | Roth, Germany |
| SYBR Green PCR Mastermix | Applied Biosystems, Germany |
| Taqman | Applied Biosystems, Germany |
| TRIzol TM | Life Technologies, Germany |
| Tween-20 | Merck, Germany |
| Urine-derived hCG (Pregnyl) | EurimPharm Arzneimittel, Germany |
| Urine-derived hCG (Sigma) | Sigma, Germany |
| Xylene | Roth, Germany |

### 2.1.3 Primer and probes

| Cytokine/Probe | Sequence |
|---|---|
| IL-17 | Fwd GCT CCA GAA GGC CCT CAG A |
| | Rev AGC TTT CCC TCC GCA TTG A |
| IL-10 | Fwd GAA GAC CCT CAG GAT GCG G |
| | Rev CCT GCT CCA CTG CCT TGC T |
| IL-10 probe | Fam-CGC TGT CAT CGA TTT CTC CCC |
| | TGT GA-Tamra |
| IL-4 | Fwd CTC ATG GAG CTG CAG AGA CTC TTT |
| | Rev GTG ATG TGG ACT TGG ACT CAT TCA |
| IL-4 probe | Fam-ATG CCT GGA TTC ATC GAT AAG CTG |
| | CAC CT-Tamra |

### 2.1.4 Mouse strains

| Mouse strain | Company |
|---|---|
| C57BL/6J | Janvier Laboratory, France |
| IL-10IRESeGFP (IL-10GFP) | own breeding facility, Magdeburg |

### 2.1.5 Antibodies

| Antibody | Clone | Company |
|---|---|---|
| CD4 FITC (rat anti-mouse) | RM4-4 | BD Biosciences, Germany |
| FOXP3 eFluor 660 (anti-mouse/rat) | FJK-16S | eBioscience, Germany |
| CD19 PerCp (rat anti-mouse) | 6D5 | Biolegend, Germany |
| IL-17 PE (rat anti-mouse) | eBio17B7 | BD Biosciences, Germany |
| IFN$\gamma$ PerCp-Cy5 (rat anti-mouse) | XMG1.2 | BD Biosciences, Germany |
| TNF$\alpha$ PE (rat anti-mouse) | MP6-XT22 | BD Biosciences, Germany |
| CD11c APC (hamster anti-mouse) | HL3 | BD Biosciences, Germany |
| CD80 FITC (hamster anti-mouse) | 16-10A1 | BD Biosciences, Germany |
| MHCII PE (rat anti-mouse) | M5/114.15.2 | BD Biosciences, Germany |
| IFN$\gamma$ BV421 (rat anti-mouse) | XMG1.2 | BD Biosciences, Germany |
| TNF$\alpha$ PE (rat anti-mouse) | MP6-XT22 | BD Biosciences, Germany |
| CD11c APC (hamster anti-mouse) | HL3 | BD Biosciences, Germany |
| CD80 FITC (hamster anti-mouse) | 16-10A1 | BD Biosciences, Germany |
| MHCII PE (rat anti-mouse) | M5/114.15.2 | BD Biosciences, Germany |
| IFN$\gamma$ BV421 (rat anti-mouse) | XMG1.2 | BD Biosciences, Germany |
| TNF$\alpha$ PerCp-Cy5.5 (rat anti-mouse) | MP6-XT22 | BD Biosciences, Germany |

| Antibody | Clone | Company |
|---|---|---|
| CD25 APC (rat anti-mouse) | 3C7 | BD Biosciences, Germany |
| FoxP3 PE-CF610 (rat anti-mouse) | FJK-16S | eBiosciences, Germany |
| IL-17a PE-Cy7 (rat anti-mouse) | eBio17B7 | eBioscience, Germany |
| FVD BV510 (anti-mouse) | - | Invitrogen, Germany |
| CD11c PE-CF594 (hamster anti-mouse) | HL3 | BD Biosciences, Germany |
| IL-10 PE (rat anti-mouse) | JeS5-I6E3 | BD Biosciences, Germany |
| CD19 BV605 (rat anti-mouse) | ID3 | BD Biosciences, Germany |
| CD11b BV711 (rat anti-mouse) | M1/70 | BD Biosciences, Germany |
| CD86 FITC (anti-mouse) | GL-1 | Biolegend, Germany |
| CD80 APC (hamster anti-mouse) | 16-1041 | BD Biosciences, Germany |
| CD45 AF700 (anti-mouse) | 30-F11 | eBiosciences, Germany |

### 2.1.6 Kits

| Kit | Company |
|---|---|
| B cell isolation kit | Miltenyi Biotec, Germany |
| Th1/Th2/Th17 CBA | BD Biosciences, Germany |

### 2.1.7 Software

| Software | Company |
|---|---|
| AxioVision Rel. 4.6 | Zeiss Microimaging, Germany |
| Cell Quest Pro 4bf4b | BD Pharmingen, Germany |
| FCAP Array Software V3.0 | BD Biosciences, Germany |
| FlowJo V10 | FlowJoLLC, USA |
| GraphPad Prism 6.0 | Statcon, Germany |
| Gene 5 TM Data Analysis Software | BioTek Instruments, Germany |

### 2.1.8 Buffers and solutions

complete RPMI medium

RPMI 1640

10 % FBS

1 % Penicillin/Streptomycin

1x PBS

137 mM NaCl

2.6 mM KCl

10 mM $Na_2HPO_4$

1.8 mM $KH_2PO_4$

adjust pH 7.4 using HCl

4 % PFA

4 g PFA

100 ml PBS

adjust pH to 7.4 using HCl

MACS buffer

0.25 g BSA

0.037 g EDTA

50 ml PBS

FACS buffer

10 g BSA

1 g $NaN_3$

fill up to 1 l using 1xPBS

Anesthesia

2.5 ml Ketamin

2.5 ml dest. water

0.84 ml Rompun

Lithium carbonite solution

0.05 g lithium carbonate

100 ml dest. water

## 2.2 Methods

### 2.2.1 Mouse husbandry

Animals were kept at a constant temperature of 22°C with a humidity of 45 %. All animals were exposed to a 12 h light cycle, water and food was available *ad libitum*. All animal work was approved by the ethical committee and animals were handled according to the animal protection laws by the Landesverwaltungsamt of Saxony-Anhalt (42502-2-1363 UniMD). I was an authorized person to conduct the experiments resulted in this thesis.

### 2.2.2 EAE induction in C57BL/6J mice

To study the effect of hCG on MS disease progression, the EAE mouse model was used. One vial of inactivated *Mycobacterium tuberculosis* H37 Ra (100 mg) was added to 10 ml of complete Freud's adjuvant (CFA) and mixed thoroughly. Additionally, one vial of the myelin oligodendrocyte glycoprotein (MOG) peptide 35-59, containing 4 mg of peptide, was diluted in 2 ml of PBS. Afterwards, two 5 ml glass syringes were connected using a three-way valve and 2 ml of the dissolved MOG peptide solution was added to one syringe avoiding bubbles. Then, 2 ml of the CFA mixture including *M. tuberculosis* was added and the remaining air within the apparatus was carefully removed. The mixture with a working concentration of 1 mg/ml was mixed several times throughout the day and stored at 4°C prior to injection. The next day, the emulsified peptide solution was transferred to one of the 5 ml syringes and the empty one was exchanged with a 1 ml glass syringe prior to injection. The solution was drawn up into the 1 ml glass syringe and connected to a 26 gauge cannula. 10 weeks old C57BL/6J female mice provided by Janvier Laboratory, France were injected subcutaneously (*s.c.*) with 50 µl of the MOG peptide solution behind the flanks and close to the spinal cord at the hind leg. This

procedure was done on both body sides. Finally, 200 ng pertussis toxin diluted in 200 µl PBS were injected intraperitoneally (*i.p.*). The pertussis toxin injection was repeated again two days later. Mice were weighed and scored daily throughout every experiment using the scoring system stated below. Exclusion criteria included weight loss over 40 % or a score of $\geq 4$. Only animals that showed disease signs were included in the experiments.

**Table 2.1: Disease scoring system of MOG-immunized C57BL/6J mice developing EAE**

| score | disease signs |
|---|---|
| 0 | no visual symptoms |
| 0.5 | impaired movement of the tip of the tail |
| 1 | limb tail |
| 1.5 | limb tail and slight slowing of righting from supine position |
| 2 | partial hindlimb weakness |
| 2.5 | dragging of hindlimbs without complete paralysis |
| 3 | complete paralysis of at least one hindlimb |
| 3.5 | complete hindlimb paralysis and slight weakness of forelimbs |
| 4 | severe forelimb weakness of at least one forelimb |
| 5 | moribund or dead |

### 2.2.3 Preventive treatment of MOG-immunized mice developing EAE with hCG every other day

In this study, two different approaches were investigated: the preventive treatment option and the therapeutic treatment option of hCG. At first, the preventive treatment option was investigated to study if hCG, as a strong immunomodulator of the immune system, is able to attenuate or prevent disease progression in the EAE mouse model by altering different immune cell populations (Tregs, Th17, B cell). For the preventive treatment option, two different preparations of hCG (recombinant hCG [rhCG] and urine-derived hCG [uhCG]) were injected every other day in MOG-immunized female C57BL/6J mice starting at the day of immunization (Figure 2.1 A). These two different preparations were used to identify whether there is a different effect between both hCG preparations which may show diverse effects and therefore may lead to different results in our experimental setting. Both of them

show equal effects, however, the rhCG preparation is better tolerated by patients regarding IVF [200]. Whereas the uhCG preparation is extracted from the urine of pregnant women, rhCG is produced by a chinese hamster ovary cell line [228, 229]. Another difference between both preparations is that the uhCG preparation consists of various forms of hCG. There is the hyperglycosylated and the free β-unit as well as the intact hCG form. Nonetheless, the uhCG preparation has a purity of approximately 70 % and non-hCG proteins can be found within this preparation [230]. The rhCG preparation consists of approximately 99 % of the complete hCG form and has therefore a higher purity compared to the urine-derived form. The rhCG preparation also shows a higher efficiency compared to the uhCG preparation meaning that a lower dose can be applied to the patient [229, 231]. However, the urine-derived preparation may be more dangerous due to its isoforms, because the free β-subunit and also the hyperglycosylated form is known to be carcinogenic [203, 232].

Each preparation was diluted in PBS with a concentration of 250 IU for rhCG and 250 IU or 150 IU for uhCG. Each mouse was injected *i.p.* either with 200 µl 250 IU rhCG, 250 IU, or 150 IU uhCG, or 200 µl PBS (control vehicle) every other day using a 30 gauge needle. Throughout the experiment, mice were weighed and scored daily. At day 10, 20, or 35 mice were sacrificed and spleen, inguinal lymph nodes, and CNS were collected for flow cytometry analysis; spinal cord was collected for histological evaluation.

### 2.2.4 Therapeutic treatment of MOG-immunized mice developing EAE with hCG

The second treatment option tested was the therapeutic treatment of hCG. Treatments usually start with disease onset and therefore it was studied whether hCG can alter disease progression in the EAE mouse model after disease onset. C57BL/6J female mice were immunized as stated in 2.2.2 followed by a therapeutic treatment *i.p.* using uhCG or rhCG starting at the day of disease onset. All mice were weighed and scored daily according to the table 2.1 until day 35. The

treatment started with an injection of 250 IU rhCG, 150 IU uhCG diluted in PBS or control vehicle (PBS) the day at which an individual mouse showed the first sign of EAE. Following, each mouse was injected every other day with the indicated substance (Figure 2.1 B).

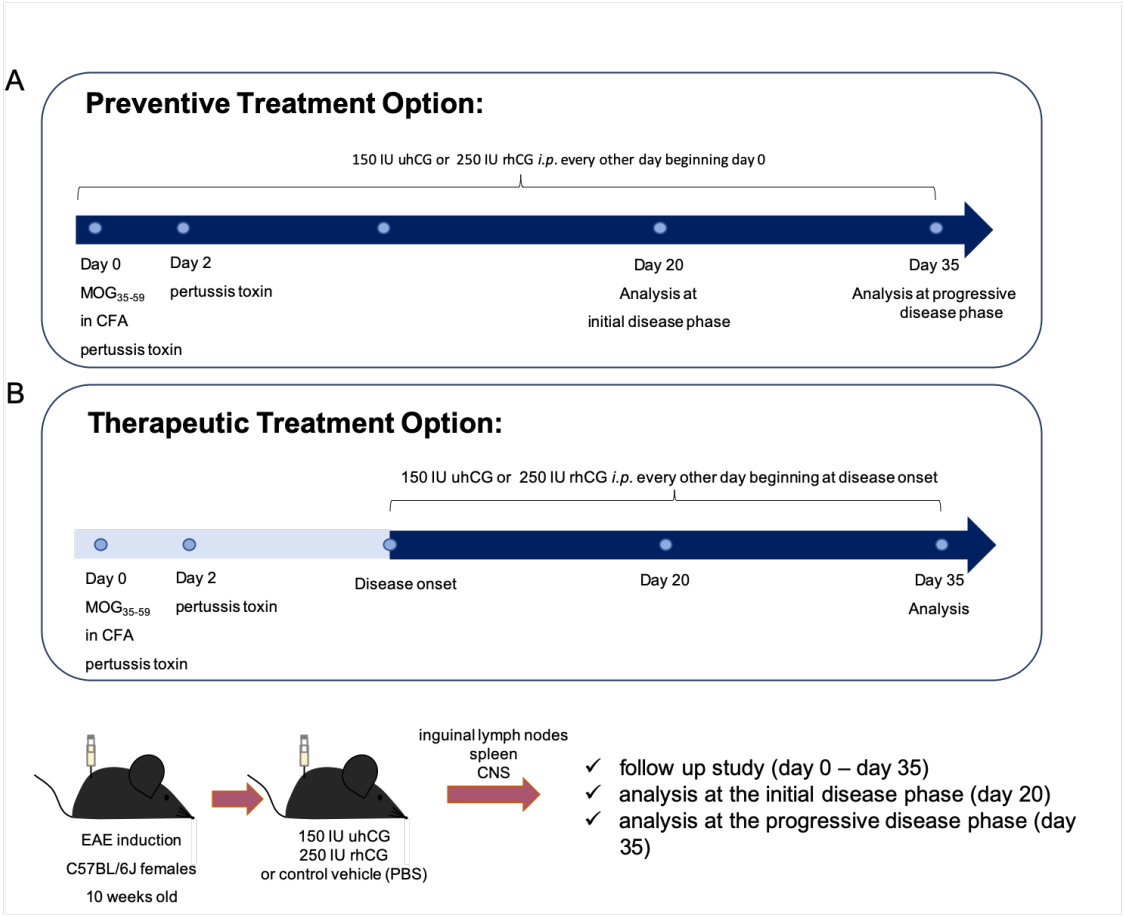


**Figure 2.1: Preventive and therapeutic treatment of MOG-immunized female C57BL/6J mice with uhCG or rhCG.**
The Preventive treatment was performed every other day with either uhCG, rhCG, or control vehicle (PBS) starting at the day of immunization (A). The therapeutic treatment started the day at which the MOG-immunized C57BL/6J female mice showed first signs of EAE (B). A follow up study was performed to analyze EAE disease progression. Furthermore, different immune cell populations of the CNS, spleen, and inguinal lymph nodes were analyzed at disease onset (day 10), the initial disease phase (day 20), and the progressive disease phase (day 35).

### 2.2.5 Tissue collection

MOG-immunized C57BL/6J mice were anesthetized with 200 µl of Ketamin/Rompun (stated in 2.1.8) *i.p.* and 100 µl *s.c.* for transcardial perfusion. When the withdrawal reflex was no longer perceptible, mice were dissected and

the heart was disclosed. The right ventricle of the heart was opened and 30 ml of ice-cold PBS was injected into the left heart ventricle under constant pressure to allow perfusion of the cardiovascular system. Afterwards, the inguinal lymph nodes, spleen, and CNS were collected. The organs were washed in PBS and placed in 2 ml reaction tubes containing RPMI 1640 supplemented with 10 % FBS and 1 % penicillin/streptomycin (complete RPMI medium) for flow cytometry analysis. To obtain the CNS, the skin of the head was removed by cutting along the midline of the head towards the ears exposing the cranium. The intraperitoneal bone line was broken and the bone plates were carefully removed at the sagittal suture without damaging the brain. The brain was placed in either 1 ml of complete RPMI medium for flow cytometry analysis (2.2.6.1) or shock frozen in liquid nitrogen for RT-PCR analysis (2.2.9.1). The spinal cord was exposed by removing the skin and the muscles along the spinal cord. The upper cranial part at the side of the atlanto-occipital joint was identified and the bone was carefully cut at the left and right side of the spinal cord. The bone was carefully removed step by step exposing the spinal cord and the spinal cord was detached using fine forceps sliding through the bone at the bottom cutting the fiber nerves. Afterwards, the spinal cord was transferred to a 2 ml reaction tube containing 1 ml of complete RPMI medium for flow cytometry analysis or shock frozen in liquid nitrogen for RT-PCR analysis (2.2.8). For histological evaluation, mice were perfused with 30 ml of cold 4 % PFA solution after PBS perfusion. Under constant pressure, the cardiovascular system was perfused with PFA for tissue fixation. The spinal cord and brain were then stored in 4 % PFA for histology (2.2.9). For histological evaluation kidney, liver, and inguinal lymph nodes of selected animals were collected and placed in 4 % PFA (2.2.9).

### 2.2.6 Lymphocyte isolation

To isolate lymphocytes from inguinal lymph nodes and spleen, density gradient centrifugation was performed using Lympholyte M$^{TM}$. Percoll gradient centrifugation was the chosen method for the CNS. The inguinal lymph nodes,

spleen, spinal cord, and brain were collected as stated in 2.2.5. All organs were kept in 2 ml reaction tubes containing complete RPMI medium. The following steps were performed under sterile conditions. The organs were transferred to one well of a 6 well plate each, containing 2 ml of complete RPMI medium. Each organ was mashed through a 100 µm strainer using a plunger from a 1 ml syringe. The mesh and the plunger were rinsed with 2 ml of complete RPMI medium and the cell suspension was transferred to a 15 ml reaction tube. 10 ml of complete RPMI medium was added and the samples were centrifuged at 300 g for 10 min at 4°C. Afterwards, the supernatant of the inguinal lymph nodes and the spleen samples were discarded and the cell pellet was resuspended in 4 ml of complete RPMI medium. The cell suspension was layered on top of 4 ml of a density gradient solution (Lympholyte M$^{TM}$) and centrifuged at 1000 g for 20 min without brake at 4°C.

For the CNS samples, the supernatant was discarded, cells were resuspended in 4 ml of 30 % Percoll diluted in PBS and layered on top of 5 ml of 70 % Percoll solution. The density gradient centrifugation was performed as stated above. Afterwards, the lymphocyte ring, visible between both layers, was transferred to a new reaction tube and the cells were washed twice with complete RPMI medium. The samples were centrifuged at 300 g for 10 min at 4°C. Cells were then resuspended in 1 ml of complete RPMI medium, counted using a Neubauer improved chamber and used for further experiments.

#### 2.2.6.1 Flow cytometry

For flow cytometry analysis, isolated lymphocytes from MOG-immunized C57BL/6J female mice sacrificed at day 35 (isolation procedure see 2.2.6) were treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 500 ng/ml ionomycin, and 10 µg/ml Brefeldin A for 4.5 h. 5 h stimulation was performed for samples of the mice sacrificed at day 20 at 37°C and 5 % $CO_2$ to ensure IL-10 detection. Cells were collected and transferred to round bottom tubes or a 96 round bottom well plate. 1 ml (tubes) or 200 µl (96 well plate) of FACS buffer was added and the cells were centrifuged at 300 g for 10 min at 4°C. The washing step was repeated

once more when using the 96 well plate. Afterwards, the supernatant was discarded and 100 µl of antibody solution (1:100 dilution for day 35 samples, 1:200 dilution for day 20 samples) was added to the samples. The samples were incubated at 4°C in the dark for 30 min. The antibody panels are listed below in table 2.2. After antibody incubation, 1 ml (tubes) or 100 µl (96 well plate) of FACS buffer was added and the cells were centrifuged at 300 g for 10 min at 4°C. The washing step was performed twice when using the 96 well plate, adding 200 µl of FACS buffer during the second step. Finally, 100 µl of fixation solution was added to all samples and the samples were stored at 4°C in the dark overnight (ON). The next day, 1 ml (tubes) or 100 µl (96 well plate) of permeabilization buffer was added to all samples. Afterwards, the samples were centrifuged at 4°C for 10 min at 300 g. When using the 96 well plate, another washing step was performed with 200 µl of permeabilization buffer. The supernatant was discarded and 100 µl antibody solution containing intracellular antibodies was added. The cells were incubated for 30 min at 4°C in the dark and 1 ml (tubes) or 100 µl (96 well plate) of FACS buffer was added afterwards, performing the washing step twice when using the 96 well plate. Finally, the cells were resuspended in up to 200 µl of FACS buffer and stored at 4°C in the dark until flow cytometry measurement.

**Table 2.2: Antibody panels for flow cytometry analysis**

| | | | |
|---|---|---|---|
| T and B cells | CD4 FITC | FOXP3 eFluor | day 35 analysis |
| | CD19 PerCp | IL-17 PE | |
| T cells | CD4 FITC | IFNγ PerCp-Cy5 | |
| | | TNFα PE | |
| Dendritic cells | CD11c APC | CD80 FITC | |
| | | MHCII PE | |
| T cell panel | CD4 FITC | IFNγ BV421 | day 10, 20 and severe illness analysis |
| | | TNFα PerCp-Cy5.5 | |
| | CD25 APC | IL-10 PE | |
| | CD45 AF700 | FOXP3 PE-CF594 | |
| | FVD BV510 | IL-17 PE-Cy7 | |
| B cell panel | CD19 BV605 | IL-10 PE | |
| | CD45 AF700 | IL-17a PE-Cy7 | |
| | FVD BV510 | | |
| APC panel | CD11c PE-CF594 | | |
| | CD11b BV711 | | |
| | CD86 FITC | | |
| | CD80 APC | | |
| | CD45 AF700 | | |
| | FVD BV510 | | |

Analysis was performed using the Cell Quest Pro software (analysis at day 35) or the FlowJo software. Gating was performed using fluorescence-minus-one controls.

#### 2.2.6.2 Counting of cells

Using the Neubauer improved chamber, cell numbers were determined. The cells were suspended in 1 ml of PBS, the chamber was covered with a cover slip and 10 µl of the cell suspension was added to the space between the chamber and the cover slip using a pipette. Cells were counted within each quadrant and the cell number was calculated as stated below:

$$cell\ number/ml = \frac{cells\ counted\ in\ all\ quadrants}{4} * 10{,}000 * volume\ of\ cell\ suspension * dilution$$

#### 2.2.6.3 Titration of MOG and hCG concentrations for B cell *ex vivo* cultures

To study the responsiveness of autoreactive MOG-primed B cells to hCG *ex vivo*, B cells were isolated from MOG-immunized C57BL/6J female mice and restimulated with MOG, hCG, or a combination of both to study if uhCG is able to suppress B17 cell frequencies and is able to induce IL-10 producing B cells. First, the appropriate concentrations of MOG and uhCG were determined. B cells were isolated as stated in 2.2.6/2.2.6.4 from MOG-immunized C57BL/6J female mice at day 20. Afterwards, 50,000 cells were transferred to a 96 well plate and cultured with different MOG (20 µg, 50 µg, 80 µg, and 100 µg) and uhCG concentrations (100 IU/ml, 150 IU/ml, 200 IU/ml, 250 IU/ml, 300 IU/ml) for 24 and 48 h.

#### 2.2.6.4 B cell cultures with MOG and uhCG

For B cell *ex vivo* cultures with MOG and uhCG, C57BL/6J female mice were immunized with MOG to develop EAE (2.2.2). 20 days after EAE induction, the spleen was collected (2.2.5) and B cells were isolated using magnetic beads. During the isolation process, all steps were performed on ice using ice-cold MACS buffer and pre-cooled separation columns as well as centrifugation steps at 4°C. The splenic cell suspension was centrifuged at 300 g for 10 min and the supernatant was aspirated. The cell pellet was resuspended in 40 µl of MACS buffer per $10^7$ cells and 10 µl of the biotin-antibody cocktail was added per $10^7$ cells mixing the cell solution and incubating it at 4°C for 5 min. Afterwards, 30 µl of MACS buffer was added per $10^7$ cells, as well as 20 µl of anti-biotin microbreads per $10^7$ cells. The cell suspension was mixed and incubated for 10 min at 4°C. A MS column was placed in a MACS separator and rinsed with 500 µl of MACS buffer. Then, a 15 ml collection tube was placed underneath the column and the cell suspension was applied onto the column. When the column reservoir was empty, the column was rinsed three times with 500 µl of MACS buffer. The cells that passed through the column were the unlabeled and enriched B cells. The unlabeled B cell population was then centrifuged at 300 g for 10 min at 4°C and the cells were resuspended

in 1 ml of complete RPMI medium. Afterwards, the cells were counted using the Neubauer improved chamber. 100,000 B cells were cultured in duplicates with 250 µl of the specified medium listed in table 2.3 in a 96 well plate for 24 or 48 h at 37°C, 5 % $CO_2$. 5 h before obtaining the samples for flow cytometry analysis, the cells were stimulated with 50 ng/ml PMA, 500 ng/ml ionomycin and 10 µg/ml Brefeldin A. Finally, antibody staining was performed (2.2.6.1).

**Table 2.3: Cell culture condition for isolated B cell culture**

| | **B cells** | **B cells** | **B cells** | **B cells** |
|---|---|---|---|---|
| complete RPMI medium | + | + | + | + |
| 20 or 50 µg/ml MOG | - | + | - | + |
| 250 IU/ml uhCG | - | - | + | + |

#### 2.2.6.5 Sorting of CD19$^+$IL-10$^+$ B cells and treatment with MOG and uhCG

The following experiment was designed to study whether CD19$^+$IL-10$^+$ cells are able to convert into CD19$^+$IL-17$^+$ cells when restimulated with the MOG peptide. Therefore, IL-10IRESeGFP (IL-10eGFP) mice were used [233]. This transgenic mouse strain contains a fusion protein of an internal ribosome entry site (IRES) with an enhanced green fluorescent protein (eGFP) downstream of the exon 5 on the *il-10* gene. Thus, all cells producing IL-10 additionally express eGFP facilitating the characterization of IL-10$^+$ cells [233]. IL-10eGFP female mice were immunized with MOG to develop EAE (2.2.2) and spleens were obtained 20 days after immunization (2.2.5). Splenic lymphocytes were isolated (2.2.6) and stained with CD19 PerCP for sorting of CD19$^+$IL-10$^+$ cells. Sorting of the cells was performed using a BD FACS Aria$^{TM}$III cell sorter in collaboration with the Department of Molecular and Clinical Immunology at the Medical Faculty of the Otto-von-Guericke University in Magdeburg. After cell sorting, the cells were distributed into 1.5 ml reaction tubes, washed in PBS and centrifuged at 300 g for 10 min at 4°C. The cell supernatant was discarded and the specified medium (Table 2.4) was added. 6,000 cells per well were plated to a 96 well plate and cultured with 250 µl of the specific medium (Table 2.4) for 48 h at 37°C, 5 % $CO_2$. Each

approach was performed in duplicates. After 43 h at 37°C and 5 % $CO_2$, the cells were stimulated 5 h with 50 ng/ml PMA, 500 ng/ml ionomycin, and 10 µg/ml brefeldin A for 5 h. Antibody staining was performed after 48 h of culture (2.2.6.1).

**Table 2.4: Cell culture condition for CD19$^+$IL-10$^+$ (B10) cell culture**

| | **B10 cells** | **B10 cells** | **B10 cells** | **B10 cells** |
|---|---|---|---|---|
| complete RPMI medium | + | + | + | + |
| 20 µg/ml MOG | - | + | - | + |
| 250 IU/ml uhCG | - | - | + | + |

### 2.2.7 Cytometric bead array (CBA)

Additionally to cell frequency analysis, cytokine levels of lymphocytes isolated of MOG-immunized C57BL/6J female mice at day 20 were analyzed to investigate a possible effect of the preventive hCG treatment *in vivo*. Isolated lymphocytes from inguinal lymph nodes and spleen (isolation procedure see 2.2.6) were treated with 50 ng/ml PMA, 500 ng/ml ionomycin and for 5 h at 37°C and 5 % $CO_2$. For removing the remaining cells and cell debris, the supernatant was collected and centrifuged at 400 g for 10 min at 4°C. The supernatant was stored at -80°C until measurement.

Th1/Th2/Th17 standards as well as a mixture of capture beads were prepared. 25 µl of the capture bead mixture was added to all assay tubes and 25 µl of the Th1/Th2/Th17 standard dilutions was added to the control tubes. 25 µl of the samples and 25 µl of Th1/Th2/Th17 PE detection reagent was added to all tubes. All tubes were protected from light and incubated for 2 h at room temperature (RT). After the incubation, 500 µl of wash buffer was added to all samples and the tubes were centrifuged at 200 g for 5 min. The supernatant was aspirated and 50 µl of wash buffer was added to each tube. Finally, the assay tubes were measured using a BD FACSCalibur$^{TM}$ and the analysis was performed using the FCAP array software.

### 2.2.8 RNA isolation

RNA expression of IL-17, IL-10, and IL-4 was investigated after the preventive treatment option with uhCG. 600 µl of TRIzol™ was pipetted to a 2 ml reaction tube including one quarter of the brain and spinal cord from MOG-immunized C57BL/6J female mice at day 20. The tissue was minced using the Ultra Turrax T8 homogenizer and 400 µl of TRIzol™ was added afterwards. All steps were carried out on ice and all centrifugation steps were done at 4°C. The tubes were inverted and 200 µl of ice-cold chloroform were added to the samples. The samples were inverted 15 times and centrifuged at 12,000 g for 15 min. Using RNAse free reaction tubes and filter tips, the aqueous upper phase was transferred to a new reaction tube and ice-cold isopropyl was added at a 1:1 ratio. The RNA was allowed to precipitate at -80°C overnight (ON). The next day, the samples were centrifuged at 12,000 g for 15 min and the supernatant was removed. The samples were washed twice with 500 µl 80 % ethanol and centrifuged at 7,500 g for 10 min. Afterwards, the supernatant was removed and the RNA pellet was allowed to dry. Finally, the RNA was suspended in 21 µl of RNAse free water and RNA quantification was done at 260 nm using a spectrophotometer. The RNA samples were adjusted to a concentration of 1 µg/µl using RNAse-free water and stored at -80°C until further use.

#### 2.2.8.1 cDNA synthesis

For cDNA synthesis, all steps were performed on ice. 2 µl of RNA was added to a RNAse free 0.5 ml reaction tube. Afterwards, 18 µl of RNAse-free water and 2 µl of oligo-desoxy-thymidin nucleotides were added. The samples were mixed and incubated at 75°C for 10 min and 2 min at 4°C. To each sample 18.5 µl of the reaction mix A (composition mixture recipe stated below) was pipetted and the samples were incubated at 37°C for 30 min followed by a DNAse inactivation step at 75°C for 5 min and an incubation step at 4°C for 2 min. Then, 2 µl of mix B

(composition mixture recipe stated below) was added and the cDNA synthesis was started at 42°C for 60 min, followed by a reverse transcriptase inactivation step at 94°C for 5 min. Finally, the samples were cooled down to 4°C and stored at -20°C until further use.

mix A per sample:

8 µl 5x M-MLV reaction buffer

4 µl RNAse free water

4 µl dNTPs (2.5 mM/l)

2 µl DNAse (2 U/µl)

0.5 µl RNAse inhibitor (40 U/µl)

mix B per sample:

1 µl reverse transcriptase (200 U/µl)

1 µl RNAse inhibitor (40 U/µl)

#### 2.2.8.2 Semi-quantitative real-time polymerase chain reaction (qRT-PCR)

The quantitative RT-PCR (qRT-PCR) is used to quantify gene expression. Two different methods were used in the present work. The first qRT-PCR was performed using a fluorescent dye (SYBR green) which intercalates with the PCR product and template amplification can be followed up at every step of the PCR cycle. The second method known as Taqman uses a specific oligonucleotide probe which consists of a quencher dye on the 3' end and a reporter fluorescent dye on the 5' end. This probe anneals to the target sequence and is cleaved by the 5' exonuclease activity of the Taq polymerase as the primer is extended. The probe is cleaved and the reporter

dye is separated from the quencher dye which induces a reporter dye signal. When the probe is displaced, the strand is further elongated. Finally, the cleavage of the reporter dye is detected with every cycle and can be measured.

For semi qRT-PCR, cDNA derived from CNS tissue at day 20 post injection (p.i.) was used. All steps were performed on ice, using RNAse-free filter tips and reaction tubes. A master mix containing either commercially available Taqman or SYBR Green, RNAse free water, a primer mix containing the forward and reverse primer (IL-4, IL-10, IL-17 or β-actin (housekeeping gene)), and either 50 nM fluorescein or 5 pMol/µl probe (IL-4, IL-10, β-actin) were prepared. 3 µl of cDNA in duplicates were placed to each well of a white 96 well PCR plate. Afterwards, 10 µl of the appropriate master mix was added and the plate was sealed. To verify that the samples are all placed on the bottom of the plate and to avoid air bubbles, the plate was shortly centrifuged. The plate was placed in the thermocycler and the following program was started.

**Table 2.5: qRT-PCR (SYBR Green)**

| | | | | |
|---|---|---|---|---|
| initial denaturation | 95°C | 10 min | 1 cycle | |
| denaturation | 95°C | 30 sec | } | |
| hybridization | 60°C | 45 sec | } | 40 cycles |
| elongation | 72°C | 30 sec | } | |
| final denaturation | 95°C | 1 min | 1 cycle | |

**Table 2.6: qRT-PCR (Taqman)**

| | | | | |
|---|---|---|---|---|
| initial denaturation | 95°C | 8:30 min | 1 cycle | |
| denaturation | 95°C | 15 sec | } | |
| hybridization/elongation | 60°C | 1 min | } | 40 cycles |
| | 50°C | 1 min | 1 cycle | |

The data of the semi qRT-PCR was evaluated, correlating the expression of the gene of interest (*IL-17, IL-4, IL-10*) to the reference gene (housekeeping gene β-*actin*). The cycle threshold (Ct) values of the reference gene and the gene of interest were adjusted which indicates the starting point of the exponential phase of the fluorescence signal. The values were normalized by subtraction of the expression

of the reference gene from the expression of the gene of interest (ΔCt). The relative expression was calculated using the following formula:

ΔCt = Ct (gene of interest) - Ct (reference gene)

relative expression = $2^{-\Delta Ct}$

## 2.2.9 Histology

### 2.2.9.1 Spinal cord preparation for sectioning

Mice were anesthetized (2.2.5) and perfused with 30 ml of ice-cold PBS followed by 4 % PFA. The spinal cord was dissected (see 2.2.5) and placed in plastic containers in 4 % PFA for 48 h. Inguinal lymph nodes, liver, and kidney were also placed in 4 % PFA for 48 h until further processing.

### 2.2.9.2 Paraffin embedding

The samples were collected for histological analysis (2.2.5; 2.2.9) to examine specific morphological differences or pathological infiltration of lymphocytes in the tissue. The tissue was fixed in 4 % PFA and processed using the protocol indicated in table 2.7..

**Table 2.7: Protocol for brain and spinal cord embedding in paraffin**

| | | |
|---|---|---|
| ON | 70 % ethanol | dehydration |
| 30 min | 80 % ethanol | |
| 60 min | 80 % ethanol | |
| 30 min | 90 % ethanol | |
| 30 min | 90 % ethanol | |
| 60 min | 100 % ethanol | |
| 60 min | 100 % ethanol | |
| 60 min | xylene | embedding |
| 60 min | xylene | |
| ON | paraffin | |

#### 2.2.9.3 Sectioning of paraffin sections

10 µm sections of the spinal cord (region L1-L3) and 5 µm sections of inguinal lymph nodes, kidney, and liver were cut using the microtome. The sections were directly transferred to pretreated slides using a water bath at a temperature of approximately 60°C. Afterwards, the sections were air dried and stored at RT until further use.

#### 2.2.9.4 Hematoxylin eosin staining

The hematoxylin eosin (HE) staining is generally used for morphological characterization of the tissue. Hematoxylin stains all acidic/basophilic structures of the cell blue (e.g. DNA, nucleus, rough endoplasmatic reticulum), whereas eosin stains the acidophilic, eosinophilic, and alkaline structures red (e.g. cell plasma proteins).

For the HE staining the slides with 5 µm sections were first deparaffinized and then dehydrated treated as stated in table 2.7, following HE staining, before covering with a cover slip using Histokitt-II-Roti.

**Table 2.8: Protocol for HE staining**

| | | |
|---|---|---|
| 15 min | xylene | deparaffinization |
| 15 min | xylene | |
| 5 min | 100 % ethanol | hydration |
| 5 min | 95 % ethanol | |
| 5 min | 75 % ethanol | |
| | rinse shortly in dest. water | HE staining |
| 6 min | hematoxylin | |
| | rinse shortly in warm tap water | |
| 5 sec | eosin | |
| | rinse shortly in dest. water | |
| dip 10x | 75 % ethanol | dehydration |
| dip 10x | 95 % ethanol | |
| dip 10x | 100 % ethanol | |
| 5 min | xylene | |
| 5 min | xylene | |

The evaluation of the samples was performed using a Zeiss microscope and the AxioVision software Rel. 4.6.

#### 2.2.9.5 Luxol fast blue staining

The Luxol fast blue (LFB) staining is used to define the demyelination within the CNS. Therefore, the 10 µm section samples of the spinal cord were processed using a deparaffinization and hydration step before staining with LFB following a dehydration step. The staining procedure is summarized in table 2.9.

**Table 2.9: Protocol for LFB staining**

| | | |
|---|---|---|
| 15 min | xylene | deparaffinization |
| 15 min | xylene | |
| 5 min | 100 % ethanol | hydration |
| 5 min | 95 % ethanol | |
| 2 h | 1 % LFB solution at 60°C | |
| | rinse in 95% ethanol | |
| 10 sec | 0.05 % lithium carbonate solution | refinement of white matter |
| | rinse in dest. $H_20$ | |
| 3 dips | 95 % ethanol | |
| 3 dips | 100 % ethanol | |
| 5 min | xylene | |
| 5 min | xylene | |

Finally, sections were covered with a cover slip using Histokitt-II-Roti.

### 2.2.10 Statistical analysis

The results were analyzed using the GraphPad Prism 6 software. Each group was tested for normal distribution using the D'Agostino-Pearson omnibus normality test. Data was displayed as means or median including the standard deviation (S.D.) or standard error of mean (S.E.M.) depending on their statistical distribution. When reaching normality, the student's $t$-test was applied (* $p<0.05$). In case the data was not normal distributed the non parametric Mann-Whitney-$U$ test

was performed. If more than two groups were compared, normal distribution was tested using the D'Agostino-Pearson omnibus normality test and One-way ANOVA (gaussian distribution assumed) or the Kruskal-Wallis test (gaussian distribution not assumed) was performed. Significant statistical differences are presented as * $p<0.05$, ** $p<0.01$.

# 3. Results

## 3.1 Establishment of the EAE model

To address the aim of the study, a chronic mouse model for EAE, mimicking MS disease progression, was established. EAE induction was performed using the MOG peptide in CFA in female C57BL/6J mice to study the demyelination and infiltration of T and B cells under hCG treatment. To find the best conditions under which a consistent EAE progression until day 35 could be observed in our animal facility, we tested the influence of different ages of female C57BL/6J mice in our model.

### 3.1.1 EAE induction using C57BL/6J female mice at 10 weeks of age resulted in a progressive disease course

Figure 3.1 shows the disease course of EAE in either 9-, 10-, and 11-weeks old female C57BL/6J mice.

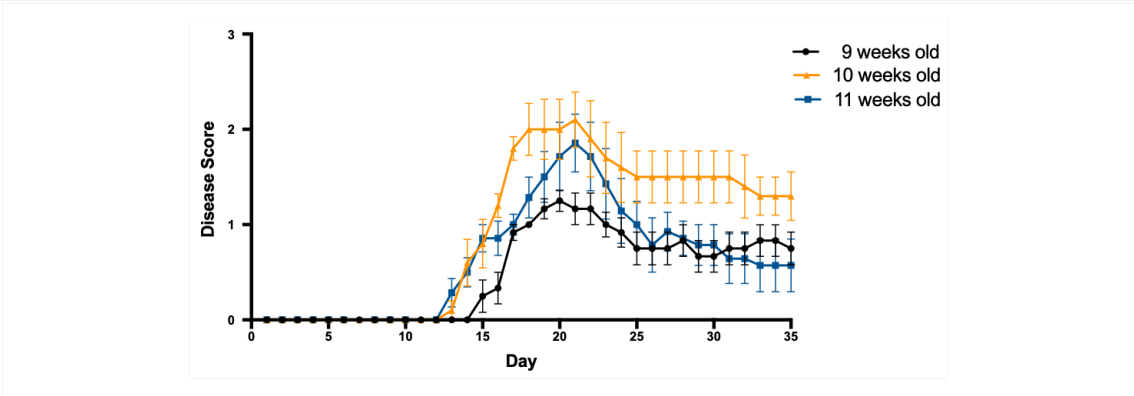


**Figure 3.1: EAE disease course using 9-, 10-, and 11-weeks old C57BL/6J female mice.** Shown is the EAE disease course (day 0 - 35) of female C57BL/6J mice at 9-weeks (n=6), 10-weeks, (n=5) and 11-weeks (n=7) of age. The 10-weeks old MOG-immunized mice showed the most pronounced EAE disease course. Data is displayed as means plus S.E.M.. Data analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

10-weeks old MOG-immunized mice showed a more pronounced EAE disease course compared to 9- and 11-weeks old female mice until day 35 (Figure 3.1). Based on this observation, 10-weeks old female mice were used in all following experiments.

## 3.2 Titration of hCG concentrations

To test whether hCG is able to change the course or outcome of EAE manifestations, two different hCG preparations (rhCG and uhCG) were administered to MOG-immunized mice developing EAE. Previous observations already suggested that rhCG is more potent than uhCG regarding the induction of ovulation in women [200]. In the first set of experiments, low concentrations of rhCG (1 IU, 2 IU, and 10 IU per mouse) were tested together with a high concentration of uhCG and rhCG (250 IU per mouse). The rhCG concentrations (1 IU, 2 IU, and 10 IU) were chosen according to previously published studies in pregnancy [25]. Here, the low concentrations of rhCG have been proven to possess beneficial effects on the immune system during pregnancy, elevating Treg cell numbers and maintaining dendritic cells in their tolerogenic state [25]. 250 IU uhCG and 250 IU rhCG were chosen because this concentration of hCG can be detected physiologically in the serum of pregnant women (25-288 IU/ml during gestation week 9-12 according to the American Pregnancy Association). First, female C57BL/6J mice were

immunized with MOG, developing EAE. Then rhCG was injected *i.p.* every other day at the mentioned concentrations, beginning at the day of EAE induction. Animals that did not show EAE signs were excluded from the data analysis, the same applied to the animals reaching one of the exclusion criteria as stated in 3.2.2. The animals that developed signs of EAE were scored and weighed daily until day 35.

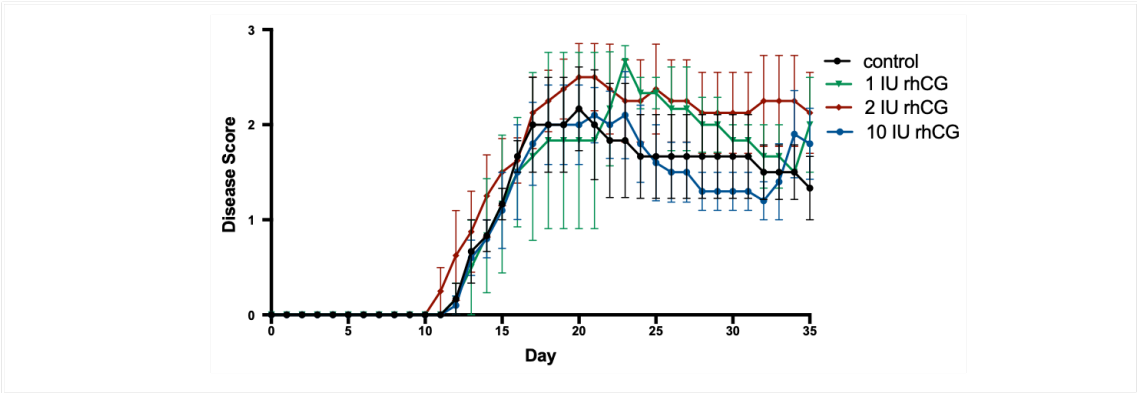


**Figure 3.2: Treatment of MOG-immunized mice with low rhCG concentrations.** MOG-immunized mice were treated every other day with 1 IU (n=3), 2 IU (n=4), 10 IU (n=5) of rhCG or treated with control vehicle (n=3). No significant differences in the EAE disease course were observed when treating MOG-immunized mice with low concentrations of rhCG every other day. Data is displayed as means plus S.E.M.. Data analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Low concentrations of rhCG had no effect on EAE development or progression (Figure 3.2). These results may indicate that the concentration of rhCG applied was too low to induce any detectable effects in the EAE mouse model used. Thus, a higher concentration of rhCG (250 IU) was applied in the next set of experiments. Furthermore, a uhCG-derived preparation (250 IU) was used due to the different properties of the preparations. The rhCG preparation consists of approximately 99 % of the complete hCG form and has therefore a higher purity compared to the urine-derived preparation. However, the uhCG preparation consists of various forms of hCG (hyperglycosylated, free β-unit, intact hCG) [230]. Therefore, a potential effect of uhCG was tested. For this, MOG-immunized mice were treated with 250 IU rhCG or 250 IU uhCG every other day for 27 days starting at the day of EAE induction (Figure 3.3).

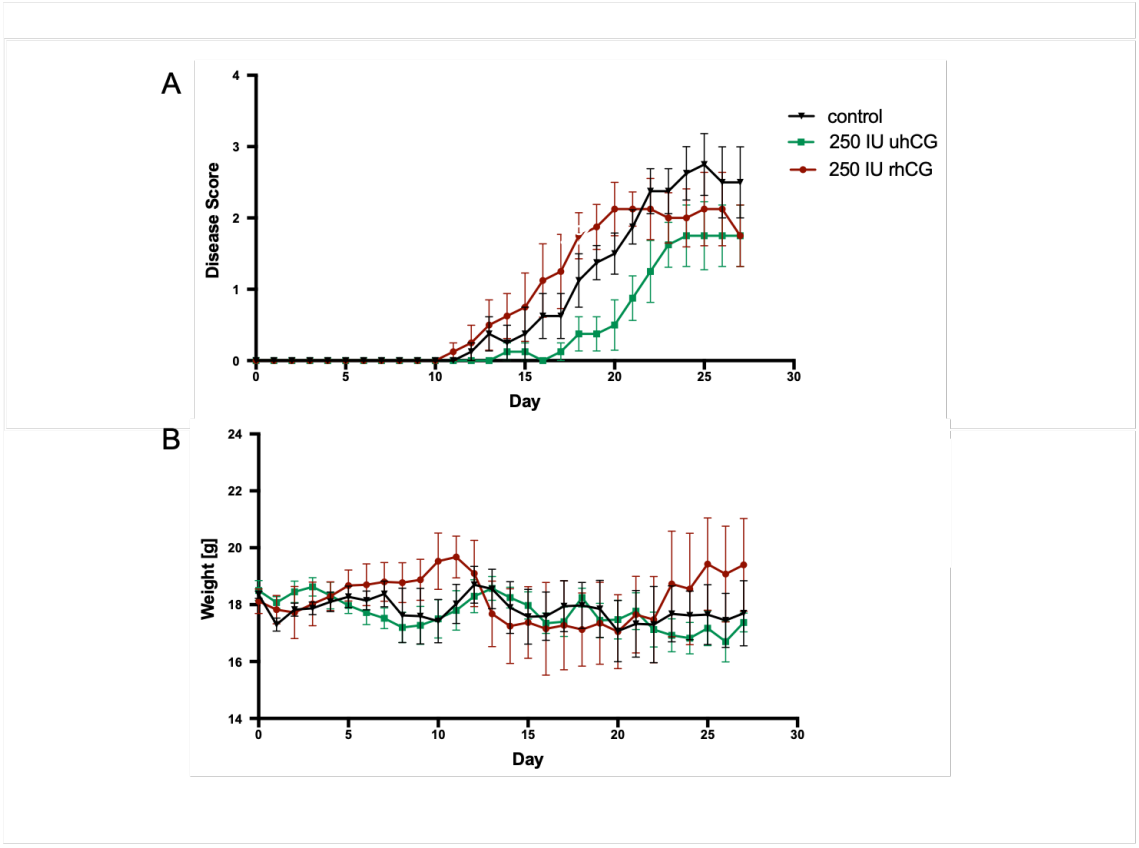


**Figure 3.3: Treatment of MOG-immunized mice with high rhCG and uhCG doses.** MOG-immunized mice, developing EAE, were treated with 250 IU rhCG, 250 IU uhCG, or control vehicle every other day. Included are all animals that reached a disease score indicating disease severity (250 IU rhCG [n=4], 250 IU uhCG [n=4], or control [n=4]). uhCG-treated animals indicated a milder EAE course compared to the control group, whereas rhCG treatment did not alter the course of EAE (A). The weight did not differ in all groups (B). Data is displayed as means plus S.E.M.. Data analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

MOG-immunized mice treated with 250 IU rhCG did not benefit from the treatment. 250 IU uhCG-treated mice showed a milder EAE disease course compared to the control group (Figure 3.3 A). Nonetheless, it was not significant. Despite uhCG treatment, it must be noted that approximately 50 % of the MOG-immunized mice died when treated with uhCG at disease onset. Also the weight did not differ among the groups (Figure 3.3 B). To discern whether the chosen dose of uhCG was harmful, a lower dose of uhCG (150 IU) was investigated for the treatment of EAE.

## 3.3 Preventive treatment of MOG-immunized mice with rhCG or uhCG

### 3.3.1 EAE disease severity was significantly ameliorated in uhCG-treated MOG-immunized mice

After considering the results shown above, treatment of MOG-immunized mice was performed with 150 IU uhCG and 250 IU rhCG per mouse every other day starting at the day of EAE induction. Animals were scored and weighed daily to analyze EAE disease progression until day 35. At day 35, T and B cell populations as well as DC populations were analyzed within the inguinal lymph nodes, spleen, and the CNS.

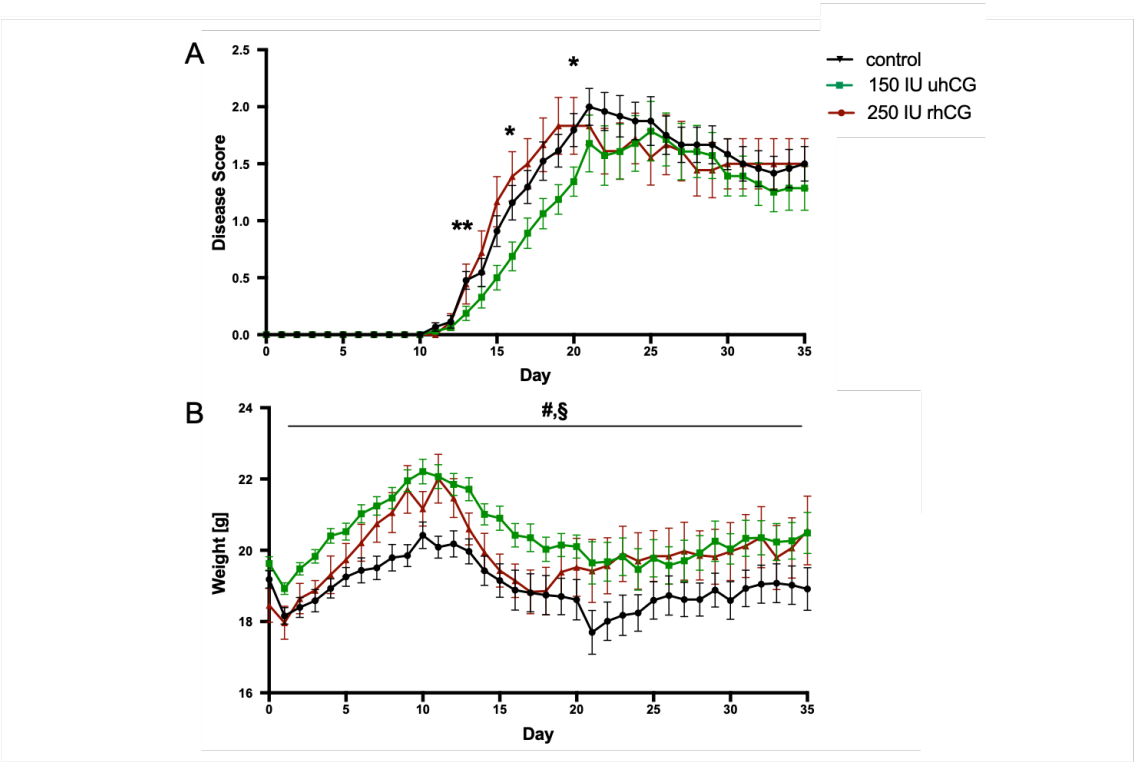


**Figure 3.4: EAE course of MOG-immunized mice after rhCG or uhCG treatment every other day.** MOG-immunized mice developing EAE were treated with 150 IU uhCG (n=32), 250 IU rhCG (n=9) or control vehicle (n=22) every other day from day 0 until day 35. Mice were scored and weighed daily throughout the experiment. The uhCG treated group showed a significant reduced EAE score at day 13, 16, and 20 compared to the control group (A). The overall weight was increased in both hCG treatment groups throughout the experiment (B). Data is shown as means plus S.E.M.. Data analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test (A: * $p<0.05$, ** $p<0.01$; B: uhCG vs. control # $p<0.0001$, rhCG vs. control § $p<0.001$).

The preventive treatment of 150 IU uhCG resulted in a significantly lower EAE score at day 13, 16, and 20 (Figure 3.4 A). uhCG-treated animals showed increased weight throughout the experiment (Figure 3.4 B). Nonetheless, the treatment of MOG-immunized mice again resulted in a 50 % lower survival rate. The rhCG treatment group did not show a significant effect on the EAE score. Additionally, the cumulative score as well as the survival rate was analyzed. To calculate the cumulative score the value of the score of each day was accumulated until day 35.

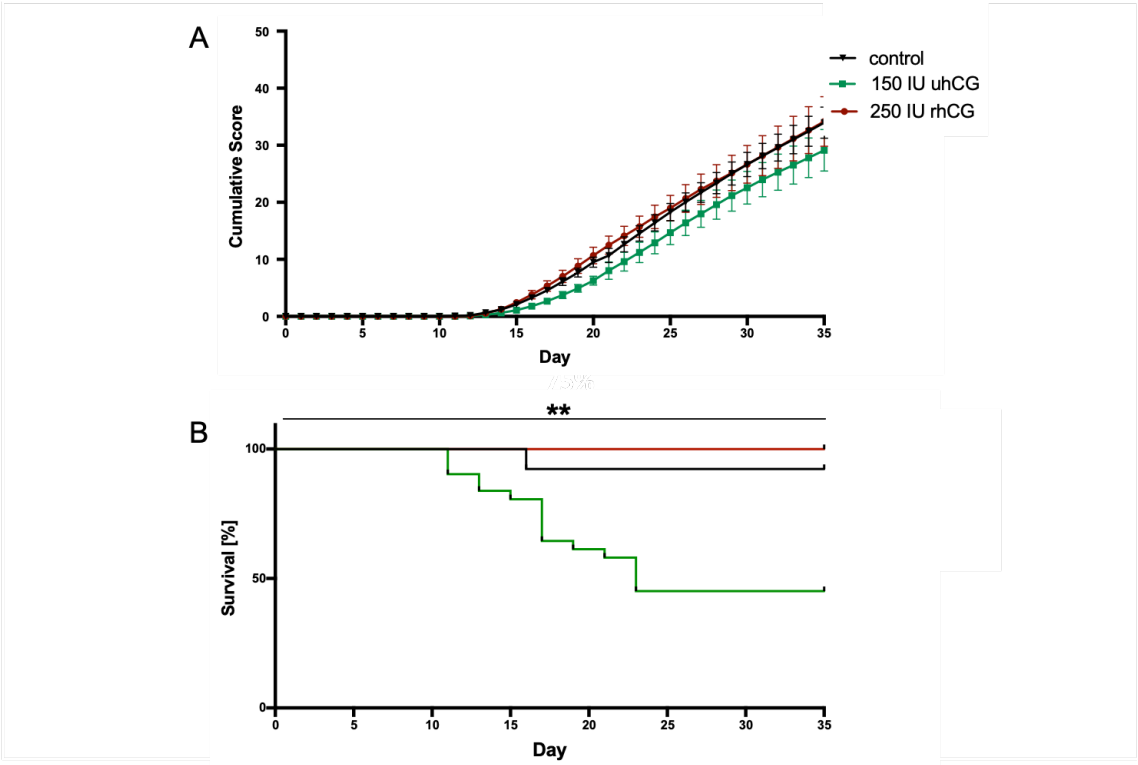


**Figure 3.5: The cumulative score and the survival rate of rhCG- and uhCG-treated MOG-immunized mice.** MOG-immunized mice were treated with 150 IU uhCG (n=32), 250 IU rhCG (n=9), or control vehicle (n=22) every other day from day 0 until day 35. The cumulative score did not result in any significant differences between the groups (A). However, the survival rate of uhCG-treated MOG-immunized mice was significantly lower with approximately 50 % compared to the control and the rhCG treatment group during EAE onset (B). Data is shown as means plus S.E.M.. Statistical analysis was performed using One-way ANOVA (A) or the Logrank (Mantel Cox) test (** $p < 0.002$) (B).

Both hCG treatment groups did not show significant differences in the cumulative score compared to the control group (Figure 3.5 A). However, uhCG treatment of MOG-immunized mice resulted in a significant lower survival rate of approximately

50 % compared to the control and rhCG treatment group. Notably, uhCG-injected animals only died, when first EAE signs appeared (Figure 3.5 B).

### 3.3.2 The treatment with uhCG of MOG-immunized mice resulted in significant reductions of B17 and reduced Th17 cell populations within the CNS at day 35

As a result of the observed EAE score reduction at day 13, 16 and 20, uhCG treatment was investigated at different stages of EAE. It was of high interest to understand whether hCG is able to modulate immune cells within the inguinal lymph nodes, spleen, or CNS at the different stages of EAE (disease onset [d10], initial disease phase [d20], progressive disease phase [d35]). First, hCG-treated MOG-immunized animals were sacrificed during the progressive phase of EAE (day 35) and lymphocytes were isolated and analyzed via flow cytometry. The frequencies of Th17 ($CD4^+IL\text{-}17^+$), Th1 ($CD4^+TNF\alpha^+$, $CD4^+IFN\gamma^+$), B17 ($CD19^+IL\text{-}17^+$) cells and Treg ($CD4^+FoxP3^+$) cells were evaluated. Furthermore, the maturation status of DCs was determined. No differences in the DC maturation state or the Th1 cell frequencies were detected in the inguinal lymph nodes, spleen, or CNS in uhCG-treated MOG-immunized mice compared to MOG-immunized control mice (Supplemental figure A.1).

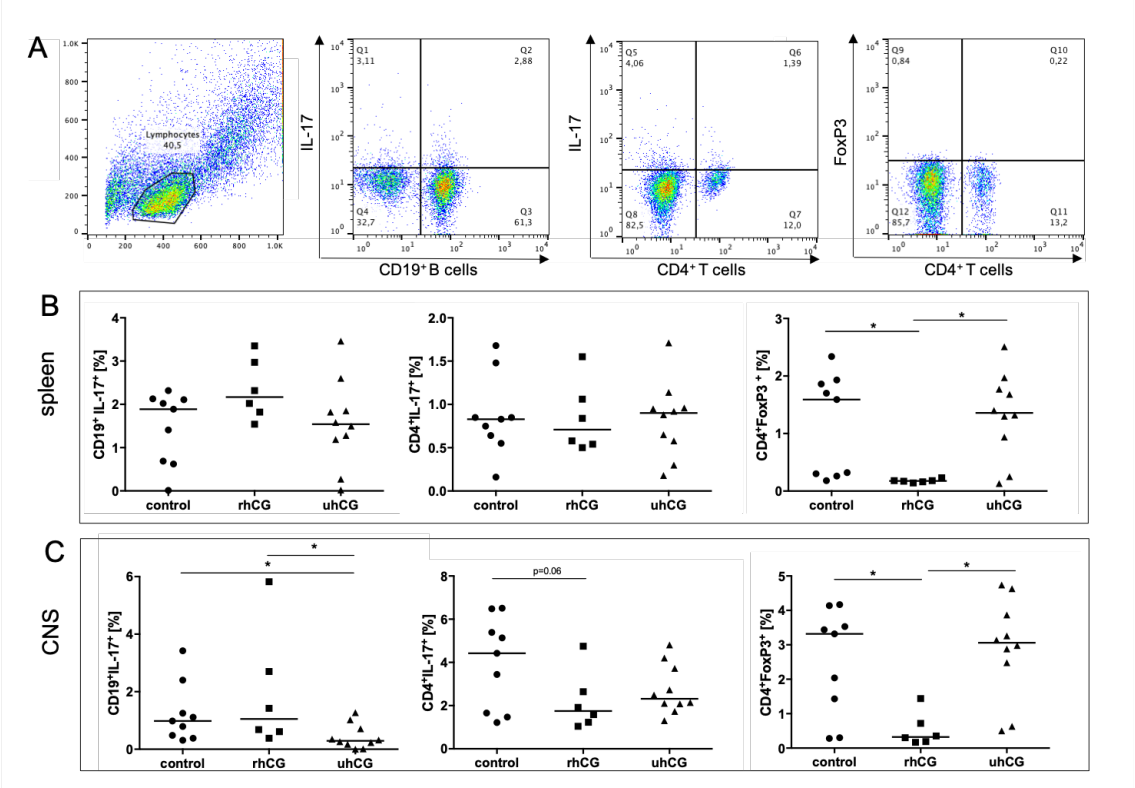


**Figure 3.6: Lymphocyte cell frequencies of rhCG- or uhCG-treated MOG-immunized female mice developing EAE at day 35.** Cell frequencies of lymphocytes isolated from MOG-immunized mice developing EAE treated with 250 IU rhCG (n=6), 150 IU uhCG (n=10) or control vehicle (n=9) at day 35. Lymphocytes were isolated and stimulated for 4.5 h with ionomycin, PMA, brefeldin A and analyzed using a four-color BD Calibur flow cytometer and the Cellquest software (A). In the spleen, neither $CD19^{+}IL\text{-}17^{+}$ (B17) nor $CD4^{+}IL\text{-}17^{+}$ (Th17) cell frequencies were altered, but a significant reduction of $CD4^{+}FoxP3^{+}$ (Treg) cells was found in rhCG-treated mice compared to control mice after EAE development (B). In the CNS, a decrease of Th17 cells in both treatment groups was observed. Treg cells were significantly reduced in rhCG-treated MOG-immunized animals in the CNS (C). In the CNS, a significant reduction of B17 cells was found in uhCG-treated animals, but not in rhCG-treated mice showing signs of EAE. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test (* $p<0.05$).

In the spleen, no alternations in the B17 and Th17 cell frequencies were found after hCG treatment. The Treg frequency was significantly lower in rhCG-treated MOG-immunized animals in the spleen (Figure 3.6 B). Analyzed DC frequencies and the Th1 cell frequency in the inguinal lymph nodes, spleen, and CNS did not reveal any differences (Supplemental figure A.1). In the CNS, a significant reduction of B17 cells in the uhCG-treated MOG-immunized mice was present (Figure 3.6 C). Additionally, Th17 cell frequencies were reduced in both treatment groups in the CNS (Figure 3.6 C). However, there was no significant difference.

Based on the results obtained during the progressive phase of EAE, it can be implied that the hCG-mediated altered B17 or Th17 cell frequencies do not result in altered EAE severity at day 35 in both treatment groups. Therefore, the question arose, whether uhCG is able to modulate different cell subsets at the initial phase of EAE (day 20), when the EAE disease score was significantly reduced.

### 3.3.3 Preventive treatment of MOG-immunized mice with uhCG at the initial disease phase

#### 3.3.3.1 B17, Th17, Th1, and Treg cell frequencies were not altered in the inguinal lymph nodes, spleen or in the CNS at day 20

MOG-immunized mice treated with uhCG every other day beginning at the day of EAE induction were sacrificed on day 20. T and B cell frequencies were analyzed using the multi-colored flow cytometer Attune NxT (Figure 3.7 A).

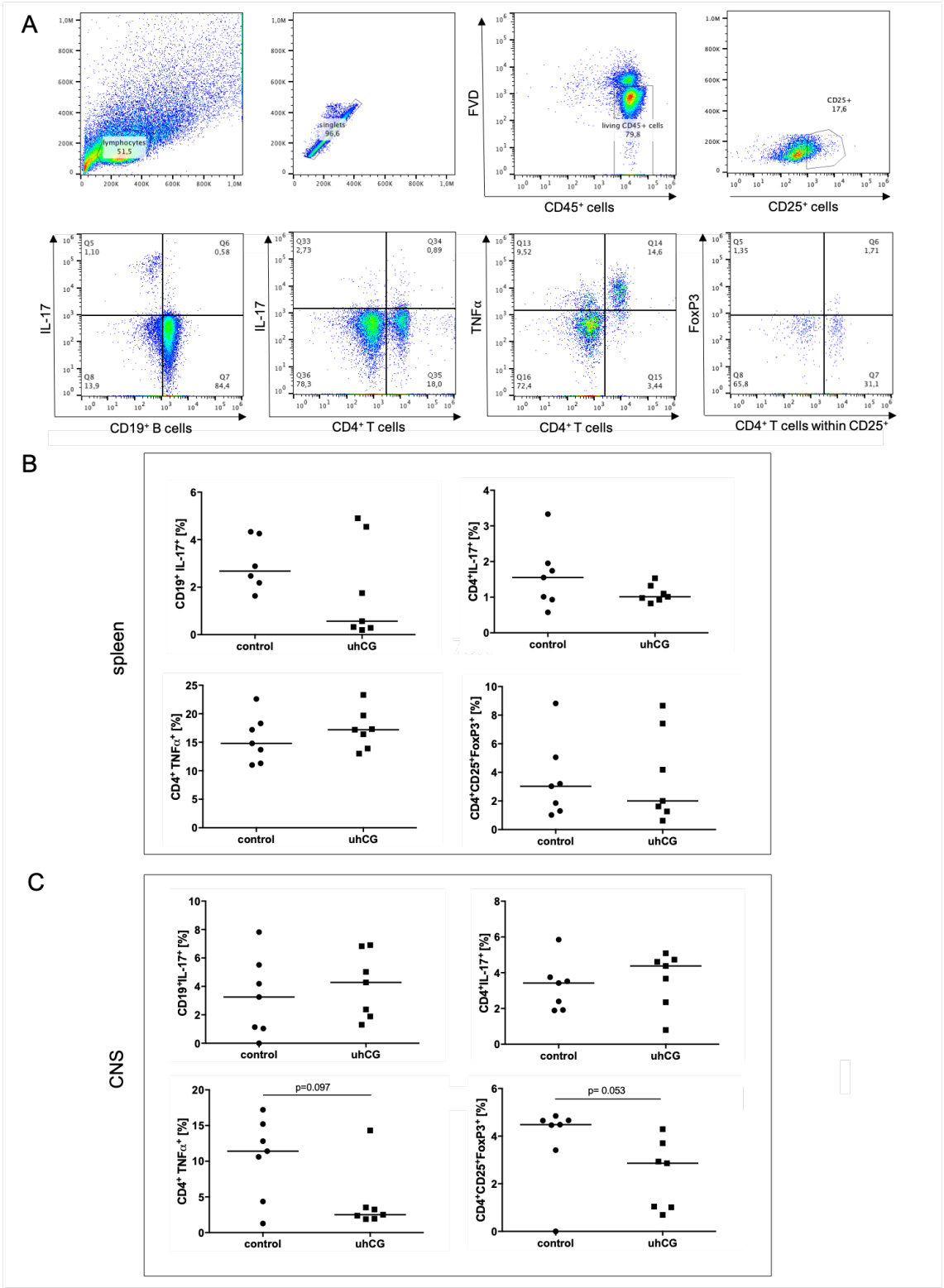


**Figure 3.7: Lymphocyte cell frequencies at day 20 p.i. after preventive uhCG treatment.** Lymphocyte cell frequencies of MOG-immunized mice at day 20 treated with uhCG (n=7) or control vehicle (n=6-7) every other day. Lymphocytes were isolated by density gradient centrifugation and stimulated for 5 h using ionomycin, PMA, and brefeldin A. For flow cytometry analysis, lymphocytes were gated, doublets and dead cells within the $CD45^+$ cell population were excluded and the cell population of interest was analyzed (A). The frequencies of B17, Th17, Th1, or Treg cells were not altered significantly at day 20 after uhCG treatment in the spleen (B). Within the CNS there was a reduced Treg population in uhCG-treated MOG-immunized animals. Nonetheless, it was not significant (C). Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Mann-Whitney-$U$ test.

As shown in figure 3.7 no significant differences in B17, Th17 and $CD4^+TNF\alpha^+$ cell frequencies were observed between uhCG-treated and control MOG-immunized mice. Interestingly, in the CNS there was a decrease of the Treg cell frequency in uhCG-treated animals compared to control MOG-immunized animals (Figure 3.7 C). However, this was not significant. Furthermore, Breg, Treg, and Th1 cell frequencies were not altered in the inguinal lymph nodes, spleen, and CNS (Supplemental figure A.2).

#### 3.3.3.2 The cytokine release of IL-4, IL-17, IL-6, and IL-10 by lymphocytes was not altered in uhCG-treated MOG-immunized mice at day 20

Although the frequencies of T and B cell populations were not altered in any organs analyzed (inguinal lymph nodes, spleen, CNS), it was of interest to investigate the *ex vivo* ability of immune cells, previously isolated from MOG-immunized mice treated with uhCG or control, to secrete cytokines. For this, lymphocytes isolated from inguinal lymph nodes and spleen of MOG-immunized mice at day 20 were stimulated for 5 h with ionomycin and PMA. The supernatant was collected and the amount of seven pro-inflammatory and anti-inflammatory cytokines was determined using CBArray technique. Unfortunately, the number of cells from CNS samples was too low, so it was not possible to analyze cytokine secretion.

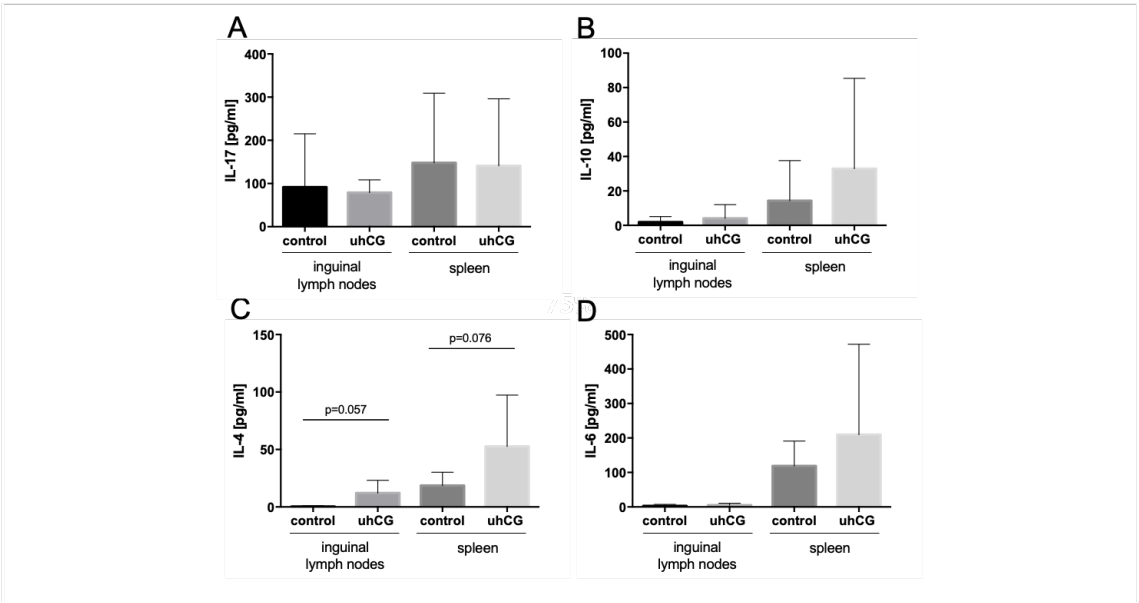


**Figure 3.8: Cytokine release by lymphocytes of inguinal lymph nodes and spleen at day 20 p.i. after preventive uhCG treatment.** Splenic (control n=7, uhCG n=7) and inguinal lymph node (control n=3, uhCG n=4) lymphocytes were stimulated with ionomycin and PMA for 5 h and the supernatant was collected. Using a Th1/Th2/Th17 CBArray several pro- and anti-inflammatory cytokines were measured. Elevated IL-4 levels were detected in the supernatant of the inguinal lymph nodes and splenic lymphocytes when treated with uhCG after EAE induction (C). However, this was not statistically significant. No differences in IL-17 (A), IL-10 (B) or IL-6 (D) cytokine secretion were found. Data is displayed as means plus S.D.. Statistical analysis was performed using the Mann-Whitney-$U$ test.

No significant differences between the uhCG-treated and control group of MOG-immunized mice were found concerning IL-2, IL-6, IL-10, IL-17, TNF$\alpha$, or IFN$\gamma$ (Figure 3.8 and Supplemental figure A.3). However, IL-4 cytokine secretion by lymphocytes increases after uhCG treatment of MOG-immunized mice at day 20, but it was not statistical significant (Figure 3.8 C).

#### 3.3.3.3 mRNA expression of *IL-17, IL-10*, and *IL-4* was not altered in samples from uhCG-treated MOG-immunized mice at day 20

Due to low numbers of lymphocytes that could be isolated from CNS tissue at day 20, *IL-17, IL-10*, and *IL-4* mRNA expression of whole tissue CNS samples was analyzed using qRT-PCR. mRNA was isolated from CNS tissue of uhCG-treated or

control MOG-immunized mice at day 20 and cDNA was synthesized. Afterwards, *IL-17, IL-10*, and *IL-4* mRNA expression, relative to the house keeping gene β-*actin*, was determined using qRT-PCR.

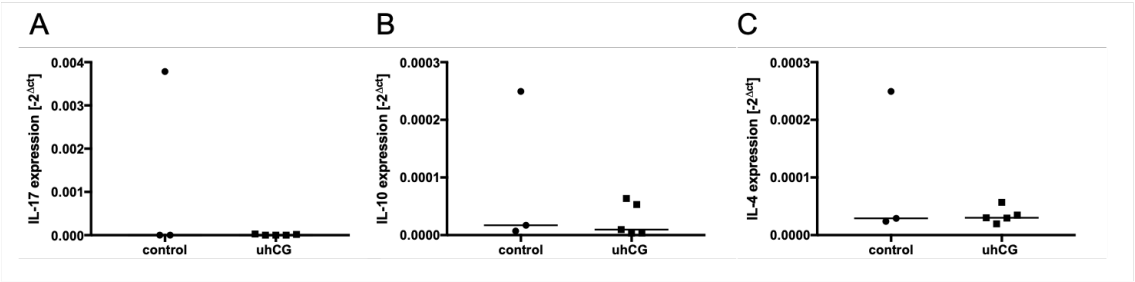


**Figure 3.9: *IL-17, IL-10*, and *IL-4* mRNA expression in CNS tissue of uhCG-treated MOG-immunized mice at day 20 p.i..** No differences were found in the expression of *IL-17, IL-10*, or *IL-4* in CNS tissue of MOG-immunized mice, developing EAE, treated with 150 IU uhCG (n=5) or control vehicle (n=3) every other day until day 20. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Mann-Whitney-*U* test.

Analysis of the CNS tissue revealed no differences in the local *IL-17, IL-10*, or *IL-4* mRNA expression in uhCG-treated MOG-immunized mice compared to the control group (Figure 3.9).

#### 3.3.3.4 No differences were detectable in the demyelination grade of the spinal cord in uhCG-treated compared to control MOG-immunized mice

To verify if uhCG has an effect on demyelination that would explain the significantly reduced disease score observed at day 20, paraffin sections of spinal cords of the control and uhCG-treated MOG-immunized animals were analyzed using LFB staining. Here, myelinated areas (white matter) are stained in violet-blue and the non-myelinated areas remain unstained. A healthy spinal cord group was included for comparison with the MOG-immunized treatment groups. One exemplary photo of the LFB staining illustrating a section of the spinal cord (region L1-L3) per group is shown (Figure 3.10).

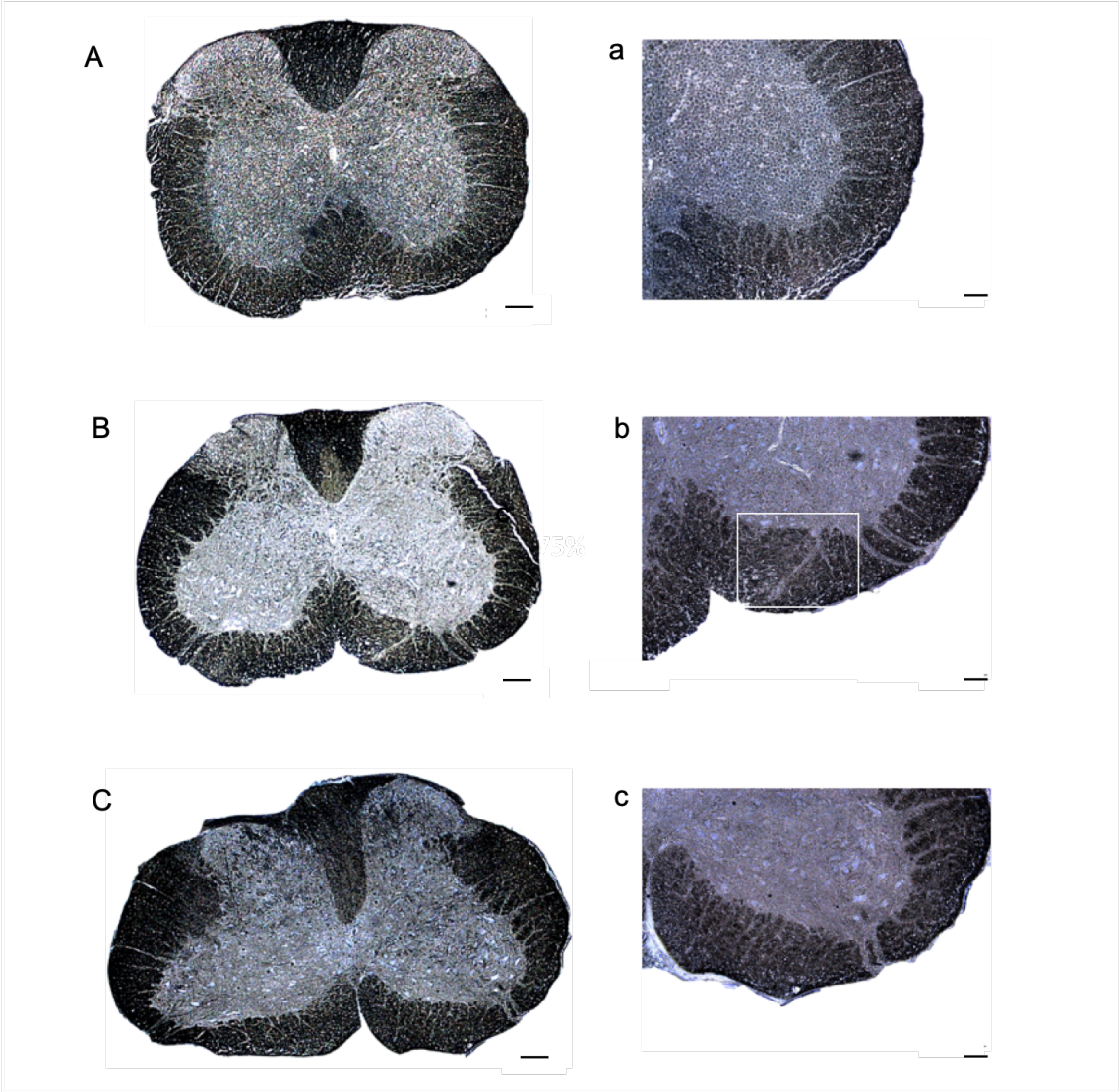


**Figure 3.10: Demyelination grade of the spinal cord of uhCG-treated or control MOG-immunized mice at day 20.** Shown are exemplary photographs of a LFB stained spinal cord section of a healthy control (A), the control MOG-immunized group (B) and the uhCG-treated MOG-immunized group (C). No differences were found in the demyelination within the spinal cord of uhCG-treated (C) and control MOG-immunized mice (B) in the L1-L3 region. The analysis revealed a diffuse morphological structure of the spinal cord of MOG-immunized mice (B, C) compared to healthy controls (A). Shown is an overview of the LFB staining of the spinal cord using a 2.5x magnification (A-C, scale bar: 200µm). A closer insight in the white matter area, reveals a possible decreased myelination in control MOG-immunized animals (b) compared to the uhCG-treated MOG-immunized animals (c) at day 20 p.i. using a 10x magnification (a-c, scale bar: 100µm).

As expected MOG-immunized mice showed a more diffuse tissue structure in the spinal cord (region L1-L3) compared to healthy mice (Figure 3.10 A-C). However, no significant changes in demyelination were found in the spinal cord of uhCG-treated MOG-immunized animals compared to the MOG-immunized controls at day 20 (Figure 3.10 B, C).

### 3.3.4 uhCG-treated animals did not show altered lymphocyte cell frequencies at disease onset

It was aimed to investigate if uhCG was able to modulate T and B cell frequencies before disease onset (day 10) that would result in an ameliorated EAE disease progression later on. Additionally, Breg cells were analyzed due to a possible interdependency of Breg cells and B17 cells which were decreased at the progressive disease phase. Thus, MOG-immunized mice were treated with uhCG every other day until day 10 when initial signs of EAE appear. Lymphocytes of the spleen, CNS (Figure 3.11 B, C), and inguinal lymph nodes (Supplemental figure A.4) were isolated and analyzed by flow cytometry using the Attune NxT flow cytometer.

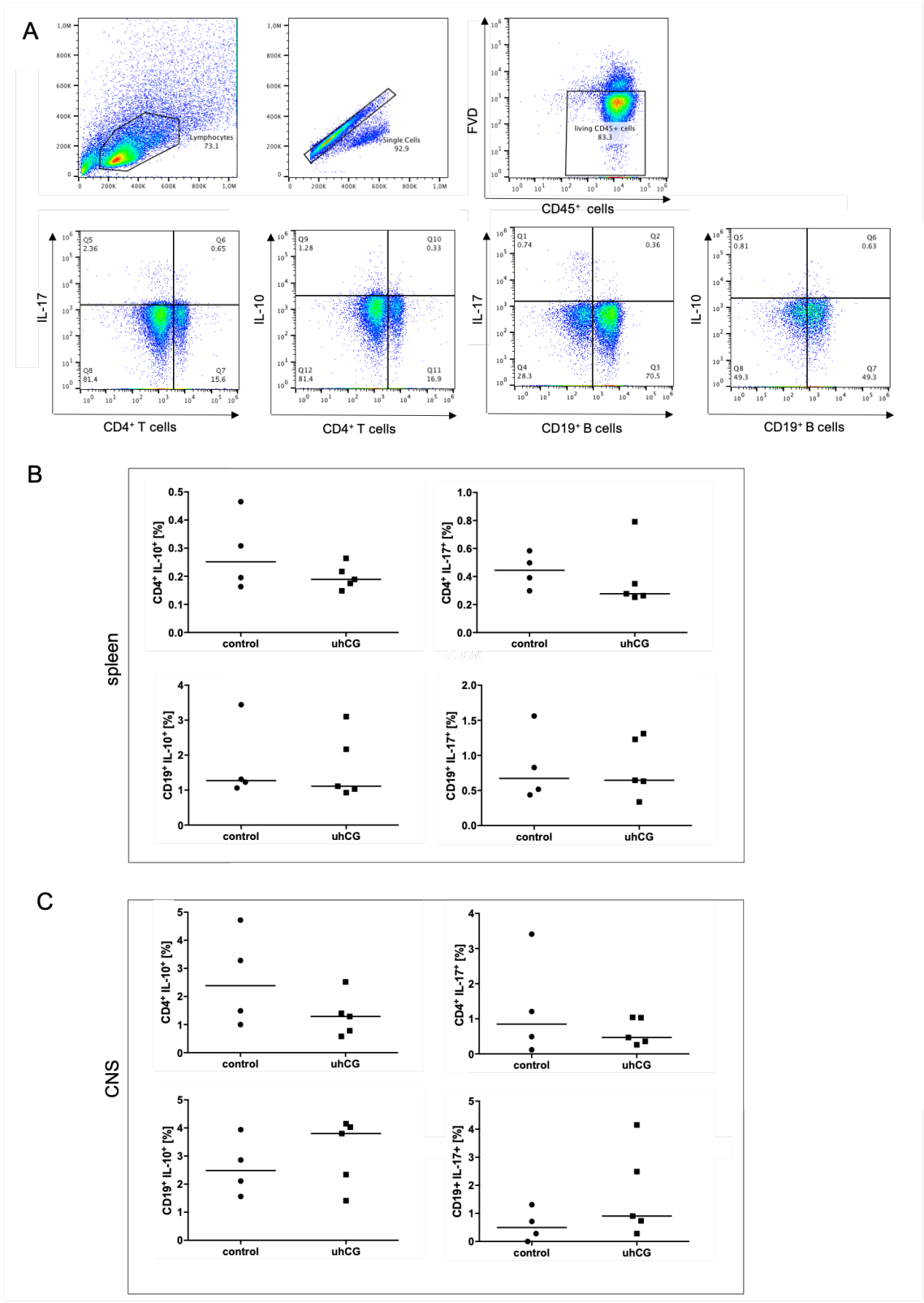


**Figure 3.11: Lymphocyte frequencies at day 10 p.i. after preventive uhCG treatment.** Lymphocytes were isolated from MOG-immunized mice at day 10 after treatment with uhCG (n=5) or control vehicle (n=4) every other day. For flow cytometry analysis, lymphocytes and single cells were gated and dead cells were excluded using FVD (A). No significant differences were found in uhCG-treated MOG-immunized mice when comparing Th17, B17, Treg, or $CD19^{+}IL\text{-}10^{+}$ (Breg) cell frequencies within the spleen (B) or CNS (C). Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Mann-Whitney-*U* test.

No significant differences were found in the cell frequencies of lymphocytes isolated from spleen, CNS (Figure 3.11 B, C), or inguinal lymph nodes (Supplemental figure A.4) regarding Treg, Th17, B17, or $CD19^{+}IL\text{-}10^{+}$ (Breg) cells. Furthermore, analysis of $CD4^{+}IL\text{-}10^{+}IL\text{-}17^{+}$ cells was performed to check whether $CD4^{+}$ T cells secreting IL-10 and IL-17 were simultaneously altered after uhCG treatment. No differences were found regarding the Th1 cell frequencies, $CD4^{+}IL\text{-}10^{+}IL\text{-}17^{+}$ cells, or Treg cell frequencies in the spleen and CNS (Supplemental figure A.5).

## 3.4 Reduced survival rate in uhCG-treated MOG-immunized mice after uhCG injection right at EAE onset

The preventive treatment of uhCG led to death of approximately 50 % of the MOG-immunized animals immediately after uhCG injection when first EAE signs appear. This led to the assumption that the uhCG preparation in correlation with the time point of EAE onset drives a mechanism resulting in the ”sudden death” of approximately half of the uhCG-treated MOG-immunized mice. To further study the underlying mechanisms for this unexpected observation an additional study group, the “sudden death” group, was included in this study.

### 3.4.1 No altered innate and adaptive immune response was observed in uhCG-treated MOG-immunized mice showing ”sudden death”

To analyze whether the uhCG treatment during EAE disease onset may cause an immune overreaction leading to the ”sudden death” phenomenon that occurred within an hour after uhCG injection (between day 10 and 20 after EAE induction), every mouse that was injected with uhCG was particularly monitored. In case of signs preceding the “sudden death” phenomenon like apathy, convulsions, or isolation from the group, animals were sacrificed and lymphocytes from inguinal lymph nodes, spleen, and CNS were isolated for flow cytometry analysis and cytokine

measurements. Furthermore, liver, kidney, and inguinal lymph nodes were collected for histology.

For the evaluation of a potential overreaction of the innate immune system after the uhCG injection in combination with EAE disease onset leading to death, specific markers for innate immune cell subsets (CD14, Ly6G) were analyzed in the inguinal lymph nodes (Supplemental figure A.6), spleen, and CNS. It must be noted that control-treated MOG-immunized mice were used as a control. The appropriate control would have been uhCG-treated MOG-immunized mice. However, the "sudden death" only occurred right at EAE onset. Therefore, it was not possible to use uhCG-treated MOG-immunized animals which later will show EAE signs because one could not be sure whether the mouse used as a control at this time point would have later developed signs of EAE. Furthermore, the number of uhCG-treated MOG-immunized animals developing EAE was limited and needed for further studies.

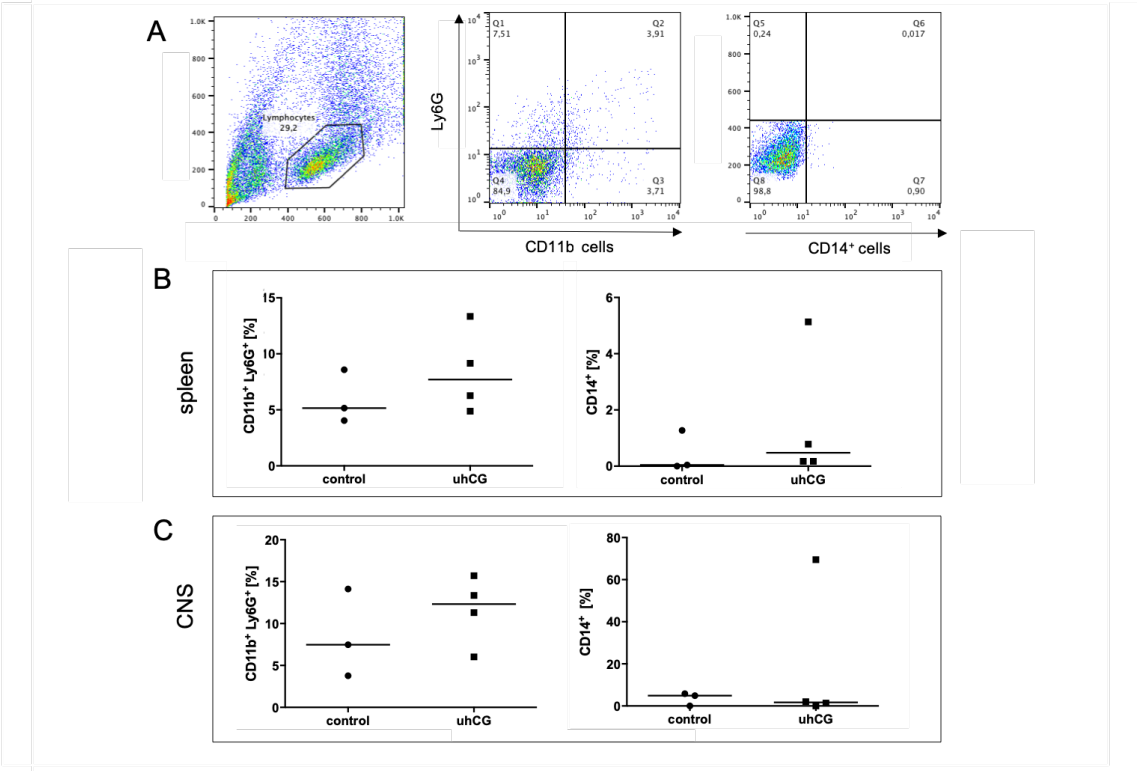


**Figure 3.12: Monocyte and neutrophil cell frequencies of MOG-immunized mice treated with uhCG or control undergoing the "sudden death" phenomenon.** For flow cytometry analysis, lymphocytes were isolated and analyzed using a four-color BD Calibur flow cytometer and the Cell Quest software (A). Monocytes expressing CD14 did not show any significant alteration in the "sudden death" group after uhCG injection in the spleen (B) or CNS (C). Neutrophils did also not show significant differences in uhCG-treated MOG-immunized animals in the spleen (B) or CNS (C) compared tio MOG-immunized controls. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Mann-Whitney-$U$ test.

No significant differences in the frequency of monocyte or neutrophils were found in the spleen, CNS (Figure 3.12 B, C), or inguinal lymph nodes (Supplemental figure A.6) in MOG-immunized "sudden death" mice compared to control MOG-immunized animals. Next, a potential overwhelming reaction of the adaptive immune system was taken into consideration. Therefore, the Th17 and Th1 cell population was further analyzed.

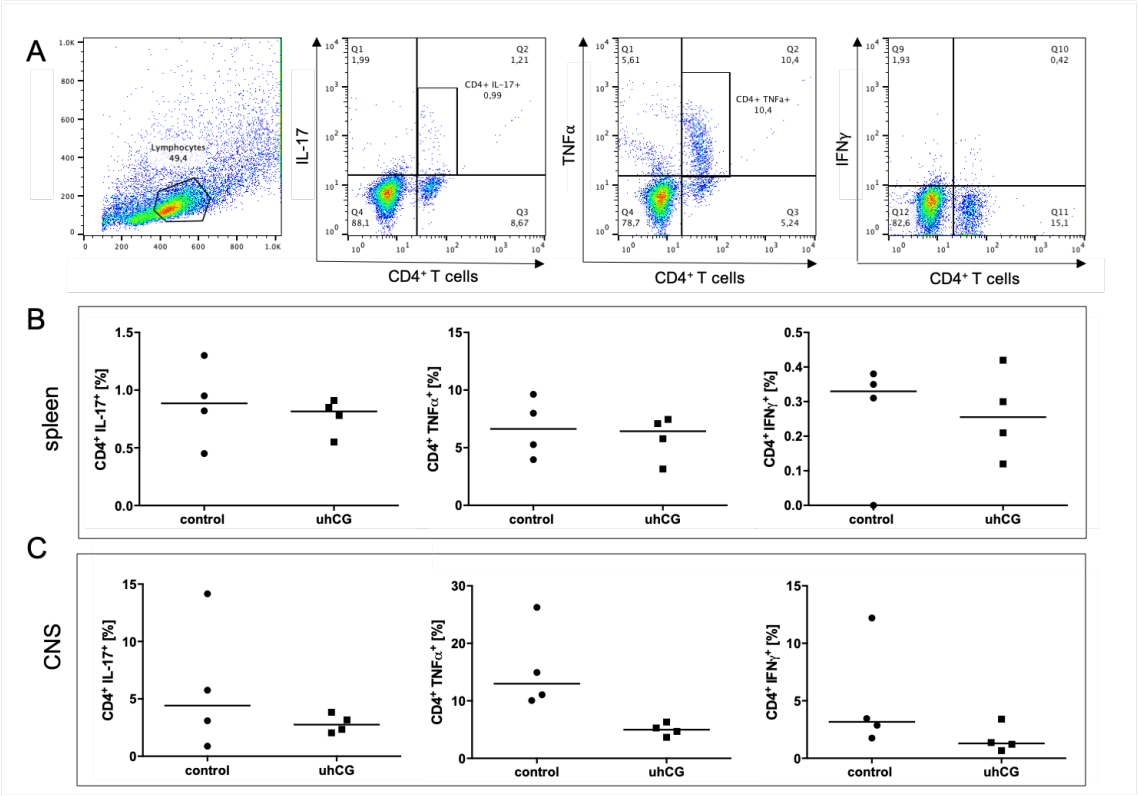


**Figure 3.13: Th17 and Th1 cell frequencies of MOG-immunized mice treated with uhCG undergoing the "sudden death" phenomenon.** For flow cytometry analysis, lymphocytes were isolated and analyzed using a four-color BD Calibur flow cytometer and the Cell Quest software (A). Th17 and Th1 cell frequencies were analyzed. Neither in the spleen (B) nor in the CNS (C) significant differences were found in uhCG-treated MOG-immunized "sudden death" animals compared to MOG-immunized controls. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Mann-Whitney-$U$ test.

The evaluation of an altered adaptive immune response (Th1 and Th17 cell frequencies) in MOG-immunized mice showing "sudden death" resulted in no changes (Figure 3.13 B,C). Also, the analysis of Th1 and Th17 cell frequencies in the inguinal lymph nodes did not show any differences (s Supplemental figure A.7). Hence, it is not clear, which mechanisms underlie this "sudden death" phenomenon *in vivo* when applying uhCG to mice developing EAE during disease onset. It is obviously not an immune phenomenon including monocytes, neutrophils, Th1 or Th17 cells.

### 3.4.2 The cytokine release of uhCG-treated MOG-immunized mice showing the "sudden death" phenomenon was not altered compared to the controls

In order to check whether "sudden death" animals suffered from an overwhelming cytokine release, the cytokine secretion of IL-17, TNF$\alpha$, IFN$\gamma$, IL-2, IL-10, IL-6, and IL-4 by lymphocytes from spleen and inguinal lymph nodes was analyzed using a CBArray.

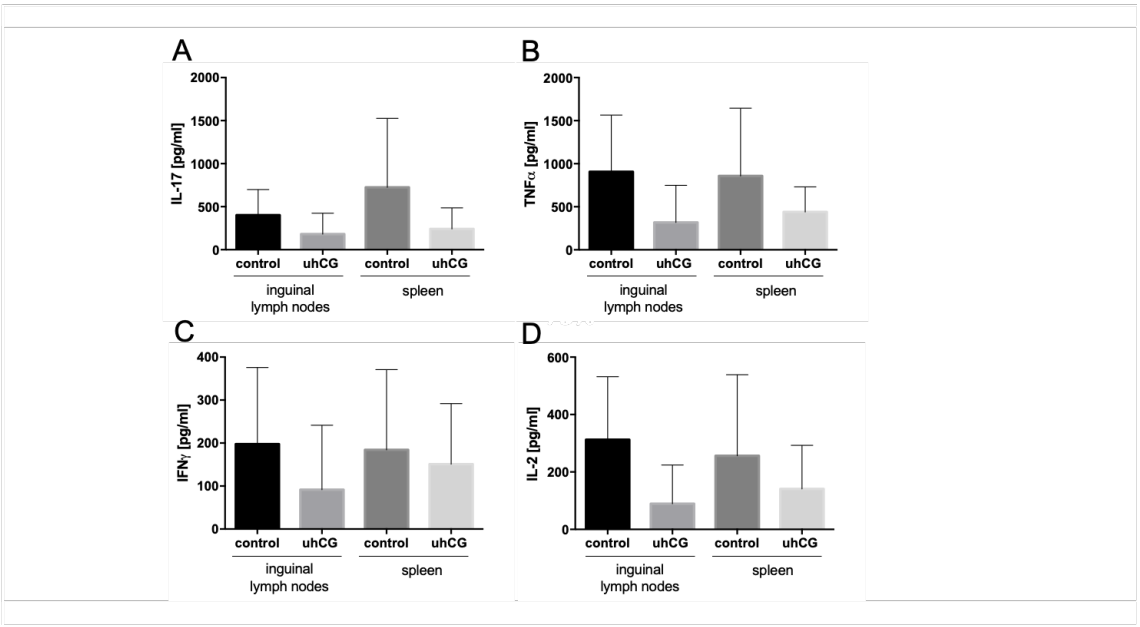


**Figure 3.14: Cytokine release by lymphocytes isolated from MOG-immunized mice showing the "sudden death" phenomenon after uhCG treatment.** Lymphocytes of MOG-immunized mice showing the "sudden death" phenomenon between day 10 and day 20 after uhCG treatment were isolated and stimulated with ionomycin and PMA for 5 h. Afterwards, the supernatant was collected and analyzed using a CBArray. No significant differences were found for all cytokines analyzed such as IL-17 (A), TNF$\alpha$ (B), IFN$\gamma$ (C), and IL-2 (D) in uhCG-treated MOG-immunized animals (n=4) compared to MOG-immunized controls (n=4). Data is displayed as means plus S.D.. Statistical analysis was performed using the Mann-Whitney-$U$ test.

IL-17, TNF$\alpha$, and IFN$\gamma$ cytokine secretion was not changed in lymphocytes in the "sudden death" group after uhCG treatment precluding an exacerbated pro-inflammatory reaction compared to controls (Figure 3.14 A-C). In the same time, IL-2 (Figure 3.14 D), IL-10, IL-6, and IL-4 (Supplemental figure A.7) secretion was not altered. Overall, no significant alterations were observed in the cytokine

secretion of the analyzed cytokines. Thus, an altered cytokine release cannot be associated with the sudden death phenomenon affecting the uhCG treatment group.

### 3.4.3 No morphological changes in the kidney, liver, and inguinal lymph nodes of uhCG-treated MOG-immunized mice showing ”sudden death” compared to the control

To analyze potential morphological changes in organs of uhCG-treated MOG-immunized mice, paraffin sections of the kidney, inguinal lymph nodes, and liver collected from the “sudden death” animals and control animals were prepared. HE staining was performed and the tissue was analyzed using the Zeiss Axio Hall 10 microscope.

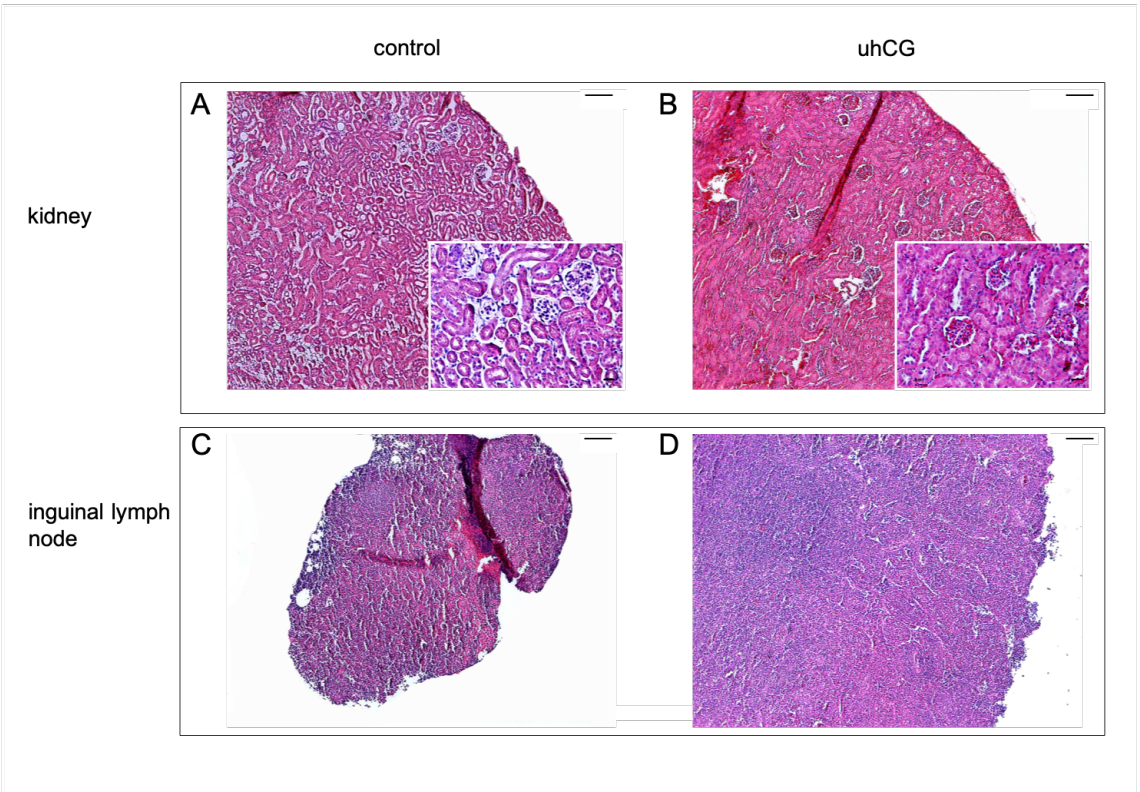


**Figure 3.15: Morphological analysis of uhCG-treated MOG-immunized mice showing "sudden death" compared to the control.** Shown are representative photos of a HE staining of the "sudden death" uhCG-treated MOG-immunized animals and the control group. No differences were present in the morphology of the kidney (A, B) and inguinal lymph nodes (C, D). Shown are detailed views of photographs with a 10x (A-D; scale bar: 200 µm) and a 20x magnification (A,B ; scale bar: 100 µm).

No significant differences in the morphological overview of the kidney, inguinal lymph nodes (Figure 3.15), or liver (Supplemental figure A.8) were found in the "sudden death" uhCG treatment group compared to the control group (Figure 3.15). Even though the same magnification was used when analyzing the tissue of the inguinal lymph nodes, it seems that the inguinal lymph nodes in the uhCG-treated MOG-immunized mice were enlarged. Nonetheless, no photo of the inguinal lymph nodes were taken right after tissue collection to confirm this result. This shows that the "sudden death" phenomenon is not accompanied with obvious morphological changes in the organs analyzed and a detailed analysis of the tissue structure was not further investigated.

## 3.5 Therapeutic treatment of MOG-immunized mice with rhCG or uhCG did not alter EAE disease course

After evaluation of the preventive hCG treatment at disease onset, the initial phase, and the progressive phase of EAE, a possible role of hCG as a therapeutic treatment option was investigated. This setting has translational value, as the treatment of a MS patient may start after first disease symptoms occur. MOG-immunized mice were treated therapeutically every other day after EAE onset. The therapeutic treatment started individually when a mouse showed the first signs of EAE. Then, MOG-immunized animals were treated with 250 IU rhCG (n=2), 150 IU uhCG (n=5), or control vehicle (n=3) every other day until day 35.

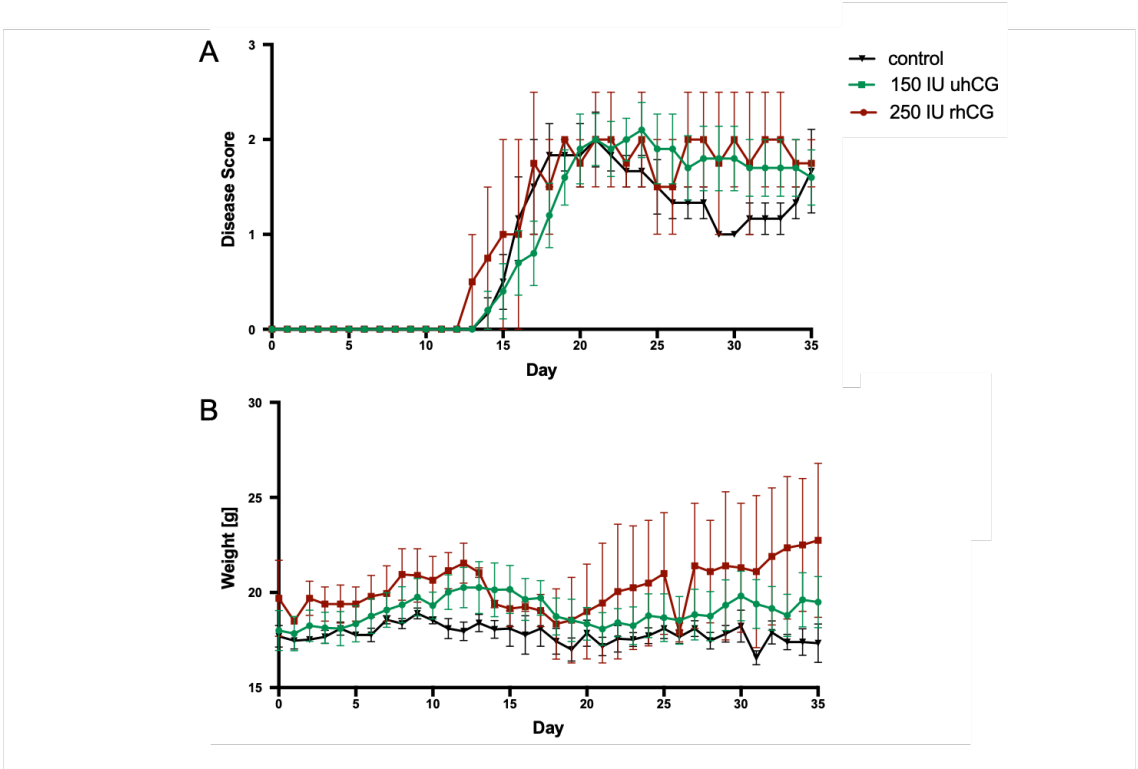


**Figure 3.16: Therapeutic treatment with rhCG or uhCG of MOG-immunized mice.** MOG-immunized mice were treated every other day until day 35 with 150 IU uhCG (n=5), 250 IU rhCG (n=2), or control (n=3) beginning when first EAE signs were visible. Each animal was scored and weighed daily. No significant differences were found in the hCG-treated groups with regard to EAE severity (A) or weight (B). Data is displayed as means plus S.E.M..

Even though the preventive treatment with uhCG showed a milder EAE disease score at the initial phase of the disease (day 13, 16, 20), the therapeutic treatment did not show any beneficial effect on EAE severity. This shows that neither

rhCG nor uhCG are able to reduce EAE severity after its onset (Figure 3.16 A). Also, the weight did not differ between uhCG-, rhCG-, or control vehicle-treated MOG-immunized mice (Figure 3.16 B).

## 3.6 B17 cells as potential mediators in EAE

The analysis of the different lymphocyte populations at the progressive phase of EAE brought an interesting novel B cell subset into focus. During the last decade, several studies concentrated on the participation of B cells on MS and EAE progression as well as pathology [109–112]. However, the majority of studies focused on the antibody production by B cells [117].

B cells are also able to produce and secrete cytokines and among these IL-17 and IL-10. IL-17 is a key regulator in MS as well as in EAE and has been shown to negatively affect disease severity in regard to Th17 cells [234]. B cells secreting IL-10 (Breg cells) are playing a rather protective role [126]. As hCG was described to induce IL-10 production in B cells and thereby enhance Breg cells, it was of interest to study if hCG can regulate the balance between B17 and Breg cells. This was already confirmed by our group highlighting that hCG can modulate the balance of Th17 and Treg cells [218]. Therefore, B cells were obtained from MOG-immunized mice from the spleen for *ex vivo* studies.

First of all, different MOG (20 µg/ml, 50 µg/ml, 80 µg/ml, 100 µg/ml) and uhCG (100 IU/ml, 150 IU/ml, 200 IU/ml, 250 IU/ml, 300 IU/ml) concentrations were tested *ex vivo* for 24 and 48 h. The effect of each concentration to alter B17 cell frequencies was analyzed and the appropriate concentration of MOG and uhCG was used for further experiments (data not shown). The titration of the different concentrations of MOG and uhCG revealed a concentration of 20 µg/ml MOG and a concentration of 250 IU/ml uhCG to be best for the following *ex vivo* assays.

### 3.6.1 uhCG reduced B17 cell frequencies within the B cell population *ex vivo*

During the preventive uhCG treatment, a significant reduction of B17 cells during the progressive EAE phase (day 35) within the CNS was found. To evaluate if B cells can be modulated by uhCG *ex vivo*, total $CD19^+$ B cells were isolated from the spleen of MOG-immunized animals that were not treated with uhCG beforehand on day 20. B cells were cultured with 20 µg/ml MOG and/or 250 IU/ml uhCG for 24 or 48 h. Afterwards, the frequencies of $CD19^+$IL-$17^+$ (B17) cells and $CD19^+$IL-$10^+$ (Breg) were measured using the Attune NxT flow cytometer (Figure 3.17).

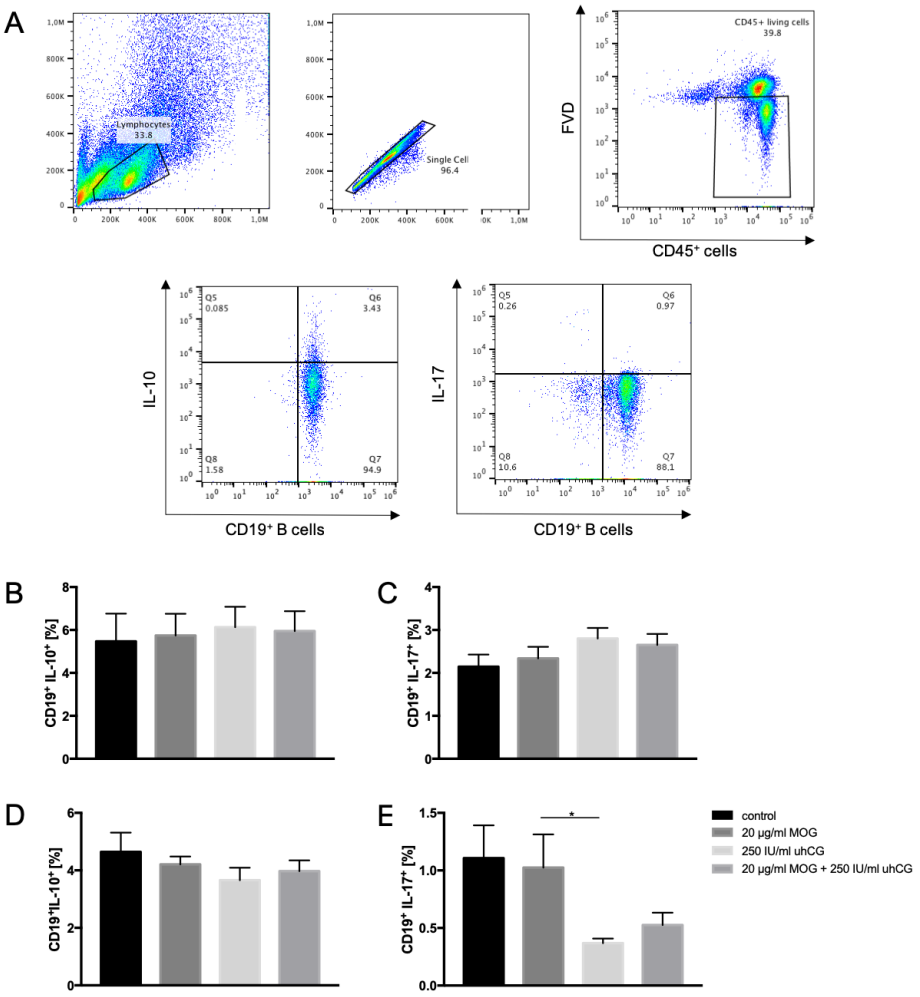


**Figure 3.17: B17 cell frequencies after uhCG treatment *ex vivo*.** For flow cytometry analysis the lymphocytes and single cells were gated and dead cells were excluded using FVD (A). After 24 h no significant differences were present concerning B17 and Breg cells cultured with MOG, uhCG, or both (B, C). The evaluation of both cell subsets after 48 h revealed that there was a significant reduction of B17 cells when treated with uhCG alone compared to MOG stimulated B cells (E). The combined culture with uhCG and MOG showed a reduction of B17 cells (E). However, this was not significant. No differences were found in the percentages of $CD19^{+}IL\text{-}10^{+}$ cells after 48 h (D). Data are displayed as means (n=5) plus S.D.. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparisons test (* $p<0.05$).

The addition of 20 µg/ml MOG did not result in an increment of the percentages of B17 cells after 24 h. Moreover, MOG did not alter the Breg cell frequencies (Figure 3.17 B, D). In line, the treatment with uhCG alone or in combination with MOG did not influence both B cell frequencies after 24 h. After 48 h, stimulation with MOG did not enhance the B17 cell frequency (Figure 3.17 E). However, the B17 cell frequency was significantly reduced in the presence of uhCG when comparing with MOG stimulated B cells (Figure 3.17 E). This data shows that uhCG is able to reduce the B17 cell frequency within the B cell population *ex vivo*.

### 3.6.2 No B17 generation of selected B10 cells was observed after MOG restimulation

Finally, the question arose if B cells display a distinct plasticity indicating the ability of B cells to convert from IL-10-producing to IL-17-producing B cells. To investigate this effect, IL10eGFP mice were immunized with MOG to develop EAE and sacrificed at day 20. This specific IL10eGFP mouse strain enables the sorting of GFP-labeled $CD19^+$ B cells secreting IL-10 for further *ex vivo* analysis. Lymphocytes from the spleen of MOG-immunized IL-10eGFP mice were isolated and stained for CD19. $CD19^+IL\text{-}10^+$ cells were then sorted and cultured with 20 µg/ml MOG, 250 IU/ml uhCG, or the combination of both for 24 h.

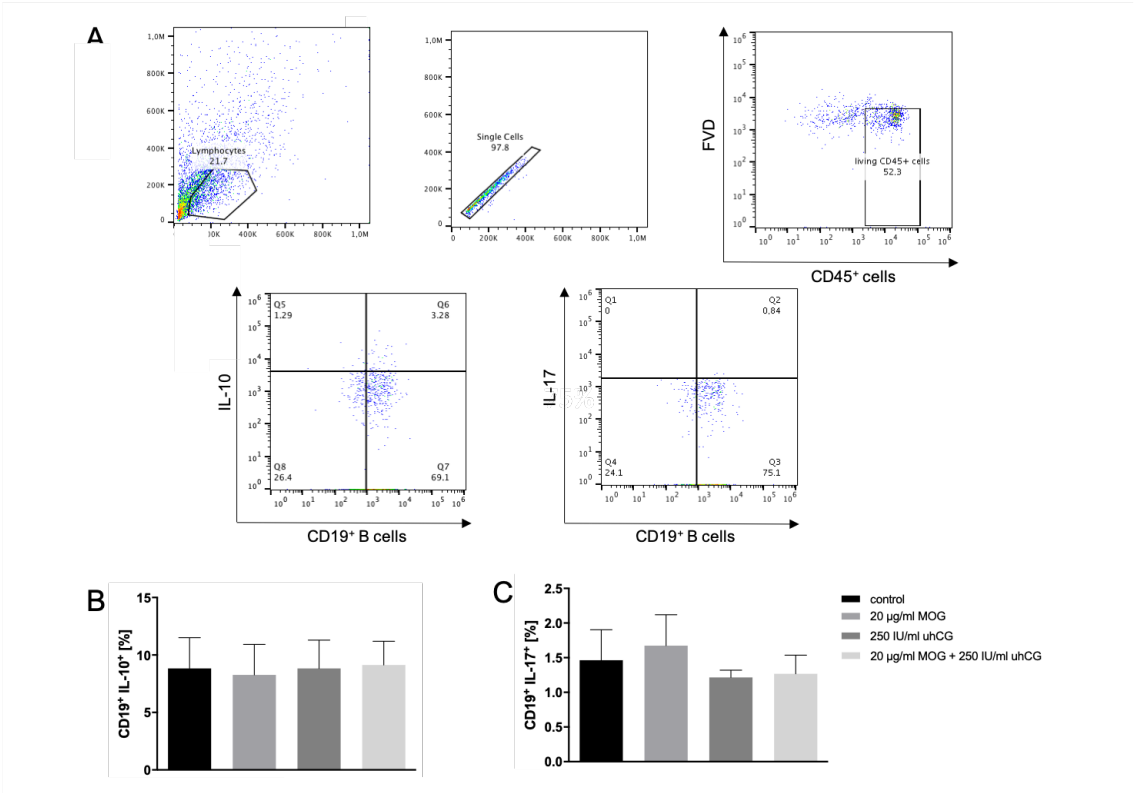


**Figure 3.18: CD19$^+$IL-10$^+$ cells and CD19$^+$IL-17$^+$ cell frequency analysis after MOG and uhCG treatment *ex vivo*.** CD19$^+$IL-10$^+$ B cells of MOG-immunized IL-10eGFP female mice showing signs of EAE at day 20 were sorted and restimulated with the MOG peptide and/or uhCG for 24 h. Gating of lymphocytes followed by an exclusion of doublets and gating of viable CD45$^+$ cells was performed (A). The overall percentages of CD19$^+$IL-10$^+$ B cells did not change throughout the experiment (B). Furthermore, no significant conversion of IL-10 producing B cells into IL-17 producing B cells was found (C). Data are displayed as means (n=6) plus S.D.. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparisons test.

Due to the low amount of cells obtained after sorting (approximately 50,000 cells per spleen), only a few number of cells was used for each condition (6,000 cells/well). The frequency of CD19$^+$IL-10$^+$ B cells was not altered after the addition of MOG, uhCG, or both. The CD19$^+$IL-17$^+$ B cells frequencies were also not changed within the CD19$^+$ cell population after uhCG addition.

To summarize the results obtained during this thesis, preventive hCG treatment of MOG-immunized mice resulted in a significantly reduced EAE disease score at the initial phase of the disease (day 13, 16, 20) and was dependent on the hCG preparation used. However, uhCG-treated MOG-immunized animals showed a significantly reduced survival rate compared to MOG-immunized control and rhCG-treated MOG-immunized animals. A potential modulatory effect of

hCG through induction of Treg cells cannot be confirmed in this experimental autoimmune setting. There was a trend towards a reduced Th17 cell population in both hCG treatment groups and a significant reduction of B17 cell frequencies in uhCG-treated animals at day 35 after EAE induction, although EAE severity was not altered at this time point. Notably, at day 20 EAE severity was significantly ameliorated in uhCG-treated MOG-immunized animals, however no significant differences were found with regard to Th17, Th1, and B17 cell frequencies. Furthermore, the demyelination in the spinal cord was not affected after uhCG treatment of MOG-immunized animals compared to control MOG-immunized mice at day 20.

Finally, a novel B cell subset, referred to as B17 cells ($CD19^{+}IL\text{-}17^{+}$), was identified in this EAE model. Here, the *ex vivo* studies using B cells from MOG-immunized mice showed that uhCG can reduce the frequency of B17 cells in the B cell population *ex vivo*. These results highlight that the application of uhCG has an effect on EAE disease severity in mice and may be able to influence immune cell population like B17 cells in an autoimmune setting. Therefore, further studies are needed to analyze the potential effect of uhCG on EAE as well as other autoimmune diseases.

# 4. Discussion

MS is one of the most common neuroinflammatory diseases with a strong heterogeneity of its symptoms and clinical course [37]. Young adults are primarily affected and the underlying processes leading to this disease are not fully understood. Moreover, the factors triggering the disease still have to be defined [37, 79]. Over the last decades, many different cell subsets have been identified which contribute to MS disease progression and various treatment options have been proposed to reduce disease severity and slow down disease progression. However, due to the complex interplay between the immune system and the nervous system as well as the lacking knowledge about potential trigger factors, a successful treatment option for MS patients has not yet been found.

The inflammatory process driven in MS results from a break down of the BBB and infiltration of autoreactive myelin-specific T and B cells as well as macrophages into the CNS [68]. T and B cells are thought to be the cell types driving the inflammatory immune reaction in the CNS following a recruitment of other cell subsets (macrophages, monocytes, neutrophils) and the activation of microglia [68]. This complex interplay of different cell types leading to neurodegeneration and resulting in MS disease progression indicates the complex immune reaction taking place. Therefore, not only the potential cell subsets driving MS disease progression need to be identified but also the interplay and activation of specific cell subsets in this neuroinflammatory process need to be understood.

Furthermore, the immune system can be altered through various circumstances. Aging, endocrine disruptors, and alternations of hormonal levels within the body can lead to altered immune responses [31–33]. hCG was shown to alter lymphocyte populations towards an anti-inflammatory environment to support fetal tolerance in pregnancy mouse models [206]. hCG supports the conversion of T cells that in turn supports an induction of Treg cells which led to an anti-inflammatory environment [25, 206]. Sha and colleagues highlighted a reduced Th17/Treg ratio after hCG treatment of women with a history of unexplained recurrent spontaneous abortion, indicating that hCG can reduce Th17 and induce Treg cell frequencies in the blood of hCG-treated patients with spontaneous abortions [235]. Additionally, hCG can prevent autoimmune diabetes by reducing $CD4^+$ and $CD8^+$ T cell populations and increasing Treg cell populations in NOD mice [224]. In RA, hCG treatment resulted in decreased arthritis as well as reduced TNF$\alpha$, IL-1$\beta$, IL-6, nitric oxide, and iNOS protein levels [223]. hCG is human and primate specific, however, mice express the highly homologous LH, which is also present in humans [236, 237]. Both species express the LH/CG receptor that binds LH and hCG. For this, even though mice lack endogenous hCG expression, exogenous hCG is able to signal through the mouse LH/CG receptor and hCG-mediated effects can be analyzed using mouse models [238]. Therefore, the analysis of immune responses by the application of hCG using the EAE mouse model is a highly interesting model to investigate the complex interplay of hormones and lymphocyte cell populations involved in neuroinflammation.

This study is based on the observed beneficial effects of pregnancy on disease severity in MS patients that might be explained by the strong increase of pregnancy hormones shown to possess a variety of immunomodulatory functions [157–159]. Previous studies have revealed that hormones like estrogens, progesterone, and testosterone are capable of modulating autoimmunity resulting in declined relapse rates and contributing to repair mechanisms in the CNS [157–159]. There are three major pregnancy hormones that can regulate different immune cell subsets towards a tolerogenic state: estrogens, progesterone, and hCG. Estradiol treatment of mice developing EAE led to increasing Breg cell frequencies and limited the recruitment

of pro-inflammatory cells into the CNS, protecting against EAE [186]. Estradiol treatment also led to a more pronounced Th2 response in an EAE mouse model [157]. Interestingly, a first trial with estradiol-treated patients, diagnosed with relapsing-remitting MS, was performed in 2002 [190]. Lesions were significantly decreased during the treatment and enhanced after estradiol treatment was stopped [190]. Additionally, elevated IL-10 and IL-5 and decreased TNF cytokine levels were present in the blood of MS patients [190]. Progesterone treatment of mice developing EAE resulted in a protection against axonal damage, reduced EAE signs, and increased IL-10 cytokine levels [180, 181]. Also, therapeutic treatment with progesterone after EAE onset resulted in a remission of EAE disease signs [181]. This highlights the potential effect of pregnancy hormones, like estrogens and progesterone, to reduce EAE signs and also disease symptoms in MS patients. However, little is known about the immunomodulatory effect of hCG on MS [226, 227]. Caspary and colleagues studied the effect of hCG in guinea pigs in two different EAE models and found no effect on EAE disease progression. An administration of 3000 IU uhCG was performed either three days before, 7, 10 or 11 days after disease onset. Furthermore, an additional group was treated with 1500 IU hCG three days in a row in guinea pigs developing EAE. In this setting, no significant differences were found. Nonetheless, Caspary and colleagues indicated a possible reduction of the mean clinical score in animals treated with 3000 IU uhCG three days before disease onset when EAE was induced using homologous spinal cord in CFA. Based on this study, it would have been of interest if the administration at additional time points would have led to reduced EAE signs, as well as if different immune cells subsets like T or B cells would have responded to the administration of hCG. Furthermore, it has to be noted that only one hCG preparation was available on the market at the time point of the study. This shows that the investigation of hCG as an immunomodulator is still highly relevant. Han and colleagues investigated the effect of hCG using an osmotic pump in an EAE mouse model and found an increased disease severity [227]. The daily dose of hCG with 5 IU/kg was rather low and it was not specified whether rhCG or uhCG was used. MMP-9, a stimulator for infiltrating immune cells into the CNS, was increased in the CNS and plasma during this study [227]. Additionally, IL-6 and IL-4 cytokine levels were increased in

hCG-treated animals developing EAE [227]. The upregulation of MMP-9 by hCG may therefore cause a higher infiltration of immune cells which are usually stimulated by MMP-9 [227]. This may cause an enhanced neuroinflammatory process [227]. These studies show that the role of hCG in MS and its EAE animal model is not well understood and further investigations are needed.

## 4.1 Establishment of the EAE model

To investigate MS pathology, the EAE mouse model represents a good tool to study the efficiency of different substances *in vivo*. Nonetheless, when using a mouse model there are advantages and limitations. Disease progression in EAE mice can vary due to different induction methods and depends on the animal strain used. For example, there are monophasic or relapsing models which need to be reconsidered for each specific experimental setup [239, 240]. Also, depending on the mechanisms investigated different models are used. EAE induction in C57BL/6J mice via MOG35-55 leads to a chronic disease progression where demyelination by T cells and macrophages can be investigated [133]. The immunization of SJL/J mice with PLP139–151 leads to a relapsing model where the spreading of T cells can be analyzed [241]. There are many other animal models available for the investigation of inflammation-induced hypoxia-like tissue injury or other mechanisms [134, 135]. Therefore, the EAE induction was performed using the MOG peptide to study T and B cells in chronic EAE.

First, the EAE mouse model had to be established and optimized under our laboratory conditions. Given the role the immune system plays in women affected by MS, the effect of hCG on female C57BL/6J mice at different ages was specifically tested. Moreover, MS is two to three times more common in females than in males, suggesting hormones such as testosterone influence the progression of EAE. Dalal and colleagues already demonstrated that the application of testosterone on female mice resulted in a reduced EAE course [159].

In our experimental setting, EAE induction in 10 week-old female mice showed the most consistent EAE course at the initial phase. Therefore, 10 week-old female mice showing a consistent disease course were used for all following experiments. However, other groups showed that animals younger than 10 weeks presented a similar clinical course [242]. A potential explanation for the different outcomes is that the hygiene-dependent microbiome of the animal facility plays an important role for the immune system of the animals in general and particularly for the development of autoimmunity [243]. Interestingly, Berer and colleagues showed that in the absence of microbiota EAE does not occur, demonstrating that commensal microbiota are required for it [244].

## 4.2 uhCG treatment reduced EAE severity at its initial phase after 13, 16, and 20 days

After the establishment of the EAE model, the hormonal effect of hCG on disease severity in MOG-immunized female mice was evaluated. Different hCG concentrations were applied to MOG-immunized mice to identify the minimum effective concentration of hCG on EAE onset and progression. Additionally, two different hCG preparations were tested: the urine-derived (uhCG), extracted from the urine of pregnant women, and the recombinant hCG (rhCG) preparation produced by a chinese hamster ovary cell line [228, 229]. Both show similar effects in the induction of ovulation, however, the rhCG preparation is better tolerated by IVF patients [200]. Of note, the uhCG preparation consists of all hCG forms present in the urine of pregnant women. These are: hyperglycosylated hCG, the free β-unit, and the intact hCG form. The uhCG preparation has a purity of approximately 70 % and non-hCG proteins can be found within this preparation [230]. The rhCG preparation consists of approximately 99 % of the complete hCG form and has therefore a higher purity compared to the urine-derived form. Also, the rhCG preparation shows a higher efficiency compared to the uhCG preparation meaning that a lower dose can be applied to the patient for the induction of final oocyte maturation [229, 231]. Both preparations are currently available on the market and used for IVF. Therefore, both preparations were used in this study

to identify whether there is a difference between both hCG preparations possibly leading to different outcomes in our experimental setting.

Concentrations from 1 IU up to 250 IU of uhCG and rhCG were tested, all of them lying within the physiological range during human pregnancy in the blood being able to affect the disease. Moreover, low concentrations of 1 to 10 IU hCG already showed an immune modulatory effect in a pregnancy mouse model with no side effects [25].

Mice were treated with uhCG or rhCG every other day, reducing the possibility of irritations at the injection sides. Moreover, hCG has a half-life of approximately 36 h ensuring that hCG is always present. The experimental time frame was set to 35 days, allowing conclusions about a potential effect of hCG with time points for disease onset (day 10), the initial phase (day 20), and the progressive disease phase (day 35).

Treatment of MOG-immunized mice with 1 IU, 2 IU, or 10 IU of rhCG did not affect disease onset and progression. The application of a high rhCG concentration (250 IU) did not alter the course of EAE while the application of 250 IU uhCG resulted in a milder EAE disease score at day 13, 16, and 20. Unexpectedly, around 50 % of uhCG-treated MOG-immunized mice (250 IU) died at EAE onset. Hence, we reduced the uhCG dose to 150 IU per mouse resulting in the same reduction of the EAE score. Even though the concentration of uhCG was reduced, around 50 % of the uhCG-treated MOG-immunized mice died during disease onset (day 10 to 20) following an uhCG injection.

In this study, uhCG treatment only resulted in decreased survival when uhCG application was accompanied with EAE onset. Han and colleagues also reported a decreased survival rate in mice developing EAE that were treated with 5 IU hCG using an osmotic pump [227]. However, they were not able to explain the effect provoked by hCG in combination with EAE development and observed the reduced survival rate at a later time point after EAE onset. Furthermore, Han and colleagues used a different hCG concentration and a different application method [227]. It is also not clear whether the hCG preparation used was a recombinant or urine-derived preparation. Notably in our study, when EAE induction did not result in EAE onset,

none of the uhCG-treated females died. Moreover, MOG-immunized mice that received uhCG therapeutically after disease onset did not die. These observations suggest that there is a direct association between uhCG treatment and disease onset. Further analysis of MOG-immunized females undergoing "sudden death" revealed no involvement of innate and adaptive immune responses in this process ruling out an overreaction of the immune system as a trigger for the severe outcome. It was hypothesized that an overwhelming immune response, such as a cytokine storm, may result in organ failure that finally resulted in the death of the animal. The deleterious consequences of a cytokine storm have been described in viral infections [245]. Here, an aggressive pro-inflammatory immune response is the result of a viral infection in combination with an insufficient anti-inflammatory immune response. The infection leads to virus proliferation and further infection of cells [246]. Due to apoptosis, this infection spreads easily inducing a strong inflammatory immune response characterized by increasing leukocytes, pro-inflammatory cytokines, and chemokines [245, 246]. This leads to fever, pain, and also death [247]. Interestingly, this was already considered in MS disease progression, but not further investigated [248]. For this, it was sought to understand whether a massive influx of cytokines after uhCG injection could explain the unexpected outcome. No changes in cytokine secretion by lymphocytes of uhCG-treated females were detected. Moreover, histological examinations revealed no pathological changes in liver, lymph node, or kidney tissue. Altogether, this data suggests that there was no overreaction of the immune system, excluding this as an explanation for the "sudden death" phenomenon.

Based on the finding that rhCG-treated MOG-immunized females did not exhibit a reduced survival rate, it can be assumed that non-hCG proteins present in the uhCG preparation and not hCG itself trigger disease severity. This has been speculated by Caspary and colleagues who found no significant alteration of disease activity upon uhCG treatment in guinea pigs developing EAE [226]. This could be due to the hCG concentration used [226]. The purity of hCG within uhCG preparations is commonly around 70 % due to the extraction method [249]. The uhCG preparation contains various hCG isoforms (e.g. β-hCG, hyperglycosylated hCG, nicked hCG, intact hCG), whereas the rhCG preparations contain only the

intact form [226, 250]. Besides hCG, these urine-derived preparations contain other proteins such as epidermal growth factor (EGF), Tamm Horsfall glycoprotein, eosinophil derived neurotoxin (EDN), early pregnancy factor (EPF), proteolytic enzymes, oxidized hCG, and gonadotropin inhibitory contaminants [251–258]. Not all contaminants were investigated in the EAE model. The application of EGF in combination with the growth hormone releasing peptide-6 led to a decreased disease progression, when administered therapeutically [259]. However, EGF alone was not able to ameliorate disease progression in rats developing EAE [259]. Furthermore, Harness and colleagues found that EPF treatment led to a protective effect on rats developing EAE [260]. They showed that lymphocytes highly secrete EPF in the spinal cord of rats developing EAE during recovery from the disease [260]. IL-10 and IL-4 mRNA expression was significantly increased after EPF treatment whereas IFN$\gamma$ mRNA expression was decreased in the spinal cord [260].

MOG-immunized females receiving uhCG that did not die showed a significant milder disease progression during the initial phase of EAE (day 13, 16, 20). This was not true for rhCG. This difference may be due to the previously mentioned disparities between both hCG preparations. Therefore, the beneficial effect observed in the EAE model was proposed to be either mediated through one of the hCG isoforms or non-hCG proteins, such as EPF, present in the preparation. uhCG may be more dangerous due to its isoforms, because the free $\beta$-subunit and also the hyperglycosylated form is known to be carcinogenic [203, 232]. Therefore, it is not clear despite the extensive investigations in our protocols, if uhCG has a protective and immunosuppressive effect or whether it can be dangerous due to its different components. By contrast, rhCG application did not result in an alteration of the EAE course which may be explained by the high purity of the preparation as well as the lack of different hCG isofoms [226, 250]. For this, the uhCG preparation needs to be analyzed in detail to define all constituents and to confirm, which substance is able to reduce disease severity. Furthermore, the impact of different hCG isoforms on EAE disease progression should be tested to clarify their potential use.

## 4.3 uhCG treatment reduced Th17 cell frequencies and significantly reduced B17 cell frequencies in the CNS

Even though uhCG application was accompanied with a lower survival rate at EAE onset speculating that the uhCG preparation, having a 70 % purity, consists of components that result in a lower survival, the uhCG administration significantly reduced the EAE severity on day 13, 16 and 20. To test whether uhCG mediates its beneficial effect through regulation of different immune cells involved in EAE pathology, lymphocytes from peripheral lymphoid organs and the CNS were analyzed 35 days after EAE induction. Autoreactive T cells are critical for MS disease progression as well as for EAE [133]. Additionally, during the last decade B cells became more emphasized and were shown to contribute to the inflammatory process [109].

In this study, the frequencies of Th17 cells were reduced in both hCG treatment groups in the CNS at the progressive EAE phase. However, this did not reach statistical significance proposing that hCG-mediated Th17 diminution alone cannot explain the positive impact of uhCG on the EAE score. The Th17 cell population is one of the main cell populations driving EAE and MS disease progression which was already demonstrated by cell transfer experiments of Th17 cells [88]. During the last decade, Th17 cells were thought to be essential for EAE and MS disease progression. Several studies demonstrated that IL-17 is a major component driving EAE disease course, but its absence cannot prevent EAE [88, 261–263]. Chen and colleagues showed that anti-IL-23 therapy reduces EAE severity [263]. This suggests that other cell subsets drive neuroinflammation in EAE. Even though rhCG was able to reduce the Th17 cell frequency in this study, it may not be strong enough to reduce EAE severity and other cell types are also responsible for the severity of disease progression.

In MS and EAE, Th1 cells are the most abundant cell population in the CNS being a major producer of IFN$\gamma$ [92, 264]. When autoreactive Th1 cells are adoptively transferred into naïve mice, these mice will develop EAE [92, 264]. Studying IFN$\gamma$-knock-out animals, EAE was exacerbated concluding that IFN$\gamma$ may also

have suppressive effects in EAE [93, 265]. In this study, the Th1 cell frequency was significantly lowered in rhCG-treated animals compared to control animals (Supplemental figure A.1), but was unaffected in uhCG-treated animals. This indicates that rhCG application does influence the Th1 and Th17 cell frequencies, but was not able to alter the EAE severity.

By contrast, uhCG was able to significantly reduce B17 cell frequencies in the CNS, while rhCG did not alter the B17 frequencies. This result is of interest because B17 cells are a novel and not well studied cell population [121]. In RA patients, this distinct B17 cell population may drive disease progression due to its IL-17 production [122]. Furthermore, Bermejo and colleagues studied B17 cells in an infection model with *Trypanosoma cruzi* [123]. These B cells produced more IL-17 than T cells in the initial phase of infection (day 10 and day 19) contributing to the establishment of inflammation [123]. In this specific infection model, IL-17 also possesses protective functions because of its capacity to recruit neutrophils producing IL-10 [123]. In MS, the role of B17 cells has not been investigated. It can be hypothesized that this specific cell population may also contribute to neuroinflammation through IL-17 secretion. In this study, the application of uhCG can lower B17 cell frequencies at the progressive EAE phase *in vivo*. How hCG may interfere with IL-17 cytokine secretion by B cells was investigated in this study using an *ex vivo* model and will be discussed in section 5.4.

After the evaluation of cell subsets contributing to inflammation, regulatory cell subsets were analyzed. hCG is a strong immunomodulator known to be able to convert T cells into Treg cells and attract Treg cells. The application of hCG provoked an increase of Treg cells for instance in pregnancy models [206, 266, 267]. However, the immunological mechanisms of pregnancy and autoimmunity differ. In this study, Treg cell frequencies remained unchanged after uhCG treatment compared to the control group. The rhCG application significantly decreased Treg cell frequencies in the CNS. Previous studies in different autoimmune disorders like RA, diabetes, and Sjögren syndrome have already focused on the role of hCG [223–225]. hCG can prevent autoimmune diabetes by reducing $CD4^+$ and $CD8^+$ T cell populations and increasing Treg cell populations in NOD mice [224]. hCG

treatment reduced TNF$\alpha$, IL-1$\beta$, and IL-6 cytokine levels and arthritis severity in a RA rat model [223]. The role of hCG and Treg induction is still not well elucidated using a MS mouse model. Interestingly, Chen and colleagues claimed that pertussis toxin, used for EAE induction, may already suppress the number and function of Treg cells in the spleen by the downregulation of FoxP3 [268]. In our model, hCG may not be able to increase the number of Treg cells in the EAE model due to impaired T cell functionality or the concentration used may not have been sufficient. However, we did not address the functionality of Treg cells in this study.

Different studies have reported that hCG is able to generate tolerogenic DCs by the downregulation of maturation markers like CD80, CD86, or MHCII in a pregnancy model [213, 269]. DCs are known to regulate the recruitment of cells into the CNS via CCL2 during EAE, however, hCG was not able to alter DC cell frequencies and therefore hinder cell trafficking into the CNS [270]. DC cell frequencies, which influence B and T cell activity via activation and suppression, were not significantly altered in any treatment group compared to the control (Supplemental figure A.1).

This indicates that uhCG can influence the Th1, Th17, and the B17 response in the EAE mouse model and leads to a reduced EAE severity in our experimental Setup.

## 4.4 uhCG reduced B17 cell frequencies *ex vivo*

Little is known about IL-17 producing B cells and their role in autoimmune disorders. Bermejo and colleagues described B17 cells in a study evaluating their role in an infection model with *Trypanosoma cruzi* [123]. Here, an infection with *Trypanosoma cruzi* was mimicked by the exposure to parasite-derived trans-sialidase *in vitro* to induce IL-17 secretion in T and B cells, whereas B17 cells were highly increased compared to Th17 cells [123]. Due to the results obtained, Bermejo and colleagues consider that B17 cells may be important in pathogen control and IL-17- and B cell-mediated autoimmunity [123].

There is supporting evidence for the role of B cells in MS. In clinical application, the depletion of $CD20^+$ B cells has shown to reduce relapse rates [271]. However, the reduced relapse rates cannot be solely explained by reduced autoantibody production

by B cells, as initially thought [271, 272]. The effect seems to be rather mediated by a reduced production of B-cell-derived cytokines as it was shown that anti-CD20 treatment did not alter autoantibodies levels [271–273].

Notably, a reduction of B17 cells within the CNS at day 35 after preventive uhCG treatment was observed. Due to the significant reduction of B17 cells during the progressive phase and the significant reduced disease score during the initial phase of EAE after hCG treatment, this specific B cell population was studied in more detail to reveal a potential relevance in EAE as well as its capacity to convert into IL-10 producing B cells.

To analyze if MOG-primed B cells react to uhCG during the initial phase by converting into an immunosuppressive phenotype, splenic B cells were isolated from MOG-immunized female mice at day 20 p.i. and stimulated with MOG and uhCG. First, the appropriate concentration of MOG and uhCG and treatment period was defined. Then, B cells were cultured with 20 µg/ml MOG and 250 IU/ml uhCG to examine whether MOG-primed B cells can be modulated by uhCG. The analysis showed that uhCG alone reduced B17 cell frequencies when compared to MOG-stimulated B cells. Furthermore, the combination of MOG and uhCG resulted in a diminution as well. However, this did not reach statistical significance. Notably, IL-10 producing B cell frequencies were neither affected by MOG nor by uhCG. This indicates that previously MOG-primed cells do not respond to uhCG by enhancing IL-10 production. Other studies addressing the effect of hCG on IL-10 production by B cells showed an increased IL-10 production when hCG was used as a stimulant [218]. The absence of B cells in the EAE model did not lead to milder EAE signs [274, 275]. It has to be noted that the B cells analyzed were obtained from MOG-immunized mice and therefore pertussis toxin was used for EAE induction. As previously described, Treg cell number and function may be impaired after pertussis toxin application [268]. Thus, it can be speculated that B10 cells may have impaired functionality after pertussis toxin injection as well. Nevertheless, uhCG was able to reduce B17 cell frequencies. This may possibly be induced through the LH/CG receptor driving these B cells towards an anti-inflammatory phenotype [218, 221].

Next, it was aimed to understand whether uhCG is able to inhibit the transition of B10 cells of MOG-immunized female mice into B17 cells *ex vivo*. Studying the effect of vaccination, B17 cells have been already reported to produce not only IL-17, but also IL-10 [276]. This depicts the plasticity of adaptive immune cell subsets. B10 cells were isolated from MOG-immunized IL-10eGFP female mice at day 20 p.i. and cultured with MOG and uhCG. MOG did not induce a conversion of B10 cells into B17 cells nor did uhCG affect this process. Unfortunately, the low cell number of B10 cells obtained from MOG-immunized IL-10eGFP females prevented further analyses (e.g. analysis of several time points). As already mentioned, it can be hypothesized that after pertussis toxin injections IL-10-producing B cells may already have impaired functions, as described for Treg cells [268]. Additionally, other stimulants like cytokines or the interaction with other cell subsets like DCs may be necessary to induce a conversion of B10 cells into B17 or further proliferation of B10 cells *ex vivo*. However, B17 cells still need further investigation and may contribute to understanding the role of B cells in EAE and other autoimmune diseases.

## 4.5 No altered lymphocyte cell frequencies or cytokine release by lymphocytes after uhCG treatment at the initial EAE phase

The initial phase of EAE was beneficially affected by uhCG treatment. Thus, T and B cell-associated immune responses were evaluated at day 20 p.i.. Here, the frequencies and functionality of T and B cell populations in different lymphoid organs (inguinal lymph nodes, spleen) and in the CNS were determined.

No significant changes were found regarding the frequencies of Th17 and B17 cells at day 20 when comparing the uhCG treatment group to the non-treated group. This differs from the observations made on day 35 after EAE induction where uhCG treatment resulted in a significant reduction of B17 cells and diminished Th17 cell numbers in the CNS. Moreover, analysis at the disease peak revealed a reduced Treg cell frequency in uhCG-treated animals in the CNS compared to the non-treated animals. No alternations of the Treg frequency after uhCG treatment *in vivo* at the

initial disease phase in the peripheral organs were found. This can be explained by the previously discussed limitations of our model, even though hCG did provoke a Treg increase in a mouse model for diabetes in the spleen or during pregnancy locally at the endometrium and in the blood [206, 224, 267]. Therefore, further studies are needed to evaluate whether and under which conditions uhCG may enhance Treg levels in the EAE mouse model.

Interestingly, the Th1 response defined by the expression of TNF$\alpha$ was decreased in the CNS of uhCG-treated mice. This might be an explanation for the reduced disease severity at day 20 and suggests that during the initial phase of the disease, Th1 responses may be more important for disease progression than Th17 cells. The number of Th1 cells is highly increased in MS patients compared to Th17 cells [277]. During inflammatory episodes, Th1 cell frequencies are not further elevated, but Th17 cells were [277, 278]. This suggests that Th1 cells contribute to the persistent inflammatory process which the progressive EAE model represents and Th17 may be more related to relapses. Another explanation is based on the fact that TNF$\alpha$ is implicated in the mechanism of lymphocyte penetration through the parenchymal border. At day 20 p.i., TNF$\alpha$ is reduced in uhCG-treated animals and this might lead to the reduced disease severity through less trafficking of autoreactive lymphocytes through the parenchymal border into the CNS [279]. Valentin-Torres and colleagues showed that anti-TNF treatment resulted in decreased EAE severity and reduced macrophage activation, demyelination, and leukocyte infiltration and the restoration of BBB integrity [280]. Overall, the inflammatory immune response was reduced, whereas TNF$\alpha$ itself activates microglia and macrophages and therefore triggers demyelination in EAE [280, 281].

In addition to lymphocyte cell frequencies at day 20, the secretion of different Th1/Th2 and Th17 cytokines was analyzed to determine whether the cytokine release by lymphocytes was altered after uhCG treatment *in vivo*. For this, lymphocytes were obtained from MOG-immunized female mice treated with uhCG or MOG-immunized non-treated mice at day 20. No significant differences were found in the cytokine secretion of cultured lymphocytes from inguinal lymph

nodes or spleen. However, IL-4 secretion tended to increase in both lymphocyte populations isolated from inguinal lymph nodes and spleen from uhCG-treated MOG-immunized mice. IL-4 is an anti-inflammatory cytokine which can reduce the secretion of IL-12 and IFN$\gamma$ [282]. It is proposed that an increased secretion of IL-4 by inguinal lymph nodes and splenic lymphocytes may participate in milder EAE progression [111, 112]. Several studies have shown that IL-4 administration is able to reduce the clinical score in MS mouse models without affecting the neuroinflammatory response [111, 112]. This study reported that amelioration of EAE does not necessarily need to be a result of differences in the neuroinflammatory pathology as seen in regard to demyelinated areas within the spinal cord at day 20. The IL-4 receptor is expressed on the axons of neurons in MS lesions, which may lead to the recovery of neurons when IL-4 is present [111, 112]. Also, IL-4 can activate microglia which can induce oligodendrogenesis and therefore can lead to recovery mechanisms of oligodendrocytes on the myelin sheath [283]. By contrast, IL-4 can also lead to increased inflammation by priming macrophages followed by an increased pro-inflammatory response and therefore it may be proposed that the beneficial effect of uhCG at day 20 will not persist after day 20 [284]. Furthermore, it has been found that specific factors like IL-4 contribute to EAE progression by rescuing autoreactive B cells and increasing their survival [110].

To further clarify if uhCG treatment leads to altered cytokine secretion in the CNS, the mRNA expression of *IL-4, IL-10*, and *IL-17* was analyzed. The evaluation of cytokine secretion within the CNS was not performed by CBArray analysis due to limited lymphocyte cell numbers available. For this reason, CNS tissue was collected and the mRNA expression of *IL-4, IL-10*, and *IL-17* was studied. The analysis revealed no differences in the mRNA expression of *IL-4* in the CNS demonstrating no effect of IL-4 that would further explain the significantly reduced EAE score. Additionally, neither *IL-10* nor *IL-17* mRNA expression was altered within the CNS after uhCG treatment.

It was of interest whether demyelination was affected in uhCG-treated animals. Sections of the spinal cord L1-L3 were stained with LFB, staining myelinated

areas [285]. Even though the same region of each spinal cord was analyzed in all groups, no severe demyelination was found in any group. However, the tissue samples of both hCG-treatment groups appeared more diffuse compared to the healthy control spinal cord. This indicates that there is a morphological dysfunction visible in the MOG-immunized mice compared to the healthy control. Furthermore, the MOG-immunized control group showed a slightly demyelinated area within the gray matter showing that demyelination took place. A possible explanation for no enlarged demyelinated areas can be that either the selected region does not show demyelination and other regions do or the demyelination has not progressed far enough to show severe demyelination at day 20 suggesting that the analysis of demyelination should be performed at a later time point of the disease. Furthermore, other studies demonstrated that the EAE model shows inconsistencies of demyelinated lesions and its location [286, 287]. Moreover, it can be difficult to identify lesions within the spinal cord when a low disease score (mean score uhCG: 1.34, control: 1.79 at day 20) is present. Therefore, in this study an effect of hCG on the demyelination in the EAE mouse model used cannot be confirmed or denied.

## 4.6 No alterations of lymphocyte cell frequencies at disease onset after uhCG treatment

After evaluations were performed at disease peak and during the progressive phase of EAE, it was of further interest to study whether the milder disease progression in the presence of uhCG was a result of immunological changes taking place already before disease onset. It is known that cell trafficking takes place through the disrupted BBB in EAE and hCG may already influence this process at disease onset resulting in reduced infiltration of lymphocytes [288]. Therefore, Treg and B10 cell frequencies were also analyzed to understand if uhCG was able to induce changes in Treg and Breg cell populations at disease onset. At the cellular level, uhCG provoked no alteration of the lymphocyte populations studied (Th17, Treg, B17, B10) in peripheral lymphoid organs (inguinal lymph nodes, spleen). Furthermore, no evaluation of the CNS was possible due to the limited number of lymphocytes

located in the CNS before disease onset that restricts the analysis. However, the data obtained is limited to the peripheral immune response and does not map immune reactions within the CNS which are critical for disease onset and progression.

Moreover, the analysis at day 10 included all MOG-immunized females even the ones that would not develop EAE signs at all. This may lead to no different results between control-treated animals and uhCG-treated MOG-immunized mice because of a possible combined group of mice which would have developed EAE later on and possible healthy mice showing no signs of EAE even though EAE was induced. This could have masked a possible effect in the analysis performed.

## 4.7 Therapeutic rhCG or uhCG treatment did not alter the course of EAE

For clinical use, it is rather important to identify a substance with ameliorating properties that can be applied therapeutically. The majority of patients already suffer from disease symptoms, when MS is diagnosed. Regarding the use of pregnancy hormones as therapeutic treatment options, estradiol and progesterone demonstrated positive effects on EAE disease progression. Kim and colleagues reported that the therapeutic treatment with estradiol led to an amelioration of EAE [157]. After estradiol treatment, higher autoantibody levels of IgG specific for the autoantigen myelin basic protein (MBP) were observed, implying an increased humoral immune response [157]. Also, IL-10 secretion by splenocytes was significantly elevated in estradiol-treated animals developing EAE [157]. Additionally, therapeutic treatment with progesterone, starting at disease onset, reduced the disease severity and decreased IL-2 and IL-17 cytokine secretion by splenocytes significantly, whereas IL-10 cytokine secretion by splenic lymphocytes was significantly increased [181]. Interestingly, the number of $CD19^+$ and $CD8^+$ cells was increased [181]. With regard to hCG, neither Han and colleagues nor Caspary and colleagues investigated the therapeutic treatment option of hCG [226, 227]. Han and colleagues used an osmotic pump and Caspary and colleagues injected hCG only once at disease onset and also used guinea pigs instead of mice [226, 227]. For this, the role of hCG as a therapeutic treatment option was investigated in this study.

The results obtained show that uhCG, when applied preventively, reduced disease severity. Hence, MOG-immunized female mice were treated with uhCG or rhCG every other day starting with the first visible signs of EAE. Both hCG preparations were tested to study whether they can alter disease progression although only uhCG ameliorated the disease when applied preventively. Neither uhCG nor rhCG interfered with disease progression suggesting that both hCG preparations were not able to reduce EAE severity after EAE onset. This observation agrees with the finding that the beneficial effect of uhCG disappeared after disease peak. The data suggests that uhCG may alter other pathways before EAE onset reducing the EAE disease score at the initial phase. However, analyses performed on day 10, only providing insights into the peripheral immune response, did not support this assumption. This does not rule out that hCG may influence immune cell populations within the CNS.

## 4.8 Summary and conclusion

In this study, the preventive and therapeutic treatment of MOG-immunized female mice with two different hCG preparations was investigated. For the first time, the effect of two different hCG preparations (uhCG and rhCG) was compared in the EAE mouse model and several immune cell populations were analyzed in neuronal and lymphoid tissue. The preventive treatment with uhCG resulted in a significantly reduced EAE score even though uhCG application was accompanied with an around 50 % lower survival rate in uhCG-treated females. The therapeutic treatment with uhCG as well as the preventive and therapeutic treatment with rhCG did not lead to significant changes of the EAE score. Analysis of Th1, Th17, Treg, B17, Breg, and DC cell frequencies showed no significant alternations after uhCG treatment during disease onset or at the initial phase of EAE. However, at the progressive disease phase uhCG provoked a significant reduction of B17 cells as well as a diminution of Th17 cells in the CNS although the EAE score was not altered at this disease phase. To further validate the *in vivo* findings, *ex vivo* studies were performed to investigate the effect of uhCG on MOG-primed B cells. This study was the first to show that uhCG was able to reduce B17 cell

frequencies within the total MOG-primed B cell pool *ex vivo*. In conclusion, the results presented in this study highlight a potential beneficial impact of the uhCG preparation for EAE treatment. The study further reveals that uhCG is able to modulate different immune cell subsets in MOG-immunized mice developing EAE and proposes B17 cells as a specific target of uhCG. The findings provide new insights into the effectiveness of pregnancy hormones as new treatment options for MS.

# 5. Outlook

The number of autoimmune disorders increases steadily and will further challenge the health care system, particularly in the northern countries. Therefore, novel treatment options need to be identified. In this study, the role of the pregnancy hormone hCG was investigated in the EAE animal model, mimicking MS disease progression. The preventive treatment with uhCG led to a significant reduction of disease severity at day 13, 16, and 20, indicating a modulatory effect of uhCG. However, the substance and mechanism leading to this decreased disease severity at the initial disease phase was not found in this study. Therefore, it would be of interest how uhCG affects EAE and if it also affects the BBB permeability. This may be possible due to the different hCG isoforms or the different molecules present in the uhCG preparation. The analysis of the specific substances is necessary to evaluate a possible target substance leading to the remission of the disease at the initial phase. When the specific molecule is identified further *in vivo* studies are mandatory to check if a remission of the disease can still be evoked. Additionally, this study was only investigating the adaptive immune response. Nonetheless, no differences were found at the time point of reduced EAE severity. Therefore, it would be interesting whether cell frequencies of the innate immune system, such as natural killer cells, mast cells, or others are altered at the initial disease phase. Additionally, the novel population of innate lymphoid cells (ILC) may be investigated. It would be of interest whether LH/CG receptor expression is enhanced after hCG treatment

in different organs (CNS, spleen, inguinal lymph nodes) or on different cells, such as T cells, B cells, ILCs, or neurons.

In this study another question arose, whether B17 cells play a role in autoimmunity and also in other experimental settings. To answer these questions further studies are needed. B17 cell transfer *in vivo* may give insights about their role in EAE progression and if these cells may be important during the initial phase of EAE. Also a possible recruitment or the expansion of B17 cells, specifically in the CNS, needs to be analyzed in the future.

In conclusion, this study is one of the first evaluating the effect of hCG on MS and characterizes a B17 cell population present in the CNS of EAE mice. For this, further studies are needed to investigate the molecular mechanisms of uhCG on EAE and B17 cells.

# A. Supplements

# A.1 No differences in Th1 and DC cell frequencies after preventive hCG treatment at day 35

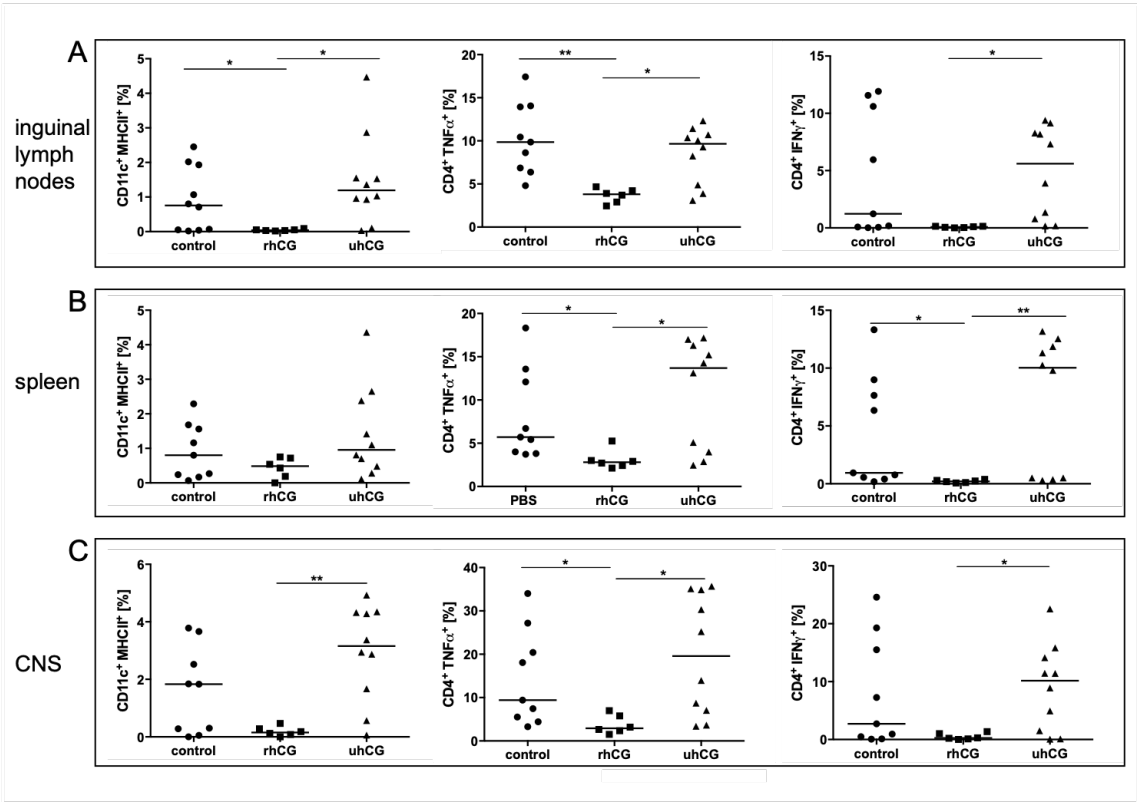


**Figure A.1: Dendritic cell maturation status and Th1 cell frequencies at day 35 after hCG treatment.** Cell frequencies of lymphocytes isolated from MOG-immunized mice treated with rhCG (n=6), uhCG (n=10), or control (n=9) every other day at day 35. Lymphocytes were isolated and stimulated for 4.5 h with ionomycin, PMA and brefeldin A. Analysis was performed using the BD Calibur flow cytometer and the Cellquest software. Within the inguinal lymph nodes (A) and the CNS (C) $CD11c^+$ cell frequencies showed an elevated MHCII expression in uhCG-treated MOG-immunized animals compared to the rhCG-treated MOG-immunized mice. The Th1 cell frequency in regard to TNF$\alpha$ was decreased in the rhCG treatment group compared to the control group in the inguinal lymph nodes (A), spleen (B), and CNS (C). Furthermore, the $CD4^+TNF\alpha^+$ cell frequency was increased in uhCG-treated compared to rhCG-treated MOG-immunized mice (A-C). $CD4^+IFN\gamma^+$ cell frequencies were increased in all organs analyzed when comparing the rhCG-treated with the uhCG-treated MOG-immunized animals (A-C). Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test (* $p<0.05$).

## A.2 Breg, Treg, B17, Th17, and Th1 cell frequencies in the inguinal lymph nodes, spleen, and CNS were not altered at day 20 after preventive uhCG treatment

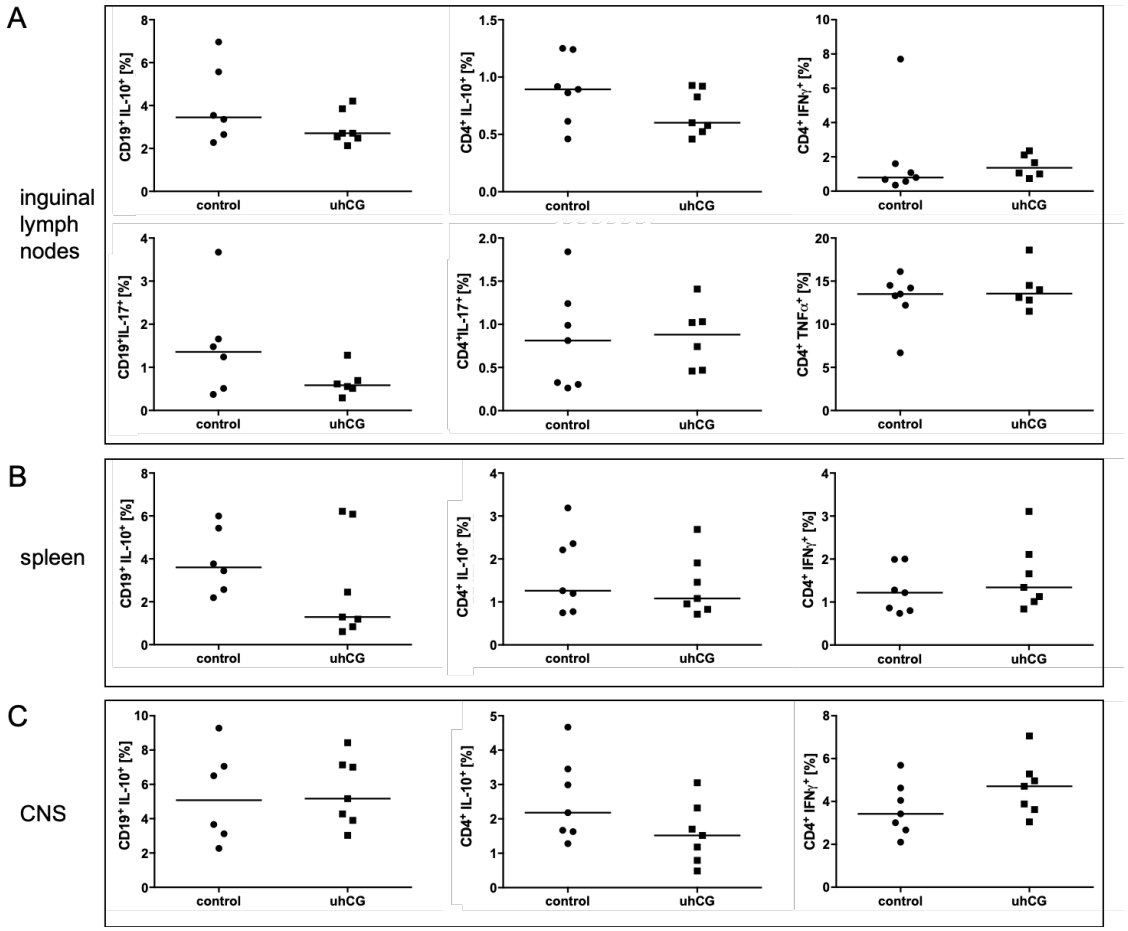


**Figure A.2: Breg, Treg, B17, Th17, and Th1 cell frequencies at day 20 after preventive uhCG treatment of MOG-immunized mice every other day.** Lymphocyte cell frequencies of MOG-immunized mice at day 20 treated with uhCG (n=7) or control (n=6-7) every other day. Lymphocytes were isolated and stimulated for 5 h using ionomycin, PMA, and brefeldin A. The frequencies of $CD19^+IL\text{-}10^+$, $CD4^+IL\text{-}10^+$ and $CD4^+IFN\gamma^+$ cells were not altered significantly at day 20 after uhCG treatment of MOG-immunized mice in the inguinal lymph nodes (A), spleen (B), nor CNS (C). Additionally, $CD19^+IL\text{-}17^+$, $CD4^+IL\text{-}17^+$ and $CD4^+TNF\alpha^+$ cell frequencies were not altered in the inguinal lymph nodes (A) after uhCG treatment at day 20. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the the Mann-Whitney-$U$ test.

## A.3 Cytokine release of lymphocytes did not change after preventive uhCG treatment at day 20

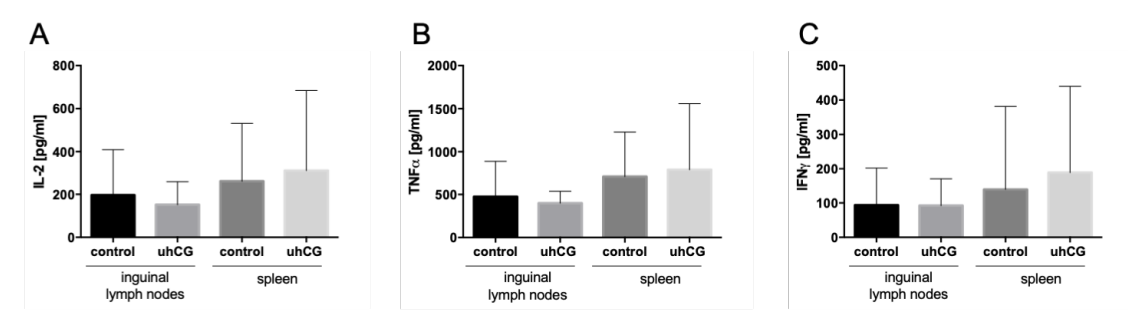


**Figure A.3: Cytokine release of lymphocytes at day 20 after preventive uhCG treatment.** Splenic (control n=7 ,uhCG n=7) and inguinal lymph node (control n=3, uhCG n=4) lymphocytes were stimulated with ionomycin and PMA for 5 h and supernatants were collected. Using a Th1/Th2/Th17 CBArray several pro- and anti-inflammatory cytokines were measured. No significant differences in IL-2 (A), TNF$\alpha$ (B), or IFN$\gamma$ (C) cytokine release were found. Data is displayed as means plus S.D.. Statistical analysis was performed using the Mann-Whitney-*U* test.

# A.4 No differences in Breg, Treg, B17, Th17, and Th1 cell frequencies in the inguinal lymph nodes at day 10 after preventive uhCG treatment

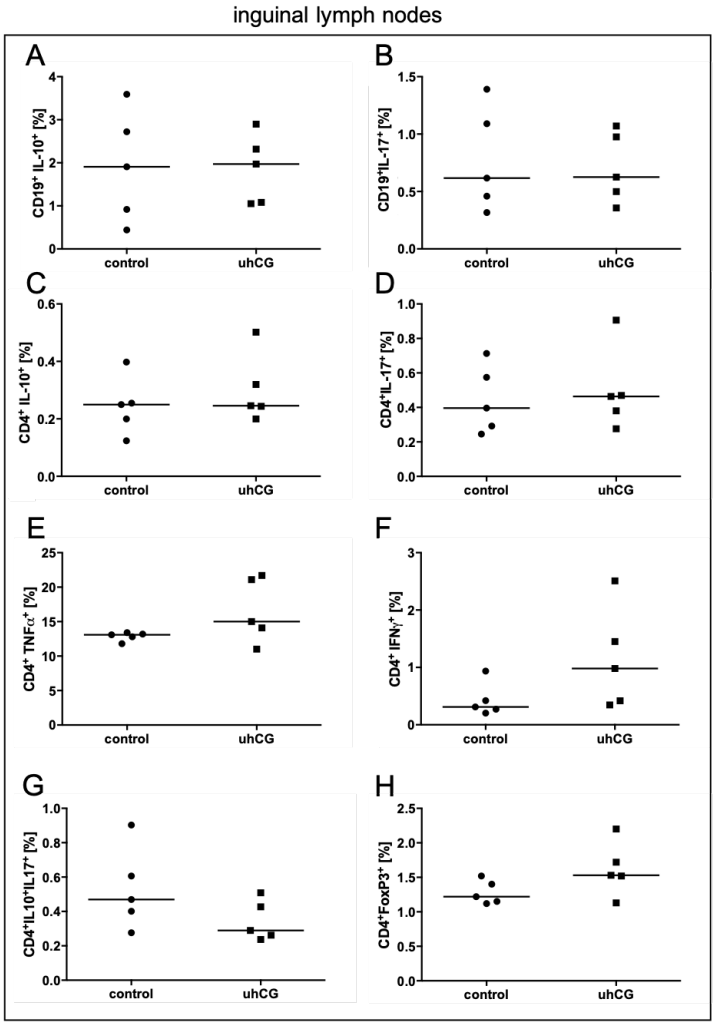


**Figure A.4: Breg, Treg, B17, Th17, and Th1 cell frequencies at day 10 after preventive uhCG treatment in inguinal lymph nodes.** Cell frequencies of lymphocytes of the inguinal lymph nodes isolated from MOG-immunized mice treated with uhCG (n=5) or control (n=5) at day 10. Lymphocytes were isolated from MOG-immunized animals and stimulated for 5 h with ionomycin, PMA, brefeldin A and analyzed using the Attune NxT. No significant differences were found in uhCG-treated MOG-immunized mice when comparing $CD19^+IL\text{-}10^+$ (A), $CD19^+IL\text{-}17^+$ (B), $CD4^+IL\text{-}10^+$ (C), Th17 (D), Th1 (E,F), $CD4^+IL\text{-}10^+IL\text{-}17^+$ (G), or Treg (H) cell frequencies in the inguinal lymph nodes. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the the Mann-Whitney-$U$ test.

## A.5 Th1, CD4$^+$IL-10$^+$IL-17$^+$, and Treg frequencies were not altered at day 10 after preventive uhCG treatment

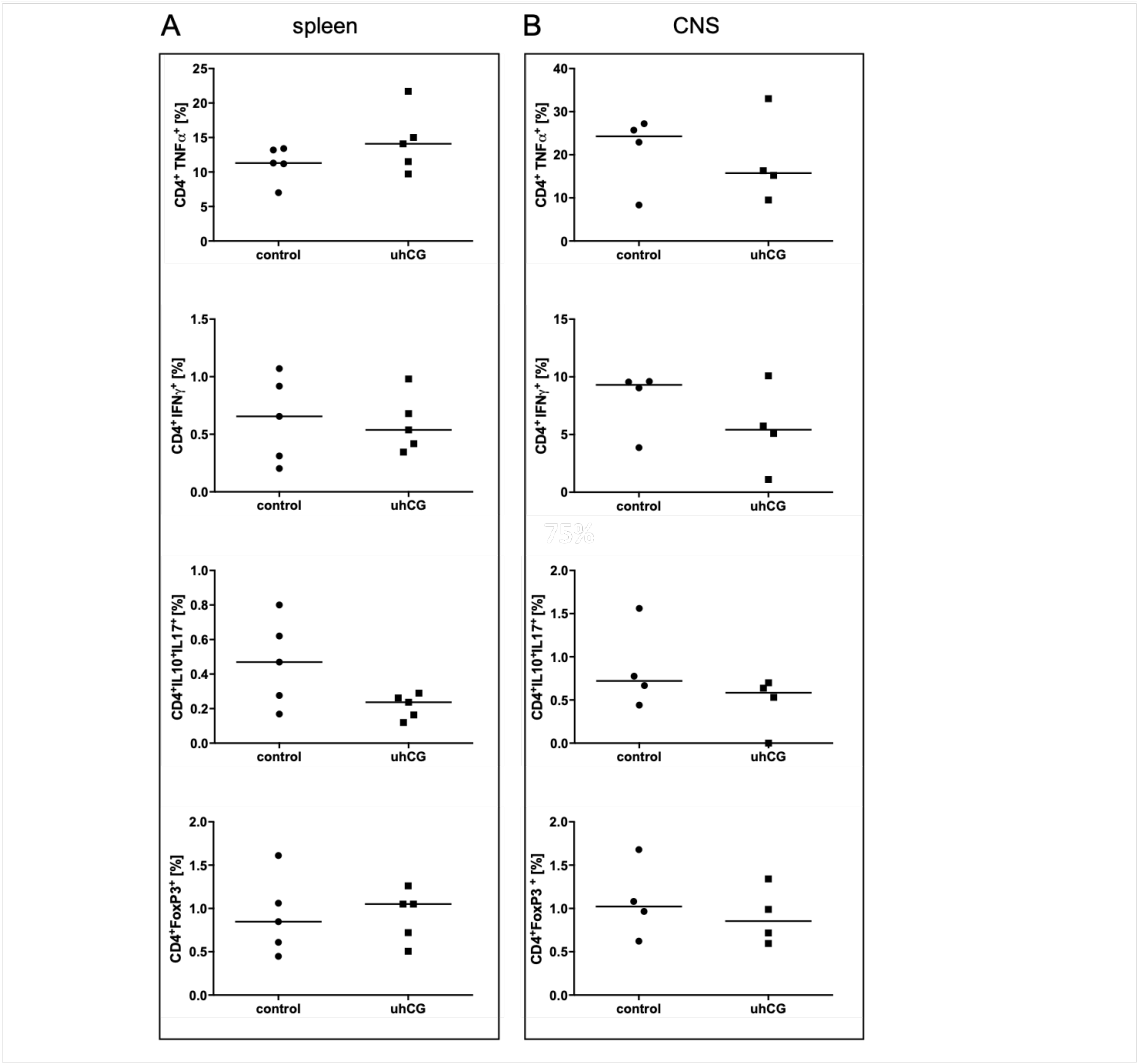


**Figure A.5: Th1, CD4$^+$IL-10$^+$IL-17$^+$ and Treg frequencies at day 10 after preventive uhCG treatment in the spleen and CNS.** Cell frequencies of lymphocytes from the spleen and the CNS isolated from MOG-immunized mice treated with uhCG (n=5) or control (n=5) at day 10. Lymphocytes were isolated and stimulated for 5 h with ionomycin, PMA, and brefeldin A and analyzed using the Attune NxT. No significant differences were found in uhCG-treated MOG-immunized mice when comparing Th1, CD4$^+$IL-10$^+$IL-17$^+$, and Treg frequencies in the spleen (A) and CNS (B). Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the the Mann-Whitney-$U$ test.

## A.6 No altered monocyte and neutrophil cell frequencies of uhCG-treated MOG-immunized mice showing the "sudden death" phenomenon

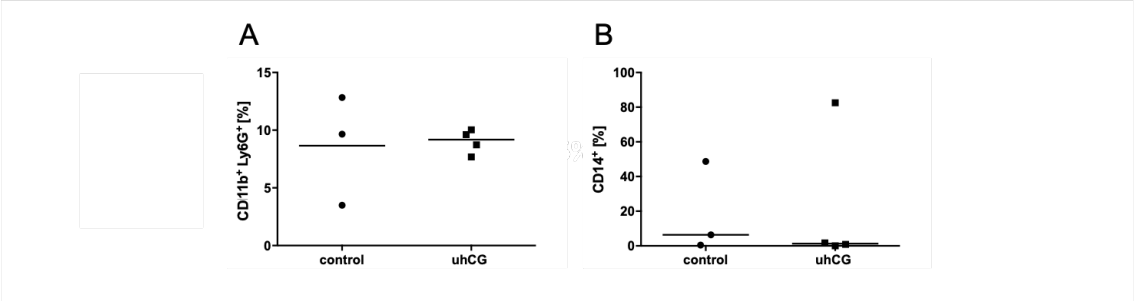


**Figure A.6: Monocyte and neutrophil cell frequencies of uhCG-treated (n=4) MOG-immunized mice showing the "sudden death" phenomenon or control mice(n=3).** Neither monocytes (A) nor neutrophils (B) showed significant differences in uhCG-treated MOG-immunized animals in the "sudden death" group in the inguinal lymph nodes. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Mann-Whitney-$U$ test.

## A.7 Adaptive immune response of MOG-immunized mice treated with uhCG and showing the "sudden death" phenomenon

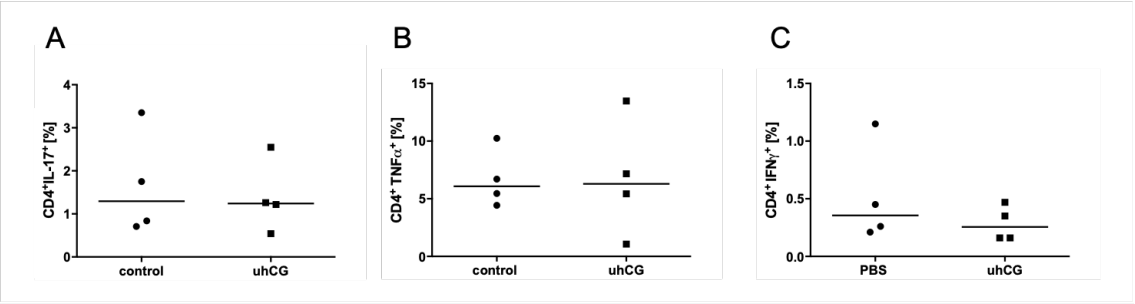


**Figure A.7: Th17 and Th1 cell frequencies in uhCG-treated MOG-immunized mice showing the "sudden death" phenomenon (n=4) compared to control mice (n=3).** The Th17 cell frequency (A) as well as the Th1 cell frequency (B, C) in the inguinal lymph nodes was not altered. Data is displayed as medians showing individual values for each animal. Statistical analysis was performed using the Mann-Whitney-$U$ test.

# A.8 No differences in the cytokine release by lymphocytes of uhCG-treated MOG-immunized mice showing the "sudden death" phenomenon.

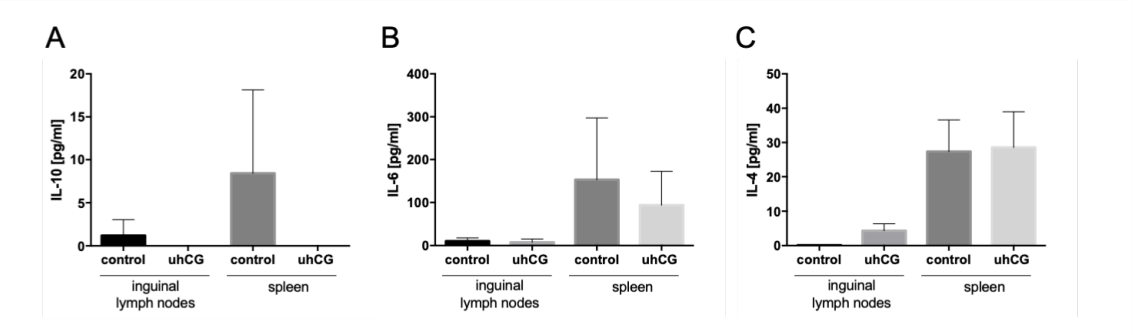


**Figure A.8: Cytokine release of lymphocytes isolated from MOG-immunized mice showing the "sudden death" phenomenon after uhCG injection.** Lymphocytes of MOG-immunized mice showing the "sudden death" phenomenon between day 10 and day 20 after EAE induction were isolated and stimulated with ionomycin and PMA for 5 h. Afterwards, the supernatant was collected and analyzed using a CBArray. No significant differences were found in IL-10 (A), IL-6 (B), or IL-4 (C) cytokine secretion by lymphocytes of uhCG-treated MOG-immunized animals (n=4) compared to controls (n=4) in the inguinal lymph nodes as well as in the spleen. Data is displayed as means plus S.D.. Statistical analysis was performed using the Mann-Whitney-$U$ test.

# A.9 Morphological analysis of organs of uhCG-treated MOG-immunized mice showing the "sudden death" phenomenon revealed no differences in the liver

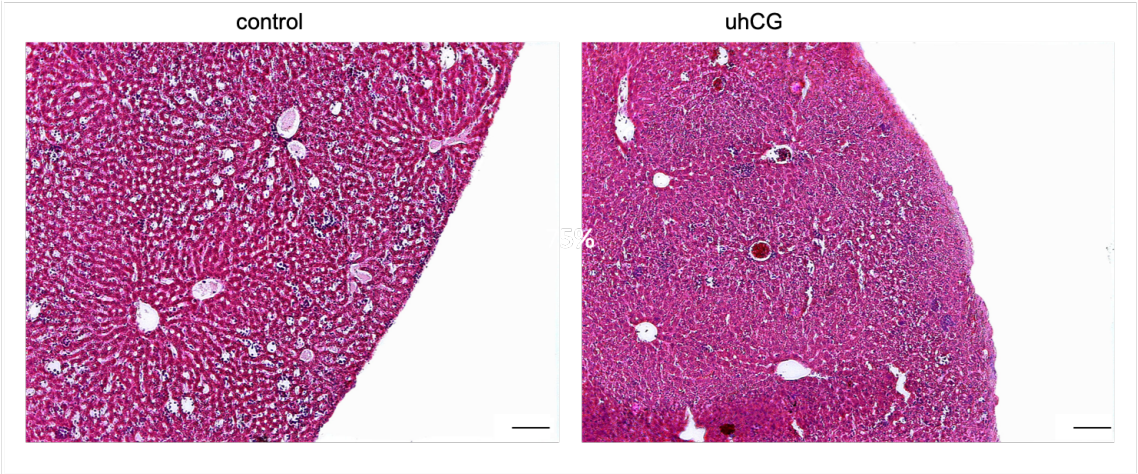


**Figure A.9: Analysis of morphological changes of liver tissue from uhCG-treated MOG-immunized mice showing the "sudden death" phenomenon or control mice.** Shown are exemplary photos of a HE staining of the liver of MOG-immunized mice showing "sudden death" and the control group. No differences were found in the morphology of the liver. Shown are detailed views photographed with a 10x magnification (scale bar: 100 µm).

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# List of Acronyms

| | |
|---|---|
| % | Percentage |
| AIRE | Autoimmune regulator |
| APC | Antigen-presenting cell |
| BBB | Blood brain barrier |
| B17 | B cells producing IL-17 |
| Breg cells | Regulatory B cells |
| CBA | Cytometric bead array |
| CCR | Chemokine receptor |
| CXCL | Chemokine C-X-C motif ligend |
| CFA | Complete freud's adjuvant |
| CD | Cluster of differentiation |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| $CO_2$ | Carbon dioxide |
| °C | Degrees Celcius |
| Ct | Cycle treshold |
| CTLA | Cytotoxic T-lymphocyte-associated protein |
| d | Day |
| DNA | Desoxyribonucleic acid |
| dNTPs | Di-nucleoside triphosphate |
| EAE | Experimental autoimmune encephalomyelitis |
| EBV | Epstein-Barr virus |
| EDTA | Ethylenediaminetetraacetic acid |

| | |
|---|---|
| eGFP | Enhanced green fluorescent protein |
| ER | Estrogen receptor |
| EVCT | Extravillous cytotrophoblasts |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| fMRI | Functional magnetic resonance imaging |
| FoxP3 | Forkhead-box-protein P3 |
| FSH | Follicle-stimulating hormone |
| FVD | Fixable viability dye |
| g | Gravity acceleration |
| GM-CSF | Granolocyte colony-stimulating factor |
| h | Hour |
| hCG | Human chorionic gonadotropin |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Sulfuric acid |
| HCl | Hydrochloric acid |
| HE | Hematoxylin eosin |
| HHV-6 | Human herpes virus-6 |
| HLA | Human leukocyte antigen |
| HRT | Hormone replacement therapy |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IFN | Interferon |
| IVF | *In vitro* fertilization |
| *i.p.* | Interperitoneal |
| IU | International unit |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Potassium dihydrogenphosphate |
| LFB | Luxol fast blue |
| LH | Luteinizing hormone |
| LH/CG | Luteinizing hormone/choriogonadotropin receptor |

| | |
|---|---|
| MACS | Magnetic cell separation |
| MBP | Myelin basic protein |
| MHC | Major histocampatibility complex |
| min | Minutes |
| ml | Milliliter |
| µg | Microgram |
| µl | Microliter |
| MOG | Myelin oligodendrocyte glycoprotein peptide |
| MMP | Matrix metallopeptidase |
| MS | Multiple sclerosis |
| NaCl | Sodium chloride |
| $Na_2HPO_4$ | Di-sodium hydrogen phosphate |
| NaOH | Sodium hydroxide |
| ng | Nanogram |
| NFκB | Nuclear factor 'kappa-light-chain-enhancer' of activated B cells |
| NOS | Nitric oxide synthase |
| NK cells | Natural killer cells |
| ON | Overnight |
| p | P-value |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered saline |
| PFA | Paraformaldehyde |
| p.i. | Post induction |
| PLP | Proteolipid protein |
| PMA | Phorbol 12-myristate 13-acetate |
| RA | Rheumatoid arthritis |
| rhCG | Recombinant human chorionic gonadotropin |
| RNA | Ribonicleic acid |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute |

| | |
|---|---|
| RT | Room temperature |
| RT-PCR | Real-time polymerase chain reaction |
| S.E.M. | Standard error of the mean |
| SD | Standard deviation |
| SLE | Systemic lupus erythematos |
| TCR | T cell receptor |
| TGF | Transforming growth factor |
| Th cells | T helper cells |
| Th1 | T cells producing IFN$\gamma$ an TNF$\alpha$ |
| Th17 | T cells producing IL-17 |
| TLR | Toll-like receptor |
| TGF | Transforming growth factor |
| TNF | Tumor necrosis factor |
| Treg cells | Regulatory T cells |
| TSH | Thyroid-stimulating hormone |
| uhCG | Urine-derived human chorionic gonadotropin |
| VCAM | Vascular adhesion molecule |
| VEGF | Vascular endothelial growth factor |
| VLA | Very-late antigen |

All units are represented in SI units.

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# Declaration of Honor

„I hereby declare that I prepared this thesis without impermissible help of third parties and that none other than the indicated tools have been used; all sources of information are clearly marked, including my own publications.

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