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Deciphering cAMP-dependent mechanisms for T cell regulation in the context of multiple sclerosis

Dissertation

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1. Introduction

1.1. Multiple Sclerosis

Multiple sclerosis (MS) is a multifactorial, complex and heterogenous disease. Affecting more than 2.5 million people worldwide with increasing number of cases, MS is the most common chronic inflammatory and neurodegenerative disease affecting the central nervous system (CNS) [1]. With women more often afflicted than men, there is a gender ration of 3:1 with disease onset mostly occurring between 20 and 40 years of age [2,3]. There is a high variation of clinical manifestations described in patients with MS, leading to substantial physical and psychological disability. These symptoms can involve deficits in motor, autonomic or neurocognitive function as well as depression, fatigue and sensation deficits [4,5]. The variety of symptoms can be explained by a spatiotemporal dissemination of CNS lesions which are caused by immune cell infiltration, an inflammatory response of brain-resident glia cells and subsequently demyelination of axons. The diagnosis of MS is based on clinical symptoms like limb weakness, loss of bladder control, dyscoordination, visual disturbances, and memory deficits [5]. Different forms of MS have been described with relapse-remitting MS (RRMS) being the most frequent form, affecting 85 to 90 % of total MS patients. Episodes of neurological dysfunctions are followed by remitting periods with clinical recovery of symptoms. The neurological dysfunctions usually last for days or weeks and are caused by early inflammation and axonal injury. Up to 80 % of patients with RRMS develop a secondary progressive MS (SPMS), marked by a lack of relapse recovery and a continuous deterioration of symptoms. About 10 to 15 % of patients suffer from primary progressive MS (PPMS), where neuroinflammation leads to continuous decline in MS symptoms. Profound axonal damage and neurodegeneration causes the non-relapsing, non-remitting progression of symptoms [4,6,7].

1.1.1. Aetiology and epidemiology

MS is understood as a disease with no single cause, but many different risk factors. Genetic as well as environmental factors have been shown to play a pivotal role in the onset of the disease. There is a geographic pattern in the occurrence of MS with a strong positive association between increasing latitudes and MS prevalence [8]. On that note, migration studies showed that migration from high- to low-risk countries lowers the risk to develop MS and vice versa [9]. These studies suggest the overall hypothesis that there is a genetic prevalence. And indeed, a genome-wide association study identified more than 150 single nucleotide polymorphisms (SNP) associated with increased

susceptibility to develop MS. Many of those SNP are in close proximity to regulatory coding regions of genes associated with immune function [10]. Especially genes within the human leucocyte antigen (HLA) complex have been shown to drive the development of MS. Variants of HLA class II genes, which are important for antigen presentation to cluster of differentiation (CD) 4+ T cells, seem to drive the disease onset [11,12].

Those genetic predispositions together with the exposure to environmental risk factors is supposed to result in the development of MS. Here, the inherited susceptibility is thought to account for at least 30 % of MS onsets [13]. However, MS susceptibility has been shown to be linked to environmental factors, such as viral infections (like Epstein-Barr virus infection) [14,15], childhood obesity [16], vitamin D deficiency [17,18] and smoking [19]. T which extend each environmental factors contribute to the development and immunopathology of MS remains an open question.

1.1.2. Immunopathology

Accumulation of CNS-infiltrating immune cells resulting in inflamed CNS is the main characteristic of MS. There are different approaches how tissue inflammation is initiated. First, the “inside-out” hypothesis suggests that inflammation occurs as a response of the immune system to CNS intrinsic events, which for instance can be environmental factors like virus infections [20,21]. These might trigger the disease development and lead to the infiltration of autoreactive lymphocytes as secondary phenomenon. The other hypothesis is the “outside-in” hypothesis or peripheral model, which suggests an autoimmune attack of the CNS by misguided, autoreactive immune cells [4,22]. Autoreactive lymphocytes are usually deleted in the thymus, but as this process is not entirely bulletproof, some autoreactive T cells might leave the thymus and migrate in the periphery. Even though there are peripheral control mechanisms keeping track of autoreactive T cell, these mechanisms can malfunction, which enables autoreactive T cells to get activated at the peripheral side and then potentially orchestrate the infiltration of immune cells to the CNS [23-25].

Infiltration of immune cells and subsequent CNS inflammation results in the occurrence of focal plaques, which are pathological identifiers of MS. These plaques, or lesions, are demyelinated areas within the brain, optic nerve and spinal cord depicting irreversible damage of neurons [26,27]. Active lesions are predominantly found in RRMS and are characterized by lymphocytic infiltration, activated microglia, macrophages, and reactive astrocytes in the white matter [28,29]. In PPMS or SPMS, active lesions are less frequent due to reduced inflammatory invasion of autoreactive immune cells. By contrast, inactive or chronically active lesions are, together with decrease in brain

volume, a hallmark of progressive MS. These are characterized by low lymphocyte infiltration but a demyelinated center and reduced axonal density, surrounded by activated microglia and macrophages [30,31]. Lesions are accompanied by the damage of the blood brain barrier (BBB), supporting a further profound infiltration of immune cells to the CNS [32-34]. The composition of the inflammatory infiltrate is similar in RRMS and progressive MS: major component are T-lymphocytes, dominantly major histocompatibility complex (MHC) class I-restricted cytotoxic T cells, CD4+ T helper cells, but also CD20+ B cells, monocytes, and macrophages. Specific effects of each cell type are diverse and not fully understood [4,34].

Next to CNS-infiltrating immune cells, CNS-resident cells like microglia and astrocytes play a major role in MS pathogenesis. Especially microglia are thought to contribute to the disease progression as they induce tissue damage in the CNS by secreting pro-inflammatory agents. However, on the other hand, microglia can switch upon activation, producing anti-inflammatory cytokines resulting in tissue repair [35-37].

The interaction between immune cells from the periphery and CNS-resident cells are thought to advance neuroinflammation. While during the RRMS immune cell infiltration is the driver of the disease onset, the contribution of the peripheral immune system decreases during progressive phase with a more profound role of CNS-resident cells [4]. Different treatment approaches try to reduce CNS lesions by the suppression or elimination of autoreactive immune cells. Currently, only drugs for RRMS have been approved, mostly preventing immune cell infiltration. These immunomodulatory therapies target the peripheral immune cell activation and limit the entry of immune cells to the CNS. These drugs are potent in reducing relapse frequencies. However, currently no drugs are approved to treat progressive forms of MS [5,38,39].

1.1.3. Animal models

MS is unique in humans. Until now no disease was described in other species which resembles the immunopathology of MS sufficiently to provide translatable insights in the disease. However, extensive research was performed from early 20th century on to study MS-like animal models in rhesus monkeys. Since then, different animal models of MS have been established in other species like guinea pigs, rats, and mice. The latter are now commonly used in MS research [40,41].

The most frequently used model of MS is experimental autoimmune encephalomyelitis (EAE) which is based on the peripheral model of MS development [42]. Therefore, it is an antigen-driven autoimmune model, where animals are immunized against a myelin autoantigen, eliciting a strong T cell response resulting in CNS demyelination. It was discovered coincidently in 1933 by Rivers et

al. as side effect of a rabies vaccination [43]. Immunization is performed by injecting two components: the myelin sheath peptides, which can be myelin basic protein (MBP), myelin proteolipid (PLP) or myelin oligodendrocyte glycoprotein (MOG). A peptide fragment of the latter, namely MOG_{35-55} is commonly used in C57BL/6J mice. The antigen, together with mycobacterium, is emulsified in Freund's adjuvant, triggering the immune activation pathways and causing potentiation of humoral immune response. In addition, pertussis toxin (PTX) can be used to enhance the immunization. Animals develop symptoms around 10 days after immunization starting with ascending paralysis from the tail to the hindlimbs, correlating with spinal cord inflammation. Direct immunization of mice is not crucial for the development of the EAE. A transfer of primed T cells to a naïve host has been shown to induce EAE as well [44]. Likewise, T cell clones which react to short peptides can induce the EAE as efficient as direct immunization [45,46]. Moreover, a transgenic mouse line with spontaneous outbreak of EAE has been generated: a transgenic T cell receptor (TCR) for MOG is enough for spontaneous EAE development [47]. The histopathology of the EAE is similar to MS, as lesions in the white matter of the spinal cord form during the disease course. However, as an animal model, the immunization C57BL/6J to induce EAE is rather mimicking the progressive disease course of SPMS and PPMS. SJL/J mice on the other hand are described to develop a relapse-remitting EAE upon immunization of MBP-derived Peptide or $PLP_{139-151}$ [48]. However, which distinguishes the EAE further from MS is the finding that EAE is predominantly a CD4+ T cell driven model. While CD8+ cells do have an important role in MS, EAE is thought to be a Th1 and Th17-dependent model with rather unaffected CD8+ T cells and B cells. Moreover, the lesions predominantly occur in the subpial spinal cord, while in MS the brain is mostly affected [41]. However, the EAE is the mostly used model mimicking the inflammatory aspects of MS and as such led to the discovery of several highly efficient therapeutics, like for instance glatiramer acetate, mitoxantrone and natalizumab [41,49,50].

Another model, which is commonly used as a model of MS in mice, is the injection with Theiler's murine encephalomyelitis virus (TMEV). TMEV is a small single stranded picornavirus which was firstly reported by Theiler to cause paralysis in mice [51]. The intracerebral infection of TMEV induces a chronic-progressive monophasic disease which peaks around one week after infection and ends two weeks later [41]. In some mice strains, a chronic demyelinating stage is reported after the initial phase [52]. This model reflects the potential "inside-out" hypothesis of MS pathogenesis that starts with demyelination of the brain, resulting in autoimmune response in the CNS [53].

Other models of MS use toxic substances to induce demyelination, enabling the investigation of remyelination processes for translational research of MS treatment. The cuprizone model for instance uses the copper chelator cuprizone which is fed to the mice for four to six weeks. It causes a dysfunction of the mitochondrial complex IV resulting in apoptosis of oligodendrocytes [54]. After

termination of cuprizone feeding, remyelination can be seen and studied [55]. Other compounds which are used to induce demyelination and to study remyelination are lysophosphatidylcholine and ethidium bromide which disrupt myelin lipids and induce oligodendrocyte loss [56-58]. An issue with the use of toxic substances is that no immune activity is seen and therefore only a fragment of the complex pathogenesis of MS involving the immune system can be studied.

1.2. Immune system

The immune system is a complex organization of cells and soluble molecules with specialized roles, evolved to protect the body against pathogens or neoplastic cells, while ideally not responding to endogenous, healthy cells. There are several major challenges for the immune system: the discrimination of infected or damaged cells and pathogens from healthy cells. After identification of potential threats, an attack follows together with the acquisition and coordination of other cellular and molecular immune mediators and finally the restoration of homeostasis [59].

To fulfill these functions, the different cell types of the immune system have evolved. Most immune cells develop from hematopoietic stem cells which then differentiate in the bone marrow to common lymphoid and myeloid progenitor cells [60]. Common myeloid progenitor cells differentiate to cells of the innate immune system, like macrophages and neutrophils. Their main purpose is to immediately prevent spreading of pathogens. Identification of pathogens relies on recognition of evolutionarily conserved patterns, called pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP), which are recognized by highly conserved pattern recognition receptors (PRR). After engagement with their respective ligand, innate immune cells attack infected cells or pathogens using a variety of strategies [61]. Even though this mode of action may be very effective as a first line of defense, innate immune cells lack the ability to respond rapid and efficient to a cognate pathogen – the so-called immunological memory. Cells of the adaptive immune system instead, which differentiate from common lymphoid progenitor cells, do have immunological memory [60]. Besides that, cells of the adaptive immune system, like B and T cells, are characterized by antigen-specific surface receptors [62]. Due to the process of somatic recombination in the genes coding for the B and T cell receptors during lymphocyte maturation, there is an extremely high diversity of antigen receptors. Upon antigen binding, a two-stage response starts: cell priming, activation and differentiation is followed by cell-specific effector response [60,63]. Even though innate and adaptive immune system do have distinct mechanisms to act against pathogens or infected and damaged cells, only the close interaction between the distinct cell types makes it possible to enable homeostasis. The balance between defeating potential threats like pathogens or

tumor cells and self-tolerance is crucial. The consequence of a disbalance can be infections, cancer, or autoimmune diseases like MS [62,64,65].

1.2.1. T cells

T cells are thought to be drivers of the MS pathomechanism and are therefore target of extensive MS research. T cells belong to the adaptive immune system and are classified by the expression of specific TCR, co-receptors, cytokine secretion and effector function. Their main function is the recognition of peptides presented by MHC molecules via their TCR repertoire [65]. Each TCR consists of an α and β or γ and δ chain with variable and constant region. The rearrangement of variable and constant regions yields in an extremely diverse TCR repertoire with the possibility to recognize millions of specific antigens [66,67]. In the steady state, T cells are marked by minimal proliferation and low cytokine secretion. Upon activation in secondary lymphoid organs, such as spleen, lymph nodes (LN), Peyer's patches or mucosal associated lymphoid tissue an immunological synapse (IS) with antigen presenting cells (APC) is formed. This IS and the activation of the T cell is dependent on MHC binding to TCRs and on co-stimulation, like the binding of CD28 to CD80/CD86, expressed on APCs [68,69]. Upon TCR and CD28 signaling nuclear factor of activated T cells (NFAT), nuclear factor k-light chain enhancer of activated B cells (NF-κB) and other downstream factors signaling pathways are initiated, resulting in the activation of the T cell whereafter proliferation and differentiation to effector and memory cells occurs [70,71]. Upon clearance of infection, most T cells undergo apoptosis while some remain in the periphery as memory T cells to secure a rapid immune response in case of reinfection [72,73].

CD8+ T cells and CD4+ T cells can be identified by binding of their TCRs to specific MHC molecules: while CD8+ T cells recognize antigens presented by MHC I, which are expressed on all nucleated cells, CD4+ T cells only bind to MHC II, expressed on specialized APC [74].

CD8+ T cells, also called cytotoxic T cells, are important for the immune defense against intracellular pathogens [75,76]. Upon activation and differentiation of CD8+ T cells, which is supported by CD4+ T cell- secreted interleukin-(IL-) 2, cytotoxic T cells migrate to inflamed tissue. Once they arrive at side of inflammation, CD8+ T cells can identify their infected target cell via its presentation of antigen by the MHC I complex [77]. Upon recognition, CD8+ T cells can kill the target cell by secretion of cytotoxic molecules like granzyme B or perforin [78]. Several subsets of CD8+ T cells with different functions, namely Tc1, Tc2, tc9, Tc17 and CD8+ regulatory T cells (Treg), have been described [77].

CD4+ T cells interact with other immune cells and thereby orchestrate the immune response via cytokine secretion and receptor-mediated contact. A well-studied CD4+ T cell subset are the

effector T cell (Teff), or helper T cells, which consist of Th1, Th2, Th17 and the less studied Th9, Th22 and follicular helper T cells [79,80]. Differentiation to each Teff subset is initiated by a distinct cytokine milieu which upregulates a subset specific transcription factor, leading to the secretion of signature cytokines with distinct function. Th1 subset differentiation from naïve CD4+ T cells is pre-dominantly initiated by IL-12, which leads to an upregulation of the transcription factor Signal transducer and activator of transcription 4 (STAT4). Increased STAT4 levels result in the secretion of interferon γ (IFNγ), IL-2 and Tumor necrosis factor α (TNFα). These pro-inflammatory cytokines enable Th1 subset to contribute to antibacterial and antiviral immunity [81-83]. In contrast, the Th2 subset is important for clearing extracellular pathogens and stimulate the repair of damaged tissue. Differentiation to Th2 cells is mainly initiated by IL-4, leading to the upregulation of the transcription factor GATA with initiated the secretion of IL-4, IL-5, IL-9, and IL-13 [79]. Th17 cell differentiation is initiated by IL-1β, IL-6, IL-21 and STAT3, leading to an upregulation of the transcription of RAR-related orphan nuclear receptor (RORγt). Th17 cells can be identified by the secretion of cytokines IL-17A, IL-17F, and IL-22. They are described to be involved in fighting bacterial or fungal infection but can also drive autoimmune diseases. Pathogenic Th17 subset, characterized by IL-23 receptor expression, can drive the pathology of different autoimmune disease [84,85].

Besides the Teff, Treg have been studied extensively. These cells have been shown to be essential for the maintenance of peripheral tolerance, the prevention of autoimmunity and the limitation of chronic diseases [86]. Treg can be divided in peripheral (pTreg) and thymic (tTreg) Treg. While tTreg are generated in the thymus by TCR interaction via negative selection, pTreg are induced in periphery and differentiate from naïve CD4+ T cells upon TCR activation with Transforming Growth Factor β (TGFβ) and high level of IL-2 [87]. Treg are characterized by high CD25 expression and their master transcription regulator Forkhead-Box-Protein 3 (Foxp3). Foxp3, also called scurfin, is essential for the immunosuppressive capacity of Treg. In absence of Foxp3, human and mice develop severe autoimmune dysfunction. In mice, a knockout of Foxp3 leads to the development of “scurfy” mice, which are characterized by the development of severe, generalized autoimmune disorders which can affect almost all organs and result in an extremely reduced lifetime [88,89]. In human, a similar effect of dysregulated Foxp3 has been described: the Immunodysregulation Polyendocrinopathy Enteropathy X-linked (or IPEX) syndrome. Patients develop severe autoimmune-associated symptoms, like enteropathy, dermatitis and type 1 diabetes due to the destruction of the pancreas by immune cells [90,91]. Primary target cells of Treg-mediated immunosuppression are Teff, dendritic cells (DC) or other APCs. There have been four mechanisms described how Treg can mediate immunosuppression [92,93]. First of all, Treg can secrete inhibitory cytokines, like IL-19, IL-35 and TGFβ, which suppress the pro-inflammatory activity of the adaptive

immune system [94]. Secondly, Treg can suppress target cells by granzyme A, granzyme B or perforin induced cytolysis [95]. Moreover, Treg can suppress their target cells by metabolic disruption, for instance via the intercellular transfer of 3',5'-cyclic adenosine monophosphate (cAMP) via gap junctions or the generation of extracellular adenosine, leading to an elevation of intracellular cAMP via adenosine receptors on target cells [96,97]. Furthermore, Treg can modulate the maturation and function of DCs via a CTLA-4 -dependent mechanism [86]. Besides Teff and Treg, there are also unconventional T cell subsets, like γδT cells, NK T cell and MAIT cells [65].

1.2.2. T cells in MS

In MS, T cells are thought to be main driver of disease onset and progression. While CD8+ T cells are predominantly found in MS lesions, CD4+ T cells play a crucial role in disease pathogenesis. In accordance with the "outside-in" hypothesis, MS is originally considered a CD4+-mediated autoimmune disease, based on CNS-infiltrating Th1 and Th17 cells [98,99]. This is supported by the finding of MS-associated variants in HLA class II genes. These genes are involved in the MHC-dependent presentation of antigens to CD4+ T cells. Carriers of the MHC II allele HLA DRB1+15:01 have a three times higher risk to develop MS compared to non-carriers [100]. Moreover, T cells appear very early in CNS lesion formation, a finding which further supports the autoimmune hypothesis [101]. Myelin-protein-derived antigens, like MBP or MOG which are also used in EAE induction, are suggested to be the main autoreactive targets. A reactivity of T cells isolated from MS patients towards these antigens has been reported [102]. But also T cells from healthy individuals show autoreactivity to myelin-protein-derived antigens, leading to the open question whether there is one distinct autoantigen in MS or not.

However, autoreactive T cells, activated by autoantigen, should be suppressed by functioning Treg. Whether a lack of immunosuppressive Treg results in a disbalance in favor of autoreactive T cells remains under debate. Several studies have been conducted to answer that. Treg frequency of MS patients versus healthy control has been shown to be comparable, leading to the question whether Treg are even involved in the development of MS [11,103,104]. An indication of such involvement is, that even frequency is similar, Treg from MS patients seem to be functionally impaired compared to those of healthy controls. MS-derived Treg were reported to show deficits in maturation and suppressive capacity [11,103]. Even though the extend T cell involvement in MS remains is still under debate, clinical trials of immune modifying drugs proof that T cells play indeed a critical role in the pathogenesis of MS. Targeting those cells of the peripheral immune system, at least in RRMS, can suppress an exacerbation of symptoms as well as frequency of relapses. Natalizumab for instance is

an ITGA-4 antibody, which blocks the extravasation of T cells, thereby limiting their migration across the BBB to the CNS parenchyma. Unfortunately, it is only effective in RRMS. As soon as a progressive state is reached and an uncoupling from peripheral immune system happens, natalizumab and other immune modulatory drugs fail [105-108].

1.2.3. T cells in EAE

In the EAE, the minimal requirement for disease induction is the activation of encephalitogenic Teff in the peripheral immune system [109,110]. Even though both, autoreactive Th1 and especially Th17 cells have been shown to be capable to induce EAE in transfer experiments, it is not entirely clarified how the T cell subsets drive the disease course [111,112].

Th1 and Th17 lead to different disease outcomes: Th1 cells have been shown to infiltrate the spinal cord and secrete IFNγ which drives the classical disease progression [113,114]. IFNγ has opposing effects on EAE pathogenesis, depending on where and when it is secreted. During the EAE induction and early phase of disease onset, IFNγ has been shown to deteriorate severity. In contrast, administration of IFNγ in later stages of EAE pathogenesis ameliorates symptoms [115]. This beneficial effect of IFNγ is also supported by studies with IFNγ or IFNγ-receptor knockout mice which showed increased EAE severity [116,117]. Moreover, IFNγ has opposing effects in brain and SC. While IFNγ in the SC induces inflammation and promotes macrophages, neutrophil, and monocyte infiltration via upregulation of CXCL2 and CCL2, it suppresses inflammation and inhibits CXCL2-dependent cell infiltration in the brain [118,119].

Th17 cells are the main CNS-infiltrating cells in the EAE, where they produce IL-17A, granulocyte macrophages-colony stimulating factor (GM-CSF) and partially also IFNγ resulting in atypical EAE symptoms [110,120]. In contrary to IFNγ, IL-17A has been described to be important for disease progression in the EAE as it leads to increasing neutrophil infiltration. IL-17A further contributes to a BBB break down via a loss of tight junction proteins by increasing reactive oxygen species (ROS) production of brain endothelial cells [121]. However, blocking IL-17A with an antibody or genetical ablation of IL-17A does not affect disease course [111,122,123]. Th17 cells derived GM-CSF is also crucial for EAE pathogenesis as mice treated with an anti-GM-CSF antibody or with genetical ablation of GM-CSF are resistant to EAE induction [124,125]. Moreover, GM-CSF can also increase the recruitment of neutrophils to the brain, leading to an atypical EAE pathogenesis [119,126].

Next to their distinct cytokine secretion profiles, Th1 and Th17 infiltration of the CNS occurs on different entry sites with Th1 subset mainly infiltrating spinal cord, and Th17 mainly infiltrating the brain [112,113]. Moreover, entry of Th1 and Th17 is mediated via the expression of different cytokine

receptors and integrins. Th1 infiltration in spinal cord requires VLA-4 expression [127]. Therefore, treatments with an anti-a4 integrin antibody completely diminished disease pathogenesis in an adoptive transfer EAE using only Th1 cells [128]. Th17 cell have lower expression of VLA-4 and therefore require other mechanisms for CNS infiltration. LFA-1 and CCR6 have been shown to be mainly involved in enabling CNS infiltration of Th17 cells [128,129]. Treg on the other hand have shown to be protective against the development of EAE. There have been several studies suggesting that the adoptive transfer of CD4+ CD25+ T cells to C57BL/6J mice ameliorates severity or even prevents spontaneous EAE in transgenic mice with anti-MBP TCR [130-132].

In summary, both Th1 and Th17, as well as Treg, can influence EAE onset and pathogenesis even though the exact contribution of each T cell subset and the secreted cytokines is not yet fully understood. Neither are the mechanisms which regulate their downstream signaling and thereby their function fully deciphered.

1.3. cAMP

cAMP is a ubiquitous second messenger, which translates signals from outside the cell to its inside, triggering downstream signaling cascades and modulating cell function. cAMP pathways have been shown to be involved in the control of a variety of cellular function in all cell types. It is therefore no surprise that malfunction of cAMP-signaling is associated with a variety of pathologic conditions like chronic kidney disease, cardiovascular diseases, cancer, and autoimmune diseases like MS [133,134]. Downstream effectors of cAMP are protein kinase A (PKA), exchange protein activated by cAMP (EPAC), and cyclic nucleotide-gated ion channels. Besides acting on multiple effectors, the amount of cAMP and its temporal and spatial compartmentalization results in tightly adjusted cAMP-mediated cell responses to a specific trigger [135,136]. These cAMP-mediated pathways and their upstream regulators are cell type specific.

1.3.1. cAMP in T cells

In the immune system, the cAMP-PKA signaling pathway predominantly affects the regulation of immune response. In T cells, the mechanisms by which cAMP regulates T cell function has not yet been deciphered entirely. Overall, cAMP is mainly described to exert a suppressive effect on Teff function [136]. Upon increasing levels of cAMP in Teff, their activity and proliferation decreases [137]. These effects are predominantly mediated via PKA. Rising levels of cAMP result in an activation of the catalytic subunit of PKA inhibiting several transcriptional factors, like inducible cAMP early repressor (ICER) and NF-κB or NFAT, thereby modulating IL-2 expression and T cell activity [138,139].

Moreover, PKA can modulate the cAMP-responsive element (CRE) binding protein (CREB). Upon phosphorylation of CREB binds to CRE, thereby modulating the expression of genes associated with T cell function [136,140,141].

However, recent studies also indicate that the EPAC-mediated pathway might be involved in T cell regulation [142]. Intracellular cAMP levels depend on G-protein coupled receptors (GPCR). The seven-transmembrane receptors can be divided in Gi-, Gs- and Gq-coupled, dependent on the associated G protein. Each G protein is trimeric and consist of α, β, and γ subunit [143]. While Gq-coupled receptors are involved in Ca^{2+} pathways, Gs- and Gi-coupled receptors affect intracellular cAMP levels [143]. Depending on the alpha subunit, GPCRs can either activate (Gs-coupled) or inhibit (Gi-coupled) adenylyl cyclases (AC), resulting in increase or decrease of intracellular cAMP. GPCRs can either get activated by a ligand or possess a constitutive activity [144]. Besides GPCR-mediated cAMP increase, an elevation of cAMP in Teff can also be reached upon cell-cell contact of Treg and Teff via gap junctions and a direct transfer of cAMP from Treg to Teff [145,146]. This mechanism is crucial for the immunosuppressive capacity of Treg on other immune cells. Moreover, extracellular adenosine can be generated upon degradation of ATP by the ectonucleotidases CD39 and CD73 which are highly expressed on Treg [147]. Upon binding of adenosine to its receptor adenosine A2A receptor on Teff, an increase in intracellular cAMP levels in Teff and a suppression of their activity occurs. Blocking or deficiency of the ectonucleotidases CD39 and CD73 results in reduced immunosuppression and several pathophysiological events like cancer or the development of autoimmune diseases [148].

The only known enzymes which can hydrolyze cAMP to 5'AMP and thereby reduce intracellular cAMP levels are phosphodiesterases (PDE). The superfamily of enzymes consists of 11 different PDE families, all with the ability to hydrolyze cAMP [137]. In T cells, PDE1-5, 7, and 8 have been found. Besides hydrolyzing cAMP to decrease intracellular cAMP levels, access to the second messenger can be limited by compartmentalization of cAMP itself as well as receptors, AC or PKA by so called A-kinase anchoring proteins (AKAP). This spatiotemporal accessibility of cAMP to its effectors is crucial for cAMP-mediated effects [149].

1.3.2. GPCRs in T cell function

Prior to compartmentalization, cAMP has to be generated, a process that is regulated by Gs-coupled GPCR. T cells do express a large variety of GPCRs which are involved in T cell function. The best studied GPCRs in the context of T cell function is the prostaglandin E2 (PGE2) receptors, namely EP1-4 [150]. Upon binding of PGE2 to its receptors EP2 and EP4, the Gs-coupled receptors get activated,

resulting in elevated cAMP levels which inhibit TCR-induced T cell activation [137]. Moreover, several chemokine receptors, which regulate T cell migration, are GPCRs. Amongst them are CXCR3 and CXCR4, both regulating T cell trafficking and formation of the IS upon binding of cognate ligands [151-153]. Besides those receptors with known ligands, also several orphan and recently de-orphanized GPCRs have been shown to be involved in T cell function. G2 accumulation (G2A) for instance has been reported to regulate T cell function and migration. G2A KO mice showed enhanced T cell proliferation and activation and suffered from systemic autoimmune disease [154,155]. GPR30, for which estrogen has just recently been identified as an endogenous ligand, has been shown to be a modulator of the EAE disease course [156]. Moreover, GPR30 activation resulted in an enhanced suppressive capacity of Treg [157]. Another recently de-orphanized GPCR is GPR83, which plays a crucial role in modulating Treg function in response to the neuropeptide PEN [158]. Even though there are controversial studies about GPR83-dependent Treg induction, an involvement of GPR83 in inflammation has been reported in several studies [159-162]. In summary, some GPCRs have been identified to be involved in T cell function and are crucial for a functioning immune system. However, there are several GPCRs which have not yet been studied in this context and neither in their ability to alter cAMP in T cells.

1.3.2.1. GPR52

One of these orphan GPCRs with unknown function in T cells is the constitutively active GPCR GPR52 [163,164]. It is a family A Gs-coupled receptor and hence its constitutive activity increases intracellular cAMP levels, which has been shown in studies using published antagonists and agonists on transfected HEK cells and primary neurons [163,165]. GPR52 is highly expressed in the brain, especially in striatal dopaminergic neurons and the nucleus accumbens [163]. Therefore, GPR52 has been connected to substance use disorders and psychiatric disorders like schizophrenia [166,167]. It has been proposed that agonism of GPR52 improves recognition memory and could be a therapeutic intervention for psychotic and cognitive dysfunctions in schizophrenia [163,168]. On the other hand, inhibition of GPR52 or genetical deletion has been reported to reduce soluble mutant Huntington level in an animal model of Huntington's disease, resulting in the rescue of behavioral phenotypes [165]. Several agonists have been synthesized with the orally bioavailable 4-(3-(3-fluoro-5-(trifluoromethyl)benzyl)-5-methyl-1H-1,2,4-triazol-1-yl)-2-methylbenzamide (FTBMT) being the most commonly used and best characterized one [163]. Two antagonists have been described by the same research group, namely E7 and compound 43 [165,169]. Moreover, the recent cannabinoid ligands cannabidiol and O-1918 have been described as inverse agonists for GPR52 [170]. In summary, GPR52

with its antagonists and agonists is a promising target in therapeutical approaches. However, it is not known whether GPR52 is also a promising target to modulate immune responses in the context of autoimmune diseases.

1.3.3. PDEs in T cell function

As cAMP is an important mediator of adequate Teff response and for the immunosuppressive capacity of Treg, PDEs have been a promising target to study in T cell function and autoimmune diseases like MS. PDE3B, PDE4, PDE7 and PDE8 have been studied in MS research and have been reported to affect the disease course of the EAE [171-173].

Blocking PDE3B via antibodies ameliorated the encephalitogenic-specific T cell response in the EAE. It reduced lymphocytic proliferation and IFNγ production in the CNS and thereby reduced severity of EAE symptoms [174]. While inhibition of PDE7 ameliorated the clinical scores of the EAE, a global KO of PDE7 had no effect on disease severity. The inhibition resulted in the increase of Foxp3 mRNA levels, reduced IL-10 and IL-17A secretion, as well as T cell proliferation, but none of these effects were observed in PDE7 KO mice [172,175,176]. A recent study focused on the involvement of PDE8 blockade in the EAE. Blocking of PDE8 with a selective antibody ameliorated EAE symptoms, probably by affecting T cell motility and regulating the LFA-1 integrin adhesion to ICAM-1 [177]. The best studied PDE in MS research is PDE4, with two clinical trials for drug development. In mouse models, inhibition of PDE4, for instance with the selective inhibitor Rolipram, ameliorates clinical EAE symptoms and the infiltration of immune cells into the CNS. Several possible factors are suggested to lead to this amelioration of EAE symptoms. First, inhibition of PDE4 has been shown to reduce T cell proliferation and secretion of pro-inflammatory cytokines like TNFα and IL-17A, while it increased secretion of the anti-inflammatory cytokine IL-10 in mice during the EAE [171,172]. Moreover, inhibition of PDE4 has been shown to strengthen the BBB, probably via affecting cAMP levels in endothelial cells [178]. Even though Rolipram was a promising drug for treatment of MS, clinical trials were closed, due to side effects like nausea and vomiting [179]. However, another PDE4 inhibitor, Ibudilast, had less side effects and showed promising result in clinical trials: in RRMS patient, Ibudilast treatment reduced brain atrophy and led to shrinkage of severe brain injury MRI signals [180]. Reduced brain atrophy was also reported in PPMS and SPMS patients treated with Ibudilast [181].

In summary, studying PDEs for their therapeutic potential to treat autoimmune diseases such as MS is appealing, but the role of some PDEs has not been addressed so far.

1.3.3.1. PDE2A

One of the neglected PDE family members is PDE2A. PDE2A, or cyclic guanosine monophosphate (cGMP) -activated PDE, has a dual substrate activity and can hydrolyze both, cAMP and cGMP. PDE2A functions as homodimer and is organized in 4 domains, namely N-terminus, GAF-A, GAF-B and the catalytic domain, the latter being a variable domain [182,183]. Alternative splicing of the N-terminal domain leads to different isoforms. Three isoforms have been described: PDE2A1, PDE2A2, and PDE2A3. The isoforms have a common domain structure and show isoform-specific localization. PDE2A1 is mostly cytosolic and highly expressed in cardiac ventricles. PDE2A2 is mostly found in the mitochondrial matrix and is suggested to regulate mitochondrial respiration via cAMP [184]. PDE2A3 is found in the plasma membrane, Golgi body, nuclear envelope and sarcoplasmic reticulum. It is highly expressed in the heart, adaptive immune cells like T lymphocytes and macrophages [185-187], and in the brain, where it is targeted to synaptic membranes [184]. PDE2A has mainly been in focus of cardiovascular research, but rising interest comes from neurobiological research. Several PDE2A inhibitors have been developed and tested *in vivo* and *in vitro*. The best studied inhibitor is BAY 60-7550 (BAY). BAY is binding to the active site of the PDE2A isoforms and interacts with the conserved glutamate residue Gln859. Gln859 is responsible for glutamine-switch, which is a key factor of the dual-substrate specificity of PDE2A. Binding to that induces a conformational change of PDE2A, leading to the formation of a hydrophobic pocket for further binding of BAY and thereby inhibiting the hydrolyzing activity of PDE2A towards cGMP and cAMP. Other inhibitors have also been developed, but BAY remains the inhibitor with a high specificity (50 to 100-fold compared to other PDEs) and inhibitory capacity (IC_{50} = 0.002µM) and is therefore often used to study the role of PDE2A in disease models [188,189].

1.3.3.1.1. PDE2A in disease

PDE2A has been in focus of cardiovascular research since the 1970s. Since then, the diverse involvement of PDE2A in cardiovascular diseases has been investigated. Altered PDE2A activity was found in several animal models of cardiovascular diseases, like a murine model of myocardial infarction, hypertrophy, and chronic pressure overload. Treatment with BAY has beneficial effects on these and other cardiovascular disease models [190,191]. In human heart failure, a twofold higher concentration of PDE2A can be observed [192]. The importance of PDE2A for a functioning heart becomes clear when looking at global PDE2A knockout (KO) mice. These mice are usually embryonically lethal, with the majority of embryos dying at embryonic day 17.5 and only a few mice

surviving until adulthood [193]. The reason for this phenomenon is a nuchal edema with enlarged hearts as well as interventricular septum and myocardial wall defects resulting in congenital heart failure [193,194].

Besides its involvement in heart function, PDE2A has been extensively studied in the context of memory and learning. Inhibition of PDE2A with BAY has been shown to enhance learning in the novel recognition task, object localization and the social recognition task in rats [195-197]. Moreover, anxiolytic effects in mice have been observed following inhibition of PDE2A with BAY or another PDE2A inhibitor, namely ND7001 [195,198]. Taken together, PDE2A is a well-studied enzyme in cardiovascular research, memory and learning. Its involvement and importance in the immune system, and more specifically in T cells, however, has not been studied.

1.3.3.1.2. cGMP/cAMP crosstalk

The alternative name for PDE2A is cGMP-activated PDE. Due to its dual substrate specificity, PDE2A can hydrolyze both, cGMP and cAMP. Upon binding of cGMP to the GAF-B domain of PDE2A, the hydrolytic activity of PDE2A towards cAMP increases, resulting in a reduction of intracellular cAMP levels [195,199]. This connection between cGMP levels and cAMP levels via PDE2A is described as cGMP/cAMP crosstalk (Figure 1). Besides PDE2A, also other PDEs like PDE3B have a dual-substrate activity [200]. cGMP can be generated via activation of guanylyl cyclases (GC). Two membrane-bound, peripheral GC (pGC) and soluble GC (sGC) have been identified. sGC gets activated via nitric oxide (NO) [201], while the two pGC, namely pGC-A and pGC-B can be activated via the natriuretic peptides (NP). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) bind with high affinity to pGC-A, which is therefore also called natriuretic peptide receptor 1 (NPR1) [202-204]. C-type natriuretic peptide (CNP) binds with high affinity to pGC-B or natriuretic peptide receptor 2 (NPR2) [203]. Upon activation of pGC or sGC, guanosine triphosphate (GTP) is converted to cGMP and can then exert its effect on PDE2A, resulting in decreasing level of intracellular cAMP (Figure 1).

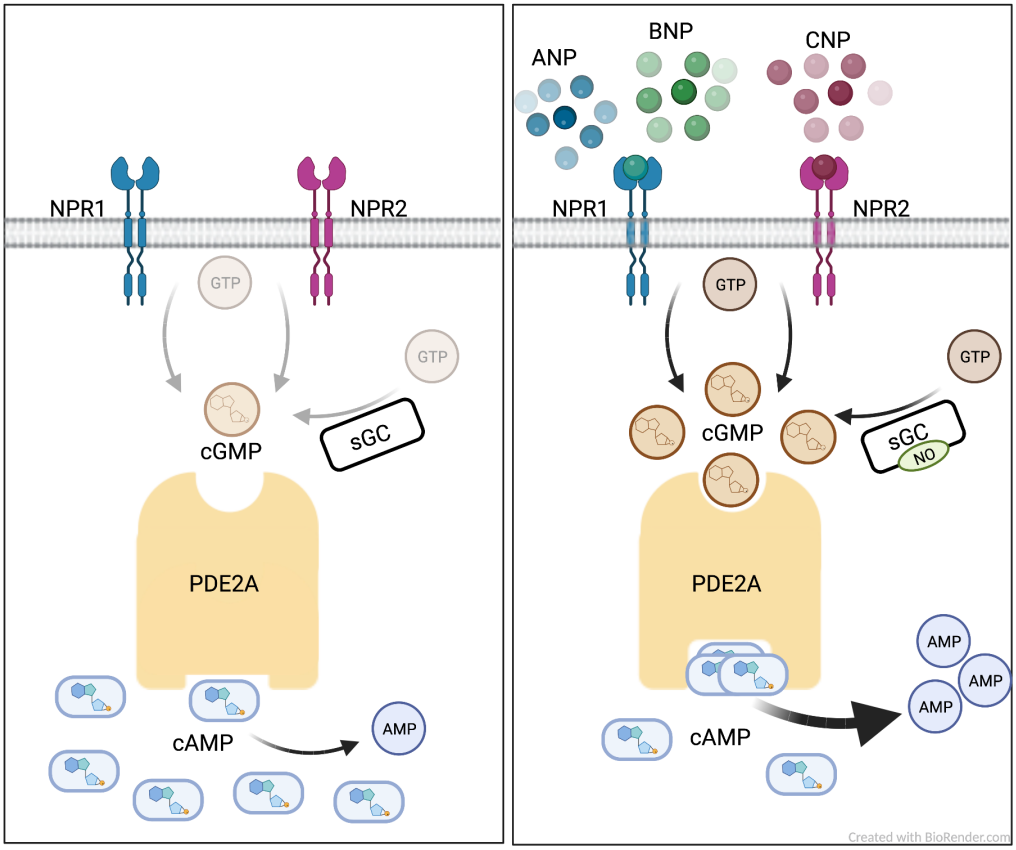

Figure 1: The cGMP/cAMP crosstalk. Binding of natriuretic peptides to their cognate receptors NPR1 and NPR2 results in an increase of cGMP. cGMP levels can also increase via activation of sGC by NO. cGMP binds to PDE2A and increases it hydrolyzing activity towards cAMP. Thereby high levels of cGMP, generated via NPRs or sGC, result in less intracellular cAMP.

1.3.3.1.3. Natriuretic peptides

NPs are evolutionary conserved peptides with a huge range of action. ANP was the first peptide discovered. De Bold described ANP as atrial natriuretic factor and identified that atrial granularity was altered upon electrolyte disbalance and showed that atrial tissues extract can promote diuresis and natriuresis in rats [205,206]. Since then, ANP has been in focus of cardiovascular research. The precursor peptide gene (*Nppa*) encodes for a 151 amino acid (AA) preprohormone which is proteolytically processed to form the 126 AA prohormone $proANP_{1-126}$. ProANP can then be cleaved by the cardiac protease corin to form $proANP_{1-98}$ and the biologically active 27 AA ANP_{99-129} [207]. ANP is primarily secreted in the atrium. During pressure increase and volume loading, plasma ANP (and BNP) concentration increase, making ANP (and BNP) accessible as biomarkers for heart failure [208,209]. Moreover, dysregulation of ANP is connected to anxiety disorders, mood disorders, PTSD and schizophrenia via a modulation of ANP release by the hypothalamic-pituitary-adrenal (HPA) axis [210].

BNP, as the name brain natriuretic peptide indicates, was firstly discovered in porcine brain. However, its main expression side is the cardiac ventricles [211]. Like ANP, it is synthesized as 132 AA prepropeptide and gets cleaved by endoproteases to the 108 AA precursor protein $proBNP_{1-108}$. It is then further cleaved in a biologically active 32 AA and a 76 AA fragment. In contrast to ANP, BNP is not stored as propeptide, but mostly abundant as 32 AA peptide [212]. Plasma BNP level increase during ventricular dysfunction and therefore have a diagnostic and prognostic significance [213].

CNP was discovered last with the endothelium and brain as major source [214]. The 103 AA propeptide is cleaved into a 53 AA fragment and the mature and biologically active 22 AA CNP_{1-22} [215-217].

NPR1 as the primary receptor for ANP and BNP can mainly be found in the cardiovascular system, but also in other organs like the kidney and lung [218,219]. NPR2, the primary receptor of CNP, is mainly expressed in chondrocytes, oocytes, endothelial cells and the brain [220-223]. It is known to be involved in chondrocyte proliferation and differentiation. Therefore, mutations in the *Npr2* gene or loss of function leads to dwarfism in mice and human [224,225]. Besides NPR1 and NPR2, a third NPR (NPR3) exists, which is thought to function as clearance receptor with no guanylyl cyclase activity [226].

An involvement of NPs in immune function has been addressed in a limited number of studies. Vollmar and colleagues was the first who studied NP in immune cells and showed that *npr1* expression is detectable in thymus, spleen and lymph nodes (LN) [227]. Moreover, they showed that ANP treatment inhibits thymocyte proliferation and TNFα secretion via an increase of cGMP resulting in the inhibition of NF-κB pathway [228,229]. BNP on the other hand has been shown to be secreted by macrophages and infiltrating T cells after heart transplantation [230]. Moreover, treatment of myocytes with cytokines like IL-1β, IL-6 and TNFα resulted in increased BNP secretion and *Nppb* expression [231-234]. *Nppc* has also been reported to be expressed in spleen, thymus and LN [235]. A controversial role of CNP in the immune system has been reported in a limited number of publications. CNP secretion by endothelial cells can be triggered by inflammatory cytokines like TNFα, IL-1β and TGFβ [236-238]. A modulatory effect of CNP has also been suggested for several diseases. For instance, CNP levels are increased in patients with septic shock [235]. Moreover, CNP reduced macrophage, neutrophil and lymphocyte accumulation in the lung in a model of pulmonary hypertension and reduced the expression of cytokines like TNFα, IL-6 and IL-1β in a model of acute renal injury [239,240]. In macrophages it was also shown that CNP treatment reduces pro-inflammatory cytokine production and inflammatory reaction in a model of epididymitis [241]. In summary, the NP are interesting peptides with a variety of functions. However, the involvement of NP T cells in the context of autoimmune diseases like MS remains an open question.

1.4. Aim of the study

Endogenous modulators of intracellular cAMP levels, like GPCRs and PDEs, are important targets to study in the context of T cell function and autoimmune diseases like MS. Pharmacological modulation or genetical depletion of several PDE and GPCR family members have been shown to alter T cell function and to modulate severity of neuroinflammatory disorders. Hence, deciphering the involvement of so far unstudied GPCRs and PDEs might decipher mechanisms for new therapeutical approaches to treat MS. The overall aim of this work is to analyze the involvement of GPR52 and PDE2A on T cell function in the context of the autoimmune disease MS. As NP can modulate the activity of PDE2A via the cGMP/cAMP crosstalk, further focus was put on the effect of NP treatment on T cell function in the context of MS.

To achieve this, following aims were addressed:

1. Identification of novel cAMP-modulating targets in T cells.
2. Deciphering the role of GPR52 on T cell function and encephalitogenic immune responses.
3. Investigating the involvement of PDE2A in T cell function.
4. Studying the impact of NPs on T cell function.

2. Material and methods

2.1. Material

2.1.1. Laboratory animals

Seven- to twelve-week-old mice were used for the experiments. Mice were housed under SFP conditions at 55 - 65 % humidity at 18 - 23 °C with a 12h light/dark cycle at the University Medical Center Hamburg-Eppendorf (UKE) in the Center for Molecular Neurobiology Hamburg (ZMNH). Food and water were provided *ad libitum*. Two weeks before *in vivo* experiments started, mice were transferred to the experimental barrier. All experiments were approved by the local ethics committee (Behörde für Justiz und Verbraucherschutz Hamburg, Tierversuchsantrag NR. 45/17 or 83/19, ORG 713 or ORG 946).

Table 1: Mouse strains

Mouse strain	*Official symbol*	*Origin*
C57BL/6J	C57BL/6J	The Jackson Laboratory
DEREG	C57BL/6-Tg(Foxp3-DTR/EGFP)23.2Spar/Mmjax	The Jackson Laboratory
2D2	C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J	The Jackson Laboratory
Gpr52 KO	$Gpr52^{tm1Kohi}$	Takeda Pharmaceutical Company Limited

2.1.2. Reagents

Table 2: Reagents for genotyping of mouse strains

Reagent	*Company*
Agarose Ultra Pure	Merck
ddH_2O	Generated in house
dNTP	Thermo Fisher Scientific
DreamTaq Green Hot Start Buffer (10x)	Thermo Fisher Scientific
DreamTaq Hot Start Green DNA Polymerase	Thermo Fisher Scientific
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific
Genotyping master mix (2x)	Thermo Fisher Scientific
QuickExtract DNA Extraction Solution	Lucigen
RotiSafe	Carl Roth
TaqMan SNP genotyping assays (40x)	Thermo Fisher Scientific

Table 3: Primer for genotyping of mouse strains

Mouse line	*Primer*	*Sequences*	*Company*
GPR52	*Gpr52* WT forward	CTAGCGTGTTTTACATGCTGTGGCTTC	biomers
	Gpr52 KO forward	CAGCCGAACTGTTCGCCAGGCTCAAGG	
	Gpr52 WT reverse	GCAGGAATTTGCCCGTCTCCTAGGTTT	
2D2	forward	CCCGGGCAAGGCTCAGCCATGCTCCTG	biomers
	reverse	GCGGCCGCAATTCCCAGAGACATCCCTCC	
DEREG	*eGFP* forward	CGGCGAGCTGCACGCTGCCGTCCTC	biomers
	eGFP reverse	CCTACGGCGTGCAGTGCTTCAGC	

Table 4: Reagents for mouse cell culture work

Reagent	*Company*
7 mm stainless steel bead	Qiagen
Bromodeoxyuridine (BrdU)	Thermo Fisher Scientific
C tubes	Miltenyi
CellTrace™ CFSE Cell Proliferation Kit	Thermo Fisher Scientific
CellTiter Glo Luminescent Cell Viability Assay	Promega
Collagenase I	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Merck
Disposable hemocytometer	NanoEntek
DNase I	Merck
Dynabeads™ mouse T-activator CD3/CD28	Thermo Fisher Scientific
EDTA	Thermo Fisher Scientific
IFNγ MAX Deluxe	BioLegend
Ionomycin	Sigma-Aldrich
NaCl	Sigma-Aldrich
PBS (1x)	Pan-Biotech/ Capricorn
Percoll	GE Healthcare
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Recombinant mouse IL-1β	Peprotech
Recombinant mouse IL-12	Peprotech
Recombinant mouse IL-2	Peprotech
Recombinant mouse IL-4	Peprotech
Recombinant mouse IL-6	Peprotech
Trypanblue solution	Sigma-Aldrich
Ultra-LEAF Anti-IL-12/IL-23	BioLegend
Ultra-LEAF Purified Anti-CD28 (Clone 37.51)	BioLegend
Ultra-LEAF Purified Anti-CD3 (Clone 145-2C11)	BioLegend
Ultra-LEAF Purified Anti-IL-4	BioLegend
Ultra-LEAF Purified anti-mouse IFNγ	BioLegend

Table 5: Reagents for human cell culture work and for staining of human samples

Reagent	*Company*
Fixation buffer	BD
Ultra-LEAF Purified human Anti-CD3 (Clone OKT3)	BioLegend

Ultra-LEAF Purified human Anti-CD28 (Clone CD28.2)	BioLegend

Table 6: Compounds for in vitro experiments

Reagent	*Concentration*	*Company*
BAY 60-7550	100nM – 1µM	Santa Cruz
Atrial Natriuretic Peptide	200nM	Bachem
Brain Natriuretic Peptide	200nM	Bachem
C-type Natriuretic Peptide	100nM – 300nM	Bachem
P19	100nM – 500nM	Phoenix Peptides
SNP	15µM – 150µM	Sigma Aldrich
SNAP	10nM – 1µM	Sigma Aldrich

Table 7: Reagents for magnetic-associated cell sorting

Reagent	*Company*
LS columns	Miltenyi Biotec
Mojosort CD4+ T cell isolation kit	BioLegend
Mojosort naïve CD4 T cell isolation kit	BioLegend
CD4+ CD25+ regulatory T cell isolation kit	Miltenyi Biotec
CD90.2 Microbeads	Miltenyi

Table 8: Reagents for animal experiments

Reagent	*Company*
CO_2/O_2 gas mixture (80% CO_2, 20% O_2)	SOL Deutschland
CO_2 gas (100%)	SOL Deutschland
DietGel Recovery	Clear H_2O
Incomplete Freund's adjuvant	BD Bioscience
Mycobacterium tuberculosis	BD Bioscience
MOG_{35-55} peptide	Peptides & elephants
PBS (1x)	Pan-Biotech
Pertussis toxin (Bordetella pertussis)	Calbiochem (Merck)
Ketanet ®S 25mg/ml (Ketamine)	Pfizer Pharma
Rompun ® 2% (Xylazine)	Bayer

Table 9: Reagents for RNA isolation

Reagent	*Company*
β-mercaptoethanol	Sigma-Aldrich
Ethanol (100%)	Carl Roth
RNeasy Micro Kit	Qiagen
RevertAid Strand cDNA Synthesis Kit	Thermo Fisher Scientific

Table 10: Primer sequences for qPCR

Gene	*Primer sequence/ Assay ID*	*Company*
Gpr52 forward	5′-TTGTCTTGCTGACATTTCTGATCA-3′	Thermo Fisher Scientific
Gpr52 reverse	5′-GGAGCACAGTGAAAGACAAAGATG-3′	Thermo Fisher Scientific
Tbp forward	5′-GTAGCGGTGGCGGGTATC-3′	Thermo Fisher Scientific
Tbp reverse	5′-CATGAAATAGTGATGCTGGGA-3′	Thermo Fisher Scientific
Pde2a	Mm01136644_m1	Thermo Fisher Scientific
Nppc	Mm01285410_m1	Thermo Fisher Scientific

Table 11: Reagents for flow cytometry and fluorescence-activated cell sorting

Reagent	*Company*
BD CompBeads (Anti-rat and anti-hamster Igκ/ negative control compensation particle set)	BD Bioscience
BD FACS Clean Solution	BD Bioscience
BD FACS Flow	BD Bioscience
BD FAC Rinse Solution	BD Bioscience
BD Trucount tubes	BD Bioscience
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Invitrogen (Thermo Fisher Scientific)
TruStain FCX anti mouse CD16/32	BioLegend
Alexa Fluor 750 NHS Ester	Thermo Fisher Scientific
UltraComp eBeads (compensation beads)	Invitrogen (Thermo Fisher Scientific)

2.1.3. Antibodies

Table 12: Antibodies for mouse flow cytometry experiments.

Antigen	*Clone*	*Fluorochrome*	*Dilution*	*Company*
BrdU	5D4	FITC	1:30	BioLegend
CD3ε	145-2C11	BV421	1:200	BioLegend
CD3ε	145-2C11	FITC	1:100	BioLegend
CD3ε	145-2C11	PerCP-Cy5.5	1:100	BioLegend
CD4	GK1.5	BV786	1:200	BioLegend
CD4	GK1.5	PB	1:100	BioLegend
CD4	GK1.5	PE-Cy7	1:100	BioLegend
CD8a	53-6.7	APC	1:100	BioLegend
CD8a	53-6.7	PerCP-Cy5.5	1:100	BioLegend
CD25	PC61	AF488	1:100	BioLegend
CD69	H1.2F3	PE-Cy7	1:100	BioLegend
CD127	A7R34	BV421	1:100	BioLegend
IFNγ	XNG1.2	BV605	1:50	BioLegend

IL-4	11B11	PE-Dazzle	1:50	BioLegend
IL-17A	TC11-18H10.1	APC	1:50	BioLegend
Foxp3	FJK-16s	APC	1:500	Thermo Fisher Scientific
Foxp3	150D	FITC	1:25	BioLegend

Table 13: Antibodies for human flow cytometry experiments.

Antigen	*Clone*	*Fluorochrome*	*Dilution*	*Company*
CD3ε	UCHT1	PB	1:300	BioLegend
CD4	SK3	BUV737	1:300	BD
CD25	BC96	AF488	1:100	BioLegend
CD69	FN50	PE-Cy7	1:100	BioLegend

2.1.4.Solutions, buffer and media

Table 14: Solutions, buffer and media

Name	*Reagent*	*Concentration/volume*	*Company*
T cell media	β-mercaptoethanol	0.01%	Sigma-Aldrich
	Fetal calf serum (FCS) (BC BW9645)	10%	Sigma-Aldrich
	Penicillin and streptomycin	1%	Thermo Fisher Scientific
	HEPES	1%	Thermo Fisher Scientific
	NEAA	1%	Thermo Fisher Scientific
	Sodium pyruvate	1%	Thermo Fisher Scientific
	Glutamax	1%	Thermo Fisher Scientific
	RPMI 1640 medium	500ml	Pan-Biotech
Erylysis buffer (pH 7.3-7.4)	Potassium bicarbonate (KHCO3)	10mM	Sigma-Aldrich
	Amoniumchloride	0.15M	Sigma-Aldrich
	Na_2EDTA	0.1mM	Thermo Fisher Scientific
	H_2O	500ml	Generated in house
FACS buffer	Bovine serum albumin (BSA)	2.5g	Merck
	Sodium azide NaN3	0.1g	Carl Roth
	PBS (1x)	500ml	Pan-Biotech
MACS buffer	BSA	0.5%	Carl Roth

	Ethylendiaminetetraaceticacid (EDTA)	2mM	Thermo Fisher Scientific
	PBS(1x)	500ml	Pan-Biotech
Human T cell media	RPMI 1640 media	500ml	Pan-Biotech
	Human serum		Capricorn
	Penicillin and streptamycin	1%	Thermo Fisher Scientific
ELISA wash buffer	PBS (1x)	500ml	Pan-Biotech
	Tween 20	0.05%	Bio-Rad
Tissue extraction buffer	Tris-HCl (pH 7.4)	100mM	Sigma Aldrich
	NaCl	150mM	Sigma Aldrich
	EGTA	1mM	Thermo Fisher Scientific
	EDTA	1mM	Thermo Fisher Scientific
	Triton X-100	1%	Sigma Aldrich
Complete tissue extraction buffer	Sodium deoxycholate	0.5%	Sigma Aldrich
	PMSF	1mM	Abcam
	cOmplete Mini EDTA-free Protease inhibitor cocktail	1:7 (1 tablet diluted in 1.5ml dd H_2O as 7x stock)	Sigma Aldrich
	Tissue extraction buffer	1ml	As described above
Nuclei incubation buffer	Sucrose	340nM	Sigma Aldrich
	$MgCl_2$	2mM	Sigma Aldrich
	KCl	25mM	Sigma Aldrich
	Glycerophosphate	65mM	Sigma Aldrich
	Glycerol	5%	Sigma Aldrich
	EDTA	1mM	Thermo Fisher Scientific
	BSA	1%	Carl Roth
	In H_2O		
Nuclei lysis buffer	Tris	10mM	PanReac AppliChem
	NaCl	10mM	Sigma Aldrich
	$MgCl_2$	5mM	Sigma Aldrich
	NP-40	0.5 %	Sigma Aldrich
	In H_2O		

2.1.5. Devices

Table 15: Devices

Name	*Company*
BD FACS Aria III cell sorter	BD Bioscience
BD FACS LSR II analyser	BD Bioscience
BD FACSymphony A3 analyser	BD Bioscience
Centrifuge	Heraeus

Computer	HP
FlexCycler2 (PCR cycler)	Analytic Jena
Freezer (-20°C)	Liebherr
Freezer (-80°C)	Sanyo
Fridge (4°C)	Liebherr
Centrifuge	Heraeus
Perfusion System	Ismatec
Pipettes	Eppendorf/Gilson
SevenCompact pH-meter	Mettler-Toledo
Fume hood	Belec Vario Lab
Gel documentary device	INTAS Science Imaging
GentleMACS™ Octo Dissociator	Miltenyi Biotec
HAT 7900 real-time PCR instrument	Thermo Fisher Scientific
QuantStudio™ 6 Flex Real-Time PCR Instrument	Thermo Fisher Scientific
INC153 incubator	Memmert
MACS cell separators (magnets)	Miltenyi Biotec
NanoDrop™ 1000 Spectrophotometer	Thermo Fisher Scientific
Sterile hood	Thermo Fisher Scientific
Tabletop centrifuge	Thermo Fisher Scientific
Thermomix	Eppendorf
Water bath with shaker	GFL
Plate shaker	IKA Labortechnick
NanoString nCounter FLEX Analysis System	Nanostring technologies

2.1.6. Flow cytometer configuration

Table 16: Flow cytometer configuration of BD FACS LSR II analyzer

Laser	*Detector*	*Dichroic Mirror*	*Bandpass Filter*	*Fluorochrome*	*Other Fluorochromes*
488 nm	E	505 LP	530/30 513/17	FITC Alternative: GFP	Alexa Fluor 488, CFSE
	D	550 LP	575/26	PE	
	C	600 LP	610/20	PE-TxRed	
	B	685 LP 635 LP	695/40 670/14	PerCP/Cy5.5 Alternative: PerCP	
	A	735 LP	780/60	PE-Cy7	
405 nm	F		450/50	BV421	Pacific Blue
	E	505 LP	525/50	AmCyan	
	D	600 LP	610/20	BV605	
	C	630 LP	660/20	BV650	
	B	690 LP	710/50	BV711	
	A	750 LP	780/60	BV786	
633 nm	C		660/20	APC	Alex Fluor 647
	B	710 LP	730/45	Alexa700	
	A	755 LP	780/60	APC-Cy7	

Table 17: Flow cytometer configuration of BD FACSymphony A3 analyzer

Laser	*Detector*	*Dichroic Mirror*	*Bandpass Filter*	*Fluorochrome*	*Other Fluorochromes*
355 nm	G	370 LP	379/28	BUV395	
	F	410 LP	450/50	DAPI	
		490 LP	515/30	Alternative: BUV496	
	E	550 LP	580/20	BUV563	
	D	600 LP	610/20	BUV615	
	C	630 LP	670/20	BUV661	
	B	690 LP	735/30	BUV737	
	A	770 LP	810/40	BUV805	
	H	410 LP	431/28	BV421	Pacific blue
405 nm	G	505 LP	525/50	BV510	AmCyan
	F	550 LP	585/15	BV570	
	E	595 LP	605/40	BV605	
	D	635 LP	677/20	BV650	
	C	685 LP	710/50	BV711	
	B	735 LP	750/30	BV750	
	A	770 LP	810/40	BV786	
	G		488/10	SSC	
	F	505 LP	530/30	Alexa 488	FITC, CFSE
			513/17	Alternative: GFP	
488 nm	E	600 LP	610/20	BB630	
	D	635 LP	670/30	BB660	
	C	685 LP	710/50	PerCP-Cy5.5	
	B	735 LP	750/30	BB755	
	A	770 LP	810/40	BB790	
	D	570 LP	586/15	PE	
561 nm	C	600 LP	610/20	PE-CF/Dazzle594	*PE-TxRed,*
	B	635 LP	670/30	PE-Cy5.5	
	A	750 LP	780/60	PE-Cy7	
637 nm	C	655 LP	670/30	Alexa 647	APC,
	B	690 LP	730/45	Alexa700	
	A	750 LP	780/60	APC-Cy7	

Table 18: Flow cytometer configuration of BD FACS Aria III cell sorter

Laser	*Detector*	*Dichroic Mirror*	*Bandpass Filter*	*Fluorochrome*	*Other Fluorochromes*
488 nm	E	505 LP	530/30	FITC	Alexa Fluor 488, CFSE
			513/17	Alternative: GFP	
	D	550 LP	575/26	PE	
	C	600 LP	610/20	PE-TxRed	PE-Dazzle594

	B	685 LP	695/40	PerCP/Cy5.5	
		635 LP	670/14	Alternative: PerCP	
	A	735 LP	780/60	PE-Cy7	
405 nm	F		450/50	BV421	Pacific Blue
	E	505 LP	525/50	AmCyan	
	D	600 LP	610/20	BV605	
	C	630 LP	660/20	BV650	
	B	690 LP	710/50	BV711	
	A	750 LP	780/60	BV786	
633 nm	C		660/20	APC	
	B	710 LP	730/45	Alexa700	
	A	755 LP	780/60	APC-Cy7	

2.1.7. General consumables

Table 19: Consumables

Consumable	*Company*
Cellstar Easy Strainer (40µm and 100µm)	Greiner
Eppendorf tubes (0.2, 0.5, 1.5, 2ml)	Sarstedt
FACS tubes (5ml)	Sarstedt
Falcon tubes (15 and 50ml)	Greiner
Liquid reservoir for multichannel pipettes	Integra
Multiwell plates (96-weel, 24-well, 12-well, 6-well)	Greiner
Parafilm N	Carl Roth
PCR plate sealing tape	Sarstedt
Pipette tips	Sarstedt
Pre-Separation filter (30µm and 70µm)	Miltenyi Biotec
Serological pipettes (5ml, 10ml, and 25ml)	Sarstedt
Syringes and needles	Braun

2.1.8. Software

Table 20: Software

Software	*Company*
Adobe Illustrator 2023	Adobe
Excel	Microsoft
FACSDiva	BD Bioscience
FlowJo (version10)	BD Bioscience
Graphpad Prism (version 8)	Graphpad
SDS 2.4	Thermo Fisher Scientific
Windows	Microsoft
NanoString nSolver Analysis Software 4. 0	Nanostring Technologies

2.2. Methods

2.2.1. Genotyping

Mouse tail biopsies were taken by caretakers of the animal facilities at the UKE. Genotyping was performed by technicians and students. Tails were lysed in 50µl QuickExtract™ DNA Extraction Solution at 65°C and 500 rpm for 6 min, followed by heat inactivation for 2 min at 98°C and 350 rpm. Extracted DNA was used for following polymerase chain reaction (PCR) to determine genotype. 2µl DNA was mixed with 10 mM dNTPs, DreamTaq Hot Start Green DNS Polymerase, 10µM primers, DreamTaq Green Hot Start Buffer (10x) and ddH_2O. PCR products were resolved by agarose gel electrophorese on a 1.5 % gel containing RotiSafe (diluted 1:5000) and visualized on a UV transilluminator.

2.2.2. Experimental autoimmune encephalomyelitis (EAE) induction

For induction of EAE, mice were anesthetized with isoflurane and received two subcutaneous injections of 50µl of a 1:1 mixture of MOG_{35-55} peptide in phosphate buffered saline (PBS) (4 mg/ml) and complete Freund's adjuvant supplemented with Mycobacterium tuberculosis (4 mg/ml) in the flank of the hind limbs. Mice furthermore received an intraperitoneal (i.p.) injection of 200 - 300ng pertussis toxin, solved in ddH_2O and diluted in PBS on day of immunization and 48h later. Weight and clinical score of mice was monitored starting six days after immunization. Clinical signs were categorized by the following system: 0 no clinical deficits; 1 tail weakness; 2 hind limb paresis; 3 partial hind limb paralysis; 3.5 full hind limb paralysis; 4 full hind and front limb paralysis were euthanized according to the regulations of local Animal Welfare Act. For tissue collection during the EAE, female C57BL/6J mice were used. For phenotype EAE, *Gpr52*-deficient mice and gender- and age-matched littermate controls were used. For FACS of T cell subsets in acute neuroinflammation, DEREG mice were used. Animals which did not get sick were excluded from statistical analysis.

2.2.3. Cell isolation

Immune cell isolation from spleen and lymph nodes:
Mice were anesthetized with O_2/ CO_2 gas mixture and killed with CO_2. Spleen and lymph nodes (inguinal, brachial, axillary, and cervical) were collected separately in ice-cold PBS. Single cell suspension was obtained by homogenizing organs separately through a 40µm cell strainer into a 50ml tube. Cells were pelleted by centrifugation (500 g, 5 min, 4°C). Supernatant was discarded. For lysis of red blood cells, spleen samples were resuspended in 2ml ice-cold erylysis buffer and

incubated at RT. Ice-cold PBS was added after 2 min to stop lysis. After centrifugation, supernatant was discarded and spleen sample was combined with lymph node sample. Cells were resuspended in either MACS buffer or T cell media depending on the follow-up application.

Immune cell isolation from CNS:

For isolation of CNS infiltrating cells, mice were anesthetized with O_2/ CO_2 gas mixture and sacrificed with CO_2. Mice were intracardially perfused with 10ml ice-cold PBS. Brain and SC were isolated and stored on ice-cold PBS. Once all samples were ready, a sterile scalpel was used to mechanically dissociate the tissue. Tissue was transferred to C tubes, filled with 2.5ml digestion solution, containing 1 mg/ml collagenase A and 0.1 mg/ml DNase in RPMI. C tubes were attached to gentleMACS Octo Dissociator with heaters. Pre-installed gentleMACS Program 37C_Multi_F was run. Tubes were removed afterwards, and sample was applied to 100µm cell strainer on a 50ml falcon. Tissue was grinded and strainer was washed with 20ml cold PBS. Suspension was centrifuged (500 g, 5 min, 4°C) and supernatant discarded. For further procedure, different Percoll solutions were needed: 90% Percoll 1 (Percoll diluted 10x PBS) was used to prepare Percoll A (78% Percoll 1 diluted in PBS) and Percoll B (30% Percoll 1 in RPMI). Pellet was resuspended in 4ml Percoll A and underlaid with 2ml Percoll B followed by 30 min centrifugation at 2.500 rpm at 4°C (low acceleration and deceleration). Interphase was collected, diluted in 14ml PBS and centrifuged for 10 min at 1.800 rpm and 4°C. Pellet was resuspended in PBS and stained as described below.

2.2.4. Cell culture experiments with murine cells

For further procedure, cells were counted in 10% trypan blue solution in a disposable hemocytometer.

For magnetic-activated cell sorting (MACS) of T cells, CD4+ T cell isolation kit, naïve CD4+ T cell isolation kit or CD4+ CD25+ regulatory T cell isolation kit were used according to manufacturer's protocol. Briefly, cells were resuspended in 100µl MACS buffer per 10^7 cells. Resuspended cells were incubated with pre-diluted antibody cocktail for 15 min on ice. Pre-diluted nanobody cocktail was added afterwards, followed by another 15 min incubation on ice. The suspension was transferred to an equilibrated LS column, equipped with a 40 or 70µm pre-separation filter, placed in a string magnet to hold back all microbead-labelled cells inside the column. The flow through containing all unlabeled (naïve) CD4+ T cells was collected in a 15ml tube. For Treg isolation, the column bound fraction contained the CD4+ CD25+ regulatory T cells. Cells were counted once more and

centrifuged (500 g, 5 min, 4°C). Supernatant was discarded and cells were resuspended in T cell media.

2.2.4.1. T cell activation

For T cell activation assay, MACS CD4+ T cells were seeded in an anti-CD3-coated 96-well plate. For anti-CD3 coating, 96-well plate was incubated with 100µl anti-CD3 in PBS (1µg/ml) overnight at 4°C. Plate was washed twice with PBS before adding cells. T cells were supplemented with soluble anti-CD28 and compound. Cells were incubated for 6h (37°C, 5% CO_2, 5% humidity). For flow cytometry analysis, cells were transferred to FACS tubes and further processed as indicated below.

2.2.4.2. T cell proliferation

For T cell proliferation, MACS CD4+ T cells were seeded in an anti-CD3-coated 96-well plate. Cells were supplemented with soluble anti-CD28, IL-2 and compound.
For BrdU procedure, cells were incubated for 56h, followed by 16h incubation with 1 µg/ml BrdU. Cells were transferred to FACS tubes and stained with surface markers, fixated and permeabilized as described below. Next, cells were incubated in PBS, supplemented with Ca2+ and Mg2+ and 40KU/ml DNase I for 1h at 3°C and 5% CO_2. Following DNA digestion, intranuclear Foxp3 and incorporated BrdU were stained as indicated below.
For CFSE staining, T cells were stained with CellTrace™ CFSE Cell proliferation kit. Briefly, cells were stained for 15 min at 37°C and 5% CO_2 with reconstituted CFSE in PBS containing 0.1% BSA. Reaction was stopped by adding 40ml RPMI supplemented with 2% FCS. Cells were centrifuged, supernatant was discarded, cells were resuspended in T cell media and counted as described above. Cells were washed once more with T cell media, supernatant was discarded and cells were seeded in T cell media.

2.2.4.3. T cell differentiation

For T cell differentiation, MACS naïve CD4+ T cells were seeded in an anti-CD3-coated 96-well plate. Cells were supplemented with soluble anti-CD28, compound as well as respective cytokines for differentiation to Th1, Th2, Th17 and Treg subset. Cytokines for each subset are indicated in Table 21. Cells were incubated for 72h at 37°C and 5 % CO_2. During the last 5h or incubation, cells were stimulated with 1 µg/ml ionomycin, 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and brefeldin A to induce cytokine production and accumulation. After incubation, cells were transferred to FACS tubes and stained as indicated below.

Table 21: Cytokines used for T cell differentiation assays

Th1	Th2	Th17	Treg
20IU/ml IL-2	50ng/ml IL-4	2ng/ml TGFβ	2ng/ml TGFβ
20ng/ml IFNγ	5µg/ml anti-IFNγ	20ng/ml IL-1β	100IU/ml IL-2
20ng/ml IL-12	5µg/ml anti-IL-12	25ng/ml IL-6	
5g/ml anti-IL-4		20ng/ml IL-23	
		40mM NaCl	
		10µg/ml anti-IFNγ	
		10µg/ml anti-IL-4	

2.2.4.4. Treg suppression assay

For Treg suppression assay, isolated Teff were stained with CellTrace™ CFSE Cell proliferation Kit as described above. Treg of *Gpr52*-deficient or proficient mice and labeled wildtype Teffs were seeded in 96-well plate together with Dynabeads™ mouse T-activator CD3/CD28 (ratio Teff:beads 1:1) and 10IU/ml IL-2. Cells were incubated for 72h (37°C, 5 % CO_2). After incubation cells were transferred to FACS tubes and stained as indicated below.

2.2.4.5. T cell assays with TCR-specific activation

For physiological TCR-specific activation of T cells, 2D2 mice with a MOG-specific TCR were used in an T cell activation and T cell proliferation assay.

MOG-pulsed feeder cells were used to present antigen to T cells derived from 2D2 mice. To generate feeder cells CD90.2 Microbeads were used. Single cell suspension of a C57BL/6J was generated as described above. Cells were centrifuged, supernatant was discarded, and cells were resuspended in 90µl MACS buffer and 10µl CD90.2 microbeads per 10 ^7 cells. After 10 min incubation at 4°C, cells were transferred to an equilibrated LS column, equipped with a 70µm pre-separation filter. Flow through was collected, cells were counted and centrifuged for 5 min at 500 g, 4°C. Supernatant was discarded and cells were resuspended in T cell media. Cells were irradiated at 30Gy before pulsing them for 30 min with MOG_{35-55} at 37°C, 5% CO_2.

CD4+ T cells were generated from 2D2 mice as described above. For T cell proliferation, CD4+ T cells were labeled with CFSE as described above. Instead of seeding CD4+ T cells on anti-CD3 coated 96-

well plate and supplement with anti-CD28, T cells were supplemented with MOG_{35-55}-pulsed feeder cells (ratio 1:1) and CNP interest. Incubation, staining of cells and analysis was performed as described above and below.

2.2.5. Cell culture experiments with human samples

2.2.5.1. Isolation of PBMCs and T cells

Isolation of peripheral blood mononuclear cells (PBMC) was performed by technicians. Briefly, blood was collected in EDTA tubes and diluted 1:1 with PBS. 35ml of diluted sample were carefully transferred to 50ml falcon, filled with 15ml Ficoll. Samples was centrifuged (2.000 rpm, 30 min, RT) and upper yellow phase was removed. Leukocytes were collected in separate 50ml falcon, diluted in 50ml cold PBS and centrifuged (1.500 rpm, 5 min, 4°C). Cells were frozen at -80°C until further processing.

For MACS of PBMCs, frozen PBMCs were thawed and centrifuged with 20ml cold PBS (1.500 rpm, 5 min, 4°C). Cells were counted in trypanblue as described above and resuspended in 40µl MACS buffer per 10^7 cells. 10µl RAN T cell Biotin-Antibody cocktail per 10^7 cells was added and sample was incubated for 5 min on ice. After incubation, 30µl MACS buffer and 20µl Pan T cell MicroBead Cocktail per 10^7 cells was added followed by another 10 min incubation on ice.

2.2.5.2. T cell activation

Frozen PBMCs were thawed and centrifuged with 20ml cold PBS (1.500 rpm, 5 min, 4°C). Cells were counted in trypanblue as described above and resuspended in 40µl MACS buffer per 10^7 cells. 10µl PAN T cell Biotin-Antibody cocktail per 10^7 cells was added and sample was incubated for 5 min on ice. After incubation, 30µl MACS buffer and 20µl Pan T cell MicroBead Cocktail per 10^7 cells was added followed by another 10 min incubation on ice.

2.2.6. Cell viability assay

To detect variation in cell survival upon compound treatment, CellTiter-Glo Luminescent Cell Viability assay was used. Briefly, MACS-purified CD4+ T cells were incubated 72h (37°C, 5% CO_2) in 200µl media supplemented with compound of interest. After incubation, 100µl media were removed and 100µl mixed CellTiter-Glo reagents were added. Plate was shaken for 2 min at 450 rpm, rested for 10 min and luminescence was measured on a Tecan plate reader.

2.2.7. Flow cytometry

Staining for analyzation:

Samples were transferred to 5ml FACS tubes containing 1ml ice-cold PBS. Cells were centrifuged (500 g, 5 min, RT) and supernatant was discarded. For surface staining of mouse cells, cells were resuspended in 50µl FACS buffer, supplemented with antibodies. For identification of dead cells, Alexa Fluor 750 NHS ester was added in addition. Nonspecific Fc receptor-mediated antibody binding was reduced by adding TruStain FCS anti-mouse CD16/32. Cells were incubated for 30 min at 4°C in the dark. After washing cells with 1ml FACS buffer, supernatant was discarded, and cells were resuspended in 300µl FACS buffer for acquisition. If intracellular staining was necessary, cells were fixated and permeabilized with eBioscience™ Foxp3/Transcription Factor Staining Buffer Set. Cells were incubated 30 min at RT in the dark. 1ml intracellular staining perm wash buffer was added and cells were centrifuged (500 g, 5 min, RT). Supernatant was discarded and cells were resuspended in 50µl intracellular perm wash buffer containing antibodies for intracellular staining. After incubation (30 min, RT, in the dark), cells were washed once with 1ml FACS buffer. After centrifugation (500 g, 5 min, RT), supernatant was discarded, and cells resuspended in 300µl FACS buffer for acquisition. For staining of human cells, cells were resuspended in 50µl FACS buffer, supplemented with antibodies. Cells were washed with 1ml FACS buffer and resuspended in fixation buffer. After 20 min incubation at RT in the dark, cells were washed with FACS buffer and resuspended in 300µl FACS buffer.

Staining for cell sorting:

Cell isolation and staining procedure as described above were performed under sterile conditions. PBS supplemented with 10µM EDTA was used instead of PBS and FACS buffer. To prevent cell aggregation, cells were resuspended and homogenized with a 70µm cell strainer after staining.

Cell sorting:

For cell sorting, collection tubes for sorted cells were coated with FCS for 5 min at RT and contained lysis buffer for RNA isolation. Stained cells were used for sorting on a BDAriaIII cell sorter. Teff were identified by their high expression of CD4 and CD3, but low expression of CD8 and CD25. Treg were identified by high expression of CD25 and low expression of CD127. For sorting of T cells from EAE, DEREG mice were used and Treg were identified by GFP signal.

2.2.8.Gating strategy

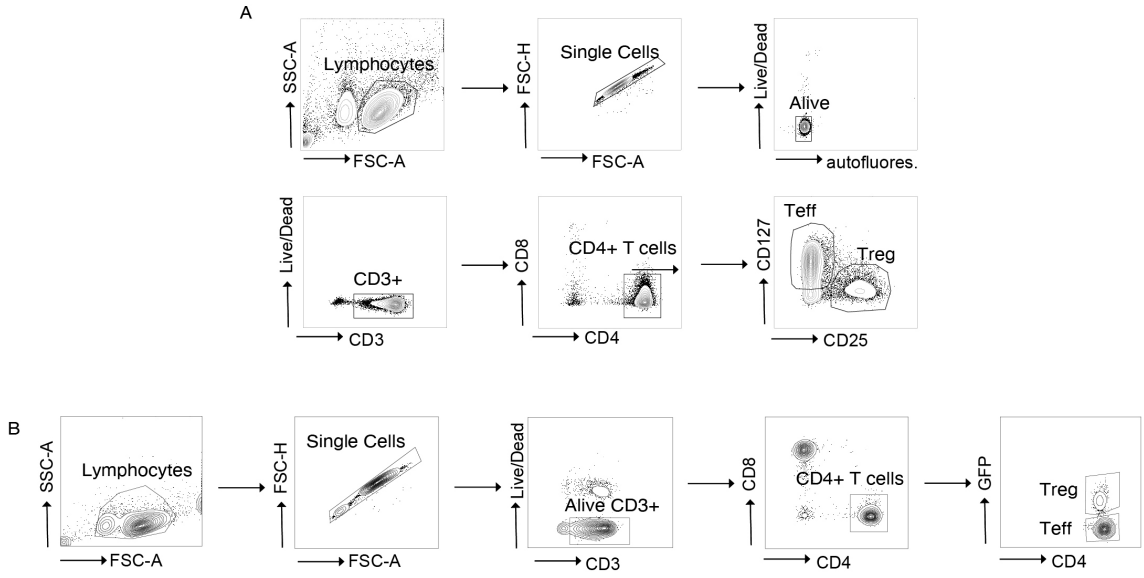

Figure 2: Gating strategy for FACS-purification of Treg and Teff. (A) For discrimination of Teff and Treg in C57BL/6J, cells were identified via CD3 and CD4 labeling. CD127 high CD4+ T cells were identified as Teff. CD25highCD127- cells were identified as Treg. (B) For cell derived from DEREG mice, the GPF signal was used to discriminate Treg from Teff.

2.2.9. Sample generation for gene expression analysis

Brain and SC samples for RT-qPCR were isolated by Christina Zeiler. Neuronal nuclei isolation was performed by Dr. Nicola Rothammer. FACS-purification of nuclei was performed by Dr. Jana Sonner.

Tissue isolation

For RT-qPCR analysis of brain and spinal cord, mice were anesthetized O_2/ CO_2 gas mixture and sacrificed with CO_2. Mice were intracardially perfused with 10ml ice-cold PBS. Brain and SC were isolated snap-frozen in liquid nitrogen. Tissue was frozen at -80°C until further processing.

Neuronal nuclei isolation and sorting for and qPCR

As described before, mice were anesthetized, sacrificed and perfused. Spinal cord was collected and dissociated with a scalpel on a cooled petri dish. Tissue was then added to 2ml nuclei lysis buffer and homogenized with a glass douncer with loose and tight pestle. After 5 min incubation on ice,

homogenized tissue was centrifuged at 500 g for 5 min at 4°C. Pellet was washed with 2ml lysis buffer, followed by washing twice with 2ml nuclei incubation buffer (500 g for 5 min at 4°C). Nuclei pellet was resuspended in 500µl nuclei incubation buffer supplemented with RiboLock and suspension was filtered through a 30µm MACS SmartStrainer. Cells were directly stained with primary labelled rabbit NeuN-AF647 (1:500) antibody and propidium iodide (1:2000). Nuclei were sorted on BD AriaIII cell sorter (BD Bioscience). Cells were sorted in 5ml tubes containing PBS with 0.02% BSA and RiboLock. Neuronal nuclei were identified by high expression of NeuN. Nuclei were then centrifuged at 1.500 g for 10 min at 4°C and pellet was directly lysed for RNA extraction as described in below.

2.2.10. Gene expression analysis

2.2.10.1. RNA isolation and cDNA synthesis

For RNA isolation of sorted cells or MACS-purified non-activated and activated (24h at 37°C, 5% CO_2 with Dynabeads™ mouse T-activator CD3/CD28 with ratio T cell:beads 1:1) T cells, cells were centrifuged, supernatant was discarded and pellet was lysed in 350µl RLT lysis buffer containing β-Mercaptoethanol (dilution 1:100). RNeasy Micro Kit was used according to manufacturer's instructions. Briefly, 70% ethanol was mixed with lysed cells in RLT buffer. Sample was then transferred to RNA isolation column and centrifuged (8.000 g, 15 sec). Flow-through was discarded and column-bound RNA was washed once with 350µl RW1 (8.000 g, 15 sec). To digest DNA, 80µl DNase 1, diluted 1:8 in RDD Buffer, was added to column-bound sample. After 15 min incubation at RT, column was washed once more with 350µl RW1 (8.000 g, 15 sec) buffer followed by a wash with 500µl RPE buffer (8.000 g, 15 sec) and a wash with 500µl 80% ethanol (8.000 g, 2 min). After each wash, flow-through was discarded. Column membrane was dried by centrifugation at 20.000 g for 2 min. To elute membrane bound RNA, 10µl RNase-free water was added to column, samples was centrifuged (20.000 g, 1 min), and flow-through was collected in a fresh RNase-free collection tube. RNA concentration was measured photometrically by Nanodrop and was stored at -80°C until further processing.

For RNA isolation of tissue, RNeasy Mini Kit was used according to manufacturer's instructions. Briefly, 600µl RLT buffer was added to frozen snap-frozen tissue and mechanically homogenized with a syringe. Lysate was centrifuged for 3 min at 20.000 g. Supernatant was removed and 700µl 70 % EtOH was added to pellet. Sample was transferred to RNeasy spin column placed on collection tube and centrifuged for 20 sec at 8.000 g. Flow-through was discarded and column-bound RNA was consecutively washed once with 700µl RW1 buffer (20 sec at 8.000 g) and twice with 500µl RPE buffer (20 sec at 2.000 g). The column was dries by centrifugation (20.000 g for 1 min) and RNA was

eluted in 30µl RNase free water into fresh RNase-free collection tube. RNA concentration was measured photometrically by Nanodrop and was stored at -80°C until further processing.
For cDNA synthesis, RevertAid First Strand cDNA Synthesis Kit was used. Briefly, 1µl random hexamer primer was added to 11µl of isolated RNA, gently mixed and denaturated at 65°C for 5min in a thermal cycler. In the second step, 8µl master mix containing 4µl 5x reaction buffer, 2µl dNTP mix, 1µl RiboLock RNase inhibitor and 1µl RevertAid H minus reserve transcriptase, was added to 12µl denatured RNA. Samples were incubated in a thermal cycler with following program: 25°C for 5min, 42°C for 60 min, 70°C for 5 min. Transcribed cDNA was diluted with RNase-free water 1:5 and stored at -20°C until further processing.

2.2.10.2. NanoString nCounter Analysis

NanoString nCounter Analysis was performed in cooperation with Prof. Viacheslav Nikolaev at the Institute of Experimental Cardiovascular research. Briefly, expression in FACS-purified Teff and Treg was measured with the NanoString nCounter FLEX analysis System. 30 - 35 ng of total RNA were used. The custom-made CodeSet for 36 genes included 6 housekeeping genes (*Actb, Gapdh, Gusb, Hprt, Rpl19, Tbp*) was hybridized to total RNA for 16h at 67°C. Expression data were analyzed utilizing NanoString nSolver Analysis Software 4.0. Raw data were analyzed and normalized to the housekeeping genes together with quality control performed using nSolver 4.0 User Manual in addition to default settings and algorithm within the nSolver Analysis.

2.2.10.3. qPCR

For gene expression analysis, quantitative real-time PCR (qPCR) was used. Briefly, 2µl cDNA were added to a master mix containing 5µl 2 x TaqMan gene expression master mix, 2.5µl RNase-free water and 0.5µl 20 x Taqman Assay. Taqman assays contained a pair of primers as indicated in Table 10 and a FAM reporter probe. cDNA samples were run in duplicates for each Taqman Assay. As purity control, RNase-free water was added instead of cDNA. Reaction was initiated by 50°C for 2 min and 95°C for 10 min, followed by 40 cycles with 95°C for 15 sec and 60°C for 60 sec, run on a QuantStudio™ 6 Flex Real-Time PCR Instrument. Gene expression was calculated as $2^{-\Delta Ct}$ relative to *Tbp* expression. Analysis was performed using QuantStudio Cloud software. Expression of *Gpr52* in Teff and Treg was performed by Dr. Roberta Kurelic.

2.2.11. IFNγ ELISA

For the measurement of IFNγ secretion of T cells, IFNγ ELISA MAX Deluxe was used according to manufacturer's instruction. Briefly, 100µl capture antibody (diluted in 1x coating buffer A) was added to each well and incubated overnight at 4°C. On the following day, plate was washed four times with 300µl ELISA wash buffer. Non-specific binding was reduced by incubation with 200µl 1x Assay Diluent A at RT for 1h on a plate shaker (400 rpm). Plate was washed again four times with 300µl ELISA wash buffer and 100µl sample (supernatant of T cells incubated 24h with aCD3/aCD28 and compound/VEH) or standards was added to well. Plate was incubated for 2h at RT on the plate shaker. After washing the plate, 100µl Detection Antibody solution was added to each well and plate was incubated for another hour at RT with shaking. Plate was once again washed four times before adding 100µl of diluted Avidin-HPR solution. After incubation of 30 min at RT on plate shaker, plate was washed five times with ELISA wash buffer. 100µl TMB substrate solution was added to each well followed by 20 min incubation in the dark. Reaction was stopped by adding 100µl Stop Solution and absorbance was reads at 450 nm within 15 min on Tecan Plate reader.

2.2.12. Statistical analysis

Data shown in bar graphs represents mean ± standard error of the mean (SEM). n represents number of mice. Number of mice was used for statistical analysis. Statistical analysis was performed using GraphPad software. Normal distribution was audited using Shapiro-Wilk test. Differences were tested using paired one-way ANOVA (followed by Dunnett's multiple comparison test), multiple T tests and Wilcoxon matched pairs signed rank test (corrected by Benjamini-Hochberg correction), Area-under-curve (AUC), Fisher's exact test, paired or unpaired Student's t-test or Mann-Whitney U test as appropriate and indicated in the figure legends. Significant differences are indicated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

3. Results

3.1. Identification of potential cAMP-modulating targets in T cells

To identify potential targets in T cells which can modulate cAMP and thus might alter T cell function, a Nanostring nCounter gene expression analysis of promising cAMP-relevant targets in FACS-purified Treg and Teff, derived from Foxp3-eGFP reporter mice (B6-DEREG), was performed in collaboration with Prof. Viacheslav Nikolaev (Figure 3A). CD4+ GFP+ cells were defined as Treg, while CD4+ GFP- were defined as Teff. Differential expression was detected for *Gpr52, Pde1b, Pde2a, Pde3b, Pde4b, Pde7a,* and *Pde7b* in pairwise comparison, but P_{adjust} was > 0.05 after Benjamini-Hochberg correction. To consolidate this findings, hits were tested in the publicly available ImmGen RNA sequencing data [242] (Figure 3B). As *Gpr52* and *Pde2a* were promising T cell targets, which have so far not been studied targets in this context and expressed in both subsets, differential expression was validated on qPCR level (Figure 3C, D).

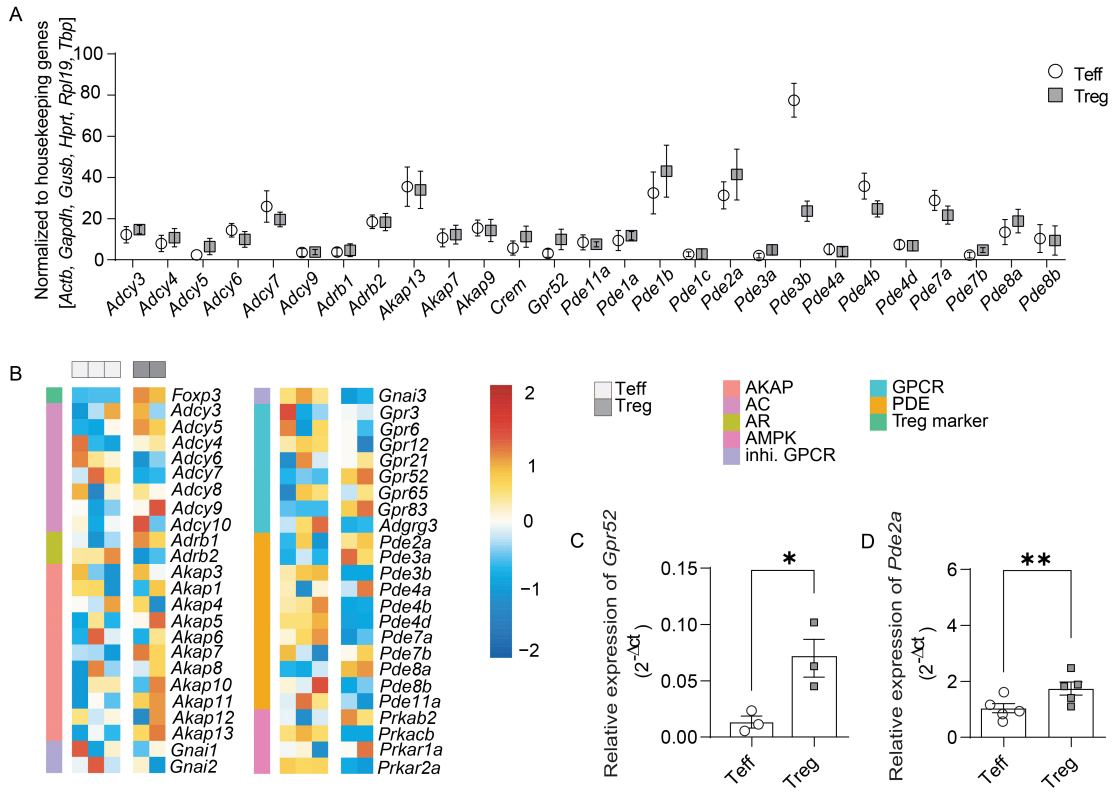

Figure 3: cAMP-relevant gene expression in Treg and Teff. (A) NanoString nCounter analysis of cAMP-relevant genes in Treg and Teff. Pde1b, Pde2a, Pde3b, Pde4b, Pde7a, Pde7b and Gpr52 were significantly differentially expressed (p= 0.031), but P_{adjust} was > 0.05 after Benjamini-Hochberg correction (n=6 mice, Wilcoxon matched pairs signed rank test with Benjamini-Hochberg correction). (B) Analysis of publicly available ImmGen RNA

*sequencing data. (C) qPCR analysis of Gpr52-expression (n= 3, paired Student's t-test, p=0.0380) and (D) Pde2a expression (n=5, paired Student's t-test, p=0.0011) in Teff vs. Treg. * P < 0.05, ** P < 0.01.*

3.2. T cell function and encephalitogenic response is independent of GPR52

3.2.1. GPR52 is dispensable for T cell function *in vitro*

First, focus was on the promising target GPR52, which was expressed in both, Treg and Teff with a higher expression in Treg (Figure 3C). *In vitro* experiments for T cell function were performed with *Gpr52* KO mice and control littermates as well as by utilizing pharmacological inhibition or activation of GPR52 with the published agonist FTBMT and antagonist E7 [163,165].

As T cell activity is crucial for their effector function and a functioning immune system, T cell activation assays were performed. T cell activation measured via the expression of the early activation marker CD69 was not affected by GPR52 deficiency (Figure 4A, B), nor by pharmacological activation or inhibition of GPR52 (Figure 4C, D).

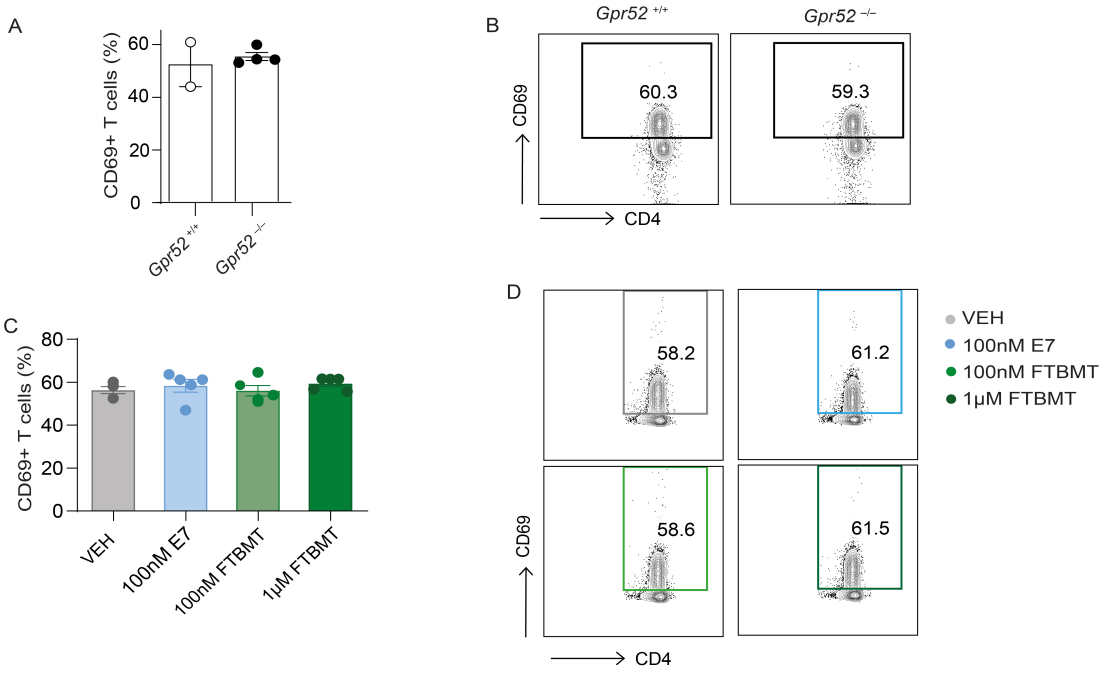

Figure 4: Neither GPR52 deletion nor pharmacological modulation of GPR52 results in altered T cell activation. T cell activation, measured as expression of early activation marker CD69, was measured after 6h with aCD3/aCD28 stimulation. (A) Effect of Gpr52-deficiency on T cell activation of CD4+ T cells (n($Gpr52^{-/-}$) = 4; n($Gpr52^{+/+}$) = 2, unpaired Mann-Whitney U test, p>0.9999) with (B) representative FACS blot depicting CD69 expression in CD4+ T cells. (C) Effect of E7 and FTBMT treatment on early activation marker CD69 expression

in CD4+ T cells (n=5, paired one-way ANOVA with Dunnett's post-hoc, p= 0.1514; Dunnett's post hoc: p(VEHvs.100nM E7)=0.4249; p(VEHvs.100nM FTBMT)>0.9999; p(VEHvs1µM FTBMT)= 0.0825). (D) Representative FACS blots of CD69 expression in CD4+ T cells upon compound or VEH treatment.

To investigate whether GPR52 is important for differentiation of naïve CD4+ T cell, a T cell differentiation assay was performed. Different cytokine cocktails as indicated in Table 21 were used to polarize naïve CD4+ T cell towards distinct subsets. As marker, intracellular IFNγ (Th1), IL-4 (Th1), IL-14A (Th17), Foxp3 were stained after incubation for three days for flow cytometry analysis. GPR52-dependent differences were not observed, as GPR52 deficiency (Figure 5A, B) or E7 and FTBMT treatment (Figure 5C-F) did not affect differentiation to T cell subsets.

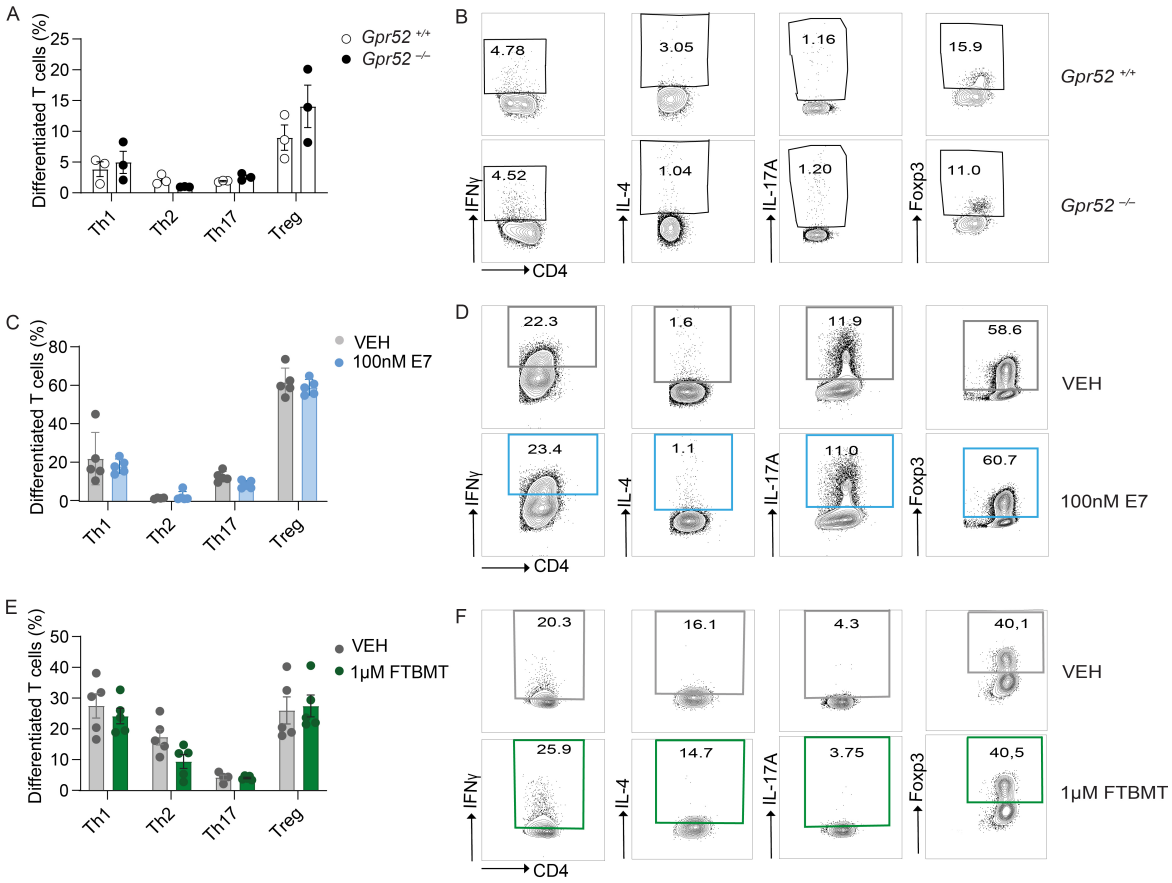

Figure 5: T cell differentiation is not affected by Gpr52-deficiency or inhibition/activation of GPR52. Naïve CD4+ T cells were incubated with cytokine mixture 72h. (A) Effect of Gpr52-deficiency on T cell subset specific differentiation of naïve CD4+ T cells (n=3, multiple paired t-tests with Benjamini-Hochberg correction, p-values before Benjamini-Hochberg correction: p(Th1)=0.6345; p(Th2)=0.0659; p(Th17)=0.1196; p(Treg)=0.2734; P_{adjust} was > 0.05 after Benjamini-Hochberg correction). (B) Representative flow cytometry blot of T cell subset specific markers IFNγ(Th1), I-L4 (Th2), IL-17A (Th17), and Foxp3 (Treg). (C) Modification of T cell differentiation upon 100nM E7 treatment (n=5, multiple paired t-tests with Benjamini-Hochberg correction, p-values before

Benjamini-Hochberg correction: p(Th1)=0.5392; p(Th2)=0.6776; p(Th17)=0.0171; p(Treg)=0.2522; P_{adjust} was > 0.05 after Benjamini-Hochberg correction). (D) Representative flow cytometry blot of T cell subset specific markers IFNγ (Th1), IL-4 (Th2), IL-17A (Th17), and Foxp3 (Treg).(E) Effect of FTBMT treatment on T cell differentiation (n=5, multiple paired t-tests with Benjamini-Hochberg correction, p-values before Benjamini-Hochberg correction: p(Th1)=0.3137; p(Th2)=0.0499; p(Th17)=0.9752; p(Treg)=0.3669; P_{adjust} was > 0.05 after Benjamini-Hochberg correction). (F) Representative flow cytometry blot of T cell subset specific markers IFNγ (Th1), IL-4 (Th2), IL-17A (Th17), and Foxp3 (Treg).

As a significantly higher expression of *Gpr52* in Treg compared to Teff was observed, an involvement of GPR52 in intracellular high cAMP levels in Treg was tested using Förster resonance energy transfer (FRET). A higher response to FTBMT in Treg, resulting in higher elevation of cAMP levels compared to Treg was observed [243]. To test whether this has a functional consequence, Treg-mediated immunosuppression of Teff, which depends on high intracellular cAMP, was tested using *Gpr52*-deficient or proficient Treg in a co-culture with wildtype Teff. GPR52 deficiency did not affect the immunosuppressive capacity of Treg as proliferation of Teff did not differ between groups (Figure 6A, B). Moreover, proliferation of T cells was not altered upon pharmacological activation of GPR52 with FTBMT (Figure 6C, D).

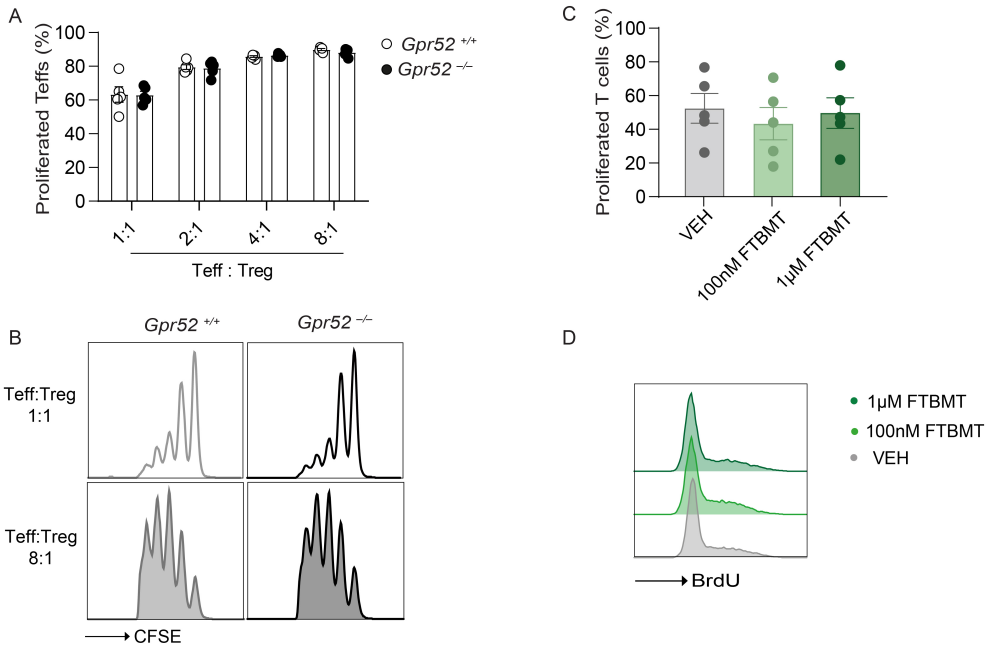

Figure 6: GPR52 deletion or agonist treatment does not affect Treg-mediated suppressive capacity or cell proliferation. (A) Immunosuppressive capacity of Gpr52-deficient and proficient cells on wildtype Teffs in different Teff:Treg ratios (n=5, multiple unpaired t-tests with Benjamini-Hochberg Correction, p(ratio 1:1)=0.9514; p(ratio 2:1)=0.7970; p(ratio 4:1)=0.3106; p(ratio 8:1)=0.1863; P_{adjust} was > 0.05 after Benjamini-

Hochberg correction). (B) Representative FACS blots of Treg-mediated suppression of Teff proliferation, measured as CFSE reduction upon proliferation. (C) Effect of FTBMT treatment on T cell proliferation (n=5, paired one-way ANOVA with Dunnett's post hoc correction, p=0.4400; Dunnett's post-hoc correction: p(VEH-100nM FTBMT)=0.9686; p(VEH-1µM FTBMT)=0.8480). (D) Representative flow cytometry blot of CD4+ T cell proliferation, measured as increased BrdU incorporation upon proliferation.

As a reduced survival of T cells was observed upon higher concentrations of E7, toxic effects of the compounds FTBMT and E7, as well as survival upon *Gpr52* deficiency was assessed. Indeed, a toxic effect upon higher concentration of E7 was detected using the CellTiter Glo viability assay after 72h incubation (Figure 7A). Of note, this decreased T cell survival was detected to a similar amount in *Gpr52*-proficient and *Gpr52*-deficient cells as determined by flow cytometry (Figure 7B).

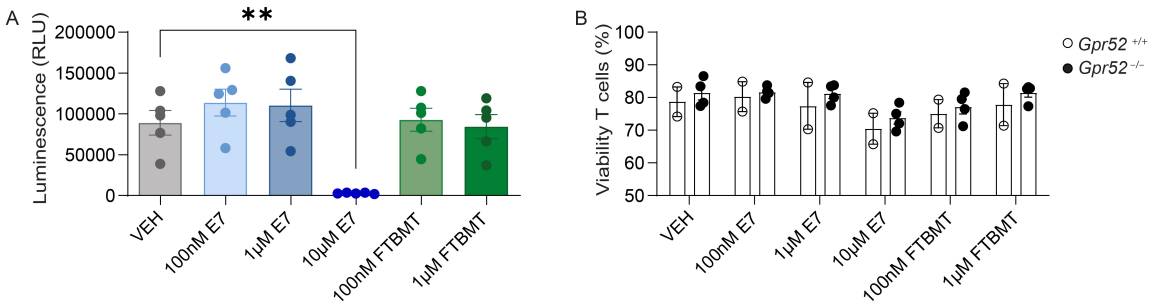

*Figure 7: E7 has a toxic effect on T cells. (A) CellTiter-Glo viability assay measurements upon 72h treatment of CD4+ T cells with different concentration of E7 and FTBMT (n=5, one-way ANOVA with Dunnett's post-hoc, p= 0.0002; Dunnett's post-hoc: p(VEH-100nM E7)=0.6476; p(VEH-1µM E7)=0.7584; p(VEH-10µM E7)=0.0017; p(VEH-100nM FTBMT)=0.9997; p(VEH-1µM FTBMT)=0.9996). (B) GPR52-mediated effects of compounds on T cell survival, measured via flow cytometry (n($Gpr52^{-/-}$) = 4; n($Gpr52^{+/+}$) = 2, multiple unpaired Mann Whitney-U with Benjamini-Hochberg correction; Benjamini-Hochberg correction: p(VEH)=0.5333; p(100nM E7)>0.9999; p(1µM E7)>0.9999; p(10µM E7)>0.8000; p(-100nM FTBMT)=0.5333; p(VEH-1µM FTBMT)>0.9999. **P < 0.01.*

3.2.2. *Gpr52*-deficiency does not affect the EAE disease course

As Teff function and the immunosuppressive capacity of Treg is dependent on intracellular cAMP, the contribution of GPR52 in autoimmunity was investigated in the EAE as animal model of MS using *Gpr52*-deficient mice and age- and sex-matched matched littermate wildtype controls. Three independent EAEs were performed and analyzed together. The course of the disease monitored as clinical score (Figure 8A) and percentual weight loss (Figure 8B) was not altered upon GPR52 loss.

Neither was incidence, disease onset, cumulative clinical score or maximal clinical score reached during the EAE affected by genotype (Figure 8C-F).

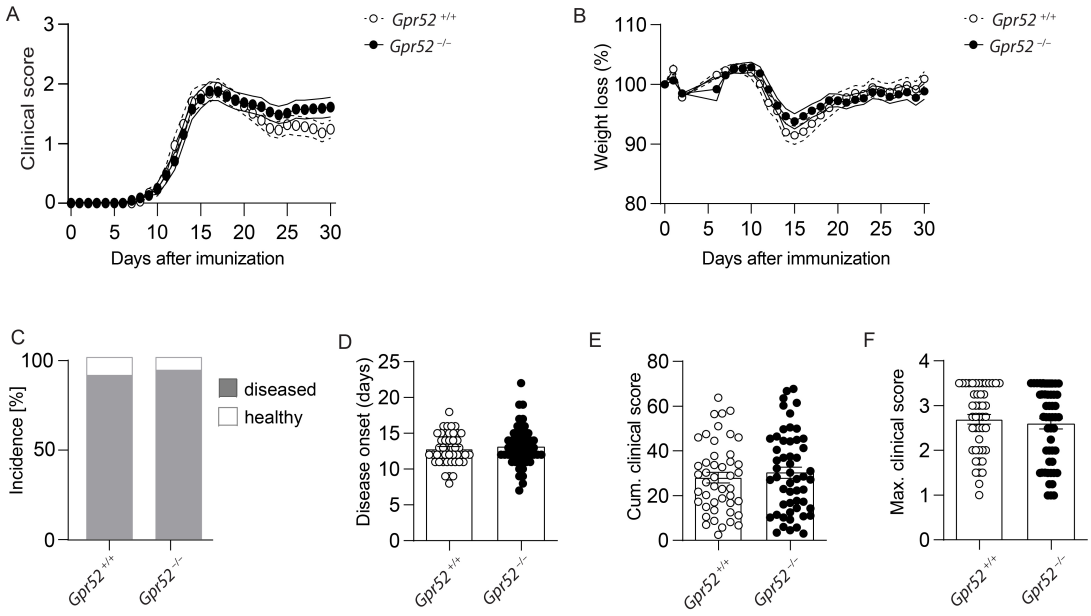

Figure 8: Global Gpr52-deficiency does not affect EAE. (A) Disease course of mice subjected to EAE with Gpr52-deficiency or proficiency (n(Gpr52$^{-/-}$) = 53; n(Gpr52$^{+/+}$) = 46, AUC followed by t-test, p=0.8654). (B) Weight loss of mice during the EAE (n(Gpr52$^{-/-}$) = 53; n(Gpr52$^{+/+}$) = 46, AUC followed by t-test, p=0.7899). (C) Incidence of disease symptoms. Mice were counted as diseased when they reached a score >0.5. (n(Gpr52$^{-/-}$) = 53; n(Gpr52$^{+/+}$) = 46, Fishers Exact Test, p=0.7341). (D) Disease onset, defined as day when first symptoms were recorded. (n(Gpr52$^{-/-}$) = 53; n(Gpr52$^{+/+}$) = 466, Mann Whitney U test, p=0.6884). (E) Cumulative clinical score of Gpr52-deficiency mice vs wildtype controls. (n(Gpr52$^{-/-}$) = 53; n(Gpr52$^{+/+}$) = 46, unpaired Student's t-test, p=0.5301). (F) Maximum score reached by each mouse during the disease course (n(Gpr52$^{-/-}$) = 53; n(Gpr52$^{+/+}$) = 46, Mann-Whitney U, p=0.6363).

In summary, GPR52 is expressed in both Treg and Teffs with higher abundance in Treg. However, neither *Gpr52*-deficiency, nor pharmacological stimulation with FTBMT affected T cell function in any of the assessed *in vitro* assays for T cell functionality. Effects observed upon E7 treatment in high concentrations can be traced back to the toxic effect of E7 on T cells. Moreover, *Gpr52*-deficiency did not result in altered clinical severity of the EAE.

3.3. PDE2A modulates T cell

PDE2A was identified as a promising cAMP-modulating target in T cell function (Figure 3A, B and D). As PDE2A activity is upregulated in activated T cells and therefore might affect T cell physiology, several *in vitro* assays were performed using the PDE2A-specific inhibitor BAY [188,189].

3.3.1. Inhibition of PDE2A affects T cell function *in vitro*

Firstly, it was assessed whether BAY affects T cell activation, quantified by the surface expression of the early activation marker CD69 on CD4+ T cells. BAY treatment for 6h, together with aCD3/aCD28 stimulation, did not affect T cell activation (Figure 9A, B). Neither did a pre-incubation with BAY (30 min) alter T cell activation (Figure 9C, D). Moreover, aCD3/aCD28 pre-activation followed by BAY treatment did not result in significantly alters T cell activation (Figure 9E, F). Secretion of IFNγ was further not affected by incubation with BAY for 24h (Figure 9G).

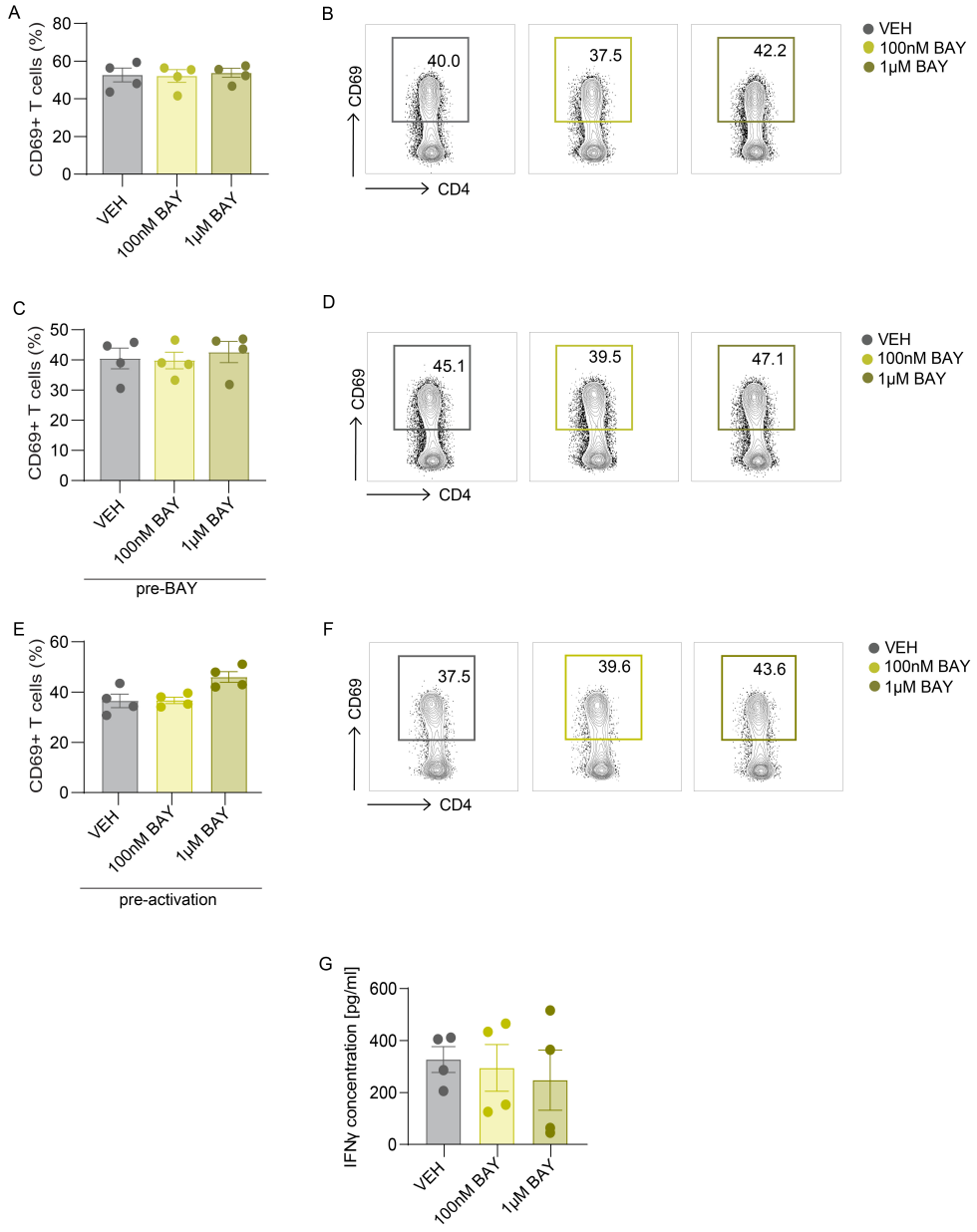

Figure 9: T cell activation is not affected by BAY treatment. T cell activation was measured using CD69 as early activation marker. (A) CD69 expression upon 6h incubation with BAY together with aCD3/aCD28 stimulation. (n=4, paired one-way ANOVA with Dunnett's post-hoc, p= 0.0724; Dunnett's post hoc: p(VEHvs100nM BAY)=0.9880; p(VEHvs1µM BAY)=0.5306). (B) Representative FACS blots of CD69 expression in CD4+ T cells upon compound or vehicle treatment. (C) CD69 expression after pre-incubation with BAY for 30 min before aCD3/aCD28 stimulation together with compound treatment for 6h. n=4, paired one-way ANOVA with Dunnett's post-hoc, p= 0.6789; Dunnett's post hoc: p(VEHvs100nM BAY)=0.9078; p(VEHvs1µM BAY)=0.9217. (D) Representative FACS blots of CD69 expression in CD4+ T cells upon compound or vehicle treatment. (E) CD69 expression after pre-activation with aCD3/aCD28 for 30 min before BAY treatment for 6h. (n=4, paired one-way ANOVA with Dunnett's post-hoc, p= 0.0545; Dunnett's post hoc: p(VEHvs100nM BAY)=0.9969; p(VEHvs1µM BAY)=0.1270. (F) Representative FACS blots of CD69 expression in CD4+ T cells upon compound or vehicle treatment. (G) IFNγsecretion upon 24h incubation with aCD3/aCD28 stimulation and BAY treatment.

(n=4, paired one-way ANOVA with Dunnett's post-hoc, p= 0.3916; Dunnett's post hoc: p(VEHvs100nM BAY)=0.0.7124; p(VEHvs1µM BAY)=0.5234).

Next, the effect of PDE2A inhibition with BAY on differentiation of naïve CD4+ T cells to Th1, Th2, Th17 and Treg subsets was measured. Different cytokines cocktails as indicated in Table 21 were used to polarize naïve CD4+ T cell towards distinct subsets. As marker, intracellular IFNγ (Th1), IL-4 (Th1), IL-14A (Th17) and Foxp3 (Treg) were stained for flow cytometry analysis. BAY treatment did not significantly affect differentiation of naïve CD4+ T cell to the T cell subsets, even though a trend towards impaired Th1 differentiation was observed (Figure 10).

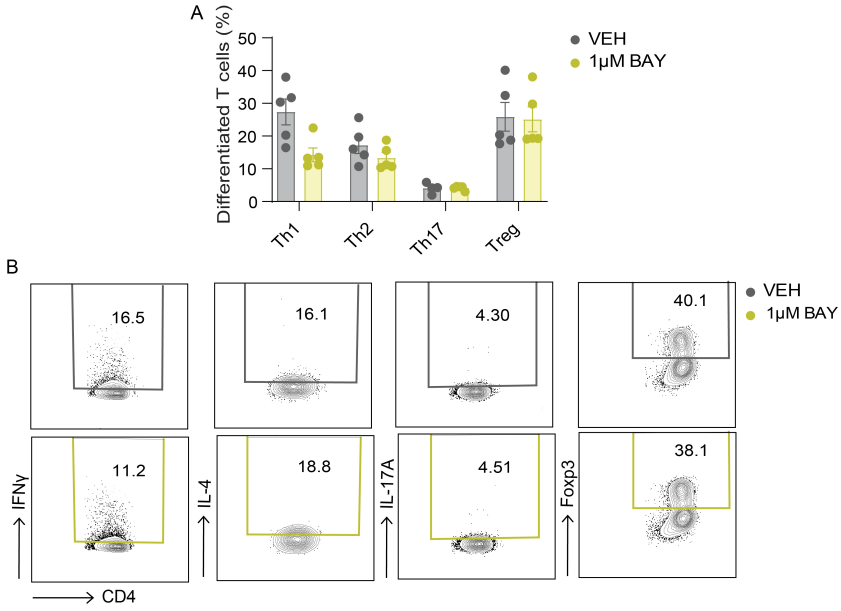

Figure 10: BAY treatment does not alter T cell differentiation. Naïve CD4+ T cells were incubated with cytokine mixture and compound for 72h. (A) Subset-specific differentiation upon BAY treatment. (n=5, multiple paired t-tests with Benjamini Hochberg correction, p-values before Benjamini-Hochberg correction: p(Th1)=0.0588; p(Th2)=0.1788; p(Th17)=0.9880; p(Treg)=0.3876; P_{adjust} was > 0.05 after Benjamini-Hochberg correction). (B) Representative flow cytometry blot of T cell subset specific markers IFNγ (Th1), IL-4 (Th2), IL-17A (Th17), and Foxp3 (Treg).

As other PDEs, like PDE7 and PDE4 [244], have been shown to affect T cell proliferation, T cell proliferation was assessed using the CFSE assay. Proliferation of CD4+ T cell was significantly decreased after 72h incubation with aCD3/aCD28 stimulation and simultaneous treatment with 100nM BAY (Figure 11A, B).

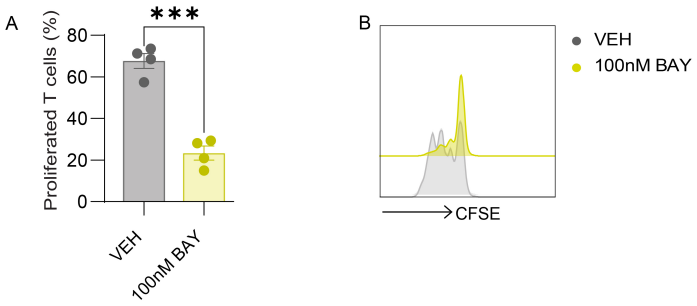

*Figure 11: BAY treatment reduces T cell proliferation. (A) T cell proliferation upon BAY treatment (n=4, paired t-test, p=0.0002). (B) Representative flow cytometry blot of CFSE stained CD4+ T cells. ***P < 0.001.*

3.3.2. *Pde2a* expression is increased in T cells in neuroinflammation

Next, the expression of *Pde2a* in the acute phase of an encephalitogenic response in FACS-purified Teff and Treg was measured. In both T cell subsets, *Pde2a* was upregulated during the acute phase of the EAE (Figure 12A, B). In contrary, *Pde2a* expression in total spinal cord (SC) and brain tissue lysate was not affected by the EAE when compared to healthy, unimmunized controls (Figure 12C, D).

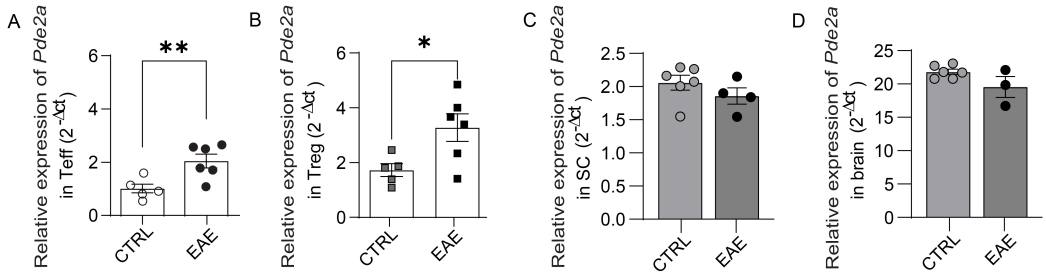

*Figure 12: Pde2a expression is increased in T cells during the EAE, but not in SC or brain. (A, B) Teff and Treg were FACS-purified during the acute phase of the EAE. Treg reporter mice were used to differentiate between Teff and Treg. (A) Pde2a expression in Teff during the acute phase of the EAE vs healthy controls. (n(CTRL)=5, n(EAE)=6, unpaired Student's t-test, p=0.0277) (B) Pde2a expression in Treg during the acute phase of the EAE vs healthy controls. (n(CTRL)=5, n(EAE)=6, unpaired Student's t-test, p=0.0105). (C) Pde2a expression during the EAE versus healthy controls in SC. n(CTRL)=6, n(EAE)=4, Student's t-test, p=0.2733). (D) Pde2a expression during the EAE versus healthy controls in SC. n(CTRL)=6, n(EAE)=3, unpaired Student's t-test, p=0.0958). *P < 0.05, **P < 0.01.*

Taken together, even though T cell activation and differentiation was not significantly affected by BAY treatment, the striking impact on T cell proliferation and the increased *Pde2a* expression in challenged T cells during the EAE together with data by Kurelic et al. [245], indicated that PDE2A should be further studied in the context of autoimmune diseases like MS.

3.4. NP modulate T cell function

PDE2A activity can be modulation via the cGMP/cAMP crosstalk, starting with the binding of the NP, namely ANP, BNP, and CNP, to its cognate receptors NPR1 and NPR2. Hence, effect of NP treatment on T cell function was assessed, again using assays for T cell activation, differentiation, and proliferation.

3.4.1. CNP predominantly affects T cell function *in vitro*

Similar as described above, surface expression of the early activation marker CD69 was used to assess the impact of NPs on T cell activation. ANP treatment significantly increased CD69 expression (Figure 13A, B) while the increase in response to BNP did not reach significance (Figure 13C, D). However, CNP treatment led to an increase of CD69 expression (Figure 13E, F). Moreover, CNP treatment increased IFNγ secretion upon aCD3/aCD28 stimulation for 24h (Figure 13G).
T cell differentiation instead was unaffected by all NP treatment (Figure 14).

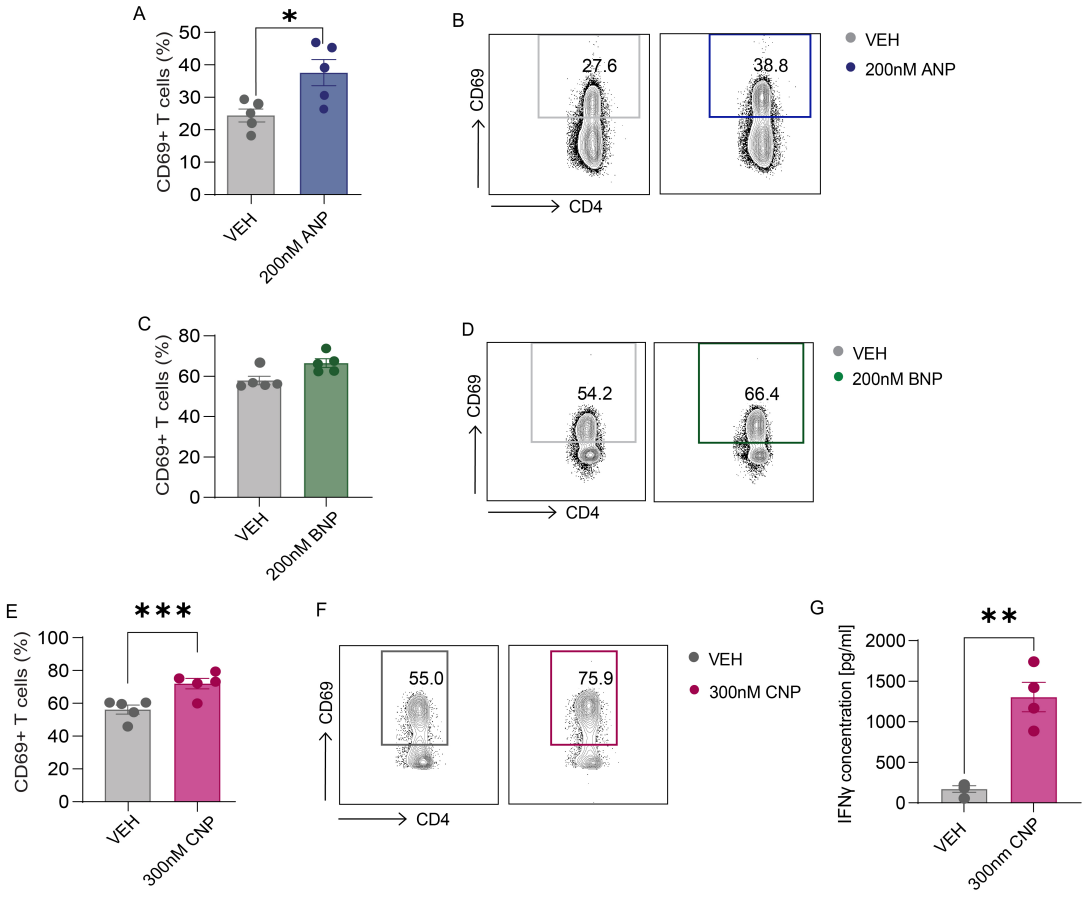

Figure 13: NP treatment increase T cell activation. T cell activation was measured as expression of early activation marker CD69 after 6h aCD3/aCD28 and NP treatment. (A) Effect of ANP treatment on T cell

*activation. (n=4, paired Student's t-test, p=0.0473). (B) Representative flow cytometry blot of CD69 expression in VEH or ANP treated CD4+ T cells. (C) Effect of BNP treatment on T cell activation. (n=5, Mann-Whitney-U test, p=0.0625). (D) Representative flow cytometry blot of CD69 expression in VEH or BNP treated CD4+ T cells. (E) Effect of CNP treatment on T cell activation. (n=5, paired Student's t-test, p=0.0001). (F) Representative flow cytometry blot of CD69 expression in VEH or CNP treated CD4+ T cells. (G) IFNγ secretion upon 24h incubation with aCD3/aCD28 stimulation and CNP treatment. (n=4, paired Student's t-test, p=0.0048). *P < 0.05, **P < 0.01, ***P < 0.001.*

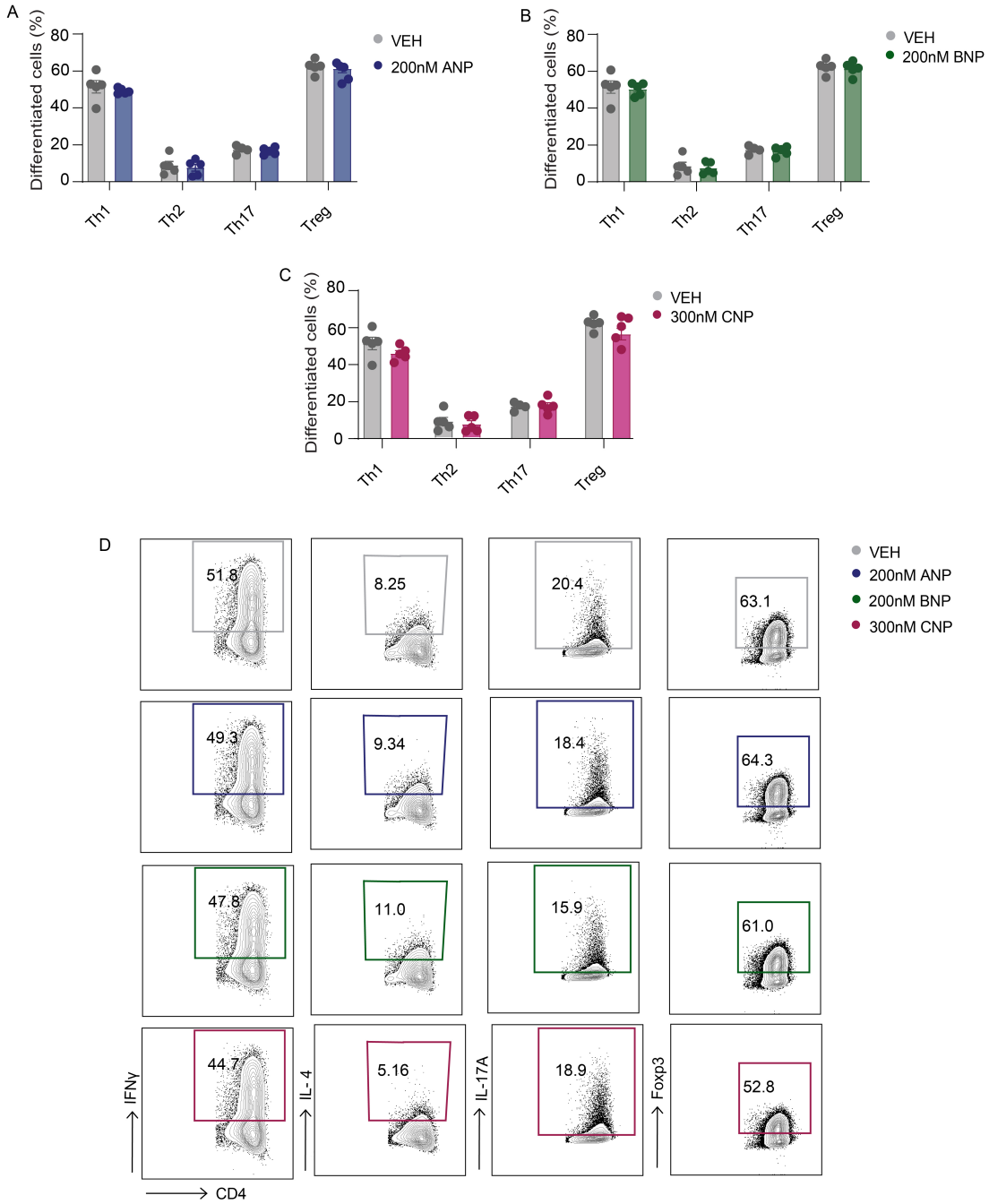

Figure 14: NP treatment does not affect T cell differentiation. (A) Subset-specific differentiation upon ANP treatment (n=5, multiple paired t-tests with Benjamini-Hochberg correction, p-values before Benjamini-

Hochberg correction: p(Th1)=0.4617; p(Th2)=0.3021; p(Th17)=0.1964; p(Treg)=0.2839; P_{adjust} was > 0.05 after Benjamini-Hochberg correction). (B) Subset-specific differentiation upon BAY treatment (n=5, multiple paired t-tests with Benjamini-Hochberg correction, p-values before Benjamini-Hochberg correction: p(Th1)=0.666; p(Th2)=0.5507; p(Th17)=0.3407; p(Treg)=0.5475; P_{adjust} was > 0.05 after Benjamini-Hochberg correction). (C) Subset-specific differentiation upon CNP treatment (n=5, multiple paired t-tests with Benjamini-Hochberg correction, p-values before Benjamini-Hochberg correction: p(Th1)=0.0423; p(Th2)=0.3470; p(Th17)=0.9708; p(Treg)=0.0668; P_{adjust} was > 0.05 after Benjamini-Hochberg correction). (D) Representative flow cytometry blot of T cell subset specific markers IFNγ (Th1), IL-4 (Th2), IL-17A (Th17), and Foxp3 (Treg).

Moreover, ANP and BNP did not affect T cell proliferation (Figure 15A-D), while CNP on the other hand led to a significant increase in proliferation of aCD3/aCD28 stimulated CD4+ T cells (Figure 15E,F). As CNP led to the most profound effect on T cell function and as this peptide has not been studied in the context of T cell function and autoimmune diseases yet, further analysis focused on the modulation of T cell function by CNP.

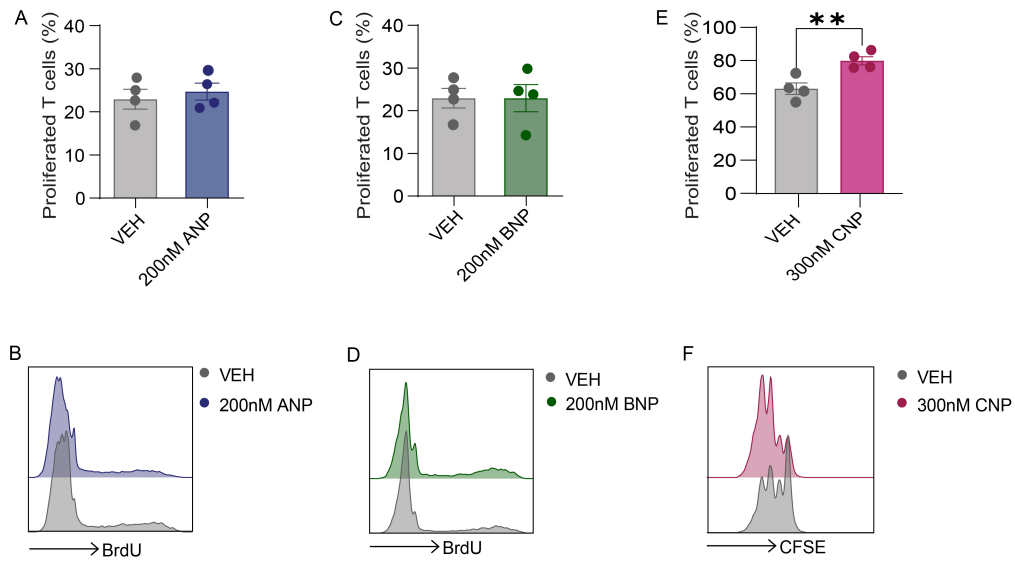

*Figure 15: CNP increases T cell proliferation while ANP and BNP had no effect on T cell proliferation. (A) Effect of ANP treatment on T cell proliferation of CD4+ T cells. (n=4, paired Student's t-test, p=0.7013). (B) Representative flow cytometry blot of BrdU labeled proliferating T cells. (C) Effect of BNP treatment on T cell proliferation of CD4+ T cells. (n=4, paired Student's t-test, p>0.9999). (D) Representative flow cytometry blot of BrdU labeled proliferating T cells. (E) Effect of CNP treatment on T cell proliferation of CD4+ T cells. (n=4, paired Student's t-test, p=0.0055). (F) Representative flow cytometry blot of CFSE labeled proliferating T cells. **P < 0.01.*

3.4.2. CNP alters T cell function upon TCR-specific stimulation

Next, the effect of CNP on T cell function was tested in a more physiological setup. In contrast to artificial and strong stimulation of T cells with aCD3/aCD28, physiological TCR stimulation of T cells derived from transgenic mice was investigated. T cells of 2D2 mice with a transgenic TCR for MOG [47] were used and cells were stimulated with MOG_{35-55}-pulsed feeder cells to enable a physiological antigen presentation to T cells. Also in this setup, CNP treatment resulted in an increase of T cell activation (Figure 16A, B) and T cell proliferation (Figure 16C, D) compared to VEH controls.

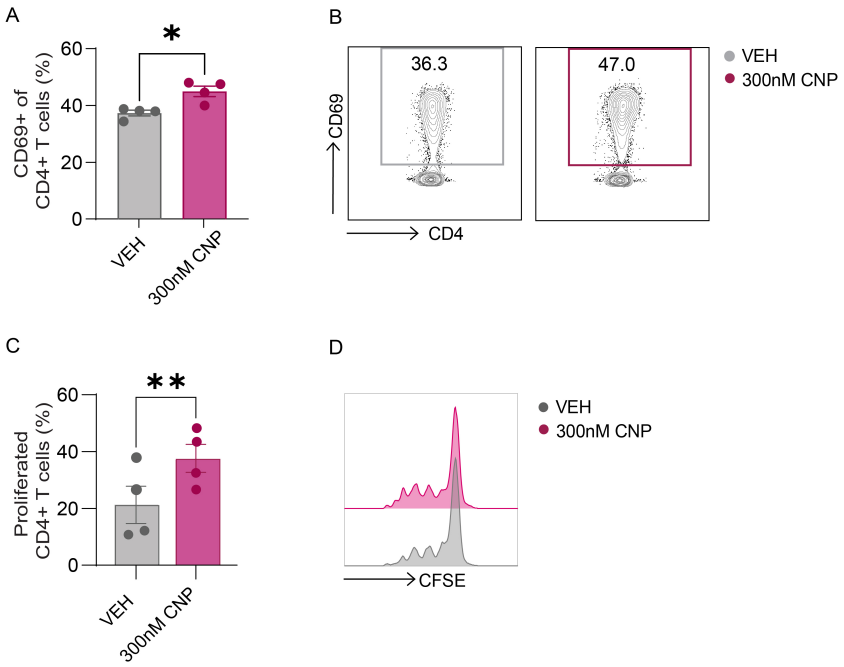

*Figure 16: CNP-mediated effects on T cell activation and proliferation occurred upon TCR-specific stimulation. 2D2 mice with transgenic MOG-specific TCR were stimulated with MOG-pulsed feeder cells. (A) T cell activation, measured as CD69 expression after 24h, upon CNP treatment in MOG-stimulated transgenic T cells (n=4, paired Student's t-test, p=0.0383). (B) Representative flow cytometry blot of CD69 expression in CNP or VEH treated CD4+ T cells. (C) Proliferation of CNP or VEH treated MOG-stimulated CD4+ T cells (n=4, paired Student's t-test, p=0.0077). (E) Representative flow cytometry blot of CFSE-labeled CD4+ T cells, treated with either CNP or VEH control. *P < 0.05, **P < 0.01.*

3.4.3. CNP-mediated effects are NPR2 dependent

As suggested in the publication by Kurelic et al. NP bind to their cognate NPR, thereby activating cGMP/cAMP crosstalk in T cells [245]. To further investigate whether NPR2 mediates the CNP-related changes in T cell function, the selective NPR2 inhibitor P19 [220] was used in the T cell activation and T cell proliferation assay. In both assays, pre-treatment with P19 reversed the CNP-mediated increase in T cell activation (Figure 17A, B) and proliferation (Figure 17C, D). Notably, P19 alone did not affect T cell activation, nor proliferation.

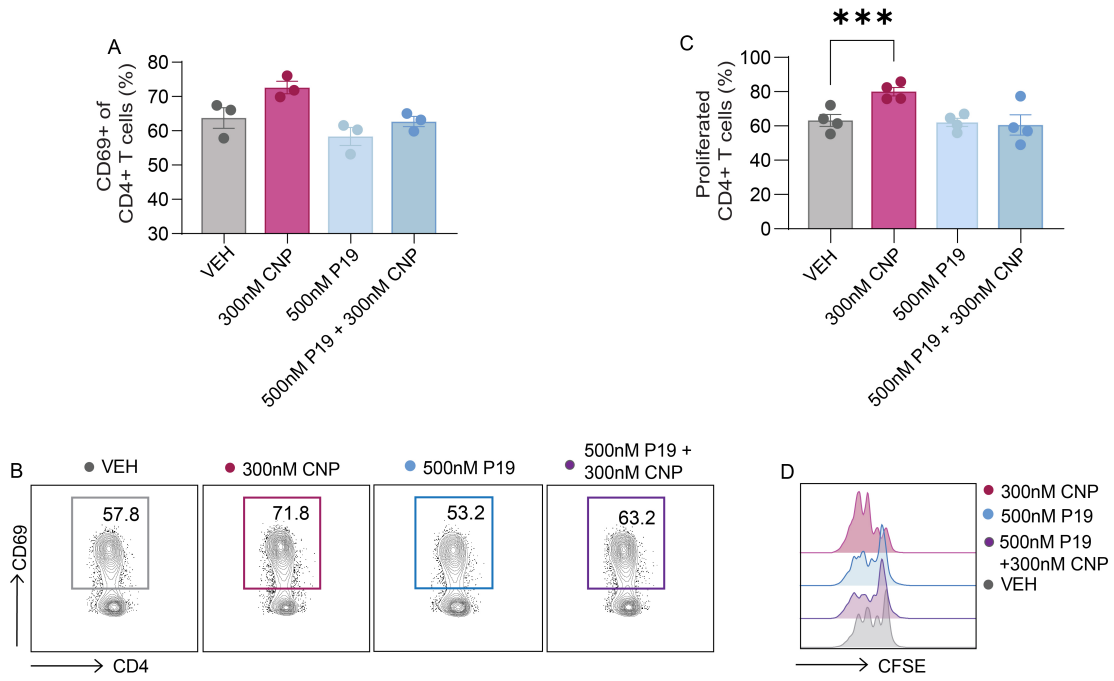

*Figure 17: P19 reverses CNP-mediated effects on T cell activation and proliferation. (A) Effect of CNP and/or P19 treatment on T cell activation. (n=3, paired one-way ANOVA with Dunnett's post-hoc, p= 0.0151; Dunnett's post-hoc: p(VEHvs300nM CNP)=0.0565; p(VEHvs500nM P19)=0.251; p(VEHvs500nM P19 + 300nM CNP)=0.9668). (B) Representative flow cytometry blot of CD69 expression in CD4+ T cells. (C) Proliferation of compound treated CD4+ T cells (n=4, paired one-way ANOVA with Dunnett's post-hoc, p= 0.0003; Dunnett's post-hoc: p(VEHvs300nM CNP)=0.0008; p(VEHvs500nM P19)=0.9584; p(VEHvs500nM P19 + 300nM CNP)=0.7146). (D) Representative flow cytometry blot of CFSE-labeled CD4+ T cells. ***P < 0.001.*

Next, it was examined whether the CNP/NPR2 mediated downstream effects on T cell function can also be triggered by activating sGC. Thus, the nitric oxide donor sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) was used. If NP-mediated effects are due to the pGC activity of their cognate receptors, also an activation of sGC by NO-donors should have the same effect.

However, neither SNP (Figure 18A, B) nor SNAP treatment (Figure 18C, D) affected CD69 expression upon aCD3/aCD28 stimulation.

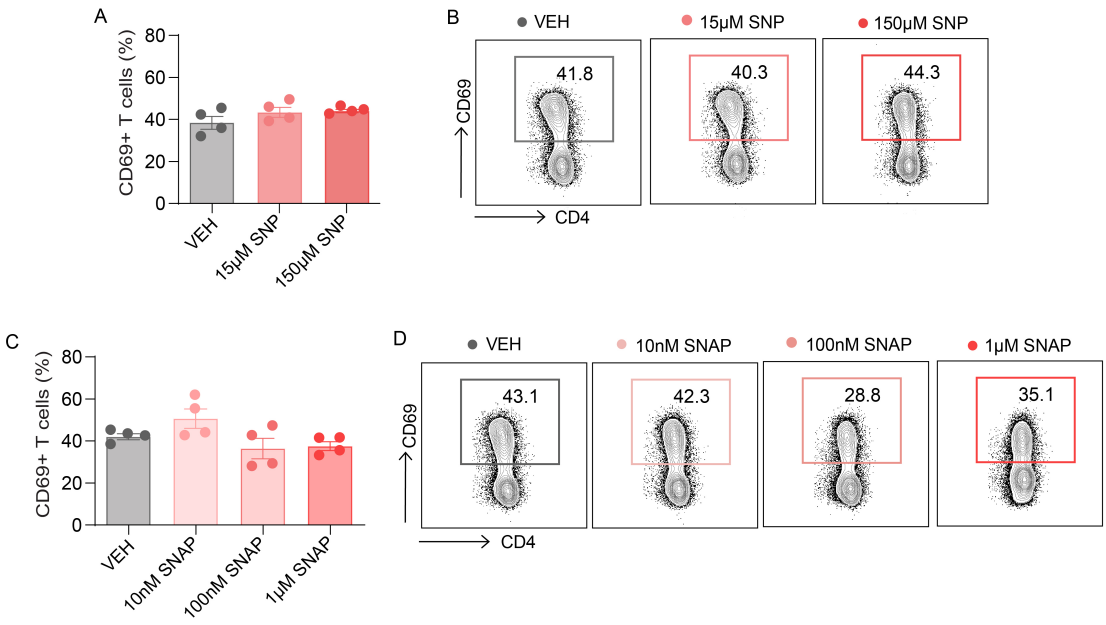

Figure 18: NO donors SNP and SNAP do not alter T cell activation. (A) Treatment with the NO donor SNP did not result in altered CD69 expression as early activation marker of T cells. (n=4, paired one-way ANOVA with Dunnett's post-hoc, p=0.2161; Dunnett's post-hoc: p(VEHvs15µM SNP)=0.2212; p(VEHvs150µM SNP)=0.3133); (B) Representative flow cytometry blot of CD69 expression in CD4+ T cells. (C) The NO donor SNAP did neither affect CD69 expression in CD4+ T cells (n=4, paired one-way ANOVA with Dunnett's post-hoc, p=0.1416; Dunnett's post-hoc: p(VEHvs10nM SNAP)=0.4155; p(VEHvs100nM SNAP)=0.6175; p(VEHvs1µM SNAP)=0.3906). (D) Representative flow cytometry blot of CD69 expression in CD4+ T cells.

Taken together, ANP and CNP treatment resulted in increased T cell activation while only CNP treatment reinforced proliferation. This effect was not only observed upon aCD3/aCD28 stimulation but also under more physiological stimulation of TCR-transgenic 2CD4+ T cells from D2 mice with MOG_{35-55}-pulsed feeder cells. Moreover, the effect of CNP on T cell function was shown to be NPR2-mediated using a pharmacological receptor inhibitor, while NO donor acting on sGCs did not result in T cell activation.

3.4.4. *Nppc* expression is reduced in SC during acute neuroinflammation

Even though it was shown that CNP affected T cell function, the source of CNP is still unknown. In literature, endothelial cells and the brain are stated as predominant sources of CNP [214]. However, it is not clear which organ secretes high amounts of CNP and whether that changes in the context of neuroinflammation.

Therefore, *Nppc* expression in SC tissue was measured by qPCR in samples collected during acute EAE and healthy controls. During the acute phase of the EAE, expression on *Nppc* was significantly decreased compared to healthy control (Figure 19A). This effect seemed to be driven by NeuN- cells. Even though not significant (p=0.0502), FACS-purified NeuN- cells showed reduced *Nppc* expression during the EAE, while *Nppc* expression in NeuN+ cells remained stable during neuroinflammation (Figure 19B, C). However, in brain tissue, a region that is characterized by minor immune cell infiltration in the MOG_{35-55} EAE model, *Nppc* expression was not altered during the acute phase of the EAE (Figure 19D). In addition, *Nppc* expression in activated and non-activated T cells was analyzed. While *Nppc* expression was not detectable in non-activated T cells, expression was detected in T cells upon 24h with Dynabeads™ mouse T-activator CD3/CD28 (Figure 19E).

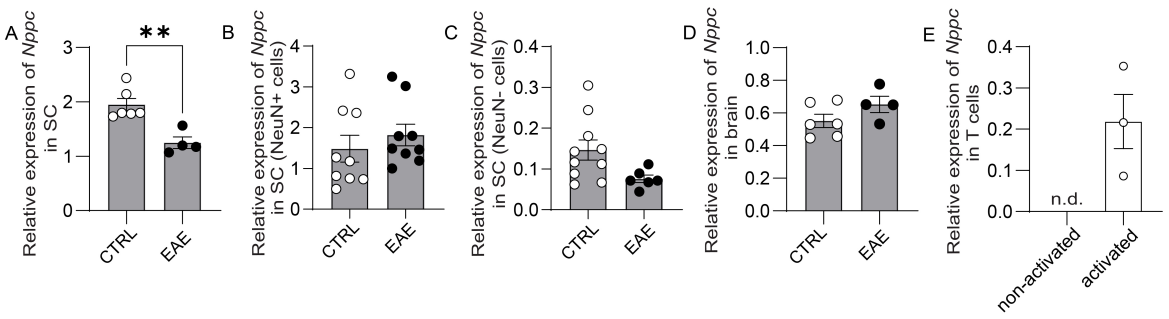

*Figure 19: Nppc expression was reduced in SC during the EAE. (A) Nppc expression in total SC samples during the acute phase of the EAE and healthy control (n(CTRL)=6, n(EAE)=4, Mann-Whitney U test, p=0.0095). (B) Nppc expression in NeuN+ cells, isolated from SC from mice undergoing the EAE and healthy controls. (n(CTRL)=9, n(EAE)=9, Mann-Whitney U test, p=0.2224). (C) Nppc expression in NeuN- cells, isolated from SC during the acute phase of the EAE and healthy controls (n(CTRL)=10, n(EAE)=6, unpaired t- test, p=0.0502). (D) Nppc expression was not altered during the acute phase of the EAE compared to healthy controls in full brain samples (n(CTRL)=6, n(EAE)=4, unpaired t- test, p=0.1572). (E) Nppc expression in non-activated vs. activated T cells. Nppc expression was not detected (n.d.) in non-activated T cells (n=3). **P < 0.01.*

3.4.5. CNP increases T cell activation in human CD4+ T cells

As the analyses performed in primary murine cells indicated a strong effect of CNP on T cells, the next step was to examine whether human T cells are also affected by CNP treatment. To clarify this question, MACS-purified T cells from healthy donors were treated with CNP. Indeed, the CNP-dependent increase of T cell activation was also observed in human T cells, where incubation for 12h with CNP resulted in higher CD69 expression compared to VEH control (Figure 20). Three independent experiments were performed and summarized.

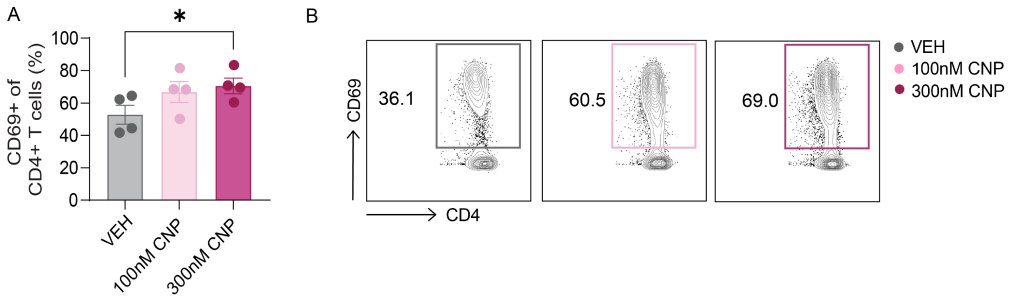

*Figure 20: CNP increased T cell activation in human CD4+ T cells. (A) Expression of CD69 in human CD4+ T cells upon CNP treatment. (n=4, paired one-way ANOVA with Dunnett's post-hoc, p= 0.0132; Dunnett's post-hoc: p(VEHvs100nM CNP)=0.0.1123; p(VEHvs300nM CNP)=0.0.0471). Data are pooled from 3 independent experiments. (B) Representative flow cytometry blot of CD69 expression in human CD4+ T cells. *P < 0.05.*

In summary, CNP has a tremendous effect on T cell function in both, murine and human primary T cells. While the endogenous source of CNP could not clearly be identified, the NPR2-dependent mechanisms was proven using the selective NPR2 inhibitor P19.

4. Discussion

The exploration of modulators of T cell function is crucial for the identification of new drug targets that may be exploited to treat T cell-driven immune disorders. As around a third of available drugs target the modification of GPCR with high specificity and efficacy, studying GPCRs and the subsequent modulation of cAMP signaling is a promising approach to decipher T cell function, especially under non-homeostatic conditions like it is found in autoimmune diseases such as MS.
In this work, three potential modulators of cAMP in T cells have been identified based on their differential expression in Treg and Teff (Aim 1) and further investigated. While GPR52 (Aim 2) was shown to be dispensable for T cell function, blocking of PDE2A (Aim 3) with its selective inhibitor BAY resulted in altered T cell function. NPs, and especially CNP (Aim 4) and its receptor NPR2, were also identified as key modulators of Teff responses.

4.1. Expression of cAMP-modulating targets in T cells

The starting point of this project was to analyze the gene expression of up- and downstream modulators of the secondary messenger cAMP in Treg and Teff. Besides the analysis of publicly available RNA bulk sequencing data [242], a more concise NanoString nCounter analysis of well-known modulators of cAMP was performed. The expression was detected in all targets with T cell subset-specific differential expression of *Gpr52, Pde1b, Pde2a, Pde3b, Pde4b, Pde7a,* and *Pde7b* on individual levels. As some of these differentially expressed targets are already well-known modulators of intracellular cAMP levels in T cells and T cell function, focus was set on two rather unknown and less studied candidates: GPR52 and PDE2A. Expression of those two targets in Treg and Teff was validated via qPCR. Both were expressed in Treg and Teff with a significantly higher expression in Treg compared to Teff. Therefore, genetical and pharmacological modification of GPR52 and PDE2A was used to determine their involvement in T cell function and the EAE.

4.2. GPR52 is dispensable for T cell function and encephalitogenic response

The Gs-coupled, constitutively active GPCR GPR52 was shown to affect intracellular cAMP levels in transfected HEK cells and primary neurons. Moreover, it has been connected to substance use disorders, psychiatric disorders and Huntington's disease [163,165]. However, GPR52 has previously not

been studied in the context of T cell physiology and acute neuroinflammation. As the expression of *Gpr52* was detected in Treg and Teff, GPR52 was chosen as first target to study. And indeed, a rise of cAMP was detected using FRET imaging in T cells upon stimulation of GPR52 with FTBMT [243,246]. As this might also affect T cell function and immunosuppressive capacity of Tregs, several *in vitro* studies were performed to assess its role in basic T cell characteristics such as activation, proliferation and differentiation. Moreover, the EAE model was used to study the impact of GPR52 on T cell physiology and encephalitogenic response *in vivo*.

To investigate whether GPR52, is crucial for T cell function, *in vitro* assays with different readouts were conducted, using published antagonist and agonist for GPR52 as well *Gpr52*-deficient T cells. Based on published reports and our recent findings using FRET imaging [243], it was hypothesized that activation of GPR52 with FTBMT results in a rise of cAMP in T cells which should increase the suppressive capacity of Tregs and inhibiting Teff response. Vice versa, antagonist treatment or *Gpr52*-deficiency should lower intracellular cAMP levels. However, none of these interventions showed any effect on T cell function *in vitro*. *Gpr52*-deficiency did not affect T cell activation or differentiation, nor did it modulate the capacity of Treg to suppress Teff proliferation. Treatment with FTBMT, the selective GPR52 agonist [163], did not alter T cell activation, proliferation, or T helper cell differentiation as well.

To inhibit GPR52, the published antagonist E7 was used [165]. While E7 treatment at low doses did not affect T cell activation or differentiation, higher concentrations, as described in the original report on E7 [165], resulted in T cell toxicity. The reduced survival of T cells upon high E7 doses was GPR52-independent as survival of T cells did not differ between *Gpr52*-deficient and -proficient cells upon E7 treatment. Whether this is a T cell specific effect or also applies to other cells remains an open question which should be considered when working with the compound. As the Gs-coupled activity of GPR52 could potentially result in altered encephalitogenic response, mice with global *Gpr52*-deficiency underwent the EAE. Differences between the KO and the wildtype mice were not observed in any of the assessed parameters. Therefore, GPR52 seems to be dispensable for the EAE disease course.

Taken together, although it was shown that GPR52 activation results in increasing cAMP levels in T cells, no effect of GPR52 modulation on T cell function could be detected.

This finding could be due to a local rise of cAMP in a GPR52-associated region. These nanodomains, so-called receptor-associated intracellular nanodomains (RAIN), orchestrate together with AKAPs the intracellular availability of cAMP [135,247]. A rise in this nanodomain or vice versa no rise due to *Gpr52*-deficiency or pharmacological modulation of GPR52 could thus be counteracted by other, more important RAINs or AKAPs in T cells.

Moreover, other cAMP modulators might be of more importance in T cells than GPR52. While GPR52 modification detectably affected intracellular cAMP levels in T cells, other regulators of cAMP, like PDE3B might be stronger modulators which overshadow the effect of one single GPCR. In the transgenic mice with a global *Gpr52*-deficiency, there is also the possibility that compensatory mechanisms were developed, covering potential effects of *Gpr52*-deficiency on T cell function. A possible solution for this problem would be the use of an inducible T cell-specific knockout of GPR52. But since the *in vitro* data did not indicate that GPR52 is a promising target in the context of autoimmune diseases like MS, no further investigations were made. However, the possibility can't be ruled out that GPR52 might be an important modulator in other cells, e.g., neurons or other cells of the immune system, or in other (T-cell driven) disease models. Therefore, further research could be conducted to evaluate this involvement of GPR52-dependent cAMP modulation in a different context.

In summary, no effect of GPR52 modulation nor deficiency on T cell function and encephalitogenic response was observed in this study indicating that GPR52 in the context of T cell function can be neglected in MS research.

4.3. PDE2A affects T cell function *in vitro*

PDEs are the only known enzymes which can hydrolyze cAMP and thereby reduce intracellular cAMP levels. While many other PDEs have been studied in the context of T cell function, there is little known about the impact of the second cAMP-modulating target in this study, namely PDE2A, on T cell physiology.

Within this thesis it was tested whether PDE2A is a potential target to modulate T cell function in the context of T cell-driven autoimmune diseases like MS. First, the effect of PDE2A inhibition on T cell activation, differentiation and proliferation was tested using the selective PDE2A inhibitor BAY 60-7550 (BAY) [189]. While T cell activation and differentiation as well as IFNγ secretion was not significantly altered, T cell proliferation was decreased by approximately 50% upon BAY treatment. In line with that, also inhibition of other PDEs, like PDE7 or PDE4, is reported to decrease T cell proliferation to a similar amount [172,248,249]. These studies suggest further that cytokine production is altered upon PDE7 and PDE4 inhibition. However, within this thesis, such effect of PDE2A inhibition on IFNγ secretion could not be shown. As PDE2A activity in T cells was found be dependent on the activation state of the T cell [245], it was also tested whether pre-activation with aCD3/aCD28 or pre-

incubation with BAY can alter T cell activation. But neither the inhibition of PDE2A before the stimulation of the cells nor vice versa did affect CD69 expression. On that note, it should be considered that only the effect of BAY treatment was studied. Even though BAY is the commonly used and highly selective inhibitor of PDE2A, also other PDE2A inhibitors exist, like for instance PF-05180999 [250], which could be tested in addition to validate the observed BAY-mediated effect.
Moreover, the use of *Pde2a*-deficient mice would clarify the function of PDE2A in T cell physiology. As a global PDE2A KO is embryonically lethal due to heart failure [193], a T cell specific PDE2A knockout should be used. The generation of this mouse line is currently ongoing. These mice could be used to test T cell function *in vitro*, to exclude potential off-target effects of BAY, but furthermore to test whether a T cell-specific KO of PDE2A affects the EAE disease course. A potential involvement of PDE2A in the MS mouse model EAE, where activated T cells drive the disease course, is underlined by the finding of Kurelic et al. showing that PDE2A is upregulated in activated T cells compared to non-activated T cells [245]. In line with that, the study revealed that *Pde2a* expression is increased in both Teff and Treg, isolated from the spleen and LN of mice during the acute phase of the EAE compared to healthy controls.
An open question is whether PDE2A additionally affects T cell migration, an essential attribute for T cells in the EAE and MS. Other studies suggest that inhibition of PDEs, like for instance PDE8 ameliorate the EAE disease course, supposedly via reducing the migration of T cells to the CNS via the BBB. This process is probably due to PDE8 inhibitor-dependent reduced integrin- and CD44-mediated adhesion to vascular endothelial cells [173]. So far it has not been shown that PDE2A is involved in T cell migration or that PDE2A inhibition alters expression of integrins, but future studies should be considered to decipher this potential involvement.
If it holds true that PDE2A inhibition not only reduces T cell proliferation but also ameliorates the EAE disease course, PDE2A also becomes a promising target for drug design in MS research. A modulation of autoreactive T cell activity during RRMS is aim of different currently approved drugs, like for instance Ofatumumab [251]. However, many of these do have severe side effects. Hence, new effective approaches with less side effects are needed. Another PDE, PDE4, has already been in clinical trials. Even though the inhibitor Rolipram had severe side effects like nausea and vomiting [179], the inhibitor Ibudilast is still under investigation as treatment for MS patients [180,181]. Inhibition of PDE2A could be another approach to ameliorate MS disease course.

In conclusion, these findings lead to the assumption, that a T cell-specific deletion of PDE2A or the pharmacological inhibition of PDE2A using BAY during the induction phase of the EAE might inhibit mounting of an encephalitogenic T cell response. Hence an attenuation of disease pathogenesis in

the mouse model of MS is likely. Similar effects have been observed for other PDEs, like i.e., PDE8, PDE4 and PDE7 [172,173,179].

4.4. NP, especially CNP, alter T cell function *in vitro*

The hydrolyzing activity of PDE2A towards cAMP can be modulated via intracellular cGMP levels. cGMP can be generated from GTP via sGC or pGC (namely NPR1 or NPR2), the latter being activated upon engagement with NP. Consequently, intracellular cGMP levels are rising, which subsequently increases the hydrolytic activity of PDE2A towards cAMP, a process known as the cGMP/cAMP crosstalk [199]. NPs have been mainly in focus of cardiovascular research. However, recent studies indicate a far more dominant role of NP, especially in the immune system, than thought. It was shown that NP treatment can trigger cytokine release and, vice versa, that NP secretion can be triggered by cytokines [229,234,236] . Despite the fact, that a few studies focused on the role of NPs in the immune system under homeostatic and pathogenic conditions, the knowledge on T cell modulation by NPs is still sparse.

To decipher the role of NPs on T cell physiology, the three NPs, namely ANP, BNP and CNP, were tested for their impact on T cell activation, proliferation and differentiation. Since CNP treatment resulted in the strongest increase of T cell activation and proliferation as well as IFNγ secretion, it was put in focus of further investigations.

First experiments to decipher CNP-mediated effects on T cells were performed upon aCD3/aCD28 stimulation which is a common method to activate T cells and thus increasing survival during culturing. In a second approach T cells from a transgenic mouse line with MOG-specific TCR [47] were co-cultured with irradiated feeder cells pulsed with MOG_{35-55} to validate these findings in a more physiological setup. The observed increase of T cell activation and proliferation indicate that under *in vivo* physiological circumstances, CNP might indeed also affect T cell physiology and reinforce T cell activation.

While it has been shown for ANP and BNP that these are released upon cardiovascular malfunction [208,209], the endogenous source of CNP in the context of neuroinflammation and the regulation of its secretion remains unknown. In literature, main production site of CNP are endothelial cells, but also brain and SC are reported to secrete CNP [214]. Due to the lack of a specific antibody, expression of *Nppc* in SC and brain was measured as first indication of CNP secretion sites. Indeed, expression of *Nppc* was found in both, SC and brain. Moreover, during acute neuroinflammation, *Nppc* expression was decreased in whole SC samples, possibly driven by the influx of NeuN- cells, which themselves

may express low levels of the transcript. In brain samples, no significant changes in *Nppc* expression were detected which is most likely due to the fact that the SC is the primary target tissue in the C57BL/6J MOG_{35-55} EAE model [46,49]. Interestingly, *Nppc* expression in T cells was only detected upon activation. While the ANP-mediated effects might indicate a connection between cardiovascular and immune system, the knowledge about CNP together with the results of this study are less conclusive. However, together with reports that CNP secretion from endothelial cells can be triggered by treatment with pro-inflammatory cytokines like TNFα and IL-1β [216,236], one could hypothesize a CNP-mediated bridge between innate and adaptive immune system. Pro-inflammatory cytokines, like TNFα and IL-1β are mainly produced by cells of the innate immune system. Upon Activation of the innate immune system, these secreted cytokines could induce secretion of CNP by endothelial cells which subsequently could lead to increased activation of T cells as part of the adaptive immune system, themselves starting the expression of *Nppc*. Interfering with this connection could alleviate immune reactions and dampen autoreactive immune cells in MS. However, to clarify this hypothesis, further experiments are needed to elucidate under which conditions and from which cells CNP is secreted. Moreover, it has to be elucidated whether the observed effects of CNP treatment on T cells are cAMP-dependent, as described by Kurelic et al. [245], or whether also the rise of cGMP levels affects T cell function via the PKG-mediated pathway [252]. Blockers and inhibitors of downstream targets can be used for this purpose in assays for T cell function. Furthermore, measurement of cGMP upon CNP treatment via ELISA or FRET would clarify which pathways are modified by CNP in T cells.

Despite modulating the availability of CNP as a ligand to activate T cells, blocking downstream signaling triggered by CNP with its receptor specifically on T cells may also be worth exploring to mitigate encephalitogenic responses. The main receptor of CNP is NPR2 [235]. But since CNP also binds to a lower extend to NPR1 and NPR3, it was necessary to validate which receptor mediates the effects of CNP in T cells. The published selective NPR2-inhibitor P19 [220] was used to validate that the CNP-mediated increase in T cell activation and proliferation was indeed mediated via NPR2. Additional experiments using the NO donors SNP and SNAP [253-255] showed, that the observed modulation of T cell function by NPR2 (pGC-B) engagement could not be mimicked by activating sGC.

In summary, these results indicate that CNP and its receptor expressed on T cells may serve as an attractive target to dampen pro-inflammatory Teff functions in autoimmune diseases. On the one hand, one could envision the peripheral supplementation with recombinant CNP or the reinforcement of local CNP expression within the CNS tissue. Yet, this requires the identification of targetable upstream regulators of CNP release, which should be explored in future studies. On the

other hand, targeting NPR2 on T cells as exemplified by P19 treatment to block this pathway could ameliorate autoimmune diseases and lead to treatment options for patients. However, also other pathways might be involved in the CNP-mediated alteration of T cell physiology. A recent paper for instance suggested that NPs arbitrate their function via their receptors NPR1 and NPR2 independently of cAMP generation, but instead via the Wnt/β-catenin pathway [256]. This pathway was described in neurons but could also account for other cell types. As the Wnt/β-catenin pathway is essential for T cell effector function and differentiation [257,258], investigating CNP-driven modulations of the Wnt/β-catenin pathway in T cells might lead to new findings. Moreover, the Wnt/β-catenin signaling was reported to be involved in neurogenesis, neuronal signaling and remyelination during EAE [259-261], opening more interesting questions to follow up in the context of CNP-mediated effects in neurons.

Based on these promising results, it was also tested whether the CNP-mediated effects observed in murine T cells also apply to human T cells and consequently could be also targeted in MS drug development. Therefore, T cells from healthy donors where purified and stimulated with aCD3/aCD28. And indeed, the CNP-mediated increase in early activation marker CD69, can also be seen in human T cells. This finding indicates that CNP-mediated effects seen in murine T cells can probably be translated to clinical research, making CNP a promising candidate to study as treatment options for autoimmune, T cell-mediated diseases. Further experiments must be performed to test whether CNP also affect other markers for T cell physiology, like T cell proliferation and migration. Moreover, it can be tested whether T cell response to CNP differs between T cells derived from MS patients versus healthy controls. If the striking effect of CNP on murine T cell function can also be observed in human T cells, targeting CNP and NPR2 becomes promising for drug development in MS research.

Taken together, the results indicate that the NP and most dominantly CNP have a tremendous effect on T cell function in mice and humans. A modulation of the CNP-NPR2-pathway is a promising target to study in the context of acute neuroinflammation and autoimmune diseases. Especially the inhibition of CNP signaling via a NPR2 blocker like P19 is supposedly beneficial for the outcome of EAE and MS and potentially also for other T cell-mediated diseases.

4.5. Conclusion and outlook

Taken together, this work identified and studied cAMP-modulating targets that can alter T cell physiology. Firstly, the orphan GPCR GPR52 was in focus. The Gs-coupled activity of GPR52 was reported in literature in many different cell types, making GPR52 a promising target to study [163,165,166]. However, neither T cell function *in vitro* nor the EAE disease course was altered upon pharmacological modulation or genetical deletion, suggesting no involvement of GPR52 in the context of T cell physiology and autoimmune diseases.

Next, the effect of PDE2A modulation on T cell function was deciphered using the selective PDE2A inhibitor BAY [189,191,195]. BAY did not alter T cell differentiation or activation but decreased proliferation of CD4+ T cells *in vitro*. Together with the finding that *Pde2a* expression is increased during the acute phase of EAE in peripheral T cells and upregulated in activated T cells [245], an involvement of PDE2A in the EAE diseases course becomes possible. To clarify this hypothesis, mice with T cell specific PDE2A KO could be used. Another option is to perform a treatment EAE, injecting BAY or other PDE2A-specific inhibitors during the EAE. Moreover, *in vitro* experiments either with T cells derived from T cell-specific *Pde2a*-specific mice or other PDE2A inhibitors will clarify how PDE2A affects T cell function and the EAE disease course.

As NP can modulate PDE2A activity and thus alter cAMP levels [199], NPs were the last target investigated within this study. All three NP, namely ANP, BNP and CNP, were tested in *in vitro* assays for T cell function. CNP had the strongest stimulatory effect on murine T cell activation and proliferation, a phenomenon that was reverted by the NPR2 inhibitor P19 and validated in human T cells. To elucidate whether CNP and NPR2 could be used as therapeutical target, further *in vitro* studies with the NPR2 inhibitor P19 or KO mice will be necessary. Applying the NPR2 inhibitor P19 in MS mouse models of MS will further address the therapeutic potential of the CNP/NPR2 axis in autoimmune diseases. Last, it must be clarified where CNP is mainly produced and how that could be modified to ameliorate MS and other autoimmune diseases. In summary, this study identified two promising targets which will be further investigated in the context of T cell function and MS.

5. Summary

Multiple sclerosis (MS) is an inflammatory, demyelinating disease with unknown etiology, leading to neurodegeneration of the central nervous system (CNS). It is the most common chronic neuroinflammatory disease, affecting around 2.5 million people worldwide. So far, there is no cure or preventive treatment for MS with treatment options only delaying the disease progression. MS is orchestrated by a misfunctioning immune system and thus commonly thought to be an autoimmune disease.

Contribution of the immune system to MS onset and progression is diverse but high impact is thought to come from autoreactive T cells which orchestrate the migration of immune cells to the CNS, guide the attack of the myelin sheath and lead to an activation of CNS-resident cells resulting in demyelination and blood brain barrier breakdown. Targeting these autoreactive, active T cells to ameliorate the disease course or even inhibit disease onset is a major goal in MS research. Hence, pathways controlling T cells physiology are important targets to decipher potential treatment options. One such pathway is the cAMP-mediated pathway, as intracellular level of cAMP can affect T cell physiology.

Within this study, different modulators of intracellular cAMP levels were studied in the context of T cell function and autoimmune diseases.

First target was the Gs-coupled G-protein coupled receptor (GPCR) GPR52. The orphan GPR52 contributes to intracellular cAMP levels via its constitutive activity which can be seen in neurons and transfected HEK cells, but also in T cells. However, its effect on T cell function has so far not been untangled. Within this thesis it was shown that neither *Gpr52*-deficiecency nor pharmacological modification of GPR52 resulted in altered T cell physiology. Moreover, *Gpr52*-deficiency had no effect on the disease course of the experimental autoimmune encephalomyelitis (EAE), the animal model of MS.

As second target, the phosphodiesterase (PDE) PDE2A was chosen. PDEs are the only known enzymes which can hydrolyze cAMP, thereby decreasing intracellular cAMP levels. Other PDEs have already been shown to affect T cell function and MS mouse models. However, PDE2A has not been studied in this context. Here, it was shown that inhibition of PDE2A with the selective inhibitor BAY 60-7550 decreased T cell proliferation while not affecting T cell activation or differentiation. Moreover, *Pde2a* expression was increased in T cells isolated from mice undergoing the EAE. Together with the finding that PDE2A is upregulated in activated T cells, an involvement in autoimmune diseases gets possible which should be determined in further *in vivo* experiments.

Third, the natriuretic peptides (NP), namely atrial NP (ANP), brain NP (BNP), and C-type NP (CNP), where tested upon their potential to alter T cell function. These peptides, which have been studied

extensively in cardiovascular research, can alter PDE2A activity via modulating the cGMP/cAMP crosstalk. While BNP had no significant effect on T cell function, ANP and CNP severely modified T cell physiology. Most dominant effect was observed upon CNP treatment, hence further focus was set on this peptide. It was shown that CNP affects T cell function via its cognate receptor NPR2. Moreover, CNP increased T cell activation also in human T cells, indicating that CNP is an interesting target in translational research.

In summary, this study deciphered the involvement of three cAMP modulators on T cell function. It was shown, that GPR52 is dispensable for T cell function. PDE2A and the NP, especially CNP, on the other hand can modulate T cell function and therefore should be considered as targets for promising therapeutical approaches to counteract autoimmune diseases like MS.

6. Zusammenfassung

Multiple Sklerose (MS) ist eine inflammatorische Erkrankung, die in einer Demyelinisierung und Neurodegeneration im zentralen Nervensystem (ZNS) resultiert. MS ist die am häufigsten vorkommende chronische, neuroinflammatorische Krankheit und betrifft rund 2,5 Millionen Menschen weltweit. Die Ursachen der Erkrankung sind bislang unbekannt, weswegen es noch keine Heilung gibt, sondern nur symptommildernde oder den Verlauf verzögernde Medikamente zur Verfügung stehen. Bei MS handelt es sich um einer Autoimmunerkrankung, was bedeutet, dass ein fehlerhaftes Immunsystem für den Ausbruch und größtenteils den Verlauf der Krankheit verantwortlich ist.

Ein vielschichtiges Mitwirken der verscheidenden Immunzelltypen wird vermutet. Insbesondere autoreaktive T Zellen tragen zur Krankheitsentstehung und -verlauf bei. Diese fehlerhaften T Zellen steuern die Migration der Immunzellen in das ZNS, die Aktivierung gewebespezifischer Zellen, und die Zerstörung der Axon-umgebenden Myelinschicht die zur Neurodegeneration führt. Aufgrund der vielfältigen Beteiligung von T Zellen an der MS stehen sie im Fokus der aktuellen Forschung. Eine verminderte Aktivität der autoreaktiven T Zellen kann den Verlauf der MS mildern. Somit ist die Erforschung von Signalwegen, die die Aktivität von T Zellen steuern, zentraler Fokus bei Autoimmunerkrankungen. Einer dieser Signalwege, der die T Zell-Aktivität steuern kann, wird durch den Second Messanger cAMP vermittelt.

Im Rahmen dieser Studie, wurden verschiedene Modulatoren des intrazellulären cAMP-Spiegels in T Zellen identifiziert und genauer untersucht.

Der erste Modulator war der Gs-Protein gekoppelte Rezeptor (GPCR) GPR52. Dieser konstitutiv aktive Rezeptor, dessen Ligand bislang unbekannt ist, trägt zur Erhöhung des intrazellulären cAMP-Spiegels bei, was bereits in Neuronen und HEK-Zellen gezeigt werden konnte. Auch in T Zellen resultiert die Aktivierung von GPR52 mittels eines selektiven Agonisten zu steigenden cAMP-Leveln. Allerdings konnte in dieser Studie kein Effekt von GPR52- Modifikation durch Antagonist oder Agonist Behandlung auf die T Zell Funktion gezeigt werden. Weiterhin zeigte die genetische Deletion von GPR52 keinen Effekt auf T Zell Funktion oder den Verlauf der Experimentellen Autoimmunen Encephalomyelitis (EAE), dem Tiermodell der MS.

Als zweites wurde die Phosphodiesterase (PDE) PDE2A untersucht. Da PDEs die einzigen bekannten Enzyme sind, die cAMP hydrolysieren können, sind bereits einige PDEs im Kontext der T Zell Funktionalität untersucht wurden. Mithilfe des selektiven PDE2A Inhibitors BAY 60-7550 (BAY) konnte gezeigt werden, dass die Inhibition von PDE2A einen negativen Effekt auf die T Zell Proliferation hat, wohingegen T Zell Aktivierung und Differenzierung nicht beeinflusst wurde. Weiterhin zeigt sich, dass *Pde2a* Expression in T Zellen während der EAE erhöht ist. Da PDE2A in

aktivierten T Zellen erhöht vorzufinden ist, könnte die Modifikation von PDE2A in Autoimmunerkrankungen einen positiven Einfluss auf deren Verlauf haben. Diese Hypothese kann durch eine EAE mit Mäusen mit T Zell-spezifischen PDE2A KO oder durch die Behandlung von Wildtyp Mäusen mit einem PDE2A Inhibitoren weiter untersucht werden.

Drittens wurden die natriuretischen Peptide (NP) auf deren Potential die T Zell Funktion zu beeinflussen untersucht. Die NP können die Aktivität von PDE2A durch den cGMP/cAMP Cross-Talk modifizieren und haben so Einfluss auf den intrazellulären cAMP-Spiegel. Besonders das C-type NP (CNP) hatte großen Einfluss auf T Zell Aktivierung, Differenzierung und Proliferation. Es konnte weiterhin gezeigt werden, dass diese Effekte über den Rezeptor NPR2 vermittelt werden und dass auch die Aktivität humaner T Zellen durch CNP moduliert werden kann.

Zusammengefasst wurden in dieser Studie drei Modulatoren des intrazellulären cAMP-Spiegels in T Zellen untersucht. Während GPR52 für T Zellen nicht relevant ist, stellte sich heraus, dass die NP, und besonders CNP, und die Modifikation von PDE2A vielversprechende Ansätze in Bezug auf Autoimmunerkrankungen und deren potenzielle therapeutische Behandlungsmethoden sind.

III. Abbreviations

AA	Amino acid
AKAP	A-kinase anchoring protein
ANP	Atrial natriuretic peptide
ANOVA	Analysis of variance
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
ATP	Adenine triphosphate
AUC	Area under the curve
BAY	BAY 60-7550
BBB	Blood brain barrier
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CNS	Central nervous system
CTLA	Cytotoxic T-lymphocyte-associated Protein
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
DAMP	Damage-associated molecular pattern
DMSO	Dimethyl sulfoxide
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid
EPAC	Exchange protein activated by cAMP
FACS	Fluorescent activated cell sorting
FRET	Förster resonance energy transfer
FTBMT	4-(3-(3-fluoro-5-(trifluoromethyl)benzyl)-5-methyl-1H-1,2,4-triazol-1-yl)-2-methylbenzamide
GC	Guanylyl cyclase

GM-CSF	granulocyte macrophages-colony stimulating factor
GPCR	G-protein coupled receptor
GPR52	G-protein coupled receptor 52
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenal
ICER	Inducible cAMP early repressor
IFNγ	Interferon γ
IL	Interleukin
i.p.	Intraperitoneal
IS	Immunological synapse
KO	Knockout
LN	Lymph nodes
MACS	Magnetic associated cell sorting
MBP	Myelin-binding protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
n.d.	Not detectable
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor k-light chain enhancer of activated B cells
NO	Nitric oxide
NP	Natriuretic peptide
NPR1	Natriuretic peptide receptor 1
NPR2	Natriuretic peptide receptor 2
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
pGC	Peripheral guanylyl cyclase
PGE2	prostaglandin E2
PKA	Protein kinase A
PLP	Myelin proteolipid

PPMS	Primary progressive multiple sclerosis
PRR	pattern recognition receptors
pTreg	Peripheral Treg
PTX	Pertusis toxin
qPCR	Quantitative real-time PCR
RM	Repeated measurements
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RORγt	RAR-related orphan nuclear receptor
RRMS	Relapse-remitting multiple sclerosis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SNAP	S-nitrose-N-acetylpenicillamine
SNP	Sodium nitroprusside
SNP	Single nucleotide polymorphism
SP	Secondary progressive multiple sclerosis
STAT4	Signal transducer and activator of transcription 4
TCR	T cell receptor
Teff	Effector T cell
TGFβ	Transforming growth factor β
Th	T helper cell
TMEV	Theiler's murine encephalomyelitis virus
TNFα	Tumor necrosis factor α
Treg	Regulatory T cell
tTreg	Thymic Treg
VLA	Very late antigen
VEH	Vehicle

IV. References

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VI. Curriculum Vitae

Lebenslauf entfällt aus datenschutzrechlichen Gründen

VII. Affidavit

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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