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Adenine dinucleotide Ca^{2+} mobilizing second messengers modulate $CD4^{+}$ T-cells differentiation and effector function

Dissertation

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Content

1. Introduction ... 7
1.1. Differentiation of naïve $CD4^+$ T cells into subsets ... 8
1.1.1. Differentiation and function of Th1 cells ... 9
1.1.2. Differentiation and function of Th2 cells ... 10
1.1.3. Differentiation and function of Th17 cells ... 11
1.1.4. Differentiation and function of regulatory T cells ... 12
1.1.5. Differentiation and function of $Foxp3^+$ T regulatory cells ... 13
1.1.6. Differentiation and function of Tr1 cells ... 13
1.2. Calcium signaling in T cells ... 14
1.2.1. Ca^{2+} signaling in T cells upon TCR stimulation ... 15
1.2.2. Effects of Ca^{2+} signaling on T-cell function ... 16
1.2.3. Effects of Ca^{2+} signaling on $CD4^+$ T-cell differentiation ... 18
1.2.4. Adenine dinucleotide second messengers and T-lymphocyte calcium signaling ... 19
1.3. $CD4^+$ T cells in pathogenesis of inflammatory bowel disease ... 22
1.4. $CD4^+$ T cells in the pathogenesis of crescentic glomerulonephritiss ... 24
1.5. Aims ... 26
2. Material and methods ... 27
2.1. Material ... 27
2.1.1. Reagents ... 27
2.1.2. Cytokines ... 28
2.1.3. Antibodies ... 29
2.1.4. Primers ... 30
2.1.5. Buffers and solutions ... 30

2.1.6. Animals 31

2.2. Methods 31

2.2.1. Genotyping 31

2.2.2. Mouse experiments 32

2.2.3. Enzyme-linked immunosorbent Assay (ELISA) of urine albumin 33

2.2.4. Histology 35

2.2.5. Cell isolation 36

2.2.6. Magnetic cell separation 37

2.2.7. Flow cytometry and Flow activated cell sorting 38

2.2.8. *In vitro* $CD4^+$ T cell differentiation 38

2.2.9. *In vitro* $CD4^+$ T cell proliferation assay 39

2.2.10. Bone marrow dendritic cell *in vitro* culture 39

2.2.11. Single-cell Ca^{2+} imaging 39

2.2.11.1. Fura2-AM loading 39

2.2.11.2. Incubation with NAADP inhibitors 40

2.2.11.3. Anti-CD3 $CD4^+$ T cell stimulation 40

2.2.11.4. Antigen specific $CD4^+$ T cell stimulation 40

2.2.11.5. Image acquisition 40

2.2.11.6. Determination of the Kd for Fura 2 40

2.2.11.7. Image processing 41

2.2.12. Statistical analysis 41

3. Results 42

3.1. Antigen affinity to TCR determines calcium signaling in $CD4^+$ T cells upon antigen presentation quantitatively and qualitatively 42

3.2. The affinity of an antigen to TCR impacts the differentiation of $CD4^+$ T cells 46

3.3. Antagonism of NAADP signaling with BZ194. 48

3.3.1. BZ194 inhibits calcium signaling after TCR stimulation in naïve $CD4^+$ T cells.....48

3.3.2. BZ194 has different effects on $CD4^+$ T cells differentiation dependent on TGF-β1 concentration, ...51

3.3.3. Impact of trans-Ned 19 on calcium signaling and proliferation of naïve $CD4^+$ T cells 52

3.3.4. Trans-Ned 19 influences the differentiation of naïve $CD4^+$ T cells *in vitro* 54

3.3.5. Trans-Ned 19 influences the differentiation of naïve $CD4^+$ T cells *in vivo* and ameliorates inflammation in CD3 specific antibody induced transient intestinal inflammation 55

3.3.6. Trans-Ned 19 affects the differentiation and function of $CD4^+$ T cells *in vivo* in an ovalbumin immunization model...58

3.3.7. Trans-Ned 19 affects the differentiation and function of $CD4^+$ T cells *in vivo* in murine nephrotoxic nephritis..60

3.4. TRPM2 channel does not play a significant role in the differentiation and function of $CD4^+$ T cells 63

3.4.1. TRPM2 channel does not have a significant effect on the differentiation of naïve $CD4^+$ T cells *in vitro* 65

3.4.2. TRPM2 channel does not have a significant impact on the differentiation of naïve $CD4^+$ T cells *in vivo* 65

4. Discussion 67

4.1. Strong TCR signals are necessary for Th17 differentiation, whereas weak TCR signals promote iTreg differentiation *in vitro* 68

4.2. NAADP antagonism influences the balance between Th17 and iTreg cells 71

4.3. BZ194 and trans-Ned 19 have opposing effects on the Th17 and Treg differentiation *in vitro* 75

4.4. Ned 19 ameliorates tissue damage in murine models of immune-mediated inflammatory diseases..77

4.5. TRPM2 channel does not play an essential role in $CD4^+$ T cell biology in the *in vitro* assays and *in vivo* experiments performed in this study 81

4.6. Conclusions and outlook 83

5. Abstract 84

6. Zusammenfassung 85

7. Abbreviations 86

8. List of Figures 89

9. List of tables 89

10. Bibliography 90

11. Acknowledgement 115

12. Curriculum Vitae 116

13. Eidesstattliche Versicherung 117

1. Introduction

One main function of the immune system is to maintain tissue homeostasis by fighting invading pathogens, eliminating malignantly transformed cells and participating in tissue remodeling. It is critical that the action of the immune system is fine-tuned so that invading pathogens or damaged cells are efficiently eliminated, and simultaneously, damage to healthy tissues are minimized [1]. The dysregulation of the immune response can lead to the development of immune-mediated inflammatory diseases (IMIDs), such as inflammatory bowel disease (IBD) or crescentic glomerulonephritis (cGN). Genome wide association studies (GWAS) have revealed specific genes that are linked to the pathogenesis of IMIDs [2], [3]. Interestingly, these studies have revealed a group of genetic loci that are shared by multiple IMIDs, many of which were associated with the development and function of adaptive immune system [2], [4]. In particular, they code for transcription factors directing the differentiation of $CD4^+$ and $CD8^+$ T cells, cytokines or cytokine receptors. IMIDs comprise a significant cause of morbidity and mortality and they lack causal therapies [5] . Immunosuppressive medication, such as corticosteroids, are effective in ameliorating the symptoms of disease, however, they do not address the cause of disease and their administration results in multiple adverse effects. Therefore, deeper understanding of the function of the adaptive immune system is required to elucidate the mechanisms governing the ethiopathogenesis of IMIDs and the development of causal therapies.

$CD4^+$ T cells are key cells of the adaptive immune system which orchestrate the immune response to pathogens, as well as developing tolerance to self, non-harmful microbes and allergens. $CD4^+$ T cells act in an antigen specific manner. This means that they express a T cell receptor (TCR) which can recognize a specific peptide presented by an antigen presenting cell (APC). $CD4^+$ T cells which have not yet recognized their cognate antigen are termed naïve. They continuously survey lymph nodes and spleen until they encounter an APC presenting a peptide that will bind their TCR with high affinity. Next, by integrating the signals from the TCR, co-stimulatory receptors and cytokine receptor naïve $CD4^+$ T cells differentiate into effector cells, which subsequently promote and direct the immune response. Alternatively, $CD4^+$ T cells differentiate into regulatory cells whose role it is to reduce inflammation and prevent tissue damage caused by an overactive immune response [1]. Elucidating the molecular mechanisms of how activation signaling events are integrated into the fate decisions of $CD4^+$ T cells is key in understanding the pathogenesis of IMIDs and in the development of new therapeutic strategies.

Signal transduction by TCR occurs via a number signaling cascades that ultimately determine cell fate. One of the early signaling events following TCR activation is the increase in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) concentration. The increase of $[Ca^{2+}]_i$ is necessary for $CD4^+$ T cells to launch a transcriptional program controlling cellular processes, such as metabolism, proliferation, differentiation and cytokine synthesis [6]. The importance of Ca^{2+} signaling for T cell biology is exemplified by multiple inflammatory, immune deficiency and autoimmune disorders caused by altered Ca^{2+} signaling in T cells [7]. Moreover, interference with calcium-dependent signaling pathways in T cells has been proven to be an effective immunosuppressive measure. For example, drugs such as cyclosporine A and tacrolimus are used in the prevention of transplant rejection and in management of IMIDs and autoimmune diseases [8]. The role of adenine-derived calcium mobilizing second messengers has previously been shown to be involved in calcium signaling in T cells upon TCR stimulation [9]. However, their role in controlling the differentiation and effector function of $CD4^+$ T cells remains unknown.

1.1. Differentiation of naïve $CD4^+$ T cells into subsets

The immune response is adjusted to the type of offending agent, so that it can be efficiently eliminated and to limit the damage to the host tissues [1]. $CD4^+$ T cells are orchestrators of the immune response. They communicate with other cells by secreting cytokines, soluble proteins which signal through specific receptors. The type of immune response is determined by the constellation of cytokines secreted by activated $CD4^+$ T cells. Naïve $CD4^+$ T cells continuously survey lymph nodes and the spleen in search of their cognate antigen [10]. Upon encounter with an APC presenting their cognate antigen, $CD4^+$ T cells are activated and integrate the signals transduced by the TCR, co-stimulatory receptors activated by APCs, and cytokine receptors [11], [12]. The combination of these signals induces a specific transcriptional differentiation program, which includes synthesis of a distinct set of cytokines. The differentiation of $CD4^+$ T cells reflects the adaptation of the immune response to the type of offending agent. Three major kinds of cell-mediated effector immune response have been described [12]–[14] . Type I immunity is directed against the intracellular bacteria, viruses and malignantly transformed cells. It is orchestrated by interferon gamma (IFN-γ)-producing Th1 cells, which promote killing of infected or transformed host cells by cytotoxic $CD8^+$ T cells and mononuclear phagocytes. In contrast, type 2 immune responses are directed against multicellular parasitic infestations. Type 2 immunity is orchestrated by Th2 $CD4^+$ T cells, which secrete interleukin 4 (IL-4), IL-5 and IL-13 that recruit basophils,

eosinophils and mast cells and promote immunoglobin E (IgE) isotype class switching. The type 2 immune response focuses on expelling invading helminthes out of the body and repairing tissue damage caused by the invading worms [14]. Finally, type 3 immune responses are directed against extracellular bacteria and fungi. It is orchestrated by Th17 cells, which activate mononuclear phagocytes, recruit neutrophils and promote epithelial antimicrobial responses [15], [16]. The immune system can, thus, tailor its responses to the type of offending agent, so that the most effective mechanisms is employed, and thus, damage to the host is minimized [12].

Naïve $CD4^+$ T cells recirculate between vascular and lymphatic systems until they encounter an APC presenting their cognate antigen. APCs stimulate the TCR and provide co-stimulatory signals to the naïve T-cells. Naïve T cells integrate TCR, co-stimulatory, and cytokine signals to start a specific transcriptional program distinct for a given lineage. $CD4^+$ T cells subsets were demonstrated to possess master transcription factors which coordinate the process of conversion from naïve to an effector cell [12]. Although, this differentiation was initially considered to be terminal, new evidence has shown that $CD4^+$ T cells can display characteristics of more than one lineage and can convert from one subset to another [17].

1.1.1. Differentiation and function of Th1 cells

Th1 cells coordinate type I immune responses, which are directed against intracellular bacteria, viruses and cancer cells [14]. The signature cytokine secreted by Th1 cells is IFN-γ. Apart from that they produce IL-2, tumor necrosis factor alpha (TNF-α) and lymphotoxin-α [14]. The differentiation of Th1 cells is driven by a strong antigenic stimulation in the presence of IL-12 [18], [19]. The IL-12 receptor consists of two subunits; IL-12Rβ1 and IL-12Rβ2, which are Jak kinases. Binding of IL-12 to its receptor results in STAT4 phosphorylation and its dimerization. Dimerized STAT4 translocates to the nucleus and activates transcription of the Th1 master transcription factor T-box transcription factor TBX21 (T-bet), which then induces transcription of *ifng* gene [20], [21]. Apart from STAT4 signaling, IL-12 and IFN-γ induce STAT1 phosphorylation, which maintain the Th1 transcriptional program [22]. Apart from activating the Th1 transcriptional program, T-bet represses the differentiation of naïve $CD4^+$ T cells into other subsets [23], [24].

Th1 differentiation was first shown to be induced by listeria-infected macrophages. IFN-γ released by Th1 cells activates mononuclear phagocytes and improves their ability to kill phagocytosed bacteria [18]. Consequently, impaired function of Th1 cells lead to increased susceptibility to

intracellular bacterial infections, in particular to *Mycobacterium tuberculosis* infection [25]. Moreover, IFN-γ activates cytotoxic $CD8^+$ T cells to promote killing of virus infected or malignantly transformed host cells. IFN-γ acts also on non-immune cells, interfering with multiple steps of viral replication [26]. Furthermore, Th1 cells are major players in the pathogenesis of multiple immune-mediated diseases, including Crohn's disease [26], glomerulonephritis [27] and multiple sclerosis [28]. However, the exact role of Th1 cells in inflammatory-mediated diseases is still not completely understood. One the one hand, mice deficient in IFN-γ were protected in the dextran sulphate sodium (DSS) induced colitis [29]. On the other hand, *Ifng*$^{-/-}$ mice exhibit exacerbated course of EAE, while administration of IFN-γ alleviates the disease severity [30], [31]. Furthermore, Th1 cells produce many other factors besides IFN-γ, such as TNF-α, which can potentially contribute to disease. Importantly, Th1 cells can also develop from other subsets of effector $CD4^+$ T cells, notably from Th17 cells, which reveals another layer of complexity in type I responses [32], [33].

1.1.2. Differentiation and function of Th2 cells

Type II immunity is directed against infestation by multicellular parasites such as helminths [1]. Signature cytokines of Th2 cells include IL-4, IL-5 and IL-13 [12]. The differentiation of Th2 cells is induced by weak TCR signal accompanied by IL-2 and IL-4 cytokine signaling [19], [34], [35]. IL-4 binding to its receptor induces STAT6 phosphorylation and its dimerization. Dimerized STAT6 induces transcription of GATA binding protein 3 (GATA3), the master transcription factor of the Th2 cells differentiation [36]. GATA3 regulates transcription of Th2 signature cytokines by binding directly to IL-5 and IL-13 promoter regions and increasing IL-4 synthesis through binding to its enhancer [12]. Moreover, IL-2 binding results in STAT5 phosphorylation, which directly promotes transcription of IL-4 [34]. GATA3 not only promotes the differentiation of Th2 cells, but also represses the transcriptional programs of the Th1 lineage [37].

Th2 cells primarily coordinate the immune response against helminthic infestations. Accordingly, mice deficient in key components of signaling in Th2 cells were unable to clear *Nippostrongylus brasiliensis* infestation [38]. Th2 cells were demonstrated to induce class switching of parasite specific antibodies to IgE class in plasma cells in an IL-4 dependent manner [39]. Eosinophils are effector cells of the innate immune system which secrete multiple parasite toxic mediators and express IgE receptor [1]. IL-5 secreted by Th2 cells induces the proliferation of eosinophils and promotes their accumulation at the site of inflammation [40]. IgE antibodies coat the parasites and

are subsequently recognized by effector cells of innate immune system, which release antiparasitic mediators. Another mechanism by which Th2 cells clear worm infestation is via action of IL-13 and TSLP. They induce contraction of smooth vasculature and in consequence expulsion of parasites [38]. Moreover, Th2 cells have been implicated to be involved in the pathogenesis of several immune-mediated diseases, such as asthma, allergy and ulcerative colitis [41]–[43].

1.1.3. Differentiation and function of Th17 cells

Th17 cells coordinate the type 3 immune response which is directed primarily against extracellular bacteria and fungi [1]. Th17 signature cytokines include IL-17A, IL-17F, IL-22 and TNF-α. Differentiation of Th17 cells was first reported to be induced by a combination of anti-inflammatory TGF-β1 and pro-inflammatory pleiotropic cytokine IL-6 [44]. In contrast, Ghoreshi *et al.* demonstrated that Th17 cells can also be differentiated in the absence of TGF-β signaling and that cells differentiated with IL-6 and IL-23 are more pathogenic in the model of transfer experimental autoimmune encephalitis compared to Th17 cells differentiation in the cytokine cocktail containing IL-6 and TGF-β1 [45]. How the interaction between TGF-β1 and IL-6 signaling results in the differentiation of Th17 cells remains incompletely understood. IL-6 binds to the IL-6Rα, which results in the phosphorylation of STAT3. Activation STAT3 signaling is necessary but not sufficient for the activation of the master transcription factor of Th17 cells, RAR-related orphan receptor gamma (RORγt) [46]. Zhou *et al.* reported that TGF-β1 at low concentrations promotes the transcription of RORγt, but high concentrations of TGF-β1 repress RORγt by favoring forkhead box P3 (Foxp3) expression [47]. IL-6 or IL-21 induced STAT3 signaling de-represses RORγt function and drives the differentiation of Th17 cells [48], [49]. IL-23 play an important role in the maintenance and maturation of Th17 cells. Naïve $CD4^+$ T cells do not express IL-23R, however its expression can be induced by IL-6, making the cells responsive to IL-23 and further supporting the Th17 differentiation program [45]. Th17 cells are plastic and they can convert both into pro-inflammatory as well as regulatory cells [50], [51]. Th17 cells differentiated in absence of TGF-β1 signaling expressed T-bet, a master transcription factor of Th1 cells, suggesting a possible conversion from Th17 to Th1 cells [45]. This suggestion was confirmed first by Lee *et al. in vitro* and later by Hirota *et al. in vivo* using a IL-17 fate tracking mouse [33], [52]. Interestingly, a study by Esplugues *et al.* suggested a possibility that Th17 can convert into IL-10 secreting regulatory cells during intestinal inflammation [53]. This phenomenon was further studied by Gagliani *et al.*

who showed that Th17 cells can lose the expression of IL-17 and transdifferentiate into Tr1 cells upon resolution of inflammation [54].

The significance of Th17 cells and type 3 immunity is highlighted by the Job's syndrome, an autosomal dominant form of Hyper-IgE syndrome. This disease is caused by a mutation in the STAT3 gene and is characterized by the impaired recruitment of neutrophils to the site of inflammation. It results in the occurence of cold *Staphyloccocus aureus* abscesses, increased susceptibility to *Candida albicans* infections and severe lung infections with formation of pneumatocelles [55]. The importance of Th17 effector cytokines was also demonstrated in mouse models using *Il17a*$^{-/-}$ mice or neutralizing IL-17A signaling with an antibody. These studies revealed that IL-17A was critical to control, among others, *Klebsiella pneumoniae*, *Salmonella enterica* serovar Typhimurium, *Bordatella pertussis* and *Candida albicans* infections [56]–[60]. Mice deficient in IL-22, another signature cytokine of Th17 cells, were in turn shown to be highly susceptible to *Citrobacter rodentium* intestinal infection [61]. Moreover, Th17 cells were shown to mediate the cross-talk between the intestinal microbiota and host immunity. Thus, segmented filamentous bacteria (SFB) in the intestine induced the emergence of Th17 in the murine small intestine lamina propria [62]. Strikingly, intestinal colonization with SFB enhanced immunity against *Citrobacter rodentium* infection [62]. The pro-inflammatory Th17 immune response is therefore beneficial in clearing infection, however, it can be pathogenic when in excess. Th17 cells have been involved in the pathogenesis of multiple immune-mediated diseases such as inflammatory bowel disease, crescentic glomerulonephritis, multiple sclerosis, rheumatoid arthritis and psoriasis [42], [63]–[66].

1.1.4. Differentiation and function of regulatory T cells

Multiple mechanisms have evolved to prevent inappropriate immune response against non-harmful and self-antigens [67]. During thymic development, T cells whose TCR reacts to self-antigens with a high affinity are either eliminated or differentiate into thymic regulatory T cells (tTregs) [68], [69]. This process is termed central tolerance and serves to eliminate potentially self-reactive T cells. However, this mechanism is not sufficient to develop tolerance towards non-harmful foreign antigens such as symbiotic microbial organisms or food allergens. Thus, regulatory T cells can also develop in the peripheral immune system at later stages in the development, when naïve CD4^{+} T cells are presented antigens in the tolerance promoting cytokine environment [70], [71]. These regulatory CD4^{+} T cells are termed induced T regulatory cells (iTreg) [72]. Moreover, a subset of

Foxp3$^-$ regulatory T cells, T regulatory cells type 1 (Tr1) have been shown to develop from effector T cells at the resolution of inflammation, thus contributing to the termination of immune response upon clearance of infection [53], [54].

1.1.5. Differentiation and function of Foxp3$^+$ T regulatory cells

Foxp3$^+$ regulatory T cells (Tregs)-mediated suppression plays a vital role in maintaining immune homeostasis. Foxp3 is a master transcription factor for Tregs [73], [74]. The indispensable role of Tregs in maintaining immune homeostasis is exemplified by the pathology observed in mice and humans with a mutation of the Foxp3 locus [75].

Tregs can emerge during thymic development of lymphocytes or be differentiated from naïve CD4$^+$ T cells in the periphery [69]. The TCR signal strength is a key signal determining the development of Tregs in the thymus. Thymocytes, whose TCR binds strongly to endogenous antigens presented by thymic stromal cells, and thus, would be predisposed to mount an immune response against self. These cells either turn anergic or they differentiate into tTregs [76]–[78]. On the contrary, the development of the Tregs in the periphery occurs in response to presentation of non-self-antigens such as allergens, food antigens or commensal microbial organisms in the tolerance promoting cytokine environment [79]–[81]. Apart from TCR signals, naïve CD4$^+$ T cells require IL-2 and TGF-βR signaling to differentiate into Tregs. Binding of TGF-β1 to its receptor activates a downstream signaling resulting in phosphorylation and translocation of SMAD proteins to the nucleus, which results in transcription of Foxp3. Zheng *et al.* , showed that conserved non-coding DNA sequence 1 (CNS1) at Foxp3 locus contains a SMAD3-NFAT response element and is critical for the differentiation of iTregs [82]. Apart from TGF-β1, IL-2 is required for the differentiation of T regulatory cells. IL-2 induces STAT5 phosphorylation, which directly regulates the expression of Foxp3 [83]. Furthermore, IL-2 opposes anti-proliferative effects of TGF-β1 and represses the differentiation of Th17 cells, whose development is promoted by TGF-β1 [84]. T regulatory cells can control the immune response by secreting the suppressive cytokines IL-10, IL-35 and TGF-β1, or by direct cell-to-cell contact through co-inhibitory receptors like CTLA-4 or PD-1 [85]–[88].

1.1.6. Differentiation and function of Tr1 cells

Tr1 cells are characterized by lack of Foxp3 expression, high secretion of IL-10 and the ability to suppress effector immune cells [89]. Although, multiple evidence regarding the cytokines and

transcription factors involved in Tr1 cells differentiation have been gathered, the identity of Tr1 cells master transcription factor remains unknown [90], [91].

IL-27 was reported to induce the differentiation of Tr1 cells [92], [93]. Nevertheless, the mechanism of how IL-27 induces the differentiation of Tr1 cells is not incompletely understood. The IL-27 receptor is a heterodimer consisting of glycoprotein 130 (gp130) and IL-27Rα (WSX-1) and [94]. Activation of IL-27R leads to phosphorylation of STAT3 through its gp130 subunit. Phosphorylated STAT3 induces expression of transcription factor c-avian musculoaponeurotic fibrosarcoma (c-Maf), which transactivates *Il10* and *Il21* genes [90]. Indeed, T cells deficient in c-Maf do not synthesize IL-10 upon IL-27 stimulation, thus indicating that c-Maf is necessary for IL-10 synthesis [92]. Moreover, c-Maf induced IL-21 in an autocrine fashion in a feed forward mechanism and maintanains expression of *c-Maf* and consequently *Il21*, and *Il10* genes [90]. IL-21 secretion is indispensable for the expansion of Tr1 cells, as the generation of Tr1 cells was impaired in $Il21r^{-/-}$ $CD4^{+}$ T cells [95], [96]. A similar IL-21 feed forward loop was described for the differentiation of Th17 cells [15]. Activation of WSX-1 subunit of IL-27R induces phosphorylation of STAT1 [97]. Phosphorylated STAT1 induces synthesis of T-bet which promotes IFN-γ production [97]. IFN-γ is a cytokine associated rather with promoting, not suppressing immune responses. Interestingly, Murugaiyan *et al.* reported that IFN-γ secreted by Tr1 cells can suppress IL-17 mediated autoimmune inflammation [98]. Nevertheless, how exactly T-bet contributes to the suppressive capacity of Tr1 cells and IL-10 production remains elusive. In addition to c-Maf, Aryl hydrocarbon receptor (Ahr) and B-lymphocyte maturation protein-1 (Blimp-1) were also described to contribute to the production of IL-10 by Tr1 cells [99]–[101]. Apart from suppressing immune responses via secretion of IL-10, Tr1 cells can also act on other cells in a contact dependent manner through Granzyme-B-mediated lysis or through co-inhibitory receptors [102], [103].

1.2. Calcium signaling in T cells

Calcium signaling is a vital signal transduction pathway in immune cells as it modulates a vast variety of downstream cellular processes. Regulated patterns of intracellular Ca^{2+} concentration control, in particular, key effector functions of lymphocytes such as proliferation, migration, differentiation, cytokine synthesis and secretion and cytotoxicity [6], [104]. Much evidence suggests that Ca^{2+} signaling is more than an on-off switch activating the T cells, as it is also critical in transducing complex information and finely controlling cellular responses [6], [104]. How Ca^{2+}

signaling is converted into meaningful cellular responses exactly has been an area of intensive investigation. The crosstalk between second messengers and a variety of calcium channels expressed on T cells allows for formation of distinct spatiotemporal patterns of Ca^{2+} concentration. In particular, highly localized spatial and temporal Ca^{2+} microdomains might be seen as a signature of Ca^{2+} signals, which through crosstalk and feedback loops might achieve functional specificity and fine tune T-cell signaling to match the needs of a complex immune response [105]. Nevertheless, our current understanding as to how different spatial and temporal intracellular Ca^{2+} signals are integrated is still very limited.

1.2.1. Ca^{2+} signaling in T cells upon TCR stimulation

An intracellular Ca^{2+} increase is a signaling event that occurs following the engagement of immunoreceptors, such as a TCR. Major transduction pathways that result in the increase of free cytosolic calcium concentration cells is Ca^{2+} release from endoplasmic reticulum (ER) and store operated calcium entry (SOCE) via calcium-release-activated calcium (CRAC) channels [106].

Following the binding of a TCR to a peptide MHC complex presented by an APC, several calcium mobilizing second messengers are synthesized: Nicotinic acid adenine nucleotide phosphate (NAADP), cyclic adenosine diphosphate ribose (cADPR) and inositol 1,4,5-triphosphate (IP_3) [6]. Within milliseconds following the TCR activation, NAADP is produced and contributes to the formation of early Ca^{2+} microdomains localized at the plasma membrane – endoplasmic reticulum (PM-ER) junction by releasing Ca^{2+} from ER via ryanodine receptor 1 (RYR1) [107]–[109]. These Ca^{2+} microdomains facilitate opening inositol-1,4,5-triphosphate receptors (IP_3R) by IP_3 through calcium induced calcium release (CICR) and further support calcium release from the ER lumen [107]–[109]. Later, within tens of minutes following the TCR ligation, cADPR acts on ryanodine receptors, thereby sustaining Ca^{2+} release from the ER [110]. Furthermore, other studies revealed that RYRs can also be activated by Ca^{2+} [111], [112]. Decrease in Ca^{2+} concentration within the ER lumen is then sensed by the stromal interaction molecule 1 (STIM1) and STIM2. Upon ER Ca^{2+} store depletion, STIMs activate CRAC channels in the plasma membrane, resulting in a significant Ca^{2+} influx with the electrochemical gradient and subsequent proliferation and cytokine production.

Although ER located Ca^{2+} channels and CRAC channels are major players mediating Ca^{2+} signaling upon TCR ligation, multiple lines of evidence have indicated that other classes of calcium channels modulate SOCE. For instance, purinergic ionotropic receptors (P2RXs) comprise another class of

ion channels which play an important function in T cell biology, with P2X4 and P2X7 being the most studied representatives [6]. P2X channels are permeable to Na^+ and Ca^{2+} and are directly activated by extracellular adenosine triphosphate (ATP) [113]. Antigen stimulated T cells increase their metabolism, and thus, the production of ATP, which can also be secreted into the extracellular space and act as a signaling molecule in an autocrine and paracrine fashion. In an autocrine manner, ATP activates the P2X channels located in the immune synapse, thereby amplifying Ca^{2+} influx [114]. In a paracrine manner ATP acts on P2X channels on neighboring resting T cells, thus slowing them down to facilitate scanning of the peptides presented by APCs [115].

Apart from endoplasmic reticulum and plasma membrane, endosomes and lysosomes have emerged to be involved in calcium signaling in T cells [116]. Upon immune synapse formation, TCR complexes and multiple other receptors are delivered from intracellular pools via vesicular trafficking [116]. Two pore channels (TPCs) comprise the most studied class of vesicular ion channels, with TPC1 being located in the endosomes, while TPC2 is located in the lysosomes [117]. The nature of ions conducted via TPCs remains controversial, as they were initially proposed to be NAADP activated Ca^{2+} channels [118], [119]. However, whole-endolysosome patch clamp studies indicated that TPCs are Na^+ selective and activated by phosphatidyloinositol-3,5-bisphosphonate [120].

1.2.2. Effects of Ca^{2+} signaling on T-cell function

Ca^{2+} signaling controls a variety of cellular processes in T cells, thus enabling them to effectively perform their function and mount an effective immune response. The consequences of increased Ca^{2+} intracellular calcium levels can be classified into short- and long-term effects. Naïve $CD4^+$ T cell survey the lymphoid organs in search of an APC, which presents their cognate antigen. TCR ligation-induced Ca^{2+} increase provides a stop signal and induces cytoskeletal rearrangements, resulting in rounding of otherwise polymorphic T cells. This process enables the formation of an immunological synapse and prolonged interaction with an APC [121]–[123]. Moreover, Ca^{2+} promotes metabolic activity of cells, thus coupling receptor activation and ATP production. Cytosolic Ca^{2+} microdomains are taken up by mitochondria through the mitochondrial Ca^{2+} uniporter and fuel cell metabolism, as the activity of three dehydrogenases in the tricarboxylic acid cycle are regulated by mitochondrial Ca^{2+} [124]–[126]. Furthermore, Vaeth *et al.* reported that SOCE directs the metabolic reprogramming of naïve T cells also on gene expression level - through activation of NFAT and mTOR pathways and regulating the expression of glucose transporters,

glycolytic enzymes and metabolic regulators [127]. Moreover, Ca^{2+} microdomains can also modulate the signaling via other signal transduction cascades. Kilipatrick *et al.* reported an essential role of NAADP released Ca^{2+} in formation and maintenance of ER-endosome membrane contact sites. ER-endosome contact sites ensured close apposition of endocytosed epithelial growth factor receptors (EGFR) with ER-localized phosphatases, thus facilitating dephosphorylation of the receptors and terminating the signaling through EGFR [128]. One can speculate that analogous mechanisms modulate cytokine signaling in T cells, thus controlling T cell differentiation.

Long term effects of calcium signaling are exerted through the changes in Ca^{2+} dependent gene expression and epigenetic changes, thereby inducing specific transcriptional programs which determine lymphocyte effector function and differentiation of naïve $CD4^{+}$ T cells [6], [104]. Strength and the duration of calcium signal following the TCR ligation can determine the pattern of gene expression as the signaling molecules and transcription factors targeted by Ca^{2+} have different activation thresholds [129], [130]. Probably the most extensive studied Ca^{2+} downstream signaling pathway involves activation of calcineurin, a phosphatase which dephosphorylates the nuclear factor of activated T cells (NFAT), thus facilitating its translocation to the nucleus and transcriptional regulation of its target genes. Decrease in Ca^{2+} concentration results in an almost immediate phosphorylation of NFAT and its export from the nucleus. Thus, NFAT-dependent gene expression requires long lasting elevation of Ca^{2+} concentration [130]. In contrast, nuclear factor-κB (NF-κB), JUN N-terminal kinase (JNK) or activating transcription factor 2 (ATF2) are successfully activated by a transient elevation in Ca^{2+} concentration. This discrepancy in the Ca^{2+} threshold for transcription factor activation might be one of the molecular mechanisms decoding Ca^{2+} spatiotemporal signatures into cellular responses [129], [130]. A vital role of the Ca^{2+}-calcineurin-NFAT signaling pathway was demonstrated by several studies, which either analyzed T cells from patients with genetic impairment of SOCE, or in which calcineurin inhibitors cyclosporine A or FK506 were used. These studies revealed, that both impaired SOCE and inhibition of calcineurin resulted in profound decrease in cell proliferation and cytokine synthesis which was accompanied by an impaired expression of several hundred genes [131]–[133]. RYRs are involved in initiating and maintaining SOCE [107]–[110]. In line with these results, pharmacological inhibition of RYRs decreased T-cell proliferation and IL-2 production [111], [112].

1.2.3. Effects of Ca^{2+} signaling on $CD4^+$ T-cell differentiation

The study of the function of components of Ca^{2+} signaling transduction networks reveals non-redundant functions of specific calcium channels regulating specific cellular processes including the differentiation of $CD4^+$ T cells [6]. Rodriguez *et al.* demonstrated that IL-2 inducible T cell kinase (Itk) plays a critical role in regulating the balance between Th17 and Treg cells. Itk is a tyrosine kinase required for full TCR-induced phospholipase C-γ activation and the synthesis of calcium mobilizing second messenger IP_3. CD4+ T cells deficient in Itk exhibited decreased IL-17A expression, which was rescued by pharmacologically induced calcium flux by ionomycin [134]. Furthermore, *Itk*$^{-/-}$ CD4+ T cells developed higher percentages of functional FoxP3 Tregs [134], [135]. In contrast, the study conducted by Nagaleekar *et al.* investigated the expression pattern and the role of IP_3 receptors for cytokine production by $CD4^+$ T cells. Interestingly, inhibition of IP_3R early during activation blocked IL-2 and IFN-γ production, but it increased the production of IL-17 by $CD4^+$ T cells [136]. Further, multiple studies revealed the critical importance of SOCE in T cell mediated autoimmunity. Genetic deletion of components of SOCE *Orai1*, *Stim1*, or *Stim2* in $CD4^+$ T cells ameliorates the disease severity in murine models of IBD, MS and organ rejection, and impairs Th17 and Th1 cells differentiation and effector function [137]–[139]. Interestingly, although Ca^{2+} dependent transcription factor NFAT is necessary for the differentiation of FoxP3 Tregs [140], genetic deletion of *Orai1* did not impair the Tregs function [137]. Moreover, SOCE promotes the metabolic reprogramming upon TCR activation and thereby supports the pathogenicity of Th17 cells [141]. $CD4^+$ T cell deficient in *Stim1* had reduced expression of genes required for mitochondrial function and oxidative phosphorylation, but enhanced reactive oxygen species production. Thus, Ca^{2+} flux controls the mitochondrial function and oxidative stress in Th17 cells [141].

Signaling through P2X channels plays a role in the differentiation of CD4 T cells, especially the balance between Th17 and Treg cells. Indeed antagonism of P2X receptors promoted conversion of naïve $CD4^+$ T cells into Tregs and activation of P2X7 channel inhibited the development and suppressive function of Tregs [142]. Further, antagonism of P2X receptors *in vivo* delayed the islet rejection in a model of islet allograft rejection. This finding was associated with reduced frequency of Th1 and Th17 cells [143]. In line with these results, Sharp *et al.* reported that mice deficient in P2X7 are protected in an EAE model [144]. Furthermore, Crohn's disease patients were found to have an increased expression of P2X7 in inflamed lesions and mice deficient in P2X7 receptor

were protected in tribnitrobenzene colitis and in DSS colitis [145]. In contrast to the above studies pointing at the proinflammatory role of P2X channels, Chen *et al.* showed that *P2x7*$^{-/-}$ mice were more susceptible to EAE and the P2X7 deficient lymphocytes were less prone to apoptosis than wild type cells [146]. Transient receptor potential channels constitute another group of cation channels expressed in T cells that control Ca^{2+} signaling in T cells [6]. For example, transient receptor potential melastatin 2 (TRPM2) was reported to be expressed in T cells and to be upregulated upon TCR activation [147]. In line with these findings, *Trpm2*$^{-/-}$ mice had a partially ameliorated course of EAE, which was accompanied by reduced proliferation of $CD4^+$ T cells and reduced secretion of Th1 and Th17 cytokines [148]. Interestingly, Ca^{2+} signaling upon TCR stimulation seems to be associated with the differentiation status of $CD4^+$ T cells, for instance Th2 cells have reduced TCR mediated Ca^{2+} influx compared with Th1 cells, which was correlated with different levels of NFAT translocation [149]. This difference was shown to be associated with higher expression of TRPM4 Na^+ channel. Ca^{2+} activates TRPM4 channels, thereby triggering Na^+ influx, which depolarizes plasma membrane and restricts further Ca^{2+} influx as a negative feedback loop [149]. Differential Ca^{2+} signaling patterns in Th1 and Th2 cells might explain the higher sensitivity of Th1 cells to FAS mediated apoptosis [150]. One other member of transient receptor potential channels: transient receptor potential vanilloid 1 (TRPV1) was shown to play an important role in CD4+ T cell biology, as *Trpv1*$^{-/-}$ mice were protected in the T cell transfer colitis and allergic rhinitis models [151], [152]. These studies indicate that a variety of calcium channels modulate $CD4^+$ T cell differentiation and effector function. Crosstalk between multiple ion channels plays a vital role in the fine-tuning of the immune response.

1.2.4. Adenine dinucleotide second messengers and T-lymphocyte calcium signaling

Ca^{2+} signaling upon TCR stimulation is modulated by the adenine nucleotide (AN) derived second messengers adenosine diphosphoribose (ADPR), 2'-deoxy-ADPR (2dADPR), cyclic ADPR (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) [9].

Although NAADP is the most potent calcium mobilizing second messenger known so far, the identity of its target channels remains elusive. It seems that the nature of NAADP signaling is cell type-dependent [153]. NAADP was discovered by Lee *et al.* in the sea urchin egg system, and so far, it is the most potent Ca^{2+}-releasing second messenger known [154]. Accumulating evidence suggests that in T cells, NAADP initiates Ca^{2+} release from ER via RYR1 and triggers formation

of Ca^{2+} microdomains. Indeed, microinjection of NAADP into Jurkat T cells induces development of Ca^{2+} microdomains within tens of milliseconds, which correlates with the finding of Gasser *et al.* who reported that endogenous NAADP is synthesized at similar time point after TCR stimulation of Jurkat T cells [107], [108]. Further, knockdown of the *Ryr1* gene in Jurkat T cells reduces formation of microdomains upon NAADP microinjection. Moreover, in primary murine *Ryr1*$^{-/-}$ T cells, the development of early Ca^{2+} microdomains upon TCR stimulation was markedly inhibited [108], [155]. The role of the Ca^{2+} release caused by NAADP was suggested to act via the CICR mechanism on IP_3R and RYRs to amplify IP_3 and cADPR induced Ca^{2+} depletion of ER [108], [155], [156]. The significance of NAADP in $CD4^+$ T cell activation and function was proved by means of the pharmacological inhibitor, BZ194, which blocks the interaction of NAADP with RYR1. BZ194 inhibited NAADP mediated Ca^{2+} release, NFAT nuclear translocation, production of IL-2 and proliferation of $CD4^+$ T cells [157], [158]. Moreover, *in vivo* administration of the compound ameliorated the clinical course of EAE in rats [159]. In this study, *in vivo* two-photon microscopy revealed, that NAADP signaling regulated T cell motility as treatment with NAADP inhibitor significantly reduced the number of stable arrests of effector T cells and their invasive ability. Further, treatment with BZ194 strongly decreased the levels of IFN-γ and IL-17A [159]. A study using another NAADP antagonist, trans-Ned 19 reported similar results in primary murine T cells [160]. In contrast, several lines of evidence suggest that in other cell types, NAADP activates endolysosomal TPC channels. First, Calcraft *et al.* showed that in HEK293 cells and primary murine β-pancreatic cells, the TPC2 channel was necessary for NAADP evoked Ca^{2+} currents [118]. Furthermore, using SKBR3 cell line, Brailoiu *et al.* reported a requirement of the TPC1 channel for NAADP-mediated calcium signaling [161]. Other studies reported that NAADP targets acidic organelles in $CD8^+$ T cells, where it is couples TCR activation and exocytosis of cytotoxic granules, and in phagocytic cells, where NAADP is produced upon Fc receptor ligation and promotes phagocytosis [162], [163]. In contrary to these results, Steen *et al.* published that in Jurkat T cells NAADP induces Ca^{2+} release from the ER and not from the acidic organelles [164]. Furthermore, other studies reported that NAADP can cause Ca^{2+} release both from the ER and acidic organelles or that NAADP activates TRPM2 channels in the plasma membrane [156], [165].In order to identify the NAADP receptor, Lin-Moshier *et al.* conducted a photoaffinity labeling study. It revealed that NAADP binds directly neither to TPC nor to RYR channels, but to a 22- and 23-kD doublet of unknown proteins termed NAADP binding protein (NAADP-BP) [166]. In line with these results, a unifying hypothesis for NAADP mediate Ca^{2+} signaling was suggested.

Distinct intracellular environments present in different cell types determine interaction of NAADP-BP with specific calcium channels [153]. In conclusion, NAADP signaling is a complex signal transduction network, involving an interplay between probably multiple NAADP-BPs and target calcium channels.

cADPR is the first discovered adenine derived Ca^{2+} mobilizing second messenger in sea urchin egg homogenates [9]. Subsequently, cADPR induced Ca^{2+} release in Jurkat T cells was also reported by Guse *et al* [167]. Detailed investigation revealed a vital role of cADPR signaling in T cell activation events. Firstly, the levels of cADPR in T cells are correlated with antigenic stimulation as cADPR levels increase within minutes upon TCR/CD3 stimulation. Secondly, Ca^{2+} signaling and expression of the activation markers, CD25 and HLA-DR, were reduced by cADPR inhibitors upon TCR stimulation in a concentration dependent manner, thereby pointing at a causal link between cADPR, Ca^{2+} signaling and activation of T cells [110], [168]. Furthermore, cADPR was suggested to activate Ca2+ release from ER via RYRs [169]. In line with these results, expression of RYR3 in Jurkat T cells was demonstrated both on transcript and protein level and cADPR specifically stimulated the association of [^{3}H] ryanodine with RYR3, thus indicating a direct influence of cADPR on RYR3 opening [110]. Moreover, gene silencing of RYR3 in Jurkat T cells significantly decreased the sustained phase of Ca^{2+} signaling upon TCR stimulation and impaired the Ca^{2+} release by cADPR in permeabilized Jurkat T cells [170]. cADPR was suggested to sustain Ca^{2+} signaling tens of minutes after TCR ligation. The experiments with antagonists of IP_3 and cADPR show that these two second messengers play non-redundant roles in Ca^{2+} signaling in T cells upon antigenic stimulation. Microinjection of IP3 antagonist after development of OKT3-induced Ca^{2+} signaling had no effect on Ca^{2+} levels, while injection of cADPR inhibitor at this time point completely inhibited Ca^{2+} signaling [110]. Thus, the two second-messengers seem to act in a temporal sequence.

ADPR is another adenine-derived Ca^{2+} mobilizing second messenger. Before its role in signaling was recognized, ADPR was considered a toxic cellular waste product. The reactive ribose of ADPR can non-enzymatically bind to amino groups of cellular proteins and impair their function [171]. Its Ca^{2+} mobilizing activity was discovered by two independent groups reporting that TRPM2, a Ca^{2+} permeable plasma membrane channel, can be activated by ADPR binding to its cytoplasmic NUDT9 domain [172], [173]. TRPM2 is expressed across multiple tissues with highest levels of expression noted in the central nervous system and the cells of immune system [150]. The

functional expression of TRPM2 was confirmed in Jurkat T cells. ADPR infusion into Jurkat T cells results in a characteristic TRPM2 ionic current and microinjection of ADPR into Jurkat T cells results in Ca^{2+} entry via plasma membrane [107]. Relevance of TRPM2 channel in T cell biology was further confirmed with the use of the *Trpm2*$^{-/-}$ mouse[174]. First, *Trpm2*$^{-/-}$ mice were protected in the DSS induced model of ulcerative colitis. The protection was correlated with TRPM2 promoting chemokine production in monocytes, while the T-cell infiltration of colonic tissue was not affected by deficiency of TRPM2 [174]. On the other hand, one study reported that $CD4^{+}$ T cells deficient in TRPM2 produce less pro-inflammatory cytokines IFN-γ and IL-17A. Moreover, TRPM2 deficient mice exhibited ameliorated EAE phenotype [147]. Protection of *Trpm2*$^{-/-}$ mice in EAE was also reported by Tsutsui *et al.* No difference in the percentages of T cells in secondary lymphoid organs or in the level of T-cell infiltration into the CNS between WT or *Trpm2*$^{-/-}$ was noted *in vivo* and an exacerbating role of TRPM2 channel was attributed to promoting CXL2 production by macrophages, thereby promoting infiltration of CNS by neutrophils.

In conclusion, adenine derived Ca^{2+} mobilizing second messengers are essential players in the T-cell activation signal transduction network. NAADP is a very early acting second messenger contributing to the development of Ca^{2+} microdomains upon TCR ligation, particularly in effector T cells. Furthermore, NAADP is involved in endolysosomal Ca^{2+} signaling regulating vesicular trafficking. cADPR plays a central role in sustaining long-lasting Ca^{2+} signaling upon TCR ligation, while the function of ADPR and TRPM2 receptors in T cells still remain unknown. The involvement of these second messengers in immune regulation makes them excellent targets for pharmacologic intervention by either antagonizing their activation of downstream ion channels or modulation of their metabolism. In fact, interference with NAADP mediated Ca2+ signaling was already successfully performed in *in vivo* models of disease: antagonism of NAADP by means of BZ194 ameliorated the EAE in rats and administration of another NAADP antagonist trans-Ned 19 controlled melanoma dissemination [159], [175], [176]

1.3. $CD4^{+}$ T cells in pathogenesis of inflammatory bowel disease

Inflammatory bowel disease (IBD) is a disease characterized by a chronic inflammation of the intestinal mucosa. It comprises two clinical entities: Crohn's disease (CD) and ulcerative colitis (UC) which share many clinical and histological features, yet are pathophysiologically distinct [177], [178]. The pathogenesis of inflammatory bowel disease is complex, multifactorial and incompletely understood. The interplay between four major factors is suggested to result in the

development of IBD, these include: dysregulated immune response, altered intestinal microbiota, genetic susceptibility and environmental factors [42]. Despite the identification of multiple pathways and mechanisms contributing to the pathogenesis of IBD, a full understanding of the pathogenesis of this disease remains out of reach. Consequently, a curative therapy of inflammatory bowel disease is still lacking.

Chronic inflammation is ultimately a dysregulated immune response, which instead of protecting the host, damages healthy tissues. Multiple studies revealed high numbers of T cells in the intestinal mucosa and secretion of large amounts of pro inflammatory T cell-derived cytokines, pointing at an involvement of $CD4^+$ T cells in the pathogenesis of IBD [42]. Early studies revealed increased levels of IFN-γ and IL-12 in lamina pretrial of CD patients, suggesting that the disease is Th1 driven [179], [180]. In contrast, ulcerative colitis was initially difficult to be classified in the Th1/Th2 paradigm, as neither Th1 associated cytokines IFN-γ and IL-12, nor IL-4 a signature cytokine of the Th2 subset, were increased in the lamina propria of UC patients [180], [181]. On the contrary, IL-5 and IL-13 were increased in UC patients [180], [182], which was termed an atypical Th2 response.

The Th1/Th2 paradigm of IBD pathogenesis was revised with the discovery of new T helper cells subsets. First, Fujino *et al.* reported that IL-17 is increased in the inflamed mucosa and serum of patients with IBD [183]. Murine models of experimental IBD proved that, what was previously thought to be Th1 driven pathology, was dependent on IL-23 driven Th17 cells [184]. Further, a possible interaction of Th1 and Th17 responses was suggested, as a cross-regulation of IL-12 by IL-23 was demonstrated in the T-cell driven model of trinitrobenzene sulfonic acid induced colitis (TNBS) [185]. Moreover, it was shown that Th17 cells can convert into Th1 cells and that these exTh17 cells are pathogenic in murine models of inflammatory bowel disease and multiple sclerosis [32], [33]. Other evidence in favor of the importance of Th17 cells in the development of IBD was provided by studies involving mice lacking IL-21, one of the key Th17 cytokines. In the dextran sodium sulphate (DSS) and TNBS colitis model *Il21*$^{-/-}$ mice were protected from colitis development. The importance of IL-23 signaling and Th17 in the pathogenesis of IBD was recapitulated in human studies. First, IL-23 receptor polymorphisms were identified as IBD susceptibility loci in multiple GWAS studies [186]. Moreover, increased numbers of IL-17 and IFN-γ co-producing cells were found in the intestinal mucosa of CD patients, which underlines the importance of Th17 and Th1 cells in the development of IBD [187].

Apart from effector T helper cells, dysfunction of T regulatory cells was also implicated in the pathogenesis of IBD [42]. Under homeostatic conditions, T regulatory cells prevent mounting of an immune response against non-harmful food antigens and intestinal microbiota [188]. Indeed, Atarashi *et al.* showed that gut microbes specifically induce differentiation of Foxp3^{+} regulatory T cells [189]. Data obtained from multiple animal studies suggest a mechanism by which dysfunction in regulatory CD4^{+} T cells leads to the development of colitis [188]. For example, Boehm *et al.* reported that deletion Foxp3 Tregs results in an exacerbated course of disease in murine models of IBD: DSS colitis and CD45Rb^{high} transfer colitis [190]. Further, Huber *et al.* showed that mice, whose T cells had impaired TGF-β signaling, had impaired T regulatory cell function and were more prone to colitis in a DSS model [191]. Moreover, mice whose T regulatory cells lack the IL-10 receptor are more susceptible to immune diseases, including colitis [192]. The role of T regulatory cells in human IBD remains poorly defined [188]. In active phases of IBD, the number of Tregs decrease in peripheral blood and increase in the intestinal mucosa [193]. Moreover, mutations in CD25 and IL-10, which are critical for Treg development and function, increase IBD susceptibility [186]. TNF-α blockade has proved to be an effective measure in IBD management [194]. Interestingly, blocking TNF-α signaling in IBD patients improves the suppressive function of Tregs [195] and reverses apoptosis of Tregs in the intestinal mucosa of IBD patients [196].

1.4. CD4^{+} T cells in the pathogenesis of crescentic glomerulonephritis

Crescentic glomerulonephritis (cGN) is an immune mediated kidney disease characterized by a formation of glomerular crescents composed of proliferating parietal cells and leukocytes which rapidly progresses to kidney failure. The pathogenesis of cGN is most widely studied using nephrotoxic nephritis model (NTN), which is induced by injecting mice with heterologous antibodies directed against glomerular basement membrane antigens and experimental anti-glomerular basement membrane glomerulonephritis (EAG). Analysis of the immune events in the animal models of cGN have suggested that kidney damage is precipitated both by the cellular and humoral immune response, both being orchestrated by CD4^{+} T cells. Initially, glomerular inflammation was thought to be driven primarily by Th1 cells, however recent discoveries in the field point at an involvement of Th17 cells [63].

Multiple evidence suggests a crucial role of type I immune response in the development of cGN. First, a study conducted by Cunningham *et al.* suggested an important role for delayed type hypersensitivity (DTH) reaction in the pathogenesis of cGN, as increased numbers of the DTH

effector immune cells (activated T-cells and macrophages) were significantly increased in the glomeruli of patients with cGN [197]. Furthermore, analysis of the IgG subclass distribution in patients with Goodpasture's syndrome revealed higher proportion of Th1-associated IgG antibody subclasses compared to healthy controlled [198]. On the contrary, mice lacking either IFN-γ or IL-12, key type I cytokines, were not protected from the development of kidney pathology in the murine models of cGN [199], [200]. On the other hand, IL-23 signaling was shown to be critical in the development of immune-mediated kidney pathology, as mice deficient in IL-23 were protected in the EAG [200]. The involvement of Th17 cells was confirmed in multiple studies. First, Paust *et al.* reported that mice lacking IL-23 p19, IL-17 or RORγt exhibited an ameliorated course of disease in the NTN, further suggesting the involvement of the IL-23/Th17 axis in the pathogenesis of cGN [201], [202]. Furthermore, using a model of antigen-specific glomerulonephritis, Summers *et al.* demonstrated that both Th17 and Th1 polarized cells were able to induce kidney damage [203]. Interestingly, the tissue damage promoting Th17 cells in NTN can migrate from the intestine and are microbiota dependent [204]. Moreover, T regulatory cells also play a role in the immunopathogenesis of cGN. First, transfer of regulatory $CD4^+$ $CD25^+$ T cells inhibited the development of NTN in mice [205]. Second, Tregs were demonstrated to migrate to the kidney and control Th1 cells as mice deficient in CCR6 exhibited impaired Treg migration to the kidney and aggravated NTN severity [206]. In conclusion, much evidence supports the notion that $CD4^+$ T cells are a major player in cGN and targeting them is a promising therapeutic approach in management of immune-mediated kidney disease.

1.5. Aims

The immune system protects the body from infection and from developing malignancy. Its dysregulation may lead to the development of multiple diseases, many of which lack curative treatment. $CD4^+$ T cells are orchestrators of the immune response. Distinct subsets of $CD4^+$ T cells have been described to play specific functions. Effector $CD4^+$ T cells coordinate defense against pathogens, removal of transformed cells or dead cells, while regulatory $CD4^+$ T cells promote tolerance and protect the body from inadequate immune response. Therefore, unraveling the molecular mechanisms which control the differentiation of $CD4^+$ T cells into the aforementioned fates and their subsequent effector function is of vital importance. This will allow to identify new targets for the treatment of diseases in which the immune system plays a role. Calcium mobilizing adenine nucleotides derived second messengers pose as promising candidates for developing new treatment strategies of immune-mediated inflammatory diseases. However, their exact role in T-cell differentiation and function was unclear. Moreover, the identity of the ion channel whose function is modulated by the adenine derived second messengers is not clear.

Therefore, the first aim of this study was to investigate the role of adenine nucleotide derived second messengers in the $CD4^+$ T cells differentiation and effector function. The second aim was to investigate the role of TRPM2, a Ca2+ channel potentially modulated by multiple adenine derived second messengers, in the CD4+ T cell biology. To this end the following research questions have been addressed:

1. How does the TCR affinity of antigens determine the Ca^{2+} signal in CD4 T cells upon recognition of an antigen?
2. How does the TCR affinity of an antigen impact the differentiation of $CD4^+$ T cells?
3. How does antagonizing NAADP signaling with the means of BZ194 impact the differentiation and function of $CD4^+$ T cells *in vitro* and *in vivo*?
4. How does antagonizing NAADP signaling with the means of trans-Ned 19 does impact the differentiation and function of $CD4^+$ T cells *in vitro* and *in vivo*?
5. What is the function of TRPM2 in the differentiation and function of $CD4^+$ T cells?

2. Material and methods

2.1. Material

2.1.1. Reagents

Basic chemicals and reagents were purchased from the companies, Merck, Sigma-Aldrich and Roth, Tocris. Specific chemicals are listed in the tables below.

Table 1 Reagents for cell isolation, cell culture, in vitro assays and animal experiments

Reagent	Manufacturer
Dimethyl sulfoxide (DMSO) anhydrous	Merck
Trans-Ned19	Tocris
Click's Medium	Irvine Scientific
β-Mercaptoethanol	Gibco
Dulbecco's Phosphate Buffered Saline (PBS), 1 x and 10 x	Sigma
Fetal calf serum (FCS)	Sigma
Penicillin/Streptomycin, 10,000 units/ml	Invitrogen
Percoll	GE Healthcare
Trypan blue solution, 0.4%	Sigma-Aldrich
L-Glutamine	Invitrogen
Ethylenediaminetetraacetic acid (EDTA)	Fluka
Collagenase IV (100 U), from Clostridium histolyticum	Sigma-Aldrich
DNase	BD Bioscience
CellTrace Violet dye Proliferation kit	ThermoFisher Scientific
Streptavidin microbeads	Miltenyi Biotech
CD4 microbeads, mouse	Miltenyi Biotech
Easysep^{TM} CD4^{+} T cell naïve isolation	Stemcell technologies

Table 2 Reagents for flow cytometry

Reagent	Manufacturer
FACS Clean Solution	BD Bioscience
FACS Flow, 20l	BD Bioscience

FACS Rinse Solution	BD Bioscience
Zombie UV Fixable Viability Kit	BioLegend
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich
Ionomycin	Sigma-Aldrich
Monensin A	BioLegend
Formaldehyde solution	Sigma-Aldrich
Nonidet P40 (NP40)	Sigma-Aldrich

Table 3 Reagents for DNA extraction and genotyping PCR

Reagent	Manufacturer
Agarose Ultra-Pure Company	Life Technologies 24
dNTP mix	Fermentas
GeneRuler 1 kb Plus DNA Ladder	Fermentas
DreamTaq DNA Polymerase	ThermoFisher Scientific
10x DreamTag Green buffer	ThermoFisher Scientific
Proteinase K	Roche
Ethidium bromide	Sigma-Aldrich

Table 4 Reagents for Ca^{2+} microscopy

Reagent	Manufacturer
Fura 2-AM	Sigma
Bovine Serum Albumin	Sigma-Aldrich
Poly-L-lysine	Sigma-Aldrich
Korasilon silicon paste	(Kurt Obermeier GmbH & Co. KG, Bad Berleburg, D).

Table 5 Reagents for murine albumin ELISA

Reagent	Manufacturer
TMB peroxidase substrate	AVIVA Systems Biology, San Diego, California, USA
Sulfuric acid (H_2SO_4)	Sigma Aldrich

2.1.2. Cytokines

Table 6 Cytokines

Cytokine	Manufacturer
Murine Interleukin-1β	Peprotech

Murine Interleukin-2	Peprotech
Murine Interleukin-6	BioLegend
Murine Interleukin-12	Peprotech
Murine Interleukin-23	BioLegend
Murine Interleukin-27	BioLegend
hTGF-β1	BioLegend
Murine IFN-γ	BioLegend
Murine GM-CSF	Peprotech

2.1.3. Antibodies

Table 7 Antibodies for animal experiments, cell culture and in vitro assays

Antigen	Clone	Manufacturer
CD3	2C11	BioLegend
CD28	37.51	BioLegend
IFN-γ	XMG	BioLegend
IL-4	11B11	BioLegend

Table 8 Antibodies for ELISA

Antigen	Clone	Manufacturer
Murine Albumin – unconjugated	polyclonal	Bethyl Laboratories Inc.
Murine Albumin – Horse Radish Peroxidase (HRP) conjugated	polyclonal	Bethyl Laboratories Inc.

Table 9 Antibodies for flow cytometry

Antigen	Clone	Staining	Dilution	Fluorochrome	Manufacturer
CD3	17A2	Surface	1:100	AF700	BioLegend
CD4	RM4-5	Surface	1:600	PacificBlue	BioLegend
CD4	RM4-5	Surface	1:600	PE-Cy7	BioLegend
CD4	RM4-5	Surface	1:600	BV785	BioLegend
CD11b	M1/70	Surface	1:400	PE-Cy7	BioLegend
CD11c	N418	Surface	1:400	PE-Cy7	BioLegend
CD8α	53-6.7	Surface	1:400	PE-Cy7	BioLegend
CD45	30-F11	Surface	1:1000	BV785	BioLegend
CD69	H1.2F3	Surface	1:200	PacificBlue	BioLegend
CD62L	MEL-14	Surface	1:600	APC	BioLegend

CD44	IM7	Surface	1:200	AF488	BioLegend
CD90.1	OX-7	Surface	1:400	APC	BioLegend
CD90.2	53-2.1	Surface	1:400	PE-Cy7	BioLegend
IL-4	11B11	Intracellular	1:100	APC	BioLegend
IL-10	JES5-16E3	Intracellular	1:100	APC	BioLegend
IL-17	TC11-18H10.1	Intracellular	1:100	AF488	BioLegend
IFN-γ	XMG1.2	Intracellular	1:100	BV785	BioLegend
Foxp3	NRRF-30	Intracellular	1:80	PE	eBioscience

2.1.4. Primers

Table 10 Primers for genotyping PCR

Genotyping primers	Sequence 5'-3'
FIR1	CAA AAC CAA GAA AAG GTG GGC
FIR2	GGA ATG CTC GTC AAG AAG ACA GG
FIR3	CAT CTT GGA GAG TCG GTG TG
IL10KOF	GTG TGT ATT GAG TCT GCT GGA C
IL10KOR1	GTG TGG CCA GCC TTA GAA TAG
IL10KOR2	GGT TGC CTT GAC CAT CGA TG
GFP-3	AAG TCG TGC TGC TTC ATG TG
GFP-5	ACG TAA ACG GCC ACA AGT TC
IL-17A KI sense	CAC CAG CGC TGT GTC AAT
IL-17 KI anti sense	ACA AAC ACG AAG CAG TTT GG
IL-17 KI IRES	ACC GGC CTT ATT CCA AGC
ROSC1-13F	CTT GGG TTG CAG TCA TAT GCA GGC
ROSC1-10R	GCC CTC ACC ATC CGC TTC ACG ATG
Pneo5'a	GCC ACA CGC GTC ACC TTA ATA TGC G
OT2 A fw	AAA GGG AGA AAA AGC TCT CC
OT2 A rev	ACA CAG CAG GTT CTG GGT TC

2.1.5. Buffers and solutions

Table 11 Buffers and solutions

10x ACK buffer	20.05 g NH4Cl, 2.5 g KHCD3, 0.093 g EDTA, ad 250 ml distilled H2O
MACS buffer	2 mM EDTA, 1% FCS in PBS
Complete medium	Click's medium supplemented with 10% FCS, 1% l- glutamine, 1% penicillin/streptomycin and 1:1000 β- Mercaptoethanol
Fix buffer	3.64% Formaldehyde in MACS buffer

Perm buffer	0.1% NP40 in MACS buffer
10x TBS	12.1 g Tris, 87.7 g NaCl, ad 1L distilled H2O
Proteinase K Buffer	12.1 g Tris, 10 ml 0.5 M EDTA, 11.7 g NaCl, 5 ml SDS (from 20% Stock), ad 1L distilled H2O
Ca^{2+} measurement buffer	140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 20 mM HEPES, 5.5 mM glucose; pH 7.4 adjusted with 7 M / 1 M NaOH
Wash solution (for albumin ELISA)	1L aqua dest., content of one package 50mM Tris buffered saline, pH 8,0 – 0,05% Tween20
Postcoat solution (for albumin ELISA)	1mL aqua dest., content of one package 50mM Tris buffered saline, pH 8,0 – 1% BSA
Sample / conjugate diluent (for albumin ELISA)	200mL of Postcoat solution, 1mL 10% (v/v) Tween20

2.1.6. Animals

Mice were kept under specific pathogen free conditions in the facility of the University Medical Center UKE. Food and water was provided *ad libitum*. C57BL/6J, OT-II transgenic, *Trpm2*$^{-/-}$ were obtained from the Jackson Laboratory, C57BL/6N from Taconic Farms. Foxp3^{mRFP}, IL-17A^{eGFP}, IL-17A^{FP635} and IL-10^{eGFP}, IFNγ^{FP635}, reporter mice are described elsewhere [54], [207]–[209]. Age- and sex-matched mice between 8 to 18 weeks of age were used for all experiments. All animals were cared for in accordance with the institutional review board 'Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz' (Hamburg, Germany). Approval was granted (numbers ORG_934, 17/12, 20/067). Mice were kept under specific pathogen free conditions, ambient temperature of 20±2°C, humidity of 55±10% and a dark/light cycle of 12 hours.

2.2. Methods

2.2.1. Genotyping

All reagents used for genotyping are listed in Table 3 and Table 10.

To determine the genotype of the genetically modified mice, the mice were genotyped by polymerase chain reaction (PCR). To this end, tail biopsies were digested at 55°C overnight using Proteinase K (in Proteinase K buffer) to extract the genomic DNA from the sample.

The master mix used for the PCR reactions contained 3µl master mix buffer, 0.6 µl dNTPs (10 mM), 0.25 µl DreamTaq polymerase, 0.9 µl of each primer (10 µM, Table 1 and 3) and 19.5 µl water. 2 µl of genomic DNA was added to the PCR master mix. Unless stated otherwise, the amplification was run in a PCR cycler with the following program: 3 min 94°C, 35 cycles of 94°C, 65°C -0.3°C/cycle and 72°C for 40 sec each, 5 min of 72°C. All PCR products were resolved by electrophoresis on a 1.5% agarose gel. DNA was stained with Ethidiumbromid and bands were visualized with a UV transilluminator.

The presence of Foxp3^{mRFP} reporter was confirmed using primers FIR1, FIR2 and FIR3. The PCR resulted in a 692 bp wild type amplicon and a 470 bp knock in amplicon.

The presence of IL-10^{eGFP} reporter was confirmed using two different PCR reactions. One using primers IL10KOF, IL10KOR1 and IL10KOR2, this PCR reaction resulted in an Il10 wild type amplicon of 340 bp, but the knock in amplicon of 550 bp could not be amplified efficiently enough. Therefore, a second PCR reaction was run using GFP-3 and GFP-5 primer to amplify the inserted Gfp.

The presence of IL-17A^{eGFP} and IL-17A^{FP635} reporter was confirmed using the primers, IL-17A KI sense, IL-17A KI anti sense and IL-17A KI IRES. The PCR reaction amplified a wild type product of 370 bp and a knock in amplicon of 300 bp.

Trpm2$^{-/-}$ was detected using ROSC1-13F, ROSC1-10R and Pneo5'a primers amplifying a 514 bp amplicon for WT and 740 bp amplicon for knock out.

OT2 Transgene was detected using OT2 A fw and OT2 A rev primers amplifying 160 bp amplicon for transgene.

2.2.2. Mouse experiments

Anti-CD3 antibody model

One group of mice were injected with anti-CD3 (clone 2C11, 0.6 µg/ml) intraperitoneally two times every other day (day 0, day 2), and sacrificed 4 hours or 48 hours after the second injection. As controls, another group of mice were injected with PBS. The mice were injected i.p. with 20 mg/kg of trans-Ned 19 in DMSO or DMSO alone 2 hours before first anti-CD3 injection.

Ova immunization model

C57BL/6N mice were immunized subcutaneously with 50 ug of OVA (Sigma-Aldrich) emulsified with CFA (Difco) in flank. Seven days after immunization, the mice were challenged with the intradermal injection of 20 µg of OVA (1 mg/ml in PBS) in the right footpad and PBS as a negative control in the left footpad. 20 mg/kg of trans-Ned 19 or DMSO alone were administered i.p. daily from day of immunization until second ovalbumin challenge. DTH was assessed by measuring the thickness of the ova injected footpad to PBS injected footpad 24 hours after the challenge by a micrometer (TECLOCK). Mice were euthanized, and single-cell suspensions of inguinal lymph nodes and spleen were prepared.

Nephrotoxic nephritis

Experimental cGN was induced by i.p. injection of 200 µl nephrotoxic sheep serum in 8- to 12-week-old male mice as described in [210]. At day 9 urinary samples were collected in a metabolic cage and analyzed for the concentration of albumin. Mice were euthanized at day 10. 20 mg/kg of trans-Ned 19 or DMSO alone were administered i.p. daily starting 2 hours before nephrotoxic sheep serum administration.

2.2.3. Enzyme-linked immunosorbent Assay (ELISA) of urine albumin

Plate preparation

A high-binding 96 well-plate was coated overnight at 4°C with anti-murine albumin antibody at a dilution 1:100. Plate was washed three times with 200 µL washing buffer per well. Then wells were incubated for 30 min, RT with a post coat solution. Afterwards, the plate was washed three times with 200µL washing buffer per well.

Sample preparation

Standard murine albumin dilutions were prepared according to the Table 12. Sample dilutions were prepared according to their proteinuria level (measured with urine sticks) according to the Table 13.

Table 12 Standard dilutions of murine albumin for ELISA

Standard No.	Standard concentration (ng/ml)
0	10000
1	1000
2	500
3	250
4	125
5	62.5
6	31.25
7	15.63
8	7.82

Table 13 Dilutions of the murine urine sample for the ELISA determination of albumin concentration

Level of proteinuria (according to urea stick)	Urinary protein content (mg/dl)	Pre dilution of urine sample
-	0 (negative)	No measurement
+/-	< 30	1:500
+	30	1:1000
++	100	1:20 000
+++	300	1:50 000
++++	>2000	1:100 000

Measurement

100 µl of sample or standard dilution was pipetted into the appropriate wells and incubated for 1h at RT. Next, the wells were washed five times with 200 µl washing buffer per well. Then, 100 µl of the HRP-coupled secondary antibody (1:50 000) was added and incubated for 1h at RT. The plate was again washed 5 times and the substrate reaction followed by addition of 100 µl of ready-to-use TMBE substrate solution to each well. The plate was incubated in the dark for 15 min at RT. The substrate reaction was stopped by addition of 100 µl of 2M H_2SO_4 per well under the fume hood. The fluorescence of samples and standard albumin dilutions were measured with a Spectrophotometer (DS11, DeNovix Inc., Wilmington, USA) at 450 nm. The calibration curve was fitted, and the albumin concentration was determined.

2.2.4. Histology

Fixation of tissue samples

Cross sections of kidneys were kept at 4 % paraformaldehyde overnight. Paraformaldehyde was removed by washing three times the tissue for 10 minutes each with fresh PBS on a shaker at room temperature.

Histological sample preparation

Next, the tissues were kept in 50 % (v/v) Ethanol at room temperature for three hours. This was the first step followed by an automatically controlled sequence. In this, samples were first transferred in a specific order into an increasing content of ethanol (60 %, 70 %, 96 %, 100 % EtOH). This process served the purpose of dehydrating the tissue. The last steps required transferring the tissues, first in Xylol, and last in paraffin. The whole process took approximately 15 hours. Afterwards, tissue samples were prepared for final embedding in paraffin.

Fabrication and treatment of samples slices

To facilitate the fabrication of tissue intersections, paraffin blocks were kept at -20°C until being processed. Using a microtome, thin tissue sections of 1 µm were cut. Before tissues were stained with PAS reagents, first paraffin was first removed followed by hydration of the tissues and washing of the glass slides with distilled water.

Periodic acid-Schiff reaction (PAS)

The whole staining-procedure was performed at room temperature. Before use, all solutions were brought to their corresponding appropriate temperatures. Deparaffinized and hydrated tissues were transferred into 0,5 % periodic acid solution and incubated for 15 minutes. The solution was washed off first under running tap water for three minutes and then with distilled water. Next, glass slides were moved into a cuvette, covered with Schiff's reagent, and stained for 40 minutes. Schiff's reagent was removed under warm running tap water followed by a wash step with distilled water. The incubation step in Hematoxylin solution for one-minute, colored nuclei, mitochondria and the cell membrane. Staining was checked microscopically and if needed, glass slides were again stained for another minute in Hematoxylin solution. After efficient staining, glass slides were washed for three minutes under warm running tap water and dipped for a few times in hydrochloric acid (HCl/EtOH) solution. Glass slides were washed with cold running tap water for three minutes

followed by one washing step with distilled water. Glass slides were dried under the hood until no remaining water was left. Carefully, tissues were coated with mounting medium and sealed with a cover glass. The staining procedure was performed with the help of Alina Borchers the technical assistant of AG Krebs.

2.2.5. Cell isolation

Immune cell isolation from spleen

Mice were first anesthetized with a mixture of 80% CO_2 and 20% O_2 and subsequently sacrificed by inhaling 100% CO_2. Spleens were harvested with sterile instruments and collected in complete medium on ice. Spleens were homogenized using 40 µm cell strainers and single cell suspensions were pelleted by centrifugation (350 x g, 5 min, 4°C). Erythrocytes were lysed by treating the cells with ACK buffer for 5 min at 4°C. The cell lysis was stopped by adding PBS in excess and cells were pelleted by centrifugation (350 x g, 5 min, 4°C). Then cells were re-suspended in MACS buffer or medium depending on the following step.

Immune cell isolation from small intestine

Mice were sacrificed as described above and the small intestine was harvested with sterile instruments. The Peyer's patches were removed, and the small intestine opened longitudinally and washed in 1% PBS/FCS. Then the small intestine was cut into small pieces of approximately 0.5 cm and incubated in the presence of 5 mM EDTA in complete medium at 37°C for 30 min while shaking. Intraepithelial lymphocytes (IEL) could be collected in the media after this step using centrifugation (350 x g, 5 min, 4°C). The tissue was collected to further isolate lamina propria lymphocytes (LPL). To this end, the small intestine was digested by collagenase IV (100 U/ml) and DNase I (10 U/ml) in complete medium at 37°C for 45 min while shaking. The digested intestinal tissue was further homogenized through a metal strainer and lymphocytes (IEL fraction and LPL fraction were pooled prior to this step) were further separated with a Percoll gradient.

Percoll gradient: The osmolality of Percoll was first adjusted by adding 1 part (v/v) 10x PBS to 9 parts (v/v) of Percoll (90% Percoll). This isotonic Percoll was further diluted with 1% PBS/FBS buffer to create 40% and 67% Percoll solutions. In a 15 ml tube 4 ml of isotonic 67% Percoll was added. Cells were re-suspended in 40% Percoll (4 ml) and gently overlaid over 67% Percoll. The gradient was centrifuged to separate the cells (400 g, 20 min, break = 1, acceleration = 1, RT). Lymphocytes were collected from the interphase.

Immune cell isolation from kidney

Mice were sacrificed as described above and the kidneys were harvested with sterile instruments. The kidney capsule was gently removed and then the kidney was cut into small pieces. The tissue was then transferred into a "C tube" (for GentleMACS dissociator), prefilled with 5 ml of complete medium supplemented with collagenase IV (100 U/ml) and DNase I (10 U/ml) and incubated for 40 minutes at 37°C while shaking. After digestion, the cell suspension was treated twice in a GentleMACS (standard programs: "mSpleen 1.01" and then "mLung 2.01"). Then, the suspension was centrifuged at 300 g for 8 min and 4°C and the supernatant was removed. For leukocyte enrichment, the cell suspension was further centrifuged for 15 min at 500 g and RT in density gradient with 37% Percoll.

2.2.6. Magnetic cell separation

Two isolation protocols were used depending on an *in vitro* assay performed. All reagents used for magnetic cell separation are listed in Table 1.

For ratiometric Ca^{2+} signaling imaging cells were enriched by negative selection with the column free magnetic system and EasySep™ Mouse Naïve CD4+ T Cell Isolation Kit according to the manufacturer's protocol (Stemcells technologies).

For other *in vitro* assays, naïve $CD4^+$ T cells were enriched with a combination of positive and negative selection using Magnetic-activated cell sorting™ and LS columns (Miltenyi).

Single cell suspension of spleens and lymph nodes were re-suspended in MACS buffer containing biotinylated antibodies against CD25 (1:200) and CD44 (1:200) and incubated for 15 minutes at 4ºC. Cells were washed by adding MACS buffer, pelleted by centrifugation (350 x g, 5 minutes, 4°C) and then re-suspended in MACS buffer with Streptavidin beads (1:40) and incubated for 30 minutes at 4°C. The cell suspension was run through a MACS LS column and washed 3 times with 3 ml of MACS buffer. The flowthrough containing $CD25^-$ and $CD44^-$ cells was collected, pelleted (350 x g, 5 minutes, 4°C) and further incubated with MACS buffer containing CD4 microbeads (1:100) for 15 minutes at 4°C. The cell suspension was run through a MACS LS column and washed 3 times with 3 ml of MACS buffer. Labeled $CD4^+$ cells, considered as naïve T cells were collected after flushing the column with MACS buffer. To collect antigen presenting cells (APCs) the flow through was further incubated with biotinylated anti-CD3 antibody for 15 minutes at 4ºC. Cells were washed by adding MACS buffer, pelleted by centrifugation (350 x g, 5 minutes, 4°C)

and then re-suspended in MACS buffer containing Streptavidin beads (1:40). After 30 minutes of incubation at 4ºC, the cell suspension was run through another MACS LS column and washed 3 times with 3 ml of MACS buffer. The resulting flowthrough containing the APCs that was pelleted and then incubated with 1ml of 1X ACK buffer to lyse the erythrocytes. After 3 minutes of incubation at room temperature, the reaction was stopped by adding MACS buffer or 1X PBS in excess. APCs were irradiated with 30 Gy to avoid proliferation during in vitro culture.

2.2.7. Flow cytometry and Flow activated cell sorting

Identification of dead cells

To identify dead cells a zombie UV staining (BioLegend) was performed. To this end cells were incubated in 0.2 ml PBS buffer including 1 µl of zombie UV staining reagent for 15 min in the dark at RT before the surface staining.

Surface staining

Lymphocytes (1 x 10^6 cells) were transferred to a 5 ml tube, centrifuged and resuspended in 100 µl MACS buffer containing Fc-block (1:100) antibody and directly fluorochrome labelled antibodies against surface markers. Cells were stained in the dark for 20 min at 4°C, washed with MACS buffer and pelleted. Then cells were either resuspended in 300 µl MACS buffer for direct acquisition or further proceeded to intracellular staining.

Intracellular staining

For intracellular cytokine staining (ICS), cells were re-stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL), ionomycin (1 µM) in the presence of Monensin A (2 µM) for 3 hours at 37°C prior to the staining. Cells were washed and pelleted and surface markers were stained as described above. Cells were fixed in 100 µl 4 % Formaldehyde (Fix buffer) for 20 min at RT, washed, pelleted and re-suspended in 100 µl 0.1 % NP40 (Perm buffer) for 4 min at RT in the dark. After the permeabilization, cells were washed with MACS buffer and stained for intracellular antigens with antibody dilution prepared in MACS buffer.

2.2.8. *In vitro* $CD4^+$ T cell differentiation

Naïve $CD4^+$ T cells and APCs were enriched from spleen and lymph nodes from OT-II or reporter mice using MACS as described above. The cells were counted in a 1 to 3 dilution of the suspension containing the cells with Trypan blue solution under the microscope using a Neubauer chamber.

Naïve cells were cultured in a concentration of 10^6 cells/ml in the presence of 96 well plates with flat bottom were used to culture the cells for 5 days in complete medium under Th17 polarization conditions: mAb IL- 4 (10 µg/ml), mAb INF-γ (10 µg/ml), mAb CD3 (3 µg/ml), mAb CD28 (0.5 µg/ml), IL- 6 (10 ng/ml), TGF-β1 (1 ng/ml), IL-23 (20 ng/ml), IL-1β (10 ng/ml)), FICZ (100mM).

After culture, cells were analyzed using flow cytometry and/or further separated using fluorescence-activated cell sorting (FACS). The preparation of differentiated cells prior sorting includes the collection of the cells from the plate, washing with FACS buffer and depletion of APCs from the cell suspension using Lympholite gradient according to manufacturer instructions. Table

2.2.9. *In vitro* $CD4^+$ T cell proliferation assay

T cells were loaded with violet dye and activated by coated CD3 specific antibodies in presence of soluble CD28 specific antibodies. Proliferation was measured at day 3 of in vitro culture by assessing the dilution of the violet dye.

2.2.10.Bone marrow dendritic cell *in vitro* culture

Femurs and tibias from four to eight-week-old C57BL/6J and B10.Br mice were manually flushed to harvest bone marrow cells, and RBCs were lysed in ACK lysis buffer. Cells were cultured in complete DMEM containing 20% of L929 cell-conditioned medium (containing M-CSF) for 8 d to obtain bone marrow–derived macrophages (BMDMs). Alternatively, to generate bone marrow–derived DCs (BMDCs), we cultured bone marrow cells in medium containing murine GM-CSF (1000 U/ml) for 8 d. DC and macro- phage yield was determined by flow cytometry.

2.2.11.Single-cell Ca^{2+} imaging

2.2.11.1. Fura2-AM loading

10 mln primary murine naïve $CD4^+$ T cells were pelleted at 350g for 5 minutes at RT and resuspended in 1 ml of fresh full Click's medium and incubated for 5 min at 37°C. Then, 4 µl (4 µM) of Fura2-AM was added and cells were incubated at 37 °C for 15 min in dark. After 15 minutes, cell suspension was diluted with 4 ml of pre-warmed medium and incubated for additional 15 minutes. After Fura2-AM incubation the cells were pelleted and resuspended with fresh medium and rested for 20 minute at 37°C for de-extrification. Following, the cells were washed twice with 5 ml of Ca^{2+} measuring buffer and resuspended in 100 µl of Ca^{2+} measuring buffer.

2.2.11.2. Incubation with NAADP inhibitors

In experiments with NAADP antagonists, after loading with Fura2-AM the cells were incubated with trans-Ned 19 (25 µM, 50µM, 100 µM) or DMSO (0.1%) for 1 hour or with BZ194 (250 µM, 500 µM, 1000 µM) or DMSO (0.25%) for 5 hours at 37°C, 5% CO_2.

2.2.11.3. Anti-CD3 $CD4^+$ T cell stimulation

In experiments with anti-CD3 antibody cell stimulation, the cells were placed within a rubber O-ring fixed on slides via Korasilon silicon paste of medium viscosity (Kurt Obermeier GmbH & Co. KG, Bad Berleburg, D). Slides were coated before cell addition with 5 µl of 5 mg/ml bovine serum albumin solution and 5 µl of 0.1 mg/ml poly-L-lysine solution (70 000 – 150 000 g/mol). Cells were allowed to settle down for 5 min on one slide. Then, 80 µl of Ca^{2+} measurement buffer was added, and the slide was placed of an inverted Leica microscope (type DM IRBE, Leica microsystems, Wetzlar, D). After 1 minute of acquisition, 10 µl of 50 µg/ml anti-CD3 was added. Images were acquired every 2 seconds for 10 minutes.

2.2.11.4. Antigen specific $CD4^+$ T cell stimulation

In experiments with antigen stimulation of OT-II cells BMDCs were cultured in microscopy Glass bottom dishes (MatTek), Fura-2 loaded OT-II cells were added to antigen pulsed BMDCs. Images were acquired every 2 seconds for 30 minutes.

2.2.11.5. Image acquisition

Images were acquired with a Leica IRBE2 microscope (40-fold magnification). A Sutter DG-4was used as a light source, and frames were acquired with an electron-multiplying charge-coupled device camera (C9100, Hamamatsu). Images (512 × 512 pixels) were acquired in 14-bit mode with twofold binning. Exposure time was 25 ms. The following filter set was used for the measurement of the Fura2 fluorescence: excitation at 340/10 nm and 380/10 nm every 2 s and emission 495/10 nm.

2.2.11.6. Determination of the Kd for Fura 2

Fura2 fluorescence was calibrated using 0.1 % (w/v) Triton X-100 for cell lysis and obtaining R_{max} as cytosolic Fura2 binds to extracellular Ca^{2+} which is efficient for saturating Fura2. After 140 s, 3 mM EGTA and 40 mM Tris were added to chelate extracellular Ca^{2+} and to obtain Rmin as EGTA binds Ca^{2+} with higher affinity than Fura2. Tris is added for pH buffering. Rmin and Rmax and the corresponding highest and lowest light intensities were calculated and determined using the actual ratio values and light intensities in Excel. For calculation of the $[Ca^{2+}]_i$, the following equation described by (Grynkiewicz et al., 1985) was used:

$$[Ca^{2+}]_i = K_d \cdot \frac{R - R_{min}}{R_{max} - R} \cdot \frac{F_{380_{max}}}{F_{380_{min}}}$$

$[Ca^{2+}]_i$ is the free cytosolic Ca^{2+} concentration. Kd refers to the dissociation constant which is 224 nM. R is the ratio of fluorescence at an excitation of 340 nm (F340) divided by fluorescence at an excitation of 380 nm (F380). Rmin and Rmax are the minimal and maximal ratio, respectively, which were obtained during calibration. F (380)max and F(380)min are maximal and minimal intensities measured at an excitation of 380 nm, respectively, which were measured during calibration. Measurements using Fluo4 were normalized to fluorescence intensity measured at the first time point (F/F0).

2.2.11.7. Image processing

Volocity software (version 6.6.2; PerkinElmer Inc.) was used for the acquisition and analysis of mean global Ca^{2+} signals. Frames taken with a Dual-View module were split into the two wavelength emission channels with Fiji 1.53c. Furthermore, Fiji was used for background correction of Fura-2. Cells were then detected by applying a threshold segmentation and their trajectories were recorded using the Trackmate Fiji plugin (version 4.0.0).

Raw data of identified cells was then exported and analyzed with R (version 4.0.1). Tracks of length shorter than 400 frames and cells in which 340 to 380 mean ratio in initial 20 frames exceeded 0.45 were excluded. Activated cells were identified as the ones with the 340 to 380 ratio over frame differential greater than 0.01 for 5 consecutive frames. The tracks were then aligned to overlay the initial slope of 340 to 380 ratio. The area under the curve was computed with the function from the Desctool package.

2.2.12.Statistical analysis

Data are single-cell measurements from three separate sets of experiments and are shown as means ± SEM. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software Inc.) by applying Mann-Whitney U tests and Kruskal-Wallis tests with Dunn post hoc tests, respectively, to the non-normally distributed data. The normality of distribution was tested by Shapiro-Wilk test.

3. Results

3.1. Antigen affinity to TCR determines calcium signaling in $CD4^+$ T cells upon antigen presentation quantitatively and qualitatively

It has been previously shown that TCR signal strength can determine the differentiation of $CD4^+$ T cells [19], [211], [212]. Calcium signaling was proven to be essential for synthesis of cytokines by $CD4^+$ T conventional cells as well as for expression of Foxp3, a signature transcription factor of T regulatory cells [213], [214]. Thus, it could be hypothesized that the control of $CD4^+$ T-cell differentiation by TCR signal strength occurs, at least partially, by evoking different spatiotemporal patterns of $[Ca^{2+}]_i$ upon TCR binding by varying doses of antigens or by binding antigens with different TCR affinity.

In order to test this hypothesis, OT-II transgenic mice, which overexpress a single TCR, and altered peptide ligands were used. OT-II cells recognize a C-Terminal ovalbumin epitope 323-339 and amino acid 333 was recognized as a primary TCR contact residue [215]. Native ova peptide, which acts as a full agonist of the OT-II TCR, has glutamate (E) at this position. Altered ova peptides with amino acids methionine (M), tryptophan (W) or aspartic acid (D) at position 333 of the ova peptide were used [215]. Naïve $CD4^+$ OT-II T cells were then co-cultured with bone marrow dendritic cells loaded with the described peptides. Single cell confocal ratiometric microscopy was then performed to unravel the temporal patterns of free cytosolic calcium concentration upon binding of antigens with differing TCR affinity. Measures chosen to describe the quantity of $[Ca^{2+}]_i$ upon TCR activation include percentage of the cells with a discernable $[Ca^{2+}]_i$, which are referred to as activated cells, maximal relative increase in $[Ca^{2+}]_i$ upon activation, and area under the curve of the time course of $[Ca^{2+}]_i$.

As expected, native Ova 323-339 peptide (peptide E) proved to be the most efficient in inducing $[Ca^{2+}]_i$ increases in naïve OT-II $CD4^+$ T-cells upon TCR stimulation considering all three named parameters. Interestingly, the plateau phase of $[Ca^{2+}]_i$ was higher after presentation of peptide E to the cells, compared to stimulating the cells with CD3 specific antibody. However, this observation did not reach statistical significance. Ova peptides with an altered amino acid sequence were significantly less potent in inducing $[Ca^{2+}]_i$ upon TCR stimulation. The Ova peptide with a Tryptophan substitution was more potent in eliciting $[Ca^{2+}]_i$ increase upon TCR stimulation than other altered peptide ligands, however, this relation did not reach statistical significance (Fig. 1).

Interestingly, the $[Ca^{2+}]_i$ temporal patterns upon TCR stimulation exhibited qualitative differences between the altered peptide ligands and the full agonist antigen. Analysis of the curves of $[Ca^{2+}]_i$ upon TCR stimulation allowed allocation of the patterns into four categories: peak followed by an oscillation in $[Ca^{2+}]_i$, peak followed by stable plateau of $[Ca^{2+}]_i$, oscillations of $[Ca^{2+}]_i$ without a discernable peak in $[Ca^{2+}]_i$ and repeated short lived transient increases in $[Ca^{2+}]_i$ (Fig. 2 A). Most of the cells activated by presentation of the native ova peptide (peptide E) and by anti-CD3 antibody exhibited either a peak and oscillation, or a peak followed by a plateau. Interestingly, cells activated by the partial agonists of TCR were much more likely to have less profound increases in $[Ca^{2+}]_i$, which were rather in form of oscillations or transient $[Ca^{2+}]_i$ increases (Fig, 2 B).

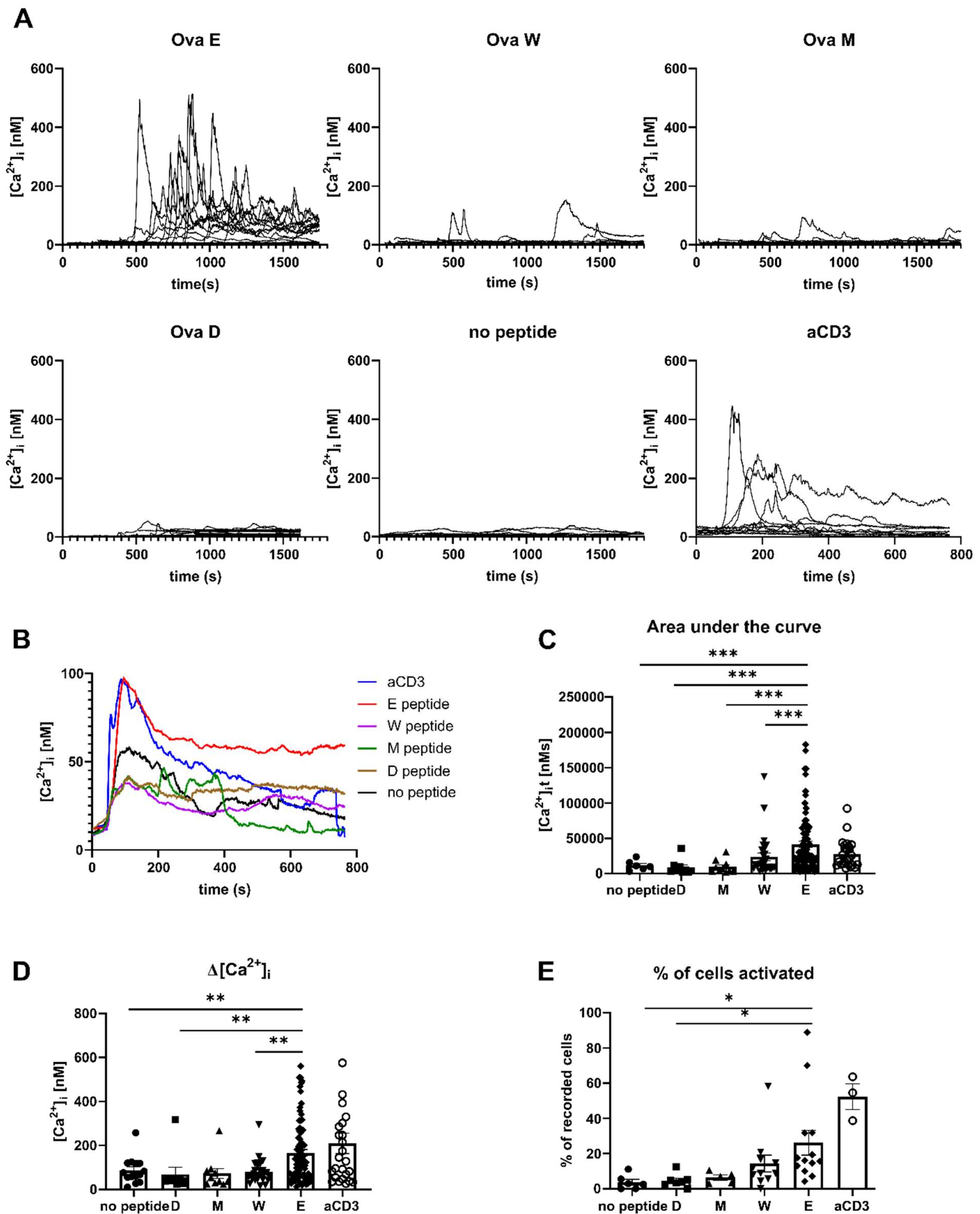


Figure 1: Affinity of the antigen to the TCR determines calcium signaling in CD4^{+} naïve T-cells quantitatively.

CD4^{+} naïve T cells were isolated from OT-II mice and loaded with Fura2. Bone marrow dendritic cells were cultured for 7 days from bone marrow of a wild type mice with GM-CSF and pulsed with peptides in presence of IFNγ overnight. The data in summary graphs comes cells with a clear global $[Ca^{2+}]_i$ increase. **(A)** Representative time course plots of

340/380 fluorescence ratio in CD4$^+$ naïve OT-II cells. **(B)** Mean time course 340/380 fluorescence ratio for the ova peptides and the cells stimulated with CD3-specific antibody **(C)** Area under the time course curves of 340/380 ratio of the activated cells **(D)** Maximal relative increase in 340/380 fluorescence in activated cells **(E)** Percentage of the observed cells in which discernable increase in $[Ca^{2+}]_I$ was observed. *$p < 0.05$; **$p < 0.01$***$p < 0.001$. Kruskal-Wallis test with Dunn's multiple comparison test was used to calculate significance.

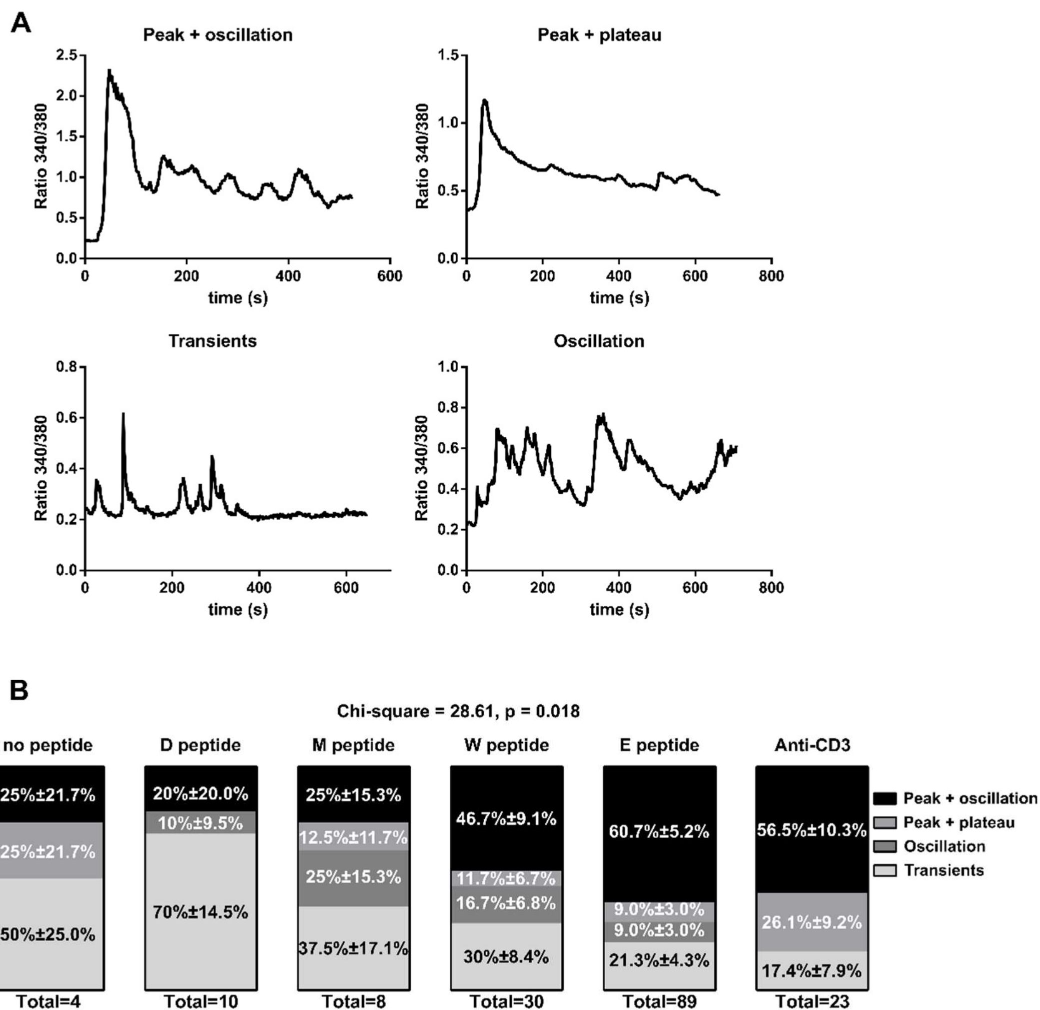


Figure 2: Affinity of the antigen to the TCR determines calcium signaling in CD4$^+$ naïve T cells qualitatively.

(**A**) Representative time course curves of the four recognized temporal patterns of $[Ca^{2+}]_i$ upon TCR activation. **(B)** Summary percentages of the patterns among activated cells. *$p < 0.05$; **$p < 0.01$***$p < 0.001$. Chi-square test was used to calculate the significance.

In conclusion, presentation of the Ova 323-339 peptide with a single amino acid substitution to a naïve CD4$^+$ OT-II cells induced calcium signals that were both quantitatively and qualitatively different from the signals when presented to a native ova peptide.

3.2. The affinity of an antigen to TCR impacts the differentiation of $CD4^+$ T cells

As single cell ratiometric microscopy revealed that altered ovalbumin peptide ligands induced different temporal patterns of $[Ca^{2+}]_i$ upon TCR stimulation, the next step was to investigate whether the differences in the Ca^{2+} signaling upon $CD4^+$ T cell activation have an impact on the differentiation of the cells at later time points. In order to answer this question, $CD4^+$ OT-II cells were stimulated *in vitro* with ova peptides in the presence of cytokine cocktails driving differentiation of Th1, Th2, Th17 and iTregs cells. Interestingly, in this setting the affinity of antigen, and consequently the strength of TCR signaling did not play a role in the differentiation of Th1 and Th2 cells (Fig. 3 A-B). In contrast, the TCR signal strength had a significant impact on the differentiation of Th17 and induced $Foxp3^+$ Tregs. Presentation of native ova peptide to the naïve $CD4^+$ OT-II cells resulted in the most effective Th17 cell differentiation compared with the presentation of altered ovalbumin peptides. The impact on the TCR signal strength was the opposite in the differentiation of $Foxp3^+$ Tregs. The original variant of the Ova peptide inhibited the differentiation of Tregs cells in Treg polarizing cytokine cocktail while a weak TCR stimulation by the altered peptide ligands resulted in more effective Treg differentiation.

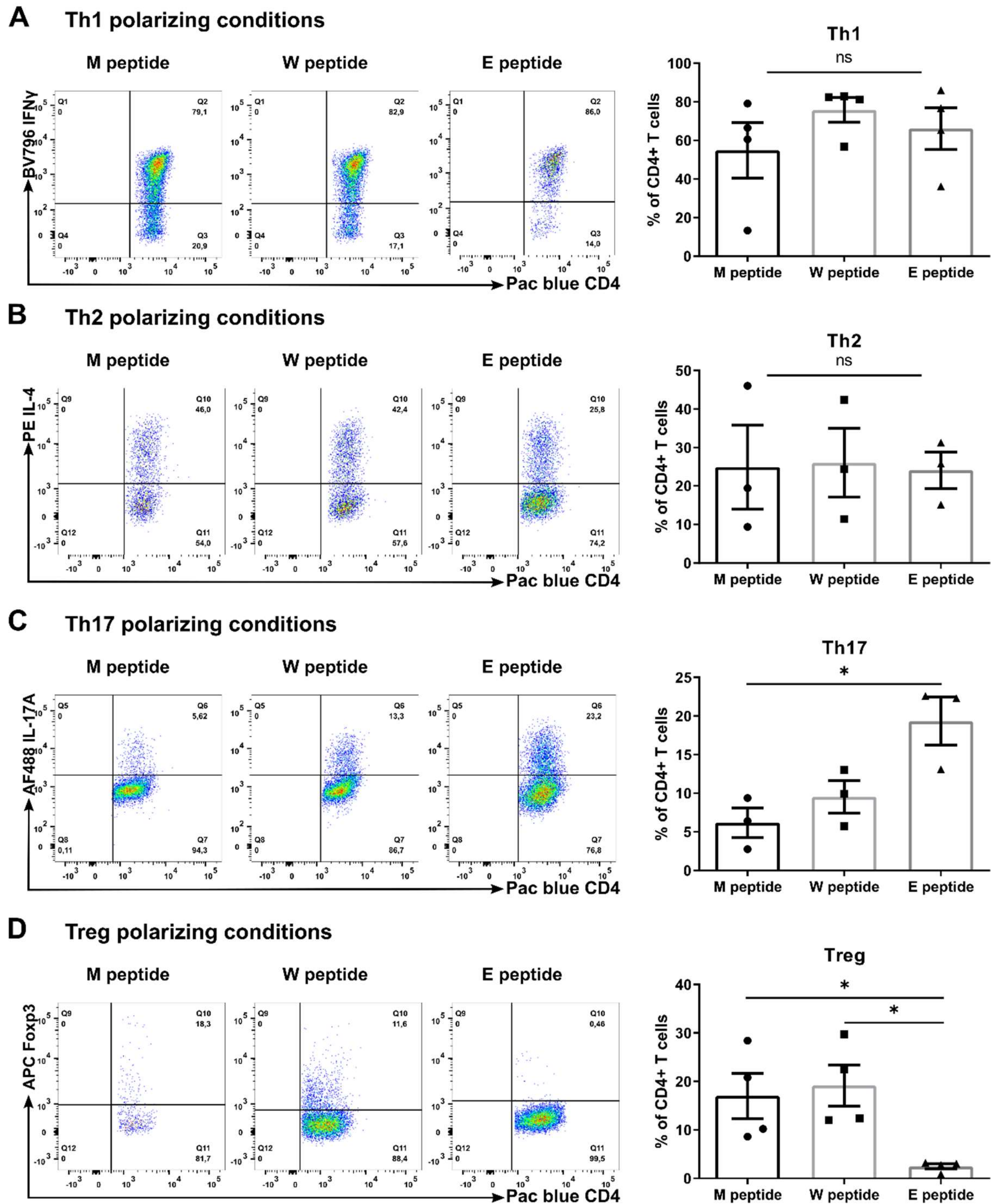


Figure 3: Affinity of the antigen to the TCR determines differentiation of CD4⁺ T cells *in vitro*

(A) *In vitro* differentiation of naïve $CD4^+$ OT-II cells stimulated by different ova peptides in presence of Th1 polarizing cytokines (IL-2, IL-12). **(B)** *In vitro* differentiation of naïve $CD4^+$ OT-II cells stimulated by different ova peptides in presence of Th2 polarizing cytokines (IL-2, IL-4). **(C)** *In vitro* differentiation of naïve $CD4^+$ OT-II cells stimulated by different ova peptides in presence of Th17 polarizing cytokines (IL-6, IL-23, TGF-β1) **(D)** *In vitro* differentiation of naïve $CD4^+$ OT-II cells stimulated by different ova peptides in presence of iTreg polarizing cytokines (IL-2, TGF-β1). *$p < 0.05$; **$p < 0.01$***$p < 0.001$. Ordinary one-way ANOVA with Holm-Sidak's multiple comparison test was used

to calculate the significance. For each point the representative dot plots are presented on the left side and pooled biological replicated on the right side.

In conclusion, the affinity of antigen binding to the TCR did not have a significant impact on Th1 and Th2 differentiation, highly affine antigens promoted Th17 fate while antigens with lower affinity to the TCR supported Treg differentiation.

3.3. Antagonism of NAADP signaling with BZ194.

3.3.1. BZ194 inhibits calcium signaling after TCR stimulation in naïve $CD4^+$ T cells

The strength of TCR activation affected both Ca^{2+} signaling events and differentiation of the $CD4^+$ T cells. Adenine derived Ca^{2+} mobilizing second messengers NAADP and cADPR are synthesized early after TCR stimulation and are critical for the activation of T cells [107], [108]. Thus, antagonism of the NAADP or cADPR signaling upon TCR stimulation could impact the differentiation of $CD4^+$ T cells. BZ194 is a synthetic antagonist of NAADP signaling. It was previously published that it interferes with the interaction of NAADP with RYR1 Ca^{2+} channels, and thus, inhibits calcium signals in T cells upon TCR/CD3 stimulation, blocks proliferation, cytokine synthesis and ameliorates the clinical course of EAE [157]–[159]. However, its impact on the differentiation of $CD4^+$ T cells has not been addressed. First, the results on the influence of BZ194 on TCR induced $[Ca^{2+}]_i$ and proliferation were reproduced in primary murine $CD4^+$ naïve T cells. Next, in order to investigate the impact of NAADP antagonism on $CD4^+$ T cells differentiation $CD4^+$ naïve T cells from a $Foxp3^{RFP}$, IL-$17A^{eGFP}$, $IFN\gamma^{Katushka}$ triple reporter mouse were cultured *in vitro* under Th1, Th17 and Treg polarizing condition in the presence of BZ194. Interestingly, BZ194 had a significant impact on Th17 and Treg differentiation *in vitro*.

To analyze the effect of a NAADP antagonist BZ194 on calcium signaling upon TCR/CD3 stimulation in primary murine naïve $CD4^+$ T cells, single cell ratiometric calcium imaging was performed. Incubation of cells with increasing concentrations BZ194 decreased both the initial spike in $[Ca^{2+}]_i$ as well as the $[Ca^{2+}]_i$ during the plateau phase (Fig. 4 A). In the next step the impact of NAADP antagonism on the proliferation of naïve $CD4^+$ T cells in response to CD3 stimulation was investigated *in vitro*. Interfering with the NAADP signaling by means of BZ194 inhibited the proliferation of naïve $CD4^+$ T cells in a concentration dependent manner (Fig. 4B).

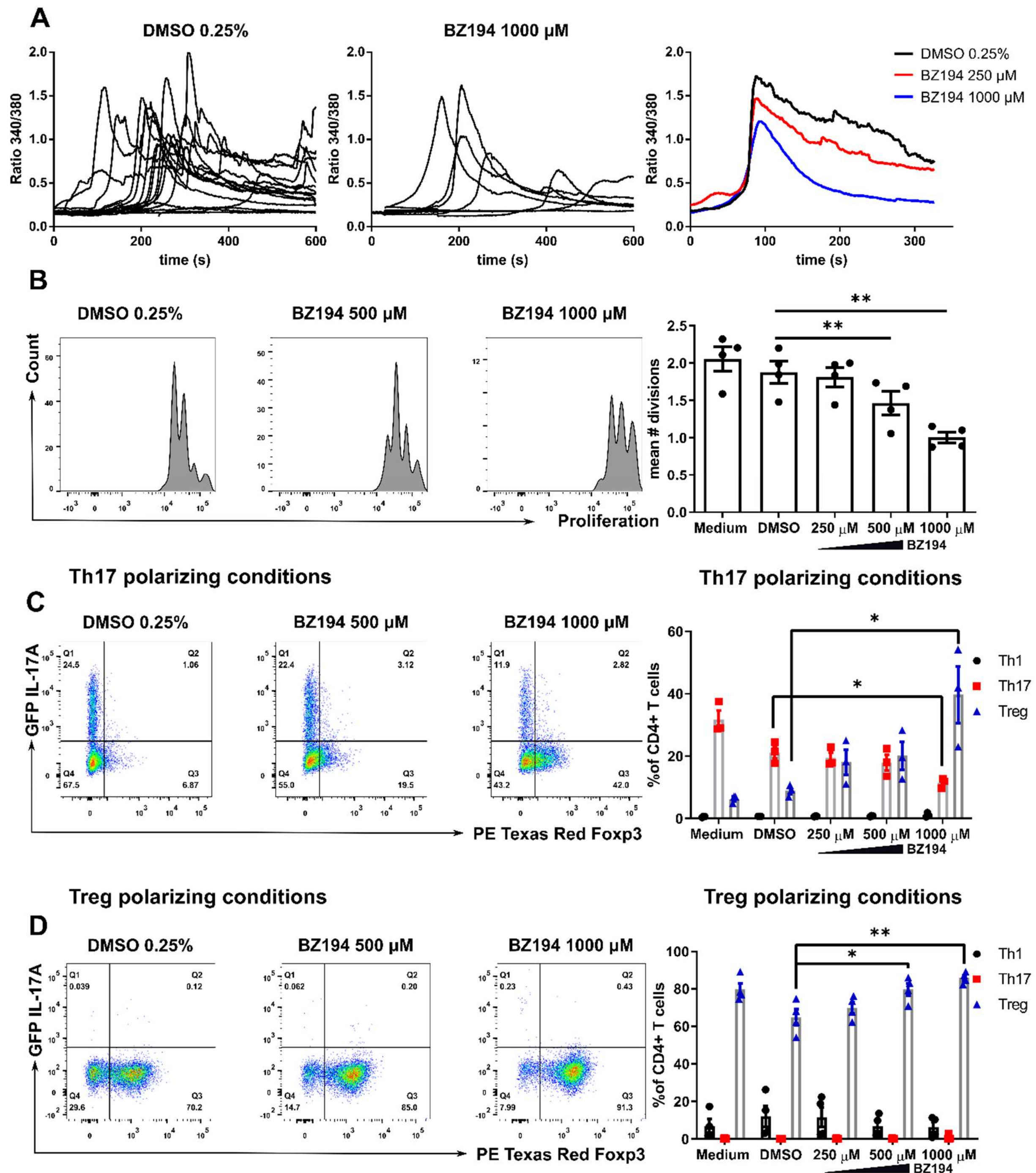


Figure 4: BZ194 influences calcium signaling, proliferation and differentiation of CD4⁺ T cells

Naïve CD4⁺ T cells were isolated from spleen and lymph nodes of a Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mouse **(A)** Cells were incubated at indicated concentrations of DMSO or BZ194 for 5 h and subsequently loaded with Fura2 and activated by soluble anti-CD3 antibody. Depicted are representative plates on the left and mean 340/380 ratio on the right. Data are cumulative of two experiments **(B)** Cells were loaded with 5 µM violet dye, incubated with the indicated concentrations of B194 or DMSO and activated with plate bound anti-CD3 antibody and soluble anti-CD28 and cultured for 48h. **(C)** Naïve CD4⁺ T cells were incubated in indicated concentrations of BZ194 or DMSO

and cultured under Th17 polarizing (IL-6, TGF-β1 0.5 ng/ml, IL-23) conditions for 96 hours. **(D)** Naïve CD4+ T cells were incubated in indicated concentrations of BZ194 or DMSO and cultured under Treg (IL-2, TGF-β1 2 ng/ml) polarizing conditions for 96 hours. *$p < 0.05$; **$p < 0.01$***$p < 0.001$. Ordinary one-way ANOVA with Holm-Sidak's multiple comparison test was used to calculate the significance. For each point the representative dot plots are presented on the left side and pooled biological replicated on the right side.

To investigate the role of NAADP signaling in the differentiation of CD4+ T cells, naïve CD4+ T cells were isolated from Foxp3^{RFP}, IL-17A^{eGFP}, IFN-γ^{Katushka} triple reporter mice and cultured *in vitro* under polarizing conditions in presence of BZ194. Interestingly, antagonizing NAADP by BZ194 promoted development of induced Tregs under iTreg polarizing conditions (IL-2, TGF-β1), under Th17 polarizing conditions (IL-6, IL-23, TGF-β1). Furthermore, BZ194 inhibited the differentiation of Th17 cells in Th17 polarizing conditions.

To sum up, BZ194 inhibited calcium signaling in CD4+ T cells and TCR stimulation. It decreased the proliferation of CD4+ T cells upon TCR stimulation and skewed the differentiation of CD4+ T cells towards regulatory fate.

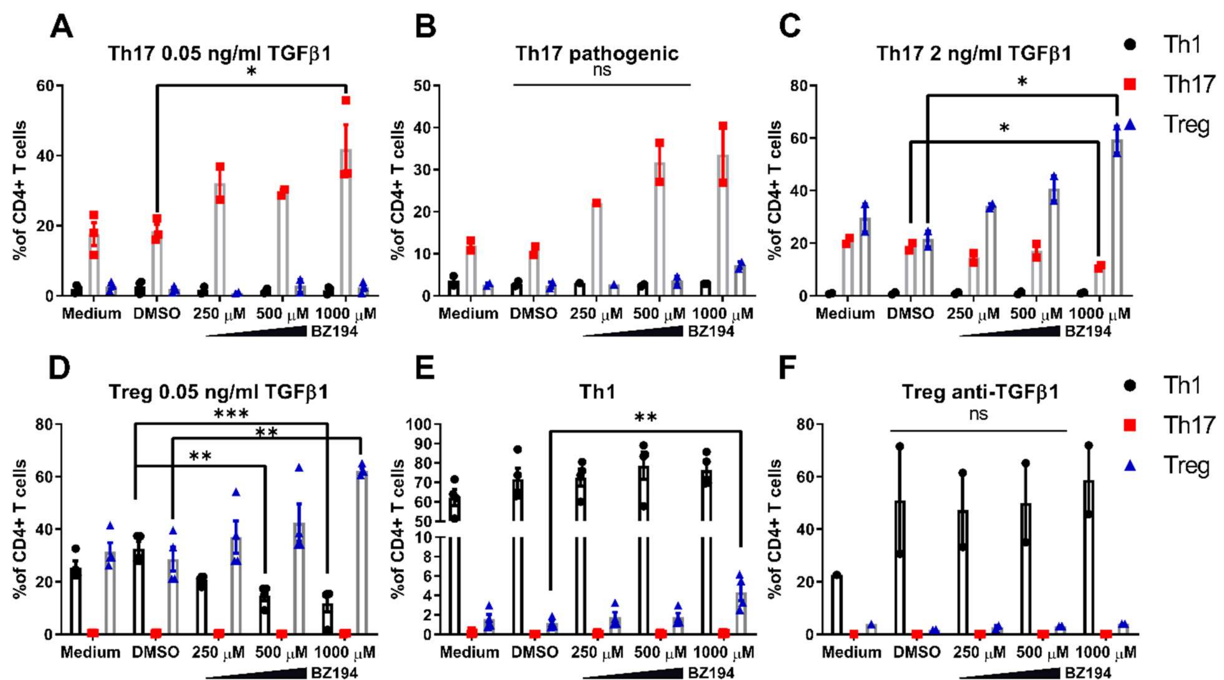


Figure 5: Effect of BZ194 on CD4+ T cells is TGF-β1 dependent

Naïve CD4+ T cells were isolated from spleen and lymph nodes of a Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mouse. Cells were incubated at given concentrations of DMSO or BZ194 for 5 h. The cells were cultured for 96 hours under in the following cytokine cocktails. **(A)** IL-6, IL-23, TGF-β1 0.05ng/ml, **(B)** IL-6, IL-1β, IL-23, **(C)** IL-6, IL-23, TGF-β1 2ng/ml, **(D)** IL-2, TGF-β1 0.5 ng/ml **(E)** IL-2, IL-12 **(F)** IL-2, anti-TGF-β1. *$p < 0.05$; **$p < 0.01$***$p < 0.001$. Ordinary one-way ANOVA with Holm-Sidak's multiple comparison test was used to calculate the significance.

3.3.2. BZ194 has different effects on $CD4^+$ T cells differentiation dependent on TGF-β1 concentration,

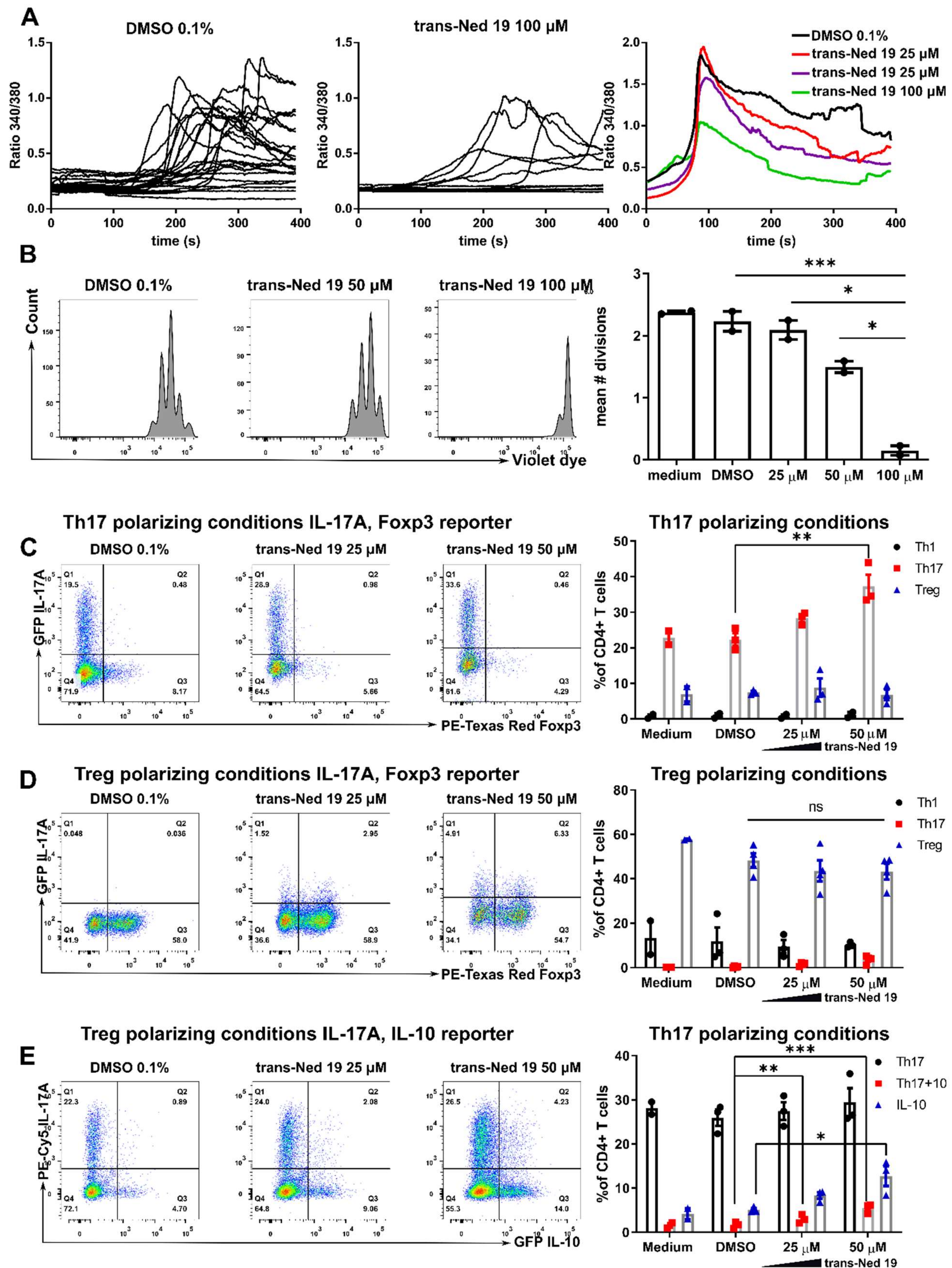
The Th17 and iTreg cell both require TGF-β1 signaling for initial differentiation, however the strength of required signaling differ: iTreg require strong TGF-β1 signals, while Th17 require moderate ones [15], [69]. Moreover, strong TGF-β1 signaling induces Foxp3 expression, which antagonizes the development of Th17 cells [49], [216]. As BZ194 promoted the differentiation of iTregs and skewed the Th17 differentiation into Treg fate, it was hypothesized that this effect is mediated by sensitization of naïve $CD4^+$ T cells to TGF-β1 signaling. In order to test this hypothesis, *in vitro* differentiation experiments were performed in which the concentration of TGF-β1 was titrated. Firstly, $CD4^+$ T cells were cultured under Th17 polarizing conditions with suboptimal concentration of TGF-β1. It resulted in a low percentage of IL-17A producing cells, which was increased by BZ194 (Fig. 5 A). Second, the cells were cultured under a pathogenic Th17 polarizing condition, which lacks added TGF- β1. As previously reported, BZ194 increased the percentage of IL-17A producing cells (Fig. 5 B). Next, the cells were cultured under a Th17 condition with an increased concentration of TGF-β1, resulting in decreased Th17 to Treg ratio (Fig. 5 C – medium). BZ194 further increased the percentage of developing Tregs and inhibited Th17 differentiation (Fig. 5 C). Next, Treg differentiation with a cytokine cocktail with a decreased TGF-β1 concentration was performed. This resulted with increased percentage of Th1 cells and a lower percentage of Treg cells compared to typical Treg polarizing conditions (Fig. 4 D). In this condition, BZ194 inhibited differentiation of Th1 cells and promoted the development of iTregs. Interestingly, under Th1 polarizing condition BZ194 did not affect Th1 differentiation of naïve $CD4^+$ T cells, however, it caused small but significant increases in iTreg differentiation (Fig. 5 E). Finally, to investigate whether BZ194 can bypass TGF-β1 in inducing Tregs, naïve $CD4^+$ were cultured with IL-2 and TGF-β1 receptor blocking antibody. In this condition, BZ194 did not induce differentiation of $Foxp3^+$ cells (Fig 5. F).

To summarize, antagonizing NAADP by means of BZ194 promoted the synthesis of Foxp3 in $CD4^+$ T cells, likely by sensitizing the cells to TGF-β1 signaling.

3.3.3. Impact of trans-Ned 19 on calcium signaling and proliferation of naïve $CD4^+$ T cells

Apart from BZ194, another antagonist of NAADP-induced calcium release was developed by the way of virtual screening, named trans-Ned 19 [217]. This antagonist was validated in cell lines and primary murine cells, where it was able to block NAADP-induced calcium release from intracellular stores [160], [175], [218]. Moreover it was shown to interfere with calcium signaling and proliferation of murine $CD4^+$ T cells upon TCR ligation [160]. However, the impact of trans-Ned 19 on the differentiation and effector function of $CD4^+$ T cells was not investigated.

At first the ability of trans-Ned 19 to block the free cytosolic calcium increase upon TCR/CD3 stimulation was reproduced. To assess the impact of trans-Ned 19 on the temporal patterns of calcium signaling in $CD4^+$ naïve T cells, single cell ratiometric microscopy was performed. This showed that, indeed, trans-Ned 19 is capable of inhibiting the calcium signal in $CD4^+$ naïve T cells upon TCR stimulation in a dose dependent manner (Fig. 6 A). Moreover, proliferation of naïve $CD4^+$ T cells upon TCR/CD3 stimulation was investigated. Pretreatment of cells with trans-Ned 19 lead to significantly impaired proliferation of $CD4^+$ naïve T cells upon TCR/CD3 stimulation (Fig. 6 B)


A
DMSO 0.1%
trans-Ned 19 100 µM
Ratio 340/380
time (s)
DMSO 0.1%
trans-Ned 19 25 µM
trans-Ned 19 25 µM
trans-Ned 19 100 µM
B
DMSO 0.1%
trans-Ned 19 50 µM
trans-Ned 19 100 µM
Count
Violet dye
mean # divisions
medium
DMSO
25 µM
50 µM
100 µM
***
*
*
Th17 polarizing conditions IL-17A, Foxp3 reporter
C
DMSO 0.1%
trans-Ned 19 25 µM
trans-Ned 19 50 µM
GFP IL-17A
PE-Texas Red Foxp3
Th17 polarizing conditions
%of CD4+ T cells
Th1
Th17
Treg
Medium
DMSO
25 µM
50 µM
trans-Ned 19
**
D
Treg polarizing conditions IL-17A, Foxp3 reporter
DMSO 0.1%
trans-Ned 19 25 µM
trans-Ned 19 50 µM
GFP IL-17A
PE-Texas Red Foxp3
Treg polarizing conditions
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Th1
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trans-Ned 19
E
Treg polarizing conditions IL-17A, IL-10 reporter
DMSO 0.1%
trans-Ned 19 25 µM
trans-Ned 19 50 µM
PE-Cy5 IL-17A
GFP IL-10
Th17 polarizing conditions
%of CD4+ T cells
Th17
Th17+10
IL-10
***
**
*
Medium
DMSO
25 µM
50 µM
trans-Ned 19

Figure 6: Trans-Ned 19 influences calcium signaling, proliferation and differentiation of CD4^{+} T cells *in vitro*

Naïve CD4^{+} T cells were isolated from spleen and lymph nodes of a Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} or Foxp3^{RFP}, IL-10^{eGFP}, IL-17A^{Katushka} triple reporter mouse **(A)** Cells were incubated at indicated concentrations of DMSO or trans-Ned19 for 1 h and subsequently loaded with Fura2 and activated by soluble anti-CD3 antibody. Depicted are representative plates on the left and mean 340/380 ratio on the right. Data are cumulative of two experiments **(B)** Cells were loaded with 5 µM violet dye, incubated with the indicated concentrations of DMSO or trans-Ned 19, activated with plate bound anti-CD3 and soluble anti-CD28 and cultured for 48h. **(C)** Naïve CD4^{+} T cells from Foxp3^{RFP}, IL-17AeGFP, IFNγKatushka triple reporter mouse were incubated in indicated concentrations of trans-Ned 19 or DMSO and cultured under Th17 polarizing (IL-6, TGF-β1 0.5 ng/ml, IL-23) conditions for 96 hours. **(D)** Naïve CD4^{+} T cells were incubated in indicated concentrations of Ned 19 or DMSO and cultured under Treg (IL-2, TGF-β1 2 ng/ml) polarizing conditions for 96 hours. **(E)** Naïve CD4^{+} T cells from Foxp3^{RFP}, IL-10eGFP, IL-17AKatushka triple reporter mouse were incubated in indicated concentrations of trans-Ned 19 or DMSO and cultured under Th17 polarizing (IL-6, TGF-β1 0.5 ng/ml, IL-23) conditions for 96 hours. Ordinary one-way ANOVA with Holm-Sidak's multiple comparison test was used to calculate the significance. For each point the representative dot plots are presented on the left side and pooled biological replicates on the right side.

3.3.4. Trans-Ned 19 influences the differentiation of naïve CD4^{+} T cells *in vitro*

As trans-Ned 19 interfered with the activation of the CD4^{+} naïve T cells, its effects on the differentiation of CD4^{+} T cells *in vitro* was investigated. CD4^{+} naïve T cells were isolated from Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mice and cultured *in vitro* in the presence of increasing concentrations of trans-Ned 19. First, trans-Ned 19 promoted the differentiation of Th17 and did not have an impact on the differentiation of Treg cells in Th17 polarizing conditions *in vitro* (Fig. 6 C). Second, antagonism of NAADP with trans-Ned 19 did not have a significant influence on the differentiation of T regulatory cells in the Treg inducing cytokine cocktail (Fig. 6 D). To further investigate the nature of Th17 cells developing with trans-Ned 19 naïve CD4^{+} T cells were isolated from Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mouse and cultured under Th17 polarizing conditions. Antagonizing NAADP under Th17 polarizing conditions had promoted the differentiation of IL-17A, IL-10 co-producing cells and IL-10 single-producing cells. Moreover, the percentage of IL-17A cells which do not produce IL-10 remained unchanged upon addition of trans-Ned 19 to the culture media (Fig. 6 E). Further, as BZ194 influenced the sensitivity of cells to TGF-β1, similar titration experiments were performed with trans-Ned 19. Trans-Ned 19 did not promote Treg differentiation in the Th1 polarizing condition, on the contrary it promoted the differentiation of IFNγ producing cells. However this effect did not reach significance (Fig. 7 A). Interestingly, in opposition to the findings with BZ194, trans-Ned 19 inhibited the differentiation of iTreg cells and promoted the differentiation of Th17 cells (Fig.7 B)

To conclude, trans-Ned 19 did not promote the differentiation of Foxp3^{+} Tregs, had no significant impact on differentiation of Th1 cell and was found to promote the differentiation of Th17 cells *in*

vitro. The increase in the differentiation of Th17 can be explained by the increase of IL-17A, IL-10 coproducing cells.

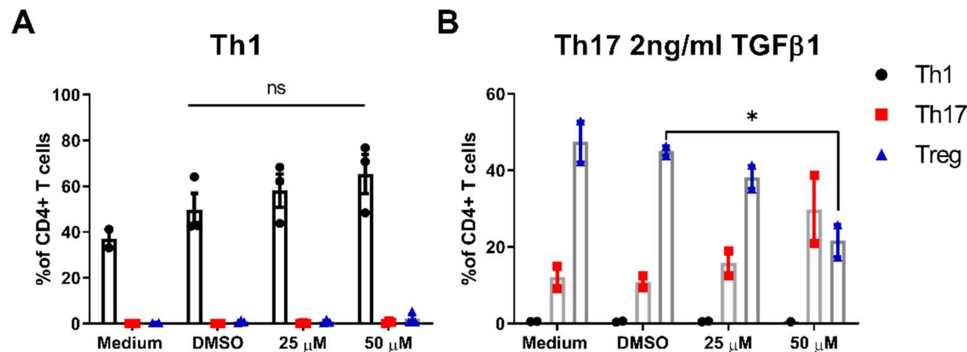


Figure 7: Trans-Ned 19 effect on the differentiation of CD4⁺ T cells

Naïve CD4⁺ T cells were isolated from spleen and lymph nodes of a Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mouse and cultured for 96 hours under the following polarizing conditions: **(A)** IL-2 and IL-12, **(B)** IL-6, IL-23 and TGF-β1 2 ng/ml. *p < 0.05; **p < 0.01***p < 0.001. Ordinary one-way ANOVA with Holm-Sidak's multiple comparison test was used to calculate the significance.

3.3.5. Trans-Ned 19 influences the differentiation of naïve CD4⁺ T cells *in vivo* and ameliorates inflammation in CD3 specific antibody induced transient intestinal inflammation

The *in vitro* findings on inhibiting the activation and proliferation of cells skewing the Th17 cells towards a more regulatory fate suggest that the NAADP pathway might be a therapeutic target in the treatment of immune-mediated inflammatory diseases. To investigate this, a model of CD3 specific antibody-induced transient intestinal inflammation was used. In this model, Th17- and Th1-mediated intestinal inflammation is transient and is accompanied by the emergence of Foxp3⁺ Tregs and exTh17 IL-10 producing cells, which makes it a suitable model to validate the *in vitro* findings *in vivo*[53], [54]. Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mice were administered trans-Ned 19 before first injection of CD3 specific antibodies (Fig. 8 A). The mice treated with trans-Ned 19 had an ameliorated course of disease, as they lost significantly less weight compared to the vehicle treated mice (Fig. 8 B). Moreover, trans-Ned 19 impacted the differentiation of CD4⁺ T cells *in vivo*. Cells from the small intestine mucous membrane were analyzed by flow cytometry (Fig. 8 C). Contrary to *in vitro* findings, the mice treated with trans-Ned 19 had significantly less IFNγ producing cells and less IFNγ, IL-17A double-producing cells. No significant difference between percentages of Th17 or Foxp3 Tregs was observed (Fig. 8 D).

The number of the cells were highly variable in the group treated by trans-Ned 19. Thus, there was no statistical significance between the overall numbers of $CD4^+$ and $CD8^+$ T cells, as well as between the specific subsets of $CD4^+$ T cells (Fig. 8 E).

To conclude, trans-Ned 19 ameliorated the course of disease in the CD3-induced intestinal inflammation model, which was accompanied by a decreased percentage of IFNγ producing cells.

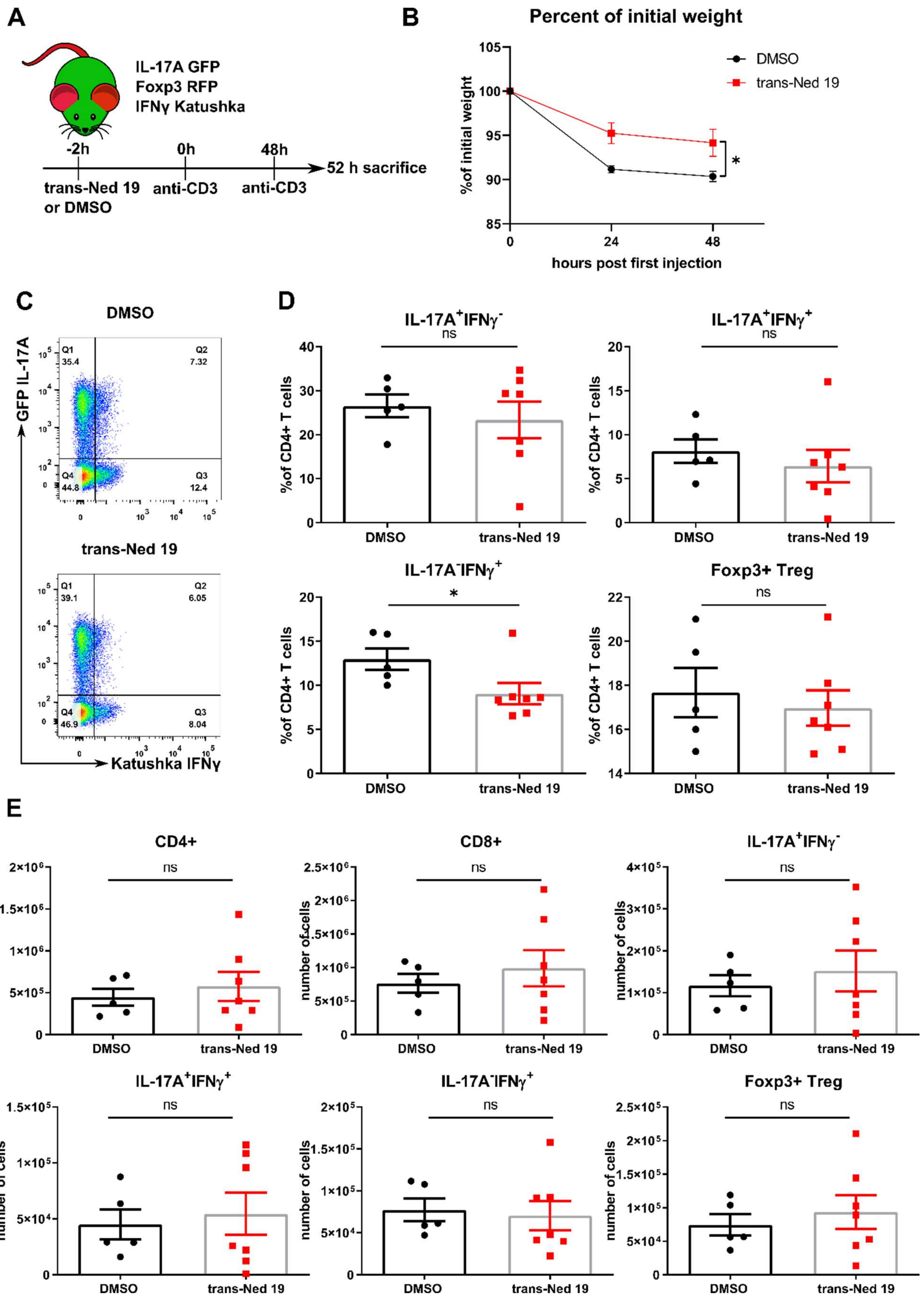

A
IL-17A GFP
Foxp3 RFP
IFNγ Katushka
-2h
0h
48h
52 h sacrifice
trans-Ned 19
or DMSO
anti-CD3
anti-CD3
B
Percent of initial weight
DMSO
trans-Ned 19
%of initial weight
hours post first injection
*
C
DMSO
trans-Ned 19
GFP IL-17A
Katushka IFNγ
Q1 35.4
Q2 7.32
Q4 44.8
Q3 12.4
Q1 39.1
Q2 6.05
Q4 46.9
Q3 8.04
D
IL-17A+IFNγ-
IL-17A+IFNγ+
IL-17A-IFNγ+
Foxp3+ Treg
%of CD4+ T cells
ns
*
DMSO
trans-Ned 19
E
CD4+
CD8+
IL-17A+IFNγ-
IL-17A+IFNγ+
IL-17A-IFNγ+
Foxp3+ Treg
number of cells
ns
DMSO
trans-Ned 19

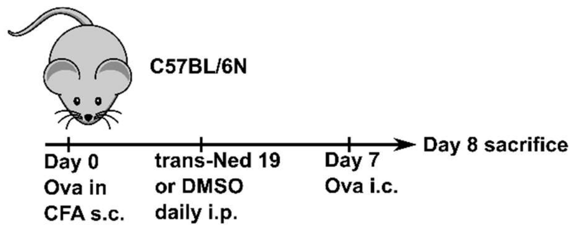
Figure 8: Trans-Ned 19 ameliorates the disease in anti-CD3 induced transient intestinal inflammation

Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mice were treated with trans-Ned 19 prior to administration of anti-CD3 antibody. Weight was monitored daily, at day 3 mice were sacrificed and leucocytes were isolated from the small intestine mucous membrane. Depicted data comes from two independent experiments **(A)** Experimental scheme. **(B)** Percent of initial weight. $*p < 0.05$ Ordinary two-way ANOVA was used to calculate the significance. **(C)** Representative flow cytometry plots plot of Foxp3 negative, CD4^{+} T cells. $*p < 0.05$ Mann-Whitney U-test was used to calculate the significance **(D)** Summary graphs of percentages of CD4^{+} T cells **(E)** Summary graphs of cell numbers of CD4^{+} T cells

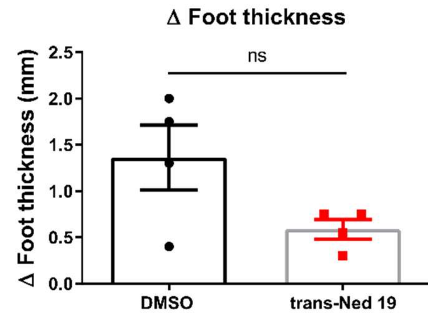
3.3.6. Trans-Ned 19 affects the differentiation and function of CD4^{+} T cells *in vivo* in an ovalbumin immunization model

As antagonism of the NAADP signaling pathway with trans-Ned 19 interfered with calcium signaling upon CD3/TCR stimulation and inhibited the proliferation of CD4^{+} naïve T cells *in vitro*, the delayed-type hypersensitivity model was utilized to validate these findings *in vivo*. In the Ova immunization model, T cells are activated in an antigen specific manner. Moreover, the immune response is mainly type 1 driven, i.e. by IFNγ producing cells [219], [220], whose differentiation was already shown to be impacted by trans-Ned 19 *in vivo* in the anti-CD3 intestinal inflammation model. Wild type mice were immunized with the emulsion of Ovalbumin in complete Freund's Adjuvant and administered trans-Ned 19 daily for 7 days. After 7 days of immunization, the mice were challenged with the ovalbumin injection into the footpad (Fig. 9 A). The swelling was measured after 24 hours and compared to the swelling of the foot injected with PBS. Interestingly, treatment with trans-Ned 19 decreased the degree of foot swelling, however this effect has not reached statistical significance (Fig. 9B). Lymphocytes were isolated from the draining lymph nodes and spleens and analyzed by flow cytometry. There were no differences in the percentages of the TNFα and IFNγ producing CD4^{+} and CD8^{+} T cells (Fig. 9 C, D).

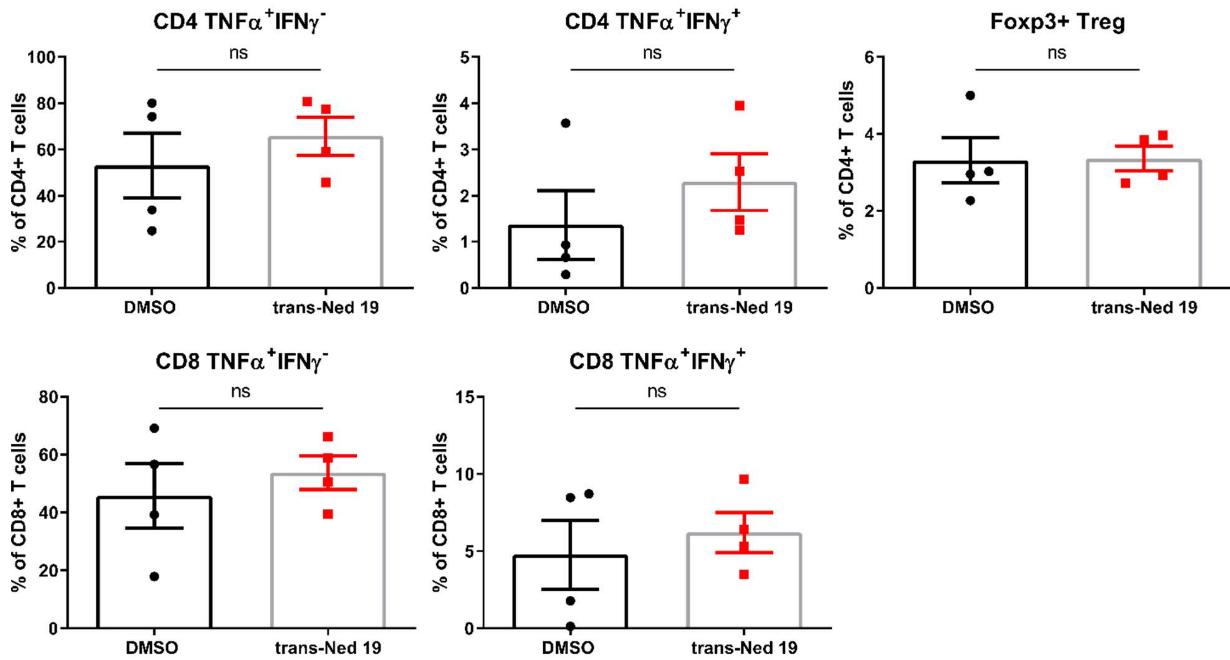
A



B



C Inguinal lymph nodes



D Spleen

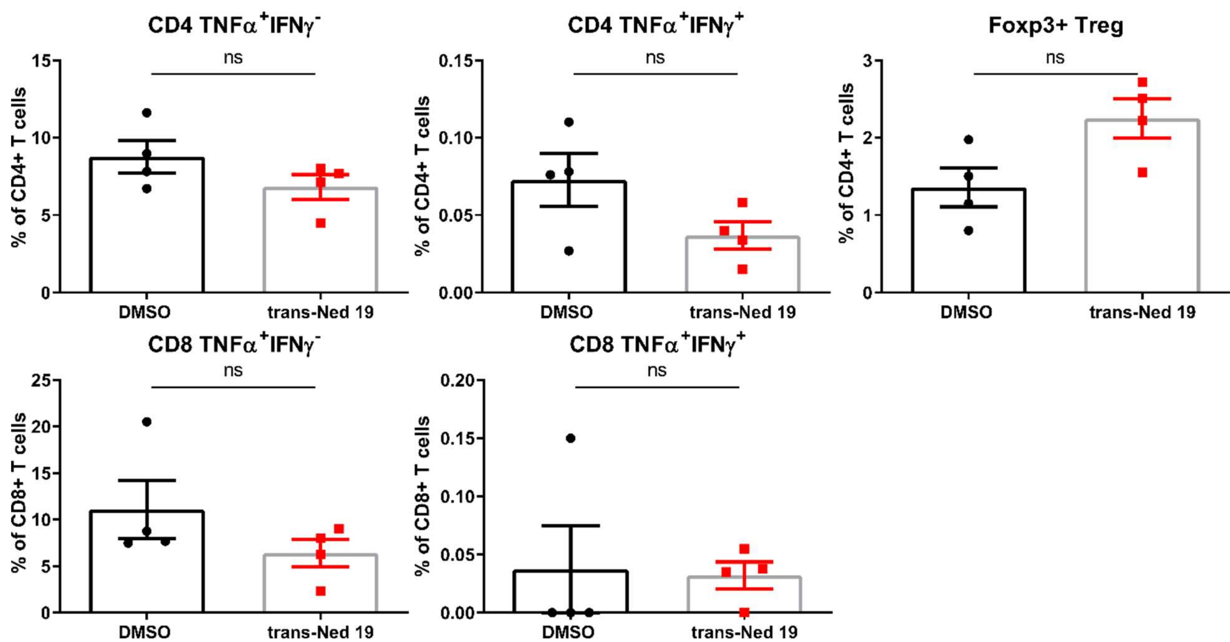


Figure 9: Trans-Ned 19 partially inhibits the antigen specific activation of T cells *in vivo* in delayed hypersensitivity model

Wild type mice were treated immunized with ovalbumin emulsified in complete Freund's adjuvant and treated with trans-Ned 19 daily for 7 days. At day 7, mice were injected intradermally with ovalbumin precipitate into one foot and PBS in the other foot. On day 8, foot swelling in response to ovalbumin injection was measured and normalized to the PBS treated foot. Data represents a result of a single experiment. **(A)** Experimental scheme **(B)** Difference in foot thickness between ovalbumin challenged foot and PBS injected foot. **(C)** Summary graphs of the flow cytometric analysis of leukocytes isolated from inguinal lymph nodes **(D)** Summary graphs of the flow cytometric analysis of leukocytes isolated from spleen. Mann-Whitney U-test was used to calculate the significance.

3.3.7. Trans-Ned 19 affects the differentiation and function of CD4^{+} T cells *in vivo* in murine nephrotoxic nephritis

To further explore NAADP signaling pathway as a therapeutic target in immune-mediated inflammatory diseases, the nephrotoxic nephritis model was used. Th17 cells are thought to play a major role in tissue damage in this model [221]. Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mice were used in order to investigate the impact of trans-Ned 19 on the differentiation and function of regulatory and pathogenic CD4^{+} T cells in kidney inflammation. Trans-Ned 19 was administered before the administration of nephrotoxic serum, and afterwards, mice were treated once daily with trans-Ned 19 or the vehicle alone (Fig. 10 A). The analysis of the biochemical indicators of kidney injury, blood urea nitrogen and albumin to creatinine ratio in the urine revealed that administration of trans-Ned 19 ameliorated kidney damage in this model (Fig. 10 B). However, analysis of the cellular composition of the lymphocyte infiltrates in the inflamed kidneys was not conclusive. Mice treated with trans-Ned 19 in trend had less IL-17A^{+}IFNγ^{-} cells and IL-17A^{+}IFNγ^{+} double-producing cells. On the other hand, treatment with trans-Ned 19 resulted in increased percentage of Th1 cells and lower percentage of Foxp3^{+} Tregs. Moreover, considering the numbers of cells, there was no difference between treated and non-treated mice in the total number of CD4^{+} or CD8^{+} cells or subsets of CD4^{+} T cells (Fig. 11 A, B).

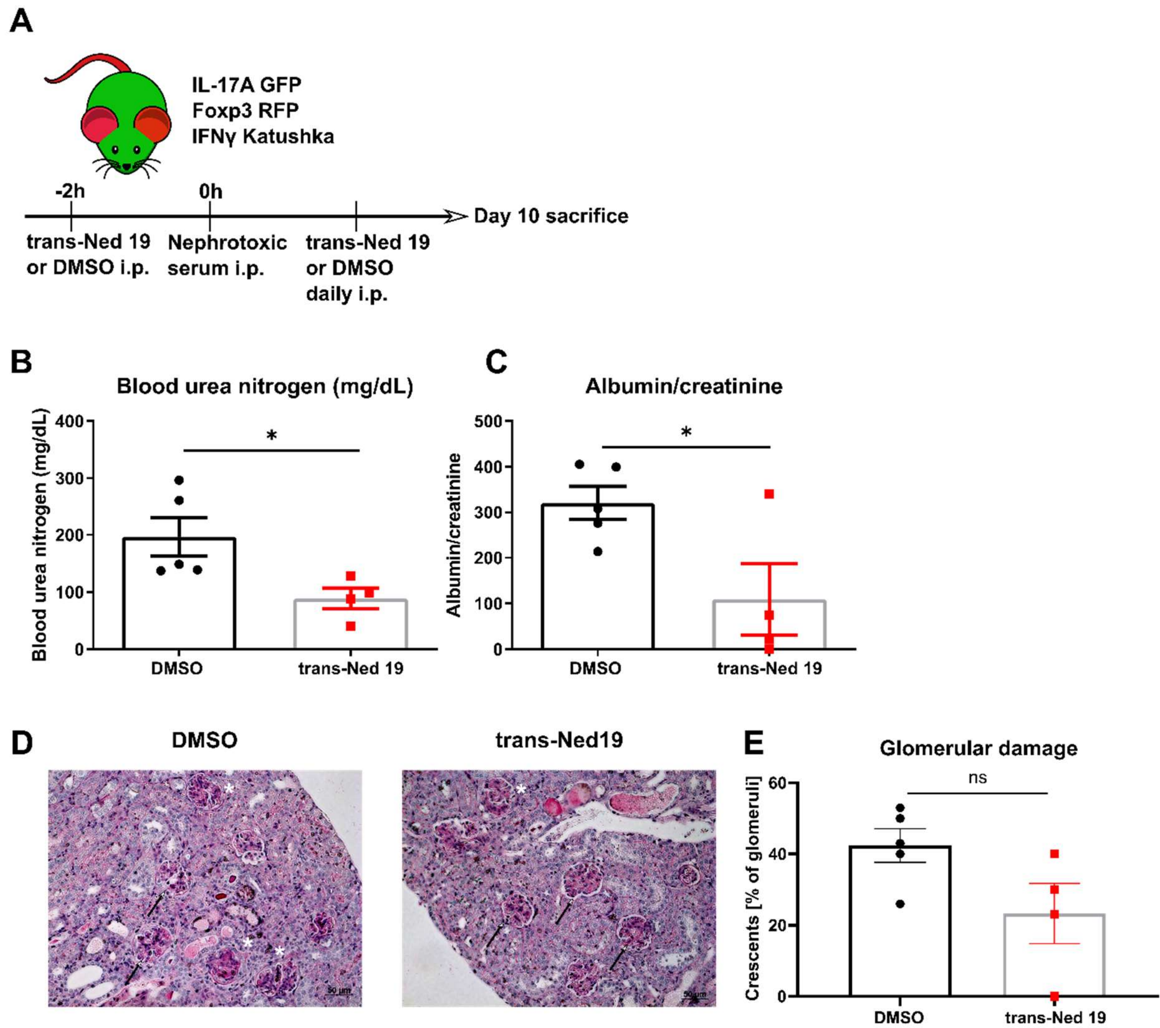


Figure 10: Trans-Ned 19 ameliorates NTN.

Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mice were treated with trans-Ned 19 prior to administration of nephrotoxic serum. At day 9, urine was collected. At day 10, mice were sacrificed and organs were harvested. Depicted data comes from one experiment **(A)** Experimental scheme. **(B)** Blood urea nitrogen. **(C)** Albumin to creatinine ratio. **(D)** Representative histology pictures of PAS-stained kidney samples, glomeruli with crescent formation are marked with an asterisk, intact glomeruli are marked with an arrow. **(E)** Cumulative histological score of glomerular damage. *$p < 0.05$ T-test for two independent groups was used to calculate the significance.

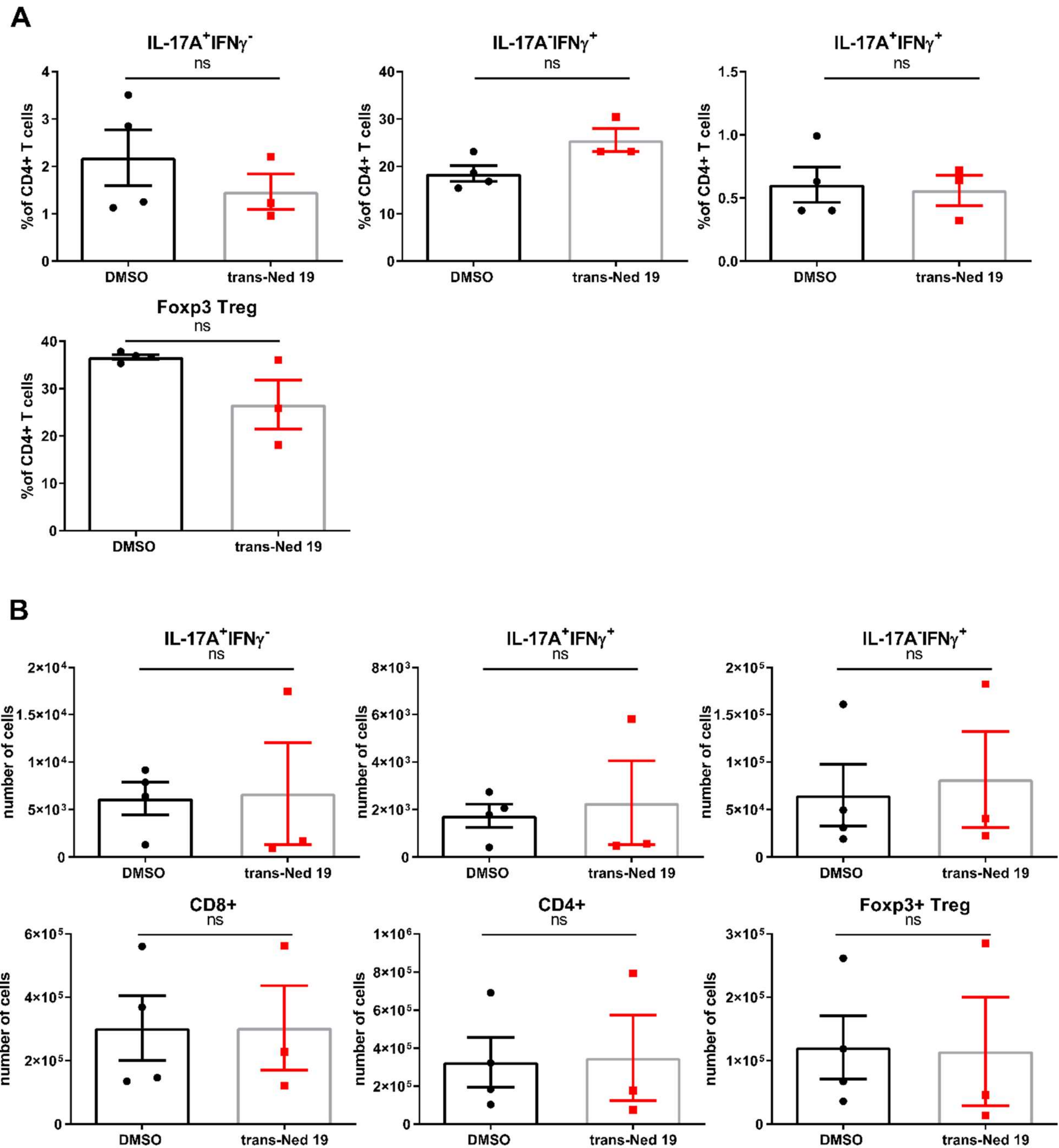


Figure 11: Trans-Ned 19 impact on the differentiation of CD4$^+$ T cells in nephrotoxic nephritis

Foxp3^{RFP}, IL-17A^{eGFP}, IFNγ^{Katushka} triple reporter mice were treated with trans-Ned 19 prior to administration of nephrotoxic serum. At day 9, urine was collected. At day 10, mice were sacrificed and organs were harvested. Leukocytes were isolated from kidneys. Depicted data comes from one experiment. **(A)** Summary graphs of CD4$^+$ T cell subsets percentages **(B)** Summary graphs of CD4$^+$ and CD8$^+$ T cells numbers. *$p < 0.05$ Mann-Whitney U-test was used to calculate the significance.

3.4. TRPM2 channel does not play a significant role in the differentiation and function of $CD4^{+}$ T cells

Blocking NAADP signaling pathway with the means of trans-Ned 19 and BZ194 had a significant impact on activation and differentiation of $CD4^{+}$ T cells *in vitro*. Moreover, administration of trans-Ned 19 ameliorated the course of disease in murine models of inflammatory diseases. In order to understand the biology of NAADP signaling in $CD4^{+}$ T cells, it is essential to find its receptor or identify calcium channels whose function are modulated by NAADP. The matter of molecular target of NAADP *in vivo* remains controversial, and so far, three possible calcium channels were suggested to be targeted by NAADP: RYR1, TPC1/2 and TRPM2 channel [9]. In this study, TRPM2 channel function in the differentiation and effector function of $CD4^{+}$ T cells was investigated.

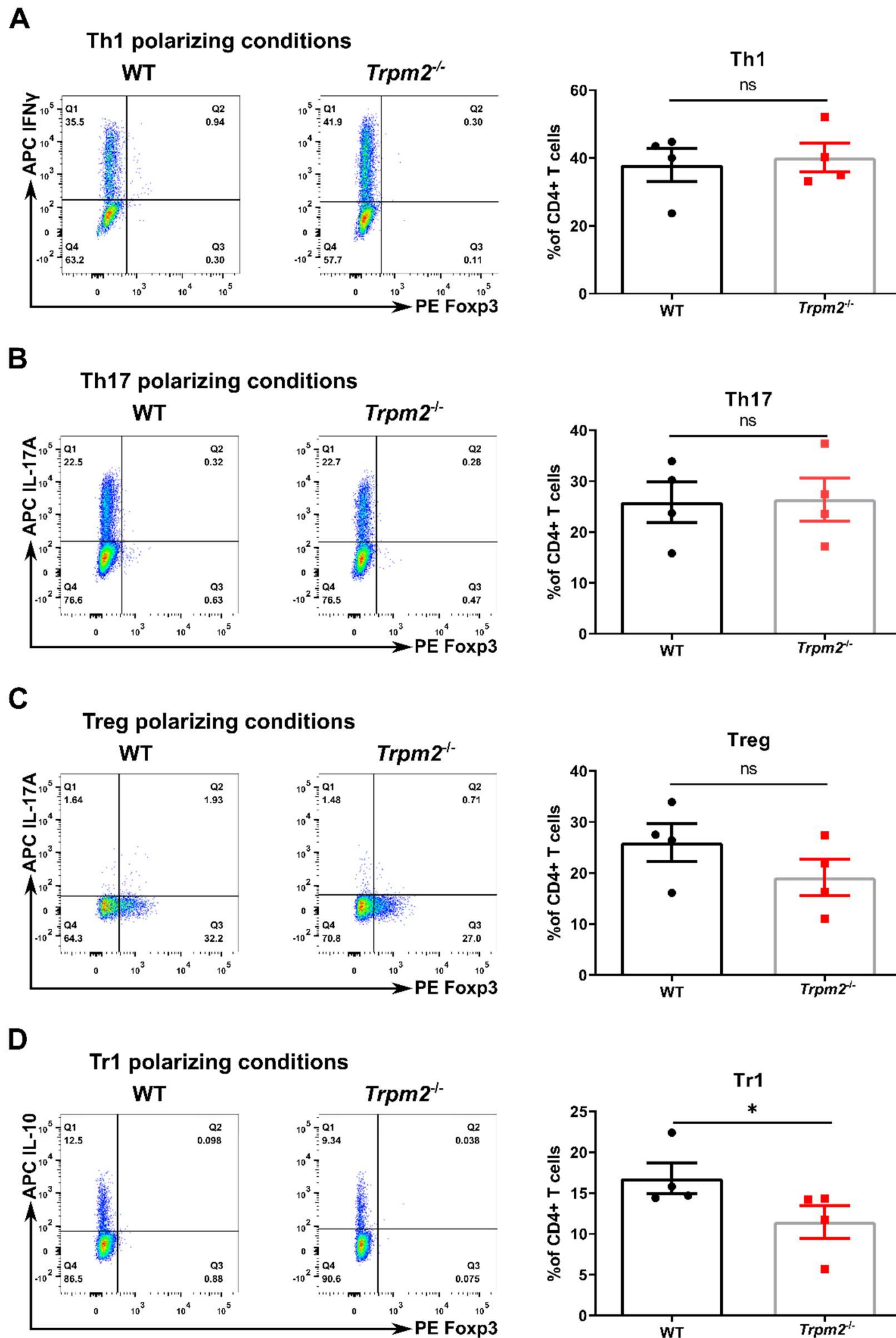


Figure 12 TRPM2 channel influence on the differentiation of CD4$^+$ T cells

Naïve CD4$^+$ T cells were isolated from spleen and lymph nodes of *Trpm2$^{-/-}$* or WT mice and cultured for 96 hours after polarizing conditions. Cytokine expression after restimulation with PMA and ionomycin for 3.5 hours. Representative

flow cytometry plots (left) and summary plots with mean ± s.e.m. are (right) Cells were cultured under the following polarizing conditions (A) Th1: IL-2, IL-12 (B) Th17: IL-6, IL-23, TGF-β1, (C) iTreg: IL-2, TGF-β1 (D) Tr1: IL-27, TGF-β1. *p < 0.05 Mann-Whitney U-test was used to calculate the significance.

3.4.1. TRPM2 channel does not have a significant effect on the differentiation of naïve $CD4^+$ T cells *in vitro*

Both studied antagonists of NAADP signaling had an impact of $CD4^+$ differentiation *in vitro*. In order to investigate the function of TRPM2 channel in the differentiation of $CD4^+$ T cell *in vitro*, differentiation assays with *Trpm2*$^{-/-}$ $CD4^+$ naïve T cells were performed. These experiments revealed that absence of TRPM2 channel in naïve cells did not have an impact on the differentiation of Th1, Th2, Th17 and Foxp3 positive regulatory $CD4^+$ T cells (Fig. 12 A, B, C, D). Interestingly, TRPM2 seemed to play a role in the synthesis of IL-10 as reduced differentiation into Tr1 cells was observed for TRPM2 cells when cultured with TGF-β1 and IL-27 (Fig. 12 E).

3.4.2. TRPM2 channel does not have a significant impact on the differentiation of naïve $CD4^+$ T cells *in vivo*

The *in vitro* findings suggested that the TRPM2 channel might play a role in the development of Tr1 cells. It was shown that Th17 cells transdifferentiate into Tr1 cells upon resolution of inflammation [54]. This phenomenon can be well investigated in the CD3 specific antibody-induced intestinal inflammation model. To test the hypothesis that TRPM2 channel plays a role in the differentiation of Tr1 cells, *Trpm2*$^{-/-}$ and wild type mice were treated with CD3 specific antibody (Fig. 13 A). TRPM2 channel proved to have no impact on the differentiation and function of CD4 T cells *in vivo* in this model. The disease severity was comparable between *Trpm2*$^{-/-}$ and wild type mice, however, the *Trpm2*$^{-/-}$ mice exhibited a less pronounced weight recovery (Fig. 13 B). Moreover, in trend, the percentage IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$, as well as IL-17A$^-$IFN-γ$^+$ cells was higher in *Trpm2*$^{-/-}$ mice (Fig. 12 C).

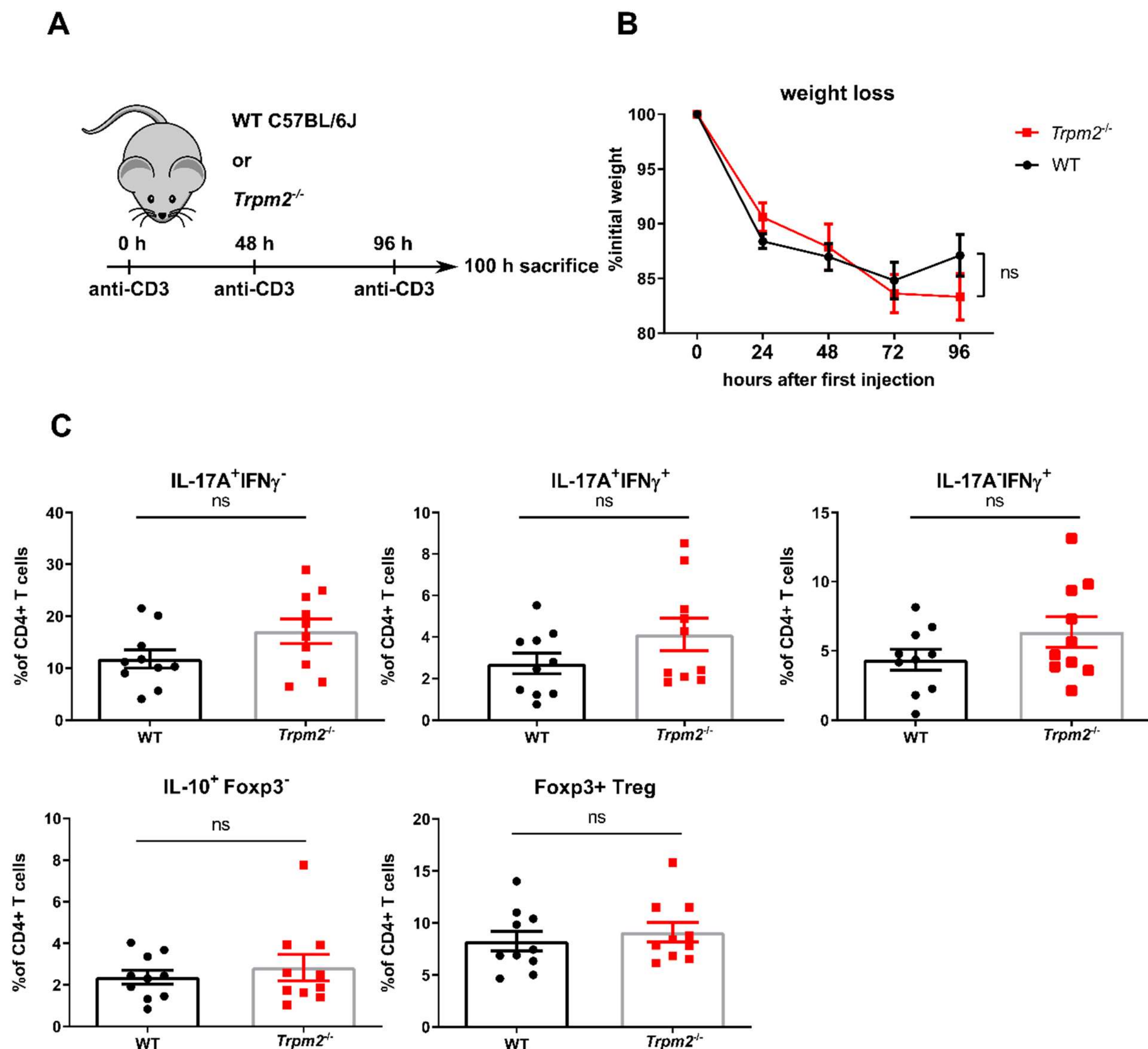


Figure 13: Total body deficiency of TRPM2 channel does not have a significant impact on the differentiation and effector function of $CD4^+$ T cells in an anti-CD3 mediated model of transient intestinal inflammation

Trpm2$^{-/-}$ and WT mice were administered anti-CD3 specific antibody 3 times every 48 hours. Weight loss was monitored daily and at day 5 mice were sacrificed and leukocytes were isolated from small intestine mucous membrane. Data comes from two independent experiments (A) Experimental scheme (B) Percent of initial weight Significance was calculated with two-way ANOVA. (C) Cytokine expression was measured after restimulation with PMA and ionomycin for 3,5 hours. Percentages of $CD4^+$ T cells isolated from small intestine. Significance was calculated with Mann-Whitney U-test.

In conclusion, the performed experiments revealed, that TRPM2 channel had no significant impact on the differentiation and effector function of $CD4^+$ T cells neither *in vitro* nor *in vivo* in the investigated model.

4. Discussion

IMIDs such as IBD or cGN comprise a major cause of morbidity worldwide [5]. The ethiopathogenesis of these disorders are complex and remain largely unknown. A general consensus is that genetic predispositions together with environmental factors promote the development of dysregulated immune responses, which in turn results in uncontrolled inflammation and tissue damage [4]. Genome wide association studies uncovered multiple susceptibility genes that are shared across more than one IMID [2]. As one could expect, most of these genes are associated with the development and function of the immune system, and in particular, include genes that are involved in $CD4^{+}$ T cell biology. These include genes encoding for interleukins (*IL-2, IL-21*), receptors for interleukins (*IL-2Rα, IL-23R*) and TCR signaling (*PTPN2, PTPN22 SH2B*) [2]. This highlights that further investigation of the adaptive immune system function, in particular, $CD4^{+}$ T cell biology is necessary to better understand the pathogenesis of IMIDs that will help to develop new treatment strategies.

The hallmark of the adaptive immune system is the antigen specificity mediated by the TCR and BCR in T and B Lymphocytes, respectively. The VDJ chain of TCR recombination results in $3 \cdot 10^{11}$ possible unique T cell receptors, which transduce signals only when bound by a cognate receptor. During the thymic development of T cells, the cells whose TCRs recognize self-antigens are either eliminated or directed towards regulatory fate (nTregs). The positive and negative selection in the thymus is conceptualized by the affinity model. Mature T cells, after thymic development still adjust the type of immune response. Naïve T cells integrate the signals from TCR, co-stimulation and cytokines to develop into several subsets of $CD4^{+}$ T cells which are specialized to fight certain types of pathogens or the other way around to promote immune tolerance. Thereby, throughout the life, the immune system learns which antigens are associated with harmful pathogens or damaged tissues and which antigens, like for instance food particles or symbiotic microbial organisms, present no danger to the host [1], [67]. The first aim of this study was to investigate the relationships between the strength of TCR signal, resulting Ca^{2+} signaling and the differentiation of $CD4^{+}$ T cells.

4.1. Strong TCR signals are necessary for Th17 differentiation, whereas weak TCR signals promote iTreg differentiation *in vitro*

By differentiating into different subsets, $CD4^+$ T cells can adjust the immune response to optimally fight against the type of the offending agent. How $CD4^+$ T cells are instructed to take on their fate has been an area of intensive research [12]. It is generally accepted that naïve $CD4^+$ T cells integrate the signals coming from the TCR, costimulatory receptors and cytokine receptors to decide on a specific transcriptional lineage program [12]. To study the role of the TCR signal strength in $CD4^+$ T-cell fate decision, OT-II transgenic mice and ovalbumin altered peptide ligands were used. T cells from OT-II mice express a single TCR which recognizes a 321-339 fragment of ovalbumin. Within the 321-339 ovalbumin peptide, the amino acids at positions 331 and 333 were found previously to determine the affinity to the OT-II TCR binding [215], therefore 321-339 ovalbumin peptides with single amino acid substitutions in the position 331 were synthesized. Ratiometric microscopy and *in vitro* differentiation assays were used in order to investigate the impact of the antigen affinity to the TCR on initial calcium signaling events and fate decisions of naïve $CD4^+$ T cells, respectively.

The native ovalbumin peptide has the highest affinity to the OT-II TCR, and as expected, we found here that naïve $CD4^+$ T cells activated with the native ova peptide showed the most robust increases in the $[Ca^{2+}]_i$ upon TCR ligation. As shown in figure 1, modified ova peptides, which acted as partially agonistic altered peptide ligands were less effective in inducing Ca^{2+} signaling upon TCR activation. Both peak $[Ca^{2+}]_i$ increase and area under the $[Ca^{2+}]_i$-time curve were significantly lower when compared with signal evoked by binding of a strong agonistic peptide. Interestingly, qualitative differences in the Ca^{2+} signaling patterns upon TCR stimulation with full or partial agonists were observed. Signals following binding of a full agonistic peptide were more in a peak-plateau or peak-oscillation form, while weaker agonists tended to evoke oscillations in Ca^{2+} signaling without a discernable peak or transient increases in$[Ca^{2+}]_i$. These findings are in accordance with previously published results regarding the calcium signaling in $CD4^+$ T cells following antigenic stimulation. Sloan-Lancaster *et al.* showed that the altered peptide ligands induce $[Ca^{2+}]_i$ increases following TCR stimulation and the plateau of $[Ca^{2+}]_i$ increase was lower than Ca^{2+} signal induced by TCR activation with a strong agonist [222]. Another study performed on OT-I $CD8^+$ T cells with a genetically encoded calcium sensor showed that both signal quality and quantity is dependent on the affinity of the antigen to the TCR [223].

Both quantitative and qualitative characteristics of Ca^{2+} signaling following antigen stimulation of the TCR play a role in the activation and effector function of $CD4^+$ T cells [6], [104], [224]. For instance, Dolmetsch *et al.* demonstrated that the oscillations of $[Ca^{2+}]_i$ upon TCR ligation are necessary for the nuclear translocation of NFAT. NFAT is a key transcription factor controlling the activation of T cells and interestingly distinct NFAT isoforms differentially regulate the $CD4^+$ T cell development and differentiation [8], [225]. Moreover, $CD4^+$ T cells with dysfunctional mechanisms of Ca^{2+} entry upon TCR stimulation have impaired effector function [6], [104]. For example, genetic defects of SOCE significantly decrease the synthesis of pro-inflammatory cytokines, and consequently, T cell mediated autoimmunity in *in vivo* models [138], [139]. Moreover, differential impact of Ca^{2+} signaling on effector and regulatory T cells was reported. For instance, Kaufmann *et al.*, showed that specific $CD4^+$ T cell deficiency of *Orai1* reduced the cytokine synthesis by effector $CD4^+$ T cells, whereas the proliferation and suppressive function of Tregs remained unaffected [137].

To further investigate whether the observed differences in Ca^{2+} signaling have an impact on the fate decisions in $CD4^+$ T cells, *in vitro* differentiation assays were performed in our study. In order to vary the TCR signal strength, OT-II cells were stimulated either with the native ovalbumin peptide or with the altered peptide ligands. Surprisingly, as shown in figure 3, the affinity of the antigen to the TCR did not significantly impact the differentiation of Th1 and Th2 cells. In contrast, the TCR signal appeared to be a decisive factor in Th17 cells and Treg fate decisions. Indeed, Th17 differentiation was promoted by the strong TCR signal, whereas Treg differentiation was favored by a weak TCR signal. The notion of TCR signal strength directing the differentiation of $CD4^+$ T cells has been previously extensively investigated and reviewed in [19], [226]–[228]. Several reports revealed that TCR signaling can play a dominant role over cytokine signal in controlling the differentiation of $CD4^+$ T cells [19], [227]. According to the literature, strong TCR signals drive the differentiation of Th1 cells, while weak TCR signal strength promotes the differentiation of Th2 cells [229], [230]. As depicted in figure 3, performed *in vitro* assays revealed that TCR stimulation with two higher affinity peptides (W and E) promoted Th1 differentiation when compared with lowest affinity peptide (M). However, intermediately affine peptides (W) resulted in the most efficient Th1 differentiation. One can speculate that the decrease of the percentage of Th1 cells in the condition with the native ova peptide results from an over-activation of the TCR and consequently induction of the activation induced cell death.

In contrast, conducted assays proved that TCR signal strength is a critical factor in Th17 and Treg differentiation. The relationship between TCR signaling and Th17 and Treg fate decisions has been extensively investigated in the field. Indeed, Th17 cells and Treg share developmental pathways, which suggests the existence of a molecular switch regulating the differentiation into one of these two fates [231], [232]. Various mechanisms explaining this relationship were hypothesized and they were broadly reviewed in [19], [227], [233]. First, while both Th17 and Treg $CD4^+$ T cells require TGF-β1 signaling for their development, Th17 require a moderate TGF-β1 signal, and Treg a strong TGF-β1 signal. The TGF-β1 signaling cascade is conducted via SMAD proteins. SMAD signaling can be terminated by phosphorylation by mitogen-activated protein kinase kinase kinase 2 (MEKK2) and MEKK3. Notably, MEKK2 and MEKK3 are activated by TCR ligation, thus, TCR signaling counteracts TGF-β1 signaling [13], which can explain how strong TCR signal promotes Th17 differentiation and inhibits Treg differentiation. Second, IL-2/STAT5 is essential for the differentiation of Tregs and inhibits the development of Th17 cells. The responsiveness of IL-2R to IL-2 has been shown to be modulated by TCR signaling via inhibition of phosphorylation of STAT5 [84]. Moreover, Akt/mTOR signaling pathway has been reported to play a decisive role in the differentiation of T cells [234], [235]. Strong activation of the Akt/mTOR pathway due to high TCR signal strength is accompanied by impaired Treg differentiation [236], [237]. Furthermore, TCR signaling can affect the interaction of T cells and APCs which can then indirectly impact the differentiation T cells. Iezzi *et al.* reported that strong TCR stimulation induces CD40L expression on T cells, which consequently instruct dendritic cells via CD40 ligation to secrete IL-6. This supports the Th17 and inhibit the Treg differentiation respectively [238]. Moreover, Rodriguez *et al.* demonstrated TCR induced Ca^{2+} signaling plays a role in Th17 differentiation. Indeed, deficiency of *Itk* in $CD4^+$ T cells impaired Ca^{2+} signaling upon TCR ligation and decreased the synthesis of IL-17A [134]. In another study, Rodriguez *et al.* reported that a reciprocal relationship between Th17 and Treg fate is impacted Ca^{2+} signaling since *Itk* deficient $CD4^+$ T cells differentiate into iTregs under *in vitro* Th17 polarizing conditions [135]. In conclusion, multiple lines of evidence indicate that TCR signal strength is a decisive factor regulating Th17 and Treg differentiation, however, the exact role of Ca^{2+} signaling controlling this process, and in particular, adenine-derived Ca^{2+} mobilizing second messengers, remains to be unknown.

To sum up, affinity of the antigen to the TCR shapes the strength and quality of Ca^{2+} signaling in naïve $CD4^+$ T cells. Moreover, strength of the TCR signal impacts the outcome of the

differentiation of $CD4^+$ T cells. These observations allow to hypothesize that the relationship between the TCR signal strength and $CD4^+$ T-cell differentiation is, at least partially, mediated by Ca^{2+} signaling and in particular by adenine derived Ca^{2+} mobilizing second messengers.

4.2. NAADP antagonism influences the balance between Th17 and iTreg cells

The results linking TCR signal strength both with Ca^{2+} signaling and $CD4^+$ T cell fate decisions allowed us to hypothesize that the relationship between the TCR stimulus and differentiation of $CD4^+$ T cells is mediated through calcium signaling, in particular through NAADP mediated calcium release from intracellular stores.

Indeed, in Jurkat T cells, NAADP was shown before to be synthesized very early upon TCR/CD3 stimulation, and moreover, the extent of its synthesis correlated with the strength of CD3 stimulation [107]. The identity of the cytoplasmic receptor of NAADP remains to be controversial [153], [239] and the investigation of NAADP signaling relies upon the use of pharmacological blockade of NAADP[118], [157], [217]. So far, two compounds have been synthesized and validated to effectively block NAADP induced calcium release: BZ194 and Ned 19 [157], [217]. Dammerman *et al.* reported that BZ194 inhibits Ca^{2+} increase and proliferation of T cells upon TCR/CD3 stimulation in a cell line of rat myelin basic protein specific effector T cells [157]. Furthermore, BZ194 was shown to ameliorate the clinical course of EAE in rats. Inhibition of NAADP signaling was suggested to regulate T cell motility and re-activation of effector cells upon arrival in the nervous system [159]. The impact of NAADP signaling antagonism with BZ194 on $CD4^+$ T cell differentiation, however, was not investigated.

In order to investigate the role of NAADP signaling in $CD4^+$ T cell differentiation, we used a Foxp3, IL-17A and IFN-γ triple reporter mouse. In the first step, as shown in figure 4, the inhibitory effect of BZ194 on Ca^{2+} signaling and proliferation upon TCR/CD3 stimulation was reproduced using primary murine naïve $CD4^+$ T cells. Next, *in vitro* differentiation assays under Th17 and Treg polarizing conditions were performed. As depicted in figure 4, antagonizing NAADP by means of BZ194 inhibited Th17 differentiation under Th17 polarizing conditions and promoted Treg cell differentiation both in the presence of Th17 and Treg cytokine cocktails. Assuming that NAADP signaling is correlated with the strength of TCR signaling, this observation was in line with the literature and with the results obtained with OT-II mice and ovalbumin altered peptide ligands [134], [238], [240]. Namely, TCR signal strength is correlated both with the extent of NAADP

production and with the likelihood of Th17 over Treg differentiation. Antagonizing NAADP signaling by means of BZ194 reverses the effects of strong TCR signaling and thus impairs the Th17 differentiation and disinhibits the development of Tregs. Interestingly, Th17 cells and Treg share multiple molecular developmental pathways [231], [232]. For example, it is known that differentiation Th17 cells requires moderate strength TGF-β1 signaling, while strong TGF- β1 signaling inhibits Th17 fate and promotes the development of Tregs [47]. It was therefore hypothesized that NAADP suppresses TGF-β1 signaling and thus its inhibition by means of BZ194 increases the strength of TGF- β1 signal.

In order to test the hypothesis that antagonizing NAADP signaling by means of BZ194 potentiates TGF-β1 signaling *in vitro* differentiation assays were performed in which the TGF-β1 concentration was titrated. In the presence of Th17 polarizing cytokine cocktail with low TGF-β1 concentrations BZ194 promoted the differentiation of Th17 cells, which is in line with the fact that Th17 cells require moderately strong TGF-β1 signal. In addition, in the presence of a cytokine cocktail consisting of IL-2 and low concentrations of TGF-β1, naïve $CD4^+$ T cells differentiated to an equal extent into Th1 cells and Tregs. Addition of BZ194 in this condition inhibited Th1 differentiation and promoted Treg differentiation program. This result is in line with the previous findings, that TGF-β1 signaling inhibits differentiation of Th1 cells and promotes differentiation of Tregs [241]. BZ194 also induced Tregs in the presence of Th1 polarizing cocktail consisting of IL-2 and IL-12. Although the increase of Tregs population under Th1 polarizing conditions caused by BZ194 treatment was statistically significant it was small in the absolute numbers. In contrast, BZ194 did not affect the differentiation of Th1 cells in Th1 polarizing cytokine cocktail. This finding was in line with the reports showing that IL-12 antagonizes TGF-β1 signaling [242] and likely overrides the TGF- β promoting effect of BZ194. At last, when cells were cultured in the presence of anti-TGF-β1 antibody and IL-2, BZ194 failed to induce Tregs, indicating that TGF-β1 signaling is necessary for BZ194 to induce Tregs. Taken together, these results suggest that the impact of BZ194 on the differentiation of $CD4^+$ T cell might be mediated through TGF-β1 signaling pathway. This would suggest and interesting interplay between Ca^{2+}, adenine nucleotides and cytokine signaling. Nevertheless, an explicit investigation of influence of BZ194 on TGF-β1 signaling is necessary to confirm this hypothesis.

In conclusion, inhibiting the NAADP signaling pathway with BZ194 promoted Treg differentiation in Treg polarizing conditions, Th17 polarizing conditions and Th1 polarizing conditions. It is likely

that the effect of BZ194 on CD4$^+$ T cell differentiation is mediated by an increasing sensitivity of the cells to TGF-β1. However Treg promoting activity of BZ194 could occur also via modulating other signaling pathways, for example IL-2/STAT5 or Akt/mTOR. Elucidation of molecular mechanisms of BZ194 requires further investigation.

Besides BZ194, another compound: trans-Ned 19 was previously found to antagonize NAADP mediated calcium release from intracellular stores [217]. It was identified by means of a virtual screen of a library of compounds that had similar spatial properties to the NAADP [217]. Trans-Ned 19 was shown to inhibit NAADP induced Ca^{2+} release in sea urchin eggs. Moreover, trans-Ned 19 inhibited Ca^{2+} increase upon TCR/CD3 stimulation in primary murine T cells, and thus, impaired the proliferation of naïve and effector T cells and cytokine synthesis by effector T cells [160]. Nevertheless, what the impact of trans-Ned 19 on the differentiation of naïve CD4$^+$ T cells is has not been addressed so far. Therefore, this question was addressed in this study.

First the findings regarding impact of trans-Ned 19 on the TCR induced Ca^{2+} signaling and proliferation of primary murine naïve CD4$^+$ T cells were reproduced. As shown in figure 6, pre-incubation of naïve CD4$^+$ T cells with 100 µM completely blocked their proliferation upon TCR ligation. Basing on this result, further *in vitro* experiments involved using 25 µM and 50 µM concentrations of trans-Ned 19 in order to observe its modulatory effect on differentiation without compromising cell proliferation. To evaluate how trans-Ned 19 influences the differentiation of CD4$^+$ T cells, Foxp3, IL-17A, IFN-γ and Foxp3, IL-17A, IL-10 triple reporter mice were used. As shown in figure 6, trans-Ned 19 promoted the differentiation of IL-17A producing cells under Th17 polarizing conditions and did not have a significant impact on the development of Tregs under Treg polarizing condition. One could however appreciate the trend towards inhibiting the differentiation of Tregs by trans-Ned 19. These findings seem to contradict the results obtained after inhibition of the NAADP signaling pathway by BZ194 and the results obtained in *in vitro* differentiation of OT-II cells with ova APLs where lowering the TCR signal strength decreased Th17 differentiation and promoted the differentiation of Tregs.

The role of Th17 cells in the immune mediated diseases cannot be qualified as either only pathogenic or protective [243]. For instance, although Th17 cells have been suggested to play a pathogenic role in the development of inflammatory bowel disease, blocking IL-17A with a monoclonal antibody did not improve, or even exacerbated disease in patients suffering from

Crohn's disease [243]. It was therefore proposed, that Th17 cells can be either pathogenic or non-pathogenic, the latter state being characterized by co-expression of anti-inflammatory cytokine IL-10 [244], [245]. Interestingly, using an IL-10 reporter mouse it could be demonstrated, that under Th17 polarizing conditions, trans-Ned 19 promoted the differentiation of IL-17A, IL-10 co-producing and IL-10 single-producing cells. Thus, trans-Ned 19 skewed Th17 differentiation towards a more regulatory phenotype, suggesting its possible application in the management of T-cell mediated immune diseases. In contrast, an analogous IL-10 promoting effect was not observed under other *in vitro* polarizing conditions.

Moreover, as shown in figure 6, trans-Ned 19 did not affect the differentiation of Th1 cells. Ali *et al.* reported that Ned 19 inhibits synthesis of IL-17 and IFNγ from Th17 and Th1 cells, respectively [160]. In this study however, the concentration of trans-Ned19 used was 100 μM. In ours hands this concentration completely blocked proliferation of naïve $CD4^+$ T cells and to study modulatory effect of trans-Ned 19 on $CD4^+$ T cell differentiation concentrations of 25 μM and 50 μM were chosen. Furthermore, as the results obtained with BZ194 suggested a link between NAADP and TGF-β1 signaling, a Th17 differentiation assay with high TGF-β1 concentrations was performed. Interestingly, trans-Ned 19 inhibited the differentiation of Tregs, but promoted the differentiation of Th17 cells at these polarizing conditions. Noteworthy, these observations are opposite to the results obtained with use of BZ194. The impact of trans-Ned 19 reciprocates the inhibitory effect of IL-6 signaling on Treg differentiation [232]. Further, IL-6 signaling was reported to drive IL-10 synthesis in Th17 cells [245]. Together, these results may suggest that Ned-19 is exerting its effects through potentiating IL-6 signaling in T cells. Moreover, reduction of Ca^{2+} signaling by trans-Ned 19 could directly inhibit Treg differentiation, as Foxp3 expression is induced by NFAT [69], [246]. These hypotheses requires however further and more detailed investigation.

To conclude, interference with NAADP signaling pathway with trans-Ned 19 inhibits TCR evoked Ca^{2+} signaling and proliferation of naïve $CD4^+$ T cells. Moreover, trans-Ned 19 promoted the differentiation of non-pathogenic Th17 cells *in vitro* and did not significantly affect the differentiation of $CD4^+$ T cells into other subsets. One could speculate that trans-Ned 19 potentiates IL-6 signaling.

4.3. BZ194 and trans-Ned 19 have opposing effects on the Th17 and Treg differentiation *in vitro*

Ratiometric microscopy and *in vitro* proliferation assays performed with BZ194 and trans-Ned 19 revealed that both antagonists of NAADP signaling inhibit a TCR-induced increase in $[Ca^{2+}]_i$ and proliferation of primary naïve murine $CD4^+$ T cells. Moreover, BZ194 and trans-Ned 19 both influenced the differentiation of naïve $CD4^+$ T cells *in vitro*. However, two compounds differently affected the fate decisions in naïve $CD4^+$ T cells. The differential effects of BZ194 and Ned 19 on the differentiation of naïve $CD4^+$ T cells recapitulates the controversy regarding the target ion channel of NAADP [247], [248]. Some evidence suggest that NAADP causes $[Ca^{2+}]_i$ increase by opening RYR1 and releasing Ca^{2+} from the ER, while other data imply that TPC channels in endosomes and lysosomes are NAADP target channels [164], [249]. Importantly, conflicting data regarding the identity of the cytoplasmic target of NAADP emerged from the use of different cell line systems, different methods of introducing NAADP into the cells and distinct chemical antagonists of NAADP induced calcium release [153]. At least three hypotheses explaining the different impact of used NAADP antagonists on the $CD4^+$ T cell differentiation can be formulated. First, integrating existing evidence allows to hypothesize that NAADP can act both on RYR1 and TPC channels while the chemical antagonists inhibit interactions of NAADP with specific calcium channels: BZ194 with RYR1 and Ned-19 with TPCs. Then distinct NAADP dependent Ca^{2+} channels then differently affect $CD4^+$ T-cell function. Another possible explanation as to why differentiation of $CD4^+$ T cells is affected differently by BZ194 and Ned-19 is that NAADP signaling network is more complex and either contains more molecular components or additional calcium-independent NAADP signaling events occur. Lastly, different effects of BZ194 and Ned-19 on the differentiation of $CD4^+$ T cells can be explained by non-specific off target effects of these compounds.

The first hypothesis that could explain the different impact of BZ194 and trans-Ned 19 on the differentiation of $CD4^+$ T cells, despite having the same effect on TCR induced $[Ca^{2+}]_i$ increase and proliferation, is that the two compounds interact with distinct calcium channels. Dammerman *et al.* used membrane preparations from Jurkat T cells and demonstrated that BZ194 inhibits NAADP facilitated opening of RYR1 channels [157]. Naylor *et al.* in turn developed Ned 19 basing on its spatial and electrostatic characteristics and demonstrated that Ned 19, due to its intrinsic fluorescence, labels endosomes in primary murine pancreatic cells [217]. Previously, Calcraft *et*

al. reported that two pore channels located in the lysosomal membranes are targets of NAADP, and primary pancreatic cells deficient in the two pore channel 2 in the lysosomal membrane did not respond to NAADP dialysis [118]. Taken together, these results demonstrate that BZ194 and Ned 19 inhibit calcium release from different calcium channels, both of which might be targeted by NAADP in primary murine T cells. Indeed, Gerasimenko *et al.* reported that NAADP can target more than one organelle within a cell [156]. It can be therefore hypothesized, that NAADP acts on both RYR1 and TPCs in murine T cells, forming small areas of increased calcium concentration (Ca^{2+} microdomains) which are then amplified through calcium-induced calcium release by IP3 receptors, ryanodine receptors or other channels forming a positive feedback loop [155]. Thus, Ca^{2+} release via both RYR1 and TPCs contribute to the global $[Ca^{2+}{}_i]$ increase upon TCR stimulation. Nevertheless, both channels are located in different cellular compartments and Ca^{2+} microdomains in those compartments can differentially influence other signaling pathways. For instance, cytokine signaling in $CD4^+$ T cells. NAADP was shown to be involved in the formation of ER-endosome contact sites, which in turn regulates the signal strength of epidermal growth factor receptor (EGFR) signaling [128]. It exemplifies that Ca^{2+} microdomains can regulate signaling of different pathways. Such a model would explain that both antagonists of NAADP signaling impact global calcium signaling and proliferation of $CD4^+$ T cells upon TCR stimulation, yet have distinct effects on $CD4^+$ T cell differentiation. Furthermore, this would mean that an increase in $[Ca^{2+}]_i$ is not only a digital signal for T cells to start proliferating, but the specific patterns of $[Ca^{2+}]_i$ in space and time have an impact on T cell fate decisions.

A second possible explanation for the different influence of BZ194 and Ned 19 on $CD4^+$ T cell differentiation involves the finding that NAADP does not bind to RYR1 or TPC channels directly, but rather through a NAADP binding protein. Lin-Moshier *et al.* used NAADP photoaffinity probes to investigate the binding site of NAADP in several mammalian cell line systems. While neither RYR1 nor TPC channels seemed to be bound by a NAADP probe, two 22 and 23 kDa proteins were bound by a photoaffinity probe, and therefore termed NAADP binding proteins (NAADP BP) [166]. This suggests that NAADP-induced Ca^{2+} release is a more complex event than a ligand opening its target channel, and secondly that NAADP influenced $[Ca^{2+}]_i$ increase might only be a part of a more complex signaling network that could control multiple cell functions. In such a model, BZ194 and Ned-19 would interfere with NAADP BP interaction with its downstream targets, and consequently impact downstream signaling processes.

A final suggested explanation for the different effects of Ned 19 and BZ194 on the differentiation of $CD4^+$ T cells are the possible off target effects of either compounds. Both compounds were designed to inhibit NAADP-induced calcium release from intracellular stores, however they have distinctive chemical structures. For example, the nicotinic acid moiety of BZ194 can indeed inhibit calcium release through RYR1 upon increase of the intracellular concentration of NAADP, while other fragments of BZ194 molecules might themselves interfere with some cellular processes. To further characterize the binding sites of BZ194 and Ned-19, it would be beneficial to perform detailed binding studies and investigate how both of these compounds influence signaling pathways involved in the differentiation of $CD4^+$ T cells. This would provide insight into molecular targets of BZ194 and Ned-19, evaluate the existence of possible off-targets of both compounds and aid the identification of the cytoplasmic target of NAADP.

To conclude, the aforementioned models of NAADP signaling in T cells require further investigation and point at an apparent complexity in NAADP signaling. Indeed, studies of NAADP signaling require detailed investigations of the identity of target calcium channels which mediate NAADP induced Ca^{2+} increase. To approach the problem with a different perspective, one could perform binding assays and identify the cellular targets of BZ194 and Ned 19 and analyze how calcium-independent signaling pathways are influenced by these compounds.

4.4. Ned 19 ameliorates tissue damage in murine models of immune-mediated inflammatory diseases

The data obtained in *in vitro* experiments with NAADP antagonists showed that NAADP plays a vital role in $CD4^+$ T cell biology and suggested that manipulation of NAADP signaling in $CD4^+$ T cells could be a therapeutic approach in the therapy of immune-mediated inflammatory diseases. It revealed that inhibiting NAADP signaling with Ned 19 inhibited TCR-induced global increase in $[Ca^{2+}]_i$ and proliferation of $CD4^+$ T cells. Moreover, the performed experiments indicated that the NAADP signaling pathway is involved in integrating signals from TCR, costimulatory receptors and cytokine receptors to steer the differentiation of $CD4^+$ T cells. Fine tuning Ca^{2+} signaling in T cells by means of modulating the function of NAADP dependent Ca^{2+} channels might be therefore considered a therapeutic approach in the management of immune-mediated inflammatory diseases.

One of the main aims of this thesis was to explore the possibility of inhibiting the NAADP signaling pathway in the management of immune-mediated diseases. The NAADP antagonists have already

been used *in vivo* previously. Cordglieri *et al.* reported that inhibiting NAADP signaling *in vivo* in rats with BZ194 ameliorated the clinical course of EAE [159]. However, the toxicity of BZ194 prevented us from using it *in vivo* in mice. Ned-19 in turn, was reported to be administered chronically in mice in a study conducted by Favia *et al.*, where antagonizing NAADP signaling resulted in inhibition of tumor growth and metastasis of melanoma cells [175]. Furthermore, chronic administration of Ned-19 did not result in significant changes in pulse rate, weight or diastolic blood pressure suggesting low toxicity [175]. These published results have prompted us to use Ned 19 in preclinical models of immune-mediated inflammatory diseases.

Administration of Ned 19 ameliorated the clinical course of anti-CD3 induced transient intestinal inflammation. This model is characterized by an excessive activation of T cells, followed by their apoptosis and a cytokine storm which induces development of Th17, Th1, Tregs and Tr1 cells [53], [54]. Intestinal inflammation is associated with weight loss and is followed by an induction of tolerance. Tolerance was suggested to be mediated by IL-10 producing T cells, including Tr1 cells that originate from Th17 cells [33]. Inhibiting NAADP *in vivo* in an anti-CD3 model resulted in an ameliorated disease course; the mice treated with Ned 19 lost less weight than the mice treated with the drug vehicle. However, the composition of leukocyte infiltrates did not mirror the *in vitro* findings. First, the *in vitro* data indicated that treatment with Ned 19 promotes the Th17 differentiation and does not affect Th1 differentiation. *In vivo,* however, upon administration of trans-Ned 19 in the anti-CD3 mediated intestinal inflammation model, the percentage of Th17 cells was unaffected and the percentage of Th17 cells co-producing IFN-γ and Th1 cells was decreased in the small intestine lamina propria. This result is in line with the hypothesis that intestinal immune pathology is mainly mediated by IL-17A and IFN-γ co-producing cells, and that Th17 cells can transdifferentiate into Th1 cells [26], [42]. It can be hypothesized, that Ned-19 skews the differentiation of Th17 cells towards a more regulatory, IL-10 secreting, phenotype, which in turn diminishes the pathogenic Th17 cell fate, and in consequence, ameliorates intestinal damage and weight loss. To address this hypothesis, experiments in mice which track the fate of IL-17 producing cells and report for actively IL-10-producing cells should be performed.

As the results obtained in the anti-CD3 intestinal inflammation model suggested that NAADP plays a role in Th1 differentiation *in vivo,* an ovalbumin immunization model was used, as it is known it is mediated by a type I immune response. The foot swelling response observed in the ovalbumin immunization model is a delayed type hypersensitivity reaction, mediated mainly by Th1 cells,

$CD8^+$ T cells and activated macrophages [219], [220]. Thus, the footpad swelling model allowed to investigate the effect of Ned 19 on the antigen specific TCR activation of $CD4^+$ T cells, and specifically, Th1 cell differentiation and function *in vivo*. The results revealed that Ned-19 modulated T cell activation and function in this model, as the treated group exhibited less pronounced foot swelling. However, no significant differences in the composition of lymphocytes in the spleens or in the draining lymph nodes was observed. The experiments had a few methodological limitations. First, the cells in the secondary lymphoid organs and not at the site of inflammation were analyzed. Second, the production of cytokines was analyzed in response to the non-antigen specific restimulation with PMA and ionomycin, which in turn upregulated cytokine synthesis, not only in ova specific cells but all circulating effector cells. Restimulating cells in an antigen-specific manner with ovalbumin peptide would result in a more specific readout. Moreover, to further discriminate antigen specific cells prior to immunization, a mouse can be transferred congenically marked $CD4^+$ T cells from transgenic OT-II mouse.

Ca^{2+} is a universal second messenger in T cells, therefore it was hypothesized that NAADP is vital for T cell activation in inflammatory conditions affecting other organ systems than intestine. The therapeutic activity of Ned 19 has thus been tested in nephrotoxic nephritis (NTN), a murine model of crescentic glomerulonephritis [250]. The results indicated that blocking NAADP was protective in this model. The pathogenesis of NTN is still not completely understood, however the significant role of Th17 cells, $Foxp3^+$ Tregs and Tr1 cells was previously reported [63], [204], [206]. The *in vitro* findings of skewing Th17 cell differentiation towards the non-pathogenic $IL\text{-}10^+$ producing phenotype can partially explain the protective properties of Ned 19 in NTN. The flow cytometric analysis of the leukocyte infiltrates in the affected kidneys, however, was not conclusive in explaining the protective effect of Ned-19. The mice treated with Ned 19 had both decreased percentage of proinflammatory Th17 and Th1 cells, as well as decreased percentage of T regulatory cells. Interestingly, the number of both $CD4^+$ and $CD8^+$ T cells was not affected by Ned 19 treatment. The experiment faced several limitations. First, only T lymphocytes were considered as possible mediators of Ned 19 mediated protection in this model, although multiple other cells have been implicated in the immune-pathogenesis of crescentic glomerulonephritis [63]. Moreover, several cytokines critical for T cell-mediated responses were not analyzed, that includes IL-10, TNF-α and GM-CSF. Nevertheless, the findings suggest that NAADP signaling is critical in the

pathogenesis of nephrotoxic nephritis and further, more detailed studies, are required to elucidate the mechanism of Ned 19-mediated protection in NTN.

One of the main aims of this study was to test whether inhibiting NAADP signaling might be an effective approach to treat immune-mediated diseases. The performed experiments revealed that, indeed, inhibition of NAADP signaling can ameliorate tissue damage in murine models of inflammatory bowel disease and crescentic glomerulonephritis, and it impaired the function of adaptive immunity in the ova immunization model. Importantly, in the course of these experiments, mice showed no overt signs of toxicity. The finding of *in vivo* efficacy of Ned 19 suggests that NAADP signaling plays an important role in the function of the immune system, and in particular in the development of immune-mediated pathology. One possible mechanism suggested by results from *in vitro* assays is that Ned-19 promotes IL-10 synthesis by Th17 cells. Th17 cells play a vital role, both in the anti-CD3-induced intestinal inflammation, and in NTN [204], [207]. A hypothesis that Ned-19 is protective in this model due to increased secretion of IL-10 by Th17 cells could be tested using an IL-17A Cre IL-10 flox mouse. Nevertheless, although Ned 19 had a significant impact on $CD4^+$ T cell activation and differentiation *in vitro*, it is difficult to attribute the protection seen *in vivo* to the sole modulatory effect of Ned 19 on $CD4^+$ T cells. Ned 19 was administered systemically and it could affect any immune or non-immune cells of the body. NAADP signaling, and in particular, its inhibition by means of Ned 19, has been shown to be critical for the cytotoxic function of $CD8^+$ T cells as well for the phagocytosis [162], [163]. Moreover, it has been shown that it is also has a significant impact on the function of non-immune cells [176], [251]. Accumulating evidence indicates that NAADP triggers Ca^{2+} signaling either via RYR1 or TPC channels and suggests involvement of additional calcium channels and accessory proteins [153], [248]. To decipher the molecular mechanism of Ned 19-mediated protection seen in the *in vivo* IMIDs models, the best approach would be to perform a loss of function experiment with RYR1 and TPC conditional knockout mice. Using various Cre drivers one could remove RYR1 and/or TPC channels from different cell populations and investigate whether deficiency of any of these channels reproduces the effects of Ned 19 administration, and further, if it removes the protective action of Ned 19. In conclusion, performed experiments suggest that the NAADP signaling pathway is a potential target of novel immunomodulatory therapies and highlights the need of further investigation of the role of RYR1 and TPCs mediated Ca^{2+} signaling in immunity and inflammation.

4.5. TRPM2 channel does not play an essential role in $CD4^+$ T cell biology in the *in vitro* assays and *in vivo* experiments performed in this study

Collective *in vivo* and *in vitro* data suggest that the NAADP pathway plays a vital role in T cell activation and differentiation, and that antagonizing NAADP might be therapeutic in IMIDs. Nevertheless, mechanistic studies of NAADP the signaling pathway requires the identification of the molecular targets of NAADP. Several ion channels were previously described to respond to NAADP *in vitro* [9]. These include RYR1, TPC1 and TPC2, TRMPL1 and TRPM2 channel [9]. Here, the role of TRPM2 channel in the differentiation and effector function of $CD4^+$ T cells using a *Trpm2*$^{-/-}$ mouse was investigated *in vitro* and *in vivo*. Obtained results suggest that TRPM2 does not play a vital role in differentiation and effector function of $CD4^+$ T cells.

TRPM2 function was reported to be modulated by multiple adenine-derived second messengers, namely cADPR, ADPR and NAADP [9]. Among these interactions, TRPM2 activation by ADPR is the most investigated one and was shown to be involved in cell physiology via mediating apoptosis [9], [172], [173], [252]. In addition, a few studies reported that NAADP and cADPR can activate the TRPM2 channel causing ion fluxes in T lymphocytes [253]. TRPM2 was linked to oxidative stress-mediated cell death in the cells of central nervous system [254], [255]. TRPM2 was reported to be expressed in T cells, to be upregulated upon TCR activation and to promote the synthesis of proinflammatory cytokines [147]. In line with these findings, *Trpm2*$^{-/-}$ mice had a partially ameliorated course of EAE, which was correlated with reduced proliferation of $CD4^+$ T cells and reduced secretion of Th1 and Th17 cytokines *in vitro* [147]. In contrast, another study also reported an ameliorated EAE disease course in *Trpm2*$^{-/-}$ mouse, however *ex vivo* analysis of T cells revealed no significant differences in T cell activation. The EAE phenotype was attributed to decreased production of CXCL2 by innate cells deficient in TRPM2 [148]. Moreover, the role of TRPM2 in calcium signaling upon TCR stimulation was questioned in the study by Wolf *et al.*, who reported that TRPM2 was not necessary for the formation of Ca^{2+} microdomains upon TCR ligation [108]. The role of TRPM2 in the T-cell biology in particular in the differentiation of $CD4^+$ T cells has not been yet systematically studied.

In order to investigate the role of TRPM2 channel in $CD4^+$ T cell differentiation *in vitro* differentiation assays were performed. These revealed that under Tr1 polarizing conditions the percentage of cells producing IL-10 was lower in *Trpm2*$^{-/-}$ cells compared to wild type $CD4^+$ T cells. TRPM2 channel did not seem to play a vital role in the differentiation of other $CD4^+$ T cell

subsets. On the contrary, the results obtained with NAADP antagonists BZ194 and Ned 19, where inhibiting NAADP signaling resulted in increased production of IL-10 and affected the differentiation of Th17 and Treg cells, suggesting that TRPM2 is not the channel whose function is modulated by NAADP antagonists.

In order to characterize the role of TRPM2 in the activation and differentiation of $CD4^+$ T cells *in vivo* the anti-CD3-induced transient intestinal inflammation model was used. The deficiency of TRPM2 did not influence the disease severity expressed by weight loss. Furthermore, the analysis of the cellular composition of lymphocytic infiltrates from the lamina propria of the mucous membrane did not reveal any significant differences. Based on *in vitro* data, TRPM2 would contribute to the immune regulatory mechanisms by supporting IL-10 production by $CD4^+$ T cells. Accumulating evidence, however, pointed at proinflammatory functions of TRPM2, such as it promoted secretion of CXL2 from innate cells in DSS colitis and EAE models [148], [174]. Lack of phenotypic difference in the anti-CD3-induced intestinal inflammation could be explained by the fact that the impaired chemokine secretion by TRPM2 deficient innate cells compensated for the impaired suppressive function of $CD4^+$ T cells. In order to rule out this possibility $Rag1^{-/-}$, which do not have B cells or T cells, but have a competent innate immune system were reconstituted with $CD4^+$ T cells, isolated either from a wild type or a $Trpm2^{-/-}$ mouse. The reconstituted mice were then treated with anti-CD3. Again, when controlling for the effect of TRPM2 deficiency in innate cells, TRPM2 did not have an effect on the disease severity and cellular phenotype in the anti-CD3-mediated model of intestinal inflammation.

In conclusion, the performed experiments suggest that the TRPM2 channel does not play a vital role in the differentiation and function of $CD4^+$ T cells.

4.6. Conclusions and outlook

The aim of the study was to investigate the role of AN-derived Ca^{2+} mobilizing second messengers in the differentiation and effector function of $CD4^+$ T cells. We used here pharmacologic antagonists to answer this question. Overall our data suggest that NAADP plays a critical role in the fate decisions and effector functions of $CD4^+$ T cells. However, as shown by the use of *Trpm2*$^{-/-}$ mice, the calcium channel TRPM2 does not seem to play a critical role in the NAADP signaling network mediating this effects in $CD4^+$ T cells.

The study of NAADP signaling is hampered by the fact that the identity of the NAADP-producing enzymes, the NAADP-degrading enzymes and the targeted ion channels remain unclear. It is therefore not entirely certain whether BZ194 and Ned 19-mediated effects on $CD4^+$ T-cell biology are mediated by antagonizing NAADP or from off target effects of the pharmacologic probes used. In order to mechanistically study the role of NAADP signaling in T cells, it is necessary to identify cellular targets of NAADP and enzymes which are responsible for the formation and degradation of NAADP. Similarly, to further study the role of other adenine-derived Ca^{2+} mobilizing second messengers, the use of calcium channel knockout mice is probably the best approach.

Together these studies could then pave a way to the development of new immunomodulatory drugs which would fine-tune activation of T cells thus ameliorating the symptoms of immune mediated diseases without resulting in excessive immune suppression.

5. Abstract

$CD4^+$ T cells orchestrate the immune response and Ca^{2+} signaling is essential for their activation and effector function. Adenine-derived Ca^{2+} mobilizing second messengers: adenine nucleotides nicotinic acid adenine dinucleotide phosphate (NAADP), adenine diphosphoribose (ADPR), and cyclic adenosine diphosphoribose (cADPR) have been identified as Ca^{2+} mobilizing secondary messengers in lymphocytes. Nevertheless, what are their functions in $CD4^+$ T-cell biology remains to be unclear. Therefore, the first aim of this thesis was to address the question of importance of adenine-derived Ca^{2+} mobilizing second messenger in $CD4^+$ T-cell activation, differentiation and effector function. *In vitro* assays were used to answer this question. Using OT-II mice and altered ovalbumin peptides, revealed that different TCR signal strength induces distinct Ca^{2+} signals and strong TCR signals promoted Th17 differentiation, whereas it inhibited iTreg differentiation. The role of NAADP signaling in the $CD4^+$ T-cell biology was studied using the pharmacological blockade of NAADP by compounds BZ194 and trans-Ned 19. Both compounds diminished Ca^{2+} signaling and proliferation of naïve $CD4^+$ T cells upon TCR stimulation. Interestingly, BZ194 decreased differentiation of Th17 cells and promoted development of iTregs *in vitro*, without having a significant effect on the Th1 differentiation. The other antagonist of NAADP signaling, trans-Ned 19, promoted Th17 and inhibited iTregs differentiation *in vitro*. Second, we used *in vivo* administration of trans-Ned-19. We found that trans-Ned-19 ameliorates disease severity in anti-CD3 mediated intestinal inflammation and nephrotoxic nephritis. The second aim of this thesis was to identify the molecular targets of adenine-derived second messengers in $CD4^+$ T cells. TRPM2 channel is a putative target of NAADP and ADPR. $CD4^+$ T-cell differentiation *in vitro* assays and investigation TRPM2 channel *in vivo* in the anti-CD3 model of intestinal inflammation revealed TRPM2 channel is not important for CD4+ T-cell biology in this model. Taken together, adenine derived Ca^{2+-}mobilizing second messengers play a vital role in $CD4^+$ T-cell biology. However, the identity of their target Ca^{2+} channels remains unclear. Dissection of adenine nucleotide mediated Ca^{2+} signaling in T cells requires the use of knockout mice deficient in RYR1 or TPC, enzymes producing and metabolizing the AN-derived second messengers and the accessory molecules, such as NAADP binding protein. Knowing the mechanics of the adenine nucleotide mediated Ca^{2+} signaling can contribute to the development of a novel class of immune modulating medication.

6. Zusammenfassung

$CD4^+$ T-Zellen steuern die Immunantwort und ihr Aktivierung und die Effektorfunktion ist von den Ca^{2+} Signalwegen abhängig. Adeninnukleotide (AN) Nikotinsäureadenindinukleotidphosphat (NAADP), Adenindiphosphoribose (ADPR) und cyclische Adenosindiphosphoribose (cADPR) wurden als Ca^{2+} mobilisierende sekundäre Botenstoffe in Lymphozyten identifiziert. Welche Kalziumkanäle von diesen sekundären Botenstoffen moduliert werden und welche Funktionen sie in der $CD4^+$ T-Zellbiologie haben, muss noch geklärt werden. Das erste Ziel dieser Arbeit ist es daher, die Bedeutung der von Adenin abgeleiteten Ca^{2+}-mobilisierenden Second Messengern, für die Aktivierung, Differenzierung und Effektorfunktion von $CD4^+$ T-Zellen zu klären. Wir verwendeten ein *in vitro* System, um diese Frage zu beantworten. Unter Verwendung von OT-II-Mäusen und veränderten Ova Peptiden konnten wir zeigen, dass unterschiedliche TCR-Signalstärken unterschiedliche Ca^{2+}-Signale induzieren. Starke TCR-Aktivierung förderte die Th17-Differenzierung, während sie die iTreg-Differenzierung hemmte. Die Rolle der NAADP-Signalübertragung in der $CD4^+$ T-Zellbiologie wurde unter Verwendung der pharmakologischen Blockade von NAADP mithilfe der Substanzen BZ194 und trans-Ned 19 untersucht. Beide Substanzen verringerten das Ca^{2+}-Signal und die Proliferation von naiven $CD4^+$ T-Zellen bei TCR-Stimulation. Interessanterweise verringerte BZ194 die Differenzierung von Th17-Zellen und förderte die Entwicklung von iTregs *in vitro*, ohne einen signifikanten Einfluss auf die Th1-Differenzierung zu nehmen. Der andere Antagonist des NAADP-Signalwegs, trans-Ned 19, förderte Th17 und inhibierte die iTregs Differenzierung *in vitro*. Des Weiteren zeigte sich, dass *in vivo* die Anwendung von trans-Ned-19 zu einer verminderten Krankheitsaktivität bei anti-CD3-vermittelter Darmentzündung und nephrotoxischer Nephritis führt. Das zweite Ziel dieser Arbeit war es, die molekularen Ziele von Adenin-abgeleiteten Second Messengern in $CD4^+$ T-Zellen zu identifizieren. Der TRPM2-Kanal ist ein vermeintliches Ziel von NAADP und ADPR. Die Differenzierung von $CD4^+$ T-Zellen *in vitro* ergab, dass der TRPM2-Kanal für die IL-10-Produktion durch CD4 + T-Zellen wichtig sein könnte. Die Untersuchung des TRPM2-Kanals *in vivo* im Anti-CD3-Modell der Darmentzündung ergab, dass der TRPM2-Kanal für die $CD4^+$ T-Zellbiologie in diesem Modell nicht relevant ist. Zusammenfassend spielen die von Adenin abgeleiteten Ca^{2+}-mobilisierenden Second Messenger eine wichtige Rolle in der $CD4^+$ T-Zellbiologie. Welche Ca^{2+}-Kanäle durch diese Botenstoffe stimuliert werden, bleibt jedoch unklar. Zur Entschlüsselung der Adenin-Nucleotid-vermittelten Ca^{2+}-Signalen in T-Zellen wird es

erforderlich sein, Mäuse zu generieren, denen die RYR1 und TPC Ionkanäle fehlen. Alternativ wäre es auch möglich, Enzyme zu deletieren, die die von Adenin abgeleiteten Ca^{2+}-mobilisierenden Botenstoffe produzuieren oder metabolisieren, sowie akzessorische Moleküle, wie das NAADP-Bindungsprotein. Die Kenntnis der Mechanismen der AN-vermittelten Ca^{2+}-Signalisierung kann zur Entwicklung einer neuen Klasse immunmodulierender Medikamente beitragen.

7. Abbreviations

°C	Degree Celsius
2dADPR	2'-Deoxy-Adpr
^3H	Tritium
ACK	Ammonium-Chloride-Potassium
ADPR	Adenosine Diphosphoribose
Ahr	Aryl Hydrocarbon Receptor
AN	Adenine Nucleotide
APC	Antigen Presenting Cell
ATF2	Activating Transcription Factor 2
ATP	Adenosine Triphosphate
BFP	Blue Fluorescent Protein
Blimp1	Pr Domain Zinc Finger Protein 1
BM	Bone Marrow Cells
BMDCs	Bone Marrow Derived Dendritic Cells
BrdU	5-Bromo-2′-Deoxyuridine
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ions
cADPR	Cyclic Adpr
CCR6	Chemokine Receptor 6
CD	Crohn'S Disease
CD49b	Integrin A2
cDNA	Complementary Dna
CFA	Complete Freund'S Adjuvant
cGN	Crescentic Glomerulonephritis
CICR	Calcium Induced Calcium Release
c-Maf	C-Avian Musculoaponeurotic Fibrosarcoma
CNS	Central Nervous System
CNS1	Conserved Non-Coding Dna Sequence 1
CRAC	Calcium Release-Activated Ca2+ Channels
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
CXL2	Chemokine (C-X-C motif) Ligand 2
DC	Dendritic Cell
DMSO	Dimethylsuloxide

DNA	Deoxyribonucleic Acid
dNTP	Nucleoside Triphosphate
DSS	Dextran Sodium Sulphate
DTH	Delayed Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
EAG	Experimental Anti-Glomerular Basement Membrane Glomerulonephritis
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epithelial Growth Factor Receptor
ER	Endoplasmic Reticulum
Erg-2	Ets-Related Gene 2
ERK	Extracellular Signal-Regulated Kinases
EtOH	Ethanol
FACS	Fluorescence-Activated Cell Sorting
FAS	Fas cell surface death receptor
Fc	Fragment Crystallisable
FCS	Fetal Calf Serum
FIR	Foxp3-Ires-Mrfp
Foxp3	Forkhead Box P3
g	Gram
GATA3	Trans-Acting T-Cell-Specific Transcription Factor Gata-3
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
gp130	Glycoprotein 130
GvHD	Graft-Versus-Host-Disease
GWAS	Gene Wide Association Study
Gy	Gray
HEK293	Human Embryonic Kidney 293 Cells
HLA-DR	Human Leukocyte Antigen Dr
HLA-G	Human Leukocyte Antigen G
HRP	Horseradish Peroxidase
HSCT	Hematopoietic Stem Cell Transplantation
IBD	Inflammatory Bowel Disease
IBD	Inflammatory Bowel Disease
ICOS	Inducible T-Cell Costimulator
ICS	Intracellular Cytokine Staining
IEL	Intraepithelial Lymphocytes
IFN	Interferon
IgE	Immunglobulin E
IL	Interleukin
IMID	Immune Mediated Inflammatory Disease

IP_3	Inositol 1,4,5-Triphosphate
itk	Inducible T Cell Kinase
iTreg	Induced Regulatory T Cells
JNK	Jun N-Terminal Kinase
KO	Knockout
LPL	Lamina Propria Lymphocytes
MACS	Magnetic Activated Cell Sorting
mTOR	Mammalian Target Of Rapamycin
NAADP	Nicotinic Acid Adenine Dinucleotide Phosphate
NAADP-BP	Naadp Binding Protein
NF-κB	Nuclear Factor-Kb
NTN	Nephrotoxic Nephritis Model
NUDT9	Nudix Hydrolase 9
OVA	Ovalbumin
P2RXs	Purinergic Ionotropic Receptors
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death 1
RBCs	Red Blood Cell
RFP	Red Fluorescent Protein
ROI	Region Of Interest
RORγt	Rar-Related Orphan Receptor Gamma T
RT	Room Temperature
RYR	Ryanodine Receptor
SEM	Standard Error Of The Mean
SFB	Segmented Filamentous Bacteria
SMAD	Mothers Against Decapentaplegic
SOCE	Store Operated Calcium Entry
STAT	Signal Transducer And Activator Of Transcription
STIM	Stromal Interaction Molecule
T-bet	T-Box Transcription Factor Tbx21 Thymocytes
TCR	T Cell Receptor
TGF-β1	Transforming Growth Factor
TNBS	Trinitrobenzene Sulfonic Acid
TNF	Tumor Necrosis Factor
TPC	Two Pore Channel
Tr1	T Regulatory Cells Type 1
TRPM2	Transient Receptor Potential Melastatin 2
TRPV1	Transient Receptor Potential Vanilloid 1
tTreg	Thymic Derived Regulatory T Cells
UC	Ulcerative Colitis

8. List of Figures

Figure 1: Affinity of the antigen to the TCR determines calcium signaling in $CD4^+$ naïve T-cells quantitatively 44
Figure 2 Affinity of the antigen to the TCR determines calcium signaling in $CD4^+$ naïve T cells qualitatively 45
Figure 3 Affinity of the antigen to the TCR determines differentiation of $CD4^+$ T cells in vitro .. 47
Figure 4 BZ194 influences calcium signaling, proliferation and differentiation of $CD4^+$ T cells . 49
Figure 5 Effect of BZ194 on $CD4^+$ T cells is TGFβ1 dependent 50
Figure 6 Trans-Ned 19 influences calcium signaling, proliferation and differentiation of $CD4^+$ T cells in vitro 54
Figure 7 Trans-Ned 19 effect on the differentiation of $CD4^+$ T cells 55
Figure 8 Trans-Ned 19 ameliorates the disease in anti-CD3 induced transient intestinal inflammation 58
Figure 9 Trans-Ned 19 partially inhibits the antigen specific activation of T cells *in vivo* in delayed hypersensitivity model 60
Figure 10 Trans-Ned 19 ameliorates NTN 61
Figure 11 Trans-Ned 19 impact on the differentiation of $CD4^+$ T cells in nephrotoxic nephritis . 62
Figure 12 TRPM2 channel influence on the differentiation of $CD4^+$ T cells 64
Figure 13 Total body deficiency TRPM2 channel does not have a significant impact on the differentiation and effector function of $CD4^+$ T cells in anti-CD3 mediated model of transient intestinal inflammation 66
Figure 14 Deficiency of TRPM2 channel restricted to $CD4^+$ T cells does not have a significant impact on the differentiation and effector function of $CD4^+$ T cells in anti-CD3 mediated model of transient intestinal inflammation .... **Error! Bookmark not defined.**

9. List of tables

Table 1 Reagents for cell isolation, cell culture, in vitro assays and animal experiments 27
Table 2 Reagents for flow cytometry 27
Table 3 Reagents for DNA extraction and genotyping PCR 28
Table 4 Cytokines 28
Table 5 Antibodies for animal experiments, cell culture and in vitro assays 29

Table 6 Antibodies for flow cytometry 29
Table 7 Primers for genotyping PCR 30

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12.Curriculum Vitae

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10.2018 -	Medical Doctorate at Hamburg University
10.2011 - 6.2017	Medicine at Poznań University of Medical Sciences
10.2012 - 6.2015	B.Sc. in Mathematics at Adam Mickiewicz University in Poznań Thesis: *Measures of non-compactness in some metric spaces*

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Publications

1. Kempski J, Giannou AD, Riecken K, Zhao L, Steglich B, Lücke J, Garcia-Perez L, Karstens KF, Wöstemeier A, Nawrocki M, Pelczar P, Witkowski M, Nilsson S, Konczalla L, Shiri AM, Kempska J, Wahib R, Brockmann L, Huber P, Gnirck AC, Turner JE, Zazara DE, Arck PC, Stein A, Simon R, Daubmann A, Meiners J, Perez D, Strowig T, Koni P, Kruglov AA, Sauter G, Izbicki JR, Guse AH, Roesch T, Lohse AW, Flavell RA, Gagliani N, Huber S. IL22BP Mediates the Anti-Tumor Effects of Lymphotoxin Against Colorectal Tumors in Mice and Humans. *Gastroenterology*. 2020
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