Non classical regulators in autoimmune demyelination

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Tag der mündlichen Prüfung:

Weil was aufhört, wenn was Neues beginnt,
irgendjemand verliert, wenn ein andrer gewinnt.
Weil man Großes an Kleinigkeiten erkennt
und weniger oft mehr ist als man das denkt.
Weil ich weiß, dass man über kurz oder lang,
das was wirklich zählt nicht zählen kann.
Nichts zu riskieren find ich auch riskant,
das Leben ist nun mal ein Kontrastprogramm.

Mono & Nikitaman - Kontrast

Table of content

1 Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) affects more than 2.5 million people in North America and Europe, thereby being the second most frequently reason for neurological disability after trauma (1). Twice as many women as men are affected (2). First symptoms typically occur in young adulthood (3). It is an autoimmune, chronic, inflammatory, demyelinating disease of the central nervous system (CNS) (4). It is generally accepted that it is a predominantly T cell mediated disease (5), with T cells being autoreactive to myelin antigens, crossing BBB, and leading to neurodegenerative processes (6). The demyelination results in signal conduction block or conduction slowing (7). During recovery phases, inflammation and oedema decrease and remyelination is mediated via glial ensheathment, whereas axonal loss is irreversible (8).

Usually, first symptoms in the common relapsing-remitting MS course are reversible episodes of neurological disability (1). Clinical signs are very variable and are mainly determined by the location of inflammatory plaques. Symptoms can be sensory disturbances, unilateral optic neuritis, diplopia, Lhermitte's sign, limb weakness, clumsiness, gait ataxia, and neurogenic bladder and bowel symptoms (9). In patients, most of the lesions can be found in the periventricular white matter, cerebellum, brainstem, and optic nerves (10). Patients' brains exhibit axonal pathology along with immune cell infiltration. The structures of the so-called "black holes" (which can be detected via magnetic resonance imaging (MRI)) represent complete tissue loss within in a lesion (11).

It is common sense that patients can be classified into four types of disease which differ in their course of progression.

- About 85% of all patients are affected by relapsing remitting MS (RRMS). It is characterised by relapse phases followed by remission phases where symptoms disappear (probably not completely).
- The secondary progressive MS (SPMS) form often develops out of a previous RRMS. Here symptoms persist, so remission phases are absent.
- About 10% of all MS patients suffer from the primary progressive MS (PPMS) disease type. In this case symptoms worsen from the beginning, without remission or relapse phases.
- The progressive relapsing MS (PRMS) type affects less than 5% of all MS patients. This form is similar to PPMS but with intermitting flare ups of worsening of disease (2).

The pathological hallmark of MS are the plaques of inflammatory mediated demyelination in the white matter (1). The characteristics of lesions of acute MS differ from those of lesions of chronic MS (12). In general, myelinated nerve axons have a larger diameter than demyelinated nerve axons. This leads to increased conduction velocity, as well as the myelin sheath has protective and trophic roles for the axon. Break down of this framework appears very early in MS (13–15). Inflammatory demyelination, ultimately leading to transection and loss of axons, culminates in irreversible disability (12).

Most of the MS lesions are found in the spinal cord, optic nerve, brain stem and periventricular areas but generally they can appear everywhere in the CNS (16). Frequent mild meningal inflammation occurs, consisting of T and B cells, plasma cells and macrophages (17).

Up to now it is not known to which extent MS is a primarily autoimmune, infectious, or degenerative disease (18). But it is generally considered to be an immune-mediated disease. Genome wide association studies (GWAS) identified more than 50 susceptibility loci that are associated with MS. Most of them are related to immune cell functions (19). Nevertheless,

concordance in identical twins is less than 50%. Some environmental factors are associated with MS as well, none of those definitely proven (18). However, there is strong evidence for the contribution of sunlight, vitamin D and smoking (20–22). It has been hypothesised that genetically susceptibility along with an external/environmental trigger leads to the activation and transition out of the tolerant state of autoimmune T cells in the periphery (10).

Generally, the immune system cannot access the CNS that easily like it is able to do so in other tissues. Reasons are the existence of the blood brain barrier (BBB), absence of lymphatic vessels and, absence of parenchymal dendritic cells (DCs) (23–25). According to current disease models for MS, activated autoreactive T cells cross blood brain barrier, recruit leukocytes, leading to inflammation, demyelination and axonal loss (26). The BBB gains it barrier function by the junctional proteins present in its endothelial cells. Disruption of this function is due to activation or damage of these cellular components (27).

MS patients have the same numbers of T cells in their periphery that are directed against myelin as compared to healthy controls (12). But the characteristics of these T cells differ. T cells from patients have an activated phenotype, whereas those from healthy persons are in a naïve state (28, 29). The cytokines and receptors that reactive T cells from patients secrete and express show a more inflammatory pattern than those from healthy individuals (30, 31).

1.2 T cells

T cells are very important key players in vertebrate immunity. They mediate immunity against viral, bacterial, fungal, and parasitic threats to the individual, as well as they are able to deal with malignant cells. They act direct or indirect by secretion of cytokines, or by providing signals for macrophages, neutrophils, eosinophils, basophils and B cells. Aberrant recognition of autoantigens can lead to autoimmune diseases (32). T cells arise from hematopoietic stem cells that migrate to the thymus. There they get “educated”, herein undergoing positive and negative selection. This process is mainly based on their specific TCR with the reason to

delete T cells not restricted to MHC (positive selection) and T cells with specificity for autoantigens (negative selection) (33). The specificity of the TCR arises from a complex process in the thymus (somatic rearrangement by usage of recombinase activating genes) (34), building a repertoire of approximately 10^{13} possible specificities (35).

Naïve T cells become activated when encountering their TCR specific antigen presented on MHC molecules. They mature from naïve to activated T cells. Several types of T cells exist and are mainly distinguished due their expression of either CD4 or CD8 molecule on their surface. T cells carrying CD4 are termed T helper cells (Th) and respond to antigens presented on MHCII. Those T cells release cytokines to the recognised cell to stimulate it. T cells carrying CD8 are termed cytotoxic T cells and respond to antigens presented on MHCI. Those T cells can induce cell death of the MHCI-carrying recognised cell directly through the release of preloaded granules. Usually, MHCI present internal antigens and MHCII present serum antigens. $CD4^+$ T cells recognize peptides that consist of 10 and 25 amino acids, and CD8 T cells recognize peptides that consist of 8 and 10 amino acids (36, 37).

T cell recognition is mediated by systematic scanning of cell surfaces of surrounding cells. After recognition, cell surface molecules rearrange to a pattern that is effective for building a strong synapse, the so called immunological synapse. Here TCR/CD3, CD28 and CD4 or CD8 accumulate in the centre, with LFA-1 and CD45 building an outer ring (38–42). Although the role of this architecture is not yet completely understood, it is clear that this structure is needed for proper T cell activation (43). For Th cells, coupling with the antigen presenting cell can last for 12 hours, whereas only 2-3 hours being necessary for irreversible activation (44, 45). After a few hours mRNAs for cytokines are detectable, and, at the same time, secretory vesicles get loaded (46). Additionally, the cell starts to proliferate, which might be a reason for the immunological synapse to break. Cytotoxic T cells respond much

quicker, namely in a range of few minutes and target killing can occur (due to preloaded vesicles) in less than 5 minutes (47).

During adaptive immune response $CD4^+$ T cells play several critical roles. (48) They promote class switching and affinity maturation of B cells and they recruit and activate $CD8^+$ T cells, macrophages, neutrophils, eosinophils, basophils and other cell types. They can as well act directly on many other tissue cells (49).

Naïve CD4 T cells get activated after encountering their specific antigen presented on a dendritic cell and develop into T helper cells after massive proliferation. In this phase it is also determined into which Th subset they will develop. Th cells can be subdivided into groups mainly due to their cytokine expression profile and their set of transcription factors. The determination to which distinct Th subset a naïve T cell will develop, derives from the cytokines it produces itself during activation, from cytokines of the dendritic cell it recognises and other cells that are involved during differentiation (50). This process is believed to consist of two phases. One phase is the TCR driven induction phase (induction and activation of key transcription factors) and the other phase is the cytokine driven polarization phase (amplification of the expressed key factors). At least four distinct Th subsets exist and all of them have their specific marker cytokine profile. This is for Th1 IFN-γ, Th2 IL-4, Th17 IL-17, and for Treg TGF-β and IL-10. Furthermore, the subsets have their certain master regulatory transcription factor and a certain signal transducer and activator of transcription (STAT) that is for Th1 (T-bet and STAT4), for Th2 (GATA3 and STAT5), for Th17 (RORγt and STAT3), and for Treg (Foxp3 and STAT5) (51).

Major subpopulations known to be involved in MS (and EAE) and many other autoimmune diseases are Th1 and Th17. Signature cytokines of these Th subsets are mainly IFN-γ and IL-17, respectively. In Th1 cells the activation of STAT1 via IFN-γ upregulates the expression of T-bet, which again leads to upregulation IL-12 receptor β2 subunit. IL-12 acts via STAT4

and prolongs survival and IFN-γ expression of the Th1 cell. In Th17 cells the cytokines TGF-b, IL-6, and IL-21 are required for differentiation, and the factors IL-23, STAT3, RORγt and RORα are required for stabilisation (52). So Th1 as well as Th17 require activation by two related heterodimeric cytokines. This is for Th1 IL-12 and for Th17 it is IL-23 (53).

Importantly, Th cells may "switch" regarding the subpopulation they belong to. This phenomenon is termed Th cell plasticity. So Th cells under certain conditions may change the cytokine expression profile that defines them from one lineage to another (49). For example, the plasticity of Th1/Th2 cells depends on the differentiation state. The more differentiated a cell is, the less it will switch to the other subset (54). Th17 cells, for instance, are very unstable in relation to Th1 and Th2 cells (55). They seem to be plastic during all differentiation stages (49).

The aforementioned definition for Th subset based on their signature cytokines may not represent the reality found in organism, as, for instance, it is well reported that IFN-γ^+ IL-17^+ producing cells exist *in vivo* (49). They were for example found in brains of MS patients (56). In the recent years more and more Th subsets were found and then defined due to the cytokine they mainly produce (Th9 producing IL-9, Th3 producing TGF-b, Tr1 producing IL-10, Th22 producing IL-22, etc.).

Another important T helper cell type is the group of regulatory T cells. Their main task is believed to be controlling of autoimmune T cells that were not eliminated by negative selection in the thymus and are now in the periphery, thereby representing a threat for the organism (57). Treg cells are determined by their transcription factor Foxp3 and mainly produce TGF-β and IL-10 (58–60).

In blood and CSF cells from MS patients during disease exacerbation an increased IL-17 response was found (61–63). As well it was shown that deletion of IL-17 reduced severity and

recruitment of Th17 cells to inflamed tissues (64). Moreover, it was observed that in mice the induction of a Th17 response promotes experimental autoimmune encephalomyelitis (EAE, see below) (65).

1.3 EAE

Experimental autoimmune encephalomyelitis (EAE) is the most common animal model for MS because it shares clinical and pathological features (8). It was first discovered in the 1930s by immunising rabbits with spinal cord homogenates from humans, which lead to spinal cord inflammation (66). Based on this model, the procedure was refined to achieve a model more similar to the pathology of MS, including demyelination, axonal damage, and as well including episodes of relapse and remission (67). Important achievements were obtained out of animal studies using EAE. Relevant therapies were discovered by EAE. Therapy with Glatiramer acetate, mitoxantrone or natalizumab were developed after promising results obtained from EAE (18).

The principle of EAE is that immunisation of mice leads to activation of peripheral T cells by antigen presenting cells (APCs) which then cross the BBB, are restimulated within the CNS, resulting in inflammation causing demyelination, astrogliosis and recruiting of further lymphocytes (68).

Of course, there are problems with this animal model when applying it to the human disease. Generally, the evolutionary distance between mice and men results in differences in innate and adaptive immunity (69), the microbiological clean conditions the rodents are kept in leads to immunological immaturity which is different in humans (70), and genetically homogeneous mice are used, which, of course, does as well not represent the reality found in patients. A specific problem regarding EAE is the fact that in MS lesions $CD8^+$ T cells outnumber $CD4^+$ T cells, whereas in EAE $CD4^+$ T cells mainly drive the disease (71). Another important

fact is that MS occurs spontaneously, whereas in usual (non-transgenic) EAE models the disease is induced by immunisation along with distinct external triggers.

These triggers are designed to activate parts of the innate and adaptive immune system. The usage of adjuvants results in activation of innate immune mechanisms. Such an adjuvant is complete Freund's adjuvants (CFA). It usually consists of mineral oil with inactivated mycobacteria and inactivated particles or toxins of *Bordella pertussis*. Latter can be injected additionally to the adjuvants in order to enhance the action of proinflammatory T cells, or the antigen presentation, or the crossing of T cells over the blood brain barrier (72–75). It is supposed that the effect of adjuvants on T cells is mediated by APCs. APCs provide the required stimuli for the T cells by possessing pathogen recognition receptors (PRR) (76).

Several models for EAE exist. The previously mentioned procedure with the usage of adjuvants is found in the active models of EAE. Here animals get immunised directly with the specific particular antigen. As a model for relapsing remitting EAE, SJL mice are suitable using the myelin proteolipid protein ($PLP_{139-151}$) peptide (77). As a model for chronic EAE, C57BL/6 mice are used, immunising them with the myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide (78). Depending on the detailed immunisation protocol, variations of disease progression can be generated in C57BL/6 as well (79).

Further models used in research are the models that are termed ‘passive’ EAE. Here myelin specific T cells from actively immunised mice are transferred into naïve mice. Recipients, for example, can be mice from the same strain or mice that lack functional T cells. The transferred cells then induce EAE in the recipients (80–82).

Another model was constructed with transgenic mice that overexpress the TCR specific for MOG_{35-55} (83). Those mice develop an EAE spontaneously, but only at low incidence (84).

Importantly, in all models the spinal cord is the part that is attacked the most by inflammation and mice develop ascending flaccid paralysis. This again reveals a difference to the human disease MS (10).

As mentioned before, the Th cell subsets provide the two proinflammatory subsets Th1 and Th17, being mainly responsible for immunopathogenic mechanisms in EAE. Th1 clones that are adoptively transferred induce EAE, whereas mice deficient for T-bet are resistant to EAE (85–87). IL-12 is dispensable for induction of EAE. This is different for IL-23. IL-23 is required for EAE development (88). It was shown then that Th17 clones can induce EAE as well (89). So far it is not known which one of the two signature cytokines with their particular subsets is more important or whether they are equally involved (8, 90). It seems that the different cells types and their ratio correlate with different disease types and inflammatory patterns (62). This was shown in EAE similarly: Th1 mediated EAE results in classical symptoms, whereas Th17 mediated EAE results in non classical symptoms like ataxia, rolling, and proprioception defects, additionally (91, 92). Surprisingly, neither IFN-γ nor IL-17 are required for EAE (10). Consequently, studies were carried out that identified GM-CSF as a central cytokine required for EAE, as genetically deficient mice showed resistance to EAE (93). It was shown as well, that the required production of GM-CSF is that from T cells (94). Indeed, T cells producing GM-CSF can induce EAE with lack of IFN-γ and IL-17 (95). It is supposed that GM-CSF recruits inflammatory macrophages and promotes the production of IL-23 by DCs (10).

The importance of Tregs in EAE and MS was shown by adoptive transfer of those. The transferred Tregs are able to decrease disease severity (96). As well it was observed that Tregs from patients compared to healthy controls have less suppressive function (97–99).

1.4 Proteasome and immunoproteasome

The proteasome is an enzyme complex that aroused quite early in evolution, as a simplified form can be found in archaebacteria (100). Proteasomes are constitutively expressed in all cells (101) and represent the most important pathway for intracellular non-lysosomal protein degradation in eukaryotic cells (102). They were discovered more than 30 years ago (103). The proteasome consists of large macromolecules are found in the nucleus as well as in the cytosol (104). The 26S proteasome (2.5 MDa) is composed of the barrel shaped 20S (670 kDa) proteasome and of two 19S (900 kDa) regulators, which are attached to the ends of the 20S particle (105). The 19S subunit recognizes and unfolds the substrates prior to degradation. It acts like a gate to the core of the 20S complex (103, 106). The 20S proteasome has a cylindrical complex formed of four heptameric rings with a dyad symmetry structure, with the outer rings consisting of seven α-type subunits and the inner rings consisting of seven β-type subunits. The 20S subunit is responsible for the proteolytic activity of the proteasome. The active subunits are 6 of the 14 β-subunits (105). Its active subunits are β1, β2 and β5. The subunits have preferred cleavage sites and face the central chamber of the proteasome. β1 cleaves after acidic residues (caspase-like activity), β2 cleaves after basic residues (trypsin-like activity), and β5 cleaves after hydrophobic residues (chymotrypsin-like activity) (107). The proteasome generates peptides that consist of 8-9 amino acids (108). Origin of these peptides can be proteins from pathogens or cellular proteins (109), mostly derived from poly-ubiquitylated defective ribosomal products (DRIPs) (110). By removing abnormal and/or aggregated proteins in cells, proteasomes play a very important proteostatic role within the cell (103).

Proteasomes are involved in a variety of cellular processes, including cell cycle control, stress response, intracellular signalling, and MHCI antigen processing (111, 112). Latest was thought to be the unique function of proteasomes, which in the last years was found out to not

be true. The generated peptides are translocated to the endoplasmatic reticulum via TAP1 and TAP2 (transporter associated with antigen processing) where they bind to MHC I/β_1-microglobulin dimers. These complexes are then presented on the cell surface (113–115). These proteins are absent in cells of the CNS, but the expression can be induced in astrocytes and microglia under pathological conditions (116).

Cytokine exposure (which can be type I IFNs, TNF-α, or IFN-γ (117–120)) leads to rapid changes of the catalytic active subunits which are integrated into the 20S core (121). Now the subunits belonging to the so called immunoproteasome (IP) take the former positions of β1, β2, and β5 and are now termed β1i, β2i, and β5i. Synonyms of these units in mice are LMP2, LMP10, and LMP7, respectively. The referring genes are *PSMB9*, *PSMB10* and *PSMB8*. The term of the subunits came from their location in the genome. β1i and β5i were found to be within the region of MHCII (122). The neosynthesis of proteasomes under the given conditions nearly completely shifts to formation of IPs (120, 123). Moreover, the integration of the subunits results in structural changes, suggesting an optimized accessibility of the active sites (124). Generally, the induction of IPs leads to an enhanced presentation of peptides on MHCI molecules (125).

Furthermore, the IFN-γ inducible proteasome activator 28 (PA28 or 11S REG, 28kDa) can associate with the 20S IP which results in formation of the so-called hybrid proteasome (126). PA28 itself consists of PA28α and PA28β subunits and is heteroheptameric (see below).

In addition to the previously mentioned induction of the IP through cytokines, IPs are constitutively expressed in most of the immune cells, including T cells (101, 127, 128). Current research is ongoing whether IPs supplement proteasomes or replace them completely (129). As well it was found out that mixed 20S proteasomes can occur, being build-up of standard subunits and immuno subunits (130, 131). The repertoire of peptides that proteasome and IP produce is overlapping. The difference here lies in the cleavage of the termini of the

peptides (132). However, the peptidase activity of the IP is more efficient for many MHCI epitopes (133), resulting in an improved MHCI presentation, thereby triggering an efficient CTL response (134–137).

Immunoproteasomes are found to degrade poly-ubiquitylated substrates faster than standard proteasome, thereby advantaging the protein homeostasis in tissues where IPs are constitutively expressed (103).

Relative high levels of IPs are found in lymphoid tissues and immature dendritic cells and these levels can only slightly be upregulated (125, 138, 139). T cells, B cells and macrophages were also found to have high levels of IPs (125). It was shown as well that LMP2 and LMP7 mRNA are detectable in human brain (140), and that in this tissue immunoproteasomes are present additionally to standard proteasomes. In this study data furthermore point to the existence of heterogeneous immunoproteasomes (141).

IP subunits are furthermore induced by heat stress, arsenic trioxide, nitric oxide, and under conditions found in neurodegenerative diseases (142–145).

Extensive studies were performed to get insights into functions of the distinct immuno subunits. Mice deficient for fully functional IP show no general effects on T cell priming (146), but have defects in clearance rates upon infection associated with impaired survival (147–149). T cells that lack one or more immune subunit show altered proliferation upon activation by cognate antigens (135) or mitogens (deficient for LMP7 and LMP10) (37) and differ in homeostatic proliferation (150). Mice deficient for only one subunit show no hyperproliferation of T cells (37). Additionally, mice lacking LMP2 and therefore having mixed proteasomes, have an impaired lymphocyte survival as well as their DCs produce less cytokines (129). In these mice oxidised proteins are increased in brain and liver as well (151). Cells that are deficient for LMP7 show a defect in peptide supply which leads to reduced

MHCI cell surface levels (152). LMP2 poorly assembles into 20S complex, when LMP7 is missing (153). Furthermore, proteomic analyses on mice deficient for LMP7 and for LMP10 reveal a decrease in abundance and diversity of MHC I ligands (154). And additionally, immunoproteasomes in general have functions in cell proliferation and differentiation. LMP2 and LMP10 deficient mice show decreased numbers of naïve CD8 T cells (137, 155), and LMP10 deficient mice have a higher CD4:CD8 T cell ratio (150).

Moreover, several studies suggest that there is an important function for IPs to prevent auto-inflammatory diseases (156–159). IPs are supposed to have a function in controlling T cell expansion in infection. Indeed, studies on mouse models for arthritis and diabetes using a selective LMP7 inhibitor showed suppression of autoreactive immune response (160).

Dissimilar data were obtained from EAE in mice deficient for one immuno subunit: LMP2 is not required for establishing EAE (161), whereas deficiency of LMP7 exacerbates disease (162).

From all these data it is nowadays obvious that IP has an enormous wide range of functions that exceeds the historically known function in antigen presentation to $CD8^+$ T cells. IPs seem to be at the intersection of innate and adaptive immunity, by maintaining cellular proteostasis (innate) and processing pathogenic proteins for MHCI presentation (adaptive) (105) and by cleaning up upon inflammation (162). Moreover, gene expression profiling showed that the immuno subunits LMP2, LMP7, and LMP10, as well as PA28 are upregulated in the spinal cord of EAE mice (163).

1.5 PA28

PA28 was discovered independently by four different groups in the early 1990s (106, 164–166). As mentioned before PA28 is induced as well under cytokine exposure (167). It is formed of homologous α- and β-subunits that alternating build a heteropolymeric ring

structure that attaches to both ends of the 20S proteasome (168) and activates proteolysis by increasing the ability for the entrance of the peptides (169–171). The α- and β-subunits are identical in about 50% of their amino acid sequence (172). Their corresponding genes *Psme1* and *Psme2*, respectively, seem to have emerged by tandem duplication (173). In difference to the chromosomal location of the immuno subunit genes, *Psme1* and *Psme2* do not map to the MHC. In humans as well as in mice, *Psme1* and *Psme2* are located close to each other (174, 175).

Attaching of PA28 to the 20S is fully reversible and PA28 does not get modified during association (106). This attaching results in coordinated double cleavages and it leads to an increased turnover of short peptide substrates (176). PA28 itself seems to has no catalytic activity (177) and is ATP-independent itself (178). It has a half-live time of 30h (179).

The highest levels of PA28 in mice are found in spleen, thymus, and lung, but PA28 is almost absent in brain (180), as one might expect concerning MHC I distribution in these tissues. Analyses on the distribution of PA28 within the cell reveal that it is mainly located in the cytoplasm and not in the nucleus and that IFN-γ seems to have no effect on the subcellular localization (181).

Mice with a disrupted *PA28β* gene lack both subunits, α and β (182). PA28 deficient mice have no clear defect in $CD8^+$ T cells responses to certain viruses (183). PA28 deficiency reduces the level of MHCI binding peptides. Missing of immunoproteasome in these cells leads to a further increased reduction of expression of MHC, so it seems that the effects are additive, most likely due to different mechanism via PA28 and the immuno subunits act (184). Interestingly, peritoneal macrophages from PA28 deficient mice do not upregulate MHCI upon IFN-γ treatment (185). Furthermore, PA28 is found to be upregulated in dendritic cells during maturation (139).

1.6 Adenosine

Adenosine is an endogenous purine nucleoside known to be an important physiological regulator in the immune system (186–188), which leads to suppression of inflammatory response (189–194). Four adenosine receptors are identified, namely A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R (195). These receptors are coupled to heterotrimeric guanosine triphosphate-binding proteins (G proteins) and contain seven transmembrane domains (196). The physiological level from about 1µM is sufficient to activate A_1R, $A_{2A}R$ and A_3R, but not $A_{2B}R$. A_1R and A_3R couple with $G_{i/o}$ proteins which results in inhibited adenylyl cyclase (converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP)) and decreased cAMP levels, whereas $A_{2A}R$ and $A_{2B}R$ couple with G_s proteins which results in stimulated adenylyl cyclase and increased cAMP levels (197). Immune cells that acquire expression of all four adenosine receptors, recruit them stepwise in the order A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R (195). Generally, the expression pattern of the distinct receptors is very different among different cell types (198). Activated T cells express $A_{2A}R$ and $A_{2B}R$ but not, A_1R and and A_3R (186, 199).

Adenosine is typically produced intracellularly, but the physiological low extracellular levels rise due to stressed or damaged cells that release ATP (200, 201), which also leads to decreased tissue oxygen tension (187, 188). ATP is degraded to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by CD39 (apyrase) and then to adenosine by CD73 (5'-nucleotidase) (202). Extracellular adenosine has a half-life of a few seconds (203). Elevated levels of adenosine can occur as well through tissue damage resulting from inflammation. Further possibilities for adenosine level regulation are degradation to inosine by adenosine deaminase, salvage to AMP by adenosine kinase or flux through membrane nucleoside transporters when intracellular adenosine levels rise (187, 188, 204).

CD39 is as well expressed on DCs, thereby being involved in recruitment, activation and polarization of naïve T cells (205). Lymphocytes from CD73 genetically deficient mice that

were transferred adoptively into T cell deficient mice developed a more severe EAE (compared to wild type transferred lymphocytes) (206). Adenosine receptor signalling mediated migration of lymphocytes was shown to be required for EAE development. Mice deficient for CD73 were protected from EAE, as well as wild type mice treated with broad spectrum antagonist. Mills *et al.* proposed the adenosine signalling at the choroid plexus, the most likely point of entry of lymphocytes from blood to CSF, as explanation for altered migration of lymphocytes (206).

Under hypoxic conditions, CD39 and CD73 are upregulated by hypoxia-inducible factor (HIF-1) α (202, 204, 207). Furthermore, adenosine kinase is downregulated in this scenario. These facts lead to the idea that the increase in extracellular adenosine in inflammatory environment is caused by local hypoxia (208). Furthermore, damaged microcirculation and diminished blood supply can lead to the situation, that adenosine reports damage and then impedes additional damage by suppressing activated immune cells (187). This effect is mediated by the $A_{2A}R$ pathway.

Elevated levels of adenosine were found in inflammatory processes like asthma and sepsis (198), but as well in solid tumors (209–211). It is known for lymphocytes (202, 212–214), neutrophils (204, 215, 216), monocytes/macrophages (217–219), and dendritic cells (220, 221) that extracellular adenosine has inhibitory effect on these cell types.

Besides immunological functions, adenosine signalling is known to be very important in the communication of the cells of the nervous system. Adenosine is not released via exocytosis, but it acts like a extracellular signal molecule without being a neurotransmitter. Therefore, adenosine is a neuromodulator and the results of adenosine receptor activation can enhance or inhibit neuronal communication. So adenosine signalling has impact on diverse neurophysiological processes, e.g. regulation of sleep and arousal, locomotion, anxiety, cognition and memory, and furthermore on neuronal damage, maturation and degeneration

(222). Generally, adenosine is considered to be neuroprotective, but in some immunological scenarios adenosine receptor signalling worsens tissue damage mainly via $A_{2A}R$ in a delayed fashion (223, 224). Physiological concentration of adenosine in the brain is in the range of 30 to 300nM (225) but it can rise to 10 to 50µM after 15min ischemia (226). Neurons and glial cells express $A_{2A}R$ at high levels as well (196).

The $A_{2A}R$ pathway is a mechanism that downregulates inflammation (186–188). $A_{2A}R$ is known to be predominantly expressed on T cells (and immune cells generally). Binding of adenosine to $A_{2A}R$ inhibits T cell activation by inducing intracellular cAMP (194, 195). cAMP acts like an OFF signal by inhibiting several signalling pathways and interrupts proinflammatory processes (187). For example it in turn activates protein kinase A (PKA), which inhibits TCR-triggered signalling pathways (199, 227). Phosphorylation by PKA of the COOH terminal of the Src kinase (Csk) leads to phosphorylation and negative regulation of Lck kinase which consequently inhibits T cell activation (228). Culmination of $A_{2A}R$ expression in T cells is 4-6 days post activation *in vitro* (190). B cells express less $A_{2A}R$ than T cells (229).

Mice genetically deficient for $A_{2A}R$ or treatment with antagonists show increased inflammatory tissue damage (186, 230, 231). Studies on T cells showed that agonists result in impaired cytokine production and cytotoxicity, but that proliferation is diminished only marginally (208). The effect of decreased proliferation of $A_{2A}R$ signalling in T cells can be explained by inhibition of the TCR-triggered IL-2 receptor upregulation (199). The inhibition of cytotoxicity is mediated by down regulation of FasL and granule exocytosis (227, 232–234). Furthermore, $A_{2A}R$ deficient mice suffer stronger from acute hepatitis, ischemia-reperfusion injury, sepsis and toxin induced lung inflammation (230, 235–237). $A_{2A}R$ agonists suppress inflammatory bowel disease (202) and attenuate damage in colitis and ileitis (190). Importantly, in healthy $A_{2A}R$ deficient mice (in lymphoid and non lymphoid tissues) there is

no compensatory effect of the other three adenosine receptors (238). Mice heterozygous for $A_{2A}R$ show a gene-dose effect and no reserve for $A_{2A}R$ (239).

A_1R deficient mice develop severe EAE than wild type mice and those mice have more activated macrophages in the brain parenchyma. These mice show more demyelination and axonal injury (240). Interestingly, macrophages from MS patients' brains show a decreased expression of A_1R (241).

2 Material and methods

2.1 Mice

Mouse strains were as follows: PA28C57BL/6;B6CBF1(ICR)-PA28^{tm} (referred to as PA28$^{-/-}$), B6;129-Adora2atm1Dyj/J (referred to as $A_{2A}R^{-/-}$), B6.129S7-Rag1^{tm1Mom}/J (referred to as RAG1$^{-/-}$), B6.SJL-*Ptprca Pepcb*/BoyJ (referred to as CD45.1$^{+/+}$). Strains were purchased from The Jackson Laboratory (#002014 and #008461) or were kindly provided from the Charité Universitätsmedizin, Berlin (Dr. Antje Voigt, department of biochemistry and Dr. Christine Brandt, department of neuroanatomy).

2.2 Experimental autoimmune encephalomyelitis (EAE)

2.2.1 Active EAE

Protocol was as follows: female mice aged 6-10 weeks were immunised subcutaneously with 200µg myelin oligodendrocyte glycoprotein$_{35-55}$ (MOG_{35-55}, Pepceuticals, amino acid sequence: MetGluValGlyTrpTyrArgSerProPheSerArgValValHisLeuTyrArgAsnGlyLys and 800µg mycobacterium tuberculosis (H37RA, Difco #263810) emulsified by sonification on ice in 100µl complete Freund's adjuvant (CFA, Sigma #F5881) and 100µl phosphate buffer saline (PBS, Gibco #14190-144). Subcutaneous injection spots were at base of tail and flanks on left and right side. On day of immunization (d0) and on day 2 after immunization (d2) mice received 400ng pertussis toxin (PTX, List Biological Laboratories #181) intraperitoneally (i.p.).

2.2.2 Transfer EAE into RAG1$^{-/-}$

For transfer EAE, donor mice got immunized as for active EAE (but without PTX) and then sacrificed 10 days post immunization. Spleens and lymph nodes were harvested and mashed through a 70µm cell strainer (BD #352350). Erythrocytes got lysed by treating single cell

suspension with isotonic ammonium chloride solution (pH7.4). Cells were then cultured in 24 well cell culture plates (Greiner #662102) at a concentration of $4x10^6$/ml in 2ml RPMI 1640 (Gibco #21870-076) supplemented with Penicillin-Streptomycin (Gibco #15070063), glutaMAX (Gibco #35050-061) fetal calf serum (FCS, Gibco #10106-169) and β-mercaptoethanol (Gibco #31350-010) and in presence of 10µg/ml MOG_{35-55} in an incubator at 37°C with 5% CO_2.Cells were harvested on day 4, washed twice with PBS and 10 million cells in 200µl PBS got transferred i. p. into recipient mice ($RAG1^{-/-}$).

2.2.3 EAE in bone marrow chimera

Bone marrow chimeras were generated by irradiation of recipients with 10.5Gy. Donors ($CD45.1^{+/+}$) got sacrificed and tibiae and femora were taken out to harvest bone marrow via flushing them with ice cold PBS. Recipients were then narcotized sublethally with isoflurane (Actavis, PZN-7253744) and $25x10^6$ bone marrow cells were injected intracardially (i.c.) in 100µl PBS. Those mice were then held under germ free conditions in individually ventilated cages (IVC) and received drinking water with borgal 24% (Virbac, PZN-5355771) at 1ml/l ad libitum. Active EAE got induced 7 to 9 weeks after bone marrow transfer.

2.2.4 Evaluation of EAE scores

Mice were scored daily as follows: no disease = 0, tail weakness = 1, paraparesis = 2, paraplegia = 3, paraplegia with forelimb weakness or paralysis = 4, moribund or dead animals = 5, as well as intermediate steps (242).

2.3 Sacrification

Animals were sacrificed by lethal inhalation of isoflurane (Actavis, PZN-7253744). All procedures were carried out according to protocols approved by the local welfare committee.

2.4 Primary cell cultures

All cell cultures were supplemented with penicillin-streptomycin (Gibco #15070063), glutaMAX (Gibco #35050-061) fetal calf serum (FCS Gibco #10106-169) and placed in an incubator with 5% CO_2 at 37°C.

2.4.1 Bone marrow derived dendritic cells (BMDC)

Bone marrow cells were obtained as described in 2.2.3. $3x10^6$ cells were then cultured in bacterial petri dishes (BD #351029) in 10ml RPMI 1640 (Gibco #21870-076) with β-mercaptoethanol (Gibco #31350-010) as well as 10ng/ml recombinant murine granulocyte macrophage colony stimulating factor (rmGM-CSF, ImmunoTools #11343122). On day 3 another 10ml media was added to each dish and on day 6 10ml media were changed. If indicated, lipopolysaccharid (LPS, Sigma #L2762) was added at 1µg/ml on day 9 for 24 hours for activation and analysed on day 10. Cells were collected by placing petri dishes on ice for 5min and then harvested with cell scrapers (Greiner #541070).

2.4.2 Bone marrow derived macrophages (BMDM)

Bone marrow cells were obtained as described in 3.2.3. $4x10^5$ cells were then cultured in cell culture dishes (Greiner #664160) in 5ml DMEM/F-12 (Gibco #21331-020) with 20ng/ml recombinant murine macrophage colony stimulating factor (rmM-CSF, ImmunoTools #12343113). On day 3 another 5ml media was added to each dish and on day 7 cells were used as indicated. Cells were harvested by incubation with StemPro Accutase cell dissociation reagent (Life Technologies #A11105-01) according to manufacturer's protocol.

2.4.3 $CD4^+$ T cell cultures

Single cell suspensions of splenocytes were obtained as described in 2.2.2. Media was RPMI 1640 as described above. $CD4^+$ cells were negatively isolated via $CD4^+$ T cell isolation kit II, mouse (Miltenyi Biotech # 130-091-155) or via EasySep mouse $CD4^+$ T cell enrichment kit (Stemcell Technologies #19752) according to manufacturer's protocol with an outcome of at

least 85% purity of $CD4^+$ cells verified by flow cytometry. Cells were then cultured either alone at $3x10^5$/200µl/96well (Greiner #650180) or for T helper cell (Th) polarisation with irradiated (2000rad) splenocytes as antigen presenting cells (APCs) at ratio 1:5 with $3x10^6$/200µl/96well, 1µg/ml α-CD3 (ebioscience #16-0032-81), 1µg/ml α-CD28 (ebioscience #16-0281-85) and cytokines for a) Th_1 polarisation: 20ng/ml rmIL-12 (R&D Systems #419-ML-010) and b) Th17 polarisation: 10ng/ml rmTGF-β (R&D Systems #7666-MB-005), 30ng/ml rmIL-6 (R&D Systems #406-ML-005) and 10µg/ml α-IFN-γ (eBioscience #17-7313-81).

2.5 ^{3}H-thymidin assay

Splenocytes or lymph node cells were obtained as described in 2.2.2. Cells were put into 96well plates (Greiner #650180) at $3x10^6$ per well in 200µl RPMI 1640 media as described above with different stimuli as indicated. On day 2 cells were pulsed with 0.5µCi ^{3}H-thymidin per well and harvested onto filterplates on day 3. ^{3}H-thymidin incorporation was measured on a β-counter (PerkinElmer) in counts per minute (cpm). Proliferation index was determined as relative to unstimulated controls. All experiments were carried out in quadruplicates.

2.6 Phagocytosis Assay

Myelin fraction was obtained from adult mouse similarly as described by Norton and Podulso (243) and labeled with 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes #D-282). Myelin-DiI was added to BMDM at 5µg/ml for 1h in incubator.

2.7 Enzyme linked immunosorbent assay (ELISA)

Cell culture supernatants were harvested at the indicated time points and frozen (-80°C) immediately. Samples were measured in triplicates with Standard ELISA Development Kit (mIFN-γ #900-K98, mIL-17 #900-K392, IL-1β #900-K47, mTNF-α #900-K54, GM-CSF

#900-K55, all Peprotech) according to manufacturer's protocol and measured on GENios Pro plate reader (Tecan Group Ltd).

2.8 Flow cytometry

$3x10^5$ cells were stained with flow cytometry antibodies in PBS with titrated amount for each antibody for 20 min. For intracellular staining cells were stimulated with Leukocyte Activation Cocktail (BD #550583) and then fixed and permeabilised with Cytofix/Cytoperm kit (BD #554714) according to manufacturer's protocols. Flow cytometry was performed on a FACS CantoII and a FACS Calibur (both BD) with FlowJo 7.6.1 (Tree Star) software and FACSDiva 6.1.3 (BD) software, respectively.

2.9 Quantitative real time polymerase chain reaction (qRT-PCR)

RNA was isolated with TRIzol reagent (Life Technologies #15596-026) or with Direct-zol RNA MiniPrep Kit (Zymo Research #R2052) according to manufacturer's protocols. Quality and amount of isolated RNA was assessed by NanoDrop 2000 (Thermo Scientific) measurement. cDNA was synthesized with TaqMan Reverse Transcription Reagents (Applied Biosystems #N8080234) on a TPersonal Thermocycler (Biometra). Programme was 10min at 25°C, 45min at 48°C, 5min at 95°C. qRT PCR was performed with Power SYBRGreen PCR Master Mix (Applied Biosystems #4368708) and TaqMan Universal PCR Master Mix (Applied Biosystems #4364340) on a 7500 Real Time PCR System (Applied Biosystems). Programme was 2min at 50°C, 10min at 95°C and then 40 cycles with 15sec at 95°C and 60sec at 60°C. Dissociation curve was performed from 95°C to 60°C. Each sample was measured in duplicates and ΔCT method was used for calculation of fold induction values relative to houskeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.10 Primer

5'→3'

mCD80	F: TGTCCAAGGCTCATTCTTCTCTT R: CTGACTTGGACAGTTGTTCATCAAC
mCD86	F: GGCAGCACGGACTTGAACA R: TCCACGGAAACAGCATCTGA
mGAPDH	F: CCAGCCTCGTCCCGTAGAC R: CCATTCTCGGCCTTGACTGT probe: (Fam) CGGATTTGGCCGTATTGGGCG (TAMRA)
mIFN-γ	F: TGCATTCATGAGTATTGCCAAGT R: GCTGGATTCCGGCAACAG
mIL-17	F: CTCCAGAAGGCCCTCAGACTA R: GCACTGAGCTTCCCAGATCAC
mIL-1β	F: TGGGCCTCAAAGGAAAGAATC R: GCTGATGTACCAGTTGGGGAACT
mTNF-α	F: GGGCCACCACGCTCTTC R: GGCTTGTCACTCGAATTTTGAGA

2.11 Adenosine agonists and antagonists

5′-(N-Ethylcarboxamido)adenosine (Sigma # E2387), referred to as NECA

CGS-21680 hydrochloride hydrate (Sigma # C141), referred to as CGS

2.12 Immunohistochemistry

Mice were perfused through left cardiac ventricle with ice cold PBS. Brain and spinal cord were taken out and placed into 10ml 4% paraformaldehyde (PFA, Sigma #P6148) in PBS overnight. After that samples were washed three times with 10ml PBS and were then left overnight in 10ml 30% sucrose (Sigma # S0389) in PBS. They were then embedded into Tissue-Tek O.C.T. Compound (Sakura #4583), frozen at -80°C and cut on a cryostat (Leica) into 20µm thick slices. Permeabilisation of slices was achieved by treatment with 0.5% Triton X-100 (Sigma #X100) with simultaneously blocking via 1% bovine serum albumin (BSA, Sigma #A2153) and 5% normal goat serum (NGS, Sigma #G9023) in PBS for 1h at room temperature (RT). Staining was performed with primary antibodies incubated overnight and

secondary antibodies incubated for 1h at RT. Nuclei were counterstained with Hoechst 33258 (Molecular Probes #H3569). Microscopy was done with BX51 (Olympus) and quantification was made with Photoshop 5.0 (Adobe) and ImageJ 1.47 (National Institutes of Health).

2.13 Statistics

Data represent mean ± standard error of the mean (SEM). Statistics were performed with GraphPad Prism 5.04 (GraphPad Software). $p \leq 0.05$ was determined to be significant by unpaired Student's t test or by by two-way ANOVA, Bonferroni multiple comparisons (in EAE scores).

2.14 Shared data and mice

Daily evaluation of EAE animals was performed by several group members. Graphs of EAE scores are used as well for projects that stress other aspects. Furthermore, different tissues were used for different projects from the very same mice.

3 Results

3.1 PA28

It is generally accepted that the immunoproteasome has a central role in variety of processes, especially during inflammatory processes. So far, it is not fully elucidated which role PA28 in a chronic inflammation of the CNS, especially MS, plays. For this reason, the role of PA28 was investigated by the mouse model of MS, namely EAE.

Therefore active EAE in $PA28^{+/+}$ and $PA28^{-/-}$ mice was induced. As can be seen in figure 1, $PA28^{-/-}$ mice developed a significantly higher mean clinical score at the indicated days compared to $PA28^{+/+}$ mice. In addition to that, $PA28^{-/-}$ mice showed an earlier onset of disease relative to control group.

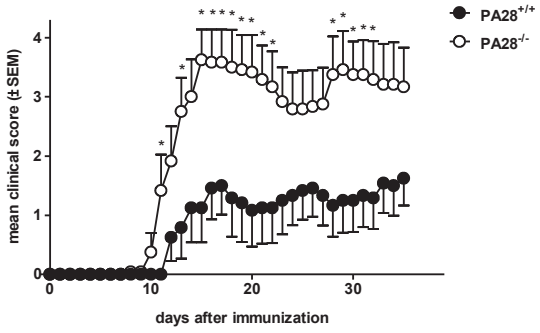


Figure 1:

Active EAE in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=6

$PA28^{-/-}$: n=6

incidence $PA28^{+/+}$: 4/6

incidence $PA28^{-/-}$: 6/6

In order to investigate basic characteristics of immune cells active in the late phase of this EAE (d35), immune cells from these mice were subsequently analysed, with focus on T cells.

Flow cytometric analysis of composition of splenocytes revealed that there were no differences in percentages of T and B cells (assessed by staining with α-$CD4^+$, α-$CD8^+$, and α-$CD19^+$ respectively) which is shown in figure 2.

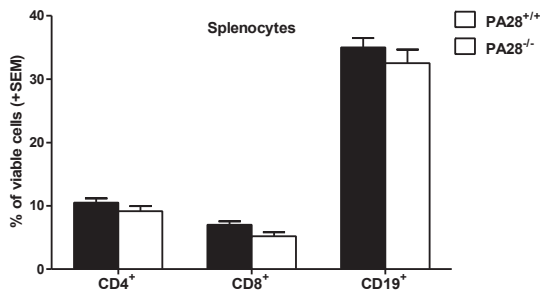


Figure 2:

Flow cytometric analysis of splenocytes from actively induced EAE in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=4

$PA28^{-/-}$: n=6

When splenocytes were restimulated antigen specifically 3 days ex vivo by culturing them with MOG_{35-55} and assessing proliferation with ^{3}H-thymidin incorporation assay, a slightly significant lower proliferation was observed in $PA28^{-/-}$ mice at a concentration of 10µg/ml MOG_{35-55} (fig. 3). However, this effect could not be confirmed when splenocytes were restimulated with a higher MOG_{35-55} concentration (50µg/ml). Besides, antigen unspecific stimulation via α-CD3 resulted in equal levels of proliferative response. Due to that, from these data no impact of PA28 in proliferative potential of encephalitogenic T cells can be concluded.

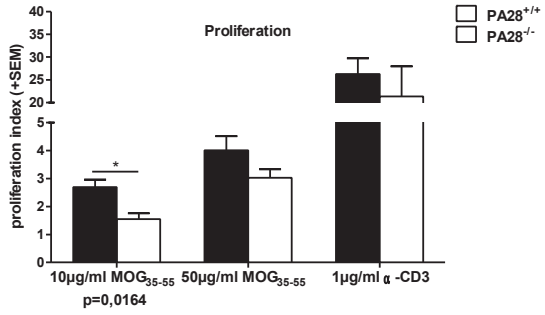


Figure 3:

Proliferative response upon restimulation of splenocytes from actively induced EAE in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=4

$PA28^{-/-}$: n=6

Additionally $CD4^{+}$ T cells were examined for their ability to produce distinct activation markers upon antigen specific and antigen unspecific stimulation. These data were obtained by culturing splenocytes with 10µg/ml MOG_{35-55} or 1µg/ml α-CD3 for two days and by assessing the expression of activation markers on after 48h by flow cytometry. As seen in

figure 4 neither the expression of CD54, CD44, CD62L, CD69, nor CD25 on $CD4^+$ T cells revealed differences between $PA28^{+/+}$ and $PA28^{-/-}$. So the potential of $CD4^+$ T cells to get activated seemed unaffected in $PA28^{-/-}$ mice, at least regarding the examined parameters.

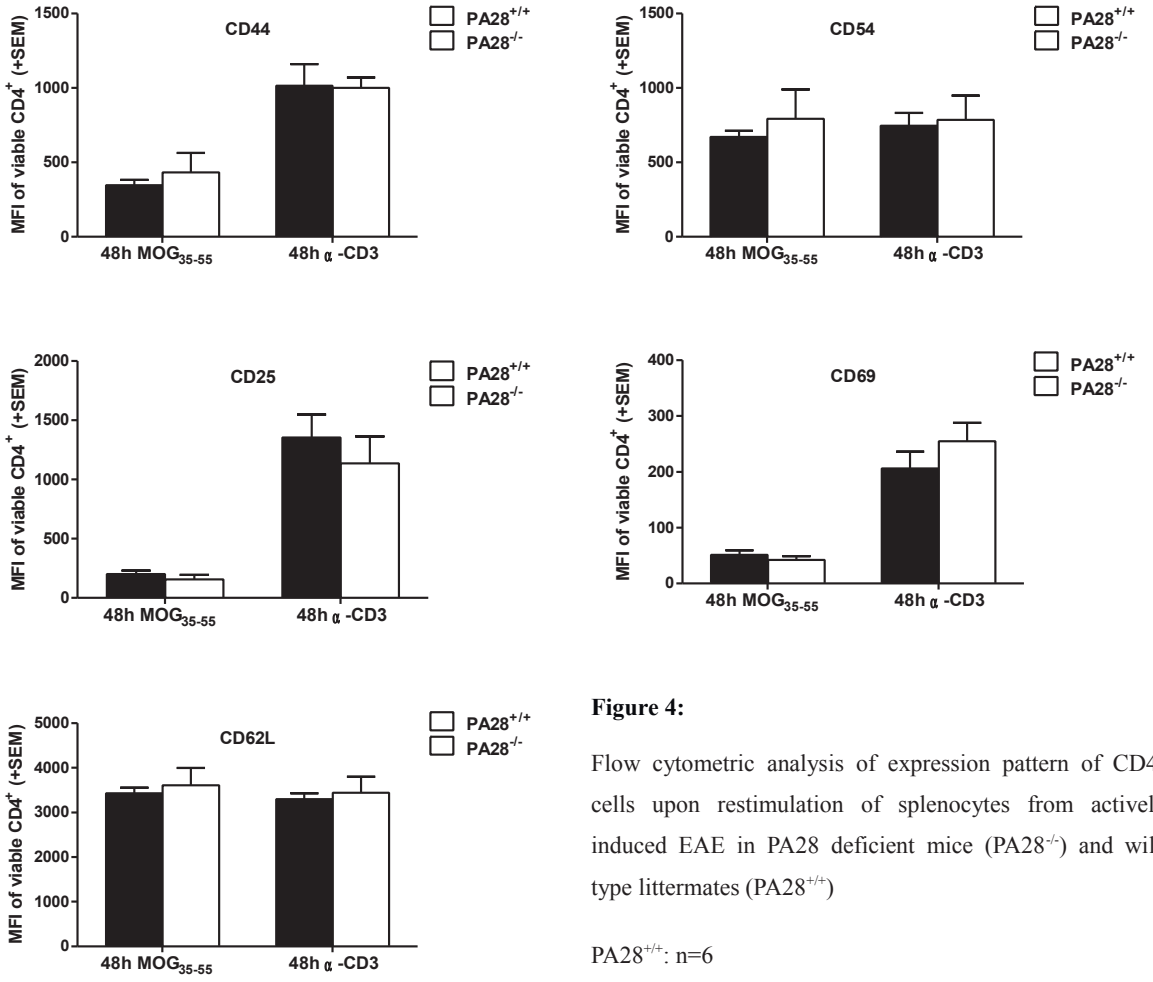


Figure 4:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=6

$PA28^{-/-}$: n=4

Flow cytometric measurement of number of $CD4^+$ T cells after stimulation showed equal amounts (see fig. 5). These results reveal as well no explanation for differences observed in the EAE phenotype.

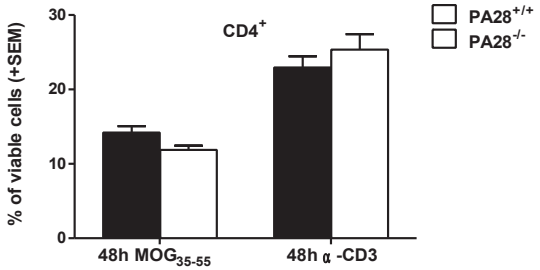


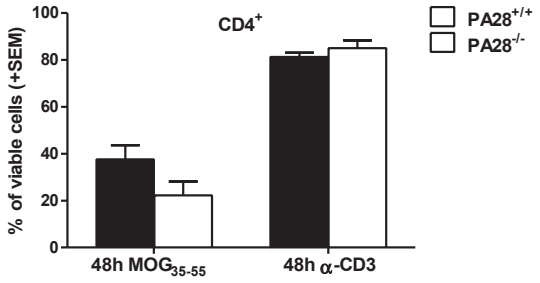
Figure 5:

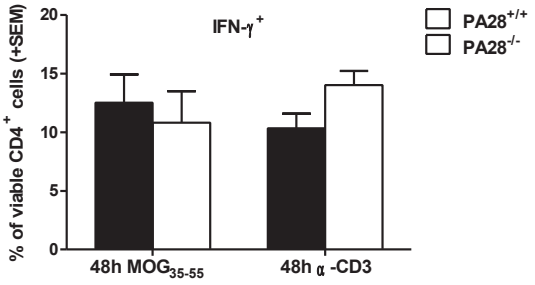
Flow cytometric analysis of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=6

$PA28^{-/-}$: n=4

Furthermore negatively isolated $CD4^+$ T cells (purity at least 93%) were tested for their capacity of production of signature cytokines of MS/EAE (IFN-γ, IL-17) upon culturing with antigen specific (MOG_{35-55}) or unspecific stimuli (α-CD3). Data were acquired by flow cytometry subsequent to fixation and permeabilisation. Results can be seen in figure 6. $PA28^{+/+}$ and $PA28^{-/-}$ cells produced same levels of indicated cytokines with no significant differences for both culture conditions. Numbers of $CD4^+$ T cells in these cultures were equal.





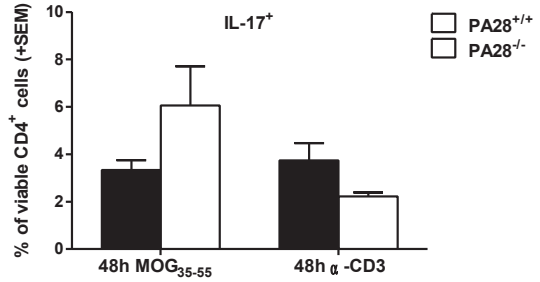


Figure 6:

Flow cytometric analysis of intracellular IFN-γ and IL-17 production in $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=6

$PA28^{-/-}$: n=4

Histological analysis of the spinal cord from these mice revealed that the Iba^{+} (macrophages/microglia) area was higher in PA28$^{-/-}$ compared to those from wild type mice as one would expect from the score and progression of disease. Same tendency was found in brainstem of the examined mice brains (see figure 7).

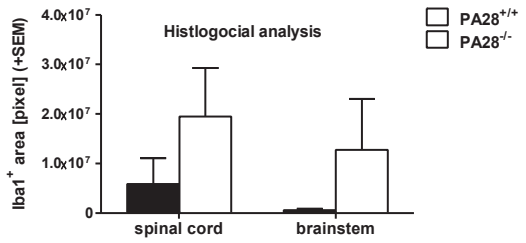


Figure 7:

Histological analysis of brainstem and spinal cord from actively induced EAE in PA28 deficient mice (PA28$^{-/-}$) and wild type littermates (PA28$^{+/+}$)

PA28$^{+/+}$: n=3

PA28$^{-/-}$: n=3

To exclude possible factors in basic immune components of PA28$^{-/-}$ mice in general that might affect EAE curve, naïve mice were tested for basic immunological parameters. For this lymph nodes (inguinal and axillar), spleen and thymus of 6 week old naïve mice were examined by flow cytometry. As one can see in figure 8 the results showed no differences in cell number in any of the immune compartments between PA28$^{+/+}$ and PA28$^{-/-}$ mice.

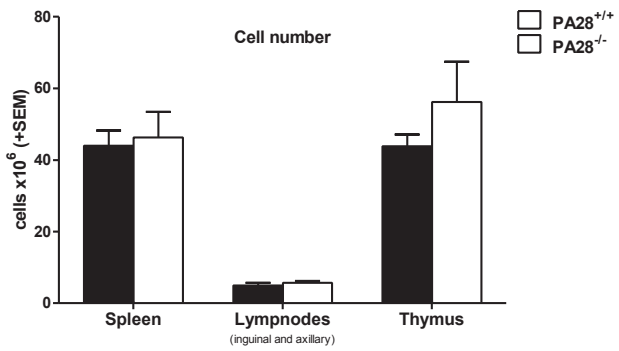


Figure 8:

Absolute cell number from lymphoid organs in naïve PA28 deficient mice (PA28$^{-/-}$) and wild type littermates (PA28$^{+/+}$)

PA28$^{+/+}$: n=4

PA28$^{-/-}$: n=4

When looking at figure 9, it can be seen that there were equal amounts of overall T cells (stained with α-CD3), CD4^{+} T cells (stained with α-CD4), CD8^{+} T cells (stained with α-CD8), double positive CD4^{+} CD8^{+} T cells (stained with α-CD4 and α-CD8) and B cells (stained with α-CD19) in all investigated organs in both mouse strains.

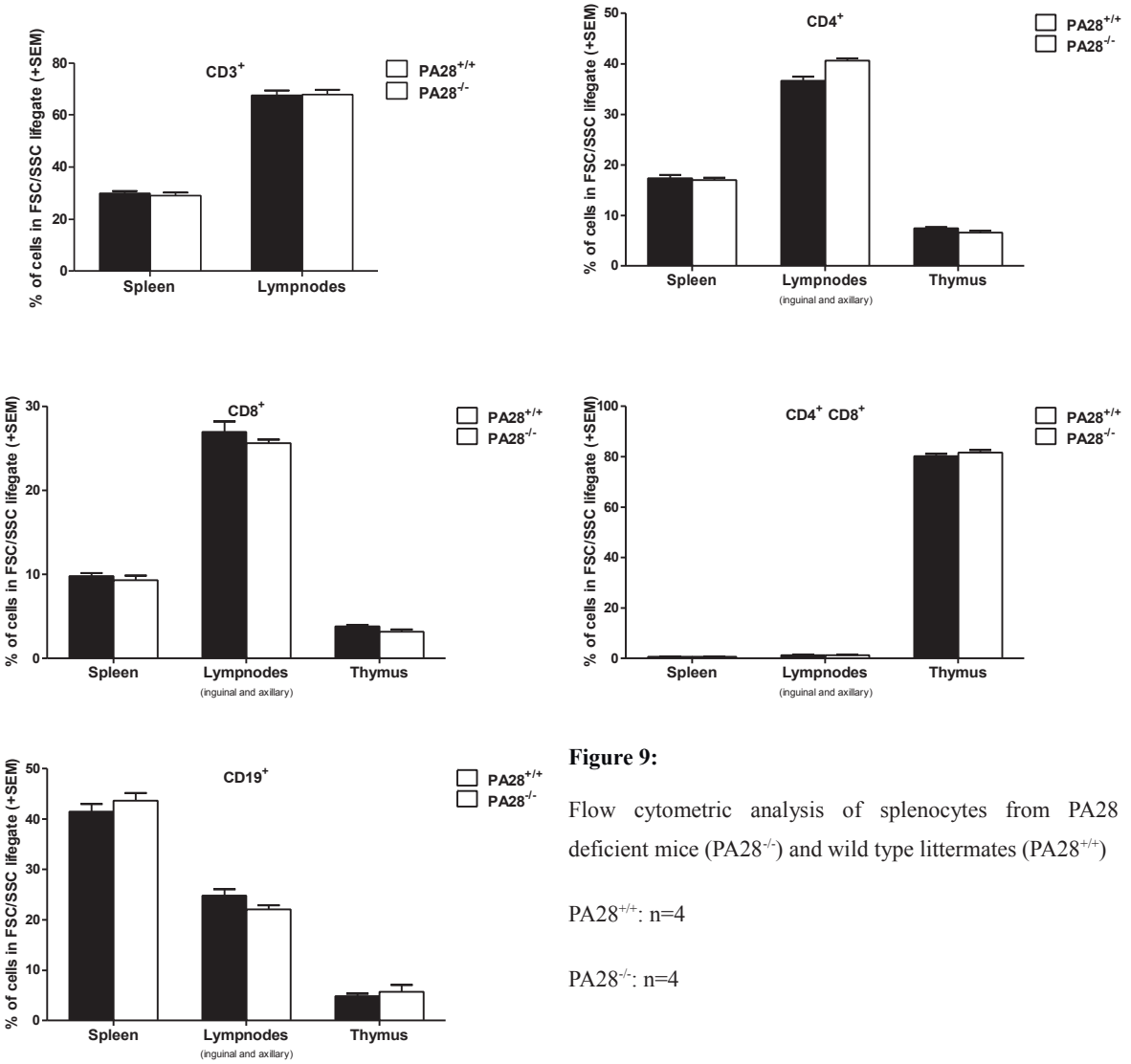


Figure 9:

Flow cytometric analysis of splenocytes from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=4

$PA28^{-/-}$: n=4

As well when looking at cells from myeloid lineage no significant changes were observed (fig. 10). $PA28^{+/+}$ and $PA28^{-/-}$ had equal percentages of macrophages (stained with α-CD11b and α-F4/80), dendritic cells (stained with α-CD11c and α-F4/80) and neutrophils (stained with α-CD11c, α-CD11b, α-F4/80 and α-Gr-1). From these data it was concluded, that in immune cell numbers of $PA28^{-/-}$ mice there were no reasons that might influence phenotype in EAE.

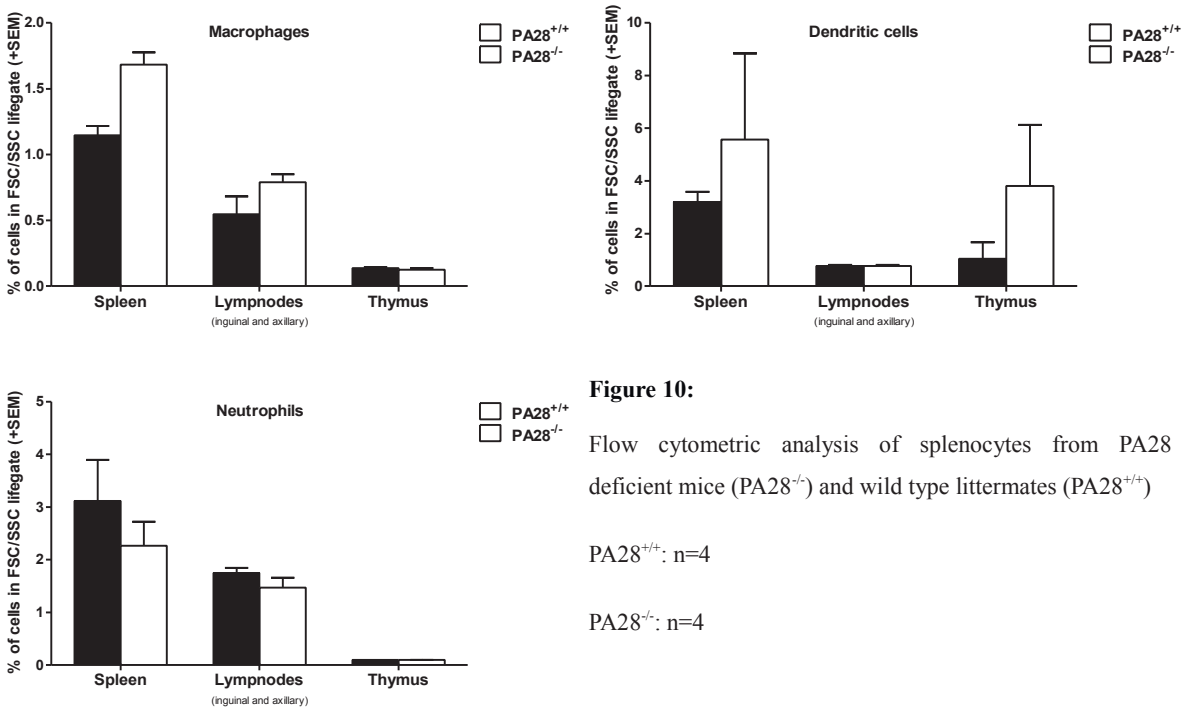


Figure 10:

Flow cytometric analysis of splenocytes from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=4

$PA28^{-/-}$: n=4

Since it is known that $CD4^+$ T cells play a major role in progression of EAE, a specific focus was given to this cell type. To minimize possible impacts of T cell characteristics from naïve mice for different EAE curves, $CD4^+$ T cells were studied more detailed. For this naïve $CD4^+$ T cells were negatively isolated from spleens from $PA28^{+/+}$ and $PA28^{-/-}$ mice and cultured for 24h without any stimulus or with 1µg/ml α-CD3 to stimulate them clonally. At the beginning and at the end of the experiment cultures were measured by flow cytometry for assessing expression of their surface molecules to get information about their abilities of $CD4^+$ T cells to get activated. Also here no significant differences were observed (figure 11). The measured $CD4^+$ T cells had comparable levels of expression for CD44, CD54, CD25, CD69 or CD62L (stained with the respective antibody). Again, these results gave no explanation for the clinical EAE phenotype.

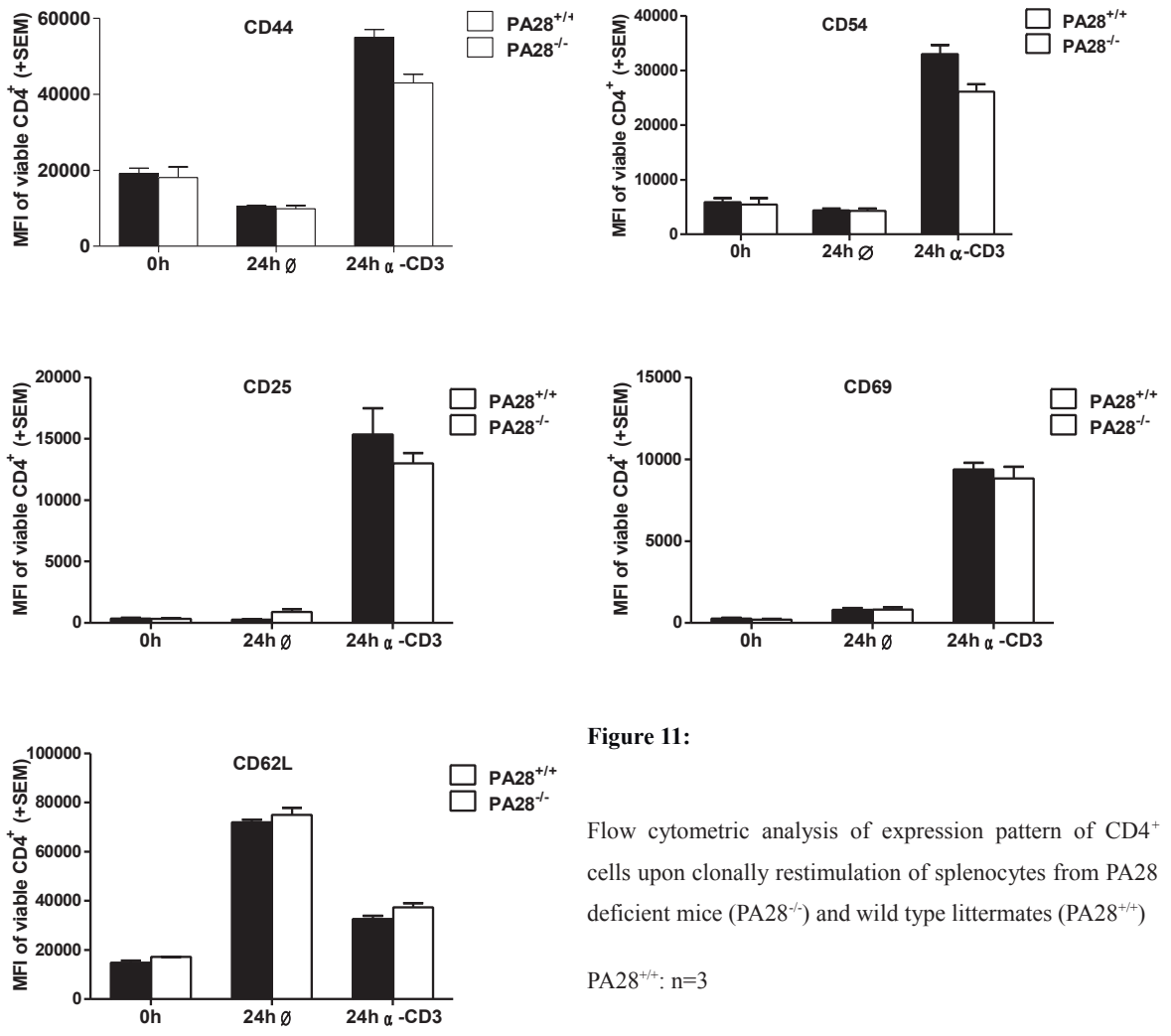


Figure 11:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon clonally restimulation of splenocytes from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

To extend the knowledge of $CD4^+$ T cell characteristics in the two mouse strains, $CD4^+$ T cells were analysed for their cytokine profile. To test this, splenocytes from $PA28^{+/+}$ and $PA28^{-/-}$ mice were taken, naïve $CD4^+$ T cells were isolated negatively and put into culture with irradiated (3000 gray) splenocytes as APCs in a ratio of 1:5. For polarisation towards Th1 rmIL-12 and for polarisation towards Th17 rmTGF-β, rmIL-6 and α-IFN-γ were used. Cultures without polarising agencies are referred to as Th0. After 5 days of culturing, cells were fixed and permeabilised and stained intracellularly with α-IFN-γ and α-IL-17. Under none of the different conditions any difference in cytokine expression levels between $PA28^{+/+}$ and $PA28^{-/-}$ was observed as well as in the percentages of $CD4^+$ T cells producing their signature cytokine (figure 12).

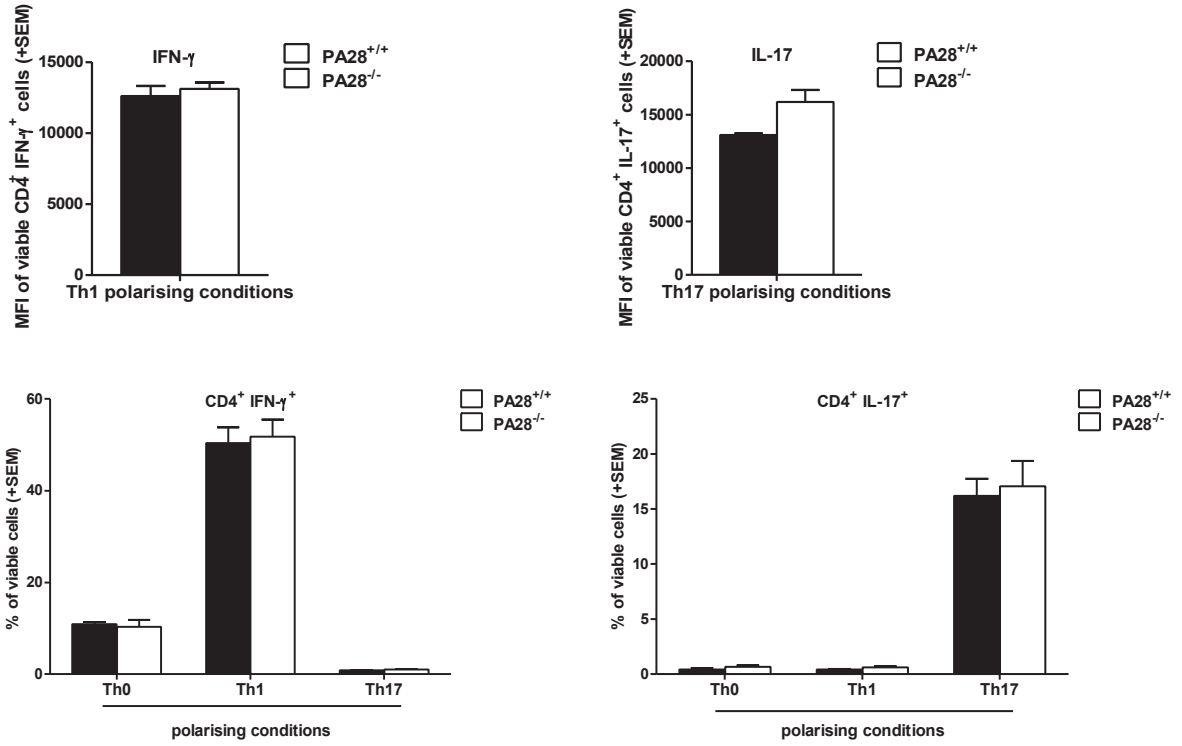


Figure 12:

Flow cytometric analysis of intracellular IFN-γ and IL-17 production in $CD4^+$ cells upon cytokine driven polarisation of splenocytes from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

Numbers of $CD4^+$ T cells were the same, too (figure 13). So these data indicated, that $CD4^+$ T cells from both mouse strains had the same potential to get polarised towards a certain direction and then to produce same amounts of their signature cytokine.

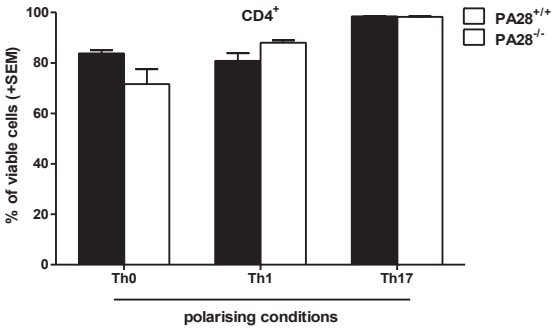


Figure 13:

Flow cytometric analysis of number of $CD4^+$ cells upon cytokine driven polarisation of splenocytes from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

When on these cells surface molecules important for activation (CD45, CD44, CD62L, CD69, CD25) were measured by flow cytometry, it became obvious that there were no differences in numbers of these surface molecules expressed on $CD4^+$ T cells between $PA28^{+/+}$ and $PA28^{-/-}$ (see figure 14).

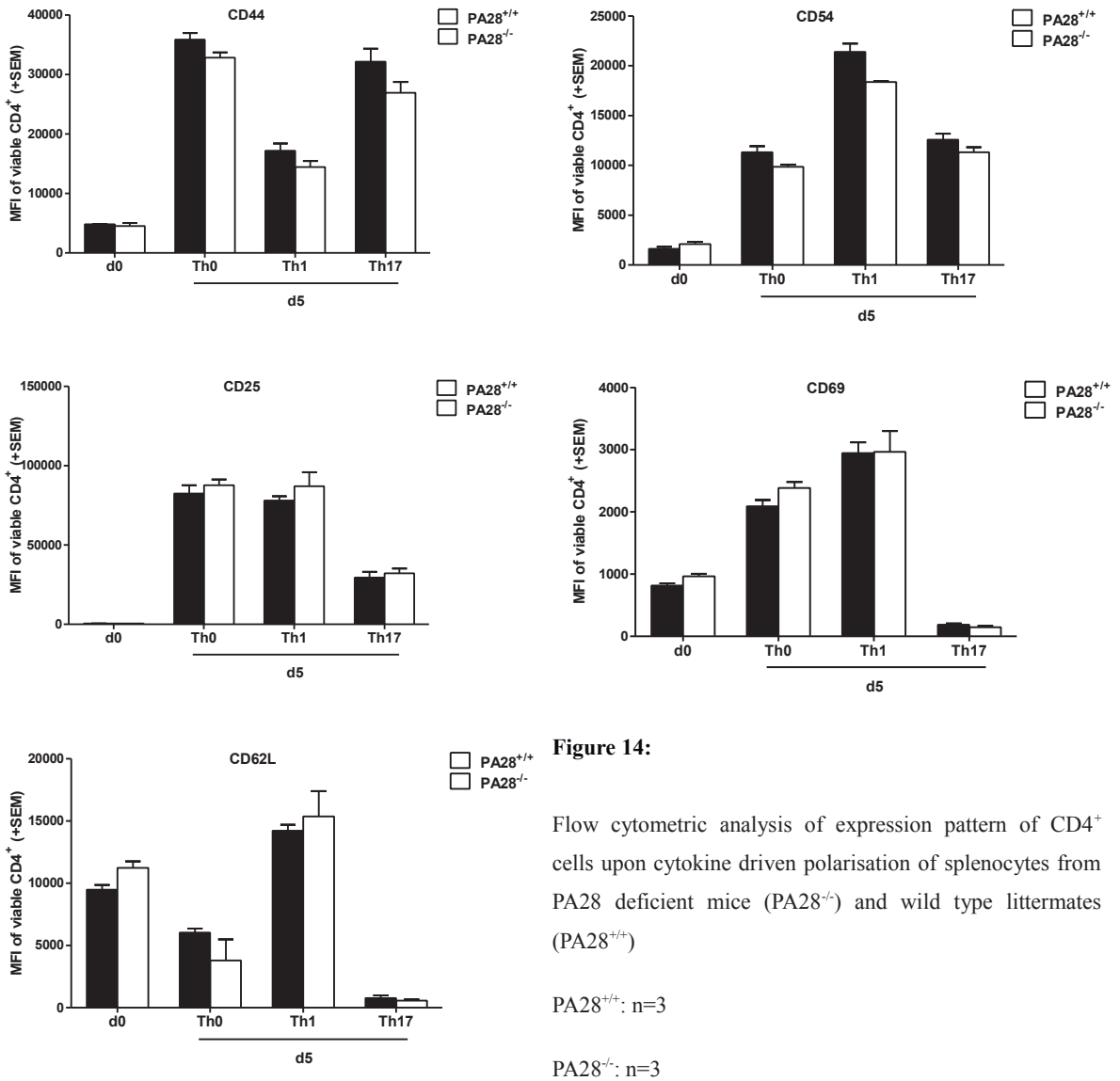


Figure 14:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon cytokine driven polarisation of splenocytes from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

Taken together, these data revealed no reason for different EAE curves between $PA28^{+/+}$ and $PA28^{-/-}$ lying in the field of basic immune cell type or basic functions of $CD4^+$ T cells.

The time point in EAE that is known to be most influenced by immune cells is in the very beginning when T cells are supposed to become encephalitogenic T cells.

So in the next step it was investigated what impact deficiency for PA28 has on immune cells (with focus on $CD4^+$ T cells) in early phase of EAE, namely before onset of disease. For this, active EAE was induced in $PA28^{+/+}$ and $PA28^{-/-}$ mice and those were analysed on day 7 after immunisation.

Flow cytometric analysis of splenocytes revealed no differences in number of cells as well in the composition of splenocytes when looking for T cells (see figure 15). Percentages of $CD3^+$ T cells, as well as the percentages of $CD4^+$ T cells and $CD8^+$ T cells were at identical levels.

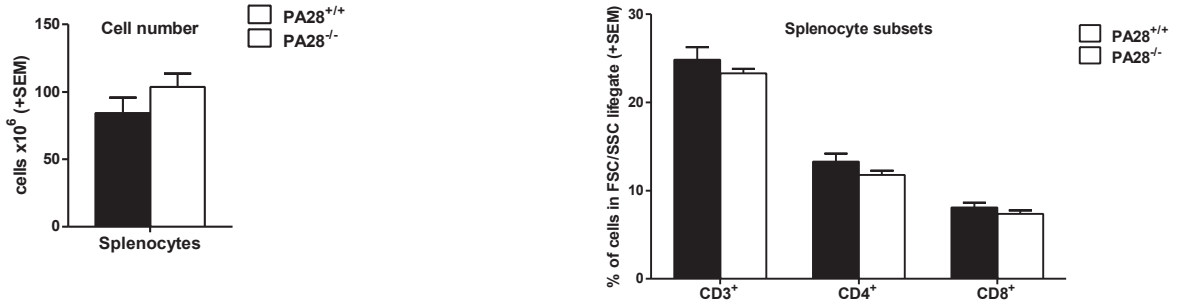


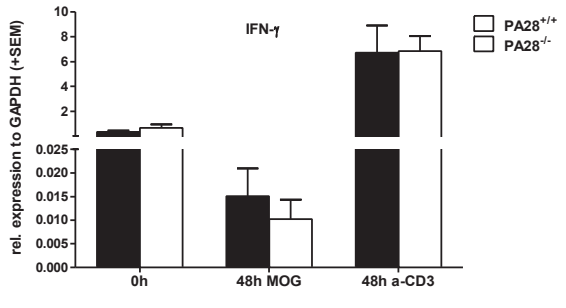
Figure 15:

Cell number and flow cytometric analysis of splenocytes from actively induced EAE prior to onset in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=5

$PA28^{-/-}$: n=5

Further investigations of these splenocytes showed that they produce same levels of IFN-γ and IL-17 which was assessed by qPCR. As well when these cells were put into culture for 48h with restimulating agencies (MOG_{35-55} or α-CD3) the levels detected via qPCR were the same (see figure 16).



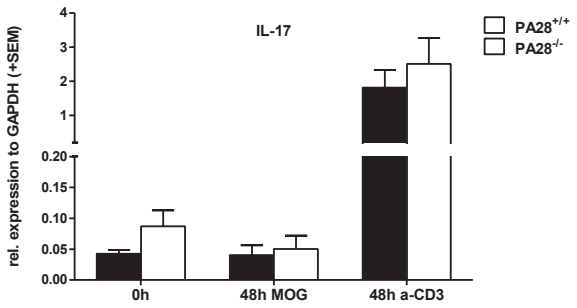


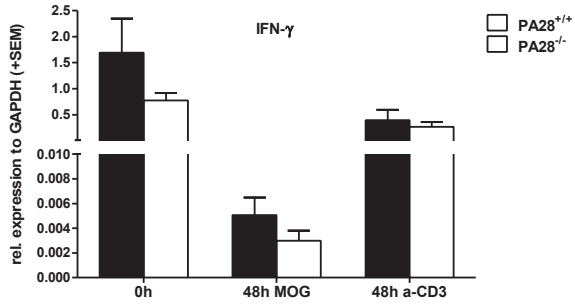
Figure 16:

qPCR analysis of IFN-γ and IL-17 upon restimulation of splenocytes from actively induced EAE prior to onset in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=4

$PA28^{-/-}$: n=4

The same was true for isolated $CD4^+$ cells: on day 0 as well under the mentioned culture conditions, $PA28^{+/+}$ and $PA28^{-/-}$ showed same levels of the important cytokines IFN-γ and IL-17 (see figure 17).



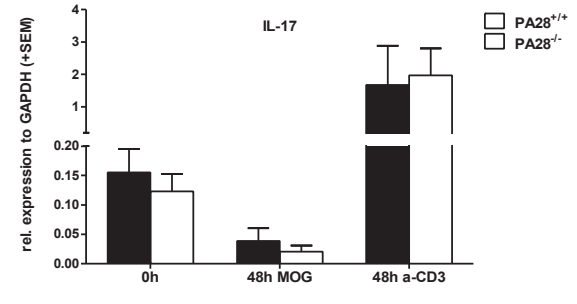


Figure 17:

qPCR analysis of IFN-γ and IL-17 upon restimulation of $CD4^+$ cells from actively induced EAE prior to onset in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=4

$PA28^{-/-}$: n=4

qPCR analysis of lymph node cells from these mice resulted in the same relative expression of IFN-γ and IL-17 to GAPDH between $PA28^{+/+}$ and $PA28^{-/-}$ (see figure 18). All these data indicate that PA28 surprisingly has no influence in early phase of EAE on the examined parameters.

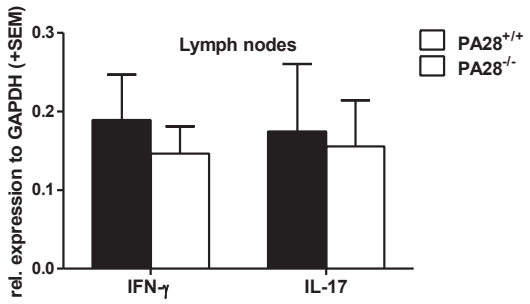


Figure 18:

qPCR analysis of IFN-γ and IL-17 upon restimulation of lymph node cells from actively induced EAE prior to onset in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=4

$PA28^{-/-}$: n=4

Looking at the proliferative potential from the isolated splenocytes revealed that they responded to the same amount when restimulated antigen specifically ($MOG_{35-5535-55}$) or unspecifically (α-CD3), which was assessed by ^{3}HT incorporation. Graphs can be seen in figure 19.

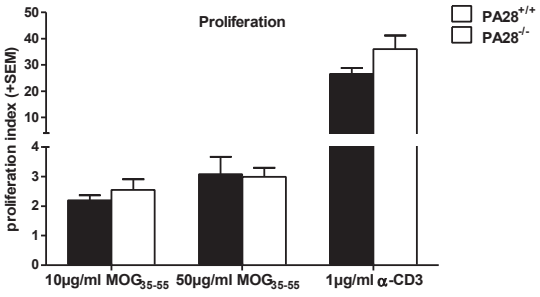


Figure 19:

Proliferative response upon restimulation of splenocytes from actively induced EAE prior to onset in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=5

$PA28^{-/-}$: n=5

Flow cytometric analysis of non T cells from splenocytes in early phase of EAE showed that $PA28^{-/-}$ mice had significantly more macrophages (stained with α-CD11b and α-F4/80), but same levels of DCs (stained with α-CD11b and α-CD11c) compared to $PA28^{+/+}$ (figure 20).

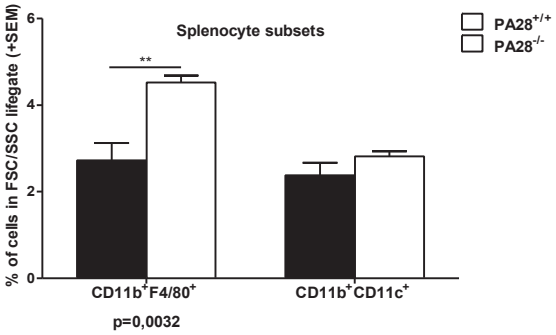


Figure 20:

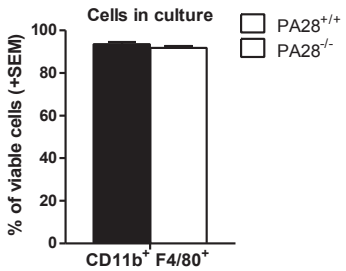
Flow cytometric analysis of splenocytes from actively induced EAE prior to onset in PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=5

$PA28^{-/-}$: n=5

As equal amounts of macrophages were found in the late phase of EAE, their potential to be generated from bone marrow cells and characteristics of these so called bone marrow derived macrophages (BMDM) were examined.

For this bone marrow cells were harvested from tibiae and femora from $PA28^{+/+}$ and $PA28^{-/-}$ mice and cultured for 7 days in presence of M-CSF (20ng/ml). After these 7 days more than 90% $CD11b^+$ $F4/80^+$ cells were present in cell cultures (stained with α-CD11b and α-F4/80) from cells of both mouse strains (figure 21). The expression levels of these surface molecules were also identical. So deficiency of PA28 does not alter the ability of bone marrow cells to generate BMDM.



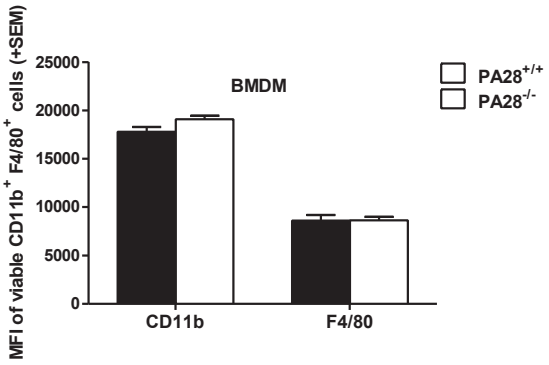


Figure 21:

Flow cytometric analysis of BMDM from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

To check the ability of these BMDM to phagocytose, the cultured cells were incubated 1h with DiI labelled myelin (myelin-DiI), which is supposed to induce phagocytosis. Again no difference between $PA28^{+/+}$ and $PA28^{-/-}$ was observed in the amount of phagocytosed myelin. Measurement was done by flow cytometry as well by TECAN reader to exclude possible methodological bias by harvesting procedures (figure 22 and 23).

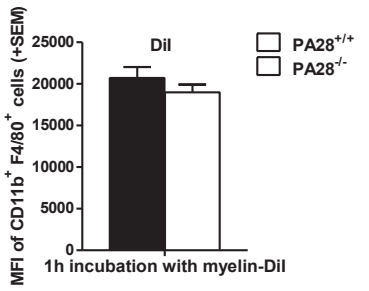


Figure 22:

Flow cytometric analysis of myelin-DiI uptake of BMDM from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

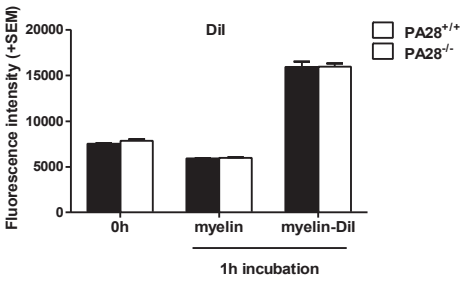


Figure 23:

Analysis (via TECAN reader) of myelin-DiI uptake of BMDM from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

Measurement of percentages of $MHCII^{+}$ cells by flow cytometry resulted in identical values between $PA28^{+/+}$ and $PA28^{-/-}$ before and after incubation with myelin (figure 24).

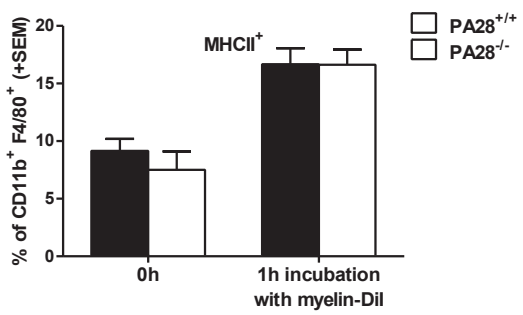


Figure 24:

Flow cytometric analysis after incubation with myelin-DiI of BMDM from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

qPCR analysis of these cells showed that IL-1β and TNF-α were upregulated in the same amount and that as well expression of CD80 and CD86 were equal at indicated time points (figure 25) between $PA28^{+/+}$ and $PA28^{-/-}$.

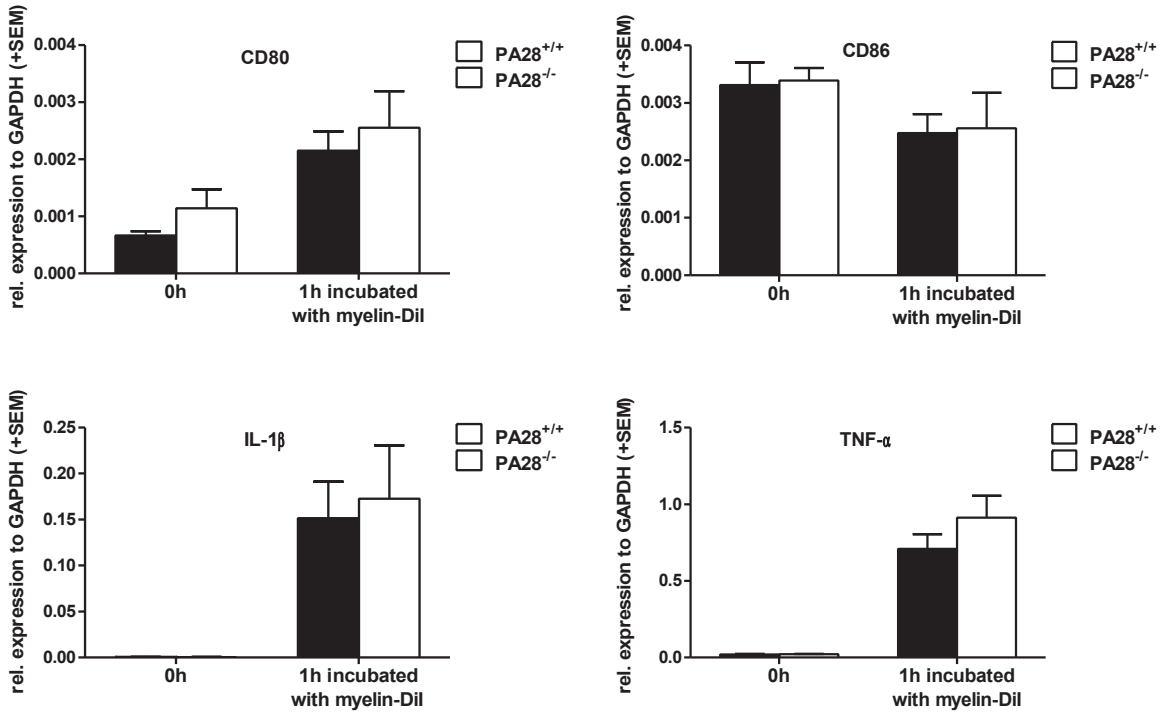


Figure 25:

qPCR analysis after incubation with myelin-DiI of BMDM from PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$)

$PA28^{+/+}$: n=3

$PA28^{-/-}$: n=3

From these data no difference was observed that would be able to serve as a hint for differences in EAE curves seen in $PA28^{-/-}$ compared to $PA28^{+/+}$ mice. Of course, a conclusion from these general data to the specific case EAE cannot be made.

To condense all these data, it seemed that factors beyond immune system are responsible for clinical EAE differences between $PA28^{+/+}$ and $PA28^{-/-}$ mice. To test this hypothesis, BM

chimera were generated in order to obtain animals expressing PA28 in the immune system but not in the other parts of the organism. $PA28^{+/+}$ and $PA28^{-/-}$ mice were irradiated and reconstituted with identical immune system by intracardial transplantation of bone marrow cells from CD45.1 mice. After 7 weeks of transplantation, active EAE was induced.

As can be seen in figure 26, $PA28^{-/-}$ mice reconstituted with $PA28^{+/+}$ immune system had a five day earlier onset of disease compared to $PA28^{+/+}$ mice reconstituted with $PA28^{+/+}$ immune system. So it seemed confirmed, that it was rather a non immune component that was responsible for differences between $PA28^{+/+}$ and $PA28^{-/-}$ mice for phenotype seen in EAE from the beginning. The late phase in this BM chimera EAE showed that differences seen in the beginning became absent later on.

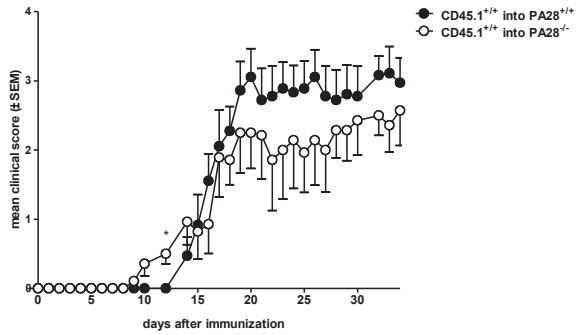


Figure 26:

Active EAE in bone marrow chimera (irradiated PA28 deficient mice ($PA28^{-/-}$) and wild type littermates ($PA28^{+/+}$) reconstituted with $CD45.1^{+/+}$)

$PA28^{+/+}$: n=9

$PA28^{-/-}$: n=7

incidence $PA28^{+/+}$: 9/9

incidence $PA28^{-/-}$: 7/7

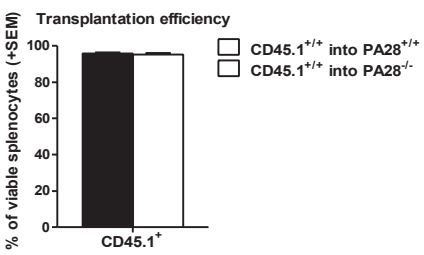


Figure 27:

Efficiency of transplantation from bone marrow chimera

$PA28^{+/+}$: n=9

$PA28^{-/-}$: n=7

3.2 Adenosine

Adenosine is a very important factor in extracellular milieu. It has an important impact on an enormous number of cellular processes. The attempt of this part of the work was to examine the impact of one of the four adenosine receptors, namely $A_{2A}R$, in immune mediated demyelinating disease of the CNS, focussed on the immune system. For this, active EAE was induced to observe disease progression in mice deficient for $A_{2A}R$ compared to their $A_{2A}R^{+/+}$ controls.

Results show a two days earlier onset in $A_{2A}R^{-/-}$ mice as well as a significantly higher peak about day 15 compared to $A_{2A}R^{+/+}$. In late phase of EAE (from day 20 on) no significant differences were visible (see figure 28). A difference in onset of disease usually points to reasons lying in the field of immune system that in this case is supposed to be altered in function by deficiency of $A_{2A}R$. Therefore analysis of immune cells was conducted at different time points of disease, i. e. during peak and remission phases. At both time points mice were sacrificed and analysed.

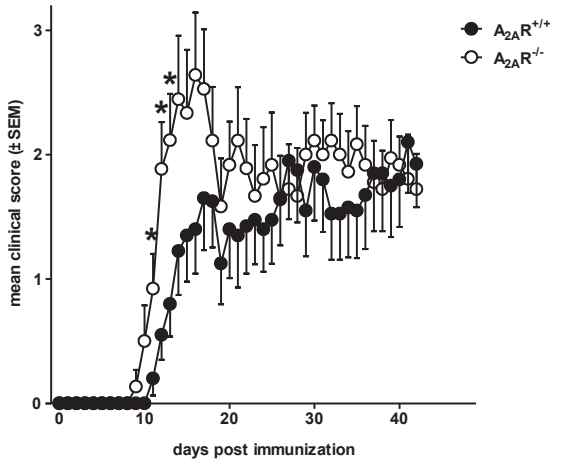


Figure 28:

Active EAE in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=10

$A_{2A}R^{-/-}$: n=9

incidence $A_{2A}R^{+/+}$: 8/10

incidence $A_{2A}R^{-/-}$: 9/9

The number of T and B cells found in splenocytes was assessed by flow cytometry (stained with α-CD4, α-CD8 and α-CD19) to examine possible reasons for differences in EAE coming there from. As can be seen in figure 29 (peak) and 30 (remission), analysis revealed equal amounts of both, T and B cells.

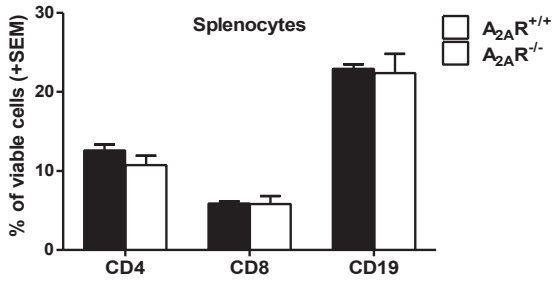


Figure 29:

Flow cytometric analysis of splenocytes from actively induced EAE during peak in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=4

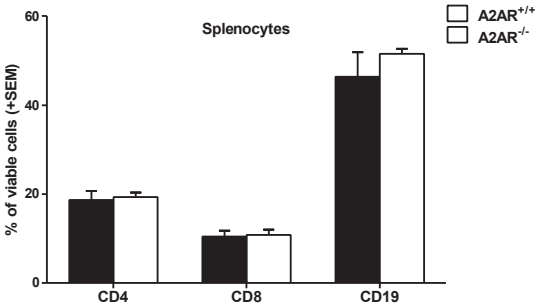


Figure 30:

Flow cytometric analysis of splenocytes from actively induced EAE in remission in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

Cultures from these cells for 48h showed that upon antigen specifically restimulation with MOG_{35-55} as well upon clonally stimulation with α-CD3, number of $CD4^+$ T cells in cultures were unaffected by deficiency of $A_{2A}R$ in cells of $A_{2A}R^{-/-}$ mice (figure 31 for peak and 32 for remission).

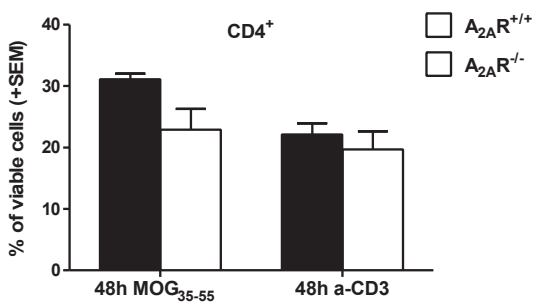


Figure 31:

Flow cytometric analysis of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE during peak in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=4

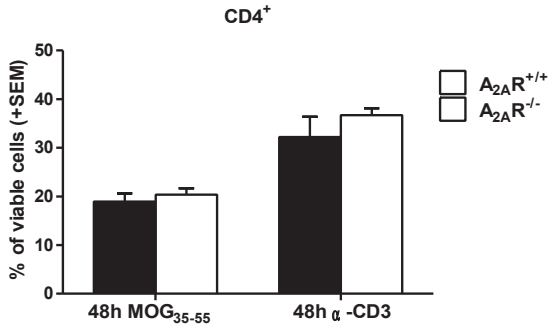


Figure 32:

Flow cytometric analysis of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in remission in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

Under all tested conditions, numbers of $CD4^+$ T cells staining positive for CD25, CD44 and CD69 were identical comparing $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ in peak and in remission (displayed in figure 33 and 34, respectively). So capability of $CD4^+$ T cells to express markers relevant for activation status seemed unaffected concerning deficiency of $A_{2A}R$.

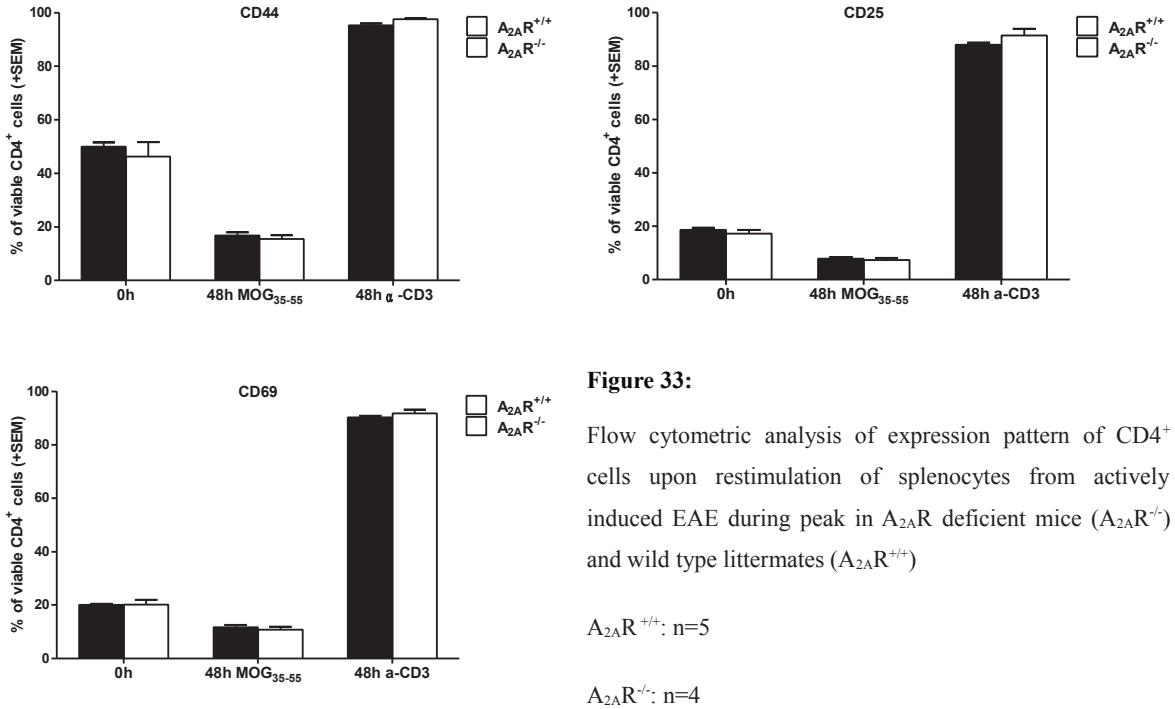


Figure 33:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE during peak in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=4

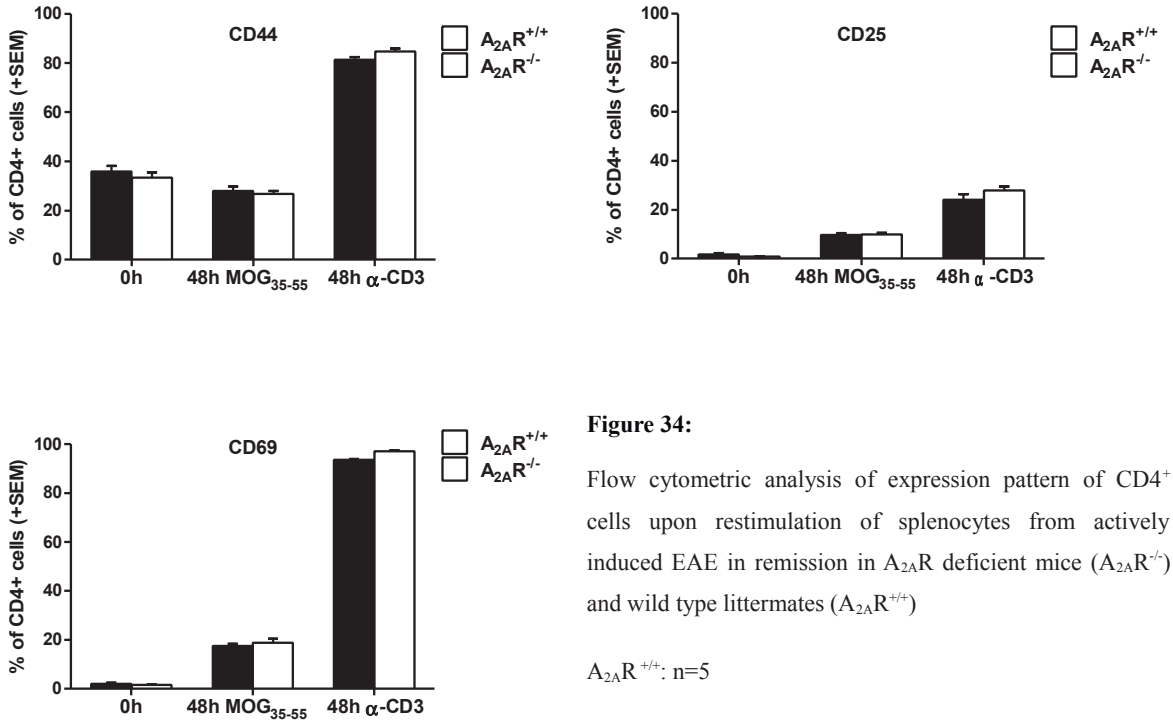


Figure 34:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in remission in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

To test proliferation potential of cells restimulated antigen specifically with MOG_{35-55}, from mice at peak of disease lymph node cells and from mice in remission splenocytes were taken for proliferation assay. This approach was chosen due to the fact that in earlier phase of disease it is assumed, activated T cells having at that time point migrated to lymph nodes, whereas in late phase they have already evaded this spot. In both cases antigen specifically restimulated cells as well as cells restimulated clonally with α-CD3 from $A_{2A}R^{-/-}$ mice proliferated to the same amount compared to $A_{2A}R^{+/+}$ mice (see figure 35 for peak and 36 for remission). So proliferative potential generally, as well as proliferative potential antigen specifically seemed unaltered by missing of $A_{2A}R$ in any of the two examined phases of disease.

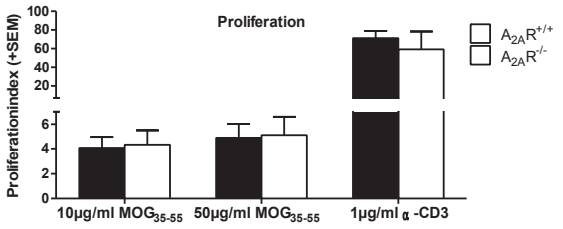


Figure 35:

Proliferative response upon restimulation of lymph node cells from actively induced EAE during peak in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=4

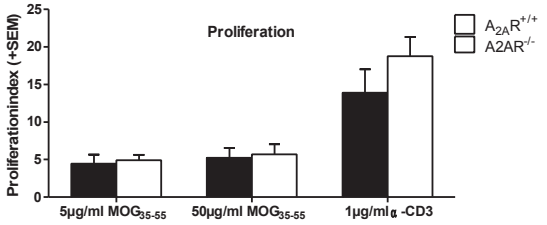


Figure 36:

Proliferative response upon restimulation of lymph node cells from actively induced EAE in remission in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

Next to test was the ability of $CD4^+$ T cells present in peak of disease to produce the cytokines that are thought to be most important in acute phase of disease. So isolated $CD4^+$ T cells were cultured with irradiated antigen presenting cells for 5 days with antigen specific stimulus and measured for intracellular cytokines (after fixation and permeabilisation) via flow cytometry. Results are given in figure 37.

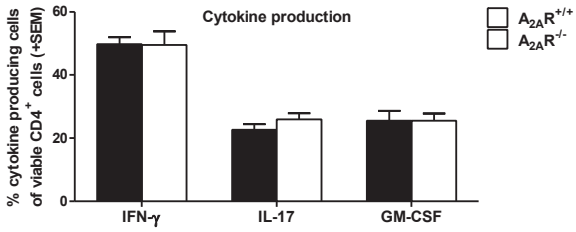


Figure 37:

Flow cytometric analysis of intracellular IFN-γ and IL-17 production in $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE during peak in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=4

Both mouse strains produced identical amounts of IFN-γ, IL-17 and GM-CSF under the previously mentioned conditions. Numbers of $CD4^+$ T cells in these cultures were uniform between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ (see fig. 38). So a difference in production of these cytokines making it possible to explain earlier onset of disease and higher peak, respectively, was not detectable.

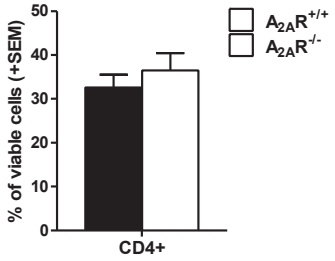


Figure 38:

Flow cytometric analysis of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE during peak in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=4

Due to the fact, that the peak as time point to investigate functions of major immune cells responsible for induction of EAE might be too late, another active EAE in $A_{2A}R^{-/-}$ and $A_{2A}R^{+/+}$ mice was induced. Here, mice were sacrificed before onset of disease and cells were examined, by this providing a possibility to look more exactly at that time point, where T cells get activated through the active immunisation protocol.

In this EAE as well, basic characteristics of immune cells were assessed revealing unaltered numbers of T and B cells of spleen and lymph node compared between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice (can be found in figure 39 and 40; flow cytometry of cells stained with α-CD4, α-CD8 and α-CD19).

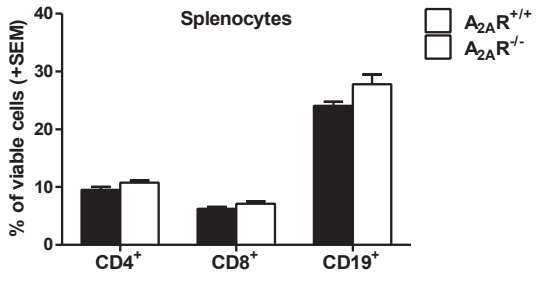


Figure 39:

Flow cytometric analysis of splenocytes from actively induced EAE prior to onset in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

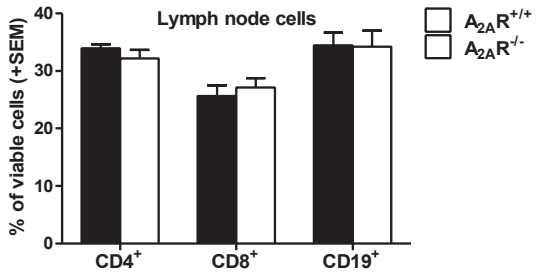


Figure 40:

Flow cytometric analysis of lymph node cells from actively induced EAE prior to onset in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

When the splenocytes were restimulated either with MOG_{35-55} or with α-CD3 they showed the same amount of CD44, CD62L, and CD69 expressed on surface of $CD4^+$ T cells (see figure 41; flow cytometry of splenocytes stained with α-CD44, α-CD62L and α-CD69).

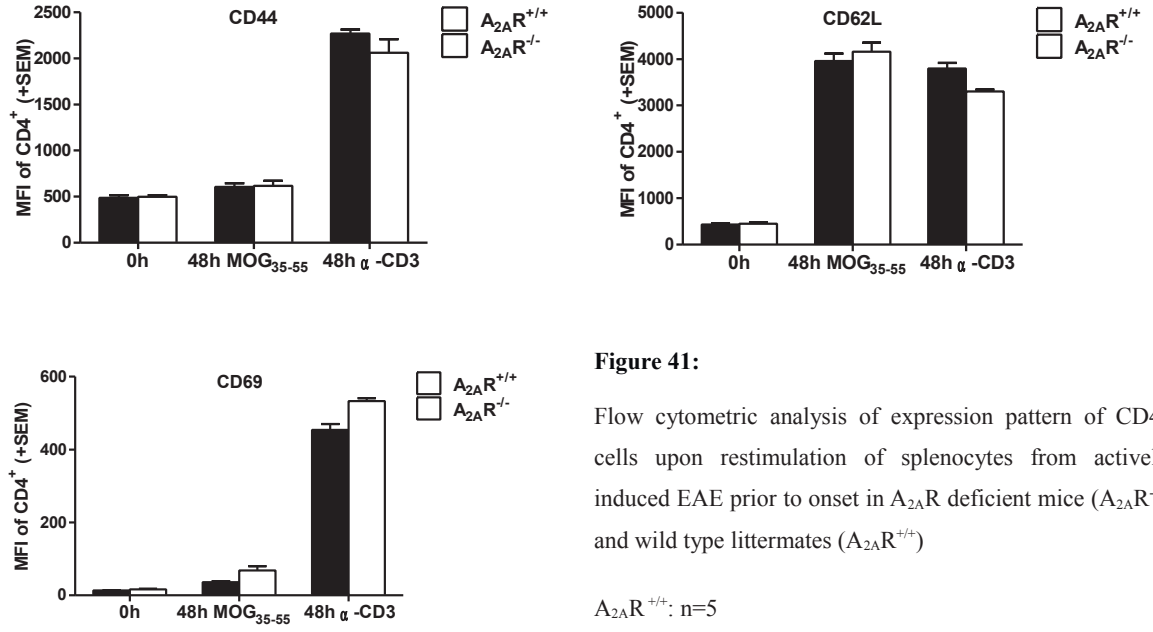


Figure 41:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE prior to onset in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

Interestingly, when isolated $CD4^+$ T cells were cultured with the antigen specific stimulus of MOG_{35-55} (10µg/ml) in presence of irradiated (3000 gray) antigen presenting cells (splenocytes) and then stained intracellularly for the cytokines IFN-γ, IL-17 and GM-CSF measured with flow cytometry, the cells from $A_{2A}R^{-/-}$ mice showed significant increased amounts of $CD4^+$ T cells producing these cytokines in relation to those from $A_{2A}R^{+/+}$ mice. This finding might serve as a first hint for an explanation of earlier onset and higher peak of

disease in $A_{2A}R^{-/-}$ mice compared to $A_{2A}R^{+/+}$ that was observed in first EAE (referring graphs can be seen in figure 42).

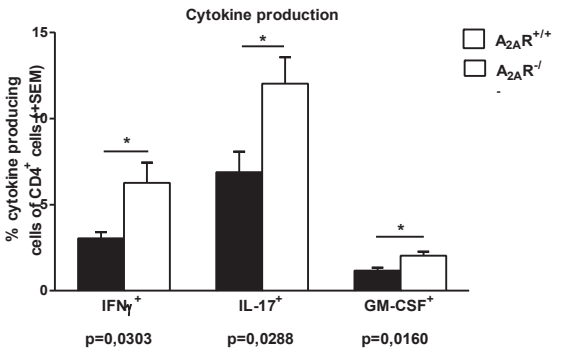


Figure 42:

Flow cytometric analysis of intracellular IFN-γ and IL-17 production in $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE prior to onset in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=5

$A_{2A}R^{-/-}$: n=5

The outcome was confirmed by ELISA from supernatant of these cultures, representatively carried out for IL-17 (figure 43).

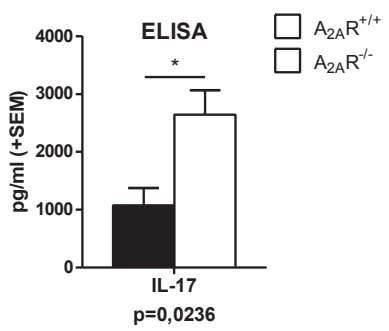


Figure 43:

ELISA from supernatant from restimulated splenocytes from actively induced EAE prior to onset in $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=4

$A_{2A}R^{-/-}$: n=5

To test whether this was an effect specifically found in EAE or whether cells from $A_{2A}R^{-/-}$ mice produced more of these cytokines in general, cells from naïve $A_{2A}R^{-/-}$ and $A_{2A}R^{+/+}$ mice were investigated.

Proliferative capacity from splenocytes was tested by ^{3}H-thymidin incorporation assay. The assay revealed (as shown in figure 44) that clonally activated splenocytes (for 3 days) from both mouse strains result in equal values (given in counts per minute). So $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ splenocytes seemed equally able to proliferate.

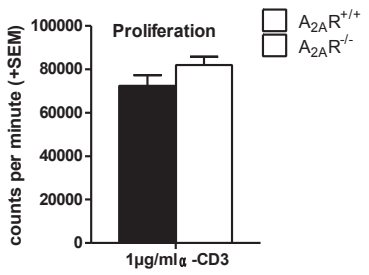


Figure 44:

Proliferative response upon restimulation of splenocytes from $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=3

$A_{2A}R^{-/-}$: n=3

Interestingly, when now tested for the ability of naïve $CD4^+$ T cells to develop into a certain type of T helper cell (and thereby producing its respective signature cytokine IFN-γ and IL-17) it was possible to see that naïve $CD4^+$ T cells were polarized to the same percentage in $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ cells to the different T helper cell subtypes, characterised by the production of the corresponding signature cytokine. Results are shown in figure 45.

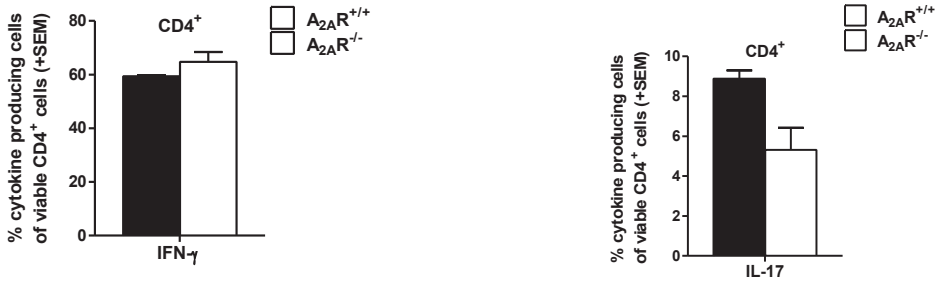


Figure 45:

Flow cytometric analysis of intracellular IFN-γ and IL-17 production in $CD4^+$ cells upon cytokine driven polarisation of splenocytes from $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$)

$A_{2A}R^{+/+}$: n=3

$A_{2A}R^{-/-}$: n=3

As described above, isolated $CD4^+$ T cells were cultured with irradiated antigen presenting cells. $CD4^+$ T cells were activated via α-CD3 and α-CD28 and polarised towards a certain T helper cell subtype by conditions mentioned in methods section, stained and analysed on flow

cytometer. Here as well, results were confirmed by ELISA for IL-17, representatively (fig. 46).

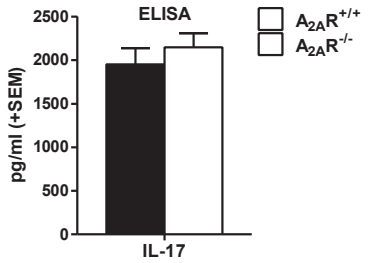


Figure 46:

ELISA from supernatant from splenocytes upon cytokine driven polarisation in A2AR deficient mice (A2AR-/-) and wild type littermates (A2AR+/+)

$A_{2A}R^{+/+}$: n=3

$A_{2A}R^{-/-}$: n=3

So $CD4^+$ T cells from $A_{2A}R^{-/-}$ mice did not show a general defect to develop to different T helper cell subsets that are active in EAE.

Taken together, these results provided no clear explanation for the altered T helper cell subsets caused by the deficiency of $A_{2A}R$ observed in EAE, but the fact, that the T helper cell subsets are affected in $A_{2A}R$ deficient mice, revealed an explanation for earlier onset and higher peak of disease in $A_{2A}R^{-/-}$ mice that was observed.

Further approaches were carried out to get a more detailed understanding of $A_{2A}R$ signalling in immune system components. For this, a series of experiments with agonist selective for $A_{2A}R$ was performed. As selective agonist for $A_{2A}R$, CGS21680 was chosen.

At first active EAE was induced in daily CGS treated mice to see what kind of effect permanent over activation of $A_{2A}R$ has on disease progression. In one experiment mice were treated from d0 of immunisation until peak of disease and in another experiment mice were treated from peak of disease until end of the experiment, therefore providing a method to look for preventive and therapeutic effects of CGS.

In the preventive approach, mice treated with vehicle had an earlier onset of disease compared to CGS treated mice as well as a significantly higher peak of disease about d19. When daily injections were stopped at d19, the mice that were treated with CGS before reached a mean

clinical score similar to that of the mice treated with vehicle before (see fig. 47). So in this case treatment with agonist seemed to have a protective potential on disease progression.

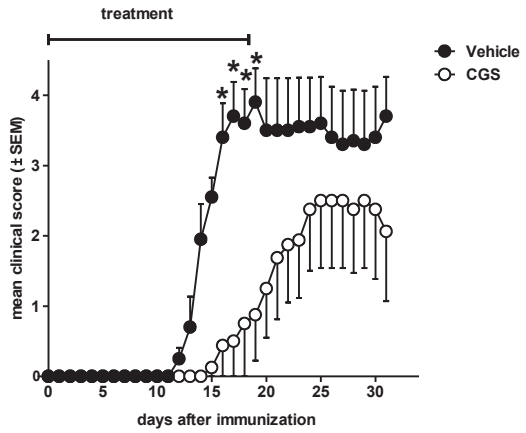


Figure 47:

Active EAE in wild type mice treated with vehicle or CGS

Vehicle: n=5

CGS: n=4

incidence vehicle: 5/5

incidence CGS: 3/4

In contrary, in the therapeutic approach mice that were treated with CGS from peak on at d12 of immunisation showed in comparison to then vehicle treated mice no remission phase and remained at a significantly higher mean clinical score (fig. 48). In this model it seemed that treatment with agonist has a detrimental effect on disease progression.

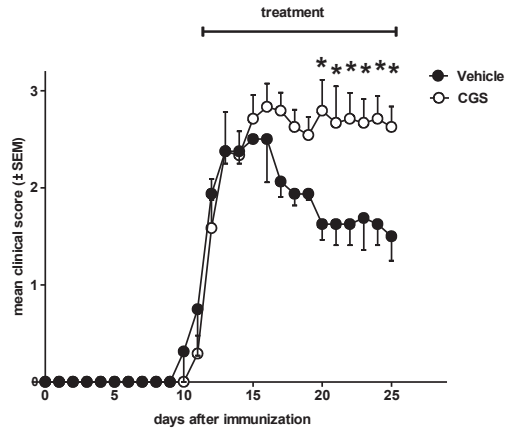


Figure 48:

Active EAE in wild type mice treated with vehicle or CGS

Vehicle: n=4

CGS: n=6

incidence vehicle: 4/4

incidence CGS: 6/6

Taken together, these results fitted to the EAE performed in $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice. Activation of $A_{2A}R$ in early phase has a protective effect that can of course not take place in $A_{2A}R^{-/-}$ mice which for they had an earlier onset and higher peak of disease. In late phase activation of $A_{2A}R$ has an opposite effect which was of course as well not observed in $A_{2A}R^{-/-}$ mice and therefore no difference between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice was given.

Immunological analysis of both previously mentioned CGS vs. vehicle EAE was performed at the endpoints. It revealed that antigen specifically restimulated (MOG_{35-55}) splenocytes showed no significant differences in their proliferative potential assessed by incorporation assay with ^{3}H-thymidin (see figures 49 for preventive and 50 for therapeutic).

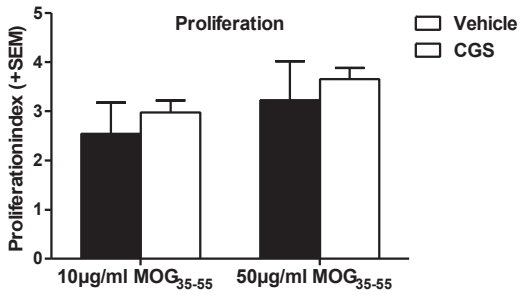


Figure 49:

Proliferative response upon restimulation of splenocytes from actively induced EAE in wild type mice treated preventively with vehicle or CGS

Vehicle: n=3

CGS: n=4

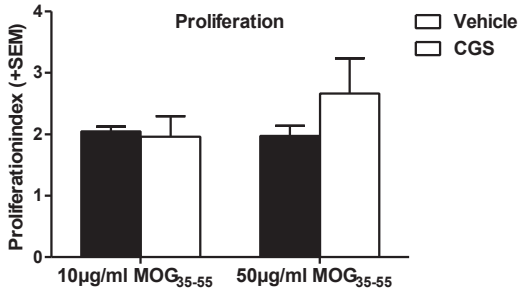


Figure 50:

Proliferative response upon restimulation of splenocytes from actively induced EAE in wild type mice treated therapeutically with vehicle or CGS

Vehicle: n=4

CGS: n=4

The number of T and B cells (measured by flow cytometry of splenocytes stained with α-CD4, α-CD8 and α-CD19) was unaffected by treatment of mice as well in both EAEs (see fig. 51 for preventive and fig. 52 for therapeutic).

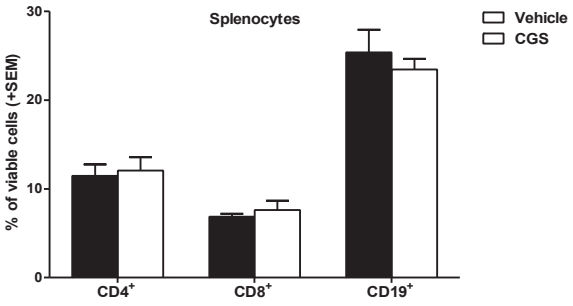


Figure 51:

Flow cytometric analysis of splenocytes from actively induced EAE in wild type mice treated preventively with vehicle or CGS

Vehicle: n=3

CGS: n=4

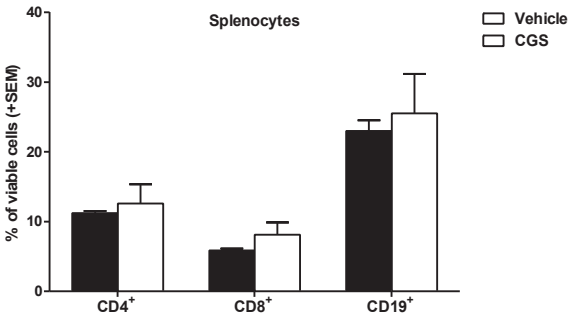


Figure 52:

Flow cytometric analysis of splenocytes from actively induced EAE in wild type mice treated therapeutically with vehicle or CGS

Vehicle: n=4

CGS: n=3

When isolated $CD4^+$ T cells were cultured in presence of MOG_{35-55} or α-CD3 for 48h, no differences were found on level of surface markers used as indicators of activation of $CD4^+$ T cells (flow cytometry measurement of cultured and stained $CD4^+$ T cells with α-CD4, α-CD44, α-CD69, and α-CD25) as can be seen in figure 53 (for preventive) and 54 (for therapeutic).

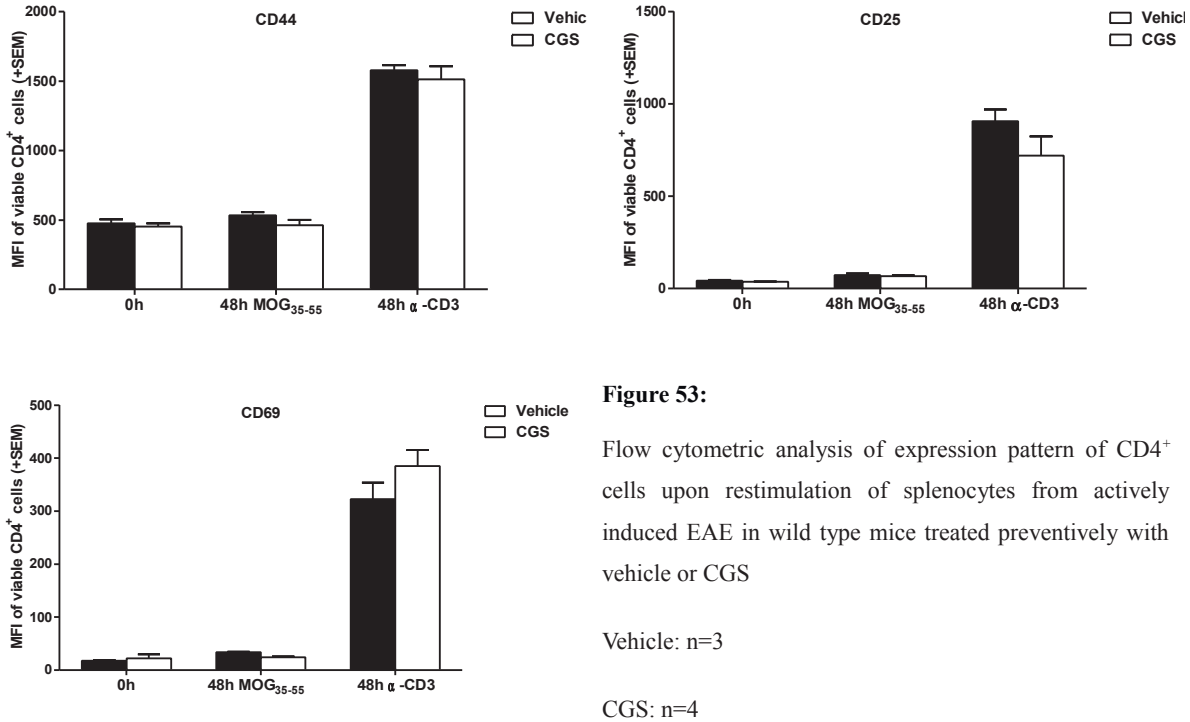


Figure 53:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in wild type mice treated preventively with vehicle or CGS

Vehicle: n=3

CGS: n=4

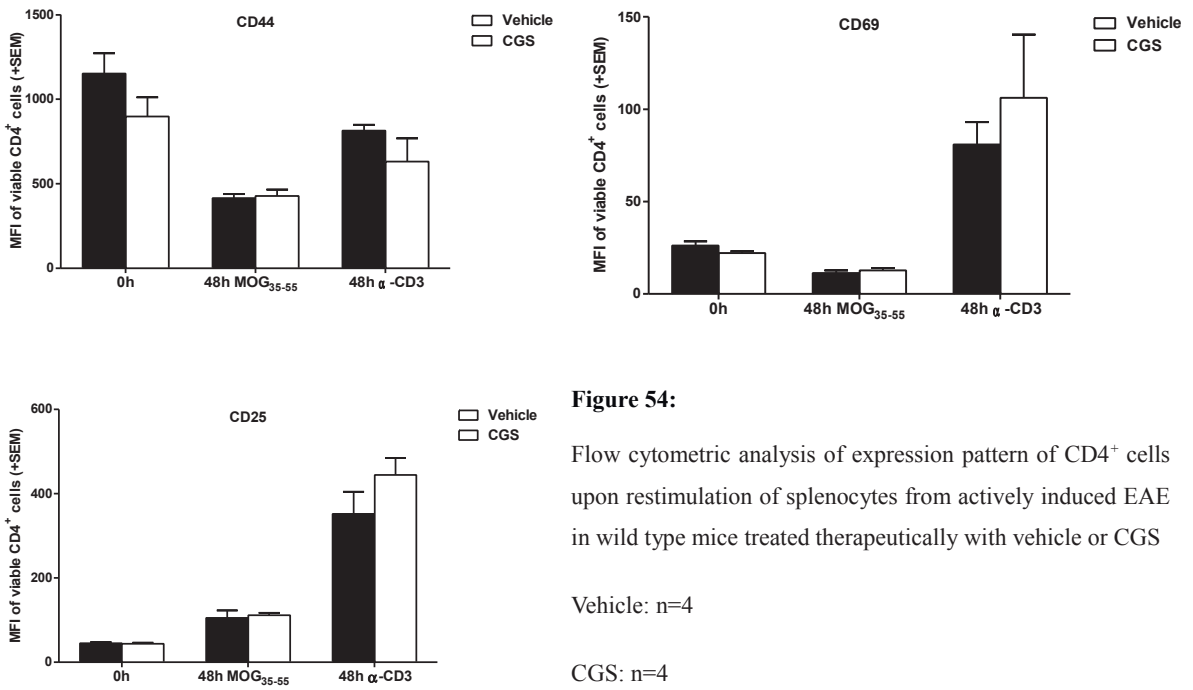


Figure 54:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in wild type mice treated therapeutically with vehicle or CGS

Vehicle: n=4

CGS: n=4

Survival of cells in cultures was assayed as well via flow cytometry (staining with α-CD4 and fixable viability dye). Numbers of viable cells and viable $CD4^+$ T cells in culture were identical between the examined groups (see fig. 55 for preventive and 56 for therapeutic).

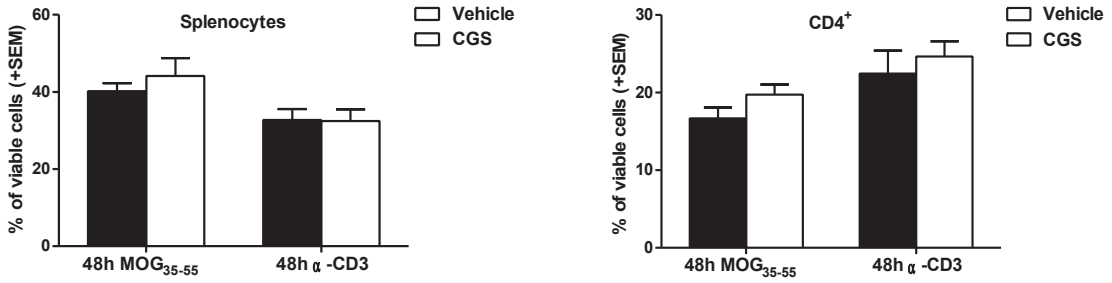
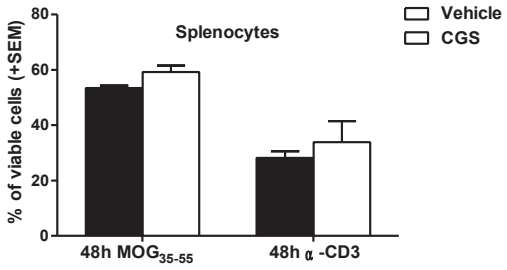


Figure 55:

Flow cytometric analysis of splenocytes and $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in wild type mice treated preventively with vehicle or CGS

Vehicle: n=3

CGS: n=4



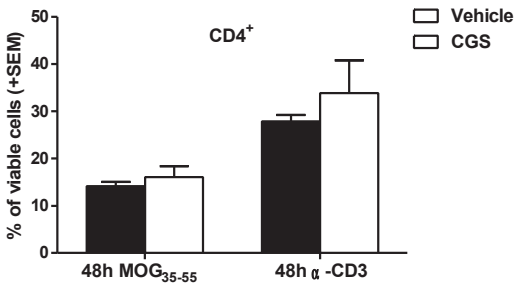


Figure 56:

Flow cytometric analysis of splenocytes and $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE in wild type mice treated therapeutically with vehicle or CGS

Vehicle: n=4

CGS: n=4

As the before described data were derived from a later time point when immune cells derived from the periphery are less relevant, active EAE was induced in mice treated daily with CGS or vehicle and sacrificed and analysed on day 7 prior to onset of symptoms. To get a closer look into immune system active at time point of induction of disease during agonistic treatment, this approach was chosen.

The number of B and T cells was not significantly affected (see fig. 57), as well as markers used for assessment of activation status of $CD4^+$ T cells (flow cytometry of (cultured) splenocytes stained with α-CD4, α-CD44, α-CD69, α-CD54, α-CD25, α-CD8, and α-CD19) when restimulated clonally (α-CD3) or antigen specifically (MOG_{35-55}) for 48h (see fig. 58).

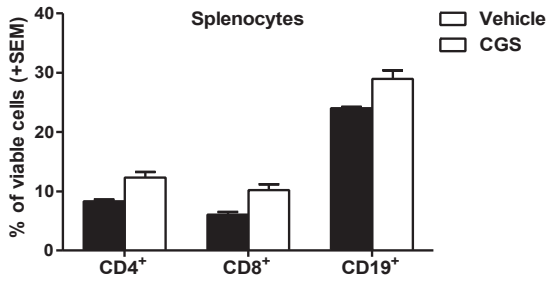


Figure 57:

Flow cytometric analysis of splenocytes from actively induced EAE prior to onset in wild type mice treated with vehicle or CGS

Vehicle: n=3

CGS: n=4

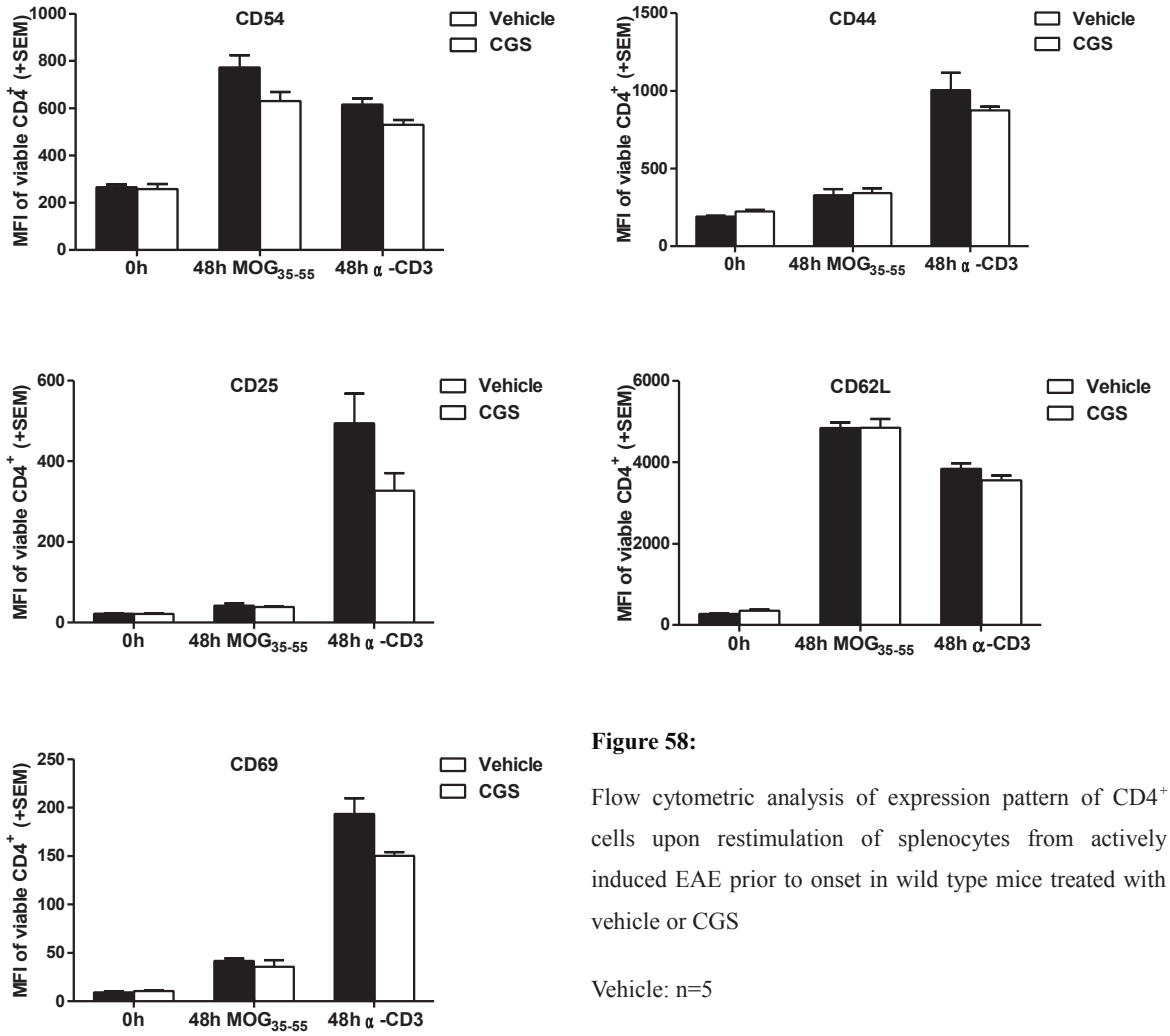


Figure 58:

Flow cytometric analysis of expression pattern of $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE prior to onset in wild type mice treated with vehicle or CGS

Vehicle: n=5

CGS: n=5

Interestingly, here the numbers of cells producing IFN-γ, IL-17 or GM-CSF was unaltered between vehicle and CGS group when splenocytes were stimulated antigen specifically or clonally for five days with following fixation, permeabilisation and intracellular staining for flow cytometry (staining with α-CD4, α-IFN-γ, α-IL-17 and α-GM-CSF) as can be seen in figure 59.

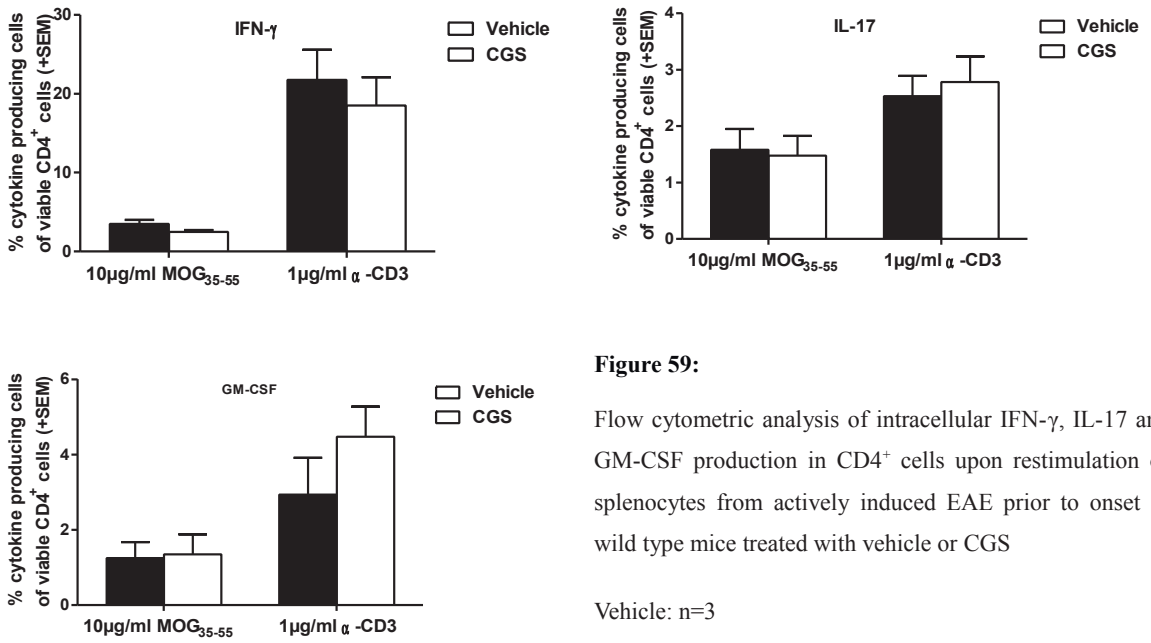


Figure 59:

Flow cytometric analysis of intracellular IFN-γ, IL-17 and GM-CSF production in $CD4^+$ cells upon restimulation of splenocytes from actively induced EAE prior to onset in wild type mice treated with vehicle or CGS

Vehicle: n=3

CGS: n=3

This was confirmed representatively for IL-17 by ELISA from supernatant from theses cultures (fig 60).

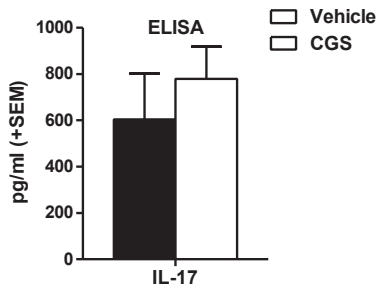


Figure 60:

ELISA from supernatant of splenocytes upon restimulation from actively induced EAE prior to onset in wild type mice treated with vehicle or CGS

Vehicle: n=3

CGS: n=3

Another remarkable fact is shown in figure 61. Lymph node cells were assayed for their proliferative potential in response to antigen specific or unspecific stimulus. Here lymph node cells from CGS treated mice showed a dramatically decreased proliferation in 3H-thymidin incorporation assay compared to lymph node cells from vehicle treated mice. However, this effect was not observed when splenocytes were tested in the same manner. So here again, presence or activation of $A_{2A}R$ seemed to have influence on activated $CD4^+$ T cells or on $CD4^+$ T cells that are in the activation process.

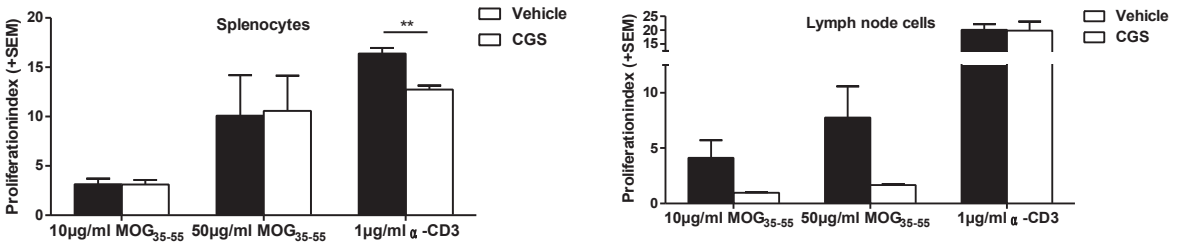


Figure 61:

Proliferative response upon restimulation of splenocytes and lymph node cells from actively induced EAE prior to onset in wild type mice treated with vehicle or CGS

Vehicle: n=3

CGS: n=3

To address the question whether the previously mentioned effects were specific for EAE or agonist treatment contributes to a general alteration, negatively isolated $CD4^+$ T cells from naïve WT mice were cultured in presence of $A_{2A}R$ agonist CGS at a concentration of 10nM and 100nM for 5 days under polarising conditions, restimulated, stained and measured by flow cytometry. Polarisation of isolated $CD4^+$ T cells towards Th1 and Th17 resulted in production of equal numbers of $CD4^+$ T cells secreting the relevant cytokine, so independently of CGS presence in culture (see fig. 62).

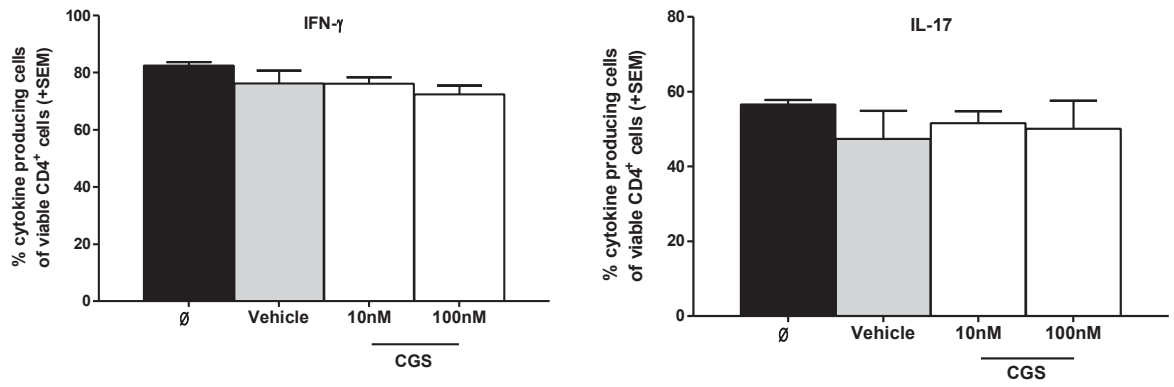


Figure 62:

Flow cytometric analysis of intracellular IFN-γ and IL-17 production in $CD4^+$ cells upon cytokine driven polarisation of splenocytes from wild type mice with presence of vehicle or CGS in cell cultures

n=3

Again, from these cultures supernatants were taken for ELISA, which confirmed the results representatively (fig. 63).

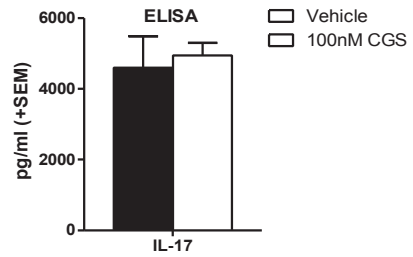


Figure 63:

ELISA from supernatant from splenocytes upon cytokine driven polarisation from wild type mice with presence of vehicle or CGS in cell cultures

Vehicle: n=3

CGS: n=3

As well the impact of CGS in cultures on proliferation was assessed. It revealed that with CGS in cultures, proliferation from WT splenocytes was significantly reduced compared to vehicle (see fig. 64).

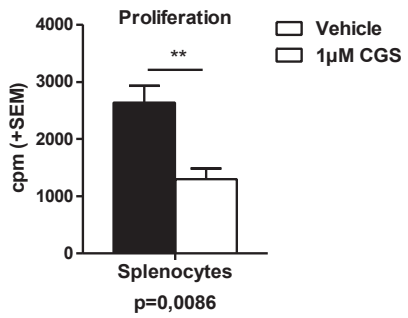


Figure 64:

Proliferative response of splenocytes from wild type mice with presence of vehicle or CGS in cell cultures

Vehicle: n=4

CGS: n=4

So the pharmacological activation of $A_{2A}R$ on splenocytes has impact on the proliferative potential of these cells.

Next approach was to work with NECA, another agonist binding non selectively to all four adenosine receptors. So NECA was used to compare the effects of missing $A_{2A}R$ between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ by activating all adenosine receptors. Simplified, when now observing differences between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$, this must be due to the deficiency of $A_{2A}R$, though activation of all adenosine receptors represents a quite artificial situation. As well therefore, only limited conclusions about the specific function of $A_{2A}R$ can be drawn by these results.

First, NECA was tested for its impact on polarisation of isolated $CD4^+$ T cells towards Th1 and Th17 from naïve wild type mice (shown in figure 65). Unexpectedly, activation of all adenosine receptors by NECA (2µM and 20µM) seemed to not influence the number of cytokine producing $CD4^+$ T cells measured in this assay (isolation of $CD4^+$ T cells, culture for 5 days with indicated cytokines, restimulation, fixation, permeabilisation and staining with α-CD4, α-IFN-γ, and α-IL-17).

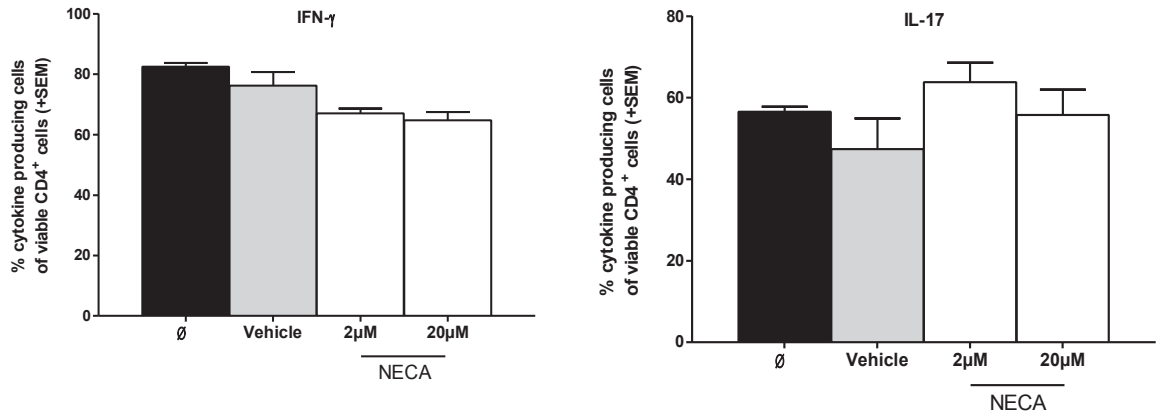


Figure 65:

Flow cytometric analysis of intracellular IFN-γ and IL-17 production in $CD4^+$ cells upon cytokine driven polarisation of splenocytes from wild type mice with presence of vehicle or NECA in cell cultures

n=3

To again focus on proliferation, naïve $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ splenocytes were cultured for 3 days with or without NECA (100µM) in culture and then underwent 3HT Assay. When first comparing $A_{2A}R^{+/+}$ vehicle vs. $A_{2A}R^{+/+}$ NECA in figure 66, it can be seen that NECA reduced proliferation. This effect was not observed between $A_{2A}R^{-/-}$ vehicle vs. $A_{2A}R^{-/-}$ NECA. Significant difference in proliferation was observed between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ treated with NECA.

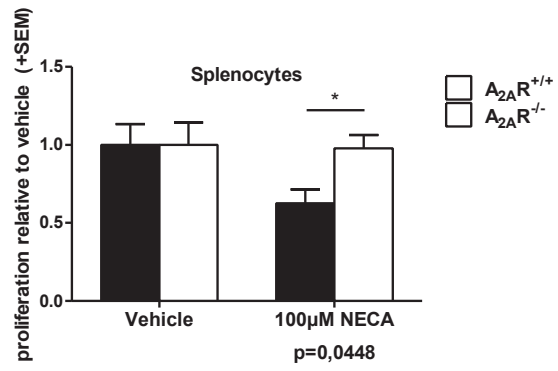


Figure 66:

Proliferative response of splenocytes from $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$) with presence of vehicle or NECA in cell cultures

$A_{2A}R^{+/+}$: n=3

$A_{2A}R^{-/-}$: n=3

In $A_{2A}R^{-/-}$ the $A_{2A}R$ cannot get activated which results in a higher proliferation compared to these conditions in $A_{2A}R^{+/+}$. So here obviously $A_{2A}R$ was responsible for this effect of less

proliferation. Or to formulate it the other way round: deficiency of $A_{2A}R$ leads to increased proliferation relative to existence of $A_{2A}R$ when activating the other adenosine receptors. This effect could be enhanced with increasing concentrations of NECA to 200µM. These data were consistent with the results obtained from proliferation assay from naïve splenocytes cultured with CGS or vehicle (figure 67).

With the intention to exclude possible factors from active immunisation procedure that has influence on immune cells and to see whether differences in T cells are in the end responsible for results seen in active EAE, transfer EAE into $RAG1^{-/-}$ mice was performed. In this model, mice that are deficient for recombinant activating gene 1 and because of that have no functionally T cell receptors, receive cells from cultures that were set up of splenocytes with MOG_{35-55} for restimulation from actively immunised mice. By this the RAG1 deficient mice receive activated encephalitogenic T cells and develop EAE symptoms. One group of $RAG1^{-/-}$ mice received cells from CGS treated wild type mice and one group of $RAG1^{-/-}$ mice received cells from vehicle treated wild type mice.

Results showed no difference in onset as well in further progression of disease between groups as shown in figure 67.

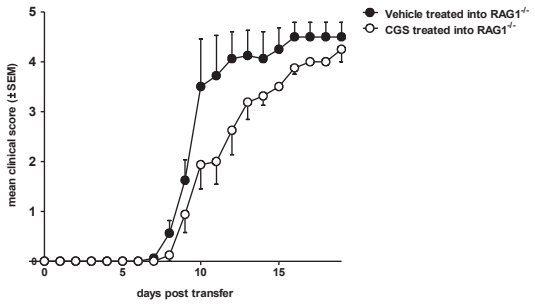


Figure 67:

Transfer EAE into RAG1$^{-/-}$ with splenocytes from actively induced wild type EAE mice (treated with vehicle or CGS) as donors

vehicle treated into RAG1$^{-/-}$: n=4

CGS treated into RAG1$^{-/-}$: n=4

incidence vehicle treated into RAG1$^{-/-}$: 4/4

incidence CGS treated into RAG1$^{-/-}$: 4/4

So from a first look it seemed that not the differences in T cells were responsible for disease progression seen in the active EAE of vehicle and CGS treated mice. One interesting finding was that the percentage of cells that were harvested from these cultures differed between vehicle and CGS treated. Whereas in CGS cultures 11.7% of the cells survived upon antigenspecific restimulation, in vehicle cultures 18.4% of the cells survived. A possible explanation is that in splenocytes from mice treated with CGS the number of MOG_{35-55} specific cells was lower than in splenocytes from mice treated with vehicle. These data were not able to serve as statistical evaluable data, but supported the tendency seen in the previous experiments. The method used here reveals an explanation by itself, why unexpectedly no differences were seen in EAE curves between vehicle and CGS group: recipient mice received same numbers of cultured cells, thereby levelling the effect CGS had in donor mice.

So from all these data it seemed that in early phase of disease the effect of deficiency of $A_{2A}R$ is due to alterations in proliferative potential (and along going cytokine production) of immune cells but is not due to this in late phase of disease. To check another immune cell type import in EAE, relevance of $A_{2A}R$ in macrophages was assessed upon activation of bone marrow derived macrophages. Therefore, BMDM were generated and activated via IFN-γ and IFN-γ along with LPS. As can be seen in figure 68, $A_{2A}R$ was found to be upregulated on mRNA level upon activation.

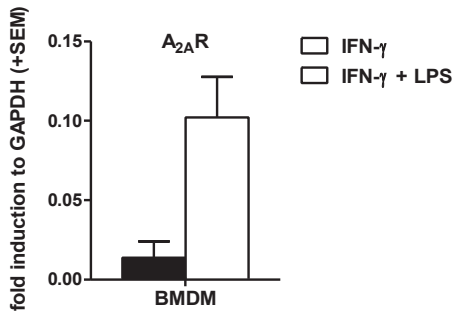


Figure 68:

qPCR analysis of $A_{2A}R$ upon stimulation of BMDM from wild type mice

$A_{2A}R^{+/+}$: n=2

$A_{2A}R^{-/-}$: n=3

When generating BMDM from $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ it was shown that activated BMDM produced the same amount of IL-1β mRNA. Treatment of these BMDM with 20µM NECA had no effect on levels of IL-1β mRNA detected in these cells from both strains, suggesting this effect being independent of $A_{2A}R$ (see fig. 69).

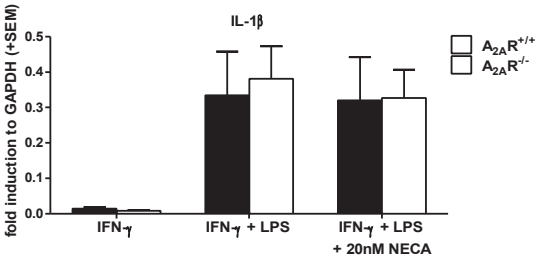


Figure 69:

qPCR analysis of IL-1β upon stimulation of BMDM from $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$) and wild type littermates ($A_{2A}R^{+/+}$) with or without presence of NECA in cell culture

$A_{2A}R^{+/+}$: n=3

$A_{2A}R^{-/-}$: n=3

Taken together, these data revealed no reason for differences in late phase of EAE between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice caused by the deficiency of $A_{2A}R$ in BMDM, although $A_{2A}R$ itself is upregulated in activated BMDM.

4 Discussion

This work provides insight into two different pathways that obviously contribute to the pathogenesis of an animal model for autoimmune mediated demyelinating disease. On the one hand there is the intracellular component PA28 and on the other hand there is the extracellular component $A_{2A}R$. Both pathways are linked to metabolic changes and both factors are non classical mediators which were usually not related to this scenario.

In awareness of the importance of the maintenance of metabolic equilibrium in health and disease, knowledge of the exact function of the two examined parameters, might contribute to novel therapeutic approaches by providing an opportunity to modulate those factors.

4.1 PA28

Immunoproteasomes were supposed to have their main function in producing epitopes for antigen presentation. Little is known about the effects of deficiency of PA28. In this part of the work, the influence of PA28 in the course of autoimmune mediated demyelination was investigated by means of PA28 deficient mice.

Active EAE induced in mice deficient for PA28 and in the corresponding wild type littermates revealed that PA28 deficient mice developed a significantly severe phenotype than wild type mice, whereas beginning of disease was unaltered. Analysis of number of B cells and T cells, proliferation of restimulated splenocytes, activation status of $CD4^+$ T cells, survival of $CD4^+$ T cells in cultures, and cytokine production of purified $CD4^+$ T cells from these mice showed no differences that would be able to explain the unequal disease progression. The histological analysis of macrophages/microglia in spinal cord and brainstem reflects the disease score of the two examined groups. Furthermore, general characterisation of naïve $PA28^{+/+}$ and $PA28^{-/-}$ mice showed uniform results in basic immune parameters like cell number of spleen, lymph nodes, and thymus, as well as the distribution of T and B cells, macrophages, dendritic cells,

and neutrophils in these organs. Furthermore, investigations of characteristics of $CD4^+$ T cells, namely activation status of naïve and different Th subsets and the cytokine production (IFN-γ, IL-17, and GM-CSF) within these cells revealed undifferentiated values. Moreover, monitoring basic immune parameters influenced by PA28 deficiency at early time point in EAE showed comparable data concerning number of B cells, T cells, and DCs, cytokine production (IFN-γ and IL-17) of CD4+ T cells, splenocytes, and lymph node cells, and proliferation of restimulated splenocytes. The only statistical difference was found in the number of macrophages, wherefore *in vitro* analysis of BMDM from both mouse strains was performed, that again revealed no differences in any of the examined parameters, which were the ability to be generated, their ability to phagocytose myelin and due to that upregulate MHCII, as well as the co-stimulatory molecules CD80 and CD86, and moreover, the production of IL-1β and TNF-α. So to summarise, no immunological explanation for the dissimilar EAE progression between PA28 deficient and wild type mice was found. Furthermore it is remarkable that PA28 seems to have no impact on processes of T cell proliferation, differentiation and cytokine production in cells from naïve mice as well as from EAE mice.

Regarding possible future directions based on the current work, it would be interesting to assess the (immuno)proteasomal activity of mice deficient for PA28 and whether the composition within the three subunits differs compared to wild type mice. Second, it would be of interest, whether in mice deficient for PA28, the 19S units are active/attached to the 20S proteasome. And third, it might make sense to investigate these factors in mice heterozygous for PA28.

4.2 Adenosine

The aim of this part of this work was to investigate the function of $A_{2A}R$ mediated signalling in demyelinating diseases, such as MS. To further investigate this topic, several experiements

were carried out for this work, mostly in the animal model EAE. It was shown that in mice deficient for $A_{2A}R$, EAE had an earlier onset, as well as a higher peak of disease, whereas in the late phase no differences were observed, compared to the wild type control group. Immunological analysis revealed that there were no differences in the number of B and T cells, in the activation status of restimulated splenocytes, in the proliferation of encephalitogenic cells, as well in the cytokine production of restimulated $CD4^+$ T cells. Surprisingly, when looking at a time point before onset if disease, $CD4^+$ T cells from mice deficient for $A_{2A}R$ produced more cytokines (IFN-γ, IL-17, GM-CSF) upon restimulation as compared to the wild type control group. Again, no difference was observed in the number of B and T cells and in the status of activation of $CD4^+$ T cells. Importantly, splenocytes from naïve $A_{2A}R$ deficient and wild type mice exhibited no difference in the potential to proliferate to antigen unspecific stimulus and their $CD4^+$ T cells produced the same amount of cytokines.

Furthermore, when investigating the pharmacological effect of a selective $A_{2A}R$ agonist (CGS) on the course of EAE, it was observed that the disease progression of the two groups matched the data obtained from wild type (CGS: activation of $A_{2A}R$) vs. $A_{2A}R$ deficient (vehicle: no activation of $A_{2A}R$) mice. This work was carried out in a preventive and in a therapeutic scheme. In both cases no difference was found in basic immunological parameters between the CGS and the vehicle group (proliferation, number of B and T cells, activation status of $CD4^+$ T cells). A difference in proliferation was found when animals were treated again from immunisation on with CGS, but then analysed before onset of disease. Here, lymph node cells from CGS treated mice proliferated less than those from the vehicle treated group, whereas again no differences were seen in the other parameters tested. When investigating the effect of CGS on proliferation on splenocytes of naïve wild type mice, it was shown that CGS was able to reduce the proliferative potential *in vitro* compared to the vehicle treated cells, whereas no difference in cytokine production was detectable. Some general observations can be drawn from these results: All observed effects were at an early time point of disease or at the early

phase during activation of T cells. Furthermore, all observed effects concerned proliferation and cytokine production. It is possible to hypothesis that the activation of the $A_{2A}R$ leads to suppressed proliferation, leading to higher cytokine levels in $A_{2A}R$ deficient mice compared to wild type control, as it was seen in the analysis of active EAE prior to onset of symptoms. This leads to another general effect observed in this work: no differences were observed in any approach when investigating immunological parameters at the late phase of EAE, although this of course might be a general issue. Furthermore, all differences found in the experiments were in "inflamed" conditions: either in EAE or when using CGS and thereby generating a level of adenosine/activation of $A_{2A}R$ that is closer to that of inflammation. That was shown as well when utilizing NECA to generate an artificial scenario of massive over activation of all adenosine receptors (like in inflammation). In absence of $A_{2A}R$ it will not be possible for the other adenosine receptors to trigger the production of cAMP and thereby to interrupt the activities of immune cells, leading to tissue damage. By this, $A_{2A}R$ has a negative feedback mechanism that downregulates inflammation. This is of relevance when cells get destroyed and their intracellular (high) level of adenosine (244) is released to the neighbouring cells.

NECA in *in vitro* cultures revealed that splenocytes from $A_{2A}R$ deficient mice proliferated more, due to the lack of the down regulatory effect of $A_{2A}R$. This scenario might be the scenario closest to that found in EAE, where $A_{2A}R$ maybe usually downregulates proliferation and thereby protects from overwhelming immune response, as it was as well described here in another study (208). Another hint for that theory is the fact that splenocytes from CGS treated mice survived less *in vitro* compared to splenocytes from vehicle treated mice. These results lead to two critical points of the experiments concerning the agonists CGS and NECA. In general, it is difficult to apply a concentration at a physiological range (in cell cultures and animal experiments) due to half life time of the pharmacological substances (*in vitro* different than *in vivo*). Furthermore, a detail that should be considered is that the physiological level of

adenosine is sufficient to activate $A_{2A}R$, but not $A_{2B}R$ which is expressed on activated T cells as well. Due to the circumstance that the exact concentration found in the animals cannot be determined, it cannot be excluded that the observed effects were not mediated by $A_{2B}R$ either. Furthermore, the impact of leakage of the BBB during EAE on the physiological adenosine level in the brain should be considered. The concentration of adenosine in the animal studies is not known for reasons that I) it is not known exactly what half-life time the agonists have, II) how they are distributed in the organism when applied to it, and, importantly in the scenario examined here, III) how much adenosine/ATP is released additionally by destroyed cells/tissue in the CNS. Another problem is the fact that the vehicle needs to be chosen thoroughly, and that no ligand is fully selective (197), especially when the concentration in the organism is unknown due to the afore mentioned reasons. Another critical aspect on this work is the fact that culmination of $A_{2A}R$ expression in T cells is 4-6 days post activation *in vitro* (190). This leads to question whether mice would have been needed to be pre-treated for this time period prior to EAE, as well how much conclusions one can draw from proliferation assays from naïve mice that were cultured only for three days. Furthermore it is only known, that there is no compensatory effect of the adenosine receptors in healthy $A_{2A}R$ deficient mice, but the situation under pathological conditions is unknown. Therefore effects mediated by other adenosine receptors than $A_{2A}R$ cannot be excluded.

If one would assume that the effect of proliferation ($A_{2A}R^{-/-}$ proliferate more) is the responsible effect for different EAE curves seen in $A_{2A}R$ deficient vs. wild type mice, it would explain why no differences were visible in the experiments of transfer EAE with splenocytes from mice treated with CGS or vehicle: the number of cells injected into the recipients was adapted to an identical level, so the effect of altered proliferation fails to appear. Furthermore, the increased cytokine production by restimulated encephalitogenic $CD4^+$ T cells from $A_{2A}R$ deficient mice from early time point EAE fits to the hypothesis, that the effects mediated by $A_{2A}R$ are during early events of immune response.

Additionally, it was observed that $A_{2A}R$ is upregulated in BMDM upon activation with LPS, but deficiency of $A_{2A}R$ has no effect on IL-1β production.

All the observations generally fit to the knowledge that adenosine signalling has effects at different time points in immune response (197). The observed effect in this experiments is at the time point when T cells get or are activated/encephalitogenic. This furthermore supports the anti-inflammatory effect, but not the promigratory effect of $A_{2A}R$ signalling on lymphocytes which was described elsewhere (206, 245). Consistent with another recent study from Mills *et al.* was the observed effect of impaired cytokine production from $A_{2A}R$ deficient T cells, whereas in their work no impairment in proliferation of these cells was found (246). Generally, taking pharmacological influence on proliferation of activated/activating T cells represents an appropriate target for treatment of MS patients and the possibility to influence T cells at this specific time point via adenosine signalling might be considered in future studies.

Under inflammatory conditions as found in EAE, large amounts of adenosine flood the body. The reduced proliferation upon $A_{2A}R$ activation found under these conditions might serve as a protective mechanism for over activation of immune cells. Through deficiency of $A_{2A}R$ this protective mechanism is not active anymore which resulted in higher proliferation and thereby in an earlier onset of disease as well as in a higher peak of disease in $A_{2A}R^{-/-}$ compared to $A_{2A}R^{+/+}$ and gives by that a possible explanation for differences observed. Assessing proliferation of splenocytes from EAE prior to onset between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice would be needed to confirm this hypothesis.

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6 Appendix

6.1 Summary

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system in Western countries affecting more than 2.5 million individuals worldwide. It is generally suggested that MS is mediated by autoreactive T cells, leading to demyelination, neuronal damage and subsequent irreversible neurological disability. The most common animal model is experimental autoimmune encephalomyelitis (EAE) induced by immunisation with myelin antigens in susceptible species. Here, I investigated the contribution of two non classical regulators of autoimmune neuroinflammation, i.e. (i) the proteasome activator 28 (PA28) and (ii) the adenosine receptor 2A ($A_{2A}R$) to the clinical phenotype and associated immunological and pathological alterations in different EAE paradigms. (i) PA28 is known to attach to an enzyme complex termed immunoproteasome under inflammatory conditions, leading to an increased turnover of short peptide substrates by this complex. Previously, gene expression of PA28 was shown to be upregulated in the spinal cord of EAE mice, but the precise role of PA28 remained elusive. (ii) For adenosine, existing studies suggested the modulation of immune processes via its receptor $A_{2A}R$. $A_{2A}R$ had been shown to couple with G_s proteins, resulting in increased cAMP levels and downregulation of inflammatory responses. However, the contribution of $A_{2A}R$ to autoimmune neuroinflammation had not been known so far.

For PA28, naïve C57BL/6 animals lacking this factor showed no immune deviation as compared to wild type littermates, as reflected by cell number and percentages of T and B cells of lymphoid organs and by the examined cytokines and surface markers on activated T cells. However, upon EAE induction, an increased disease severity was seen as compared to wild type littermate controls. Again, no difference was observed for the primary immune response, as a comparable cytokine profile and *ex vivo* autoimmune T cell response was observed at different time points during EAE, including investigation prior to disease onset. Concomitant histopathological analysis confirmed increased immune infiltration in the spinal cord and brainstem of affected animals. These experiments demonstrate for the first time the critical contribution of PA28 to autoimmune neuroinflammation *in vivo*. Considering the lack of any detectable immune regulation, further research is needed to clarify possible neurobiological explanations for this observation.

For $A_{2A}R$, mice deficient for this specific receptor showed an earlier onset of symptoms, as well as a higher peak in disease upon EAE induction compared to wild type littermate controls. Here, no differences in primary immune response were found between the groups. Remarkably, the investigated CD4 T cells isolated from $A_{2A}R$ deficient mice upon EAE induction before the onset of disease symptoms were found to produce significantly increased levels of relevant cytokines as compared to wild type littermate controls. This tendency was absent between naïve $A_{2A}R$ deficient and wild type littermate controls. Furthermore, preventive and therapeutic treatment in the EAE model with a selective $A_{2A}R$ agonist (CGS) confirmed the previous findings, i. e. the examined immunological parameters and the progression of disease. Investigations of splenocytes isolated before onset of EAE symptoms revealed the decreased proliferative potential of those from CGS treated mice compared to the referring control group. This result was confirmed by in vitro models of naïve splenocytes treated with CGS. Hence, A2AR seems to influence CD4 T cells at early phases in proliferation and cytokine production.

6.2 Zusammenfassung

Multiple Sklerose (MS) ist die häufigste chronisch-entzündliche Erkrankung des zentralen Nervensystems in westlichen Ländern und betrifft weltweit mehr als 2,5 Millionen Menschen. Es wird allgemein angenommen, dass MS durch autoreaktive T Zellen vermittelt wird und Demyelinisierung, neuronalen Schaden und schließlich irreversible neurologische Behinderung zur Folge hat. Das am häufigsten verwendete Tiermodell ist die experimentelle autoimmune Enzephalomyelitis (EAE), die durch die Immunisierung mit Myelinantigenen in empfänglichen Spezien induziert wird. Ich habe die Beteiligung zweier nicht klassischer Regulatoren autoimmuner Neuroinflammation in Bezug auf den klinischen Phänotyp und die damit assoziierten immunologischen und pathologischen Veränderungen in verschiedenen EAE-Paradigmen untersucht. Diese sind (i) der Proteasomaktivator 28 (PA28) und (ii) der Adenosinrezeptor 2A ($A_{2A}R$). (i) Es ist bekannt, dass PA28 bei inflammatorischen Bedingungen an einen Enzymkomplex namens Immunoproteasome andockt und dadurch den Umsatz dieses Komplexes an kurzen Peptidsubstraten erhöht. Es wurde gezeigt, dass PA28 auf Ebene der Genexpression im Rückenmark von EAE-Mäusen erhöht ist. Die präzise Rolle von PA28 blieb dabei unbekannt. (ii) Für Adenosine deuten Studien auf die Modulierung von Immunprozessen mittels des Rezeptors $A_{2A}R$ hin. Es wurde gezeigt, dass $A_{2A}R$ an G_s-Proteine bindet, was zu erhöhten cAMP-Leveln und der Herunterregulation von inflammatorischen Prozessen führt. Die Beteiligung von $A_{2A}R$ in autoimmuner Neuroinflammation ist bisher nicht bekannt.

Naïve C57BL/6 Mäuse defizient für den Faktor PA28 zeigten keine immunologische Deviation im Vergleich zu Wildtyp-Wurfgeschwistern, was sich in der Zell- und Prozentzahl von B- und T- Zellen der lymphoiden Organe und der untersuchten Zytokine und Oberflächenmarker von aktivierten T-Zellen zeigte. Jedoch wiesen diese Tiere in der EAE eine erhöhte Krankheitsschwere im Vergleich zu Wildtyp-Wurfgeschwistern auf. Auch hier wurden keine Unterschiede der primären Immunantwort (vergleichbares Zytokinprofil und *ex vivo* Reaktion von autoimmunen T-Zellen) zu verschiedenen Zeitpunkten der EAE, inklusive der Untersuchungen vor dem Einsetzen der Symptome, beobachtet. Die entsprechende histopathologische Analyse bestätigte die erhöhte Immuninfiltration in Rückenmark und Stammhirn der betroffenen Tiere. Diese Experimente zeigten zum ersten Mal die kritische Beteiligung von PA28 an autoimmuner Neuroinflammation *in vivo* auf. Aufgrund mangelnder detektierbarer Unterschiede der Immunregulation ist weitere Forschung von Nöten, die eine mögliche neurobiologische Begründung für diese Beobachtungen abklärt.

Naïve C57BL/6 Mäuse defizient für den spezifischen Rezeptor $A_{2A}R$ zeigten nach Auslösen der EAE ein früheres Einsetzen der Symptome sowie einen höheren Peak in der Krankheit verglichen mit Wildtyp-Wurfgeschwisterkontrollen. Dabei wurden keine Unterschiede in der primären Immunantwort zwischen den beiden Gruppen gefunden. Bemerkenswerterweise jedoch produzierten die vor dem Einsetzen der Symptome isolierten CD4 T-Zellen in der EAE der $A_{2A}R$ defizienten Mäuse signifikant erhöhte Level der relevanten Zytokine verglichen mit Wildtyp-Wurfgeschwisterkontrollen. Diese Tendenz war absent zwischen naïven $A_{2A}R$ defizienten und Wildtyp-Wurfgeschwisterkontrollen. Darüber hinaus konnten diese Ergebnisse (die untersuchten immunologischen Parameter und der Krankheitsverlauf) durch therapeutische und präventive Behandlung mit einem selektiven $A_{2A}R$ Agonisten (CGS) im EAE-Modell bestätigt werden. Untersuchungen an vor dem Auftreten der Symptome isolierten Splenozyten zeigten ein vermindertes proliferatives Potential der Splenozyten der CGS behandelten Mäuse in Vergleich zur Kontrollgruppe. Dieses Ergebnis wurde mittels *in vitro* Modellen an mit CGS behandelten Splenozyten naïver Mäuse bestätigt. Somit scheint

$A_{2A}R$ CD4 T-Zellen zu frühen Zeitpunkten der Proliferation und Zytokinproduktion zu beeinflussen.

6.3 Declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist.

Die Dissertation wurde noch nicht bereits einer anderen Fakultät vorgelegt.

Ich habe keine vorherigen erfolglosen Promotionsversuche gemacht.

Düsseldorf, den 29.9.2014

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