BACE1 IS A NOVEL REGULATOR OF TH17 FUNCTION IN EAE

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Th17 cells are implicated in autoimmune disease, including attack of the central nervous system (CNS) in multiple sclerosis. β-site APP-cleaving enzyme 1 (BACE1) is a membrane protease expressed in neurons and astrocytes. BACE1 is best known for its role in promoting neurodegeneration in Alzheimer's disease by cleaving amyloid precursor protein, although it also plays a critical role in driving myelination of the central and peripheral nervous system. In addition, BACE1 has been reported to contribute to lesion severity following brain injury, as has IL-17A, although these two molecules have not previously been linked.

Here, we show that in vitro-differentiated BACE1$^{-/-}$ Th17 cells exhibited reduced IL-17A and CD73 production despite regular RORγt upregulation. Expression of IL-17F was mildly reduced while other prototypic Th17 molecules remained unaltered, such as RORγt, IL-23R or GM-CSF. BACE1 regulation of IL-17A and CD73 occurred in a T cell intrinsic manner and its deficiency impaired the pathogenic function of Th17 cells in different models of EAE. Although affected by BACE1 deficiency, CD73-deficient animals did not exhibit decreased IL-17 production or reduced encephalitogenicity.

Mechanistically, BACE1-deficiency resulted in reduced expression of PTEN and increased production of cAMP by the adenylate cyclase (AC). Concomitantly with imbalanced PTEN, BACE1$^{-/-}$ T cells exhibited higher phosphorylation of Akt upon T cell activation. Accordingly,

forskolin-induced activation of the AC as well as PTEN hemideletion or pharmacological blockade phenocopied the findings observed in BACE1$^{-/-}$ Th17 cells.

In summary, our data demonstrate that BACE1 is a novel regulator of Th17 function but does not impact Th17 differentiation. By modulating cAMP and PTEN levels, BACE1 can couple early signaling events, such as T cell activation and Ca^{2+} signaling, with the specific regulation of IL-17A and CD73 expression in Th17 cells. These findings highlight BACE1 as a novel potential therapeutic target to treat IL-17A-driven autoimmune disorders.

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LIST OF ABBREVIATIONS

Ab	Antibody
AC	Adenylate Cyclase
AD	Alzheimer's Disease
APC	Antigen Presenting Cells
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
CD	Cluster of Differentiation
CNS	Central Nervous System
DAG	Diacylglycerol
DC	Dendritic Cell
EAE	Experimental Autoimmune Encephalomyelitis
Fox	Forkhead Box
Hif	Hypoxia-Inducible Factor
i.p.	intra-peritoneally
i.v.	intra-venously
IFN	Interferon
IL	Interleukin
IP_3	Inositol triPhosphate
Itk	Interleukin-2-inducible T-cell kinase
KO	Knock-Out
Lck	Lymphoyte-specific protein tyrosine Kinase

LN	Lymph Nodes
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
mTORC	mammalian Target of Rapamycin Complex
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
PD-K1	Phosphoinositide-dependent kinase-1
p.i.	post-immunization
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-Kinase
PIP2/ $PI(4,5)P_2$	Phosphatidylinositol 3,4-bisPhosphate
PIP3/ $PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5)-trisPhosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	PhosphoLipase C
PLP	ProteoLipid Protein
PTEN	Phosphatase and Tensin homolog (on chromosome 10)
RA	Rheumatoid Arthritis
ROR	RAR-related orphan receptor
STAT	Signal Transducer and Activator of Transcription
TCR	T Cell Receptor
TF	Transcription Factor

TGF	Tumor Growth Factor
Th	T helper
TNF	Tumor Necrosis Factor
Tregs	Regulatory T cells
WB	Western Blot
WT	Wild Type

1.0 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The term immunity derives from the Latin word *immunitas*, a word coined to refer to legal protection from prosecution. In the context of health sciences, immunity historically referred to protection against disease, and later on, against pathogens and transformed cells. The immune system is the set of cells, molecules, and their interactions, that collectively generate a coordinated response (immune response) to prevent infectious diseases and development of cancer cells as well as to mediate tissue repair under conditions of sterile inflammation. In case of infection, the innate immune system provides an early response against pathogens. It is composed of pre-existent cellular and biochemical mechanisms that react to broad pathogen and damage signals that will trigger a response to (1) block advance of the pathogen and (2) to destroy the invading agent. The innate immune system is constituted by very diverse mechanisms such as physical barriers like epithelial cells, clotting agents, the complement system, phagocytic cells (neutrophils, macrophages), natural killer (NK) cells and dendritic cells (DCs).

In contrast, the adaptive immune system is the branch of the immune system responsible for mounting an immune response against **specific** antigens or pathogens and to develop immunological **memory** to rapidly control subsequent infections with the same specific pathogen.

This degree of specificity requires detection of specific molecules (antigens) that are present only in each infectious agent, and is made possible by the T cell receptor (TCR). During their development in the thymus, T cells express a vast array of TCRs with different and random specificities and affinities. This diversity is possible due to a complex mechanism of genetic recombination in T cells known as somatic recombination, during which gene segments encoding the variable (V and J or V, D and J) and constant (C) regions of the TCR chains recombine and assemble a functional TCR gene. TCR diversity, and the ability to recognize diverse antigens, thus derives from the large amount of V, D and J segments that can recombine. After TCR rearrangement, T cells will go through a couple of checkpoints to ensure that their TCR is fully functional (positive selection) and does not detect peptides from its own cells/tissues (self-antigens/autoantigens). At the end of T cell development in the thymus, each T cell will end up expressing a single and unique TCR. Negative selection is a critical process to prevent autoimmunity whereby T cells recognizing self-antigens are eliminated. During cancer development, malignant cells typically end up expressing proteins that slightly differ from those in healthy cells. Therefore, the immune system evolved to generate cells that can mount immune responses against pathogens and malignant cells from the same individual while still preventing attack of healthy cells (autoimmunity).

Unfortunately, sometimes T cells harboring autoreactive TCRs escape the control mechanisms in the thymus and exit to the periphery and secondary lymphoid organs. Autoimmune disorders are triggered when these self-reactive T cells are activated after encountering autoantigens and other anti-inflammatory mechanisms fail to regulate the inflammatory response. Other mechanisms of T cell activation or exacerbation in autoimmune disorders include molecular

mimicry and bystander effect. Molecular mimicry refers to the structural similarity between antigens found in exogenous organisms or particles, for which an immune response is necessary, and peptides expressed by host's healthy cells. This similarity between antigens can trigger an autoimmune attack against healthy tissues upon T cell activation, after encountering alien immunogenic antigens. Once the immune response is initiated, T cells recognizing epitopes different from the initial ones can become activated by cytokines produced during the antigen-specific T cell response, therefore amplifying the pathogenic effect of the immune response. Depending on the type of immune response and the organ/tissue targeted, a vast array of autoimmune disorders can arise, such as type-1 diabetes, systemic lupus erythematosus, inflammatory bowel diseases, psoriasis, rheumatoid arthritis (RA) or multiple sclerosis (MS).

1.2 T HELPER SUBSETS: TH1, TH2 AND TREGS

The adaptive immune response is initiated by antigen-presenting cells (APCs) that have encountered the source of insult. Activated DCs and other APCs present at the site of damage/infection, can internalize and process different antigens that will be presented to T cells in the secondary lymphoid organs. Here, naïve T cells that specifically recognize the peptides that are being presented will become activated and proliferate, in a process known as clonal expansion (1).

There are different types of T cells in the body, but one major group are αβ $CD4^+$ T helper (Th) cells. These cells represent the main orchestrators of the adaptive immune response (2). Th cells are responsible for activating and recruiting effector cells, such as neutrophils and

macrophages to the site of infection/inflammation and to provide B cell help. At the moment of T cell activation, Th cells will recognize antigens presented by APCs as well as additional secondary co-activating signals to ensure their proper activation. These two processes are commonly known as signal 1 and signal 2. (1,2). Depending on the type of insult, APCs will be primed to secrete different types of cytokines (3). The different amounts and combinations of cytokines, as well as other factors in the T cell activating microenvironment, will determine the fate of the new forming Th cells. The presence of these soluble molecules, or signal 3, is crucial for the development of Th cells into different subsets (4,5).

1.2.1 Th1 and Th2 cells

During the late 1970's and early 1980's two different types of antigen-specific (clones) $CD4^+$ Th cells were identified (6), but it was not until 1986 that works from Mosmann and Coffman characterized and defined these two subsets as Th1 and Th2 (7). Th1 cells are the T helper subset involved in responses against intracellular pathogens, such as intracellular bacteria/protozoa or viruses. Th1 differentiation is primarily triggered by interleukin 12 (IL-12) (8,9). Upon engagement to its receptor on the T cell surface, IL-12 signaling through signal transducer and activator of transcription 4 (STAT4) will result in upregulation of the Th1 lineage-specifying transcription factor, T-box transcription factor (Tbet) (10). Expression of Tbet induces the expression of the Th1 effector cytokines (11) interferon gamma (IFNγ), interleukin 2 (IL-2) and tumor necrosis factor alpha (TNFα) (12,13), as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) (14). The expression of these cytokines results in a cellular host response tailored against intracellular pathogens, based on the activation and recruitment of

macrophages, $CD8^+$ cytotoxic T cells, Natural Killer (NK) cells and B cells. In addition, Th1-secreted IFNγ and IL-2 can further favor the development of Th1 cells through STAT1 and STAT5 signaling, respectively (15). The development of Th1 cells against autoantigens or excessive activation of these cells has been shown to cause Type 4 delayed-type hypersensitivity and is associated with autoimmune disorders such as Type-I diabetes, multiple sclerosis (MS), systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) (5).

Th2 cells are mostly implicated in the host defense against multicellular pathogens, including helminths (16,17). Thus, Th2 cells are broadly found on epithelial surfaces such as the gut and the lungs. The expression of IL-4 in these tissues is the principal factor that leads to Th2 differentiation (18), but other cytokines like IL-25 (19–21) and IL-33 (22,23) also play a role in the induction of Th2 cells. IL-4R signaling results in activation of STAT6 transcription factor that induces the expression of the Th2 master regulator, GATA3 (24,25). GATA3 expression induces the production of the Th2-effector cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 (24–26). These cytokines initiate what is known as a humoral response, characterized by the activation of IgE-producing B cells, eosinophils and mast cells that will release soluble factors to destroy and expel the pathogen (12) and increase mucus production (27,28). An exacerbated Th2 response has been implicated as the cause of Type-I IgE-mediated allergy and hypersensitivity disorders such as asthma and atopic diseases (5).

1.2.2 Regulatory T cells

In the 1970's Gershon and Kondo identified that T cells could also inhibit the immune response (29) and the term suppressor T was minted to describe this potential new type of T cells (29).

Despite much effort, challenges to identify a specific population (several different types of T cells could become suppressive) or specific mechanism of suppression (a plethora of very diverse mechanisms were suggested) resulted in a skepticism that suppressor cells even existed. In addition, the dichotomy of the Th1/Th2 paradigm, further reinforced by the findings that signature cytokines and signaling mechanisms specific for one subset suppress the polarization of the other one (30,31), partially explained the observed suppression by T cells.

It was not until 1995 that Sakaguchi and colleagues definitively identified a population of T cells with a regulatory or inhibitory function (32). This subset of $CD4^+$ T cells was identified as $CD25^+$ and named regulatory T cells or Tregs (32,33). Further studies demonstrated that Treg differentiation and function was dependent on the expression of the transcription factor forkhead box P3 (Foxp3) (34–36).

Tregs can be divided into two major groups: thymus-derived Tregs (tTregs) or peripheral Tregs (pTregs). Thymus-derived or thymic Tregs are a population of regulatory T cells that develop in the thymus during T cell generation and result in an endogenous long-lived population of T cells specific for self-antigens (37). These cells are positively selected in the thymus cortex and upon encountering self-antigens in the medulla do not get negatively selected. Thymic Treg progenitors bind self-antigens with high affinity (1,37). The signaling resulting from this high affinity interaction does not induce cell death or T cell anergy but triggers their polarization toward a regulatory phenotype. The generation of these cells is critical in the prevention of autoimmunity (38).

Peripheral Tregs are a population of regulatory T cells that derive from mature naïve T cells in the periphery that underwent normal positive selection and negative thymic selection (39). During activation in secondary lymphoid organs in the presence of anti-inflammatory molecules, primarily the cytokines IL-10 and TGF-β1 (40,41), they differentiate to a regulatory program.

Both tTregs and pTregs confer protection against autoimmunity by inhibiting the inflammatory effector functions of neighboring immune cells (37,42). The mechanisms by which Tregs can dampen inflammatory responses are very diverse and include direct physical interaction with other cells in a cell-to cell fashion and secretion of soluble factors (43,44). One of the main mechanisms by which Tregs exert their function is the secretion of IL-10 and TGF-β1. TGF-β1 is a key cytokine in the induction of pTregs by inducing the expression of Foxp3 (40,45), therefore being critical for initiation and maintenance of the Treg phenotype. Secretion of IL-10, can promote the differentiation of Tr1 cells, an inducible subset of $Foxp3^-$ regulatory T cells that express high levels of suppressive IL-10 (46). Nevertheless, most of IL-10 immunosupressive functions target APCs, decreasing the expression of MHC-II and co-activating molecules CD80/CD86 (47,48) and therefore dampening T cell activation.

Other immunoregulatory mechanisms of Tregs involve the direct interaction of these cells with effector Th cells or APCs. Tregs express high levels of immunosuppressive co-receptors like CTLA-4, GITR, Neuropilin-1 or PD-1 (44) that upon binding to their counter-receptors on T effector cells and APCs can dampen the activation state of these cell types. Other Treg immunosuppressive mechanisms include metabolic deprivation (49,50), control of the purinergic halo (43,51–53) and direct competition during antigen presentation (43).

The critical role of Tregs in autoimmunity and their polarizing and effector cytokines (and transcription factors) have been extensively studied (38) and, in fact, transfer of Treg-depleted T cells or thymocytes deprived of Tregs are used as models for a variety of autoimmune diseases (54). In this regard, the induction or expansion of antigen-specific Tregs could be very beneficial in the treatment of autoimmune diseases or in the prevention of organ rejection after transplantation. On the contrary, due to their immunosuppressive functions, Tregs have been shown to be detrimental in the immune response against cancers and understanding their metabolism and functions in the tumoral environment is being actively studied.

1.3 MULTIPLE SCLEROSIS AND THE EAE MODEL – DISCOVERY OF TH17 CELLS

1.3.1 Multiple Sclerosis

Multiple sclerosis is an autoimmune disease that affects the central nervous system (CNS). MS is characterized by CNS demyelination, axonal damage and subsequent neurologic disability. It is estimated that 2.5 million people worldwide are affected, with more women suffering this disorder, at a ratio of 2:1 compared to men. The name of this disease refers to the presence of multiple sclerotic plaques (lesions/scars) in the brains of MS patients. MS symptoms vary widely depending on what area of the CNS is damaged, but this disease is generally characterized by progressive deterioration of sensory and motor functions. According to the type of symptom progression, MS has been classified in 4 subtypes: relapsing-remitting, primary-progressive, secondary-progressive

and progressive-relapsing. Relapse-remitting MS is the most common form of multiple sclerosis, affecting 85% of the patients (55).

1.3.2 Etiology

Although the etiology of MS is not fully understood, genetic mutations as well as environmental factors play an important role in modulating susceptibility to disease. Genome-wide association studies (GWAS) showed evidence that specific HLA genes (constituents of the major histocompatibility complex in humans) correspond to enhanced or reduced susceptibility to MS (56–58). Other genes with mutations that have been correlated with MS involve IL2R and IL7R (59,60), reinforcing the immunological basis of this disease.

As with all autoimmune diseases, genetics only account for a small proportion of susceptibility. Twin studies revealed that if a patient with MS has an identical twin sibling, that twin's risk of developing MS can be increased up to 25% in comparison to an increased risk around 5% in fraternal twins (61–63). However, no genetic or genomic differences have been found between twins where only one sibling develops disease (61,64). A large number of environmental factors have also been linked to the prevalence of MS. Most notably, proximity to equator has been associated with reduced susceptibility to MS, due to the lower incidence of MS cases in those latitudes (65). This effect has been attributed to exposure to sunlight and synthesis of vitamin D (66,67). However, it has also been linked to the prevalence of different viral infections. No immunization or vaccination has ever been demonstrated to induce exacerbation of MS (68), suggesting that the actual infections and the natural immune response are to be held accountable

for the demyelinating trigger. Although some viral infections can directly cause demyelinating encephalopathies, it is believed that viral infections might increase MS susceptibility through molecular mimicry (69–71) or bystander effect (72). The most frequent seroprevalence from MS patients correspond to viral infections caused by Torque Teno Virus, Epstein-Barr Virus, or Human Herpes Virus 6 (73–75).

1.3.3 Characteristics of MS Lesions

The diagnosis of MS is based on its clinical presentation. In order to diagnose a patient with MS, the clinical symptoms have to be presented as two independent neurological events or flares, basing the clinical diagnosis in the generation of at least two independent lesion in the CNS, at different times (76). Currently, analysis of specific markers in the cerebrospinal fluid (CSF) and magnetic resonance imaging (MRI) can substitute for a clinical relapse for diagnosis, allowing earlier treatment (77). In general, MS lesions are characterized by cellular aggregates of $CD8^+$ and $CD4^+$ T cells, activated neutrophils, macrophages and B cells, and the presence of autoantibodies in the CNS and CSF (78,79). Indeed, the presence of high titers of autoantibodies in the CSF can be used to confirm MS diagnosis (oligoclonal bands) (79). Most commonly, these immune infiltrates tend to be periventricular and in close proximity to blood vessels (perivascular), correlating with their sites of entry into the CNS (80). The immune response results in myelin degradation and axonal damage accompanied by destruction of oligodendrocytes (81,82). The advancement of these lesions, particularly into the white matter, leads to the formation of conglomerates of activated and dying cells, sphingolipids and scar tissue, generated in response to damage by glial cells (82). Depending on the relative importance of each one of these immune

players and the type of damage caused, MS lesions have been classified into four different patterns: CNS lesions with predominant T cell infiltrates (pattern I), B cell infiltrates with complement deposits (pattern II), loss of oligodendrocytes in the absence of prominent inflammatory infiltrates (pattern III), or altered myelination in the periplaque white matter suggestive of primary oligendrocyte disorders (pattern IV) (83). This heterogeneity in lesions was indeed expected due to differences in antigen specificity from both T and B cells and autoantibody titers in MS patients. In addition, the development of different courses of disease also hinted that the types of lesions could potentially be different from patient to patient.

1.3.4 Current and developing therapies

MS is an autoimmune disorder caused by immune attack directed to antigens present in the myelin sheath and oligodendrocytes. Hence, the first line of action is to reduce autoimmune inflammation. The effectiveness of each treatment varies between MS patients, suggesting heterogeneity in disease pathogenesis. Nevertheless, the overall benefits of immune targeted therapies provide insight on the functional immune responses involved in the development of the disease.

β-interferons were the first group of disease modifying drugs to be approved for MS. These molecules are a family of cytokines that play crucial roles in the inhibition of viral replication and they are also important immunomodulators. β-interferons can reduce autoantigen presentation by reducing MHC-II expression. They can also dampen the inflammatory reaction by inducing IL-10

production and inhibiting Th1 and Th17 development (84,85). In addition, β-interferons can decrease the blood-brain barrier (BBB) permeability, limiting the traffic of leukocytes into the CNS (86).

Due to its relatively low frequency of side effects, the most commonly prescribed therapy to treat relapsing forms of MS is glatiramer acetate (GA). Frequently, the myelin-directed autoimmune attack is targeted towards Myelin Basic Protein (MBP) antigens. GA is a mix of peptides and amino acids that mimic MBP antigens that, when administered subcutaneously, is thought to induce a bystander suppression by promoting the development of MBP-specific Tregs in the periphery (87,88). Moreover, some peptides in GA can directly bind into the groove of MBP-presenting MHC-II molecules, and therefore preventing the autoimmune antigen presentation of MBP (88,89).

CNS inflammation usually results in increased BBB permeability that facilitates the entry of new immune cells and the exacerbation of the immune response. Therefore, targeting lymphocyte trafficking is an effective way to prevent CNS autoimmune attack. CNS–migrating lymphocytes express the integrin/adhesion molecule VLA-4, composed of the subunits α4 and β1 (90). Natalizumab is a humanized monoclonal antibody that binds and blocks α4, hence preventing lymphocytes from penetrating the BBB (91). In a similar approach, Fingolimod, a sphingosine-1 phosphate receptor modulator, has recently been approved for MS treatment (55). Fingolimod prevents lymphocyte egress from the lymph nodes and therefore reduces their mobilization to the CNS (55,85).

B cells are also important contributors in MS pathology (92). Rituximab is a monoclonal antibody that blocks CD20, to efficiently delete B cells and pre-B cells, without affecting stem cell progenitors or antibody-producing plasma cells (93–95). Although high titers of autoantibodies are present in the CSF and serum of MS patients (96), it has been shown that targeting $CD20^+$ B cells is more effective than attacking plasma cells (94,95). The contribution of B cells to MS pathology has been more recently highlighted by the approval of a humanized monoclonal antibody targeting CD20, Ocrelizumab (55,97).

In severe cases of MS, such as in the progressive types or during relapses, strong immunosuppression is required. Antineoplastic drugs such as Mitoxantrone or Methotrexate are commonly used to inhibit lymphocyte proliferation and reduce severe autoimmune attacks (55,98). In addition, broad-spectrum anti-inflammatories like corticosteroids are also commonly prescribed to ameliorate MS exacerbations and, in very extreme situations, plasmapheresis can be performed on MS patients (55,98,99). The potential benefits of these therapies are limited by systemic adverse events, such as increased risk of malignancy and opportunistic infections and, therefore, the doses and effects must be carefully monitored.

Because of the disabling effects of MS, besides reducing immune responses, most MS patients are also treated with drugs or therapies focused on reducing neurological disability by directly targeting the symptoms (pain, incontinence, depression, sexual dysfunction, etc.) or by enhancing the transmission of action potentials. This is the case of dalfampridine (100), a potassium channel inhibitor that enhances axon conductance showing improvement in motor and

sensory functions. In this regard, physical therapy is also recommended to help reduce the impairment of the motor function and to gain some functional independence.

Development of new MS therapies are aimed at improving the specificity of immune inhibition. Laquinimod is a small molecule in the pipeline to potentially treat MS. Laquinimod is a small molecule that favors Th2 polarization, reducing the frequencies of Th1 and Th17 cells. In addition, it seems to promote the generation of neuroprotective molecules in the CNS as well as decrease antigen presentation by inhibiting the expression of MHC-II (101).

Dimethyl fumarate is a new potential drug that inhibits T and B cells by mechanisms that are not fully understood, but in vitro experiments point to antioxidant effects and decrease in NF-κB signaling that can lead to induction of Th2 cells and production of IL-10 (102,103).

Current and developing MS therapies highlight the immunological basis of this disease. However, more specific treatments are necessary in order to prevent general immunosuppression and prevent the outcome of opportunistic infections. With the development of biologicals, in particular blocking monoclonal antibodies, more specific therapies are being investigated to effectively restrain MS development, with particular interest of the IL-23/IL-17 axis (discussed in detail in the next section). In this regard, Secukinumab, an αIL-17A monoclonal antibody is currently being tested in Phase II clinical trials to deter IL-17A driven inflammation in MS (104).

1.3.5 Experimental Autoimmune Encephalomyelitis model (EAE)

The experimental autoimmune encephalomyelitis (EAE) is an animal model of MS based on immunization with a myelin antigen to induce an immune response against a self-antigen in the CNS (105,106). This model evolved from the incidental observations during the early development of the rabies vaccine by Louis Pasteur. By the end of the XIX century, primitive rabies vaccines were based on spinal cord emulsions from rabbits intra-cranially infected with rabies. In order to obtain more immunogenic preparations and to cover the high demand, Pasteur and colleagues had to obtain the emulsions from spinal cords that were dried/processed for only one day. Injection of these preparations resulted in neuropathies similar to rabies and MS. Works from Rivers in the 1930s, demonstrated that it was the spinal cord components and not the rabies virus that induced the neurological defects and paralysis in these patients (107–109), therefore establishing the molecular basis for the EAE model (105,106).

The most commonly used and prototypical model of EAE is immunization of C57BL/6 mice with MOG_{35-55}, an immunogenic myelin antigen, with complete Freund's adjuvant (CFA), a mix of mineral oil and inactivated *Mycobacterium* to strengthen the immune response. This reproduces a monophasic inflammatory episode in the CNS that replicates the initial stages of MS. CNS inflammation and lymphocyte entry into the CNS are facilitated by administration of Pertussis toxin (PTx). Around day 6-8 post-immunization (p.i.) the immune response is localized in the draining lymph nodes where $CD4^+$ T cells are primed and differentiated. Both in MS and EAE, autoreactive T cells activated in the periphery will migrate into the CNS, where they will be re-activated by local or infiltrating APCs (80). This secondary activation triggers their clonal expansion and production of cytokines such as IL-17, IFNγ, IL-6 and TNFα as well as chemokines

in the CNS (110–112). Secretion of these effector molecules attracts and activates other immune populations, especially neutrophils and macrophages that phagocyte myelin (113,114). This immune attack results in the degradation of the myelin sheath. The loss of myelin insulation hinders the propagation of neuronal action potentials, preventing the transmission of neuronal signals. In addition, the lack of myelin sheaths exposes a naked nerve/axon, making them susceptible to the cytotoxic effects of cytokines and other molecules in the inflammatory milieu (105,115). The resulting axon injury culminates in the degeneration of the whole neuron, even in areas distant from the initial injury site. This process, named Wallerian degeneration, constitutes the main mechanism of damage spreading in MS (116,117). At early stages of disease, the deletion of the myelin sheath is compensated by the *de novo* formation of myelin. Oligodendrocytes are the cells responsible for generating both the developmental myelin sheath as well as re-myelinating processes after injury (118). Unfortunately in MS, oligodendrocytes are targeted by autoreactive macrophages/monocytes (119,120) and IL-17A can directly induce their apoptosis (121,122), resulting in the destruction of any compensatory/regenerative mechanism.

Although EAE does not mimic all of the processes that take place in human MS, EAE recapitulates the major features of MS. Both EAE and MS lesions are histopathologically similar in structure, location and time distribution (105,106). In addition, this model has proven to be of exceptional relevance in the understanding of the roles of T helper cells in autoimmunity as well as their differentiating mechanisms (105,106,123,124).

MS is a heterogeneous disease evidenced both by different courses of disease as well as different types of lesions. Importantly, different antigen-specificities have been observed in both

B and T cells from MS patients (125,126). By using different peptides or proteins in different strains of mice we can replicate different types of MS (106,119,124,127–134). A relapse-remitting model of EAE has been developed by active immunization of SJL female mice with MBP or myelin proteolipid protein (PLP) peptides or full protein (124). Modulation of relapses can be achieved by administering different doses of PTx (106,124,135). In this model, a new T cell response against a different immunodominant epitope is generated with each relapse, highlighting the concept of epitope spreading in autoimmunity (69,136). Epitope spreading can be triggered by the presentation of other domains from the original antigen or by the ability to present new antigens released by tissue damage.

Both C57BL/6 acute monophasic and SJL relapse/remitting EAE models are driven by autoreactive $CD4^+$ T helper cells and are primarily used to study important molecules for T helper development and function for autoimmune pathogenicity. Active immunization after adoptive transfer of specific T cells into $Rag1^{-/-}$ recipients (that lack B cells or T cells) can be used to study the role of particular molecules in $CD4^+$ T cells for the development of EAE (124). Similarly, passive transfer of specific autoreactive Th subsets have been used to induce EAE without immunization, to study the relative relevance of different Th subsets or cytokines necessary for their proper differentiation (123).

MS susceptibility and viral infections have been linked in the past (65,70,73–75). In addition, cellular infiltrates in MS lesions are abundant in $CD8^+$ T cells (137), a lymphocyte population key in the clearance against viral infections (138). To study the role of this T cell population, as well as to better understand MS mechanisms derived from viral replication in the

CNS, scientists make use of the Theiler's murine encephalitis model. In this model a *Picornavirus*-induced demyelinating disease is generated in SJL/J mice that allows to study both acute and chronic viral effects (70,127).

1.3.6 Discovery of Th17 cells

Historically, MS was considered a Th1 disease (139–141). In addition to self-reactivity to myelin antigens, other factors, such as the ability to produce IFNγ or TNFα as well as specific adhesion molecules proved essential for the encephalitogenicity of T cells (139–142). High levels of IFNγ were described in MS cerebral lesions and in the CNS of EAE mice at the peak of disease (141,143), with waning levels during the recovery phase (143,144). In addition, systemic administration of IFNγ in MS patients resulted in exacerbated symptoms (145). EAE can be induced by active immunization with myelin antigens, as previously described, or by transfer of autoreactive T cells that have been expanded and polarized *in vitro* (passive transfer EAE). Transfer experiments of autoreactive Th1 cells into naïve mice were sufficient to induce EAE (142,146), further supporting the role of Th1 in EAE development. Moreover, genetic ablation of Tbet (147) or triggering signaling events that suppressed Th1 responses (148) resulted in ameliorated disease. These findings, together with the deleterious effect of IFNγ in other autoimmune conditions (12), made it logical to conclude that IFNγ-producing Th1 cells were responsible for orchestrating the immune response in MS/EAE. Contrary to expectations, knock-out mice for IFNγ or IFNγ receptor resulted in increased EAE severity (149,150). Altogether, it

looked like generation of Th1 cells but not IFNγ were critical for EAE, prompting further research to find other potential Th1 cytokines playing a role in MS.

Early studies that reported that Th1 cells were necessary for EAE were based on the blockade of IL-12 or IL-12R during *in vitro* polarization of Th1 cells, prior to adoptive transfer of EAE (151,152). According to these reports, blockade of IL-12 signaling abrogated Th1 differentiation that resulted in resistance to EAE. However, IL-12 is a cytokine composed by two subunits, IL-12p35 and IL-12p40 (153). In 2000, Oppmann *et al.* cloned and described a new cytokine, IL-23, composed of IL-12p40 and IL-23p19 (154). Interestingly, the antibodies used to block Th1 *in vitro* polarization in those early transfer experiments targeted the common subunit to both IL-12 and IL-23. Further work involving specific blockade of the exclusive subunits of IL-12 (IL-12p35) and IL-23 (IL-23p19) revealed that IL-12 is dispensable to induce EAE whereas IL-23 is required (155–157). In fact, IL-12p35-deficient mice develop more severe EAE (158).

In 2003, Aggarwal et al. showed that IL-23 promotes the production of IL-17 by activated T cells (159). Further in vivo experiments with genetic depletion and blocking the specific subunits of IL-23 confirmed that IL-23, and not IL-12 was necessary for EAE induction (155–157). In 2005, Langrish *et al.* observed that IL-23 induced the generation of novel subset of T helper cells that produced high quantities of IL-17A and that were sufficient of induce EAE (157), subsequently confirmed as Th17 cells (160,161).

Concomitant with the suppressive effects of Th1 cytokines and signaling molecules on Th2 differentiation, and vice-versa, Th1 and Th2-specific transcriptional regulators and effector

cytokines could suppress Th17 differentiation (160,161). Importantly, both IL-12 and IFNγ inhibit IL-17 expression (162) and KO mice for both Th1-cytokines resulted in exacerbated EAE (150,163). These findings shed some light into the confounding initial role of IFNγ in EAE, now being advantageous in EAE via suppression of Th17 cells.

It has been shown that TGF-β1 and IL-6 are necessary expression of RAR-related orphan receptor gamma thymus (RORγt) in T cells and their differentiation into Th17 cells (164–167). Similar to Tbet in Th1 cells and GATA3 in Th2 cells, overexpression of RORγt was sufficient to polarize cells to Th17 and to induce IL-17 expression (167). TGF-β1 is a regulatory cytokine with pleiotropic functions in T cell development, homeostasis and tolerance (168). Although regarded more as an anti-inflammatory molecule for its role in Treg development and effector functions (168), TGF-β1 was described to be necessary for Th17 polarization and for their pathogenic role in EAE (164–166). In particular, genetic ablation of TGF-β1 or its receptor, resulted in lack of Th17 generation and protection from EAE (165,169). TGF-β1, through induction of RORγt, is necessary for the initial induction of IL-17 and expression of IL-23R (170), which allows further maturation of Th17 cells after STAT3-dependent IL-23 signaling (157,164,171–173). Subsequently, RORγt and STAT3 were shown to induce the production of IL-17A and IL-17F as well as IL-23R in Th17 cells (167,174).

Importantly, TGF-β1 can induce the polarization of both Tregs and Th17 cells (40,162,164,166). In this regard, high concentration of TGF-β1, in the absence of other pro-inflammatory molecules, can inhibit Th17 polarization by inducing Foxp3 expression and the

subsequent inhibitory effects caused by direct interaction between Foxp3 and RORγt (40,164). In this regard, the effect of IL-6 proved critical in favoring the Th17-polarizing role of TGF-β1, rather than a suppressive effect (175). IL-6 is a pro-inflammatory cytokine secreted by a plethora of cells, including immune cells from both the innate and adaptive responses (176). IL-6 was found to be crucial for Th17 generation, since Th17 *in vitro* polarization with supernatants from LPS-stimulated DCs failed to induce IL-17 expression in the presence of IL-6 blocking antibodies (164,165). On the contrary, addition of recombinant IL-6 was very potent in suppressing the induction of Foxp3 by high concentrations of TGF-β1, thereby enhancing Th17 differentiation and IL-17 production (164). Activation of naïve $CD4^+$ T cells in the presence of IL-6 triggers STAT3 activation and induction of RORγt and RORα (172,177). Although overexpression of RORγt is sufficient to induce and maintain a Th17 phenotype, IL-6 on its own or STAT3 are absolutely required but not sufficient (178), and IL-6 can only generate Th17 cells in the presence of TGF-β1(45) or IL-1β (179). Instead it appears that apart from inducing STAT3/RORγt, IL-6 plays a secondary role in Th17 polarization by preventing the induction of Foxp3 as well as inducing IL-21 expression (164,173), as discussed below.

After induction of RORγt, T cells are committed to the Th17-lineage. However, these newly generated Th17 cells are not fully inflammatory and, indeed, show a high plasticity towards Treg conversion (164). At this stage, Th17 cells express IL-17A and IL17F, IL-21 and high levels of IL-10 (175,180). Production of IL-21 is dependent on IL-6 and STAT3 signaling (181) but not RORγt (173). IL-21 acts in a positive feedback loop, amplifying the Th17 "precursor" population in an autocrine/paracrine fashion (173,182,183). IL-21, similar to IL-6, acts through activation of STAT3 signaling and induction of RORγt expression (184). Although both TGF-β1+IL-6 and

TGF-β1+IL-21 can induce the expression of RORγt, and therefore Th17 polarization (173), it is believed that IL-6 plays a dominant role in this function, and only when IL-6 levels are limiting, IL-21 may have a more important role in Th17 induction (182). Therefore, IL-21 is normally considered a cytokine necessary for the maintenance of the Th17 pool rather than inducing the differentiation (182,183).

IL-1β has been described as another cytokine that promotes the conversion of naïve T cells into IL-17-producing cells (185). IL-1β has also been reported to be necessary for the pathogenic function of Th17 cells in EAE (186). IL-1β synergizes with IL-6 and IL-23 to induce the expression of IL-17A and IL-17F (187) as well RORγt, both by active transcriptional and epigenetic regulation (188). Indeed, IL-1β has been shown to overcome the defects in Th17 generation in the absence of TGF-β1 in *in vitro* cultures (165). IL-1β also induces the expression of IRF4 (189), another Th17 transcription factor critical for the production of IL-21 (190). In addition, IL-1β has been shown to promote Th17 polarization by inducing changes in DCs, which express a more Th17-skewing phenotype (191), as well as corrupting Treg polarization by inducing alternative splicing of Foxp3 (192).

IL-6 and IL-21 have been shown to induce the expression of IL-23R (180,193,194) and TGF-β1 can further enhance it (195). Interestingly, Th17 cells differentiated with IL-6 and TGF-β1, despite upregulating RORγt, failed to induce EAE in passive transfer models (175) . In addition, KO mice lacking IL-23 (IL23p19 subunit) or IL-23R failed to fully mature and expand inflammatory Th17 cells and were resistant to EAE (157,164,171,175). Concomitant with IL-6 and IL-21, IL-23R signals through STAT3 activation and further induction of RORγt gene

expression (172,196). Although the exact role in Th17 cells remains elusive, IL-23 is absolutely necessary for maturation of Th17 progenitors into fully pro-inflammatory Th17 cells (171,175,197). Therefore, Th17 differentiation has been described as a sequential process. First, TGF-β1and IL-6 commit naïve T cells to the Th17 lineage by inducing RORγt expression (in a Smads and STAT3 dependent manner). Moreover, IL-6 antagonizes any TGF-β1-driven skewing towards Tregs and induces expression of IL-21. In turn, IL-21 expression results in an auto-amplification of the Th17 phenotype. All three Th17-polarizing cytokines, STAT3 and RORγt result in the expression of IL-23R, that upon encountering its cytokine partner stabilizes the Th17 lineage, shuts off IL-10 expression, enhances IL-17A and IL-17F expression and induces the production of other Th17-signature cytokines, such as IL-21, IL-22, TNFα and granulocyte macrophage colony-stimulating factor (GM-CSF) (198).

Since the discovery of Th17 occurred in the context of EAE and autoimmunity research, Th17 cells have been extensively studied for their role and importance in autoimmunity. Th17 cells have been found to be critical players in other autoimmune diseases such as psoriasis, rheumatoid arthritis, colitis and SLE, among others. In fact, current (and developing) therapies against autoimmune disorders are targeting Th17 cells, by either preventing their differentiation or blocking their effector cytokines (180,193).

Most of the effector functions of Th17 cells have been attributed to the secretion of IL-17A and IL-17F. Both IL-17A and IL-17F are homodimeric cytokines that can also form a heterodimeric form, IL-17A/F. IL-17 cytokines acts mostly in non-immune cells like fibroblasts, endothelial and epithelial cells and keratinocytes. In these tissues and cell types, IL-17 signaling

typically triggers the activation of these cells to produce antimicrobial peptides, such as defensins (199). In addition, IL-17 induces chemokine production, like CXCL1, CXCL2, CXCL5 or CCL20 (200,201), to attract macrophages and neutrophils to clear the infection (200,202).

The role of IL-17 cytokines, especially IL-17A, has been extensively studied in both the context of autoimmunity and fungal infections (193). Mice lacking the cytokine or their receptor subunits exhibit reduced severity of EAE (203,204) as well as resistance against other autoimmune disease models such as psoriasis or arthritis (205,206). Also, blockade of IL-17 with specific antibodies resulted in ameliorated EAE (207) and secukinumab, a monoclonal antibody targeting human IL-17A, is currently approved for the treatment of psoriasis (193,207), as well as being tested in Phase II clinical trials as a therapy for MS ((208)). In contrast, deficiency in IL-17 cytokines or signaling results in enhanced susceptibility to some bacterial and fungal infections (209).

IL-22 is an important Th17-cytokine for its role in protective immunity, especially at mucosal sites (210,211). Nonetheless, this cytokine can be expressed by many other cells from both the adaptive and innate immune system (212). Induction of IL-22 production is tightly regulated by Notch and aryl hydrocarbon receptors (AHR) (213–215). IL-22 signals through STAT3 phosporylation as well as the Akt/mTORc cascade (212). Similar to IL-17, IL-22 signaling results in induction of antimicrobial peptides and mucins (216). In addition, IL-22 has been shown to have a protective role by preserving the gut mucosal integrity and preventing commensals from causing infection (217). As with IL-17, IL-22 has also been described to play a detrimental role in autoimmunity. High levels of IL-22 have been observed in MS and neuromyelitis optica (NMO)

patients (217–219), but contrary to expectations, IL-22$^{-/-}$ mice were not protected from EAE (220). Data obtained from human cell lines suggest that IL-22 might be playing an important role in the disruption of the BBB during MS/EAE (221,222). The role of IL-22 in promoting autoimmunity was further confirmed in psoriasis (223,224).

As can be inferred from its name, GM-CSF is a growth factor for granulocytes and macrophages (198). Th17-secretion of GM-CSF results in the activation and maturation of dendritic cells and monocytes (225). GM-CSF causes the upregulation of MHC-II from DCs as well as induces the expression of IL-6 (226), therefore perpetuating the Th17 response. The detrimental effects of GM-CSF expression have been observed in several autoimmune diseases like MS or rheumatoid arthritis (227–229). The depletion of GM-CSF in mice, genetically or with blocking antibodies, in the EAE model resulted in lower myeloid infiltration in the CNS and reduced severity of disease (230,231). It is important to note that some Th1 cells can also express GM-CSF and that these cells can induce EAE (232). Thus, GM-CSF expression from Th1 cells could explain why adoptively transferred Th1 can induce EAE.

As expected, Th17 cells did not evolve to just cause autoimmunity. Indeed, Th17 cells play a key role in battling infections, especially those caused by extracellular bacteria and fungi (193). Th17 cells and IL-17 have been shown to be critical in the clearance of pathogens as diverse as Gram-positive bacteria like *Propionibacterium* acnes, Gram-negatives such as *Citrobacter rodentium*, *Klebsiella pneumoniae*, pathogenic fungi like *Pneumocystis* or *Candida* species and even playing a role in immune responses in intracellular bacteria like *Borrelia*, *Bacteroides* or *Mycobacterium* (233).

1.3.7 Other T helper subsets

The aforementioned are not the only T helper subset present in the human body. The detailed discussion of some specific subsets was necessary due to their importance in EAE/MS (Th17, Tregs and Th1) or for historical reasons (Th1/Th2). Other important Th subsets include Th3 or Tr1, which can be included as subtypes of Tregs (5), Th9 or follicular helper cells (Tfh). Detailed discussion of these T helper subsets falls out of the scope of this dissertation.

1.4 IMMUNE RESPONSES IN THE CNS

1.4.1 CNS architecture: the Blood-brain barrier and glial cells

Due to their functional specificity, neurons need the help and support from other cells in order to survive. Early in evolution, animals developed a set of cells that give both physical and biochemical/nutritional support to neurons: glial cell. The glia (or glial cells) is constituted by three main populations: astrocytes, oligodendrocytes and microglia. Astrocytes correspond to 20-40% of glial cells and is the population with more known functions in the CNS. Astrocytes provide structural support to the brain by forming a physical scaffold where neurons can grow and develop into complex networks. Through direct control of the BBB, astrocytes control the uptake of nutrients and other molecules from the bloodstream into the CNS. In addition, they exert important roles in the metabolic support of neurons. Importantly, astrocytes play key roles in CNS repair

after injury, filling up the space formed upon nerve injury and forming what is known as the glial scar. Both positive and negative effects for astrocytes in this matter have been described.

Oligodendrocytes are the most abundant cells in the glia, representing 80% of this compartment. Although oligodendrocytes can also provide structural and trophic support to neurons, their most important function in the CNS is to generate the myelin sheath. Hence, these cells are absolutely essential for the proper function and insulation of nerves.

Unfortunately, these cells are particularly sensitive to inflammatory cytokines and therefore, the CNS has mechanisms to prevent inflammation (234). The first one is the physical separation of the CNS from the rest of the organism. Blood vessels that infiltrate the CNS are organized in a specially tight and regulated architecture to prevent free trafficking of molecules and cells between blood and the CNS: the blood-brain barrier (235). This structure is kept tightly packed thanks to endothelial tight junctions and the presence of a thick basal membrane. Moreover, BBB endothelial cells are associated to pericytes, a set of contractile cells that help sustain the structure and regulate other homeostatic and hemostatic functions of the brain (236). Therefore, most of the molecular transport between the bloodstream and the CNS is actively regulated through specific transporters.

Despite this separation of bloodstream and CNS, surveillance of neuronal tissues is necessary to protect against pathogens and aberrant cells. This compartment still is a common target for pathogenic agents. Infectious diseases of the CNS include fungal infections like *Criptococcus* (237), protozoal infections such as malaria or toxoplasmosis (238) and complication

or late stages of some bacterial infections like tuberculosis and neurosyphilis. However, the most common infectious threat in the CNS is viral: viral meningitis, rabies, poliomyelitis, Epstein-Barr and Herpes viruses as well as many other encephalitic viruses can target the CNS (236,239,240). Despite the anatomical barriers that isolate the brain and spinal cord from other tissues, immune-surveillance of the CNS is thus required to prevent infections and to scan for the presence of malignant cells. The CNS, as any other tissue, requires the functions of the immune system.

Historically, it was believed that immune cells were absent in a healthy brain, and that immune surveillance in the CNS was conducted by microglia. The microglia is the third type of neuroglia and accounts for 10-15% of the total cells in the brain. This cell type is considered the macrophage equivalent of the CNS, since it is constantly scavenging for plaques or damaged tissue and clearing unnecessary or malignant/infected neurons and other glial cells. Quite recently, it has been discovered that in steady state there is a constant transit of lymphocytes within the CNS and the rest of the body. Sentinel immune cells infiltrate the CNS from the CSF, via the choroid plexus. Inside the CNS, anti-inflammatory mechanisms keep the infiltrating surveilling leukocytes under control. One of these mechanisms is the production of IL-10 by infiltrating Tregs and other CNS-resident cells.

1.4.2 Role of IL-17A in MS

Both pathophysiological studies of MS lesions and CSF samples, as well as the insights derived from the EAE model underscored the importance of Th17 cells and IL-17A expression in the development and progression of this autoimmune disorder (180,218,236).

Expression of IL-17A by myelin-reactive Th17 cells can activate glial cells, especially astrocytes, to secrete chemo-attracting molecules like CCL20, CXCL2, CXCL9, CXCL10 and CXCL11(241), matrix metalloproteinases (MMPs) and nitric oxide (242). This facilitates the recruitment of macrophages (241,243–245) and, especially, neutrophils (246–248) that will target and destroy the myelin sheath and oligodendrocytes (119,120), the cells responsible for the generation and re-generation of the myelin sheath in the CNS (249). This destruction of the myelin sheath leads to deficient axon potential transmission and renders naked axons susceptible to neurotoxic effects driven by inflammation. In this regard, it has been shown that IL-17A can drive direct Ca^{2+}-induced neurotoxicity that results in neuronal death (ref). In addition, IL-17A expression can induce the expression of pro-inflammatory cytokines like IL-6, IL-1β, TNFα and IL-23 from astrocytes, microglia and oligodendrocytes (250–252), resulting in a positive feedback loop in Th17 function, reinforcement of glial activation and an overall exacerbation of CNS inflammation. More importantly, IL-17A signaling can induce the apoptosis of oligodendrocytes during EAE (121,122), therefore directly impacting the regeneration of the myelin sheath and promoting further neurodegeneration.

Moreover, IL-17A can directly act on endothelial cells and prevent the formation of tight junctions, resulting in increased BBB permeability. In addition, IL-17A induces the production and secretion of IL-6, CCL2 and CXCL2 by endothelial cells as well as promotes the expression of ICAM-1 by these cells (222,253). Together, all these different effects of IL-17A on CNS endothelial cells compromise BBB integrity, facilitating the infiltration of more immune cells and

perpetuating the cycle of inflammation. The different roles of IL-17A in MS inflammation and neurodegeneration are summarized in Figure 1.

Although Th17 cells and IL-17A expression are the initial orchestrators of the immune attack in the MS lesions is, in fact, the response from CNS-resident cells that results in secondary neurodegeneration. Exacerbation of the inflammation driven by glial cells as well as the neurotoxicity associated with their response in the absence of the myelin sheath acts in a loop to promote neurodegeneration, even at distant points from the initial inflammatory insult (116,117).

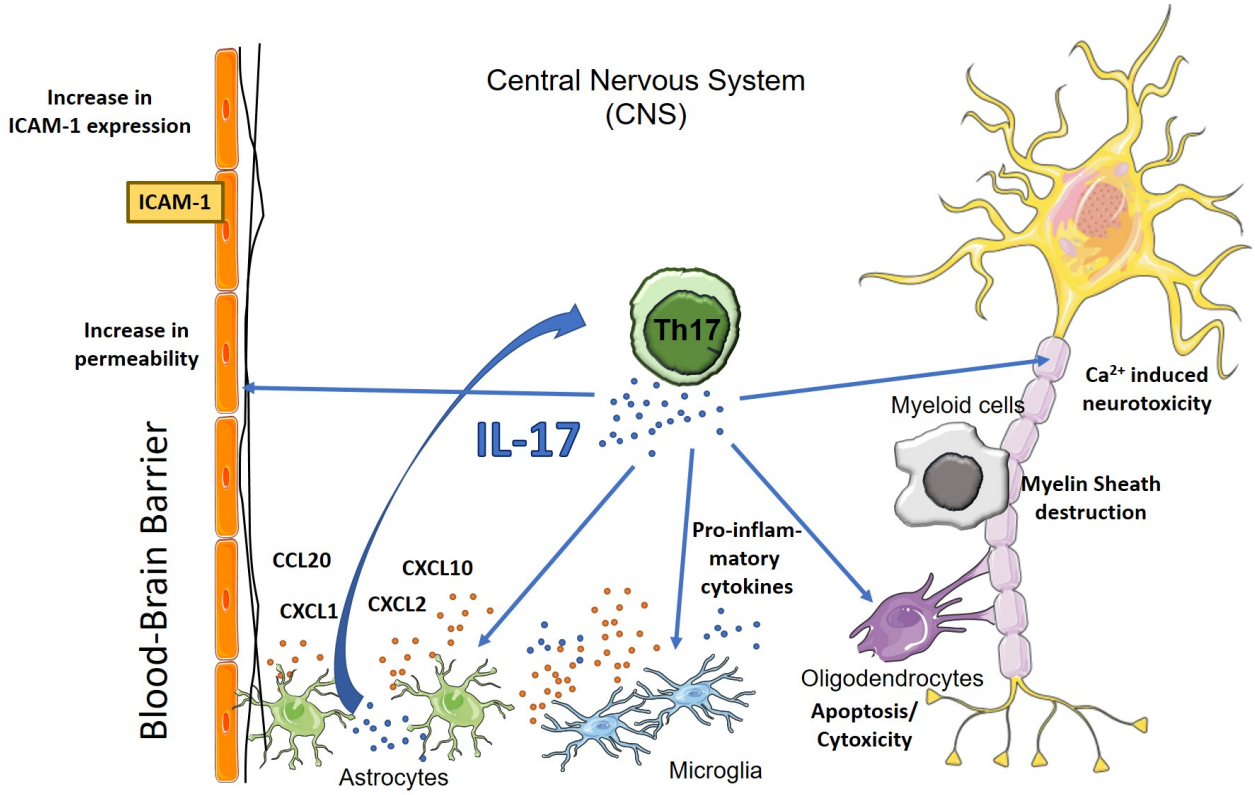

Figure 1. IL-17A-driven inflammation in MS lesions.

1.4.3 Role of IL-17A in other inflammatory CNS disorders

In addition to the detrimental roles of IL-17A during initiation and development of MS inflammation and disability, Th17 cells and IL-17A have been shown to play crucial roles in other inflammatory CNS disorders, like traumatic and ischemic insults. The primary outcome after an ischemic episode is the cellular death of the affected area (236). This tissue decay provokes secondary inflammation, responsible for the overall pathophysiology of CNS damage after stroke. Increases in IL-17 expression and IL-17-expressing cells have been observed in the brains of ischemic patients as well as in mouse models (254–256). Early neutralization of IL-17 with monoclonal antibodies resulted in reduced lesions and improved neurological outcome (257). Different mechanisms can lead to the generation or expansion of Th17 cells in the ischemic brain such as the cellular damage, but it is believed that hypoxia, through induction of Hif-1α, is the main mechanism of Th17 generation in ischemia (258–260).

Interestingly, IL-17 expression and Th17 cells have also been linked to neurodegeneration in AD. Studies in AD models, demonstrated that the presence of amyloidogenic Aβ42 peptides resulted in increased recruitment and expansion of Th17 cells in the amyloid deposits. In fact, Aβ42 triggered the expression of RORγt, IL-17A and IL-22 from these cells (261). Interestingly, peripheral blood mononuclear cells from AD patients stimulated with Aβ antigens showed increased expression of IL-21, IL-22, RORγt from Th17 cells compared to healthy donors, and the accumulation of effector T cells and reduced frequencies of naïve and memory T cells (262). Similar findings were obtained from T cells isolated from the hippocampus and CSF from rat models of AD, accompanied by increased BBB permeability and increased expression of Fas ligand by Th17 cells (261).

Together, these findings suggest that IL-17-driven inflammation plays an important role in neurodegenerative diseases. Most of current therapies to treat MS are based on limiting inflammation (ref), a strategy that may be extended to treatment of other neuroinflammatory disorders. However, the progression of neurodegeneration following immune attack is less understood, and therefore there is a very limited amount of therapies to slow down disease progression (ref). In this regard, we wanted to elucidate molecular links between neuroinflammation and subsequent neurodegeneration. One potential candidate is BACE1, a protease highly expressed in neurons and astrocytes in Alzheimer's disease that is upregulated under inflammatory conditions.

1.5 BACE1

1.5.1 BACE1: description and role in pathogenesis of Alzheimer's disease.

Beta-amyloid precursor protein cleaving enzyme 1 (BACE1) is a transmembrane aspartic protease that is related to the pepsin family and retroviral aspartic proteases (263). Although *Bace1* mRNA can be found ubiquitously, biologically relevant protein levels are primarily detected in neurons and astrocytes (263–265). Its name is derived from its role in the amyloidogenic cleavage of amyloid precursor protein (APP) in Alzheimer's disease (263,266). AD is a neurodegenerative disease characterized by the accumulation of amyloid plaques and neurofibrillary tangles in the brain. The latter are formed by intracellular aggregates of aberrantly processed

hyperphosphorylated Tau protein. In contrast, amyloid plaques are caused by the cerebral accumulation of hydrophobic amyloid beta (Aβ) fibrils, the hallmark of AD. It is believed that Aβ accumulation is a critical early step in tangle formation, neuroinflammation, synaptic loss and neuron death, ultimately resulting in dementia (267).

The BACE1 catalytic domain is located in the extracellular compartment. It contains two signature aspartic acid active motifs (DS/SGS/T) that are spaced approximately 200 residues apart but sit close together in the tridimensional conformation of BACE1 (263,268). The presence of both aspartyl residues in each motif is indispensable for BACE1 enzymatic activity (269). BACE1 is typically expressed on the plasma membrane, predominantly in lipid rafts (270–272). Moreover, BACE1 is localized within early endosomes and the trans-Golgi network (TGN) (273–276). Interestingly, BACE1 presents optimal catalytic activity at low pH, correlating with its expression in these low pH intracellular compartments (263,271,277). Although BACE1 activity has been described in the lipid rafts and the TGN, especially in AD-prone patients (APP Swedish mutation), most APP-cleavage by BACE1 has been described in early endosomes (278–280).

BACE1 is synthesized as a zymogen, which pre- and pro-peptide domains are removed in the endoplasmic reticulum (ER) and TGN by signal peptidase and pro-protein convertases (furin), respectively (269,281). During its transit through the ER to the plasma membrane, BACE1 is heavily post-translationally modified, including N-glycosylation of four Asp residues (282,283), cross-linking of the catalytic domain by disulfide bonds (282), phosphorylation of Ser498 (284), ubiquitination of Lys501 and S-palmitoylation at four cysteine residues at the junction of the

transmembrane and cytosolic domains (270). Although these mechanisms are not fully understood, it is known that these modifications regulate the localization and activity of BACE1 (285).

From a historical perspective, it is relevant to note that the proteolytic cleavage of APP by a β-secretase was described a decade earlier than the discovery of BACE1. In 1999, five independent groups discovered the molecular identity of the B-secretase and gave it different names: BACE1, memapsin2 or aspartyl protease 2 (Asp2) (286–290). In order to better understand the function of BACE1 in AD, different BACE1 knock out mice were generated by deleting critical exons (291) or by ending protein translation prematurely (292). Although initially described as viable and phenotypically normal (291,293,294), it was soon discovered that BACE1$^{-/-}$ mice exhibited congenital defects (292,295). BACE1$^{-/-}$ pups present an extremely high mortality rate during the first 3-6 days of life. In addition they show growth retardation compared to WT littermates, exhibiting increased death rate up to 3-4 weeks after birth (292). Thanks to the generation of BACE1$^{-/-}$ mice and the defects these mice presented, new substrates and functions of BACE1 have been elucidated.

1.5.2 BACE1 and developmental myelination

One of the best-understood physiological functions of BACE1 is its role in the proteolytic processing and activation of Neuregulin 1 (Nrg1) type III. BACE1$^{-/-}$ mice exhibit congenital problems in developmental (post-natal) myelination in both the CNS and the peripheral nervous system (PNS), with a more striking effect in peripheral axons (295–298). In particular, BACE1-deficient mice displayed a quantitative and structural hypomyelination phenotype accompanied by

an imbalance in the number and structure of small unmyelinated nerves (Remak bundles) (299,300). Neuregulins are a family of transmembrane neuronal proteins that signal through their epidermal growth factor domain (EGF), which binds and activates ErbB receptors. Nrg1 is expressed on the neuronal membrane along the axons and upon interaction with ErbB receptors on the surface of oligodendrocytes (ErbB2/3) and Schwann cells (ErbB4), the myelinating cells in the CNS and PNS, respectively (299,301). This interaction allows recruitment and coordination of myelinating cells to properly form the myelin sheath around axons (302). Nrg1 contains two transmembrane domains that generate a hairpin-like structure, preventing the EGF domain to be properly exposed and precluding it from interacting with its receptors. Similar to its role in shedding APP, BACE1 is able to proteolytically cleave Nrg1 to induce its mature form: a single transmembrane protein with all extracellular functional domains properly exposed (271,302). Further experiments confirmed the role of BACE1, Nrg1, ErbB receptors and their downstream Akt signaling as the main orchestrating molecules during developmental myelination of CNS and PNS (303–306) and post-injury remyelination of peripheral nerves (296,307).

1.5.3 Identification of new BACE1 substrates in BACE1$^{-/-}$ mice

Pharmacological inhibition of BACE1 bears a high potential as an effective target to prevent further neurodegeneration in patients with AD (308,309). Indeed, selective inhibitors have been developed and are currently in phase2/3 clinical trials (263). For this reason and the unexpected phenotypes described in BACE1$^{-/-}$ mice, it is crucial to better understand all BACE1 functions and to identify all potential substrates. In this regard, extensive research has been conducted in the past decade (263,310) and different BACE1 substrates have been identified and classified as: (1)

proteins that participate in synapse function, (2) proteins that interact with the environment to modulate axon growth and formation of microdomains and (3) substrates with non-neurological functions and (4) identified substrates with unknown physiological function or relevance (263).

In addition to APP, BACE1 can also cleave amyloid precursor-like protein 1 and 2 (APLP1 and APLP2) but the biological function of their shedding remains unclear although it is believed to play a role in neuromuscular formation (311–314). In the context of neuromuscular interactions, it has been shown that BACE1 plays a critical role in the formation of the muscle spindle (sensory receptors within the muscles), through proteolytic maturation of Nrg1 type I; in this case, instead of opening a hairpin structure, BACE1 shedding generates a soluble EGF domain (315–317). The aberrant muscle spindle results in defects in coordinated movement, both in young and adult individuals highlighting its role in development and maintenance of muscle bundles (318).

BACE1$^{-/-}$ mice also exhibit spontaneous seizures and hippocampal neuron loss that increases with age (319–321). Interestingly, BACE1 expression is highest in the hippocampus, where neuronal plasticity is maintained throughout life. BACE1$^{-/-}$ neurons display elevated sodium currents and action potentials due to increased numbers of voltage-gated sodium channels (Nav) (322–324). Interestingly, the expression of Nav can regulate the localization of conventional sodium channels (322,325). In addition, Nav β-subunits 2 and 4 are BACE1 substrates (324,326) and their cleavage regulates their cell-surface density, neuronal excitability and susceptibility to seizures (327). Interestingly, BACE1 can also modulate the activation of Nav channels by mechanisms independent of the proteolytic activity, however the specific mechanisms remain unknown (328).

Other neurological defects driven by BACE1 deficiency include impaired spatial working memory, increased anxiety behavior and reduced pain sensitivity and motor coordination (292,295,318,329,330). Further works revealed that these symptoms are due to the absence of processing of neurexin1 alpha and members of the neuroligin and latrophilin families (331–333). As stated before, $BACE1^{-/-}$ mice present defects in axon outgrowth and axoglial interactions (263,334,335). New BACE1 substrates have been identified in these roles such as L1, CHL1, contactin-2 and the SEZ6 family (334,336,337). BACE1 has also been more recently identified to play a role in regulating the ratio of astrocytes and neurons in the hippocampus through cleavage of Jagged1 and regulation of Notch signaling (337,338).

Besides its direct impact in neural development, BACE1-deficiency also resulted in reduced body growth (292). Some defects in the growth rate of $BACE1^{-/-}$ mice were associated to deficient processing of APP and homolog proteins (339,340). However, their resistance to diet induced obesity was explained by their increased insulin sensitivity in skeletal muscle and livers of $BACE1^{-/-}$ mice (341). Although the mechanism is not fully understood, insulin activated muscle and liver cells responded with increased phosphorylation of Akt in the absence of BACE1 (341).

1.5.4 Immune-related substrates of BACE1

In addition to the expression of BACE1 in neurons and its roles in CNS function and development, BACE1 protein levels have been identified, albeit to a lower extent, in the liver, skeletal muscle and pancreas (341,342). The TGN-resident β-galactoside α2, 6-sialyltransferase (ST6Gal1) is

primarily expressed in the liver, with enhanced expression during acute phase reactions (343,344). BACE1 was identified as the major protease responsible for the release of ST6Gal1 into serum (345,346). ST6Gal1 is the enzyme responsible for the addition of sialic acid residue on galactose residues during the N-glycosylation of some proteins. Interestingly, one product of this sialyltransferase reaction is the generation of the GL7 epitope, a commonly used marker of germinal centers (areas of B cell maturation in secondary lymphoid organs) (347).

P-selectin glycoprotein ligand-1 (PSGL-1) is expressed on most leukocytes and mediates adhesion to endothelial cells by binding to P-selectin during inflammation. This ensures efficient leukocyte recruitment and subsequent transmigration into damaged or infected tissue in the CNS and peripheral organs (114,348,349). PSGL-1 has been shown to be cleaved by BACE1, in a fashion similar to that of Nrg1 (350). However, in an acute model of peritonitis the composition and number of leukocytes transmigrating into inflamed tissues did not differ between BACE1$^{-/-}$ and WT mice (292).

A third inflammatory protein also shed by BACE1 is the interleukin-1 receptor II (IL-1R2) (351). Mainly expressed in Langerhans cells and bone marrow-derived neutrophils (352), IL-1R2 binds to IL-1α, IL-1β and IL-1R antagonist (IL1-RA) acting as a decoy receptor (352). This means that soluble IL-1R2 can prevent the aforementioned molecules from binding to IL-1R and trigger signaling events. The IL-1R2 can be shed by all three secretases (α, β, γ) and it is not known whether shedding of this interleukin-1 decoy receptor occurs under endogenous conditions or not (351).

Most BACE1 partners are substrates of the proteolytic activity of BACE1. However, non-proteolytic roles of BACE1 have been described, like the activation of Nav channels in neurons (328). In addition, it has been reported that BACE1 negatively regulates the activity of the adenylate cyclase (AC), in a protease-independent fashion. Hence, $BACE1^{-/-}$ neurons have the potential to generate higher levels of intracellular cAMP. This BACE1 activity is dependent of the transmembrane domain, but the exact mechanism remains unknown (353).

1.5.5 BACE1 in other neuroinflammatory disorders

Apart from its roles in neurological function and pathology in AD, BACE1 expression and function has been described in other inflammatory conditions of the CNS. In particular, BACE1 expression is increased upon CNS damage in traumatic brain injury (TBI) (354,355) and by ischemia in animal models of stroke (356–358). In addition, BACE1 deficiency correlated with reduced size of CNS lesions and better recovery in a mouse model of TBI (354). Increased levels of APP are commonly seen as a result of neuronal damage but generation of Aβ is not typically seen in disorders other than AD (359–361). Therefore, it is unlikely that BACE1 is acting in an amyloidogenic way in TBI or stroke. These findings indicate that BACE1 may play a broader role in neuroinflammatory or neurodegenerative processes besides its pathogenic function in AD. BACE1 has also been shown to play a role in myelin and debris clearance after axonal injury (362). Although the mechanism is not fully known, BACE1-deficient mice showed enhanced clearance and recovery compared to WT littermate controls (362). Interestingly, increased levels of Aβ, the APP product of sequential BACE1/γ-secretase cleavage, have been found in CNS lesions from MS patients and EAE mice (81,363,364). Intriguingly, inducing an antigen-specific immune response against Aβ

seemed to show therapeutic potential in EAE models (365). However, the expression patterns and potential roles of BACE1 in MS remain elusive.

1.5.6 Immune regulators control the expression of BACE1

Taking a deep look in the regulation of BACE1 expression it strikes as quite a surprise that BACE1 has not been studied more intensely from an immunology perspective. One of the first TF described to control BACE1 expression was specificity protein 1 (Sp1). Sp1 facilitates BACE1 promoter activity and the sole overexpression of this TF is able to induce BACE1 production (366). Interestingly, Sp1 has been shown to be important for TGF-β1-driven but Smad-independent polarization of Th17 cells (367). In addition, Sp1 induces the expression of the aryl hydrocarbon receptor (AhR) (214,368), which in turns induces Th17 in STAT1-dependent manner (369).

NF-κB, another TF that plays crucial roles in immune cells (370) regulates the expression of BACE1. Although it has been described that induction of the NF-κB pathway results in the inhibition of BACE1 transcription (371–373), NF-κB enhances BACE1 translation and reduces its lysosomal degradation, therefore increasing the overall levels of BACE1 (374–376). Indeed, NF-κB activation is the main pathway involved in the exacerbating positive loop exerted by Aβ (377).

The BACE1 promoter contains binding sites for other immune regulators, besides NF-κB. Another Aβ-induced pathway is the phosphorylation of cAMP response element-binding protein (CREB), which can bind to the BACE1 promoter and induce its expression (378,379). In addition, the prototypical Th1/CD8 TF STAT1 can also bind to BACE1 promoter (380). NFAT, an

important TF in the synthesis of pro-inflammatory cytokines like IL-17, TNFα or IFNγ as well as the proliferation-inducer IL-2, can also bind to BACE1 promoter and induce the expression of this secretase (381,382). The links between BACE1 expression and inflammation go even further; long-term treatment with non-steroidal anti-inflammatory drugs reduces the risk for Alzheimer's disease (383). Furthermore, pro-inflammatory cytokines like IFNγ (384) and TNFα (385) have been reported to promote BACE1 synthesis and activity. More importantly, the activation of the Th17-signature transcription factor STAT3 results in increased transcription and translation of BACE1 (356,386).

BACE1 expression is tightly linked to cellular stress conditions. Oxidative stress induces the expression of BACE1 through JNK-AP1, another signaling cascade associated with the production of inflammatory cytokines (387). Hypoxic conditions can also trigger BACE1 production through an initial ROS burst and induction of the JNK-AP1 pathway, and the later activation of hypoxia-inducible factor 1-alpha (Hif-1α) (356,358). Energy deprivation (glucose deprivation in cell culture) also leads to a post-transcriptional increase of BACE1 levels (388). Notably, glucose deprivation also results in impaired Th17/Treg balance (259). In low energy environments, Th17 responses are mainly mediated by Hif-1α, upregulating the glycolytic capacity of these cells to extract more energy from a scarce source as well as upregulating RORγt and favoring the polarization of Th17 cells (258,259). Interestingly, excessive glucose levels can impair Th17 responses through induction/activation of peroxisome proliferator-activated receptor gamma (PPARγ) (389,390). PPARγ dampens Th17 differentiation by inhibiting STAT3 signaling (391), resulting in reduced levels of RORγt, IL-17A and partial resistance to EAE (389). In neurons, PPARα and PPARγ exert a direct suppression of BACE1 expression after binding to the

BACE1 promoter (383,385). In fact, it is believed that the beneficial effects of NSAID treatment in AD are due to enhancement of PPAR production (385).

Finally, it has been reported that in a rat model of LPS-induced neuroinflammation, both BACE1 and IL-17A expression was upregulated. Interestingly, IL-17A blockade with monoclonal antibodies resulted in decreased BACE1 expression and reduced LPS-induced memory impairment (392).

In light of the newly discovered roles of BACE1 in neuroinflammatory diseases, in which IL-17A can be an important component, the numerous immune pathways promoting BACE1 expression and the role of BACE1 in myelination, we hypothesized that BACE1 may play a role in Th17-driven neurodegeneration in MS.

2.0 MATERIALS AND METHODS

2.1 MICE

C57BL/6 (WT), BACE1$^{-/-}$, CD73$^{-/-}$, APP$^{-/-}$, WT CD45.1^{+} and WT 2D2^{+} were originally purchased from The Jackson Laboratories (Bar Harbor, ME) and colonies maintained in the animal facilities of the University of Pittsburgh (South BST Facility). Rag1$^{-/-}$ mice were always purchased from The Jackson Laboratories for every experiment (Table 1).

WT and BACE1$^{-/-}$ mice were crossed to WT 2D2^{+} in order to obtain BACE1$^{-/-}$ 2D2^{+} mice and breeding pairs kept as BACE1$^{-/-}$ 2D2^{+} x BACE1$^{-/-}$.

PTEN$^{fl/+}$ mice were acquired from Dr. Lawrence Kane, University of Pittsburgh, and were originally CD4^{Cre} PI3KIP$^{fl/+}$ PTEN$^{fl/+}$. After a three generations of back-crossing we obtained the CD4^{Cre} PTEN$^{fl/+}$ strain that we used in our experiments. Kane Lab originally purchase PTEN$^{fl/fl}$ mice from The Jackson Laboratories (see Table 1).

For all experiments, animals used were in the C57BL/6 background and matched according to gender and age. All used animals were at least 4 weeks old when used. Animal protocols were approved by the University of Pittsburgh IACUC, and adhered to guidelines in the Guide for the Care and Use of Laboratory Animals of the NIH.

Common name	Mouse strain	Stock number
WT	C57BL/6J	000664
BACE1$^{-/-}$	B6.129-*Bace1tm1Pcw* /J	004714
WT CD45.1^{+}	B6.SJL-*Ptprca Pepcb* /BoyJ	002014
Rag1$^{-/-}$	B6.129S7-*Rag1tm1Mom* /J	002216
CD73$^{-/-}$	B6.129S1-*Nt5etm1Lft* /J	018986
WT 2D2^{+}	C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J	006912
APP$^{-/-}$	B6.129S7-*Apptm1Dbo* /J	004133
PTEN$^{fl/fl}$	B6.129S4-*Ptentm1Hwu* /J	006440

Table 1. Strain names and Stock numbers (The Jackson Laboratories) of all mouse strains used in the development of this project.

2.2 TISSUE PROCESSING AND CD4^{+} T CELL ISOLATION

Single cell suspensions from spleen and lymph nodes of naïve and immunized mice were obtained by passing the tissues through a 70 μm cell strainers. After erythrocyte lysis, washes and counting total lymphocytes, CD4^{+} T cells were purified using CD4^{+} (L3T4) magnetic separation (Miltenyi Biotec, Germany), following the manufacturer's instructions. Final CD4^{+} T cell suspensions were counted and re-suspended in Complete RPMI: Roswell Park Memorial Institute (RPMI 1640) medium (GE HylClone) was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-β-mercaptoethanol (Gibco), HEPES and Na pyruvate, before further use. In all experiments, CD4^{+} purity was always ≥ 95%.

For *in vitro* cultures, T cells were obtained from combining the spleen and lymph nodes (inguinal, brachial, axillary, submandibular, cervical, mesenteric and para-aortic lymph nodes) of individual naïve

mice. For EAE *ex vivo* studies, single cell suspensions from draining lymph nodes (inguinal and brachial) were obtained as previously described, without previous isolation of $CD4^+$ T cells.

2.3 *IN VITRO* T HELPER SUBSET DIFFERENTIATION

All T cells were activated with 5 μg/mL plate-bound α-CD3 (clone 145-TC11, BioXcell) and α-CD28 (clone 37.51, BioXcell) in complete RPMI. For Th17 differentiation, cells were cultured in the presence of recombinant mouse TGF-β1 (10 ng/mL), IL-6 (100 ng/mL), IL-1β (40 ng/mL) and IL-23 (20 ng/mL); all cytokines were purchased from R&D Systems, MN. In all Th0 cell cultures, $CD4^+$ T cells were cultured in the presence of 10 μg/mL anti-IFNγ neutralizing antibodies (clone XMG1.2, BioXcell). For Th1 cultures, IL-12 (PeproTech, NJ) was added at a final concentration of 10 ng/mL. For Treg differentiation, T cells were cultured in the presence of recombinant mouse TGF-β1 (20 μg/mL), recombinant human IL-2 (100 U/mL) and anti-IFNγ neutralizing antibodies (10 μg/mL).

In order to identify WT and $BACE1^{-/-}$ T cells in co-culture experiments, we used $CD4^+$ T cells isolated from naïve *B6.SJL-Ptprca Pepcb/BoyJ* (CD45.1 WT) mice. This is a congenic strain of C57Bl/6 background that expresses CD45.1 instead of the molecule CD45.2 found in the C57BL/6 background. CD45.1 WT and CD45.2 $BACE1^{-/-}$ $CD4^+$ T cells were co-cultured at a 1:1 ratio to a total cell density of $0.25x10^6$ cells/well, and differentiated to Th17 cells as described above.

Subset	Cytokines/Antibodies	Final Concentration
Th0	αIFNγ	10 μg/mL
Th1	IL-12	10 ng/mL
Th17	TGF-β1	10 ng/mL
	IL-6	100 ng/mL
	IL-1β	40 ng/mL
	IL-23	20 ng/mL
Tregs	TGF-β1	20 ng/mL
	αIFNγ	10 μg/mL
	human IL-2	100 U/mL

Table 2. Cytokine cocktails used for *in vitro* Th differentiation experiments

2.4 OTHER REAGENTS

In order to test the functional requirement of different signaling pathways during Th17 cell activation and differentiation, we *in vitro* polarized T cells from naïve WT and BACE1$^{-/-}$ mice as described before, in the presence of different compounds. In general, CD4^{+} T cells were pre-incubated with these inhibitor/activator molecules for 30 min to 2 hours before the addition to plate/bound α-CD3/α-CD28 stimulation in the presence of Th17-polarizing conditions. A complete list of the reagents used in in vitro cultures and their sources can be found in Table 3.

Compound	Source	Cat. #
InSolution BACE1 Inhibitor IV	Calbiochem/EMD Millipore	565794-500UG
LY2811477 - BACE1 Inhibitor	Selleckchem	S1528
Ionomycin - Ca^{2+} Ionophore	Fisher	BP2527-1
SF 1670 - PTEN Inhibitor	Tocris	2020-10mg
Akti1/2 Inhibitor VIII - Akt Inhibitor	Calbiochem/EMD Millipore	124018
Forskolin - AC Activator	EMD Millipore	344270-10mg
Adenosine	Fisher	AC16404-0050
NECA - Adenosine receptor Agonist	Abcam	ab120440-10mg
SCH 5826 - Adenosine receptor Antagonist	Tocris	2270-10mg
cAMP	Fisher	AC22580-5000

Table 3. Reagents and references used in *in vitro* experiments

2.5 FLOW CYTOMETRY

Single cell suspensions obtained *ex vivo* from naïve/immunized mice or $CD4^+$ T cells obtained from *in vitro* cultures (Th cultures) were washed once with PBS and incubated for 20 min on ice with Ghost Dye™ Violet 510 (TONBO biosciences, CA) to exclude dead cells. Next, cells were washed in PBS containing 2% FBS and 1% EDTA (FACS Buffer) and stained for surface markers at a 1/100 dilution.

The following FACS antibodies were purchased from BD Biosciences: CD4 (RM4-5), CD25 7D4, CD44 (IM7), CD45.1 (A20), CD45.2 (104), Ki67 (B56), IFNγ (XMG1.2), IL-17A (TC11-18H10), pSTAT3 pY705 and $2D2^+$ TCR-Vα3.2 (RR3-16). The following were purchased from eBioscience: CD73 (eBIOTY/11.8), RORγt (AKFJS9), Foxp3 (FJK-16s), IL-17F

(eBio18F10), GM-CSF (MP1-22E9) and pSTAT3 pY705. Antibody clone information is shown inside parenthesis. Typically, all antibodies were used at a 1:100 dilution, except pSTAT (1:20).

To determine intracellular cytokine production, single cell suspensions were stimulated in Complete RPMI with 50ng/mL of phorbol-myristate acetate (PMA) and 500 ng/mL ionomycin in the presence of Golgiplug for 3-4 hours, at 37ºC, before staining with Ghost Dye™ Violet 510. After surface marker staining, cells were fixed for at least 20 minutes with Cytofix/Cytoperm kit (BD Biosciences) and stained for cytokine production using specific antibodies. Staining of other intracellular markers, such as transcription factors did not require PMA/ionomycin stimulation, and fixation/permeabilization was performed using the Foxp3/Transcription Factor staining kit from eBioscience. For intracellular staining, all cells were incubated for 40 min with specific antibodies at a 1/100 dilution. All staining/fixing steps were performed at 4ºC.

In order to identify phosphorylated targets, pre-warmed phosphoFlow fixative reagent (contains Formaldehyde) was directly added onto cell suspensions at a 1:1 (vol:vol) ratio and incubated for 10 min at 37ºC. After washing with FACS buffer, cells were resuspended with 200 µL of ice-cold phosphoFlow reagent III (contains Methanol), and incubated for 20 minutes on ice. It is important to add the permeabilizing agent while vortexing the cells. Next, cells were washed with FACS buffer and stained with specific antibodies for 1 hour at room temperature using the manufacturer's recommended antibody dilutions.

All flow samples were resuspended to a final volume of 200uL with FACS buffer and samples were collected using a BD FACSARIA or BD LSRFORTESSA (both from BD Biosciences). All flow cytometry data analysis was performed using FlowJo v.10.0.7.

2.6 CYTOKINE DETECTION BY ELISA

In order to assess the secretion capacity of different cytokines by T cells we performed Enzyme-Linked ImmunoSorbent Assay (ELISA) from culture supernatants. We specifically measured IFNγ, IL-17A (homodimer), IL-17F (homodimer) and GM-CSF secretion from *in vitro*-differentiated T helper subsets, as well as from antigen-specific re-challenge responses. All cytokines were detected using Ready-SET-Go! ELISA kits from eBioscience, following the manufacturer's instructions. Samples were diluted accordingly using the diluent buffer included in the kits.

2.7 RNA EXTRACTION AND QRT-PCR

In order to analyze gene expression we performed quantitative real-time polymerase chain reaction (qRT-PCR). In this technique, mRNA was first converted to cDNA before quantification and amplification.

At the time of harvest, cell suspensions were washed and lysed/stored in RLT buffer (QIAGEN, Netherlands). Total RNA was isolated from cell suspensions using the RNeasy Mini Kit (QIAGEN, Netherlands) and resuspended in RNAse-free water before quantifying RNA by Nanodrop. When samples were to be used for RNAseq, in-column DNAse treatment was performed during RNA isolation, as per the manufacturer's instructions. Reverse Transcriptase reaction was used to convert RNA molecules into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosciences). During this step, all samples were normalized to the same amount of initial RNA (200 ng). In order to quantify the number of transcripts of a particular gene, we performed qRT-PCR using specific RT^2 qPCR primers from QIAGEN (see section XX) and the SYBR Green detection method. In this method, SYBR Green (Excella™, WorldWide Life Sciences Division, Inc., PA) acts as a DNA intercalating dye, allowing fluorescent detection of individual copies of DNA. PCR amplification was performed with a 7300 Real Time PCR System (Applied Biosciences). The expression of all genes of interest was normalized to constitutive expression of *Gapdh* (housekeeping gene) and calculated/represented as: $(2^{-\Delta CT})$ x 1,000,000.

2.8 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) – MOUSE MODEL OF MULTIPLE SCLEROSIS

2.8.1 Active immmunization

Naïve mice were immunized subcutaneously with 100µg MOG_{35-55} (Bio synthesis, Lewisville, Texas, USA) emulsified with 100µL of Complete Freund's Adjuvant (CFA) (Difco Laboratories, Detroit, Michigan, USA) containing 100µg of heat-killed *Mycobacterium tuberculosis* (strain

H37Ra) (Difco Laboratories, Detroit, Michigan, USA) distributed in four sites on the flanks. 200 ng Pertussis toxin (List Biological Laboratories) was given intraperitoneally on day 0 and 2, in sterile PBS. Mice were assessed daily for symptoms of paralysis and disease severity was evaluated using the following scoring system: **1**, flaccid tail; **2**, impaired righting reflex and hindlimb weakness; **3**, partial hindlimb paralysis; **4**, complete hindlimb paralysis; **5**, hindlimb paralysis with partial forelimb paralysis; **6**, moribund/dead. For humane reasons, any animals with a score of 5 for two consecutive days or moribund were euthanized immediately. All cages with mice with a score higher or equal to 3 were given soft mash and water in a petri dish to facilitate their food and water intake.

In order to determine the *in vivo* relevance of a T cell-specific BACE1 deletion, WT or BACE1$^{-/-}$ CD4^{+} T cells were obtained as previously described (Section 2.2.1). 10-14 x 10^{6} CD4^{+} T cells were transferred intraperitoneally to naïve Rag1$^{-/-}$ recipients, which were immunized one day post transfer, as described before.

2.8.2 Passive transfer model of EAE

Spleen and lymph nodes were harvested from WT or BACE1$^{-/-}$ 2D2^{+} TCR transgenic mice and stimulated *in vitro* using an adaptation of the protocol described by Jager *et al*. (123). Spleen and lymph nodes (LN) were collected and cell suspensions were obtained as previously described. Total splenocytes were activated with MOG_{35-55} (20ug/mL) in the presence TGF-β1 and IL-6 (5 and 50ng/mL respectively) for 4 days, in T75 flasks. Cells were washed, split and resuspended to equivalent cell densities in Complete RPMI containing recombinant human IL-2 (10U/mL). After

three days of resting, cells were reactivated in 24-well plates with plate-bound α-CD3 (1ug/mL) and soluble IL-23 (20ng/mL) for two days before transferring to recipient mice. To verify Th17 polarization, expression of IL-17A was determined by flow cytometry at the end of the first activation stage (Day 4).

In this model, transferring MOG-specific autoreactive Th17 cells is sufficient to induce EAE, and no immunization or injection of Pertussis Toxin is necessary. Due to the exacerbated Th17 response some mice developed atypical EAE. Atypical EAE is characterized by advanced ataxia, circling movements or head tilt rather than the archetypal ascending paralysis that characterizes typical EAE. In our experiments, most mice that developed atypical symptoms also showed some degree of paralysis. In order to standardize the scoring system between mice that developed atypical symptoms and those that did not, we followed the regular typical EAE scoring system described before, except that all atypical EAE mice were recorded with a minimum score of 2.

2.8.3 Adoptive transfer of $2D2^+$ T cells

WT CD45.1 recipient mice received, via intraperitoneal injection, 10^5 $CD4^+$ T cells obtained from WT $2D2^+$ or $BACE1^{-/-}$ $2D2^+$ TCR transgenic mice. One day after T cell transfer, recipient mice were immunized with 100 μg MOG_{35-55} emulsified in CFA. On day 8 post-immunization, mice were euthanized and draining lymph nodes were harvested to obtain single cell suspensions. *Ex vivo* flow cytometric analysis was performed following cell stimulation with PMA and ionomycin for 3-4 hours in the presence of Golgiplug.

2.9 ANTIGEN-SPECIFIC RE-CHALLENGE RESPONSES

Draining lymph nodes and CNS from mice immunized with MOG_{35-55} were processed to obtain single cell suspensions. Total lymphocytes were cultured in flat bottom 96-well plates at a cell density of 1M cells/well and cultured in the presence of MOG_{35-55} (50 μg/mL) in order to evaluate cytokine production after an antigen specific response. In order to skew this reaction towards a Th1 or Th17 response, IL-12 (10 ng/mL) or IL-23 (20 ng/mL) were added in the media, respectively. Cells were cultured for 3-5 days and culture supernatants were used for analysis of the cytokine production by ELISA.

2.10 T CELL ACTIVATION ASSAYS

$CD4^+$ T cells from both naïve WT and $BACE1^{-/-}$ mice were collected as previously described and resuspended to a cell density of $20x10^6$ cells/mL. $4x10^6$ $CD4^+$ T cells were added to the wells of round bottom 96-well plates, with all conditions plated at the same time. T cells were activated at different times with the addition of biotinylated αCD3 (2ug/mL) and αCD28 (2ug/mL) pre-mixed with cross-linking streptavidin (10ug/mL). All stimulated $CD4^+$ T cells were collected at the same time, washed with cold PBS and lysed with 0.1% NP40 lysis buffer in the presence of protease inhibitors. Whole cell lysates were stored at -80C before analysis. In collaboration with Lyndsay Avery (Kane Lab) and Dr. William Hawse, lysates were immunoprobed by Western Blot to detect and quantify the levels of phosphorylated Akt and total PTEN with specific antibodies.

2.11 DETECTION OF ADENYLATE CYCLASE ACTIVITY – QUANTIFICATION OF INTRACELLULAR CAMP LEVELS

$CD4^+$ T cells from both naïve WT and $BACE1^{-/-}$ mice were collected as previously described and stimulated with 10uM Forskolin, an adenylate cyclase activator, for 30 minutes. Cells were pelleted and washed with cold PBS to stop the reaction. Total levels of intracellular cAMP were detected and colorimetrically quantified with cyclic AMP Complete ELISA kit (Abcam, Cambridge, UK). Cell lysis, cAMP acetylation and detection with specific antibodies were performed with the kit reagents, according to the manufacturer's instructions.

2.12 RNAseq

WT and $BACE1^{-/-}$ $CD4^+$ T cells, isolated as described above, were polarized *in vitro* to Th17 cells with plate-bound αCD3 (5ug/mL) in the presence of TGF-β1 (10ng/mL) and IL-6 (100ng/mL) for two days. Cells were carefully washed and media was substituted by Complete RPMI containing TGF-β1 (10ng/mL), IL-6 (100ng/mL), IL-1β (40ng/mL) and IL-23 (20ng/mL) for two more days. At day 4 post-differentiation, Th17 cells were harvested and RNA extracted using RNeasy Mini Kit (QIAGEN, Netherlands) with in-column DNAse treatment. RNA concentration was quantified by Nanodrop and samples given to the Health Sciences Sequencing Core. Evaluation of RNA quality (RIN), library preparation and RNA sequencing was performed by William Horne and collaborators and expression data (fpkm (Fragments Per Kilobase of transcript per Million mapped reads) values) were uploaded to GeneSifter (PerkinElmer, Waltham MA), an online platform that allows pairwise analysis. Further statistical analyses and generation of hierarchical clustering of

differentially regulated genes between BACE1$^{-/-}$ and WT Th17 cells was performed with Partek Genomics Suite (Partek Inc., Chesterfield MO).

2.13 STATISTICS

Parametric values were analyzed using Student's t-test, or ONE-WAY ANOVA (with Tukey's correction for multiple comparisons) when more than two groups were compared. EAE clinical scores and date of onset were analyzed using Mann-Whitney test (daily scores were analyzed separately). P values are shown as * = ($p < 0.05$), ** = ($p < 0.01$), *** = ($p < 0.001$) and **** = ($p < 0.0001$), where statistical significance was found.

3.0 RESULTS

3.1 BACE1: A NOVEL PLAYER IN TH17 FUNCTION

3.1.1 Introduction

A key component of MS is the occurrence of episodes of clinical worsening with either new symptoms or an exacerbation of older symptoms over several days or weeks, known as exacerbations or flares (72,98). Most of current MS therapies that target the immune response are focused on the treatment of the acute inflammatory attack that occurs during these relapse episodes. MS relapses have been attributed to the generation of new white matter lesions caused by the activation, migration or expansion of autoreactive immune cells in the CNS, particularly Th17 cells (112,393). Th17 cells and their cytokines have been linked with neurodegenerative states due to inflammatory triggers, exacerbating CNS lesions and promoting disability (180,193). Due to the damaging impact of inflammatory Th17 cells on neurons and glial cells, we sought to identify new molecules linking CNS damage or neurodegeneration to local inflammation and immune responses.

BACE1 is a transmembrane aspartyl protease expressed on the surface of both neurons and astrocytes and is responsible for the initial cleavage of amyloid precursor protein (APP) in Alzheimer's disease (AD) (263). In AD mouse models, it has been shown that BACE1$^{-/-}$ animals are resistant to the development of disease (286–290) and BACE1 blockade significantly reduces

both formation of amyloid plaques and cognitive impairment (394). In fact, BACE1 inhibitors targeting its catalytic activity are currently being tested in clinical trials to treat AD (309,395).

In AD, BACE1 expression is tightly linked to inflammation. Inflammatory cytokines, including IFNγ and TNFα (384,385), or hypoxia resulting from ischemic insults (356–358,396) contribute to the upregulation of BACE1. In contrast, chronic use of non-steroidal anti-inflammatory drugs reduces BACE1 expression and is associated with reduced amyloid plaque burden (383,385). Interestingly, BACE1 is upregulated in other neuroinflammatory disorders, such as ischemia (357,358,396) and traumatic brain injury (354,355). Taken together, these data suggest that BACE1 may play a broader role in CNS inflammation than previously considered. Concomitantly, the IL-23-IL-17 axis has been shown to promote neurodegeneration and impair recovery after brain ischemia (256). Interestingly, IL-23R polymorphisms with reduced IL-23 signaling have been correlated with decreased incidence of AD (111,397). In AD mouse models, IL-23$^{-/-}$ mice showed reduced levels of amyloid markers and plaques both in the CNS and the CSF, which resulted in reduced cognitive deficits (398). However, any connections between BACE1 and Th17 cells have never been addressed.

Although initially regarded as a Th1-driven autoimmune disease, evidence collected in the past decade using the EAE model demonstrated that IL-23-driven Th17 cells are predominantly responsible for the demyelinating autoimmune attack in MS/EAE (156,157,171). In addition to its role in amyloid plaque formation, BACE1 plays a critical role during developmental myelination. This is achieved by promoting the proteolytic maturation of Neuregulin-1, a molecule expressed on the axonal membrane that guides myelin production from oligodendrocytes and Schwann cells

(299,307). For this reason, both Neuregulin-1 and BACE1$^{-/-}$ mice exhibit hypomyelinated nerves (295,299). Although these mice display some cognitive defects, increased tolerance to pain and an increased rate of perinatal death (292), they do not develop spontaneous MS/EAE symptoms, suggesting that their myelin levels are sufficient to properly transmit motor signals. Nevertheless, one could speculate that reduced myelin levels should render these mice more susceptible to EAE induction, since a lower degree of myelin damage could be sufficient to reach the threshold required for the onset of clinical symptoms.

We therefore sought to investigate two things: (1) whether BACE1 plays a role in the autoimmune response during EAE and (2) whether hypomyelination caused by BACE1-deficiency in the CNS results in increased susceptibility or severity of EAE.

3.1.2 BACE1 regulates production of IL-17A in autoreactive Th17 cells in EAE

In order to determine the role of BACE1 in CNS inflammation, we made use of the EAE model, a mouse model of multiple sclerosis, commonly used to study autoimmunity. We immunized WT and BACE1$^{-/-}$ mice subcutaneously with MOG_{35-55}, a myelin peptide, in CFA. Mice were also injected with PTx, intraperitoneally, at days 0 and 2 post immunization. We observed that BACE1$^{-/-}$ mice developed EAE symptoms slightly but consistently earlier than the WT group, presenting an onset of disease 1-2 days earlier (Figure 2A and C). This findings suggested that BACE1$^{-/-}$ mice may have increased susceptibility to autoimmune-mediated CNS damage. However, the frequency of mice that developed EAE (Figure 2B) and overall severity (Figure 2A and D) were not increased

in BACE1$^{-/-}$ mice, despite strain-inherent CNS defects and the apparent enhanced susceptibility of these mice to develop EAE.

This phenomenon led us to study the immune response in BACE1$^{-/-}$ mice. For that purpose, we re-challenged lymphocytes from the draining lymph nodes, spleens and CNS of MOG_{35-55}-immunized mice with the same peptide in the presence of IL-12 or IL-23 to skew the immune response towards Th1 or Th17, respectively. Interestingly, we found that MOG-induced T cell IL-17A production was significantly reduced in the absence of BACE1 (Figure 2E). During EAE, Th17 cells also produce IFNγ, but the production of this cytokine remained unaltered (Figure 2F). These results were consistent, independent of the time after immunization at which analysis was carried out (pre-onset, onset or peak of disease).

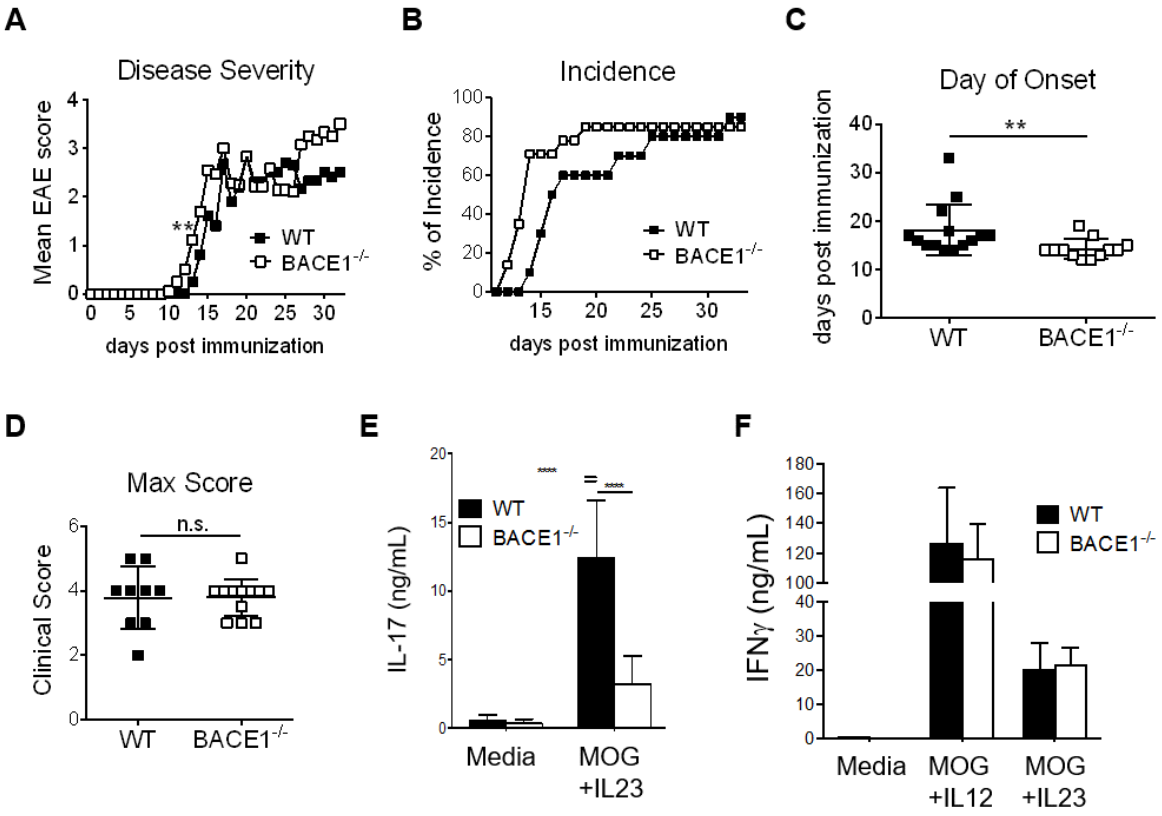

Figure 2. BACE1 regulates production of IL-17A in autoreactive Th17 cells in EAE

WT and BACE1$^{-/-}$ mice were immunized to induce EAE and the course of disease was evaluated: we measured (**A**) clinical score (**B**) incidence, (**C**) day of onset and (**D**) maximum score. Data pooled from 4 independent experiments with a total of 18 WT and 18 BACE1$^{-/-}$ mice represented in the graphs. **E-F.** On day 16 p.i., draining LN cells were stimulated *in vitro* with 50μg MOG_{35-55} in the presence or absence of IL-23 or IL-12 for three days and production of IL-17A (**E**) or IFNγ (**F**) from culture supernatants was measured by ELISA. Data representative of at least three independent experiments.

The role of BACE1 has been extensively studied in neurons and astrocytes, but no function of BACE1 in the immune system has been reported to date. Our data suggest that BACE1 participates in the development or function of pathogenic Th17 cells in EAE. This was a completely novel and unexpected role of BACE1. Due to the strong interest in Th17 immunology

of our lab, we focused our efforts on exploring the impact of BACE1 deficiency in Th17 development and effector function, both *in vitro* and *in vivo*.

3.1.3 CNS-specific BACE1 deficiency results in increased susceptibility to EAE whereas a BACE1-deficient immune compartment confers EAE protection

Neuronal BACE1 is necessary for the correct maturation of Neuregulin-1 (306), and therefore it is crucial during developmental myelination (295). Although the spinal cords (CNS) and sciatic nerves (PNS) of BACE1$^{-/-}$ mice exhibit hypomyelination (Figure 3A and B), the magnitude of this defect is not sufficient to cause spontaneous MS or EAE symptoms. However, inducing EAE, an autoimmune demyelinating model, in full BACE1$^{-/-}$ mice could lead to mixed results due to the antagonistic pleiotropy of BACE1, through its actions on different cell types.

In order to eliminate any confounding effects of BACE1 deficiency in the CNS, we decided to study the *in vivo* effect of restricting deficiency of BACE1 to CD4^{+} T cells. To this end, WT or BACE1$^{-/-}$ CD4^{+} T cells were transferred into Rag1$^{-/-}$ recipients, followed by EAE induction (Figure 3C). Rag1$^{-/-}$ mice lack T and B cells, and therefore all of the T cells in these recipients originate from the donor. Interestingly, only mice that received WT CD4^{+} T cells developed EAE, whereas all animals transferred with BACE1$^{-/-}$ CD4^{+} T cells were fully protected (Figure 3D).

It was very striking that despite the reduced IL-17A response to myelin antigens, complete BACE1$^{-/-}$ mice developed quasi-normal EAE clinical signs, in contrast to T cell-specific BACE1 deficiency. EAE is a demyelinating autoimmune model, therefore defects in developmental myelin

can influence the course of disease. EAE signs appear when action potentials cease to fully propagate from neuron to neuron and the lower the amount of nerve insulation (myelination) the more likely these signs will appear. We hypothesized that BACE1 deficiency in the CNS, and therefore CNS hypomyelination, poises these animals for enhanced susceptibility to EAE. Namely, minor autoimmune attack of the myelin sheath in these mice would suffice to cause EAE signs, whereas in a WT CNS environment, more extensive myelin damage would be required before EAE signs can develop. To test this hypothesis and to better dissect a differential role of BACE1 in the CNS and in the immune compartment we generated bone marrow chimeric mice. WT or $BACE1^{-/-}$ recipient mice were sub-lethally irradiated with a single dose of 900 rad, before being transferred intravenously with bone marrow cell suspensions from WT or $BACE1^{-/-}$ mice (Figure 3E). After 6 weeks of reconstitution, mice were immunized with MOG_{35-55} in CFA to induce EAE.

We found that mice with BACE1 deficiency in the CNS but not the immune compartment ($WT \rightarrow BACE1^{-/-}$) presented earlier disease onset compared to mice that expressed BACE1 in both compartments ($WT \rightarrow WT$) (Figure 3F), suggesting that lack of BACE1 in the CNS confers enhanced susceptibility to EAE. Conversely, mice with $BACE1^{-/-}$ immune system but normal BACE1 expression in the CNS ($BACE1^{-/-} \rightarrow WT$) exhibited slightly delayed onset of disease, conferring partial protection. Of note, this was quite a minor effect compared to the full protection conferred by $BACE1^{-/-}$ T cells during transfer experiments into $Rag1^{-/-}$ mice. This difference may be partially explained by the enhanced susceptibility to EAE displayed by irradiated mice. More importantly, we were able to distinguish donor T cells from recipient T cells by checking the expression of congenic markers (CD45.1 vs CD45.2) and observed that, whereas most B cells in recipient mice originated *de novo* from the donor bone marrow, T cells showed a mix of WT and

BACE1$^{-/-}$ immune cells in mice that received BACE1-deficient hematopoietic stem cells (Figure 3G). Hence it appears that the irradiation was not fully successful. It has been previously shown using mixed bone marrow chimeras, that the presence of WT cells can compensate almost completely for defects in (IL-23RA$^{-/-}$) Th17 cells (171) and therefore explain the reduced effect we observe when recipient WT are still present in WT→ BACE1$^{-/-}$ chimeric experiments. One possibility to increase the efficacy in depleting the recipient T cell compartment is to irradiate recipient mice with a split dose of 450 rad with a time-lapse of 6 hours. This period of time allows for all progenitor cells to be found in a proliferative state and therefore more sensitive to radiation killing (399). Although our bone-marrow chimera results were less impressive than the adoptive transfer experiments, one goal of these experiments was still accomplished. We found proof of concept that reduced myelination poised BACE1$^{-/-}$ recipients more susceptible to EAE induction. Due to the large numbers of mice required and the extensive time needed to complete bone-marrow chimera EAE experiments we decided not to proceed with split-dose experiments and focus our efforts and resources in the identification of mechanisms underlying BACE1 modulation of Th17 cells (section 3.3).

Interestingly, mice with BACE1 deficiency in both compartments presented an onset and course of clinical signs between WT into BACE1$^{-/-}$ and BACE1$^{-/-}$ into WT (Figure 3F), further highlighting that the effects we observed in full BACE1$^{-/-}$ mice were due to a combination of enhanced susceptibility resulting from lower developmental myelination and the reduced inflammatory response from BACE1$^{-/-}$ IL-17-producing T cells.

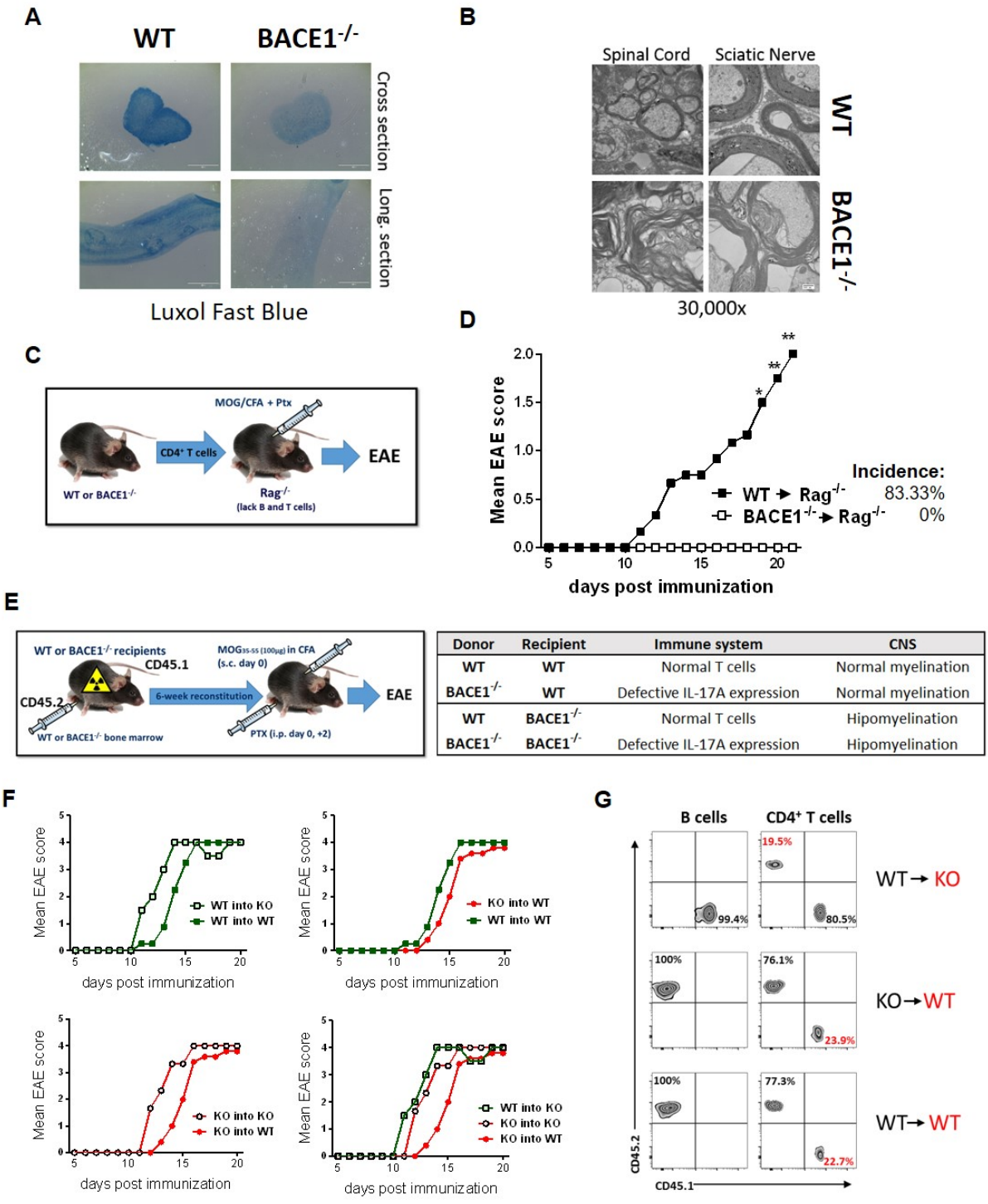

Figure 3. CNS-specific BACE1 deficiency confers increased susceptibility to EAE, whereas a BACE1-deficient immune compartment results in EAE protection.

A. Spinal cords from WT and BACE1$^{-/-}$ mice were excised and sectioned and myelin levels were detected using Luxol Fast Blue staining. **B.** Transmission electron microscopy micrographs from naïve spinal cords and sciatic nerve from

naïve WT and BACE1$^{-/-}$ mice. Spinal cords processed and electron micrographs taken by Jonathan Franks (Center for Biologic Imaging, University of Pittsburgh). **C-E.** 10^6 CD4$^+$ T cells from naïve WT or BACE1$^{-/-}$ mice were transferred intraperitoneally into Rag1$^{-/-}$ recipients one day before immunization with MOG_{35-55} in CFA to induce EAE. **D.** Clinical scores and frequency of EAE-affected mice were measured daily. Results are representative of three independent experiments with similar results. **E.** Timeline of the generation of bone-marrow chimeras and active induction of EAE (right) and description of the differences in the immune and CNS compartments in the various bone-marrow chimeras we generated. **F.** EAE severity was measured daily after active immunization with MOG_{35-55} in CFA. **G.** Reconstitution of the immune compartment was checked 6 weeks after bone-marrow transplantation. Expression of donor CD45.2 and recipient CD45.1 congenic markers were checked from peripheral blood B cells and T cells. Data representative from three independent experiments where both male and female mice were tested.

3.1.4 BACE1 is dispensable for Th1 and Treg differentiation

In order to test whether the reduced IL-17A expression from BACE1$^{-/-}$ autoreactive T cells was a specific deficiency and not a more general defect in T cell activation or development, we differentiated *in vitro* CD4$^+$ T cells from naïve WT and BACE1$^{-/-}$ mice into the three most relevant T helper subsets for EAE development: Th1, Th17 and Tregs.

CD4$^+$ T cells were differentiated into Th1 cells in the presence of IL-12. After two days, we measured the expression of their signature cytokine, IFNγ, to find that there were no differences between WT and BACE1$^{-/-}$ Th1 cells (Figure 4A and B). In addition, the mRNA expression of the Th1 lineage-defining transcription factor, T-bet, was not altered in the absence of BACE1 (Figure 4C).

Induction of Tregs (iTregs) was achieved by culturing $CD4^+$ T cells with TGF-β1 and IL-2 in the presence of IFNγ-blocking antibodies. BACE1 deficiency had no effect on Foxp3 gene expression levels from iTregs (Figure 4D and E), measured by qRT-PCR (Figure 4E), or protein levels (Figure 4D), analyzed by flow cytometry.

To further exclude a possible role of BACE1 in Tregs, we also studied the naturally-occurring populations of regulatory T cells from naïve mice. We found that the frequencies of lymph node and splenic $Foxp3^+$ T cells were very similar between WT and $BACE1^{-/-}$ mice (Figure 4F). In addition, expression of CD25 from $Foxp3^+$ T cells remained unchanged (Figure 4G), showing that regulatory T cells can develop and persist normally in the absence of BACE1 *in vivo*.

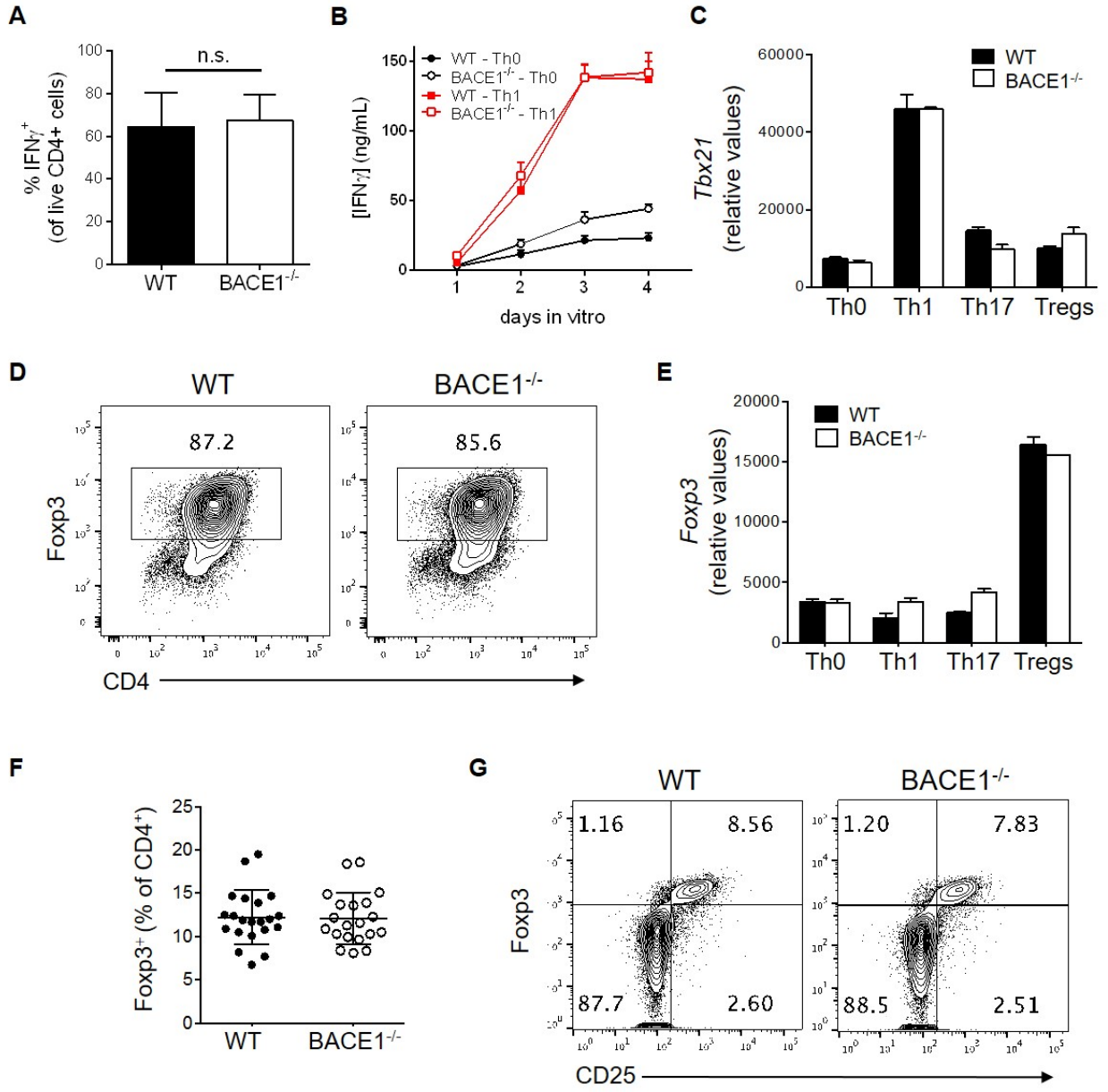

Figure 4. BACE1 is dispensable for Th1 and Treg differentiation.

A. Intracellular IFNγ expression assessed by flow cytometry in *in vitro*-polarized Th1 cells for two days, from WT or BACE1$^{-/-}$ CD4^{+} Tcells. **B.** Accumulative secreted IFNγ measured by ELISA, at indicated times, from culture supernatants. **C.** *Tbx21* gene expression in T cells cultured under the indicated differentiation conditions for two days. **D.** Intracellular Foxp3 expression was assessed by flow cytometry after three days of *in vitro* Treg polarization. **E.** *Foxp3* gene expression in T cells cultured under the indicated differentiation conditions for two days, normalized to *Gapdh* expression. **F.** Proportion of Foxp3^{+} T cells assessed by flow cytometry in LN and spleen of naïve WT and

BACE1$^{-/-}$ mice. **G.** Foxp3 and CD25 co-expression by Tregs in LN from naïve WT and BACE1$^{-/-}$. Data representative of at least four independent experiments.

3.1.5 BACE1 is necessary for IL-17 expression but does not affect general Th17 differentiation

Th17 cells were polarized for up to four days with TGF-β1, IL-6, IL-1β and IL-23 in the culture media. Cells were harvested and re-stimulated every day with PMA/ionomycin in the presence of Golgiplug to perform flow cytometric analysis of intracellular cytokine production. By this means, we found that BACE1$^{-/-}$ Th17 cells exhibited significantly reduced frequencies of IL-17A producing cells compared to WT Th17 cells (Figure 5A). Not only was the percentage of IL-17A^{+} cells reduced, but also the mean fluorescence intensity (MFI) of IL-17A signal (Figure 5B), showing that IL-17A expression was also reduced in a per cell basis.

The peak of IL-17A expression occurred, as expected, around day 3 of *in vitro* polarization. However, the defect in IL-17A expression from BACE1$^{-/-}$ Th17 cells could be identified as early as IL-17A cytokine levels could be detected (Figure 5D).

To verify that the defects in IL-17A expression were not due to enhanced plasticity of BACE1$^{-/-}$ Th17 cells with conversion to other T helper subsets, we analyzed the expression of Foxp3, T-bet and IFNγ from Th17 cells. We observed that the levels of Th1 and Treg markers remained low in both WT and BACE1$^{-/-}$ Th17 cells (Figure 4C and E), further proving that the defect in IL-17A expression is due to the specific regulation of this cytokine and not a defect in

Th17 polarization or conversion to other T helper subsets. This finding was further confirmed during RNAseq analysis (data not shown).

In order to address whether BACE1 regulation of IL-17A expression occurred at the gene expression level or post-translationally, we performed qRT-PCR analysis of *Il17a* gene expression at day 3 post differentiation, one day prior to the peak of IL-17A protein expression. BACE1$^{-/-}$ Th17 cells also showed a significant reduction in IL-17A mRNA (Figure 5C) and, interestingly the difference was more profound than that observed by flow cytometry (Figure 5A). Interestingly, ELISA analysis of culture supernatant to measure IL-17A secretion also revealed a more dramatic difference than what was detected by flow cytometry (Figure 5D).

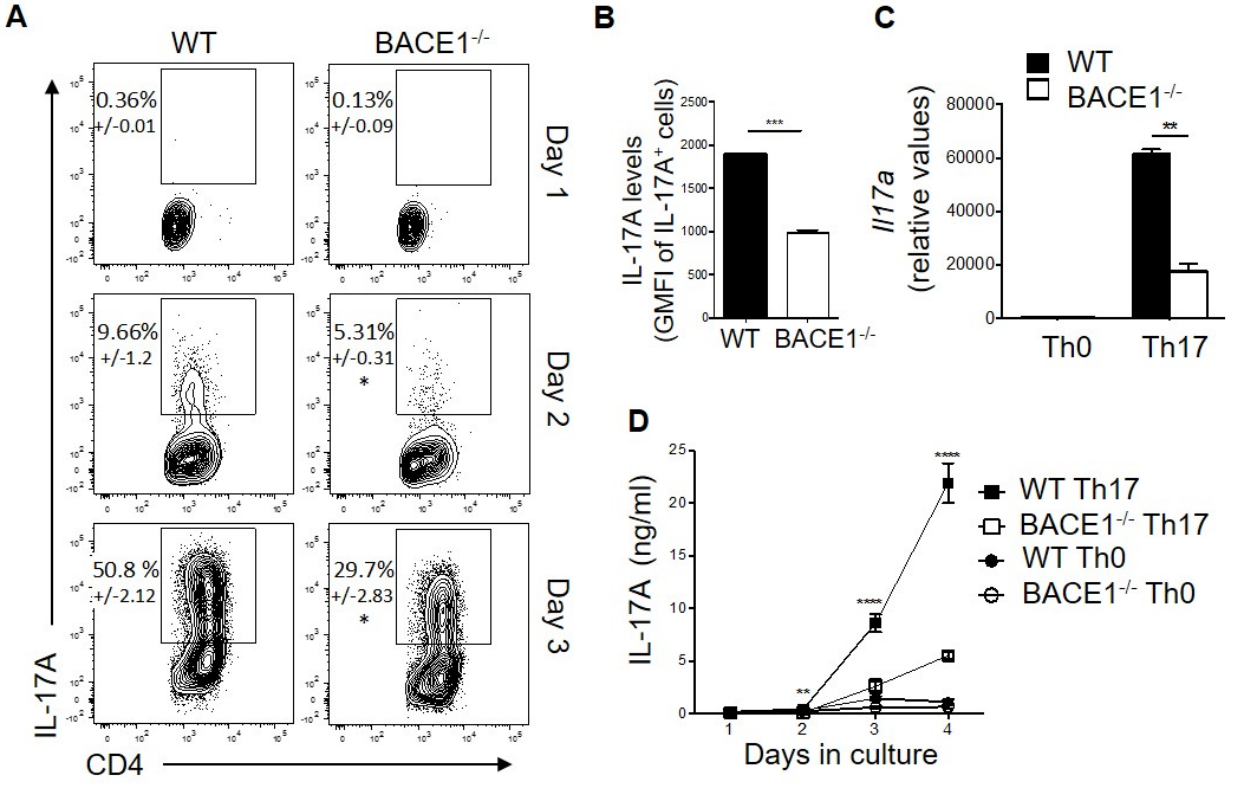

Figure 5. BACE1 is required for IL-17A expression by Th17 cells *in vitro*.

A. $CD4^+$ T cells from naïve WT or $BACE1^{-/-}$ mice were differentiated *in vitro* for three days under Th17-differentiating conditions and intracellular IL-17A expression was analyzed by flow cytometry, at the indicated times. **B.** Geometric mean fluorescence intensity of $IL\text{-}17A^+$ WT and $BACE1^{-/-}$ $CD4^+$ T cells, measured by flow cytometry on day three of Th17-polarization. **C.** IL-17A gene expression in Th0 and Th17-polarized WT and $BACE1^{-/-}$ T cells cultured for three days, normalized to *Gapdh* expression. **D.** IL-17A in culture supernatants was analyzed at the indicated times, reflecting accumulated cytokine production over time. All data are representative of at least three independent experiments with similar results.

To test whether BACE1 is specifically regulating IL-17A expression or is necessary for Th17 development overall, we analyzed Th17 markers and the expression of molecules that are necessary for Th17 polarization, by flow cytometry and qRT-PCR. RORγt, the master regulator of Th17 cells, is expressed at similar levels in both WT and $BACE1^{-/-}$ Th17 cells (Figure 6A-C). In some experiments, RORγt was slightly lower in $BACE1^{-/-}$ Th17 cells compared to WT, but this finding was not consistent across experiments (Figure 6C). Further experiments are needed to elucidate the functional status and subcellular localization of RORγt in $BACE1^{-/-}$ Th17 cells. Due, perhaps, to their close location in the genome, the expression of IL-17A is typically tightly bound to expression of IL-17F. We found that Th17 cells lacking BACE1 also exhibited reduced IL-17F expression, when measure by ELISA and qRT-PCR but not by Flow cytometry (Figure 6D-F). This defect was very minor compared to the reduced IL-17A expression observed in these cells, suggesting that BACE1 has a more important role in the regulation of IL-17A expression than it does for IL-17F.

IL-23 signaling is critical for the generation of inflammatory Th17 cells (155,157,171) and defects in this pathway can lead to reduced IL-17A expression. We found that the expression of IL-23R in $BACE1^{-/-}$ Th17 cells did not differ from WT levels. IL-23 as well as IL-21 and IL-6,

three key cytokines in the formation of Th17 cells, upon engaging their receptors on the T cell surface, signal through phosphorylation of STAT3 (pSTAT3) (178,400). After stimulating both WT and BACE1$^{-/-}$ CD4^{+} T cells with Th17-polarizing cytokines for different periods of time, we did not observe any differences in pSTAT3 (Figure 6H), suggesting that BACE1 deficiency does not affect Th17 polarization *per se*, but acts downstream of RORγt to finely regulate the expression of IL-17A and IL-17F.

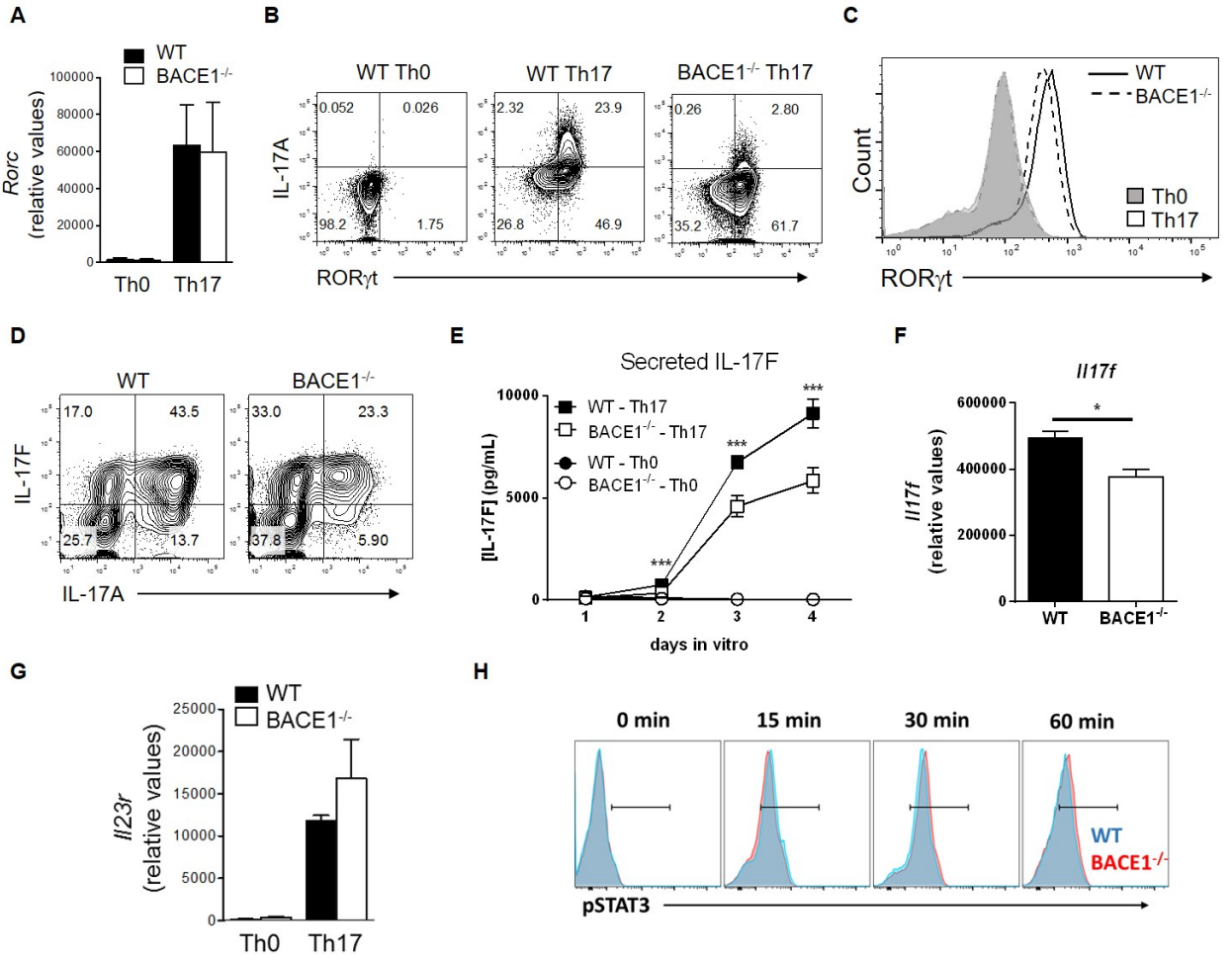

Figure 6. BACE1 deficiency does not affect general Th17 differentiation.

A. *Rorc* gene expression from *in vitro*-differentiated WT and BACE1 CD4^{+} T cells cultured for two days, normalized to *Gapdh* expression. **B.** Intracellular co-expression of RORγt and IL-17A, measured by flow cytometry at day three of Th17 polarization. **C.** Histogram representation of RORγt expression in Th0 and Th17 from WT and BACE1$^{-/-}$

$CD4^+$ T cells, at day three of Th17 differentiation. **D.** Intracellular IL-17A and IL-17F co-expression was analyzed by flow cytometry on day three post-differentiation, following PMA/ionomycin stimulation. **E.** Secreted IL-17F measured from Th0 and Th17 culture supernatants at the indicated times, measured by ELISA. **F.** *Il17f* gene expression from WT and $BACE1^{-/-}$ $CD4^+$ T cells, after two days of *in vitro* Th17 differentiation, normalized to *Gapdh* levels. **G.** Levels of *Il23r* mRNA from WT and $BACE1^{-/-}$ T cells at day 4 under the indicated differentiating conditions. Gene expression and flow cytometric data are representative of at least three independent experiments. **H.** $CD4^+$ T cells from naïve WT or $BACE1^{-/-}$ mice were activated with biotinylated activating αCD3 and αCD28 antibodies (2ug/mL, both) in the presence of cross-linking streptavidin, under Th17 polarizing conditions for different times, and phosphorylation of STAT 3 was measured using flow cytometry. Results are representative from two independent experiments.

3.1.6 RNAseq analysis of $BACE1^{-/-}$ Th17 cells

In order to perform a more detailed analysis of Th17 polarization, we performed next generation RNA sequencing (RNAseq) to reveal differences in global gene expression. Th17 cells differentiated *in vitro* for four days were used for RNA extraction and further processing for sequencing at the Health Sciences Sequencing Core (Children's Hospital of Pittsburgh). Reads per kilo base per million mapped reads (RPKM) values were uploaded to GeneSifter (Perkin Elmer), an online platform to analyze RNAseq data and provide informational gene expression. Expression levels were further analysed by Partek in order to confirm statistical relevance of dysregulated genes as well as to generate heat maps and hierarchical clustering.

Confirming our qRT-PCR findings, pairwise analysis of $BACE1^{-/-}$ vs WT Th17 cells did not show any major differences in Th17-signature markers other than IL-17A (Figure 7A). The expression of some chemokines appeared to be higher or lower in $BACE1^{-/-}$ Th17 cells, possibly

playing a role in the homing of these cells *in vivo*. However, their expression level was rather low and the role of these molecules is almost irrelevant for the expression of IL-17 cytokines in pure $CD4^+$ T cell cultures. Hierarchical clustering of the differentially expressed genes in the absence of BACE1 provide us with a list of dysregulated genes, both up- and down-regulated (Figure 7B). Besides IL-17A, the reduced expression of Nt5e in $BACE1^{-/-}$ Th17 cells was an interesting finding. Nt5e encodes for CD73, a transmembrane ectonucleotidase that converts adenosine monophosphate (AMP) into adenosine (401). CD73 is expressed on the surface of Tregs and regulatory Th17 cells, suggesting that perhaps some anti-inflammatory mechanism explained the reduced expression of IL-17A in $BACE1^{-/-}$ Th17 cells. The next section (chapter 3.2) is dedicated to the study of CD73 expression and functions in the production of IL-17A and development of EAE.

It was interesting to see that a few dysregulated genes in $BACE1^{-/-}$ Th17 cells corresponded to chemokines and chemokine receptors (Figure 7A and B). In most of the cases, the expression of these transcripts occurred at very low levels. However, CCR2 was expressed at biologically relevant levels and it was significantly reduced in the absence of BACE1. CCR2 has been shown to be expressed by CNS-infiltrating GM-CSF-producing Th17 cells, and to be crucial for the development of EAE (402). Nevertheless, defects in chemokine receptor expression does not explain the defects in IL-17A when pure $CD4^+$ T cells are being cultured. In addition, total numbers of CNS infiltrating cells were not significantly different between WT and $BACE1^{-/-}$ mice, emphasizing that the limited pathogenicitiy of $BACE1^{-/-}$ Th17 cells is due to reduced IL-17A expression rather than trafficking defects.

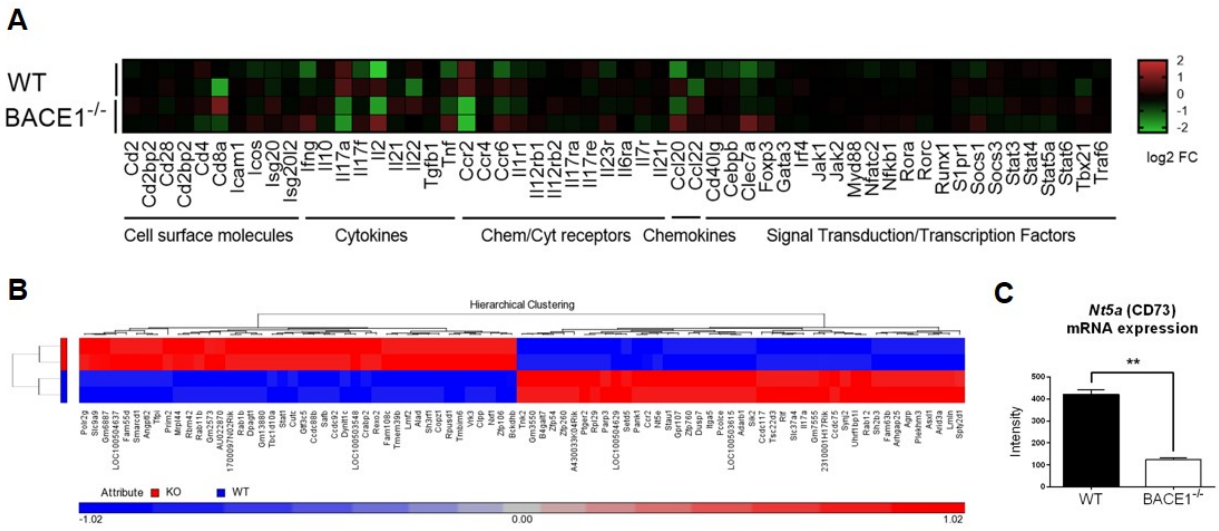

Figure 7. RNAseq reveals no major differences in Th17-sginature markers in the absence of BACE1.

WT and BACE1$^{-/-}$ CD4^{+} Th17 cells were differentiated *in vitro* for 4 days and RNAseq analysis was performed. Low expression values (fpkm) were filtered out and differences analyzed with a cut-off of at least 20% increase or decrease. **A.** Heat map of differential expression of Th17-related genes. **B.** Hierarchical clustering of differential genes between WT and BACE1$^{-/-}$ Th17 cells. Asterisks mark the two genes that we studied in detail due to their potential in Th17 pathogenesis. **A and B**. Ratio of expression is expressed as the $\log_2$ fold change to the average of both groups. **C.** RPKM values of Nt5e (CD73) mRNA expression obtained by RNAseq analysis. All data contains two samples per group, representative of two independent experiments.

3.1.7 BACE1 acts intrinsically in T cells

BACE1 is typically expressed at the plasma membrane of neurons and astrocytes. BACE1 protein has also been found in the trans-Golgi network and in early endosomes during mobilization to and from the plasma membrane. Since BACE1 can potentially be expressed on the cell surface of Th17 cells, we wondered if the action of BACE1 was cell intrinsic, or on the contrary, the expression of BACE1 on one cell could impact the function of a neighboring cell.

To test this, we first co-cultured BACE1$^{-/-}$ CD4^{+} T cells with CD45.1^{+} WT CD4^{+} T cells at a 1:1 ratio, and differentiated them to Th17 cells for three days before analyzing their cytokine profile (Figure 8A). We found that BACE1$^{-/-}$ Th17 cells retained their IL-17A deficiency even in the presence of WT cells (Figure 8B and C). Co-culturing WT and BACE1$^{-/-}$ T cells did not rescue IL-17A secretion from BACE1-deficient Th17 cells (Figure 8D), indicating that BACE1 exerts its function in T cells in a cell-intrinsic manner.

To confirm this result *in vivo*, we generated BACE1$^{-/-}$ 2D2^{+} mice, in which T cells bear a MOG-specific transgenic T cell receptor. 2D2^{+} WT or BACE1$^{-/-}$ CD4^{+} T cells were transferred into CD45.1 congenic WT recipient mice, followed by MOG_{35-55} immunization (Figure 8F). *Ex vivo* analysis of draining lymph nodes (inguinal) at day eight post-immunization revealed that BACE1$^{-/-}$ Th17 cells that developed in an otherwise BACE1-competent environment still expressed reduced percentages and per cell levels (MFI) of IL-17A (Figure 8F-H), confirming our previous *in vitro* results (Figure 8A-C). As expected, the immune response from recipient WT CD4^{+} T cells was inferior to 2D2^{+} donor T cells (Figure 8F). Moreover, there was no difference in the induction of IL-17 expression from recipient WT T cells when they were activated in the presence of WT or BACE1$^{-/-}$ 2D2^{+} donor T cells. (Figure 8F bottom).

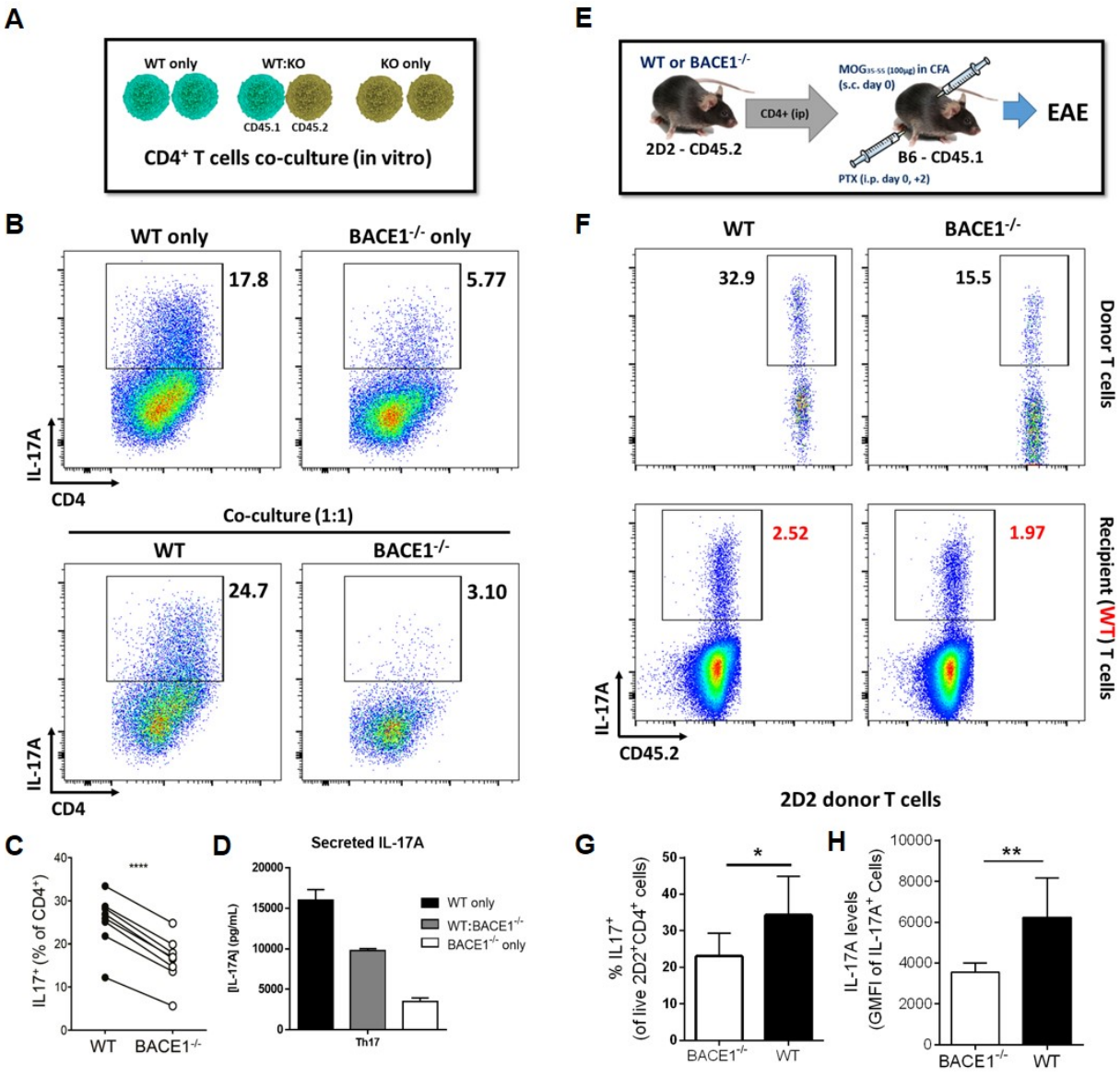

Figure 8. BACE1 acts intrinsically in T cells.

A-D. CD45.1 WT and CD45.2 BACE1$^{-/-}$ CD4^{+} T cells were cultured alone or co-cultured at a 1:1 ratio under Th17 differentiating conditions for three days. **B.** Flow cytometric analysis of co-localization of intracellular IL-17A expression and CD4, on indicated cell populations. Data representative of at least three experiments with similar results. **C.** Frequencies of IL-17A^{+} CD4^{+} T cells from co-cultured WT and BACE1$^{-/-}$ Th17 cells, distinguished by CD45.1/CD45.2 expression. Data pooled from three separate experiments. P value < 0.00001, coupled Student's *t* test. **D.** Secreted IL-17A was measured from culture supernatants at day three post-differentiation, by ELISA. Results are representative of three different experiments. **F-J.** WT or BACE1$^{-/-}$ 2D2^{+} CD4^{+} T cells were transferred

intraperitoneally into CD45.1 WT recipients and immunized with MOG_{35-55} in CFA, one day later. On day 8 p.i. intracellular IL-17A expression from donor (CD45.2) and recipient (CD45.1) draining lymph node cells, was analyzed by flow cytometry. **G.** Representative flow cytometry plots of intracellular IL-17A expression from WT and BACE1$^{-/-}$/- donor T cells, gated on CD45.2$^+$ CD4$^+$ cells. **H.** Intracellular IL-17A expression in WT recipient T cells, gated on CD45.2$^-$ CD4$^+$ T cells. Data representative of three independent experiments **I.** Frequencies of intracellular IL-17A expression from WT or BACE1$^{-/-}$ donor CD45.2 2D2$^+$ CD4$^+$ T cells. **J.** Mean fluorescence intensity of IL-17A expression in IL-17A$^+$ CD45.2$^+$ CD4$^+$ donor T cells. **I-J.** Data pooled from two experiments with similar results.

3.1.8 Retroviral overexpression of BACE1 enhances IL-17A expression in Th17 cells

Our data revealed that BACE1 expression in Th17 cells is necessary for optimal IL-17A and IL-17F production, in a cell-intrinsic manner. We next wanted to determine if overexpressing BACE1 in T cells was sufficient to induce IL-17A production from T cells. We generated a retroviral expression vector encoding for BACE1, with Thy1.1 as a transduction efficiency reporter. WT CD4$^+$ T cells were transduced with BACE1 or empty vector-containing viral particles before differentiation to Th0 or Th17. We found that Th17 cells that overexpressed BACE1 (Thy1.1$^+$ cells) showed a higher proportion of IL-17A producing cells compared to Th17 cells that did not overexpress BACE1 (Thy1.1- cells) (Figure 9A and B). Overexpression of BACE1 had no effect on T cells cultured in the absence of Th17 polarizing conditions (Figure 9A and B), indicating that BACE1 is necessary for complete IL-17A expression but, on its own, is not sufficient to drive the expression of the cytokine. It is important to highlight that these experiments lacked the control of retroviral transduction with an empty vector. Although it is very unlikely that Thy1.1 expression on its own will induce IL-17A expression, future experiments will include these controls as well

as to study whether the overexpression in BACE1$^{-/-}$ Th17 cells is sufficient to restore IL-17A expression.

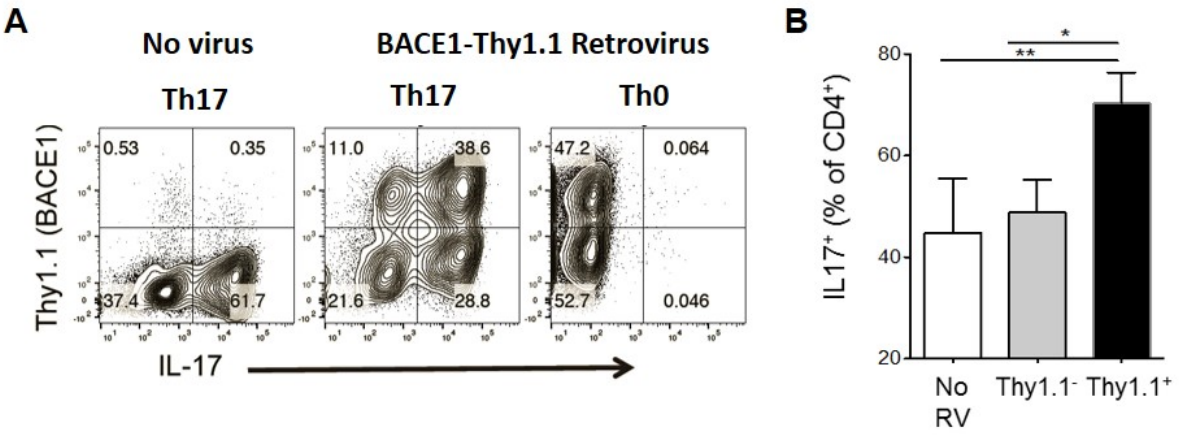

Figure 9. Retroviral overexpression of BACE1 enhances IL-17A expression in WT Th17 cells.

CD4$^+$ T cells were activated with plate-bound αCD3/αCD28 in the presence of blocking αIFNγ antibodies for 24 hours before transduction with GP$^+$E86 cell supernatant, containing retroviral particles overexpressing BACE1 and Thy1.1. T cells were then differentiated to Th0 or Th17 for three more days. **A.** Representative Flow plots of IL-17A and Thy1.1 expression from WT CD4$^+$ T cultured under different polarizing conditions in the presence or absence of BACE1-overexpressing viral particles. **B.** Intracellular IL-17A expression from un-transfected or BACE1-overexpressing (Thy1.1$^+$) T cells cultured under Th17-skewing conditions. Data pooled from two independent experiments with similar results.

3.1.9 BACE1 is required for pathogenic function of Th17 cells *in vivo*

The importance of Th17 cells in the development of EAE has been extensively described (156,157,236). Our *in vivo* data so far proved that expression of BACE1 in CD4$^+$ T cells is necessary for the pathogenesis of EAE. In addition, our results of *in vitro* polarization suggested that BACE1 is necessary for the effector function of Th17 cells, whereas Th1 and Tregs are

seemingly not affected by BACE1 deficiency. Hence, we wanted to further interrogate the requirement for BACE1 on autoimmune Th17 cell function. For this purpose, we used the passive transfer model of EAE, in which cell suspensions from spleens and lymph nodes of WT or BACE1$^{-/-}$ 2D2^{+} mice were *in vitro* differentiated to Th17 cells before transfer to WT or Rag1$^{-/-}$ recipients. Because we transferred activated autoreactive Th17 cells, recipient mice developed EAE signs without requiring immunization. We first confirmed that T cells were properly polarized to Th17 cells by measuring IL-17A expression at day 4 of culture. As expected, WT T cells expressed normal amounts of IL-17A whereas BACE1$^{-/-}$ T cells exhibited the characteristic reduced expression of the cytokine (Figure 10A). Another Th17 signature molecule critical for EAE development is GM-CSF (198,403,404). Unlike for IL-17A expression, we did not observe any differences in GM-CSF expression between WT and BACE1 2D2^{+} T cells (Figure 10B).

Upon transfer of equal numbers of WT or BACE1$^{-/-}$ 2D2^{+} Th17 cells into Rag1$^{-/-}$ naïve recipients, we observed that BACE1$^{-/-}$ 2D2^{+} Th17 cells were significantly impaired in their ability to induce EAE (Figure 10C, Table 4A). Similarly, transfer of BACE1$^{-/-}$ 2D2^{+} Th17 cells into WT recipients showed reduced severity compared to transfer of WT 2D2^{+} Th17 cells (Table 4B). Interestingly, a large proportion of WT recipients that received WT 2D2^{+} Th17 cells developed signs of atypical EAE, including severe ataxia and circling behavior, with higher mortality rates (Table 4B). However, WT mice that received BACE1$^{-/-}$ 2D2^{+} Th17 cells were more resistant to the development of atypical EAE and almost completely protected from EAE-related death (Table 4B). Atypical EAE has been associated with very strong Th17 responses (405–408). *Ex vivo* analysis of 2D2^{+} T cells from spleens and CNS of diseased mice confirmed that similar frequencies of 2D2^{+} cells were present but IL-17A production was impaired in mice that received BACE1$^{-/-}$

cells (Figure 10D and E). These data confirm that BACE1 is necessary for complete functional IL-17A production from pathogenic Th17 cells, but has no effect on the development or migration capacity of these cells into the CNS. In contrast, IFNγ and GM-CSF were present at high levels in both WT and BACE1$^{-/-}$ 2D2^{+} T cells (Figure 10F and G), corroborating the specific effect of BACE1 deficiency on IL-17A. These data also explain the incomplete resistance to EAE in this model, since GM-CSF levels are equivalent in WT and BACE1$^{-/-}$ Th17 cells after *in vitro* re-stimulation prior to their transfer and can partially compensate for the reduced IL-17A production from BACE1$^{-/-}$ Th17 cells.

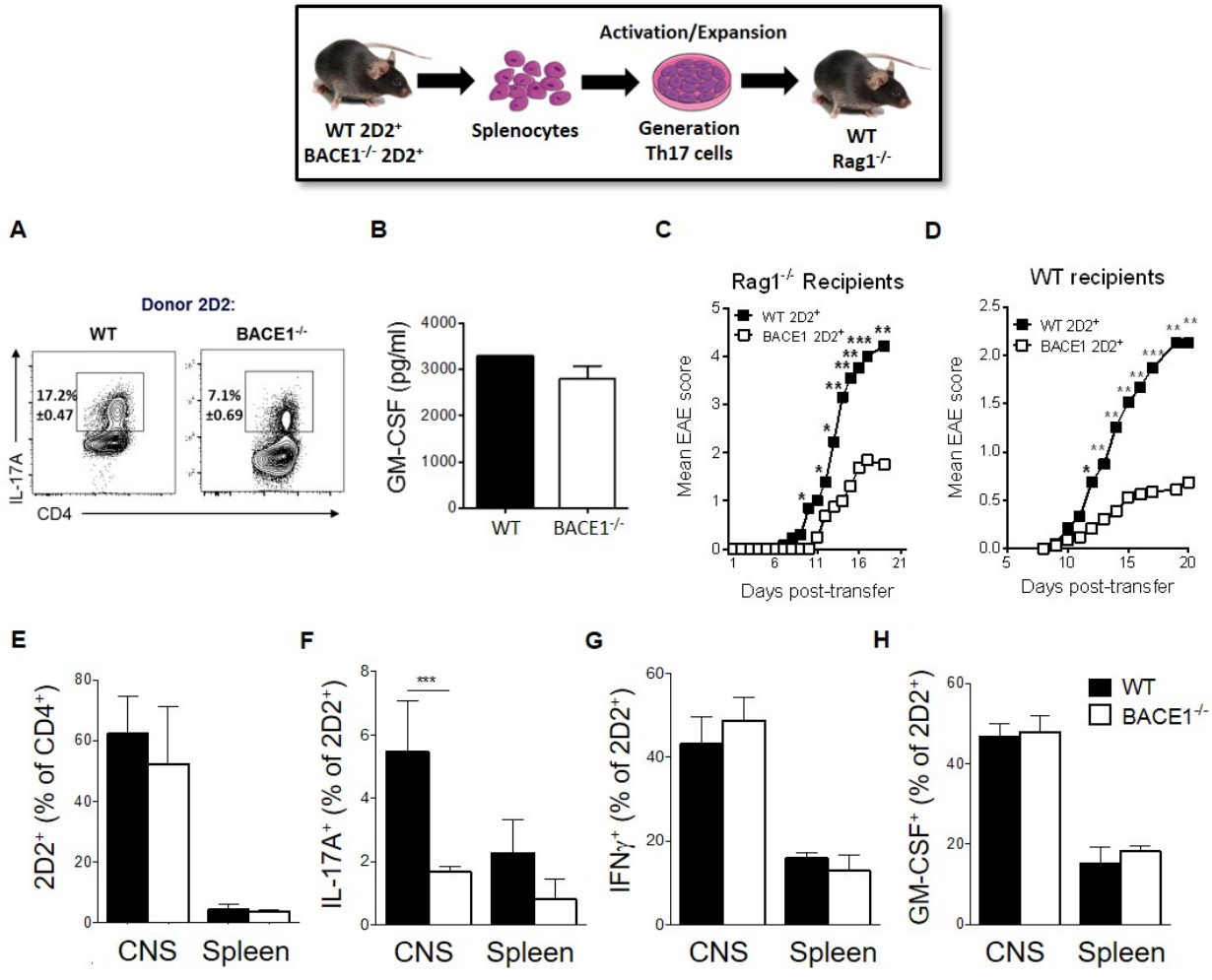

Figure 10. BACE1 is required for pathogenic function of Th17 cells *in vivo*.

Total splenocytes and lymph node cells from WT and BACE1$^{-/-}$ naïve mice were activated *in vitro* with MOG$_{35-55}$ in the presence of TGF-β1 and IL-6 for four days. Th17 polarization was addressed by intracellular staining of IL-17A **(A)** and secreted GM-CSF levels **(B)**, and rested for three days with IL-2 before re-stimulating for three days with plate-bound αCD3 and IL-23. Re-stimulated Th17 cells were transferred intraperitoneally into WT or Rag1$^{-/-}$ recipients. A-B are representative of three independent experiments where cytokines were checked before transfer (n= 1-2 mice per group and experiment). **C** and **D.** Clinical EAE scores following transfer of WT or BACE1$^{-/-}$ 2D2^{+} Th17 cells into Rag1$^{-/-}$ (**C**) or WT (**D**) recipients. Pooled data from 4 independent experiments in C and six experiments in D (number of mice used are shown in Table 2). **E-H.** Analysis of donor cells infiltrating the CNS and in the spleens of WT recipient mice 12 days post-transfer. **E.** Proportion of 2D2^{+} cells, identified by expression of TCR-Vα3.2 (gated on CD4^{+} T cells). **F.** Proportion of IL-17A^{+} cells from 2D2^{+} cells **G.** Proportion of IFNγ^{+} cells from 2D2^{+} cells. **H.** Proportion of GM-CSF^{+} cells from 2D2^{+} cells. E-H data represents mean ± standard deviation and is representative of four experiments with similar results (n=5 mice per group).

A - Rag1$^{-/-}$ Recipients

	WT 2D2^{+}	BACE1$^{-/-}$ 2D2^{+}	P-value
Number of recipients	13	16	
Incidence	13 (100%)	9 (56%)	0.0084
Atypical EAE	1 (8%)	0 (0%)	0.4483
Death	3 (23%)	0 (0%)	0.0783

B - WT Recipients

	WT 2D2^{+}	BACE1$^{-/-}$ 2D2^{+}	P-value
Number of recipients	39	34	
Incidence	26 (67%)	11 (32%)	0.0049
Atypical EAE	19 (49%)	2 (6%)	< 0.0001
Death	8 (21%)	1 (3%)	0.0316

Table 4. BACE1$^{-/-}$ 2D2^{+} Th17 cells induce milder EAE compared to WT.

Autoreactive BACE1$^{-/-}$ and WT Th17 cells were generated *in vitro* and transferred to Rag1-/- recipients (A) or WT recipients (B). This table summarizes the clinical observations over the course of the passive transfer EAE experiments.

3.1.10 Discussion

Th17 cell differentiation occurs in a step-wise manner (180,409), with initial upregulation of RORγt, IL-21 expression and some IL-17A production mediated by TGF-β1 and IL-6 (165,169,173,175,410). Subsequently, IL-21 stabilizes and expands the Th17 lineage in an autocrine/paracrine fashion (173,182,183,190). Further development of effector Th17 cells is mediated through IL-23 signaling, (171,175) with IL-1β playing roles in both initiation and effector stages of Th17 cell responses (192). IL-6, IL-21 and IL-23 cytokines induce the activation of STAT3, a key transcription factor in the Th17 program. Here, we show that BACE1, an aspartyl protease normally expressed in neurons and astrocytes, is a novel regulator of Th17 function that specifically targets IL-17A and to a lesser extent IL-17F production.

Typically, impaired production of effector cytokines is caused by disruptions in the transcriptional profile during differentiation. However, BACE1 regulation of IL-17A and IL-17F expression did not affect key transcription factors or the expression of other Th17 signature cytokines. In some experiments, we observed a minor decrease in RORγt protein levels in the absence of BACE1. This finding was not consistent in all experiments and more importantly, co-staining for RORγt and IL-17A confirmed that BACE1$^{-/-}$ Th17 cells had reduced IL-17A expression within the RORγt^{+} Th17 cell population. In the absence of BACE1, Th17 cells express

reduced levels of IL-17A and IL-17F mRNA, indicating that BACE1 regulates their expression prior to their protein synthesis.

The differential impact in IL-17A and IL-17F production in the absence of BACE1 indicates an interesting fine-regulation of these two cytokines by BACE1. This highlights the potential of BACE1 as an anti-inflammatory target very selective for IL-17A expression. Although uncommon, there is evidence in the literature of molecules that can impact IL-17A expression without affecting RORγt levels. A more detailed discussion of these mechanisms and how they may be involved in BACE1-IL-17 regulation will be given in chapter 3.3.

The observation that defects in BACE1$^{-/-}$ Th17 cells also occur in purified CD4^{+} T cells indirectly demonstrated that BACE1 is expressed in T cells. We were able to detect BACE1 mRNA levels in both activated T helper cells and naïve CD4^{+} T cells. However, our data was very variable between experiments and we could not draw any confident conclusions on the relative levels of BACE1 expression in each T cell subset. Studies with neuronal BACE1 have shown that due to the abundant post-transcriptional regulation of BACE1 maturation and function, there tends to be a poor correlation between mRNA levels and BACE1 activity (411). Therefore, we needed to evaluate the expression of BACE1 at the protein level.

In order to understand the mechanism by which BACE1 can regulate IL-17A expression, we need to determine when and where BACE1 is being expressed in T cells. Knowing the cellular compartment and the kinetics of BACE1 expression during T cell activation or differentiation will help narrow down the list of possible interacting partners. Since natural levels of BACE1 in T cells

proved hard to detect, we purchased a Myc-tagged BACE1 construct. In addition to overexpressing BACE1 to levels detectable by WB or ImageStream, this tag will ensure that the antibodies used can, indeed, detect the protein using different techniques.

Preliminary WB analysis of *in vitro*-polarized WT T cells (performed by Saikat Majumder) showed increased BACE1 expression in Th17 cells compared to Th0, albeit at very low levels (data not shown). Enrichment of the BACE1 fraction seems to be required to properly address the relative abundance between different Th subsets and the expression pattern of BACE1 over time in Th17 cells. Perhaps BACE1 is indeed expressed at low levels in T cells and this quantity is sufficient to modulate IL-17A expression in Th17 cells. Using the same monoclonal antibodies against BACE1, we tried to detect protein levels in T cells, by flow cytometry, with no success. Since BACE1 protein levels seemed to be the limiting factor, we repeated the staining using ImageStream. This technique combines microscopy detection and flow cytometry, therefore increasing the resolution as well as giving spatial information of protein localization. Although we obtained more promising results, with punctate expression of BACE1 (as seen in BACE1 expression in early endosomes in neurons) we were not able to consistently replicate these results.

BACE1 function seems to be dispensable for Th1 and Treg development, since analysis of their signature markers revealed no changes between WT and BACE1$^{-/-}$ T cells. However, proper functional assays need to be performed in order to confirm unaltered function in these T helper subsets. Infection models of *Listeria monocytogenes* and *Toxoplasma gondii* are commonly used to address Th1 responses. Transfer of WT or BACE1$^{-/-}$ CD4^{+} T cells with WT CD8^{+} T cells into Rag1$^{-/-}$ recipients can give a final answer on the role of BACE1 in Th1 cells. Although technically

more complex, the immune response of BACE1$^{-/-}$ Th1 cells could be addressed using *Mycobacterium tuberculosis* infection. To test whether BACE1 is necessary for the regulatory functions of Tregs, *in vitro* suppression assays of antigen-specific responses can be performed with WT Teff and WT or BACE1$^{-/-}$ Tregs. Use of Treg-depleted autoimmune transfer models of colitis with co-transfer of WT or BACE1$^{-/-}$ Tregs would give definitive proof of Treg functionality.

The impaired expression of IL-17 cytokines from BACE1$^{-/-}$ Th17 cells resulted in reduced pathogenicity in EAE. Interestingly, Rag1$^{-/-}$ recipients that received autoreactive WT Th17 cells but not BACE1$^{-/-}$ Th17 cells developed atypical EAE, a very severe form of EAE. Atypical EAE is associated with a strong Th17 response that preferentially targets the cerebellum and the spinal cord, characterized by ataxia and loss of motor coordination, often resulting in death (408). Of note, mice transferred with BACE1$^{-/-}$ 2D2^{+} Th17 cells were only partially protected in comparison to the complete protection observed in the Rag1$^{-/-}$ adoptive transfer. GM-CSF produced by Th17 has also been shown to promote EAE (230,231), and contrary to IL-17A, we did not observe differences in GM-CSF production by BACE1$^{-/-}$ 2D2^{+} Th17. 2D2^{+} Th17 cells express high quantities of GM-CSF, explaining the complete resistance to EAE in Rag1$^{-/-}$ recipients that received BACE1$^{-/-}$ T cells in comparison to only ameliorated disease symptoms in the passive transfer model of EAE.

Despite the clear effect of BACE1 deficiency in transfer models of EAE and the impaired IL-17A expression in *in vitro* differentiated Th17 cells, when EAE was actively induced in full BACE1$^{-/-}$ mice we did not observe major differences in the course of disease. In fact, in this model, onset of disease appears to occur slightly earlier in BACE1$^{-/-}$ mice despite the impaired IL-17A

response. BACE1 is important during developmental myelination due to its role in Neuregulin-1 maturation (295,306) and both BACE1$^{-/-}$ and Neuregulin-1$^{-/-}$ mice present defects in their myelin levels. Although their hypomyelination does not lead to spontaneous MS/EAE signs, we hypothesized that these mice would be more susceptible to EAE since less myelin destruction is necessary to achieve the level of damage necessary to exhibit clinical signs. Our findings using bone-marrow chimeras indicated that this was the case but the increase in susceptibility in BACE1-deficient CNS was much less than expected. The EAE model is a good model to test and identify molecules and mechanisms that confer protection. Nevertheless, subtle effects or mechanisms that surpass a certain threshold (regular induction in control mice) might escape detection (all mice becoming sick). This was the case while studying the role of MCPIP1, a negative regulator of IL-17 signaling, in EAE, in collaboration with Dr. Sarah Gaffen's lab. Absence of MCPIP1 results in enhanced IL-17 signaling and therefore confers increased susceptibility to Th17-driven autoimmunity (412). Notably, we could only appreciate these functional differences *in vivo*, by reducing the overall severity of the EAE induction (lower dose of antigen and PTx and treatment with anti-inflammatory drugs). Another possibility for the lack of more evident differences is the existence of inflammatory roles of BACE1 in other non-hematopoietic cells, especially in the CNS. Despite introducing BACE1-competent immune cells, full exacerbation of the immune cascade may be prevented by BACE1 deficiency on other cell types. This possibility, although very interesting, lies beyond the scope of this project. Finally, it is important to highlight that our irradiation dose seemed to be inefficient in depleting the original WT population of T cells. Therefore, "contaminating" host's T cells could be compensating for the defects in BACE1 donor Th17 cells, a phenomenon that has been previously described with IL-23RA$^{-/-}$ mice (171).

Taken together, our data show that BACE1 deficiency impacts EAE development in a dual fashion. BACE1 deficiency in the CNS leads to defects in developmental myelination that poises these mice to become more susceptible to EAE induction. BACE1 is also necessary for a fully functional Th17 response and, therefore, $BACE1^{-/-}$ Th17 cells present functional defects that result in protection from EAE.

BACE1 has been widely studied in the context of Alzheimer's disease, since it is responsible for the amyloidogenic cleavage of APP (263). Upregulation of BACE1 has also been described in other neurodegenerative disorders with an important inflammatory component such as ischemia and traumatic brain injury (354,356). Increases in IL-17A expression have been described in ischemic human lesions (255,413) and early blockade of this cytokine in mice models resulted in reduced lesions and improved neurological outcome (257). Hence, our findings call for a re-evaluation of the current roles of BACE1 in the development of CNS inflammation and neurodegeneration in which IL-17 expression plays key roles. It is possible that the dual effect of BACE1 on Th17 cells and, in particular, IL-17A expression, and its known classical role in driving neuronal cell toxicity, act together to promote neurodegeneration.

In conclusion, our findings demonstrate an unexpected requirement for BACE1 expression in Th17 cells to exert their inflammatory functions. In particular, we found that BACE1 differentially regulates the expression of IL-17A versus IL-17F at the transcriptional level, through a process that does not involve changes in overall Th17 differentiation or expression of other signature cytokines. The relevance of these findings is twofold, since we describe a new role for BACE1 in T cells and we found a new player in the fine-tuning of IL-17A expression and Th17

pathogenicity without majorly affecting the Th17 program. Together, these intriguing data raise the question of the specific mechanisms through which BACE1 modulates IL-17A expression. In the next chapters, we describe our efforts to elucidate the mechanisms by which this novel regulator of Th17 cells exerts its effects.

3.2 CD73 UPREGULATION IN TREGS AND TH17 CELLS IS DEPENDENT OF BACE1 BUT NOT NECESSARY FOR TH17 CYTOKINE EXPRESSION OR PATHOGENICITY IN EAE

3.2.1 Introduction

In the previous chapter we described a surprising new role for BACE1 in the immune system. Specifically, we found that the absence of BACE1 in Th17 cells resulted in an impairment in IL-17 cytokine production. In the process of elucidating what immune-related molecules were dysregulated in the absence of BACE1, we performed RNAseq analysis of BACE1$^{-/-}$ Th17 cells at day four post-differentiation. One of the primary molecules downregulated in BACE1$^{-/-}$ Th17 cells, apart from IL-17A was the *Nt5e* gene transcript.

The *Nt5e* gene encodes for a transmembrane ecto-5'-nucleotidase, also known as CD73 (414,415). This transmembrane enzyme is expressed in a wide variety of tissues and cell types, including endothelial cells, tumors, γδ T cells, Tregs and regulatory Th17 cells (416–420). CD73, in conjunction with ectonucleoside triphosphate dyphosphohydrolase-1 (CD39), is responsible for the transformation of pro-inflammatory ATP into immunoregulatory adenosine (421,422). During tissue damage, the enzymatic activities of CD39 and CD73 play strategic roles in regulating the duration, magnitude, and chemical nature of purinergic signals delivered to immune cells through the conversion of inflammatory ADP/ATP to AMP and AMP to adenosine, respectively (421). Generation of adenosine by CD73 has been shown to downregulate the immune response, through pleiotropic effects, such as enhancing Treg function (elevating Foxp3 and CTLA4 expression)

(53), inducing anti-inflammatory antigen presentation (423,424) or repressing cytokine production from Th1/Th2 cells (425).

Because of the immunosupressive function of adenosine, both adenosine and CD73 have been regarded as anti-inflammatory molecules. Indeed, upregulation of CD73 expression by cancer cells has been observed as a possible mechanism by which tumors evade or diminish the immune response (426). In this context, the detrimental effect of CD73 expression by Tregs and regulatory Th17 cells has been extensively studied: expression of high levels of CD73 by regulatory Th17 cells resulted in impaired anti-tumor responses in mouse models (427) and, in humans, high expression of CD73 correlated with poor prognosis in cancer patients (428).

The expression and function of CD73 can be induced under hypoxic conditions (429), as well as by the presence of different pro-inflammatory cytokines such as TGF-β1, TNFα, IL-1β or prostaglandins (401,430,431). Notably, TGF-β1 is a critical cytokine for the development of both Th17 cells and Tregs (410). In particular, during regulatory Th17 cells development, IL-6-driven STAT3 phosphorylation induce the expression of CD73 and CD39 and TGF-β1 downregulates the expression of the transcriptional repressor Gfi1, thereby allowing CD73 and CD39 expression (419).

Despite the great amount of literature focusing on the immunoregulatory role of CD73, through the production of adenosine, in recent years an increasing body of literature suggested that CD73 might be also playing a role in inflammatory cells. Expression of CD73 has been reported in inflammatory Th17 cells from patients with Crohn's disease (420), and CD73 is upregulated in

multiple sclerosis lesions (432) and in mouse EAE models (433). Interestingly, indirect evidence indicates that adenosine may play inflammatory roles in Th17 cells in addition to immunosuppressive functions on Th1 and tumor-infiltrating $CD8^+$ T cells (434). It has been shown that adenosine can promote the differentiation of pro-inflammatory Th17 cells by inducing the expression of IL-6 from dendritic cells (435) and that adenosine blockade ameliorated disease severity in EAE (422,433).

On the other hand, and consistent with the role of adenosine as an immunosuppressive molecule, mice deficient in the adenosine receptor A2A show accelerated onset and severity of EAE, along with increased production of inflammatory cytokines (422,436). Due to the variable, and sometimes opposing, roles of CD73 and adenosine in EAE it becomes rather difficult to hypothesize the effect of CD73 in autoimmune CNS inflammation. If adenosine plays an immunosuppressive role, $CD73^{-/-}$ mice, which have a reduced ability to generate adenosine, should develop more severe EAE. However, contrary to this expected outcome, Mills *et al.* showed that $CD73^{-/-}$ mice were protected from EAE development (433). Interestingly, $CD73^{-/-}$ Th17 cells produced more pro-inflammatory cytokines than WT Th17 cells and, in transfer experiments, these CD73-deficient Th17 cells were able to induce EAE in CD73-competent recipients. This suggested a requirement for CD73 in non-immune cells to control EAE susceptibility. In a follow-up paper, the same authors indicated that adenosine signaling was required later on in the choroid plexus to facilitate the entry of lymphocytes into the CNS, through induction of the chemoattractant CX3CL1 (437).

All things considered, a final and convincing picture of the role of CD73 in EAE is still yet to be established. Despite evidence for CD73 being expressed in Th17 cells during EAE, the role of the nucleotidase in the development and function of Th17 cells has not been carefully addressed. In addition, due to the pro- and anti-inflammatory duality of adenosine generation, we hypothesized that the reduced expression of CD73 in BACE1$^{-/-}$ Th17 cells could explain the impaired expression of IL-17A in these cells. Therefore, we sought to validate the expression of CD73 in pro-inflammatory T cells in autoimmunity and to elucidate the effect of CD73 deficiency in Th17 differentiation and function *in vitro* as well as during EAE.

3.2.2 CD73 expression is reduced in BACE1$^{-/-}$ Th17 and Tregs

Our RNAseq analysis revealed that, in addition to defects in IL-17A expression, BACE1-deficient Th17 cells expressed markedly reduced levels of *Nt5e* mRNA (CD73), compared to WT Th17 cells (Figure 7C). In the immune compartment, CD73 is typically expressed in Tregs and regulatory Th17 cells (415,419) but it was interesting to find high expression of CD73 in Th17 cells polarized in the presence of IL-23.

In order to verify the expression of CD73 in non-regulatory T helper subsets, and to confirm the impact of BACE1 deficiency in CD73 upregulation, we differentiated CD4^{+} T cells from naïve WT and BACE1$^{-/-}$ mice into different T helper subsets, for three days. We found that Th17 and Treg, but not Th0 or Th1-polarizing conditions dramatically induced the expression of CD73 (Figure 11 A-C), but this upregulation was significantly reduced in BACE1-deficient T cells (Figure 11A-C). We found that the frequencies of CD73^{+} T cells were reduced in all T helper

subsets (Figure 11 A and B), but due to the high levels of CD73 in WT Th17 and Tregs, BACE1 is probably more relevant in the control of CD73 expression in these two subsets. Concomitantly, the expression of CD73, in a per cell basis, was significantly lower in BACE1$^{-/-}$ Th17 and Tregs, but not in Th0 or Th1 cells (Figure 11 C).

In WT mice, virtually all *in vitro*-polarized Tregs expressed CD73, while the frequencies of CD73^{+} Th17 cells, although very high, were slightly lower compared to Tregs (Figure 11 B). Mean Fluorescence intensity of CD73 expression in Th17 cells was about 50% less compared to Tregs (Figure 11 C), confirming that Tregs express more CD73 than Th17 cells.

TGF-β1, through repression of Gfi1, induces the expression of CD73. Interestingly, TGF-β1 is the only common cytokine in our Treg and Th17-differentiating cytokine cocktails. In order to determine if the expression of CD73 in Th17 cells was driven by TGF-β1, we activated T cells from naïve mice with plate-bound αCD3/αCD28 in the presence of individual Th17-polarizing cytokines or different combinations. CD73 expression was only upregulated in those conditions that contained TGF-β1 (Figure 11 D). Moreover, TGF-β1 was sufficient to induce CD73 in the absence of any other cytokines (Figure 11 D). These results confirm that TGF-β1 is responsible for the upregulation of CD73 in Th17 cells.

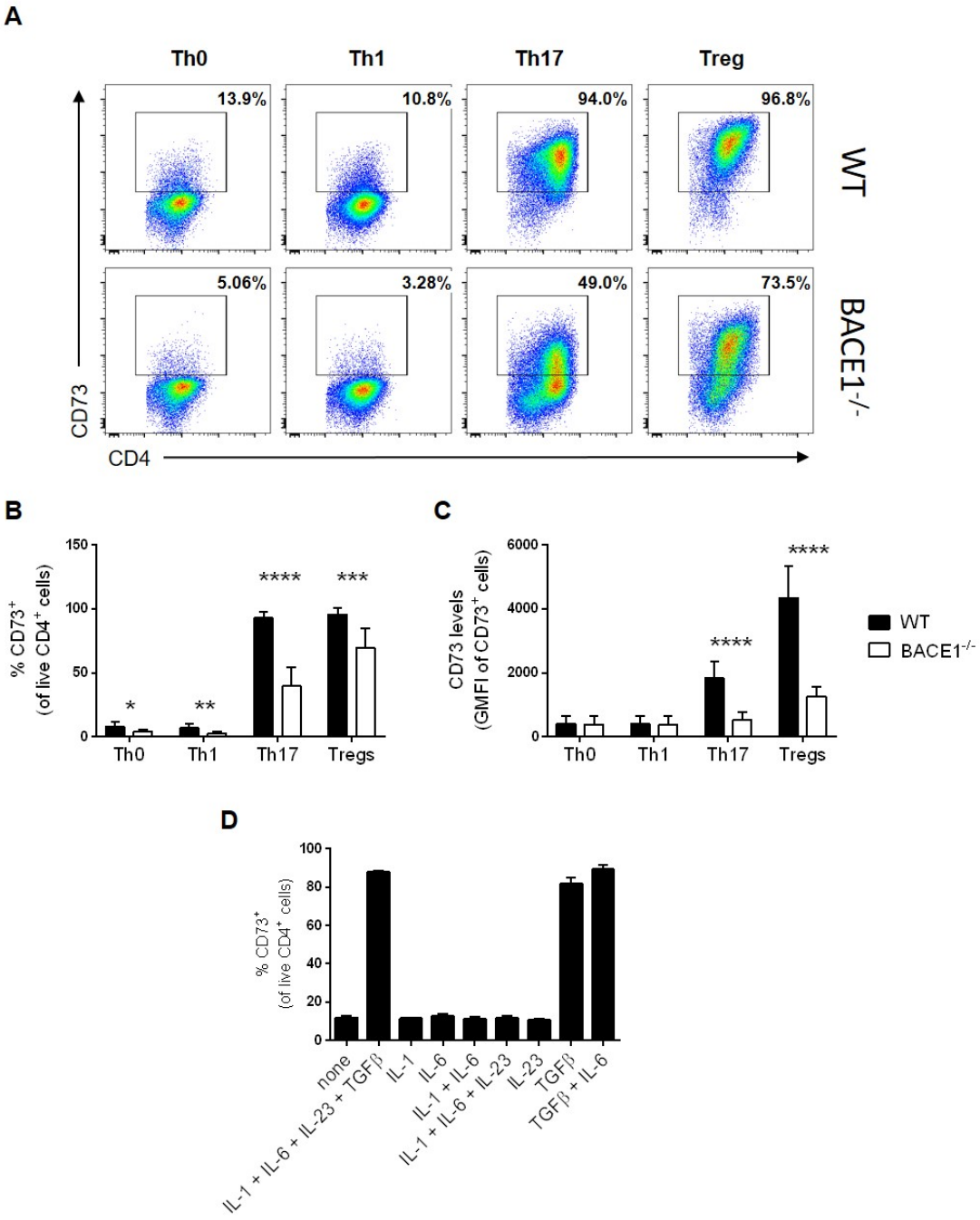

Figure 11. BACE1 deficiency in CD4$^+$ T cells results in reduced upregulation of CD73 expression in Th17 cells and Tregs.

A-C. CD4$^+$ T cells from naïve WT and BACE1$^{-/-}$ mice were differentiated *in vitro* for three days to different T helper subsets and CD73 protein expression was assessed by flow cytometry. **A.** Frequencies of CD73$^+$ cells from different *in vitro* differentiated T helper subsets. FACS plots are representative of at least four independent experiments with

similar results. **B.** Combined data from four independent experiments of frequencies of $CD73^+$ cells (gated from $CD4^+$ T cells). **C.** Geometric Mean Fluorescence Intensity of CD73 cellular expression from live $CD4^+$ T cells. Results pooled from four independent experiments. **D.** Frequencies of $CD4^+$ T cells that express CD73 after three days *in vitro* differentiation in the presence of different cytokines. Results are representative of one experiment.

3.2.3 CD73 is expressed by different T cell populations in naïve mice

Conversion of AMP into adenosine is a key mechanism in the regulatory function of Tregs (414,438–440). Indeed, CD73 has been proposed and used as a potential marker for regulatory T cells (420). In addition to Tregs, our *in vitro* data showed that CD73 expression increased not only in regulatory T cells but also in IL-17A producing Th17 cells. In order to study the relevance of CD73 expression in a living system, as well as to study whether effector T cells also express CD73 *in vivo*, we decided to analyze the expression of CD73 in naturally occurring T cell populations in naïve mice.

As expected, the vast majority of $Foxp3^+$ Tregs in naïve mice expressed high levels of CD73 (Figure 12A-E). Interestingly, we found that $Foxp3^-$ $CD44^{hi}$ effector/memory T cells expressed moderate-to-high CD73 whereas naïve T cells ($Foxp3^-CD44^{lo}$) only expressed it at low levels (Figure 12A-C). These findings are very relevant, because contrary to prediction, we found that in an *in vivo* naïve environment, CD73 is not only expressed by regulatory T cells but virtually all $CD4^+$ T cells express this nucleotidase at low levels. Our data suggest that CD73 expression in $CD4^+$ T cells is upregulated upon activation/differentiation *in vivo*.

Ex vivo analysis of naturally-occurring Tregs from naïve BACE1$^{-/-}$ mice revealed that CD73 levels were lower in BACE1-deficient Foxp3^{+} cells (Figure 12D and E), but overall frequencies of Foxp3^{+} T cells remained unaltered.

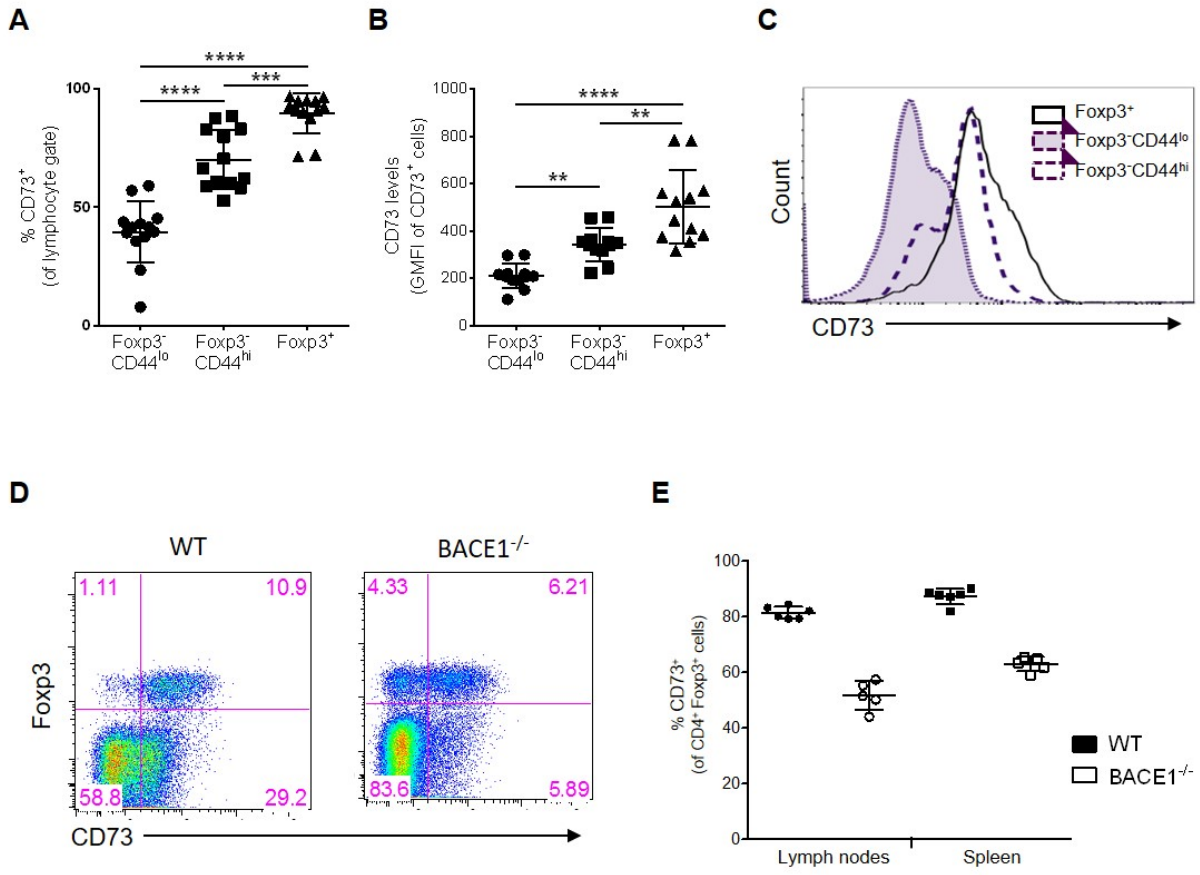

Figure 12. CD73 expression in different T cell population in naïve mice.

A. Mean percentage of CD73 expression by Tregs (Foxp3^{+}), naïve T cells (Foxp3^{-} CD44^{lo}), or effector/memory (Foxp3^{-} CD44^{hi}) T cells in lymph nodes from naïve WT mice. **B.** CD73 Geometric Mean Fluorescence Intensity of CD73 expression in the same populations T cell populations as in A. **C.** Representative histogram of CD73 expression by Tregs, naïve or effector/memory T cells from naïve WT mice. Data pooled from four independent experiments (each point represents an individual mouse). Error bars indicate standard deviation. **D.** Relative FACS plot of co-expression of CD73 and Foxp3 in *ex vivo* CD4^{+} T cells from naïve WT and BACE1$^{-/-}$ mice. Numbers indicate frequencies from one experiment with six independent mice. **E.** Frequency of Foxp3^{+} Tregs (Foxp3^{+}CD4^{+} T cells) in

the spleen and lymph nodes of naïve WT and BACE1$^{-/-}$ animals. Pooled results from 6 WT and 5 BACE1$^{-/-}$ naïve mice; error bars indicate standard deviation.

3.2.4 Inducing signaling pathways downstream of CD73 does not rescue IL-17A expression in BACE1$^{-/-}$ Th17 cells

We hypothesized that reduced levels of adenosine in the media, due to lower expression of CD73 in BACE1$^{-/-}$ Th17, could lead to the impaired IL-17A expression observed in these cells. We *in vitro* differentiated CD4^{+} T cells from naïve WT and BACE1$^{-/-}$ mice into Th17, for three days, in the presence of adenosine. However, addition of adenosine directly into the differentiating media did not rescue IL-17A expression from BACE1-deficient Th17 cells (Figure 13A). It has been shown that adenosine can act in a paracrine fashion to induce the expression of CD73 on endothelial cells (441). Addition of adenosine did not alter the frequencies of CD73-expressing Th17 cells from either WT or BACE1$^{-/-}$ cultures (Figure 12A).

In T cells, adenosine binds to Adenosine 2A receptor (A2AR), a G-coupled protein receptor that signals through cAMP as a second messenger (442,443). In mouse blood, the half-life of adenosine is ten seconds (444,445). It is therefore possible that the lack of IL-17A rescue by adenosine was due to the limited action of adenosine in such a short period of time. Therefore, we differentiated WT and BACE1$^{-/-}$ Th17 cells in the presence of 5'-N-Ethylcarboxamidoadenosine (NECA), an adenosine receptor agonist, or cAMP. We did not observe significant changes in IL-17A production or expression of CD73 in WT or BACE1$^{-/-}$ Th17 cells with the addition of NECA in the differentiating media (Figure 13B). Addition of cAMP did not impact the expression of

CD73 in either group (Figure 13A-D), while IL-17A levels remained low in BACE1$^{-/-}$ Th17 cells (Figure 13C). Interestingly, high concentrations of extracellular adenosine and cAMP, seemed to reduce the expression of IL-17A from WT Th17 cells (Figure 13A and C). The role of cAMP will be addressed more thoroughly in chapter 4. Despite not seeing any changes in CD73 expression, we wanted to ensure that the cAMP effects on IL-17A production were direct and not through a loop of CD73-adenosine regulation. For that purpose, WT and BACE1$^{-/-}$ Th17 cells were differentiated in the presence of cAMP and SCH-58261, a potent and selective A2A adenosine receptor antagonist. Although a bit more variable, results replicated the findings observed in the presence of cAMP alone (Figure 13D).

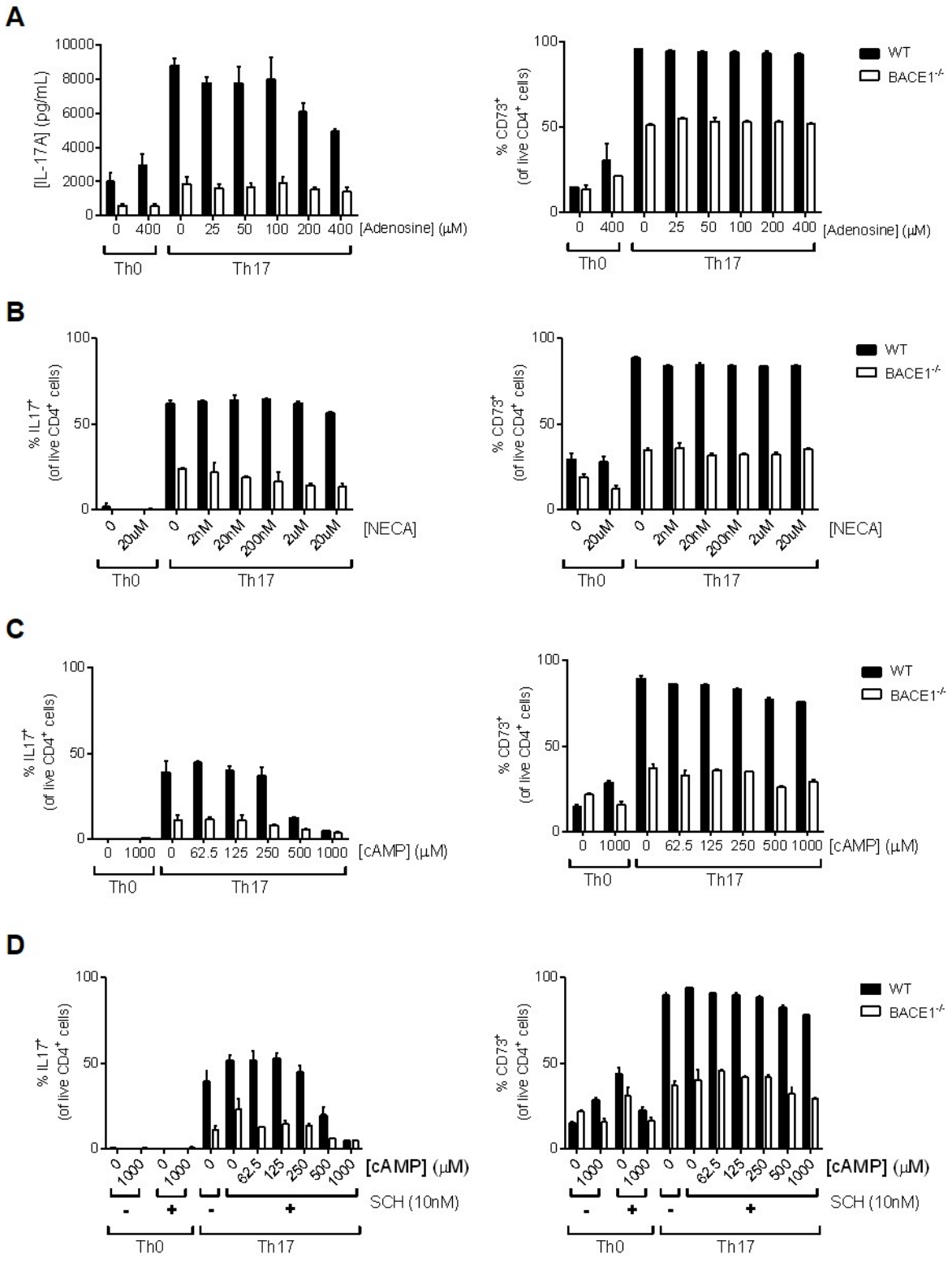

Figure 13. Induction of adenosine signaling does not rescue IL-17A expression from BACE1$^{-/-}$ Th17 cells.

A-D. $CD4^+$ T cells from naïve WT and $BACE1^{-/-}$ mice were differentiated to Th17 cells in the presence of increasing concentrations of Adenosine (**A**), NECA (**B**), cAMP (**C**) or cAMP+SCH (**D**) for three days, and expression of IL-17A and CD73 was measured by Flow cytometry (or ELISA in the case of Adenosine). Data representative from at least two independent experiments except Adenosine (A) that is representative of a single experiment.

3.2.5 Expression of CD73 during EAE development

Our *ex vivo* and *in vitro* data demonstrated that not only Tregs express CD73 but upon activation, Teff cells also upregulate this molecule. In particular, IL-17A producing Th17 cells notably upregulated CD73 *in vitro*. Given the importance of Th17 cells in the development of autoimmunity, we wanted to study the dynamics of CD73 expression in EAE as well as to address what T cell populations express this enzyme. To this aim, we immunized WT mice with MOG_{35-55} in CFA and analyzed the expression of CD73 on Th17 cells during the induction and effector phases of the inflammatory response in EAE. At the peak of T cell activation in the draining lymph nodes (day 8 p.i.), around 40-50% of Th17 cells expressed CD73 (Figure 14A). Interestingly, the proportion of IL-$17A^+$ $CD4^+$ T cells that expressed CD73 in the LN increased as the response progressed (Figure 14B). Concomitantly, the expression of CD73 from $IFN\gamma^+$ (Figure 14C) and GM-CSF^+ (Figure 14D) $CD4^+$ T cells showed a similar increasing pattern with disease progression. Corresponding with the increasing expression of CD73 by cytokine-producing $CD4^+$ T cells in the draining lymph nodes, cytokine-producing T cells that infiltrate the CNS during the peak and chronic phases of EAE also expressed CD73 (Figure 14H). The expression of CD73 in CNS-infiltrating T cells also increased from onset to peak and chronic phases of disease (Figure 14E-G).

Our data bring more evidence that CD73 is not only a marker of regulatory T cells but that all activated $CD4^+$ T cells can express it. Indeed, in the autoimmune activation and differentiation occurring in EAE, we observe an upregulation of CD73 in all cytokine-producing cells as the inflammatory response progresses, suggesting a potential link between inflammation and CD73 expression.

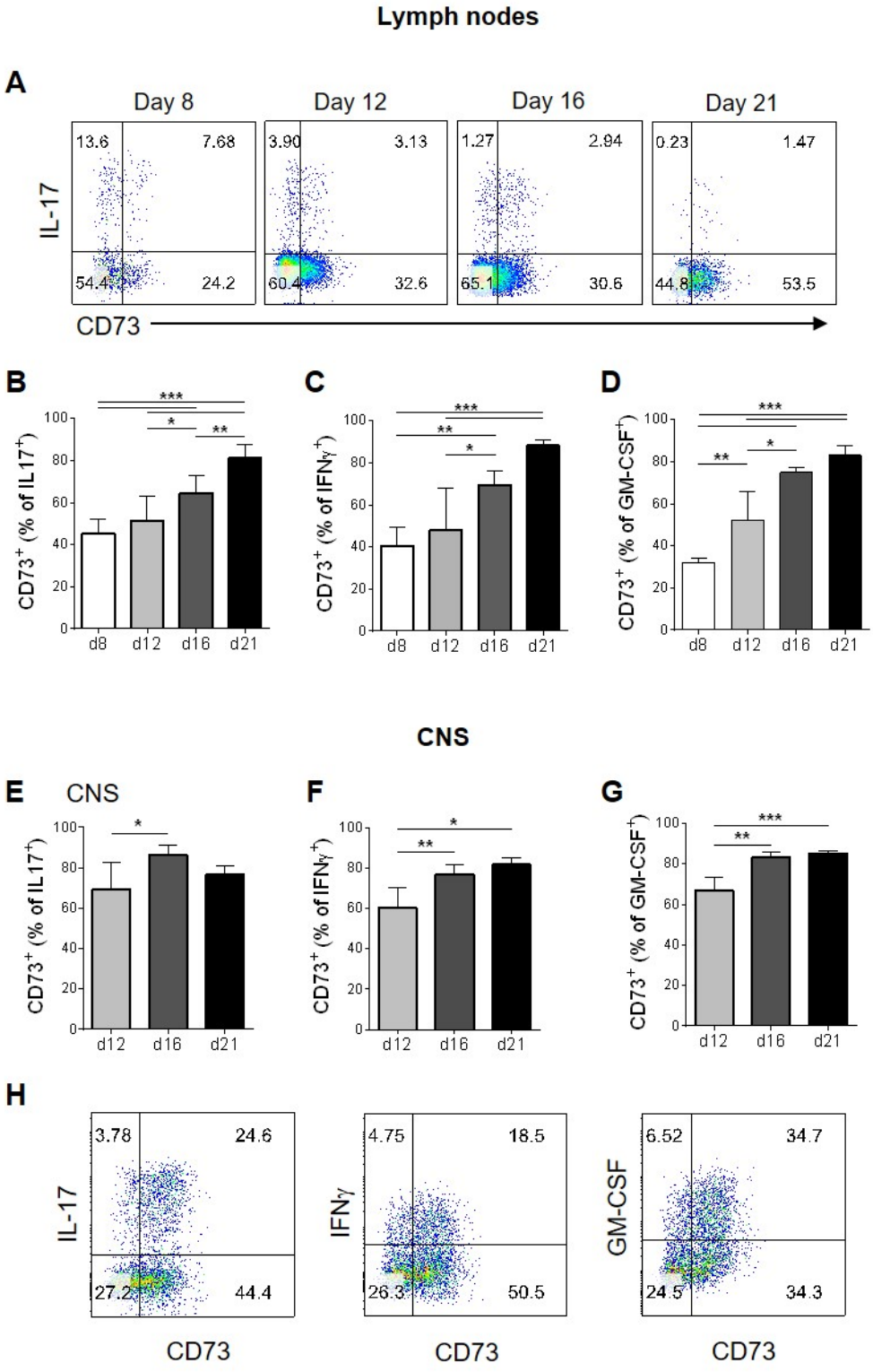

Figure 14. Th17 cells increase expression of CD73 during EAE progression.

A-D. Analysis of co-expression of CD73 on cytokine-producing cells in draining lymph nodes, gated on live $CD4^+$ T cells, at indicated time-points post EAE induction. **A.** Representative FACS plots of CD73 and IL-17A expression from EAE $CD4^+$ T cells. **B.** Percentage of IL-17^+ T cells that express CD73. **C.** Percentage of IFNγ^+ T cells that express CD73. **D.** Percentage of GM-CSF^+ T cells that express CD73. **E-H.** Analysis of co-expression of CD73 on cytokine-producing cells in CNS, gated on live $CD4^+$ cells, at indicated time-points post EAE induction. **E.** Percentage of IL-17^+ T cells that express CD73. **F.** Percentage of IFNγ^+ T cells that express CD73. **G.** Percentage of GM-CSF^+ T cells that express CD73. **H.** Representative FACS plots showing CD73 and cytokine staining in live $CD4^+$ T cells from CNS on day 16 of EAE. Values in graphs correspond to mean ± standard deviation. n = 5–13 mice/time-point pooled from 2–3 experiments (except GM-CSF^+ T cells in the CNS at day 16 that is representative of a single experiment).

3.2.6 CD73 deficiency in *in vitro*-differentiated Th17 cells and Tregs

To ultimately test the requirement for CD73 for proper IL-17A production in Th17 cells, we *in vitro* differentiated $CD4^+$ T cells isolated from $CD73^{-/-}$ and WT naïve mice. Th17 cells that lacked CD73 were able to produce regular levels of IL-17A and RORγt, compared to WT cells (Figure 15A and B), proving that CD73-deficiency does not impact Th17 development *in vitro*. These data correlate with our previous data in which downstream signaling molecules of the CD73-adenosine pathway did not rescue IL17A expression from $BACE1^{-/-}$ Th17 cells. Taken together, our data show evidence that the reduced expression of CD73 in $BACE1^{-/-}$ Th17 cells is not responsible for the impaired expression of IL-17A in this T helper subset.

From all the $CD4^+$ T helper subsets studied, Tregs were the population with the highest expression of CD73 both *in vitro* and *in vivo*. Hence, we wanted to address the impact of CD73 deficiency in Treg development. After three days of culture, *in vitro*-differentiated $CD73^{-/-}$ Tregs showed a small but significant reduction in the frequency of $Foxp3^+$ T cells (Figure 15C). In order to better understand the roles of CD73 in both Th17 and Tregs, and to interrogate whether CD73 deficiency had the same impact on Foxp3 expression from Tregs, we performed EAE studies in $CD73^{-/-}$ mice.

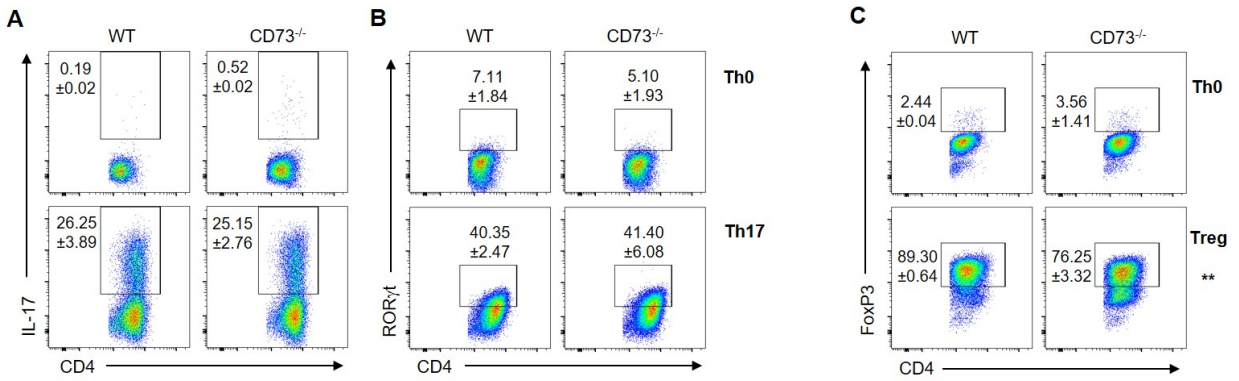

Figure 15. CD73 deficiency does not impact Th17 polarization *in vitro* but is required for Foxp3 expression in induced Tregs.

A-B. WT and $CD73^{-/-}$ $CD4^+$ T cells were differentiated for three days under Th0 (top panels) or Th17 (bottom panels) polarizing conditions and measured, by flow cytometry, the levels of IL-17A (**A**) and RORγt (**B**). Data representative of at least three independent experiments with similar results. **C.** WT and $CD73^{-/-}$ $CD4^+$ T cells were differentiated *in vitro* for three days to Th0 (upper panels) or Treg (bottom panels) before flow cytometric analysis of Foxp3 expression in $CD4^+$ T cells. Numbers indicate average percentage ± standard deviation (two technical replicates), representative of three independent experiments with similar results.

3.2.7 CD73 deficiency does not affect EAE development

The relatively high expression of CD73 on inflammatory $CD4^+$ T cells suggested that CD73 may play a role in promoting Th17 function during the induction phase of EAE. Conversely, the upregulation of CD73 as disease progresses and stabilizes could imply a potential limiting role of CD73 in later phases of EAE. Hence, we tested the requirement of CD73 expression in autoimmune Th17 cells by immunizing both WT and $CD73^{-/-}$ mice with MOG_{35-55} in CFA to induce EAE. Unexpectedly, we found no differences in severity (Figure 16A) and incidence of disease (Figure 16B) between CD73-deficient and WT mice. It appeared that $CD73^{-/-}$ mice presented a slightly delayed onset of clinical signs (Figure 16C), but this difference was not statistically significant.

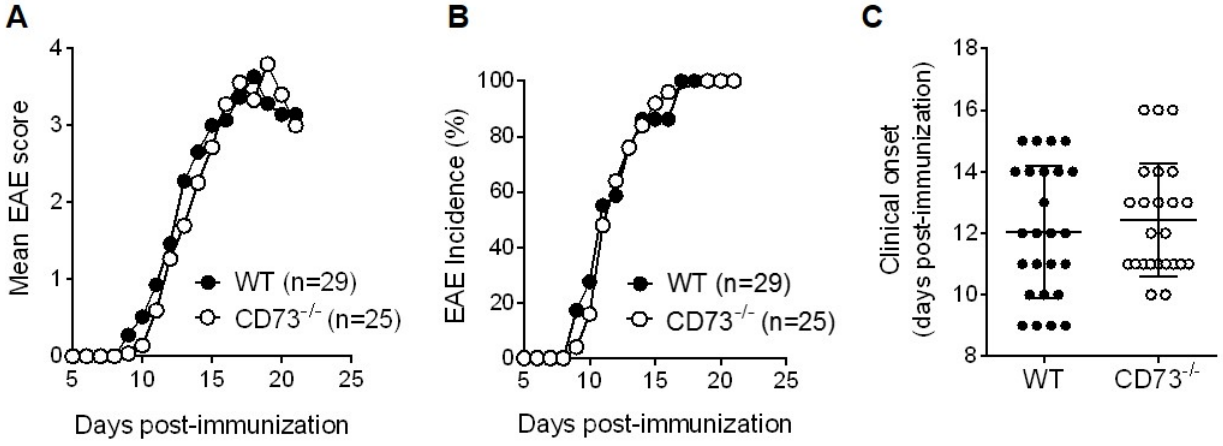

Figure 16. CD73 deficiency does not affect EAE clinical course of disease.

A. Mean clinical scores following EAE induction in WT and $CD73^{-/-}$ mice. **B.** Percentage of mice that had developed EAE clinical signs on indicated days after EAE induction. **C.** Day of EAE onset in WT and $CD73^{-/-}$ mice that

developed signs of EAE by day 16 post-immunization. Data pooled from four independent experiments is represented as means (**A-B**) ± standard deviation.

One could speculate that the lack of CD73 in Tregs could result in an enhanced Th17 response in EAE, due to an impaired regulatory function of $CD73^{-/-}$ Tregs. It has been previously reported that CD73 expression in the CNS is necessary for normal entry of lymphocytes into this compartment (433,437). Therefore, a hypothetical Th17 enhancement could be mitigated by a deficient entry of these cells into the CNS due to the lack of CD73 expression in the CNS and endothelial cells. However, a detailed analysis of cytokine-producing T cells after PMA/ionomycin stimulation revealed no significant differences in frequencies of cells producing IL-17A, IFNγ or GM-CSF in the draining lymph nodes (Figure 17A) or numbers of cytokine-producing cells infiltrating the CNS (Figure 17B), at any phase of EAE disease course. These results not only suggested that the absence of CD73 did not affect lymphocyte infiltration into the CNS, but that it had no effect on cytokine expression from $CD4^{+}$ T cells in the lymph nodes and CNS during EAE. Similarly, analysis of the MOG-specific IL-17A response from lymphocytes taken at onset (day 12 p.i.) and peak of disease (day 16 p.i.) from draining lymph nodes of WT and $CD73^{-/-}$ showed no differences in the capacity of $CD73^{-/-}$ Th17 cells to produce IL-17A (Figure 17C). These data confirmed that CD73 is not required for proper Th17 priming and function during EAE.

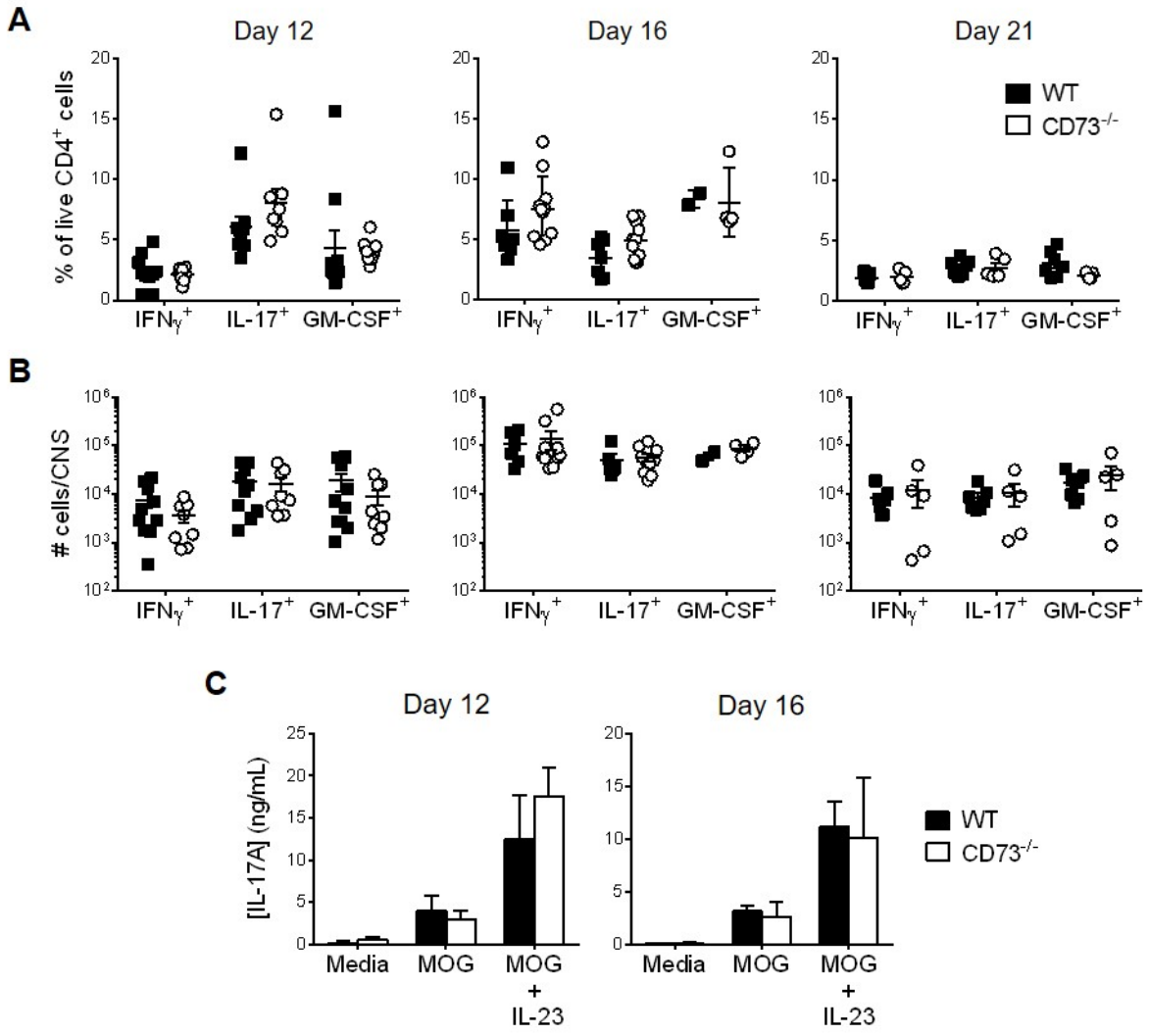

Figure 17. CD73 deficiency does not alter cytokine expression in during EAE development.

A-C. Cytokine production and Treg frequency were analyzed by FACS in draining LN and CNS of WT and CD73$^{-/-}$ mice at day 12 (onset), day 16 (peak) and day 21 (chronic/resolution) phases of EAE. **A.** Frequencies of IL-17, IFNγ and GM-CSF expressing T cells, analyzed in draining lymph nodes on indicated days after EAE induction. **B.** Numbers of IL-17, IFNγ and GM-CSF expressing T cells infiltrating the CNS at the indicated time points after immunization. A-B show mean ± SEM of pooled data, each point representing an individual mouse. **C.** Cells from draining lymph nodes at days 12 (n = 4-5/group) and 16 (n = 2-3/group) post-immunization were re-challenged *in vitro* with MOG35–55 for three days in the presence/absence of IL-23 (20ng/mL), and IL-17 expression was measured by ELISA.

3.2.8 CD73 deficiency does not impact *in vivo* generation of Tregs

In vitro-differentiated Tregs that lacked CD73 resulted in reduced frequencies of Foxp3$^+$ T cells (Figure 15C). We wanted to know if this impaired development translated into reduced frequencies of Tregs during EAE. We found that Foxp3$^+$ Treg frequencies in draining lymph nodes and the CNS similarly increased between onset and peak of disease in both WT and CD73$^{-/-}$ mice, (Figure 18A-C). These data confirm that both Th17 and Tregs function equally well *in vivo* in the absence of CD73. Taken together, these results correspond with the clinical scores, thereby confirming that CD73 does not play a critical role in either promoting or limiting the inflammatory response induced during EAE.

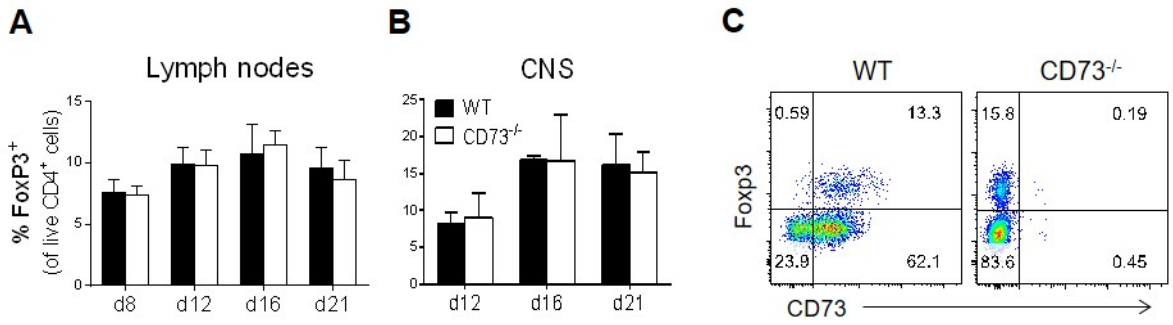

Figure 18. CD73$^{-/-}$ mice do not exhibit decreased frequencies of Foxp3$^+$ Tregs

Percentage of Tregs in the draining lymph nodes (**A**) and CNS (**B**) at the indicated time-points of EAE. **C.** FACS plots show representative staining of Foxp3 and CD73 in CNS on day 16 post-immunization, shown as mean ± standard deviation from 4–7 mice/group (except day 16 WT has n = 2 mice). Data are representative of two-three independent experiments with similar results.

3.2.9 Discussion

One of the main mechanisms by which Tregs can exert their immune-regulatory function is through the upregulation of CD73 (414,415,446) and generation of adenosine (438,439). In addition, CD73 has previously been described as an immunosuppressive molecule expressed by regulatory Th17 cells (419). Among other stimuli, TGF-β1 is a key inducer of CD73 expression (419,430). Interestingly, this cytokine is indispensable for the differentiation of Tregs and Th17 cells (45,164,447). TGF-β1-driven polarization of Th17 cells is commonly considered to induce regulatory Th17 cells (170,171,175). However, TGF-β1 is required *in vivo* for the generation of pathogenic Th17 cells in EAE(169–171) , highlighting the importance of combined stimuli in T cell differentiation. During differentiation, TGF-β1 production by early Th17 cells is necessary for further development and pro-inflammatory Th17 functions (171,448). Confirming previous studies, we observed high expression of CD73 by Tregs and Th17 cells differentiated *in vitro* in the presence of TGF-β1; but more importantly, we identified high levels of CD73 in pro-inflammatory Th17 cells during the course of EAE, showing evidence of CD73 expression not only in regulatory populations.

We hypothesized that reduced levels of CD73 caused by BACE1-deficiency could explain the impaired IL-17A expression in BACE1$^{-/-}$ Th17 cells. However, our data showed that CD73 deficiency had no implications in IL-17A expression or pathogenesis of these cells in EAE. Therefore, our data suggest that CD73 impaired production is just a sign of BACE1 deficiency and rules out this reduction in CD73 as a mechanism regulating IL-17A expression in BACE1$^{-/-}$ Th17 cells.

The widespread expression of CD73 on inflammatory Th17 cells during EAE, particularly in the CNS at onset of clinical signs, argued against a purely immunosuppressive role for this molecule in Th17 cell function. These results are in agreement with studies reporting the upregulation of CD73 in inflammatory Th17 cells in Crohn's patients (420).

Mills *et al*. observed that $CD73^{-/-}$ mice exhibited reduced severity of EAE but, paradoxically, $CD73^{-/-}$ Th17 cells expressed higher levels of pro-inflammatory cytokines (433). These results were attributed to the requirement for CD73 expression in CNS-resident cells. In a second report they suggested that contrary to the immunosuppressive role of adenosine during the initial phases of EAE, adenosine can facilitate lymphocyte entry to the CNS by promoting the expression of CX3CL1 in the choroid plexus (437). However the specific function or requirement of CD73 in Th17 cells was never addressed.

Upon induction of EAE in $CD73^{-/-}$ mice, we did not see any differences in the course of disease or cytokine production from Th17, in contrast to Mills *et al.* results (433). These differences can be attributed to different variables. First of all, different animal facilities have different microbiota communities that can influence the outcome of autoimmunity (449–453). The amounts of MOG_{35-55} and PTx used to induce EAE were also slightly different between our and their labs, a fact that can result in differences in the proportions of Th17 versus Th1 cells induced. In our hands, EAE is associated with strong induction of Th17 cells and the response is dependent on IL-17 and IL-23. We did not find any effect of CD73 deficiency on Th17 induction during any phase of EAE, as measured both by non-specific PMA/ionomycin stimulation and by stimulation with the immunizing antigen MOG_{35-55}. Differentiation of Th17 cells *in vitro* further supported our

unexpected finding that CD73 does not play a dominant role in either inhibiting or promoting Th17 differentiation. Interestingly, Mills *et al.* also reported that mice deficient in the adenosine receptor A2A showed exacerbated EAE with increased IFNγ and proliferation in response to $MOG_{35–55}$, supporting the immunosuppressive role of adenosine on Th1 responses (433,436). However, IL-17 responses were not impaired in these experiments, corresponding to our current study results and suggesting that the balance between Th17 and Th1 induction in EAE could determine the requirement for CD73 in disease susceptibility.

Development of functional Tregs is crucial in the control of autoimmune diseases (454). Our data, as well as previous reports (415,420), confirmed that this T cell subset expresses high levels of CD73. However contradictory roles for these cells in EAE have been described. Depletion of Tregs results in exacerbated disease severity (455,456) but interestingly, Tregs are not able to suppress the immune response during the early stages of lymphocyte infiltration into the CNS (457). Paradoxically, Tregs have been found to promote, rather than suppress, the differentiation of inflammatory Th17 cells, through the absorption of IL-2 (458,459) or secretion of TGF-β1 (162). Therefore, to address the impact of CD73 deficiency in Tregs to global inflammatory response is a complex task. Although *in vitro* Treg polarization of $CD73^{-/-}$ T cells yielded decreased percentages of $Foxp3^{+}$ $CD4^{+}$ Tcells, this was a small effect and the absence of the enzyme did not affect the frequencies of Tregs in the draining lymph nodes or CNS of EAE mice.

CD73 works with CD39 to generate adenosine from ATP, in what is known as the control of the purinergic halo. Although the focus is often on the CD73-driven generation of adenosine as an immunosuppressive molecule, the CD39-mediated removal of ATP from the local environment

also serves to reduce inflammation (421). Extracellular ATP activates P2X receptors as a damage-associated molecular pattern (DAMP) signal to elicit inflammatory responses such as inflammasome activation and release of IL-1β from monocytes and macrophages (460,461).In our experiments, we did not observe any change in CD39 expression in the absence of CD73 *in vitro* or *in vivo* (data not shown). Hence, it is likely that the first arm of the CD39/CD73 processing of ATP still acts to control inflammatory responses during EAE. In this context, it was recently reported that Th17 cells have the surprising ability to produce their own IL-1β through activation of the ASC-dependent inflammasome pathway, and ATP is one molecule capable of activating this pathway (461). Hence, we speculate that Th17 cells may indeed limit their own activation through the upregulation of the CD39/CD73 enzymatic complex, but that removal of ATP rather than generation of adenosine may play a more important role. In such a situation, the regular levels of CD39 expressed in $CD73^{-/-}$ T cells are enough to induce and control a normal immune response. In fact, regulatory Th17 cells express CD39 that can efficiently hydrolyze ATP (51,421,461,462), and its deficiency resulted in reduced expression of IL-10 from Th17 cells, with increased pathogenic function in colitis (462). In addition, it has been reported that administration of *Bacteroides fragilis PSA* increases $CD39^{+}$ Tregs and protects from EAE (57,450). CD39-deficient mice in this model developed greatly exacerbated disease severity compared to WT controls, and it is possible that this was due to effects on Th17 cells as well as Tregs. Separately, CD39 expressed by dendritic cells during EAE also plays an important role in limiting Th17 cell expansion and resulting EAE severity (463).

In summary, we report here that CD73 is expressed on a high proportion of Th17 cells during EAE development, including on cells in the CNS. However, CD73 deficiency did not affect

differentiation, recruitment or function of Th17 cells as assessed by EAE clinical signs, flow cytometry and antigen recall assays. We published these findings earlier this year in PLoS One (464). These data were unexpected given the known role of CD73 in regulating inflammatory immune responses, and suggest that in the face of a strong inflammatory stimulus, such as what occurs during induction of EAE, the immunosuppressive role of CD73 becomes insufficient to prevent Th17 generation and onset of autoimmune inflammation.

3.3 BACE1-DEFICIENCY RESULTS IN ABERRANT SIGNALING PATHWAYS IN CD4$^+$ T CELLS

3.3.1 Introduction

In the previous chapters we described a major impact on IL-17A expression in comparison to IL-17F, suggesting a differential regulation of these two cytokines by BACE1. In addition, other Th17 markers, like RORγt were expressed at normal levels. Although uncommon, there is evidence in the literature of molecules that can impact IL-17A expression without affecting RORγt. Serum amyloid A produced by inflamed epithelial cells has been shown to promote IL-17A production from RORγt$^+$ effector cells (465). It has also been reported that TGF-β1-driven inhibition of Gfi1 is required for Th17 development, with a marked effect on IL-17A expression but comparatively minor impact on RORγt and IL-17F levels (466). Interestingly, Gfi1 acts as a transcriptional repressor of CD73 (419), which was aldo found down-regulated in BACE1$^{-/-}$ Th17 cells. However we did not observe differences in Gfi1 levels, suggesting that BACE1 acts through a different mechanism.

We initially speculated that reduced CD73 levels in BACE1$^{-/-}$ Th17 cells would explain the defects in IL-17A expression. *In vivo* and *in vitro* experiments with CD73$^{-/-}$ mice demonstrated that the ectonucleotidase is not necessary for the expression of IL-17A and Th17 pathogenicity in EAE. Hence, we needed to investigate what pathways are dysregulated in the absence of BACE that can explain the defect in IL-17A expression.

In 2009, Gomez-Rodriguez et al. published a study where they found that in the absence of IL-2-inducible T cell kinase (Itk) expression Th17 cells expressed lower IL-17A whereas other Th17-signature molecules, including RORγt and IL-17F, remained unchanged (467). They proved that uncoupled TCR/CD28 signaling via defects in Ca^{2+} signaling impaired IL-17A production but not IL-17F. Their findings highlighted the connections between earlier signaling events and the expression of specific cytokine production. Due to the similarities with our findings in $BACE1^{-/-}$ Th17 cells their results prompted us to take closer look at the T cell signaling.

3.3.1.1 TCR signaling – early events

T cell receptor (TCR) molecules possess very short cytosolic tails that are incapable of signaling on their own. Therefore, the TCR is associated with the CD3 complex, constituted by δ, γ, ε and ζ chains. After TCR-peptide-MHC-II engagement, the intracellular domain of CD4 associates with the lymphocyte-specific protein tyrosine kinase (Lck), a Src family kinase. The association of Lck with the complex allows it to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) located on the CD3 chains. In turn, this phosphorylation recruits the Syk family tyrosine protein kinase ZAP70 to the TCR–CD3 complex. ZAP70 can propagate the TCR signal by phosphorylating multiple targets, including the membrane-associated scaffold molecule linker for activation of T cells (LAT). Phosphorylation of LAT results in the amplification of TCR signaling through the recruitment and phosphorylation of other adaptor proteins and the final recruitment and activation of effector molecules, such as phospholipase Cγ (PLCγ) and the TEC family kinase Itk (468).

3.3.1.2 PI3K/PTEN-Akt signaling in Th17 cells

Lck can recruit and phosphorylate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), leading to its activation. This enzyme subsequently catalyzes the phosphorylation of phosphatidylinositol 3,4-bisphosphate ($PI(4,5)P_2$ or PIP2) into phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$ or PIP3) (469). PIP3 serves as a docking site for proteins that harbor pleckstrin-homology (PH) domains, including Akt and its upstream activator phosphoinositide-dependent kinase-1 (PD-K1) (470). At the membrane, constitutively active PD-K1 phosphorylates Akt at Thr308 (471), leading to activation of Akt. Phosphorylation of Akt at Ser473 by mTORC2 further stimulates its enzymatic activity (472), but the mechanisms involved in mTORC2 activation are not fully understood (466,473).

Activation of Akt leads to the activation of different signaling cascades that result in enhanced T cell survival and proliferation, as well as regulation of metabolic pathways and cell cycle (474). In immune cells, the two main signaling cascades triggered by Akt activation are the induction of the mammalian Target of Rapamycin Complex 1 (mTORC1) pathway and the cytosolic sequestration of members of the forkhead box O (FoxO) family (473).

During the past decade, an increasing body of literature has demonstrated that TCR-driven signals can determine T cell fate (475–478). Strong signals induced by TCR ligation are required for the differentiation of effector Th subsets whereas low TCR signals favor Treg differentiation (470,475–477). In this regard, the Akt pathway has been shown to be particularly important in mediating the balance between Th17 and Treg commitment.

Phosphorylation of Akt drives the activation of this kinase. Specifically, Akt can be phosphorylated at two residues: Thr308 and Ser473. Thr308 phosphorylation is mediated by PDK1, and results in Akt activation (466). This enables the indirect activation of the mTORC1 complex via suppression of the mTORC1 inhibitory proteins TSC1/2 and PRAS40 (479,480). It has been reported that preventing PI3K and mTORC1 signaling using inhibitors results in increased Treg induction (481,482), whereas increasing mTORC1 signaling enhances Th17 generation (466,483). mTORC1, which is composed of the proteins Raptor, mTOR and mLST8, can specifically regulate Th17 development in a number of ways.

Firstly, mTORC1 induces activation of the transcription factor Hif-1α (258,484), which increases RORγt expression (259,260). In addition, Hif-1α can cooperate with RORγt and p300 to facilitate binding to the IL-17A promoter, with concomitant promotion of gene expression (259). Furthermore, Hif-1α enhances glycolytic activity, which is necessary for activation-induced proliferation (258).

mTORC1 also promotes EGR2 expression, which leads to the inhibition of Gfi1 expression (466). Gfi1 is a negative regulator of Th17 development, and, intriguingly, Gfi1deficiency or overexpression specifically impacts IL-17A expression, with only a minor effect on RORγt and IL-17F production (466).

In addition, mTORC1 promotes nuclear translocation of RORγt via activation of S6K2, a nuclear localization signal (NLS)-bearing protein that effectively shuttles the NLS-deficient RORγt into the nucleus, where it can promote Th17 signature gene expression (466).

Finally, mTORC1 can regulate STAT phosphorylation. Rheb or Raptor-deficient cells, which have impaired mTORC1 activity, present reduced phosphorylation of STAT3 and STAT4, in a SOCS3-dependent mechanism (483). Rheb deficiency leads to a major impairment of Th17 differentiation and mild defects in Th1 polarization (483), while Raptor deficiency results in a specific defect in Th17 development (466).

The alternative Akt phosphorylation event at Ser473 is catalyzed by mTORC2 (485). This changes Akt substrate specificity, and results in phosphorylation and repression of FoxO1 and FoxO3a (472,481,486). In the nucleus, FoxO1/O3a promote Foxp3 expression and Treg development (487–489). However, Akt-mediated phosphorylation prevents FoxO1/O3a translocation into the nucleus, thereby limiting the acquisition of a Treg phenotype (487,489). It has been speculated that this pathway promotes Th17 development (487).

The activation of the Akt TCR-driven pathway is negatively regulated by the phosphatase and tensin homolog on chromosome 10 (PTEN). PTEN, antagonizing the enzymatic reaction of PI3K, removes one phosphate group from $PI3,4,5P_3$, converting it into $PI4,5P_2$ (490,491). PTEN-driven reduction of PIP3 levels halts the recruitment and activation of Akt. In fact, overexpression of PTEN has been shown to favor the generation of Tregs and alter the Th17/Treg balance, resulting in reduced autoimmunity (492). In addition, p53, a negative regulator of the Th17

response that inhibits STAT3 phosphorylation, can also induce PTEN expression. This has been proposed as an additional mechanism by which p53 regulates Th17 differentiation (492). PTEN has also been found to interact with and inhibit Smad3 (493), a TGF-β1-downstream TF that induces IL-17 production (494). On the contrary, Th17-promoting microRNAs have been shown to inhibit PTEN expression (495). Taken together, these data suggest that PTEN expression may play a detrimental role in Th17 differentiation.

In addition to the regulatory effects on Akt signaling, PTEN activity is required for additional TCR-driven signaling cascades. Indeed, generation of PIP2 by PTEN is necessary for both Ca^{2+} and MAPK signaling upon TCR ligation.

3.3.1.3 PLCγ and Ca^{2+} signaling in Th17 cells

The propagation of TCR signaling is largely controlled by lipid second messengers, as observed by the role of $PI(3,4,5)P_3$ and $PI(4,5)P_2$ in recruiting PD-K1 and Akt. In addition, TCR-driven phosphorylation of LAT allows the recruitment and activation of phospholipase C gamma (PLCγ). PLCγ hydrolyses $PI(4,5)P_2$ to generate the membrane-associated diacylglycerol (DAG) and the diffusible inositol-1,4,5-trisphosphate (PI3). DAG can recruit other effector proteins such as protein kinase C (PKC) or RAS guanyl nucleotide-releasing protein (RASGRP), activating the NF-κB or MAPK/ERK signaling cascades, respectively.

Alternatively, PI3 triggers an increase in Ca^{2+} by releasing Ca^{2+} from the endoplasmic reticulum, which subsequently results in the influx of extracellular Ca^{2+} mediated by the Ca^{2+} sensor stromal interaction molecule (STIM) and the Ca^{2+} channel transmembrane protein ORAI1.

This initial spike will trigger an influx of extracellular Ca^{2+} by activation of the Ca^{2+} transporter CRAC. Binding of Ca^{2+} to calmodulin activates the phosphatase calcineurin, which dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT) and allows its translocation into the nucleus.

The significance of Ca^{2+} signaling in Th17 differentiation has been shown in a number of different reports. Firstly, Th17 cells appear to exhibit a distinct pattern of Ca^{2+} signaling compared to early activation of Th1 and Th2 cells, with Th17 displaying and intermediate but sustained Ca^{2+} levels (496). This signaling pattern has been shown to promote Th17 cell differentiation through activation and nuclear import of NFAT and by reducing T cell motility, thus augmenting the overall TCR and co-stimulation signaling (496). Concomitant to these results, CRAC inhibition and reducing Ca^{2+} influx also resulted in preferential inhibition of Th17 cells compared to Th1 and Th2 (497). Reduced Ca^{2+} levels lead to defects in NFAT nuclear import and transcriptional regulation of RORγt and RORα, and therefore preventing Th17 differentiation (496,497). On the other hand, stress-induced increases in Ca^{2+} signaling seem to promote Th17 differentiation (498).

In their report, Gomez-Rodriguez et al. (467) demonstrate that the absence of Itk provokes defects in Ca^{2+} due to the inability of PLCγ to associate with the TCR complex. IL-17A expression was dependent of NFAT activity and deficient IL-17A production could be restored with ionomycin. In contrast to previous reports, in $Itk^{-/-}$ Th17 cells, deficient Ca^{2+} signaling showed only major defects in IL-17A expression but not in RORγt levels or Th17 overall differentiation. These findings suggest that Ca^{2+} signaling might influence IL-17A expression in more subtle ways than previously described.

3.3.1.4 TCR strength and Th17 cells

Most of the current knowledge on TCR signaling and T cell activation is derived from studies on early T cell development in the thymus (499). It is known that the strength of TCR signaling is a crucial element during positive and negative selection. Indeed, different affinities for self-peptides are transduced into different intensities and duration of signaling, which allows deletion of the emerging autoreactive T cells, induction of tolerogenic tTregs, while discriminating other non-reactive or anergic T cells. However, the exact mechanisms by which TCR strength dictates the fate of mature T cells is still under active investigation. In this regard, co-activation by cluster of differentiation 28 (CD28) has been proposed as a key element during T cell activation. CD28 is expressed on the surface of both naïve and primed T cells and upon binding to CD80 and CD86 on the APC membrane, it cross-links and allows the interaction and activation of different SRC family kinases, such as Lck or Fyn (500). In a mechanism similar to the early activation of TCR, CD28 signaling converges in the activation of the PI3K cascade (500). Due to this convergence in signaling events, it has been hard to distinguish between CD28 signaling and augmentation of TCR/CD3 signaling by CD28 co-stimulation. In this regard, Kane et al. showed that CD28 signaling is necessary for T cell proliferation by induction of IL-2 production (501) . In addition, CD28 signaling seems to enhance expression of IFNγ but to be completely dispensable in the PI3K–driven induction of Th2 cytokines (501).

It was initially believed that Th17 differentiation was favored under low TCR strength conditions (502,503). This hypothesis was based on the Th1-promoting effect of high TCR

signaling as well as the induction of IL-2 production (502–505). IL-2 can inhibit Th17 differentiation via STAT3 inhibition and repression of RORγt and IL-17 expression (506,507). However, *in vitro* differentiation of murine Th17 cells is usually enhanced by the presence of α-CD28 clustering antibodies. More recent reports, have shown that differentiation of Th17 cells was favored in high concentrations of antigen (477). Different mechanisms have been proposed for this outcome. The first one is that a longer duration of the immunological synapse induced by higher TCR/CD28 signaling results in increased secretion of IL-6 from DCs (508). It has also been observed that strong stimuli can also cause an attenuated ability to signal via IL-2R through inhibition of phosphorylation of the transcription factor STAT5 (508).

TCR signaling is both positively and negatively regulated in a feedback loop by the TCR activation process itself. Upon CD3 activation, PTEN levels are rapidly downregulated in order to drive or amplify the TCR signal (475,509). More recently, it has been proposed that TCR stimulation can lead to a dose-dependent repression of PTEN (477) and that low vs. high stimulation can affect Th17 polarization by different mechanisms (475,476). In contrast, TCR/CD3 activation also induces different processes to limit the extent of its signaling. TCR signaling triggers the generation of cAMP in lipid rafts (510–512), especially in the absence of CD28 co-stimulation (511). cAMP inhibits proximal TCR signaling through a pathway involving activation of protein kinase A (PKA) and C-terminal Src kinase (Csk) to inhibit Lck and to reduce the recruitment and activation of ZAP70.

Interestingly, neuronal BACE1 has been described as a negative regulator of the adenylate cyclase (AC) (353), the enzyme that converts intracellular ATP into cAMP, meaning that in the

absence of BACE1, cells have an increased potential to generate cAMP. Therefore, alterations in cAMP levels could affect early signaling events in Th17 cells.

Although it is very evident that the quality of the TCR signaling is crucial for Th17 development, we need a better understanding of the cross-talk between different signaling cascades as well as the coupling between early activating events and their impact in Th17 differentiation. Thus, we decided to investigate whether BACE1 modulation of IL-17A expression is regulated by early signaling events in T cell activation.

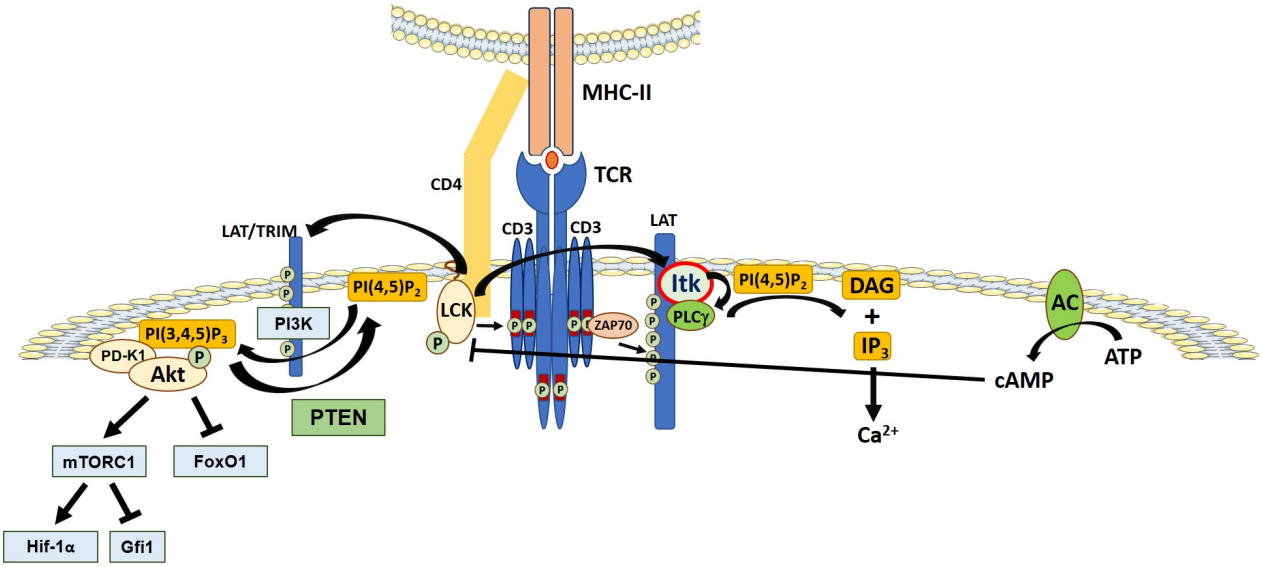

Figure 19. Early signaling events induced by TCR activation that drive Th17 differentiation and IL- 17A expression.

3.3.2 Thymic development of T cells is not altered by BACE1 deficiency.

During the isolation of $CD4^+$ T cells from the secondary lymphoid organs of WT and $BACE1^{-/-}$ mice, we observed that we often obtained more $CD4^+$ T cells from $BACE1^{-/-}$ mice (data not shown). This outcome was not always consistent and it was just noted as an observation. However, it prompted us to ask whether there could be differences in the thymic development of $BACE1^{-/-}$ T cells that may underlie the differences in $CD4^+$ T cell numbers in secondary lymphoid organs. In addition, data from the Immgen consortium revealed high expression of BACE1 in thymic progenitors of αβ T cells, prompting us to study thymic T cell development in $BACE1^{-/-}$ mice. For this purpose, we harvested thymus from naïve mice and studied, by flow cytometry, the development of T cells in both WT and BACE1 thymi. We did not observe any significant differences in the total amount of cells obtained from either WT or BACE1 thymi (Figure 19A).

In the thymus, early progenitors lack CD3, CD4 and CD8 expression and are therefore identified as double negative (DN) ($CD4^-CD8^-$) thymocytes (513,514). During this early stage, DN thymocytes can be further subdivided into four stages of differentiation: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) (513,514). Flow cytometric analysis of WT vs $BACE1^{-/-}$ thymocytes revealed no significant differences in the frequencies of $CD4^+$, $CD8^+$ $CD4^+/CD8^+$ (double positive - DP) or in the percentage of any DN developmental stage (Figure 19C-E). In addition, frequencies of $CD25^+CD4^+CD8^-$ T cells remained constant between WT and $BACE1^{-/-}$ thymi, indicating that there is no defect or enhancement in the generation of thymic Tregs (Figure 19B). These results suggest that BACE1 deficiency does not impair the production of αβ T cells in the thymus. The absence of relevant differences in the $BACE1^{-/-}$ thymus is supported by the observation that not all T helper cells are affected by BACE1 deficiency and therefore suggests that BACE1 regulation of IL-17 expression occurs at a later phase during the development of these cells.

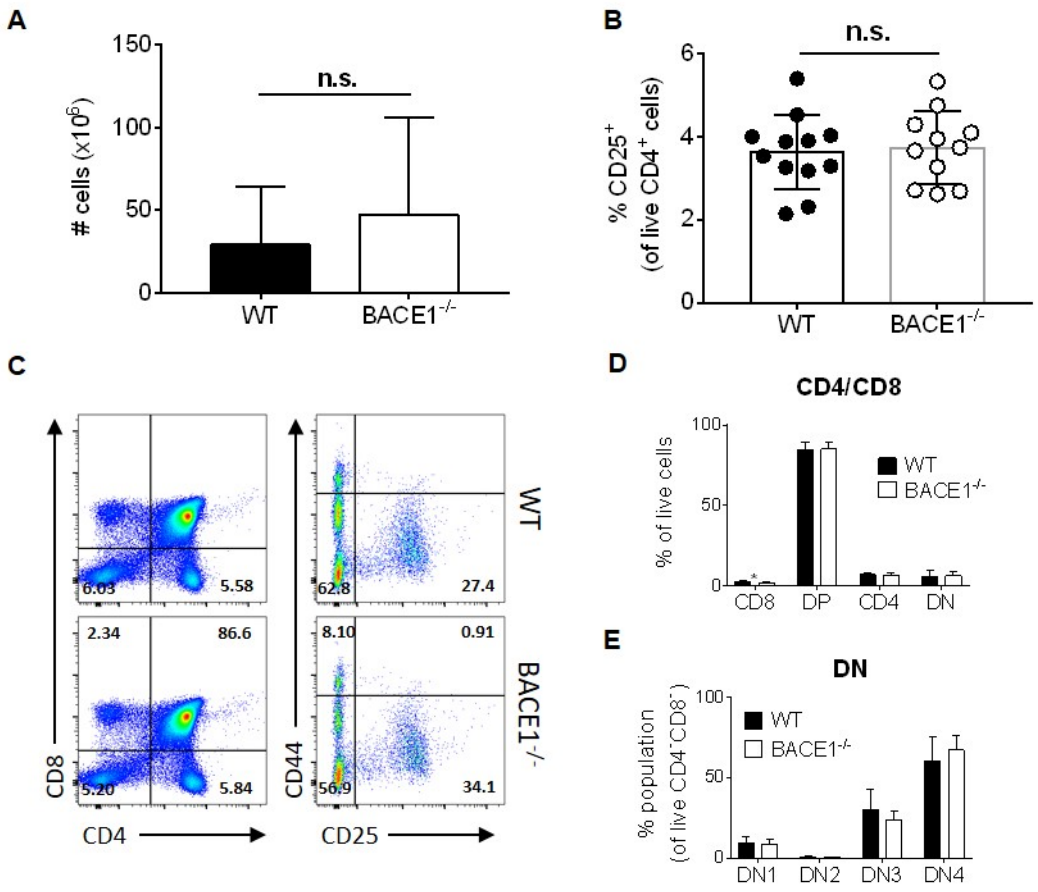

Figure 20. BACE1 deficiency does not affect thymic development of αβT cells.

Thymi from age-matched WT and BACE1$^{-/-}$ naïve mice were mechanically processed in order to obtain single cell suspensions. **A.** Total cells numbers per thymus. **B.** Frequencies of CD25^{+} tTregs, gated on CD4+ single positives. **C.** Representative FACS plot of the expression of CD4 and CD8 in total live thymocytes. **D.** Representative FACS plot of the expression of CD44 and CD25 from CD4^{-}CD8^{-} live thymocytes. E. Frequencies of single CD4^{+} or CD8^{+}, double-positive (DP – CD4^{+}CD8^{+}) or double negative (DN – C4^{-}CD8^{-}) from total live thymocytes. **F.** Frequencies of DN1 (CD44^{+}CD25^{-}), DN2 (CD44^{+}CD25^{+}), DN3 (CD44^{-}CD25^{+}) and DN4 (CD44^{-}CD25^{-}) CD4^{-}CD8^{-} live thymocytes. Values in graphs represent mean ± standard deviation. Pooled data from 8 WT and 7 BACE1$^{-/-}$ mice taken down at different times.

3.3.3 Altering the way Th17 cells are differentiated has the same effect on WT and BACE1$^{-/-}$ Th17 cells.

Differentiation of Th17 cells can be described as a multi-step process whereby TCR activation needs to be accompanied by IL-6 and TGF-β1 signaling during the initiation phase. These two crucial cytokines will induce the expression of IL-23R and IL-21R, as well as the transcription factor RORγt and the cytokine IL-21. IL-21 secretion will act in a paracrine/autocrine manner to further commit cells to the Th17 lineage. In this phase, the Th17 population will expand and stabilize the expression of RORγt and RORα (183). At this point, IL-23 (and IL-1β) are absolutely indispensable for the pathogenicity of Th17 cells, promoting and enhancing the expression of pro-inflammatory molecules like IL-17A and F, IL-22 and GM-CSF (180,193,233). Due to the multiple stages and different cytokines involved in Th17 polarization we wanted to both dissect whether BACE1 was modulating signaling of a specific cytokine and at what step of the polarization process BACE1 was necessary. To address the former, we differentiated cells using different combinations at different times. Specifically, we differentiated naïve CD4^{+} T cells for two days in the presence of IL-6 and TGF-β1. After two days, cells were washed and cultured for two more days in the presence of just complete media, IL-6 and TGF-β1, IL-23 only or IL-1β, IL-6 and IL-23 simultaneously. We found that, no matter what cytokine cocktail was used to induce IL-17A expression, the defect was always present in BACE1$^{-/-}$ Th17 cells (Figure 20A), replicating previous findings using the all-cytokine differentiating condition.

In vitro differentiation of Th17 cells can be performed with isolated CD4^{+} T cells or directly from cell suspensions obtained from processed spleens and lymph nodes (for simplicity we will call these cells splenocytes). In addition to T cells, splenocyte suspensions contain antigen-

presenting cells (APCs), which can activate T cells in a relatively more physiological fashion and can also secrete supporting factors to help in the polarization process. We induced differentiation of Th17 cells from total splenocytes by culturing 10^6 cells with soluble αCD3 in the presence of different Th17-skewing cytokine cocktails for three days. IL-17A expression and secretion, measured by flow cytometry and ELISA, mimicked the results observed in $CD4^+$ pure cultures, with $BACE1^{-/-}$ cells producing reduced levels of the cytokine (Figure 20A and B). These data not only show that APC-driven activation of T cells cannot rescue the impaired function from $BACE1^{-/-}$ Th17 cells, but more importantly, highlights the relevance of BACE1 expression in T cells. The fact that we see a phenotypic difference in experiments where only $CD4^+$ T cells were cultured, constitutes direct evidence that BACE1 is directly functioning in T cells.

Committed to determining what phase of T cell activation and differentiation to Th17 cells BACE1 is playing its role in, we addressed the effect of co-stimulation and IL-2 in $BACE1^{-/-}$ cultures. Co-stimulation with αCD28 antibodies usually enhances IL-17A expression from murine Th17 cells. We wanted to know whether the addition of co-stimulatory signals would have a different impact in WT versus $BACE1^{-/-}$ Th17 cells. We found that the addition of soluble αCD28 antibodies to $CD4^+$ cultures enhanced IL-17A expression in both WT and $BACE1^{-/-}$ Th17 cells but did not rescue the phenotype in $BACE1^{-/-}$ Th17 cells (Figure 20C).

IL-2 signaling is critical for T cell proliferation (507,515) and can be very beneficial during Th1 polarization (507,516). In contrast, IL-2 and STAT5 signaling have been shown to restrain Th17 cell generation (506,507,516). Although IL-2 production by either WT or $BACE1^{-/-}$ T cells was not assessed, addition of blocking αIL-2 antibodies in Th17-differentiating media did not

impact the defect in IL-17A expression from BACE1$^{-/-}$ Th17 cells (Figure 20C). This suggests that BACE1 is not impacting Th17 development through IL-2 regulation.

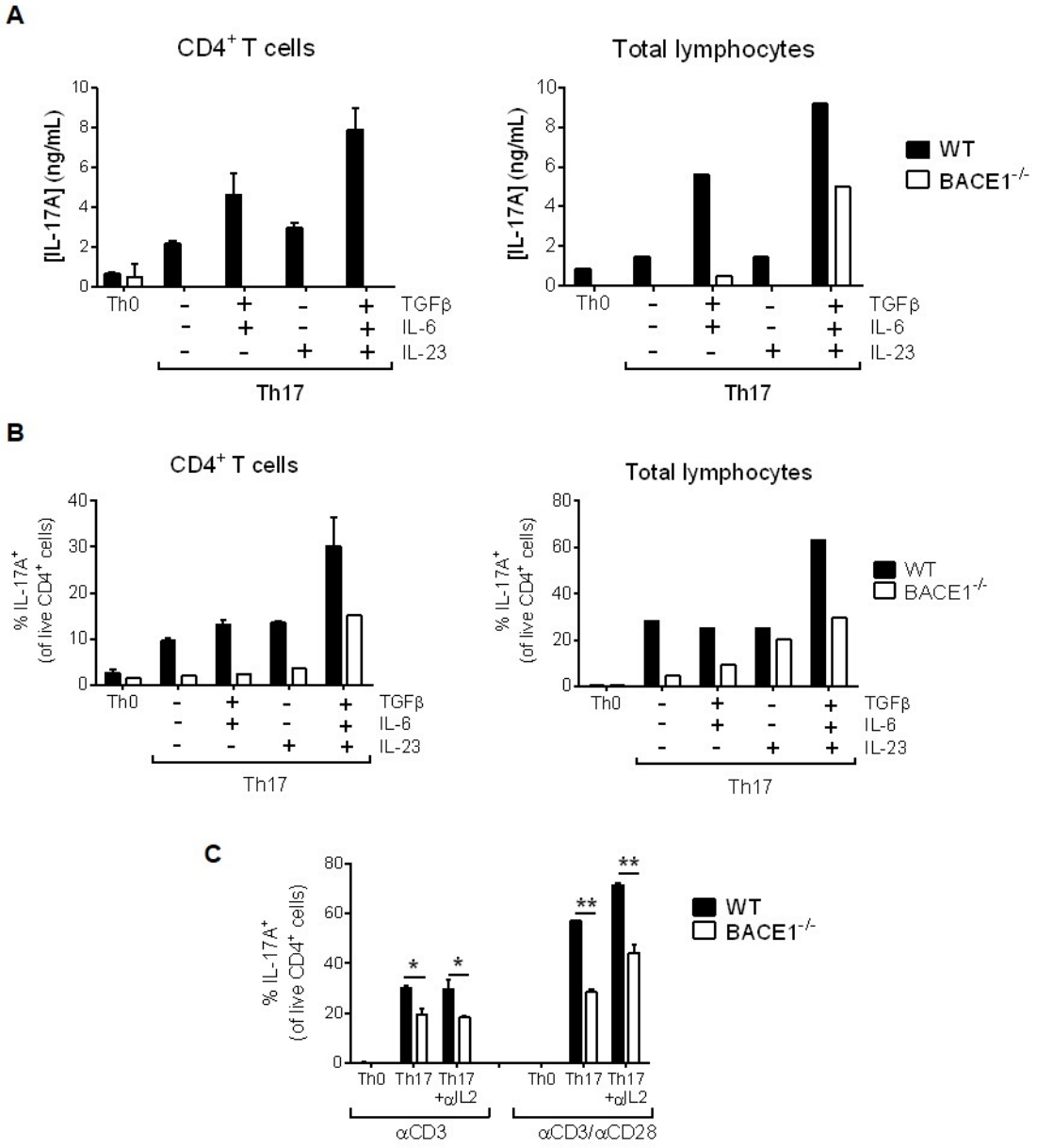

Figure 21. BACE1$^{-/-}$ Th17 cells consistently express low IL-17A despite polarization in different conditions.

A-B. Spleen and lymph nodes from naïve WT and BACE1$^{-/-}$ mice were processed and cultured for three days, under different Th17-polarizing conditions, directly as single cell suspensions (total lymphocytes) or after magnetic isolation of CD4^{+} T cells. **A.** IL-17A cytokine secretion measured in culture supernatants by ELISA from CD4^{+} only T cell

cultures (left graph) or total lymphocytes (right graph). **B.** Frequencies of IL-17A$^+$ Th17 cells differentiated with different Th17-polarizing cytokine cocktails from CD4$^+$ only T cell cultures (left graph) or total lymphocytes (right graph). Data representative of two independent experiments. **C.** Isolated CD4$^+$ T cells from naïve WT and BACE1$^{-/-}$ were activated with plate-bound αCD3 in the presence or absence of plate-bound αCD28 and with or without αIL-2 blocking antibodies. Data in graph shows percentage of IL-17A$^+$ Th17 cells under each condition, represented as mean ± standard deviation. Data is representative from at least two independent experiments with similar results.

3.3.4 APP is not required for IL-17A and CD73 expression in Th17 cells

BACE1 pathogenic role in AD is based on the proteolytic cleavage of APP (286,517). APP is highly expressed in neuronal tissues but mRNA have also been described in other tissues like the eyes or the intestines (BioGPS/Human atlas). APP expression has also been described in circulating monocytes and upregulation in CNS macrophages in neurological HIV patients (518). APP can also be produced in T cells and stimulation with PMA/ionomycin increases the levels of APP in these cells, at least at the mRNA level(519). Interestingly, Dr. Binfeng Lu observed upregulated APP levels in tumor infiltrating lymphocytes (personal communication) and we observed APP expression in our RNAseq analysis of Th17 cells (data not shown). Since APP is one of the main targets of BACE1, we decided to study whether defects in proteolytic processing of APP caused the deficient IL-17A and CD73 expression in BACE1$^{-/-}$ Th17 cells. To test this, we *in vitro*-polarized APP$^{-/-}$ CD4$^+$ T cells and found that both WT and APP$^{-/-}$ Th17 cells express regular levels of IL-17A (Figure 21A) and CD73 (Figure 21B), suggesting that there is no requirement for APP production or BACE1-processing for normal generation of IL-17A and CD73 in Th17 cells. Concomitantly, development of EAE in APP$^{-/-}$ mice did not differ from the course

of disease in control mice (Figure 21C). Hence, our data confirm that APP is not required for Th17 development and function both *in vitro* and *in vivo*.

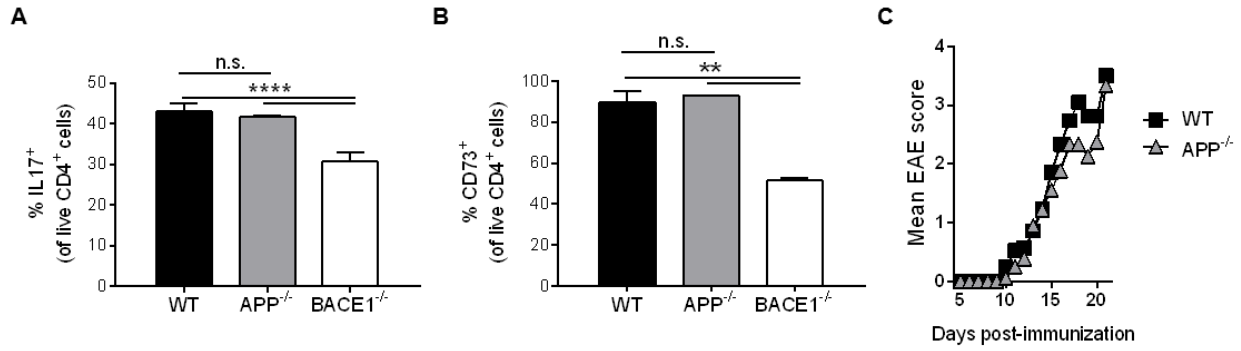

Figure 22. APP is not required for Th17 function.

A-B. WT and APP$^{-/-}$ Th17 cells were differentiated for three days *in vitro*. IL-17A (**A**) and CD73 (**B**) expression was measured by flow cytometry after PMA/ionomycin re-stimulation. Data representative of a single experiment. **C.** WT and APP$^{-/-}$ mice were immunized with MOG_{35-55} in CFA to induce EAE and disease symptoms were evaluated daily. Graph represents mean daily EAE scores. Data pooled from 5 independent experiments (21 WT and 18 APP$^{-/-}$ mice).Itay Raphael conducted some of the immunizations and provided with some of the EAE scores.

3.3.5 BACE1-dependent PTEN expression is required for IL-17A production in Th17 cells.

Previous studies have shown that differences in TCR signaling through PI3K and Akt phosphorylation can affect Th17 polarization (466). Both TCR and CD28 engagement trigger a series of phosphorylation events that result in the recruitment and activation of adaptor proteins as well as Ser/Thr kinases (520). In collaboration with Lyndsay Avery (Kane Lab) and Dr. William Hawse, we investigated whether BACE1$^{-/-}$ T cells presented any differences in the TCR/CD28-

Akt axis. WT and BACE1$^{-/-}$ CD4^{+} T cells were activated with soluble cross-linking αCD3 and αCD28 antibodies, in the presence of Th17-polarizing conditions, since the dynamics of this pathway could be different in the presence or absence of cytokines. Both WT and BACE1$^{-/-}$ T cells exhibited phosphorylation of Akt upon activation of CD3/CD28 but, interestingly, the levels of pAkt were significantly higher and sustained in BACE1$^{-/-}$ CD4^{+} T cells (Figure 22A and B). Since BACE1$^{-/-}$ CD4^{+} T cells exhibited higher phosphorylation of Akt and these cells, upon differentiation to Th17, express reduced levels of IL-17A, we hypothesized that inhibition of pAkt could rescue IL-17A expression in these cells. Consistent with reduced TCR signaling, inhibition of pAkt kinase activity resulted in reduced expression of IL-17A from WT Th17 cells, in a concentration-dependent manner (Figure 22B – left panel). Contrary to our expectations, pAkt inhibition did not restore IL-17A production from BACE1$^{-/-}$ Th17 cells (Figure 22B - right panel).

pAkt levels are dependent on the balance between PI(3,4,5)P_3 (PIP3) and PI(4,5)P_2 (PIP2) (490,520). This balance is regulated by the opposing actions of PI3K and the phosphatase PTEN (471,490,509,520). Analysis of PTEN levels from activated CD4^{+} T cells from naïve WT and BACE1$^{-/-}$ mice revealed that T cells deficient in BACE1 displayed reduced PTEN expression (Figure 22A and C). Importantly, PTEN levels were already reduced before activation with αCD3/αCD28 antibodies, suggesting a fundamental defect in BACE1$^{-/-}$ CD4^{+} T cells. Given that BACE1$^{-/-}$ Th17 cells expressed reduced levels of PTEN we wondered whether inhibition of PTEN in WT T cells would reduce IL-17A expression. Both WT and BACE1$^{-/-}$ CD4^{+} T cells were differentiated for three days to Th17 in the presence of SF1670, an inhibitor of PTEN enzymatic activity. As expected, we did not observe any significant changes in the BACE1$^{-/-}$ Th17 cells, since these cells already express reduced levels of PTEN (Figure 22E – right panel). On the other hand,

inhibition of PTEN activity in WT Th17 cells led to decreased levels of IL-17A (Figure 22E – left panel). In order to confirm the inhibition of Akt and PTEN, we analyzed the phosphorylation of S6, a downstream target of pAkt kinase activity. We observed that at high doses of Akt inhibitor, pS6 levels were significantly reduced, while there was a trend towards increased pS6 at high PTEN inhibitor concentrations (Figure 22F). Taken together, our data demonstrate that BACE1 regulates the expression of PTEN, before and during TCR/CD28 activation. Our findings also suggest that PTEN levels and activity are critical for the correct expression of IL-17A from Th17 cells.

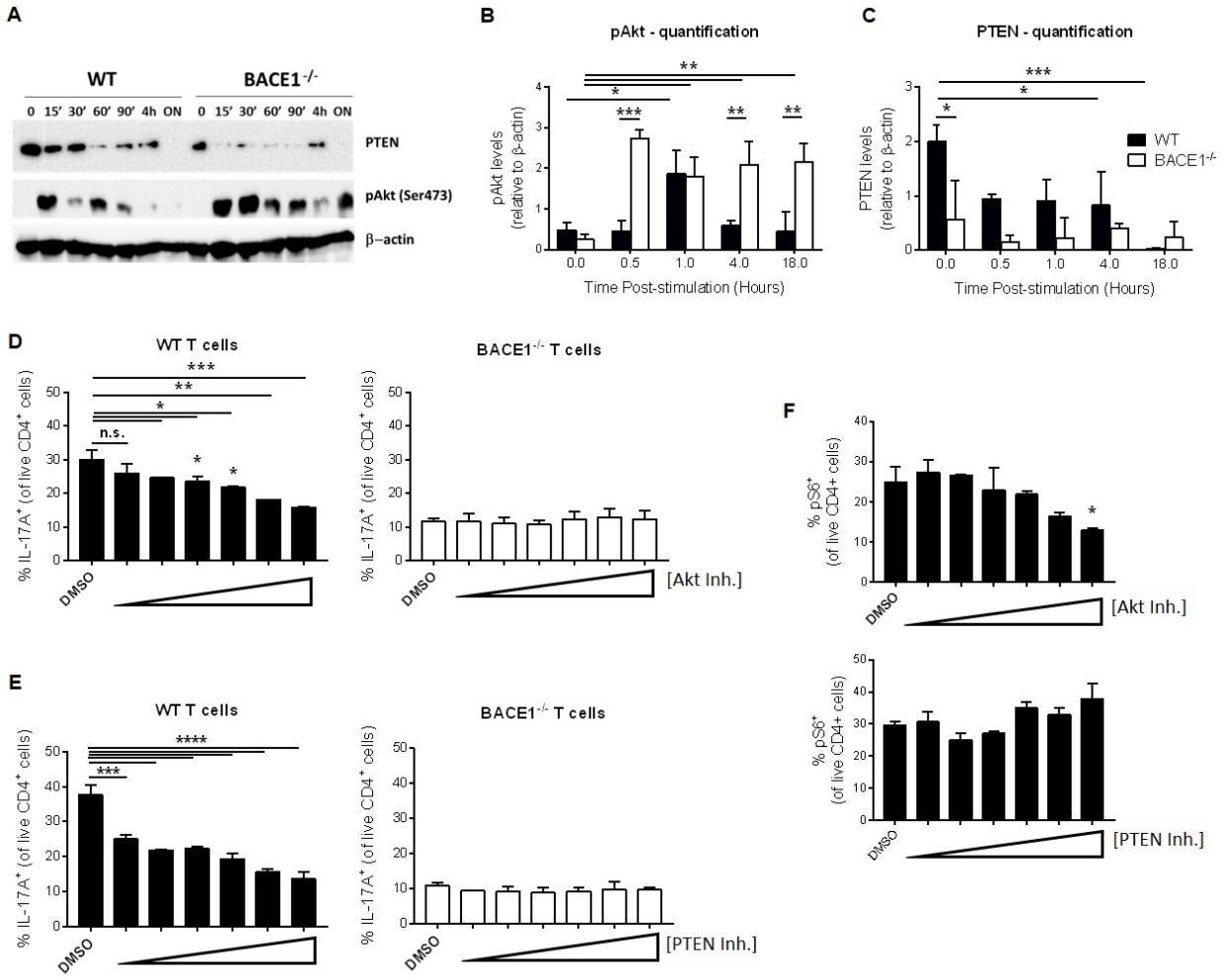

Figure 23. BACE1$^{-/-}$ CD4^{+} T cells express lower PTEN levels that result in enhanced pAkt upon T cell activation

$CD4^+$ T cells isolated from naïve WT and $BACE1^{-/-}$ were stimulated *ex vivo* with cross-linking αCD3/αCD28 activating antibodies for different amounts of time. Cells lysates were obtained and PTEN and pAKt (Ser473) were detected by immunoblot (data provided by Lyndsay Avery and Dr. William Hawse). **A.** Representative WB of the expression of PTEN and pAKt at different time-points. **B** and **C**. Quantification of pAKt and PTEN band intensity by densitometry compared to expression of β-actin (provided by Dr. William Hawse). Data pooled from at least three experiments. **D** and **E.** WT and $BACE1^{-/-}$ T cells were treated for 30 min with Akt (**D**) or PTEN (**E**) Inhibitors and differentiated for three days in Th17-polarizing conditions in the presence of the same inhibitors.IL-17A expression was measured by flow cytometry. Graphs show mean ± SD; data representative of three independent experiments with similar results. **F.** phosphorylation of S6 from WT T cells was measured at day three post-differentiation treated with Akt (top) and PTEN (bottom) inhibitors. Representative of a single experiment.

To further prove the importance of PTEN levels in Th17 function, we compared the expression of IL-17A from *in vitro* differentiated WT Th17 cells, and $PTEN^{+/-}$ Th17 cells. Heterozygous PTEN $CD4^+$ cells were obtained from naïve $CD4^{Cre}$ $PTEN^{fl/+}$ mice ($PTEN^{+/-}$). Upon expression of CD4 during T cell development in the thymus, all T cell precursors express *Cre* recombinase, an enzyme capable of removing DNA sequences flanked by the *pLox* (*flox*) sequence. Therefore, all T cells from these mice contain only a single copy of the *Pten* gene. In line with the results obtained with PTEN inhibition, $PTEN^{+/-}$ Th17 cells express reduced levels of CD73 and IL-17A, analyzed by flow cytometry (Figure 23A and B). Interestingly, profiling gene expression of different Th17 signature molecules, showed only differences in the expression of *Il17a*, *Il17f* and *Nt5e* (CD73), while other Th17 signature markers remained unchanged (Figure 23C). These data prove that PTEN expression is necessary for full expression of CD73 and IL-17 cytokines and that the reduced expression of PTEN in $BACE1^{-/-}$ Th17 cells may be responsible for their defect in Th17 function.

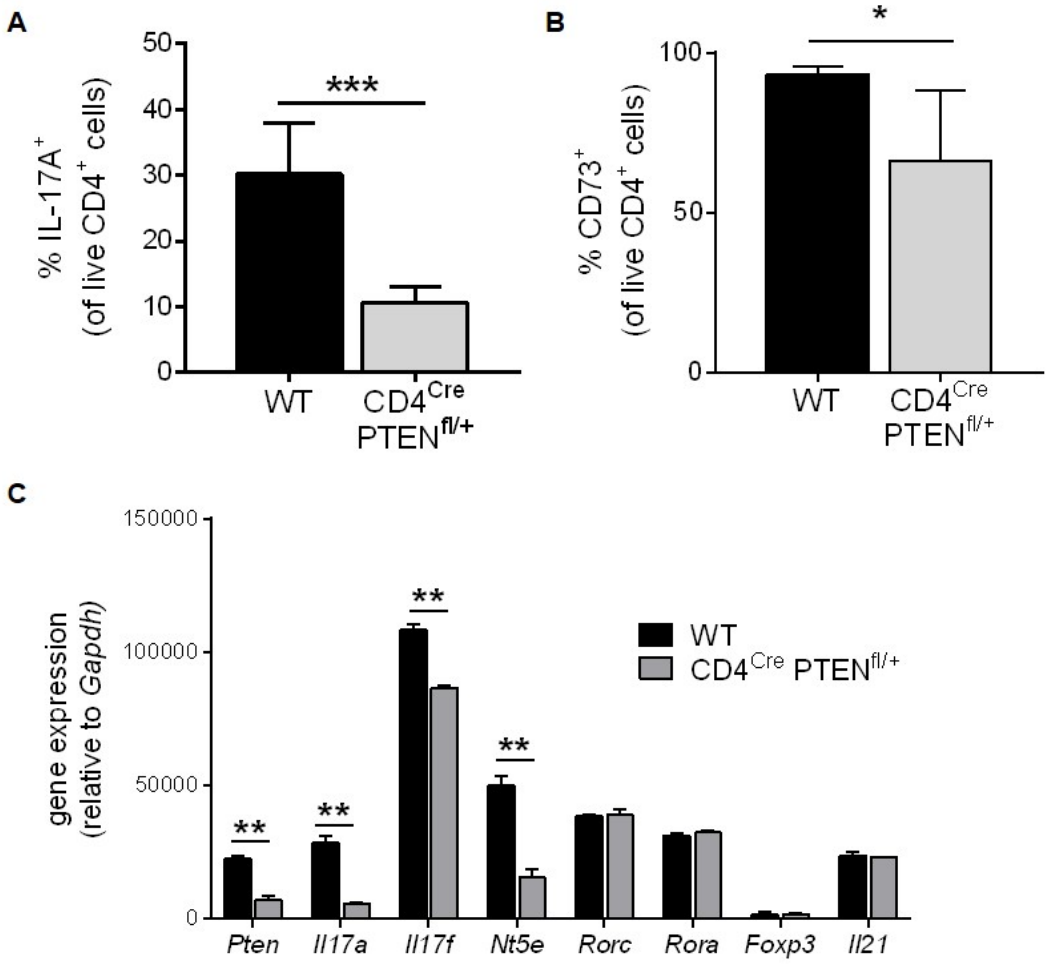

Figure 24. PTEN+/- CD4^{+} Th17 cells replicate BACE1$^{-/-}$ phenotype.

CD4^{+} T cells from naïve WT and CD4^{Cre}PTEN$^{fl/+}$ mice were cultured under Th17-polarizing conditions for three days. **A.** Frequencies of IL-17A and CD73-expressing cells from live CD4^{+} T cells, measured by flow cytometry. Data pooled from three independent experiment. **B.** RNA profiling of different Th17-signature molecules from both WT and CD4^{Cre}PTEN$^{fl/+}$ Th17 cells at day 3 post differentiation. Representative data of three independent experiments with similar results.

In 2009, Gomez-Rodriguez *et al.*, showed that Itk-deficient Th17 cells exhibit impaired IL-17A expression but normal levels of RORγt and IL-17F (467). They showed that Itk deficiency resulted in impaired TCR-induced PLCγ phosphorylation and downstream Ca^{2+} mobilization, and

that differentiation of Th17 in the presence of ionomycin, a Ca^{2+} ionophore, restored IL-17A levels.

Interestingly, in our *in vitro* Th17 cultures, the deficit in IL-17A expression was always more dramatic and consistent when measured by ELISA or gene expression (qRT-PCR) than when evaluated by flow cytometry (Figure 24A). In this latter technique, after three days of culture, $CD4^+$ T cells are re-stimulated with PMA and ionomycin in the presence of GolgiPlug before intracellular staining of cytokine production. PMA mimics DAG signaling whereas ionomycin allows an influx of Ca^{2+} from the media into the cell that triggers Ca^{2+} signaling. By using these two compounds in already committed T helper cells, we are able to induce and evaluate the expression of population-specific cytokines. The fact that $BACE1^{-/-}$ Th17 cells treated with PMA/ionomycin showed a less impressive defect compared to gene expression or cytokine secretion and the fact that Th17 polarization in the presence of ionomycin restored the IL-17 deficiency in $Itk^{-/-}$ Th17 cells, prompted us to study the effect of enhancing Ca^{2+} signaling by ionomycin treatment on IL-17A expression during in-vitro polarization of $BACE1^{-/-}$ Th17 cells.

Both WT and $BACE1^{-/-}$ $CD4^+$ T cells were differentiated *in vitro* to Th17 in the presence of different concentrations of ionomycin, for three days. Treatment with ionomycin was able to increase the expression of IL-17A and CD73 from $BACE1^{-/-}$ Th17 cells, in a concentration-dependent fashion, up to the levels of WT Th17 at the highest concentration of ionomycin (Figure 24B and C). Interestingly, when WT Th17 cells were differentiated in the presence of the Ca^{2+} ionophore, IL-17A expression was also slightly increased (data not shown), stressing the importance of Ca^{2+} in the production of IL-17A from Th17 cells.

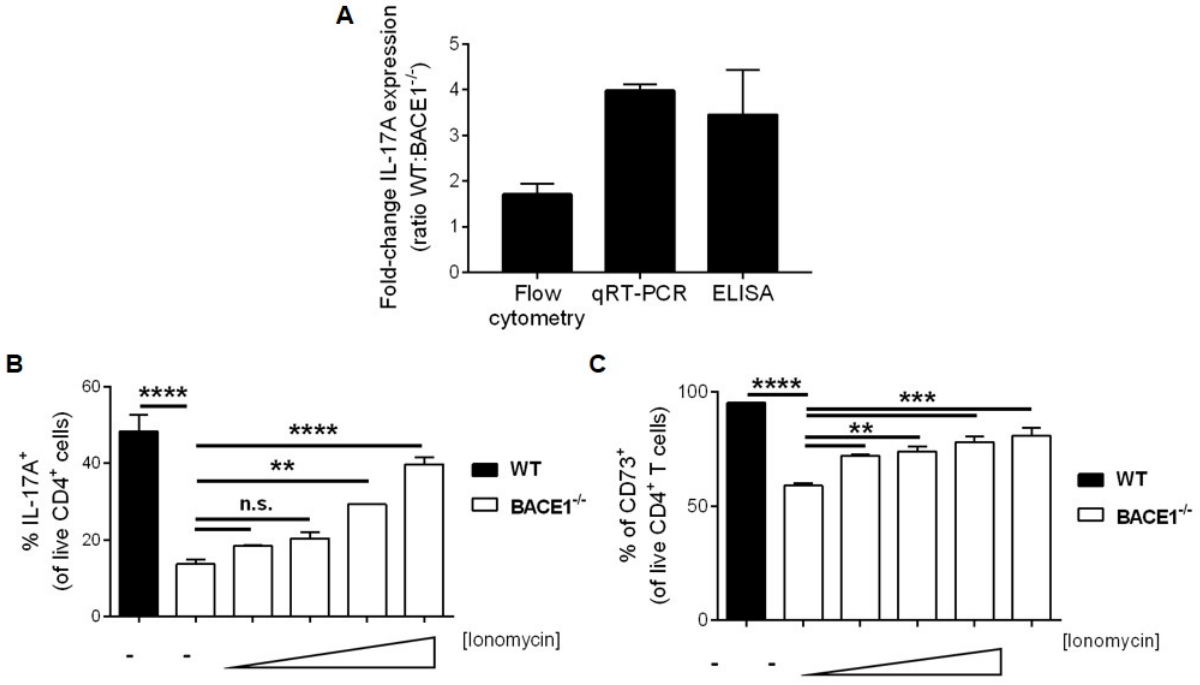

Figure 25. ionomycin treatment rescues IL-17A and CD73 expression in BACE1$^{-/-}$ Th17 cells.

IL-17A expression from WT and BACE1$^{-/-}$ Th17 was measured by flow cytometry (intracellular cytokine staining), qRT-PCR (gene expression) or ELISA (secreted protein) at day three of *in vitro* differentiation. **A.** Ratio of the expression of IL-17A in WT over BACE1$^{-/-}$ Th17 cells, from the same experiment, using different techniques. **B-C.** BACE1$^{-/-}$ CD4^{+} T cells were differentiated *in vitro*, for three days, in the presence of increasing concentrations of ionomycin. **B.** Frequencies of IL-17A expressing BACE1$^{-/-}$ Th17 cells compared to untreated WT Th17 cells. **C.** Frequencies of CD73^{+} BACE1$^{-/-}$ Th17 cells compared to untreated WT Th17 cells. Values in graphs represent mean ± standard deviation. Data representative of at least three independent experiments.

3.3.6 BACE1 protease activity does not seem to be required for control of IL-17A expression

BACE1 is an aspartyl protease, or, in other words, an enzyme that catalyzes the cleavage of other proteins (263). BACE1 has mainly been studied for its role in processing APP (517) and Neuregulin-1 (315,521). The processing of these two proteins, as well as other known substrates, is dependent on BACE1 catalytic activity (309,315). This prompted us to study the effect of various inhibitors of BACE1 proteolytic activity in $CD4^+$ T cells.

$CD4^+$ T cells from naïve WT mice were differentiated in the presence of either one of two selective inhibitors of BACE1 proteolytic activity: LY2811376 and InSolution BACE1 Inhibitor IV (InSolution), with IC_{50} of 239nM and 15nM, respectively. $CD4^+$ T cells were pre-treated with the inhibitor for 1-2 hours before culture start. Contrary to our expectations, we did not observe a significant reduction of IL-17A or CD73 expression in the presence of BACE1 inhibitors (Figure 25A and B), suggesting that the enzymatic activity of BACE1 is dispensable for Th17 function.

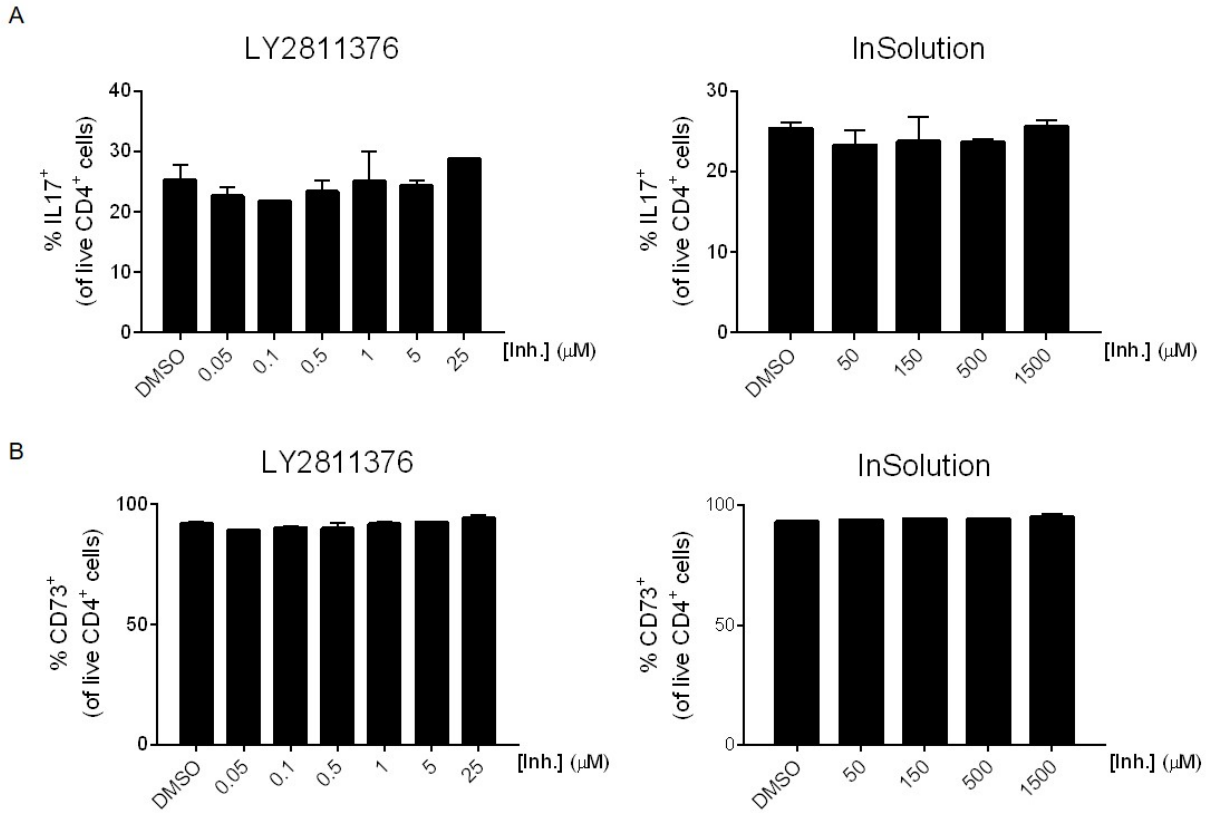

Figure 26. BACE1 inhibition does not impact IL-17A expression.

WT CD4$^+$ T cells were pre-treated for 30min with LY2811 or InSolution BACE1 inhibitors, prior to differentiation for three days under Th17-polarizing conditions in the presence of each inhibitor. Expression of IL-17A (**A**) and CD73 expression (**B**) was by flow cytometry after PMA/ionomycin re-stimulation. Data representative from two experiments with similar results.

However, these experiments lack a proper control for BACE1 inhibition. BACE1 inhibitors target the protease activity of this enzyme and, therefore, in order to assess that the inhibitors are working we would need to address BACE1 activity. In addition, these experiments should include cells known to express good levels of BACE1 such as neuronal cell lines or by inducing the overexpression of BACE1. In order to fully evaluate the requirement of BACE1 proteolytic activity in the control of IL-17A and CD73 in Th17 cells, we generated a retroviral expression

vector encoding for a protease-deficient BACE1. As an aspartyl-protease, BACE1 requires the presence of an aspartic acid in each of its two proteolytic domains(269,353). We mutated both aspartic acid residues for asparagines in order to abrogate its proteolytic function with introduction of little to no structural change (353).

3.3.7 BACE1 negatively regulates the adenylate cyclase in murine CD4$^+$ T cells

In addition to its role as a protease, BACE1 has been described to be a negative regulator of adenylate cyclase, a membrane-associated enzyme that catalyzes the conversion of intracellular ATP into cyclic AMP, in a human neuronal cell line (353). This function has been shown to occur independently of its protease activity but the exact mechanism remains unknown (353). This could be a possibility in our CD4$^+$ T cell cultures, since the absence of BACE1 resulted in reduced expression of IL-17A and CD73 but inhibition of its protease activity did not have an effect. To test this hypothesis, 4x10^6 CD4$^+$ T cells isolated from naïve WT and BACE1$^{-/-}$ mice were treated with forskolin, a potent activator of the adenylate cyclase, for 30 minutes. The levels of cAMP generated from forskolin-treated BACE1$^{-/-}$ CD4$^+$ T cells were significantly higher than those from WT T cells (Figure 26A). These results indicate that BACE1 is also a regulator of adenylate cyclase activity in CD4$^+$ T cells, and that BACE1$^{-/-}$ T cells have an increased capacity to generate cAMP. In our previous data (Figure 13), we showed that differentiation of Th17 cells in the presence of adenosine and cAMP resulted in reduced IL-17A and CD73 expression.

In order to further evaluate the role of cAMP signaling in murine IL-17A and CD73 expression, we differentiated WT CD4$^+$ T cells to Th17 cells, in the presence of increasing

concentrations of forskolin. Interestingly, we observed that the frequencies of IL-17A and CD73-expressing Th17 cells decreased with increased concentration of forskolin in the media (Figure 26B and C). Hence, our data indicate that the increased cAMP in the absence of BACE1 could be responsible for the reduced production of IL-17A and CD73 in Th17 cells.

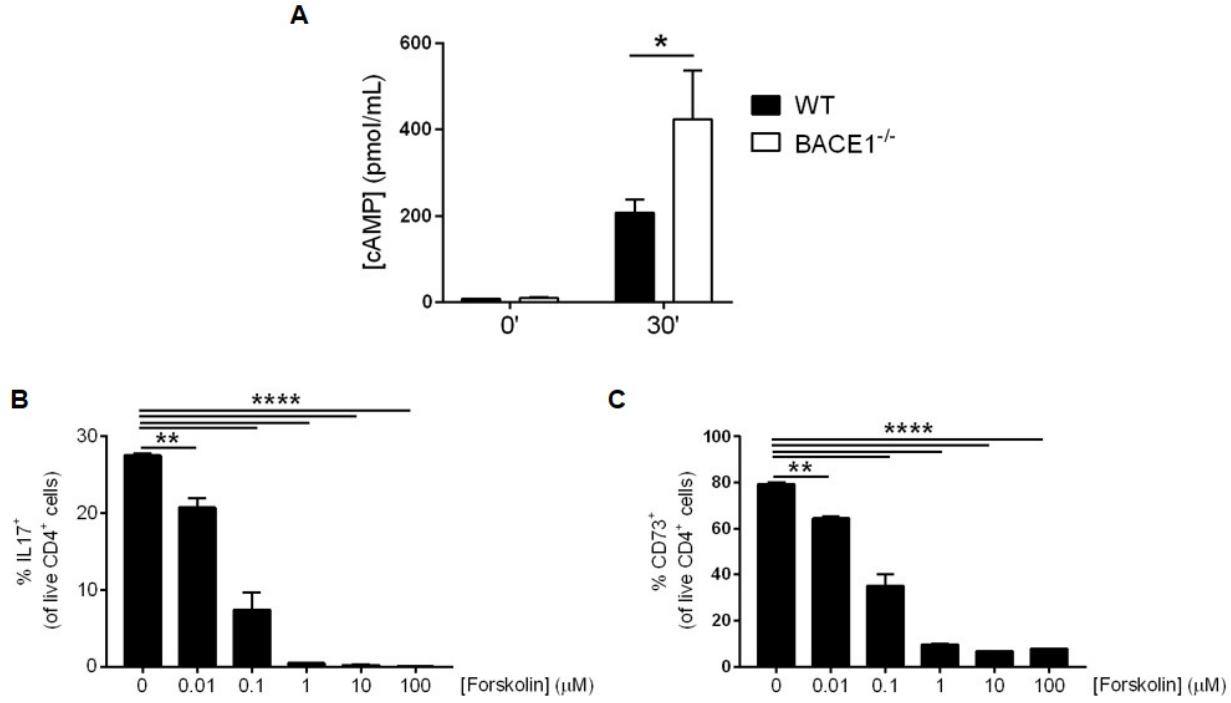

Figure 27. BACE1 negative regulation of adenylate cyclase is necessary for correct IL-17A expression in Th17 cells.

A. Intracellular cAMP concentration from WT and BACE1$^{-/-}$ CD4^{+} T cells before and after 30 min stimulation with forskolin. Data representative of three independent experiments. **B-C.** WT CD4^{+} T cells were pre-treated and differentiated to Th17 in the presence of increasing concentrations of forskolin, for three days. **B.** Frequencies of IL-17A^{+} Th17 cells. **C.** Frequencies of CD73^{+} Th17 cells. Data representative of two independent experiments with similar results.

Increases in intracellular cAMP can trigger the activation of protein kinase A (PKA), a cytosolic kinase able to phosphorylate NFAT and prevent the translocation of this transcription factor into the nucleus (522). Previous reports have shown that defects in NFAT translocation lead to reduced expression of IL-17 cytokines (467,496,523–525). Hence, we hypothesized that the enhanced capacity of cAMP generation from BACE1$^{-/-}$ T cells, could lead to defects in NFAT nuclear translocation and therefore explain the reduction in IL-17A expression. For this purpose we activated CD4^{+} T cells isolated from naïve WT and BACE1$^{-/-}$ mice with cross-linking αCD3/αCD28 for different periods of time before cell lysis and fractionation of the cytosolic and nuclear compartments. NFAT levels in both compartments were measured both by immunoblot and a semi-quantitative ELISA assay. Although initial experiments suggested that the ratio of NFAT levels translocating into the nucleus was superior in WT CD4^{+} T cells compared to BACE1$^{-/-}$ T cells, subsequent experiments did not replicate these findings (data not shown). Therefore, although possible, we do not have compelling data to support the hypothesis that BACE1$^{-/-}$ CD4^{+} T cells exhibit reduced NFAT translocation. Further experiments to investigate this possibility as well as alternative mechanism by which cAMP can limit T cell activation and TCR signaling will be described in more detail in the discussion section.

3.3.8 Discussion

During the last year, we focused our efforts on elucidating the mechanism of action of BACE1 in regulating the expression of IL-17A and CD73 in Th17 cells. Here, we demonstrated that in the absence of BACE1, CD4^{+} T cells exhibit reduced levels of PTEN that result in enhanced Akt phosphorylation. Inhibition of pAkt kinase activity did not rescue IL-17A expression from

BACE1$^{-/-}$ Th17 cells but, in contrast, Akt inhibition in WT Th17 cells resulted in reduced IL-17A expression. This corresponds to prior studies showing that Akt is important for Th17 differentiation (476,477,523).

The functional changes following phosphorylation of Akt go beyond mere activation of kinase activity, acting more like a regulatory modification. In fact, phosphorylation of Akt can increase the affinity for some substrates, while reducing it for others (475,476). Preliminary analysis of differential phosphorylation of pAkt substrates, performed in collaboration with Dr. William Hawse, revealed that in BACE1-deficient activated T cells some pAkt substrates were more phosphorylated whereas other showed reduced phospho-staining (data not shown). This unbiased analysis showed evidence that numerous molecules may be altered downstream of pAkt in the absence of BACE1. However, since pAkt inhibition did not rescue IL-17A expression in BACE1$^{-/-}$ Th17 cells, we believe that the increased levels of phosphorylated Akt are a by-product or readout of the defect in PTEN expression, rather than a major mechanism affecting IL-17A expression in BACE1$^{-/-}$ Th17 cells. Further analysis downstream of pAkt would be necessary to completely exclude this pathway as a main effector in BACE1 function. In this regard, determining the activation and localization of FoxO1 (489) or p300 (259,526) as well as the acetylation of RORγt (526,527) could prove crucial in elucidating the importance of BACE1 in other Akt-driven interactions.

The strength of TCR signaling can impact the cell fate of T cell progenitors during thymic development (37,468,499). Although we did not check the PTEN or Akt expression or the impact of their inhibition in thymocytes, we did not observe any significant differences in the development

of αβ T cells in this organ. Furthermore, the importance of TCR strength in Th1 and Th2 development has been well studied (499,505,528). Interestingly, despite the defects observed in the PI3K/PTEN/Akt signaling cascade in the absence of BACE1, we did not see any differences in Tbet or IFNγ expression in BACE1$^{-/-}$ Th1 cells. These results give rise to two possible outcomes: the first one is that in the context of Th1 polarization, BACE1 is not necessary and, therefore, this pathway remains unaffected in BACE1$^{-/-}$ Th1 cells. Another possibility is that because PI3K/PTEN/Akt signaling works differently in Th1 and Th17 cells, the role of BACE1 in Th1 has no major effect in the polarization of this subset. In this regard, this project opened new questions that go beyond the function of BACE1 in Th17 function, by implicating different roles for PTEN depending on the downstream pathways that are affected (Akt vs. Ca^{2+} signaling). An exhaustive analysis of these pathways in different cell types will help understand the intricacies of Akt signaling in different Th subsets and shed light on the cross-talk between different TCR-driven signaling events in each Th subset.

The amounts and activation state of PI3K and PTEN during TCR activation determine the balance between PIP3 and PIP2. Reduced expression of PTEN can yield increased levels in PIP3 and therefore more phosphorylation of Akt. On the other hand, the consequent reduction in PIP2 will affect Ca^{2+} signaling, which has been shown to be important for differential IL-17A expression over IL-17F in Th17 cells. In this regard, Gomez-Rodriguez et al. (467) identified that Itk$^{-/-}$ Th17 cells presented a specific defect in IL-17A expression while other Th17-cytokines, including IL-17F were expressed at normal levels. Although they observed slight differences in pSTAT3, Itk$^{-/-}$ Th17 cells expressed normal levels of RORγt and RORα. Similarly, we observed that BACE1$^{-/-}$ Th17 cells express reduced levels of IL-17A in the absence of defects in pSTAT3 signaling or

RORγt and RORα expression. GM-CSF expression was not altered in BACE1$^{-/-}$ Th17 cells, however we did see a small reduction in IL-17F expression, in contrast to the results published by Gomez-Rodriguez et al. Treating both BACE1$^{-/-}$ and Itk$^{-/-}$ Th17 cells with ionomycin during *in vitro* polarization restored IL-17A expression to WT levels, suggesting defects in Ca^{2+} signaling. Given more time it would have been ideal to test whether BACE1$^{-/-}$ Th17 cells present, in fact, defects in Ca^{2+} influx or release from ER. Increased levels of intracellular Ca^{2+} lead to activation of calcineurin and activation by dephosphorylation of the transcription factor NFAT. Concomitant to defects in Ca^{2+} signaling, they observed reduced NFAT binding to IL-17A promoter. Interestingly, they identified that, although there are putative NFAT binding sites in IL-17F promoter, NFAT did not bind to this promoter therefore explaining the differential regulation between IL-17A and IL-17F expression. We are currently testing the levels of NFAT in the nucleus by WB of cytosolic and nuclear fractions as well as with a DNA-binding assay. Preliminary data did not show any significant differences in NFAT nuclear translocation or DNA binding capability between WT and BACE1$^{-/-}$ T cells. However, a more exhaustive analysis of NFAT binding to IL-17A, IL-17F and CD73 by chromatin immunoprecipitation would be required.

Increased PIP2 can result in higher generation of IP3 and DAG, which can trigger NFAT-independent signaling. IP3 can trigger Ca2+ flux, and, together with DAG, induce activation of Protein Kinase C (PKC) (529). Interestingly, deletion of PKC θ specifically impaired Th17 differentiation by preventing the expression of STAT3 in an AP-1 and NF-κB dependent manner (530). In addition, chemical inhibition of PKC in T cells from psoriatic patients resulted in reduced IL-17A expression with no differences in RORγt expression (531). Ca2+ signaling can also induce the activation of calcium/calmodulin-dependent protein kinase IV (CaMK4), a protein required for

Th17 development that is increased in SLE mouse models (532,533). Its chemical and genetic inhibition revealed that CaMK4 promotes IL-17A expression by increasing the levels of CREM and activation of the Akt/mTORC1 pathway (532). Therefore, given that ionomycin restored IL-17A and CD73 production by BACE1-/- Th17 cells, and that our preliminary data did not reveal major differences in NFAT nuclear import, we will evaluate the aforementioned Ca2+-induced molecular pathways.

In a follow up paper, Gomez-Rodriguez et al. (477), showed that defects in TCR signaling not only affected Th17 function by Ca^{2+} defects that reduced IL-17A expression, but also by an enhanced plasticity towards the generation of Tregs. They observed that $Itk^{-/-}$ cells expressed higher levels of Foxp3 and $Itk^{-/-}$ Tregs were more suppressive than WT Tregs. Defects in $Itk^{-/-}$ TCR signaling in were triggered by decreased Akt phosphorylation caused by elevated levels of PTEN. Contrary to their results, $BACE1^{-/-}$ Th17 expressed reduced PTEN levels and concomitant enhanced pAkt. In addition, we did not observe any increase in Foxp3 levels in $BACE1^{-/-}$ Th17 cells. These differences highlight that, although there are a lot of phenotypical similarities, BACE1 and Itk do not couple early T cell activating events and IL-17A production with a common mechanism.

PTEN deficiency has been associated with defects in central and peripheral tolerance and increased autoimmunity (534). In addition, PTEN is necessary for maintaining Treg homeostasis and lineage stability (535) as well as to restrain Teff responses (536). In particular, genetic hemideletion of PTEN in $CD4^{+}$ T cells results in T cell hyperproliferation, enlarged lymphoid organs and T cell lymphomas (534). In this regard, we did not observe any enlargement of

lymphoid organs in BACE1$^{-/-}$ mice or the development of spontaneous autoimmunity. In addition, Foxp3 expression from both *in vitro* differentiated Tregs as well as *ex vivo* analysis of tTregs from naïve mice revealed no differences between WT and BACE1$^{-/-}$ mice. However, further experiments need to be conducted in order to exclude defects in Treg function in the absence of BACE1. Should we observe a deficiency in Treg function in BACE1$^{-/-}$ mice, the absence of spontaneous autoimmunity may be due to the defective IL-17A expression from effector Th17 cells that we described in these mice.

We believe, that in BACE1$^{-/-}$ Th17 cells, the reduction of inositol substrates to initiate Ca^{2+} signaling is the key defect in IL-17A production, whereas enhanced Akt signaling is a consequence of reduced PTEN expression. However, as we stated before, it would be interesting to analyze downstream impacts of increased Akt phosphorylation in BACE1$^{-/-}$ Th17 cells.

Chemical inhibition of BACE1 in WT Th17 cultures did not significantly/consistently reduce IL-17A expression. Different variables could have played a role in the absence of IL-17A reduction and, therefore, we initially hesitated on the validity of these results. At the moment, we do not know when and where BACE1 is acting in T cells. For instance, we saw reduced PTEN expression in BACE1$^{-/-}$ T cells before activation, suggesting a fundamental defect in these cells. Hence, one potential explanation for the lack of results is that the time of inhibition was not optimal. We pre-treated CD4^{+} T cells for a short period of time before activation/differentiation. Perhaps BACE1 exerts its function in T cells even before we manipulate them and therefore inhibition at this point renders no effect. Another possibility is that the experiments did not work or that the reagents did not work. In order to assure the effectivity of the treatment we used two

different BACE1 inhibitors. Moreover, we titrated both inhibitors well above and below their IC50 and compared the conditions with vehicle controls to evaluate reagent-specific cytotoxicity. In order to further prove that the inhibition assays worked correctly, titration of these compounds should be tested in BACE1 activity assays. BACE1 inhibitors target its proteolytic domain abrogating its cleaving capacity (309). Therefore, another intriguing possibility is that BACE1 is acting in a proteolytic-independent manner, such as modulating the AC activity. I did not have the time to test the protease-deficient BACE1 retrovirus that we generated, but performing these experiments will be very informative to determine the requirement of the protease function of BACE1 in Th17 cells.

In this context, non-proteolytic activities have been described for BACE1. In particular, BACE1 acts as a negative regulator of the adenylate cyclase in human neurons, in a non-enzymatic manner (353). Here, we demonstrate that BACE1 also acts as a negative regulator of adenylate cyclase in $CD4^+$ T cells, with increased generation of forskolin-induced cAMP in $BACE1^{-/-}$ T cells. In addition, we showed that increasing cAMP with forskolin during Th17 differentiation, reduced the expression of IL-17A and CD73 in both WT and $BACE1^{-/-}$ T cells. In order to fully demonstrate that the increased AC activity from $BACE1^{-/-}$ Th17 cells is indeed responsible for the defect in IL-17A expression, we will differentiate these cells in the presence of AC inhibitors. According to our findings, we hypothesize that reduction of cAMP levels by AC inhibition should restore the IL-17A expression from $BACE1^{-/-}$ Th17 cells.

Interestingly, previous reports have shown that prostaglandin E2 (PGE2) can promote IL-17A expression in human T cells by increasing intracellular cAMP levels (188,537). PGE2 can signal via EP2 and EP4 receptors in $CD4^+$ T cells (537,538). While signaling through EP2

receptors drives the activation of AC an increases in cAMP, activation of EP4 receptor can also induce Akt signaling (539). In addition, it has been described that PGE2 signaling can also increase IL-17A expression in BALB/c Th17 cells, but signaling via EP4 receptor was shown to play a more important role in IL-17A expression (538). Therefore, the discrepancy between the inhibitory effect of cAMP that we observed and the stimulatory effect on IL-17A production reported for PGE2 in Th17 cells may be due to the activation of pathways other than cAMP by PGE2. Furthermore, cAMP signaling might have different effects in different species or mouse strains. Interestingly, PGE2 exhibits opposing roles in Th1 differentiation at different concentrations (538). This may highlight different degrees of activation and kinetics between PGE2-stimulated and BACE1 deficient Th17 cells.

In conclusion, BACE1 is a novel regulator of IL-17A and CD73 expression in Th17 cells. Our data seem to indicate that BACE1 is coupling early events occurring early in the T cell activation signaling machinery with specific regulation of IL-17A expression in Th17 cells. In addition, our results highlight the importance of proper understanding of the connections between different signaling events. In this particular case, differences in the production of cAMP seem to impact the expression of PTEN that acts as a relay determining the signaling pathways to propagate TCR signals.

4.0 DISCUSSION

4.1 CONCLUSIONS

4.1.1 Results and Model

In this project, we identified a novel function for BACE1 as a regulator of Th17 function. We found that BACE1 is necessary for the correct expression of IL-17A and CD73 mRNA in Th17 cells. Reduced expression of IL-17A and CD73 in BACE1-deficient Th17 cells was accompanied by a minor defect in IL-17F expression, but other Th17-signature regulators and effector molecules remained unaltered. Due to the reduced IL-17A expression, BACE1$^{-/-}$ Th17 cells are less pathogenic in EAE. These findings demonstrate that BACE1 is required for Th17 function but dispensable for Th17 development.

By studying early signaling events downstream of T cell receptor engagement, we found that BACE1$^{-/-}$ T cells exhibited enhanced Akt phosphorylation upon T cell activation. Akt activation is dependent on the levels of PIP3, and the balance between PIP3 and PIP2 is regulated by the opposing enzymatic roles of PI3K and PTEN. Concomitantly with increased pAkt levels, we found reduced levels of PTEN in BACE1$^{-/-}$ T cells. Importantly, PTEN acts as a relay in TCR signaling events by balancing the propagation of the signal between the Akt and Ca^{2+} signaling pathways. Generation of PIP2 by PTEN allows the propagation of T cell activating signals through the Ca^{2+} signaling. Interestingly, defects in IL-17A and CD73 expression in BACE1$^{-/-}$ Th17 cells were overcome in the presence of ionomycin, a Ca^{2+} ionophore, proving that BACE1 is necessary

for regular Ca^{2+} signaling. Reduced Ca^{2+} signaling results in defective activation of calcineurin and dephosphorylation of NFAT. Defective NFAT dephosphorylation and nuclear import, could possibly explain the impaired production of IL-17A and CD73 in BACE1$^{-/-}$ Th17 cells. However, signaling pathways and molecules downstream of Ca^{2+}, like ERK/MAPK, PKC, AP-1 and NF-κB need to be analyzed in detailed to identify the exact molecular mechanism of the Ca^{2+} signaling defect in BACE1$^{-/-}$ Th17 cells.

Furthermore, our findings suggest that BACE1, by modulating the generation of cAMP by AC, controls the levels of PTEN. Correct PTEN levels distribute the TCR signal between the Akt and Ca^{2+} signaling cascades, favoring the generation of PIP2 and activation of the Ca^{2+} pathway. Ca^{2+} is necessary for activation and nuclear import of NFAT where it promotes IL-17A, IL-17F and CD73 expression. In addition, BACE1 reduction of cAMP levels can act in a feedback loop by preventing PKA activation and bolstering NFAT hypophosphorylation and transcriptional activity.

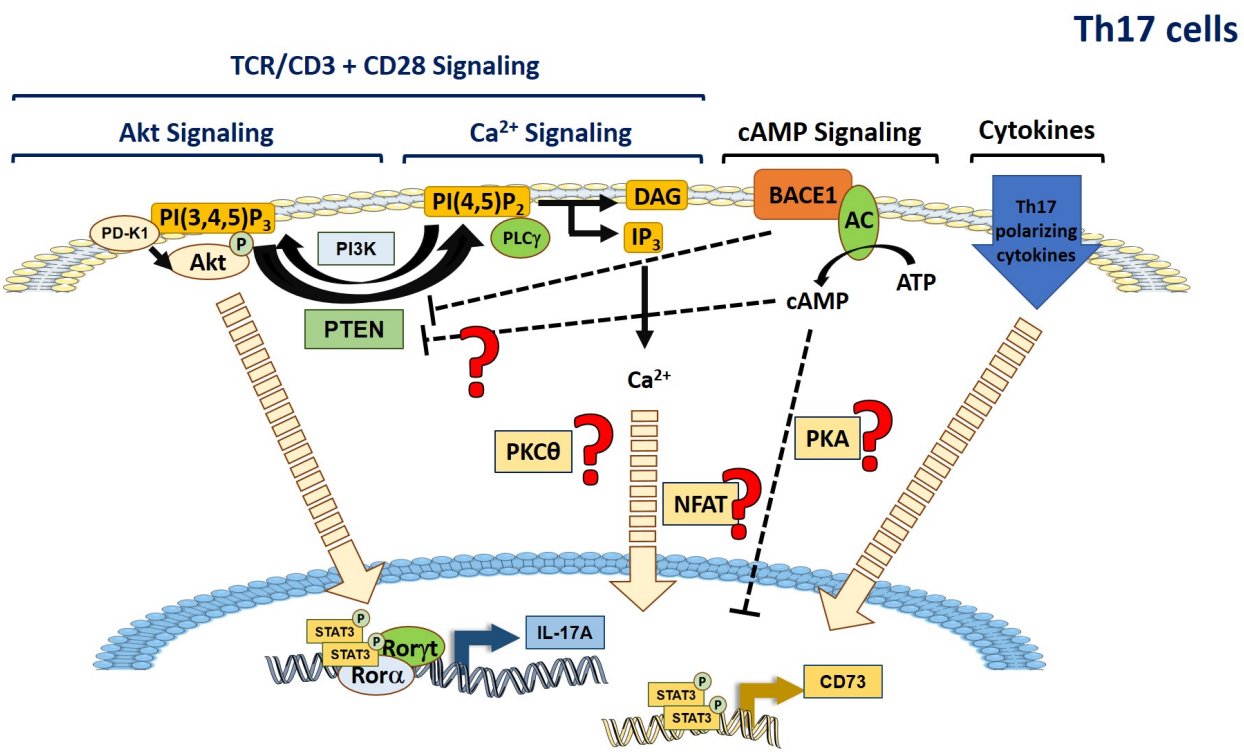

Figure 28. Schematic model of signaling events modulated by BACE1

Activation of T cells involved a complex array of signaling pathways that are interconnected. Both TCR and cytokine receptor signals are indispensable for correct Th17 differentiation. BACE1, by modulating cAMP and PTEN levels can influence Ca^{2+} signaling and regulate the expression of IL-17A, CD73 and to a minor extent IL-17F.

4.2 FUTURE DIRECTIONS

Our work convincingly shows that BACE1 regulates the gene expression of IL-17A and CD73 without majorly affecting other transcriptional regulators and effector cytokines of the Th17 program. Our findings seem to indicate that BACE1 is able to couple early events in the T cell activation and differentiation signaling machinery with specific regulation of IL-17A and IL-17F expression. In particular, we observed that BACE1-deficient T cells present enhanced TCR-induced Akt signaling due to reduced levels of PTEN. T cells also have the potential to generate

higher levels of intracellular cAMP in the absence of BACE1. Finally, we observed that artificially increasing the intracellular levels of Ca^{2+} restored IL-17A and CD73 expression in BACE1$^{-/-}$ Th17 cells. However, three major questions remained unanswered during the period of time that we developed this project.

4.2.1 How does BACE1 regulate cAMP, PI3K/PTEN/Akt and Ca^{2+} signaling?

Although we consistently observed alterations in early signaling pathways in the absence of BACE1, we have not been able to address the mechanism by which BACE1 is affecting them. It is important to understand the kinetics and subcellular location of BACE1 in T cells in order to identify the molecular mechanisms by which BACE1 may be regulating these pathways. Analysis of BACE1 protein levels before and after activation as well as upon stimulation with different Th17-polarizing cytokines will provide a better understanding on how BACE1 is expressed during Th17 development. Moreover, performing RNAseq analysis of BACE1$^{-/-}$ Th17 cells at different times during differentiation could help us determine when these differences appear. In addition, co-immunoprecipitation assays and a proteomics approach will help elucidate BACE1 interaction partners, providing more information to uncover BACE1 mechanisms of action in T cell signaling. Importantly, it is known that BACE1 can exert actions as a protease but also in a non-proteolytic fashion. A proteomics approach would not only provide quantitative evidence about protein levels but also about their size and therefore, inform about the possibility of BACE1 cleaving some partner.

As mentioned in previous chapters, the detection of BACE1 expression in T cells (especially at the protein level) proved very hard probably due to low endogenous expression of BACE1 in these cells. Nevertheless, retroviral overexpression of a Myc-tagged BACE1 in T cells should allow us to detect BACE1 protein levels with techniques that offer lower resolution. This tag will also help in the pull-down of BACE1 in co-immunoprecipitation experiments to identify interaction partners. In addition, we are interested in defining the subcellular localization of BACE1 within T cells. This knowledge will allow for a better understanding of important BACE1 interacting-partners during Th17 differentiation.

We hypothesize that BACE1 is regulating IL-17A expression by modulating the generation of cAMP by AC. BACE1 negatively regulates the activity of AC in a protease-independent manner, but the actual mechanism is not yet known (353). Testing the effects on Th17 function by overexpressing a fully functional BACE1 or a protease-deficient mutant will confirm if BACE1 is playing different roles in Th17 cells. In addition, the novel roles of BACE1 described here could be exploited to investigate the specific molecular mechanism by which BACE1 interacts and regulates AC.

4.2.2 How are cAMP, PI3K/PTEN/Akt and Ca^{2+} signaling pathways interconnected?

Early events during T cell activation are dependent on the recruitment and activation of specific kinases to microclusters in the intracellular side of the plasma membrane (540). The propagation of the TCR signal involves the activation of several signaling cascades that together result in enhanced proliferation, survival and enhanced protein synthesis (499,520,541). Broadly, TCR/CD3 signaling can stimulate the Akt/mTORC1 pathway, ERK/MAPK pathway, Ca^{2+}

signaling and NF-κB activation (466,477,481,520). In research, we tend to tackle different scientific questions in a reductionist approach, trying to minimize the variables in order to fully understand the roles of each player in a particular pathway. However, during T cell activation and differentiation, the interplay and cross-talk of all these different pathways as well as interactions with cytokine signaling events can result in different outcomes. Here, we describe that BACE1$^{-/-}$ T cells express low levels of PTEN compared to WT cells, with enhanced activation of Akt signaling. Our findings that stimulating Ca^{2+} signaling restored IL-17A expression, while Akt inhibition did not, suggest that regulating the generation of PIP2 may be the more important role of PTEN in impacting IL-17A expression. This hypothesis needs to be confirmed experimentally, by checking the levels of PIP2 and PIP3 as well by the evaluation of PLC-γ recruitment to sites of TCR clustering.

It has been recently shown that cAMP acts as a repressor of TCR signaling, especially in the absence of CD28 co-stimulation (510,511). Besides the activation of AC by G-protein coupled receptors, like the adenosine receptor (542), it is not fully understood what triggers the generation of cAMP during TCR engagement (511). cAMP, by activating PKA, prevents Lck and ZAP70 activation and halts further TCR signaling (510,511). One possible mechanism by which BACE1 is allowing TCR signaling is by preventing higher spikes in cAMP during T cell activation (negative regulator of the AC). Interestingly, cAMP has been reported to repress PTEN expression in thyroid and glial cells (543,544). This regulation of PTEN expression by cAMP argues against our findings where we see reduced PTEN but enhanced cAMP generation in the absence of BACE1. However, the regulation of PTEN expression is complex, with transcriptional regulation, chromatin remodeling, a plethora of regulatory microRNAs and various post-translational

modifications involved in the very dynamic expression and activity of PTEN (491,495,545,546). Therefore, depending on the cellular microenvironment cAMP might impact PTEN expression differently in different cell types or conditions.

Independently of the role of BACE1, our findings highlight the importance of the cross-talk between cAMP levels and TCR signaling. Therefore, a detailed analysis of the cAMP dynamics during T cell activation and differentiation may be critical in understanding the intricacies of TCR signal modulation. First of all, we would like to identify what are the initial triggers of AC during Th17 differentiation. AC activity is primarily regulated by inhibitory and activating signals from G protein coupled receptors (GPCRs). None of the cytokines used in our Th17 polarization engage GPCRs and therefore, the generation of cAMP was not expected. Usually, growth factors signal through GPCRs (547), hence nutrients present in the differentiating media could be inducing cAMP responses. However, *ex vivo* analysis of PTEN levels showed us that the defect was present very early and most likely, the action of cAMP occurs even before we obtain the cells.

Chemokines as well as nutrients and growth factors can induce AC activity during T cell development and induce fundamental differences in BACE1-deficient T cells. Although cAMP has the potential to partially halt TCR signaling, this does not seem to be the case in BACE1$^{-/-}$ T cells, since we observe enhance pAkt despite the potential of generating more cAMP. Increased pAkt correlated with low levels of PTEN in BACE1$^{-/-}$ T cells. PTEN levels were reduced in BACE1$^{-/-}$ T cells even before activation, indicating that pre-existing differences in cAMP could possibly explain the defect in PTEN expression. In this matter, we want to test whether inducing

high intracellular cAMP with forskolin (AC activator) can reduce the expression of PTEN and ultimately, affect IL-17A expression. Finally, we would like to address the actual levels of cAMP during Th17 polarization. Upon forskolin-induced activation of the AC, BACE1$^{-/-}$ T cells produce more cAMP, but testing the cAMP levels in physiological conditions will give a more definite answer of the role of BACE1 in T cells.

4.2.3 How do defects in cAMP, PI3K/PTEN/Akt and Ca^{2+} signaling impact IL-17A expression?

It was initially believed that signals 1 and 2 were responsible for T cell activation and that signals downstream of cytokine receptors (signal 3) determined final T cell effector fate (468,548). However, in the past decade, it has been shown that the strength of the activating signals (converging TCR/CD3 and co-stimulation) is important in determining T cell fate (476,477,499). Differentiation of Th17 cells is favored in the presence of high antigen concentrations that can induce stronger signals through increased TCR/CD3 clustering (466,476–478), that besides internal T cell signals also results in stronger interaction between T cells and APCs can and increased IL-6 expression by DCs (508).

Rescuing IL-17A expression from BACE1$^{-/-}$ Th17 cells with ionomycin suggested that there is a defect in Ca^{2+} signaling. PTEN is a critical player in modulating the propagation of the TCR response between the Akt/mTORC1 pathway or Ca^{2+} signaling, emphasizing the importance of PTEN regulation in T helper differentiation (466,467,475,497), and probably explaining the defect in setting the TCR signaling threshold in BACE1$^{-/-}$ T cells. In addition to activation of

NFAT, Ca^{2+} and active calcineurin and calmodulin can exert inhibiting actions on AC to reduce cAMP levels (549). Therefore, we believe that ionomycin is correcting the IL-17A expression from BACE1$^{-/-}$ Th17 by two independent mechanisms: (1) by increasing the low levels of Ca^{2+} resulting from deficient PTEN expression and (2) by reducing the cAMP levels by inhibiting AC.

As demonstrated by Gomez-Rodriguez *et al*. and others (467,497), initial TCR signals are coupled to specific IL-17A regulation. Through recruitment of PLC-γ, TCR activation induces the Ca^{2+} signaling pathway (468,520). Th17 cells exhibit a distinct Ca^{2+} profile from Th1 and Th2 cells (496,550) and defects in Ca^{2+} signaling result in reduced nuclear import of NFAT and reduced IL-17A expression (467,496,497). NFAT is thought to be a key transcription factor that enhances IL-17A expression (467,523). Defects in NFAT signaling result in reduced IL-17A expression and EAE pathogenicity from Th17 cells (467,525). In addition, overexpression of NFAT enhanced IL-17A expression in Jurkat cell lines deficient for specific TCR signaling pathways (524).

Importantly, both Ca^{2+} and cAMP signaling cascades converge in the regulation of NFAT activation state. On one side, Ca^{2+} signaling favors dephosphorylation of NFAT through activation of calcineurin. NFAT can only translocate into the nucleus in its hypophosphorylated state, and therefore calcineurin activation is necessary for NFAT transcriptional regulation of IL-17A. On the other hand, cAMP can induce the activation of PKA, which in turn can phosphorylate NFAT (510,522). Poly-phosphorylation of NFAT prevents its translocation into the nucleus and favors its sequestration in the cytosol as well as proteosomal degradation (522).

BACE1$^{-/-}$ Th17 cells have a defect in Ca^{2+} signaling and higher potential for the generation of cAMP. Both conditions should result in reduced NFAT translocation and therefore reduced IL-17A expression. We are currently addressing the nuclear translocation of NFAT in WT and BACE1$^{-/-}$ Th17 cells by WB, DNA binding assays as well as ImageStream. So far the data indicate that there are no major differences in NFAT nuclear levels but performing chromatin immunoprecipitation experiments will provide specific information on binding of NFAT to the IL-17A promoter.

Altogether, these findings suggest that a deep understanding of the roles of cAMP and Ca^{2+} signaling in T cell activation and differentiation as well as how these pathways interact at different moments during the signaling cascade is necessary to comprehend the mechanism or alternative functions of currently used treatments. We speculate that *in vitro* differentiation of BACE1$^{-/-}$ Th17 cells in the presence of AC inhibitors could restore IL-17A and CD73 expression. On the other hand, forskolin-induced inhibition of IL-17A expression should potentially be counteracted by increases in Ca^{2+} signaling with ionomycin.

4.2.4 BACE1 in other cell types

Our data suggested that BACE1 is dispensable for correct polarization to Th1 and Tregs. Th1 and Treg characterization was based on the expression of their signature markers. However, functional analysis, using *Listeria* or *Toxoplasma* infection models and suppression assays, are necessary to completely rule out any role of BACE1 in these T helper subsets. We focused our attention on the role of BACE1 in $CD4^+$ T cells due to their involvement in the development of EAE in mice.

However, infiltration of antigen-specific $CD8^+$ T cells is commonly detected in the CNS of MS patients, where they are known to cause pathology (137). Similarly, BACE1 mRNA levels have been detected in $CD8^+$ T cells (BioGPS) and, therefore, it would be interesting to address whether protein levels can be detected in these cells as well as if BACE1 is required for effective $CD8^+$ T cell function. In the future, it would be ideal to have $BACE1^{fl/fl}$ mice in order to assess the role of BACE1 in different cell populations by back-crossing to distinct *Cre* backgrounds.

BACE1 expression in Th17 cells is necessary for correct expression of IL-17A in these cells. However, "conventional" Th17 cells are not the only source of IL-17A from immune cells. Considerable recent works have identified various innate cell types that express IL-17, collectively called Type 17 cells. Type 17 subsets include certain Natural Killer cells (551) and γδ T cells (552), as well as a heterogeneous innate lymphoid cell (ILC) population known as ILC3 that lacks the TCR (551–553). Importantly, γδ T cells have been described to be the first Type 17 subset to infiltrate and produce IL-17 cytokines in the CNS upon damage (554–556). IL-17-producing γδ T cells accompany conventional autoreactive Th17 cells in the early phases of EAE, contributing to inflammation (554). Therefore, it would be very interesting if BACE1 requirement for IL-17A expression in conventional αβ Th17 also applies to the expression of this cytokine by γδ T cells.

A simple approach to test this would be to study the IL-17A response by $BACE1^{-/-}$ γδ T cells in dermal candidiasis or with the Imiquimod-induced psoriasis model. In these models, clearance of the infection or autoinflammatory skin inflammation are driven by IL-17A primarily produced from γδ T cells (557,558). In addition, we can find Type 17 ILCS and γδ T cells in the

intestinal mucosa. *Ex vivo* stimulation of both ILCs and γδ T cells with PMA/Ionomycin would provide insights on the requirement of BACE1 for the production of IL-17A in these cells.

In recent years, a growing body of work has shown the importance of the gut microbiome in shaping the immune system (449,559–566). Interestingly, it has been reported that IL-17-producing γδ T cells implicated in CNS inflammation following ischemia are, in fact, programmed by the gut microbiota (561). Interactions between gut commensals and mucosal immune cells have impacts on both sides: the microbiome primes lymphocytes to different activation programs and the immune response shapes gut ecology, therefore constantly re-shaping this environment (449,559–566). In this regard, an impaired immune response caused by BACE1 deficiency could impact the microbial populations in BACE1$^{-/-}$ mice. In the past few months, we have collected stool samples from BACE1$^{-/-}$ and WT mice that were singly or co-housed to determine changes in their gut microbiome over time. We will submit these samples for 16S sRNA sequencing. If we find differences in microbiome composition, we would like to study how the impact of reduced BACE1$^{-/-}$ IL-17A in the microbiome shapes the development of Th17-driven immune responses as well as whether it can impact other cell types such as Th1 or Tregs.

Recently, a new population of αβ T cells that can produce IL-17A was described and named natural Th17 cells (567–570). These cells, in contrast to "conventional" Th17 cells, mature and undergo functional priming intrathymically (568,569) and possess an intrinsic capacity for immediate activation in naive hosts (568). Natural Th17 are key cells in the resolution of mucosal fungal infections such as oropharyngeal candidiasis (OPC) (567). In this model, *Candida* clearance is dependent on IL-17A production (209). Initial OPC experiments with BACE1-deficient mice

showed an increase in fungal burden in BACE1$^{-/-}$ tongues (data not shown), but due to a limited amount of mice used for this experiment, the results were not statistically significant. We believe that a proper evaluation of nTh17 and γδ T cell responses in the absence of BACE1 can help elucidate if the role of BACE1 is a conventional Th17 cell-specific mechanism or if it is a conserved regulator of IL-17A expression. One must bear in mind that in contrast to conventional Th17 cells, γδ T cells and nTh17 cells do not require TCR activation to drive IL-17A production peripheral tissues (555,567) and that ILCs do not even express a TCR (553,571). Therefore we hypothesize that IL-17A expression from these cells would be less or not affected by BACE1-deficiency. However, CD73 has been identified as a crucial molecule during thymic γδ commitment (418). Due to the requirement of BACE1 for CD73 expression, γδ T cells may exhibit other developmental defects that would be important to address as well.

4.2.5 BACE1 Inhibition as a potential Th17-specific anti-inflammatory drug

In most Th17-driven autoimmune disorders, the first line of action is the use of general anti-inflammatory drugs, such as corticosteroids or methotrexate (98,572). The main side effect of these therapies is an increased susceptibility to infections due to broad immunosuppression. More specific therapies are based on the use of biologicals, typically monoclonal antibodies that block TNFα, IL-17 or their receptors (98,573–575). These therapies exhibit limited access to the CNS (576,577) as well as being expensive (572,574,575). In this regard, pharmacological inhibition of BACE1, with small molecules or peptides, would provide a cheaper and accessible IL-17A-specific inhibition without affecting other Th17 functions as well as keeping other immune cells intact.

Our findings highlight the possibility of pharmacological inhibition of BACE1 to dampen Th17-driven inflammation. *In vitro* differentiation of BACE1$^{-/-}$ T cells into Th1 or Tregs did not seem to present any defects compared to WT controls. Hence, BACE1 inhibition would result in specific targeting of the Th17 response, in particular IL-17, while other components of the immune response would remain unaffected. Several questions need to be answered before completely validating the therapeutic use of BACE1 inhibition in Th17-mediated diseases.

Due to the critical role of BACE1 in the generation of the Aβ plaques in AD, therapeutic strategies to inhibit it are being intensely pursued. Currently studied BACE1 inhibitors are designed to block proteolytic cleavage of APP and are mainly peptidomimetics, small peptides designed to mimic enzymatic substrates and block their binding, (263,309). Earlier peptides had difficulties crossing the BBB, and despite being highly effective and specific, only offered a modest reduction in Aβ formation in the brain (521,578,579). However, modified structures have been able to overcome this problem and different compounds are currently being tested in phase 2 and 3 clinical trials (263,309).

Current BACE1 inhibitors are based on the blockade of its proteolytic activity (309). BACE1 inhibition did not affect IL-17A expression *in vitro* and our mechanistic data suggest that the regulation of IL-17 generation is regulated by cAMP, in a process known to be protease-independent. Therefore, further studies need to be performed in order to ensure that the catalytic activity of BACE1 is dispensable for IL-17A expression. For that purpose, WT BACE1 and a proteolytically-deficient BACE1 will be overexpressed in BACE1$^{-/-}$ Th17 cells to study and

compare both IL-17 and CD73 expression. If proteolytically-deficient BACE1 still supports IL-17A production, the next step would be to identify which proteins BACE1 partners with, so that these interactions can specifically be targeted by new BACE1 inhibitors.

Hence, our findings impact the pharmacological research of BACE1 inhibition in different ways. First of all, they raise the possibility of repurposing BACE1 inhibitors to target Th17 cells. More importantly, they allow the possibility of rescuing some of the most specific and effective inhibitors from the pipeline that were not commercialized due to their inability to cross the BBB. This non-penetrance into the CNS may actually be desired to treat Th17-driven autoimmune diseases in other organs, such as psoriasis or RA. Secondly, it highlights the importance of evaluating the immune response in patients treated with BACE1 inhibitors. A proper analysis of immune populations, in particular Th17 cells, from patients treated with BACE1 inhibitors in AD clinical trials, will not only address the impact of BACE1 in the immune response as a side effect but also shed light on the changes in immunological processes upon systemic inhibition of BACE1.

4.2.6 PTEN and Th17 cells

Importantly, PTEN haplodeficiency phenocopied the findings observed in BACE1$^{-/-}$ Th17 cells. This important finding, aside from the BACE1 project, shows evidence of novel functions for PTEN in regulating IL-17A and CD73 expression. Studying the effects of expression of different quantities of PTEN in different Th subsets will help corroborate whether PTEN can regulate the expression different cytokines or if this is an IL-17A-specific effect. These results also highlight the intricacies of TCR signaling, by showing that depending on the pathway by which the signal

is propagated, different outcomes are possible. Indeed, it is not only a matter of strong or weak TCR signal strength, but rather how the interactions between pathways stimulated by other triggers, such as cytokines, modulate the signaling events that will lead to different T cell fates or regulation of specific Th functions.

4.2.7 Re-evaluation of BACE1 effects in neuroinflammation

Both increased BACE1 and IL-17 levels are thought to contribute to CNS inflammatory states, such as ischemia (256,257,356–358,561,580). In addition, BACE1 and IL-17A expression share transcriptional factors, suggesting a link in their expression in these inflammatory states. Therefore, our findings may call for a re-evaluation of current literature on BACE1 in neuroinflammatory states. Due to the new roles of BACE1 in Th17 function, one could speculate that some of the currently attributed functions of BACE1 in neuroinflammation could be exerted through regulation of IL-17A expression. This hypothesis is especially plausible taken into account that the precise mechanisms by which BACE1 contributes to neuroinflammation and degeneration following injury are still not clear (236). Moreover, we believe that it also is necessary to better understand how BACE1 is induced under inflammatory conditions, especially under Th17-polarizing conditions in T cells as well as other immune and non-immune cells.

[APPENDIX]

1.0 CD73 IS EXPRESSED BY INFLAMMATORY TH17 CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BUT DOES NOT LIMIT DIFFERENTIATION OR PATHOGENESIS

Attached is a copy of the manuscript:

CD73 is expressed by inflammatory Th17 cells in experimental autoimmune encephalomyelitis but does not limit differentiation or pathogenesis by Gerard Hernandez-Mir and Mandy J. McGeachy. This manuscript, published in PLoS One, contains our findings about the expression and requirement of CD73 during Th17 development and for its function/pathogenicity in EAE. This data correspond to most of the findings shown in section 3.2 of this thesis.

PLOS ONE

RESEARCH ARTICLE

CD73 is expressed by inflammatory Th17 cells in experimental autoimmune encephalomyelitis but does not limit differentiation or pathogenesis

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Abstract

CD73 works together with CD39 to convert extracellular ATP to immunoregulatory adenosine, thus inhibiting inflammation. TGFβ-mediated CD73 expression on 'regulatory' Th17 cells limits their ability to eradicate tumors, similar to the immunosuppressive mechanism described for CD73 on Tregs. However, CD73 is also expressed on Th17 cells thought to be inflammatory in Crohn's disease. CD73 has previously been reported to contribute to inflammation in the central nervous system (CNS). In experimental autoimmune encephalomyelitis (EAE), we found that inflammatory cytokine-producing Th17 cells showed increased CD73 expression as disease progressed. We therefore hypothesized that CD73 could be important for limiting the expansion or pathogenic function of Th17 cells in autoimmune inflammation of the CNS. Surprisingly, EAE development was not enhanced or inhibited by CD73 deficiency; there was correspondingly no difference in induction of Th17-associated cytokines IL-17, IFNγ or GM-CSF or recruitment of either inflammatory or regulatory cells to the central nervous system. We confirmed that CD73 was similarly not required for differentiation of Th17 cells *in vitro*. These data show that while CD73 expression is regulated during EAE, this enzyme is not absolutely required to either promote or limit Th17 cell expansion or EAE severity.

Introduction

Th17 cells produce cytokines including IL-17, GM-CSF and IFNγ that orchestrate immune and tissue inflammatory responses resulting in recruitment and activation of myeloid cells, as well as production of antimicrobial peptides and matrix metalloproteinases. These responses are beneficial in controlling extracellular bacteria and fungal pathogens such as *Staphylococcus aureus* and *Candida albicans*, as well as promoting wound healing following resolution of the infection[1, 2]. However, when Th17 responses are dysregulated or are inappropriately induced against commensal microbes or self-proteins, this can result in chronic inflammatory

diseases exemplified by psoriasis, inflammatory bowel disease and multiple sclerosis[3]. Hence there needs to be a critical balance between promoting Th17-mediated inflammation when it is beneficial, while regulating Th17 cells to prevent immunopathology.

Perhaps because of the propensity of Th17 cells to evoke potent inflammation, multiple mechanisms exist to regulate the expansion or function of Th17 cells. During differentiation, exposure to cytokines including IL-2, IL-27 and IFNγ inhibit development of Th17 cells[4]. TGFβ is required for early Th17 cell activation, and T cells deficient in TGFβ or its receptor show impaired Th17 development and function *in vivo*[5, 6]. However, Th17 cells that receive sustained stimulation from TGFβ are non-pathogenic in function, despite high production of IL-17[7, 8]. These non-pathogenic Th17 cells have also been termed 'regulatory' Th17 cells due to their capacity to suppress inflammation through production of IL-10, although there are multiple mechanisms determining their non-pathogenic phenotype[9, 10].

One of the molecules expressed both by 'regulatory' Th17 cells and Foxp3$^+$ regulatory T cells is the enzyme Ecto-5'-nucleotidase, also called CD73[11–13]. TGFβ along with STAT3 activation induces expression of CD73 along with Ectonucleoside triphosphate diphosphohydrolase-1, also known as CD39[13]. These two enzymes work together: CD39 converts extracellular ATP to AMP intermediates, which are then converted to adenosine by CD73. Generation of adenosine by CD73 on Tregs has been shown to have immunosuppressive functions on Th1 cells[13]. 'Regulatory' Th17 cells expressing high CD73 also impaired anti-tumor responses in mouse models[13, 14]. Corresponding with immunosuppression, high CD73 expression is associated with poor prognosis in human cancers[15].

The roles of CD73 in regulating autoimmune inflammation are less clear. Inflammatory Th17 cells in the intestine of Crohn's disease patients were found to express CD73[16]; whether this had any functional consequence or association with disease severity was not determined. CD73 expression has been reported in multiple sclerosis (MS) brain lesions[17]. Experimental autoimmune encephalomyelitis (EAE) is a widely used model of CNS-targeted inflammation mediated by Th17 cells. As predicted by the immunosuppressive roles of adenosine, mice deficient in adenosine receptor A2A show accelerated onset and severity of EAE, along with increased production of inflammatory cytokines[18, 19]. Hence the prediction would be that CD73$^{-/-}$ mice, having reduced ability to generate adenosine, might develop EAE with increased severity. However, contradictory findings have been reported in regards to CD73 and adenosine in the EAE model. In one report, CD73$^{-/-}$ mice were protected from active EAE, while transferred CD73$^{-/-}$ T cells induced more severe disease in wildtype (WT) recipients[20]. In contrast to the immunosuppressive effects of adenosine during EAE induction, adenosine signaling during later phases of EAE is thought to promote immune cell infiltration of the CNS through upregulation of chemokines[21]. Despite evidence for CD73 expression by Th17 cells, the role of CD73 in Th17 differentiation has not been carefully analyzed. We therefore set out to first confirm and then extend previous data by investigating expression of CD73 and its role during differentiation of Th17 cells.

Results

CD73 expression on different T cell subsets

CD73 expression is increased on TGFβ-stimulated Th17 cells and regulatory T cells *in vitro*, while remaining low on Th0 and Th1 cells (Fig 1A), confirming previous reports. *In vitro*-generated regulatory T cells expressed higher levels of CD73 than Th17 cells (Fig 1B). *In vivo*, Foxp3$^+$ Tregs in naïve mice were also CD73$^+$, and showed higher expression of CD73 than Foxp3$^-$CD44^{hi} effector/memory cells from the same animals (Fig 1C–1E). Interestingly,

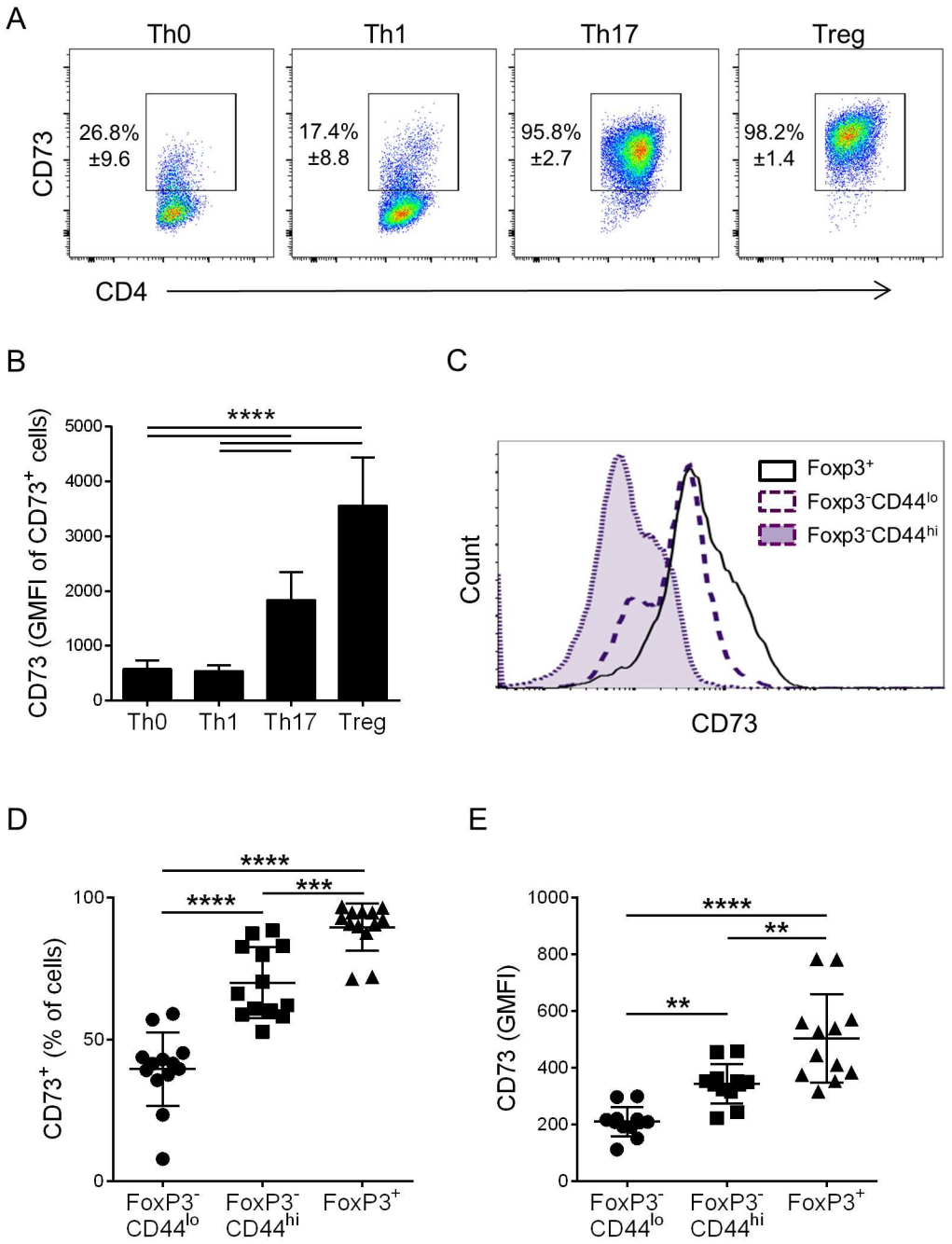

Fig 1. CD73 expression on T helper cells *in vitro* and *in vivo*. A: CD73 expression on indicated live $CD4^+$ T helper cell subsets after three days of ulture *in vitro*, numbers indicate mean % +/- S.D. from three separate experiments. B: Mean level of CD73 expression measured as Geometric Mean Fluorescence Intensity of gated live $CD4^+CD73^+$ cells, from three experiments. C: Representative histogram of CD73 expression by Tregs ($FoxP3^+$), naïve T cells ($FoxP3^-$ $CD44^{lo}$), or effector/memory ($FoxP3^-$ $CD44^{hi}$) T cells in LN from naïve C57BL/6 mice. D: Mean percentage of $CD73^+$ cells and E: CD73 Geometric Mean Fluorescence Intensity of populations indicated in C; data pooled from 4 independent experiments (each point represents a separate mouse). Error bars indicate S.D.

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Foxp3$^-$CD44^{lo} cells, which are typically considered naïve cells, also showed some expression of CD73, albeit lower percentages and levels of expression per cell (Fig 1C–1E).

Th17 cells upregulate expression of CD73 during EAE progression

These data suggest that CD73 is not only a marker of regulatory cells but is also expressed on a large proportion of effector/memory cells. Hence we investigated expression of CD73 on Th17 cells during induction and effector phases of the inflammatory autoimmune response in EAE. At the peak of T cell activation in the draining LN (day 8 following immunization), CD73 was expressed on almost half of IL-17$^+$ cells (Fig 2A and 2B). Interestingly, the proportion of IL-17$^+$ cells that expressed CD73 increased as the response progressed (Fig 2A and 2B). The expression of CD73 on LN IFNγ$^+$ cells showed a similar increase as the EAE response progressed (Fig 2C), and GM-CSF$^+$ cells followed the same pattern (Fig 2D). Corresponding with the increasing expression of CD73 by cytokine-producing cells in LN, the effector cells found in the CNS also contained high proportions of cytokine-producing cells that co-expressed CD73 (Fig 2E–2H), which increased from onset through peak and chronic phases of disease.

CD73 does not influence Th17 differentiation *in vitro*

Since CD73 is induced on both in vitro-generated 'regulatory' Th17 cells and *in vivo* inflammatory Th17 cells, as well as Tregs, we tested whether CD73 plays any role in early differentiation of these cells. WT and CD73$^{-/-}$ T cells were activated with anti-CD3 in presence of Th17-promoting cytokines. Induction of IL-17 and RORγt were comparable in absence of CD73 (Fig 3A and 3B). However, we did observe a small but significant decrease in the percentage of Foxp3$^+$ cells when CD73$^{-/-}$ T cells were activated in presence of TGFβ and IL-2 (Fig 3C).

EAE clinical course is not affected by CD73 deficiency

The relatively high expression of CD73 on inflammatory T cells during induction and onset of EAE suggests that CD73 may play a role in promoting rather than limiting Th17 function. Conversely, the increased expression of CD73 on these cells as the response progressed and stabilized in terms of clinical disease supports a potential limiting role of this molecule. We therefore tested the requirement for CD73 in autoimmune pathogenic Th17 cell function by immunizing WT and CD73$^{-/-}$ mice with MOG(35–55) to induce EAE. Unexpectedly, there was no difference in severity of EAE in CD73$^{-/-}$ mice (Fig 4A). Similarly, incidence of EAE and day of onset of clinical signs was not different in absence of CD73, although there was a slight non-significant trend towards delayed onset in CD73$^{-/-}$ mice (Fig 4B and 4C).

Th17 and Treg cell frequency and recruitment to CNS are unaltered by CD73 deficiency

It has previously been reported that CD73 expression in the CNS promotes entry of effector cells through induction of chemokines[20, 21], hence it was possible that enhanced activation of Th17 cells was mitigated by reduced entry of these cells into CNS. However, a close examination of cytokine producing T cells revealed no significant differences in frequencies of cells producing IL-17, IFNγ or GM-CSF in LN (Fig 5A) or CNS (Fig 5B) over all phases of the EAE disease course. These data were determined by intracellular cytokine staining following non-specific stimulation with PMA and ionomycin. We also cultured LN cells from WT and CD73$^{-/-}$ mice, taken at onset and peak of EAE, with MOG(35–55) and MOG(35–55) plus IL-23 to promote IL-17 and tested secretion by ELISA. Similar to the flow cytometry results,

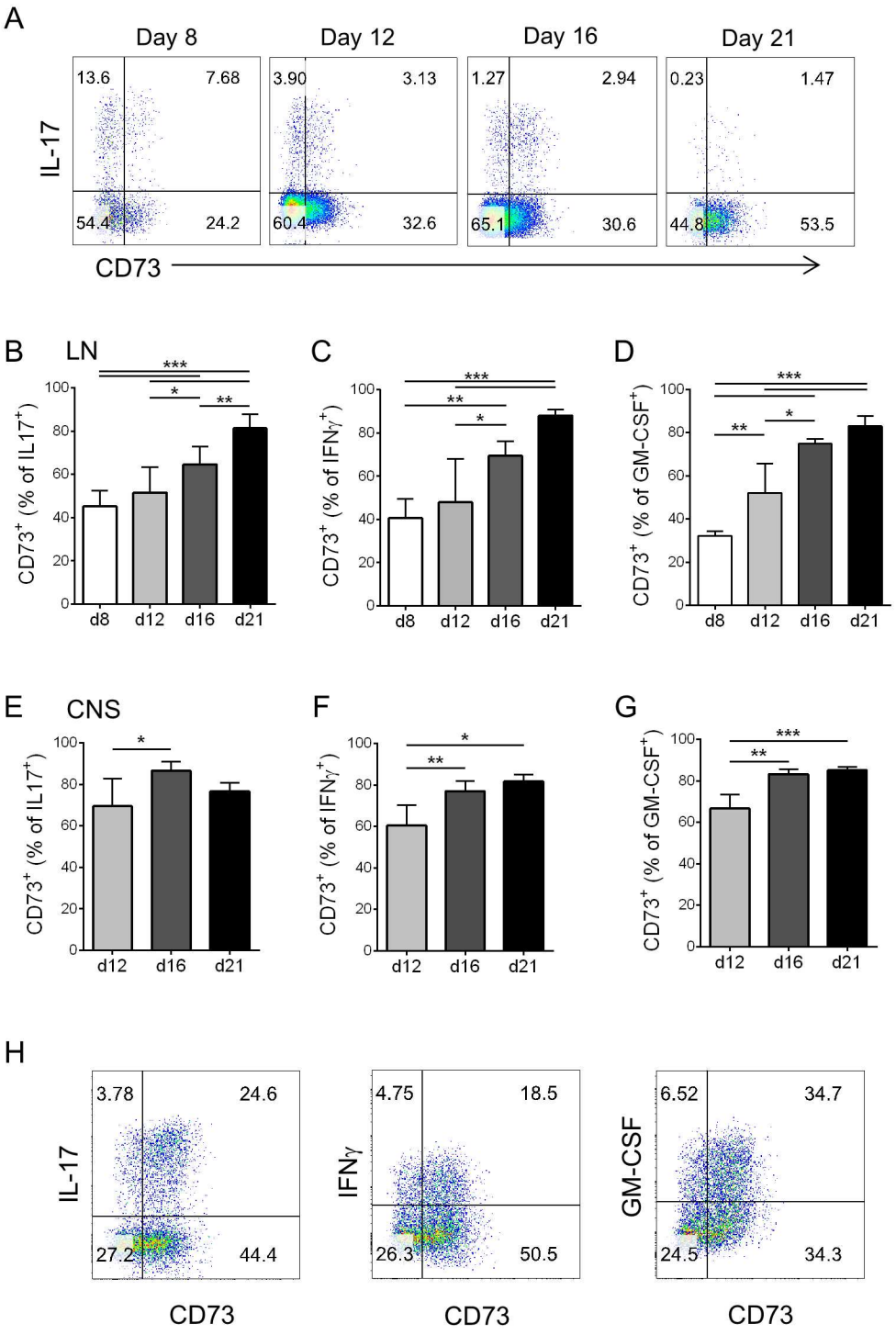

Fig 2. Th17 cells increase expression of CD73 during EAE progression. A-D: Analysis of co-expression of CD73 on cytokine-producing cells in draining LN, gated on live $CD4^+$ cells, at indicated time-points post EAE induction. A: Representative FACS plots. B: Percentage of $IL\text{-}17^+$ T cells that express CD73; C: Percentage of $IFN\gamma^+$ T cells that express CD73; D: Percentage of $GM\text{-}CSF^+$ T cells that express CD73. E-H: Analysis of co-expression of CD73 on cytokine-producing cells in CNS, gated on live $CD4^+$ cells, at indicated time-points post EAE induction. E: Percentage of $IL\text{-}17^+$ T cells that express CD73; F: Percentage of $IFN\gamma^+$ T cells that express CD73; G: Percentage of $GM\text{-}CSF^+$ T cells that express CD73. H: Representative FACS plots showing CD73 and cytokine staining in live $CD4^+$ T cells from CNS on day 16 of EAE. Values in graphs

correspond to mean +/- S.D. n = 5–13 mice/time-point pooled from 2–3 experiments (except day 16 GM-CSF is from one experiment).

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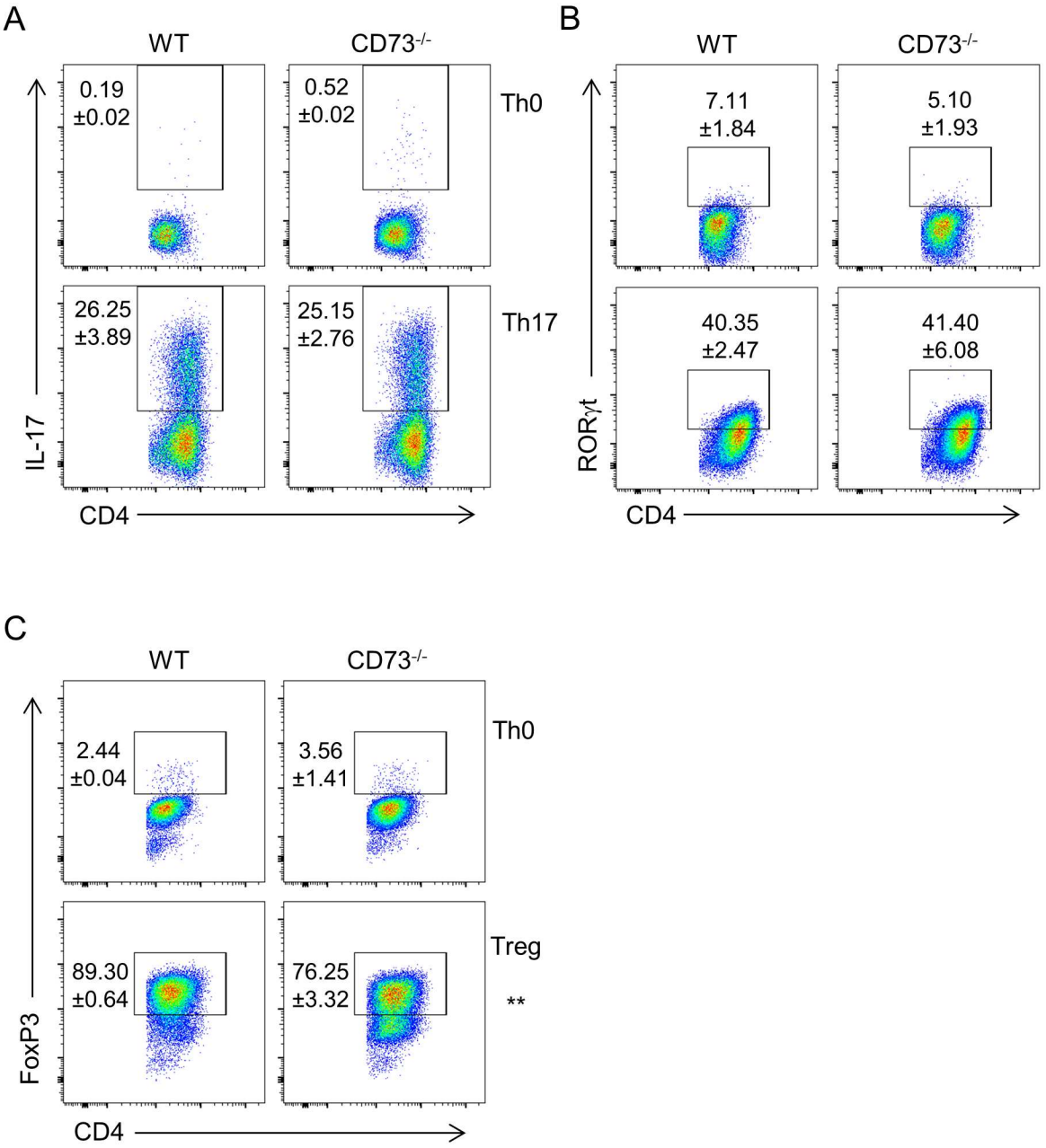

Fig 3. CD73 does not influence Th17 differentiation *in vitro*. A, B: $CD4^+$ T cells from WT or $CD73^{-/-}$ mice were cultured under Th0 (top panels) or Th17 (lower panels) differentiating conditions for three days, then expression of IL-17 (A) and RORγt (B) were analyzed. C. Expression of FoxP3 in WT and $CD73^{-/-}$ Th0 (top panel) and Tregs (lower panel) at day 3 of differentiation. Numbers indicate mean % +/- S.D. of gated cells (n = 2), representative of three independent experiments with similar results.

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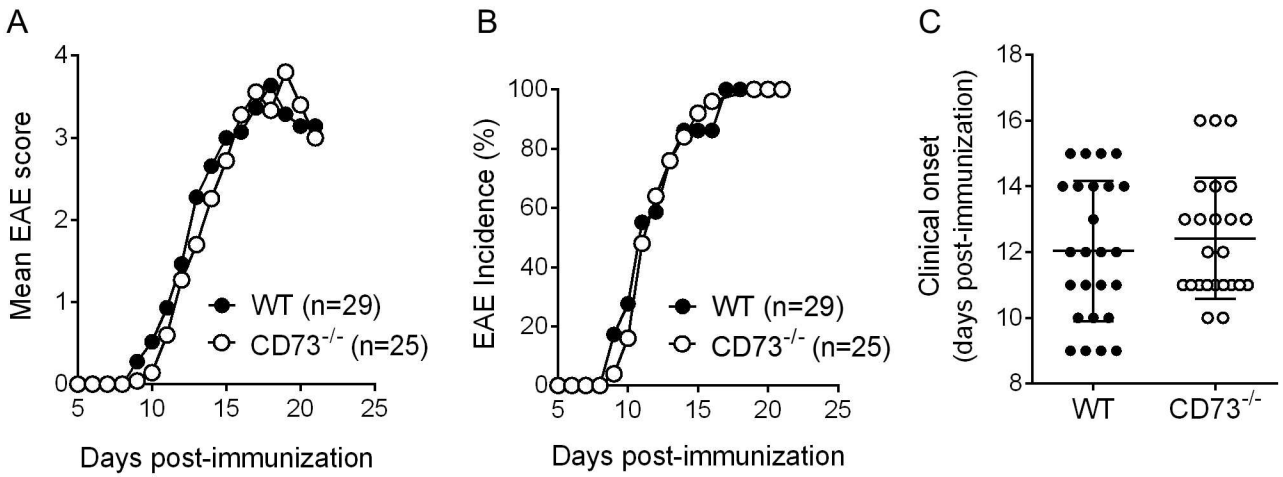

Fig 4. EAE clinical course is not affected by CD73 deficiency. A: Mean clinical scores following EAE induction in WT and CD73$^{-/-}$ mice. B: Percentage of mice that had developed EAE clinical signs on indicated days after EAE induction. C: Day of EAE onset in WT and CD73$^{-/-}$ mice that developed signs of EAE by day 16 post-immunization (mean +/- S.D.). Data pooled from four independent experiments.

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MOG-induced production of IL-17 was not different (Fig 5C), confirming that priming of the antigen specific Th17 response was not affected by CD73 deficiency. Frequencies of Foxp3^{+} regulatory T cells in LN and CNS were also not affected by CD73 deficiency (Fig 5D). Together, this data corresponds with the clinical scores to confirm that CD73 does not play a critical role in promoting or limiting the inflammatory response induced during EAE.

Discussion

The data reported here confirmed previous reports that Th17 cells differentiated in the presence of TGFβ express CD73[13]. We also demonstrated that a large proportion of Th17 cells expressed CD73 during EAE induction, and this increased as EAE progressed. More accurately, CD73 expression was similar on IL-17^{+}, IFNγ^{+} and GM-CSF^{+} CD4^{+} T cell populations; we group these together as 'Th17' since multiple studies show that all three of these cytokines are expressed by Th17 cells in an IL-23 dependent fashion in the EAE model[22–26]. CD73 has previously been described as an immunosuppressive molecule expressed by 'regulatory' Th17 cells[13]. It is important to note that while sustained high concentrations of TGFβ induce a non-pathogenic Th17 phenotype, TGFβ and STAT3 are also required for differentiation of inflammatory Th17 cells *in vivo*. Hence, CD73 expression by Th17 cells during EAE corresponds with data showing that TGFβ signals promote Th17 cells to drive EAE induction[5, 6, 27].

The widespread expression of CD73 on Th17 cells in EAE, particularly in the CNS at onset of clinical signs, argued against a purely immunosuppressive role for this molecule in Th17 cell function. However, mice deficient in the receptor for the CD73 product adenosine develop severe EAE with exaggerated cytokine responses[17, 18]. We were therefore surprised to find that CD73$^{-/-}$ mice did not develop exacerbated disease signs following EAE induction. CD73 is also highly expressed on Tregs, which are widely considered to play important roles in controlling autoimmune disease. However, conflicting data exists for the role of Tregs in EAE: on the one hand, depletion of Tregs exacerbated disease severity[28, 29], while on the other hand Tregs were found to be ineffective suppressors of inflammation in the early stages of CNS infiltration[30]. Tregs have also been found to promote Th17 differentiation through absorption of

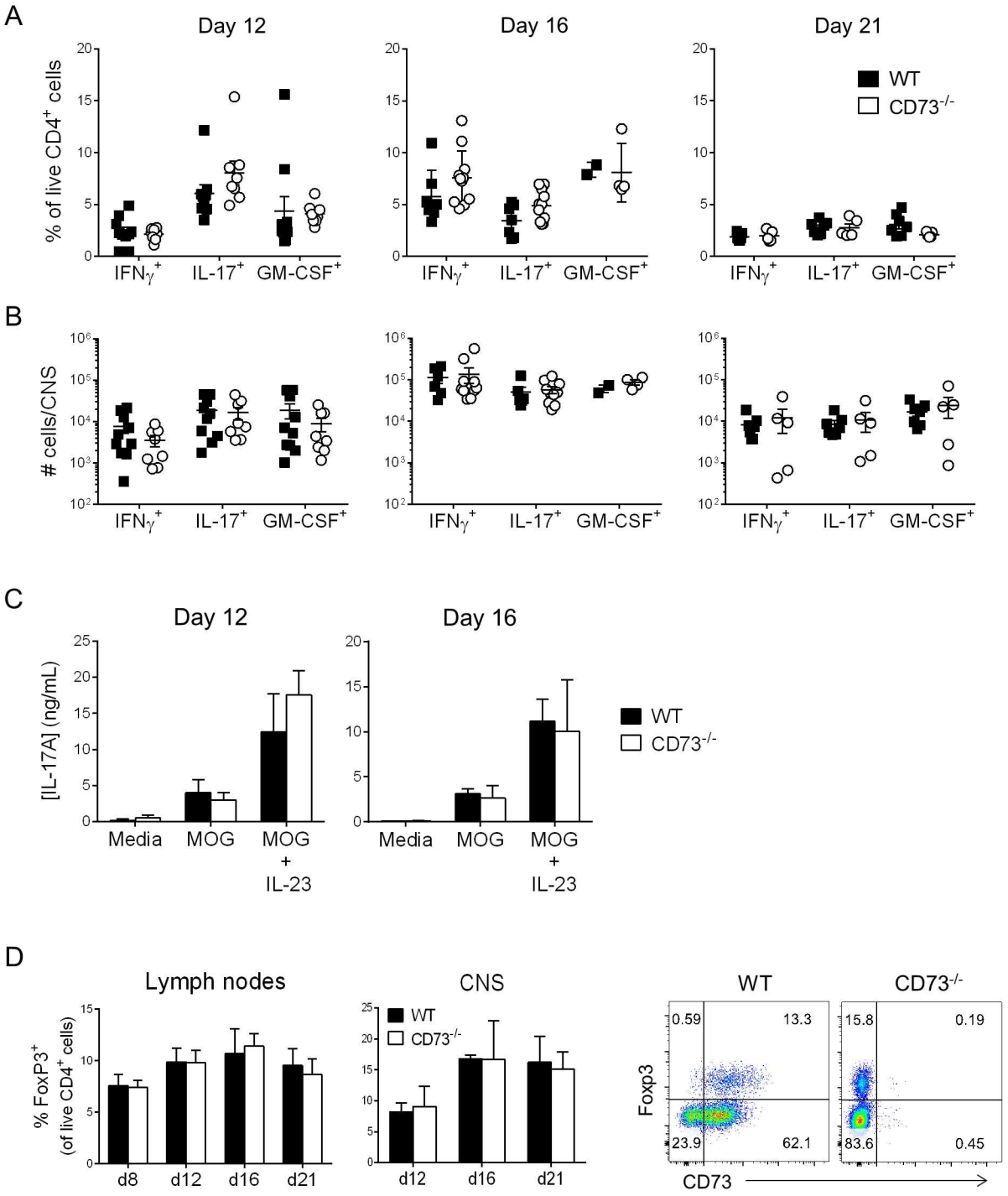

Fig 5. Th17 and Treg cells are similar in WT and CD73$^{-/-}$ during EAE. Cytokine production and Treg frequency were analyzed by FACS in draining LN and CNS of WT and CD73$^{-/-}$ mice at day 12 (onset), day 16 (peak) and day 21 (chronic/resolution) phases of EAE. A: Frequencies of IL-17, IFNγ and GM-CSF expressing T cells, analyzed in draining lymph nodes on indicated days after EAE induction. B: Numbers of IL-17, IFNγ and GM-CSF expressing T cells infiltrating the CNS at indicated time points after immunization. A-B show mean +/- SEM of pooled data, each point represents an individual mouse. C: Cells from draining lymph nodes at days 12 (n = 4-5/group) and 16 (n = 2-3/group) post-immunization were re-challenged *in vitro* with MOG(35–55) for three days in the presence/absence of IL-23 (20ng/mL), and IL-17 expression was measured by ELISA. D: Percentage of Tregs in the draining lymph nodes and CNS at indicated timepoints of EAE, FACS plots show representative staining of Foxp3 and CD73 in CNS on day 16 post-immunization, shown as mean +/- S.D. from 4–7 mice/group except day 16 WT has n = 2 mice. Data are representative of two-three independent experiments with similar results.

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IL-2[31, 32], and *in vitro* can provide a source of TGFβ[5], although this appears to be provided by Th17 cells themselves *in vivo*[6].

Our findings on EAE susceptibility were in contrast to those of Mills et al[20], who found reduced severity of EAE in CD73$^{-/-}$ mice. This was largely attributed to effects of CD73 expressed by CNS-resident cells and on adenosine actions in the CNS to promote expression of chemokines to promote lymphocyte entry to the CNS[20, 21]; T helper cell phenotypes were not intensively analyzed. There are a number of possible reasons for differences in EAE outcome. Different animal facilities have different microbiota communities that can influence outcome of autoimmunity[33]. The EAE induction protocols also vary slightly between labs, and this could result in differences in proportions of Th17 versus Th1 cells induced. In our hands, EAE is associated with strong induction of Th17 cells and the response is dependent on IL-17 and IL-23. We did not find any effect of CD73 deficiency on Th17 induction during any phase of EAE, as measured both by non-specific PMA/ionomycin stimulation and by stimulation with the immunizing antigen MOG(35–55). Differentiation of Th17 cells *in vitro* further supported our unexpected observation that CD73 does not play a dominant role in either inhibiting or promoting Th17 differentiation. Interestingly, Mills et al also reported that mice deficient in the adenosine receptor A2A showed exacerbated EAE with increased IFNγ and proliferation in response to MOG(35–55), supporting the immunosuppressive role of adenosine on Th1 responses[18]. However, IL-17 responses were not impaired in these experiments, corresponding to our current study results and suggesting that the balance between Th17 and Th1 induction in EAE could determine the requirement for CD73 in disease susceptibility.

CD73 works with CD39 to generate adenosine from ATP. Although the focus is often on adenosine as an immunosuppressive molecule, CD39-mediated removal of ATP from the local environment also serves to reduce inflammation[34]: extracellular ATP activates P2X receptors as a damage-associated molecular pattern (DAMP) signal to elicit inflammatory responses such as inflammasome activation and release of IL-1. We did not observe any change in CD39 expression in absence of CD73. Hence, it is likely that the first arm of the CD39/CD73 processing of ATP still acts to control inflammatory responses during EAE. In this context, it was recently reported that Th17 cells have the surprising ability to produce their own IL-1β through activation of the ASC-dependent inflammasome pathway, and ATP is one molecule capable of activating this pathway[35]. Hence, we speculate that Th17 cells may indeed limit their own activation through upregulation of the CD39/CD73 enzyme partners, but that removal of ATP rather than generation of adenosine may play a more important role. Indeed, 'regulatory' Th17 cells have been demonstrated to efficiently hydrolyze ATP in a CD39-dependent manner, and CD39 deficiency reduced Th17 cell IL-10 production and increased pathogenic function in colitis[36]. Administration of *Bacteroides fragilis* PSA increases CD39^{+} Tregs and protects from EAE[37]. CD39-deficient mice in this model developed greatly exacerbated disease severity compared to WT controls, and it is possible that this was due to effects on Th17 cells as well as Tregs. Separately, CD39 expressed by dendritic cells during EAE also plays an important role in limiting Th17 cell expansion and resulting EAE severity[38].

In summary, we report here that CD73 is expressed on a high proportion of Th17 cells during EAE development, including on cells in the CNS. However, CD73 deficiency did not affect differentiation, recruitment or function of Th17 cells as assessed by EAE clinical signs, flow cytometry and antigen recall assays. These data were unexpected given the known role of CD73 in regulating inflammatory immune responses, and suggest that in the face of a strong inflammatory stimulus, such as occurs during induction of EAE, the immunosuppressive role of CD73 becomes insufficient to prevent Th17 generation and onset of autoimmune inflammation.

Materials and methods

Mice

$CD73^{-/-}$ and C57BL/6 (WT) mice were purchased from Jackson Laboratories and bred and housed under SPF conditions in an AAALAC-approved facility. All animal procedures were approved by the IACUC committee at the University of Pittsburgh. Mice were age and gender-matched within experiments, both male and female mice were used in all experiments, mice were used at 7–18 weeks of age.

In vitro $CD4^+$ T cell differentiation

$CD4^+$ T cells from spleens and lymph nodes of naïve mice were purified by magnetic separation (Miltenyi Biotec, Germany). T cells were activated with 5 μg/ml plate-bound αCD3 (clone 145-TC11, BioXcell) in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-β-mercaptoethanol, HEPES and Na pyruvate. For Th17 differentiation, cells were cultured in the presence of recombinant mouse IL-1β (40 ng/ml), IL-23 (20 ng/ml), IL-6 (100 ng/ml), TGFβ1 (10 ng/ml); all cytokines from R&D Systems, MN. In all Th0 cell cultures 10 μg/ml αIFN-γ neutralizing antibodies (BioXcell) were added. For Th1 cultures, IL-12 (PeproTech, NJ) was added at 10 μg/ml. For Treg differentiation, T cells were cultured in the presence of recombinant mouse TGFβ1 (20 ng/ml), recombinant human IL-2 (100 U/ml) and αIFN-γ neutralizing antibodies (10 μg/ml).

EAE induction

Naïve WT and $CD73^{-/-}$ mice were immunized subcutaneously with 100μg MOG(35–55) (Bio synthesis, Lewisville, Texas, USA) emulsified in 200μl CFA (Difco Laboratories, Detroit, Michigan, USA) containing 100μg Heat Killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, Michigan, USA) distributed in four sites on the flanks. 200ng Pertussis toxin (List Biological Laboratories) was given intraperitoneally on day 0 and 2. Clinical scoring: Mice were monitored daily, and EAE clinical signs were scored according to the following grades: 1: flaccid tail; 2: impaired righting reflex and hindlimb weakness; 3: partial hindlimb paralysis; 4: complete hindlimb paralysis; 5: hindlimb paralysis with partial forelimb paralysis; 6: moribund/dead, in the reported experiments less than 4% of mice died from EAE. Cages in which mice were found to show signs of paralysis (grade 3 or higher) were provided access to food and water on the cage floor. At the end of the experiment, animals were euthanized by CO_2 asphyxiation.

Flow cytometry

The following FACS antibodies were purchased from BD Biosciences: CD4 (RM4-5), CD44 (IM7), Ki67 (B56), IFNγ (XMG1.2) and IL-17 (TC11-18H10). The following were purchased from eBioscience: CD73 (eBIOTY/11.8), RORγt (AKFJS9), Foxp3 (FJK-16s) and GM-CSF (MP1-22E9). For cytokine analysis, cells were cultured in complete medium (as described for T cell cultures above) with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of Golgiplug (BD Biosciences) for 3 to 4 hours followed by FACS staining and analysis. For intracellular cytokines, staining was performed using Cytofix-cytoperm kit from BD; RORγt and Foxp3 intracellular stains were performed using eBioscience Foxp3 staining kit according to manufacturer's instructions. Prior to surface staining, cells were incubated for 20 min on ice with Ghost Dye™ Violet 510 (TONBO biosciences, CA) to allow exclusion of dead cells from analysis performed in FlowJo.

MOG re-challenge and IL-17 ELISA

Draining lymph nodes from mice immunized with MOG(35–55) were processed to obtain single cell suspensions. Cells were cultured in flat bottom 96-well plates at a cell density of 1M cells/well with soluble αCD3 antibodies (clone 145-TC11, 5 μg/ml; BioXcell) in the presence or absence of IL-23 (20 ng/ml). IL-17 production was analyzed from culture supernatants three days after MOG-rechallenge using Ready-Set-Go ELISA kits (eBioscience). Samples were diluted accordingly using the diluent buffer included in the kits.

Statistics

Parametric values were analyzed using Student's *t*-test, or ONE-WAY ANOVA (with Tukey's correction for multiple comparisons) when more than two groups were compared. EAE clinical scores and date of onset were analyzed using Mann-Whitney test (daily scores were analyzed separately). *P* values are shown as * = ($p < 0.05$), ** = ($p < 0.01$), *** = ($p < 0.001$) and **** = ($p < 0.0001$), where statistical significance was found.

Supporting information

S1 File. ARRIVE checklist.
(PDF)

S2 File. Excel of data used to generate figures.
(XLSX)

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Author Contributions

Conceptualization: MM.

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Funding acquisition: MM.

Investigation: GHM MM.

Methodology: GHM MM.

Writing – original draft: GHM MM.

Writing – review & editing: GHM MM.

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2.0 GENERATION OF BACE1-OVEREXPRESSION RETROVIRUS

2.1 BACE1 ORF – FROM LENTIVIRAL TO RETROVIRAL VECTOR.

In order to achieve BACE1 overexpression in $CD4^+$ T cells, a BACE1 ORF derived originally from a lentiviral vector was inserted into a retroviral system that uses Thy1.1 as an expression reporter. The protocol, which included primer design, cloning and sub-cloning, introduction of new restriction sites and generation of transient and stably transfected cell lines was performed by Matt Henkel. Dr. Saikat Majumder also helped in subsequent cloning of the final vector generated by Matt Henkel.

1. Original BACE1 (Bace1 (NM_011792) Mouse cDNA Clone) lentiviral vector was purchased from Origene. Figure 29 shows the map of the original empty lentiviral vector from Origene. BACE1 open reading frame (ORF) (insert size: 1506bp) was inserted between AscI and NotI restriction sites within the polylinker region (full sequence not available).

Bace1 (untagged) - Mouse beta-site APP cleaving enzyme 1 (Bace1), transcript variant 1, (10ug), BC048189, 10ug (Catalog #: MC205212)

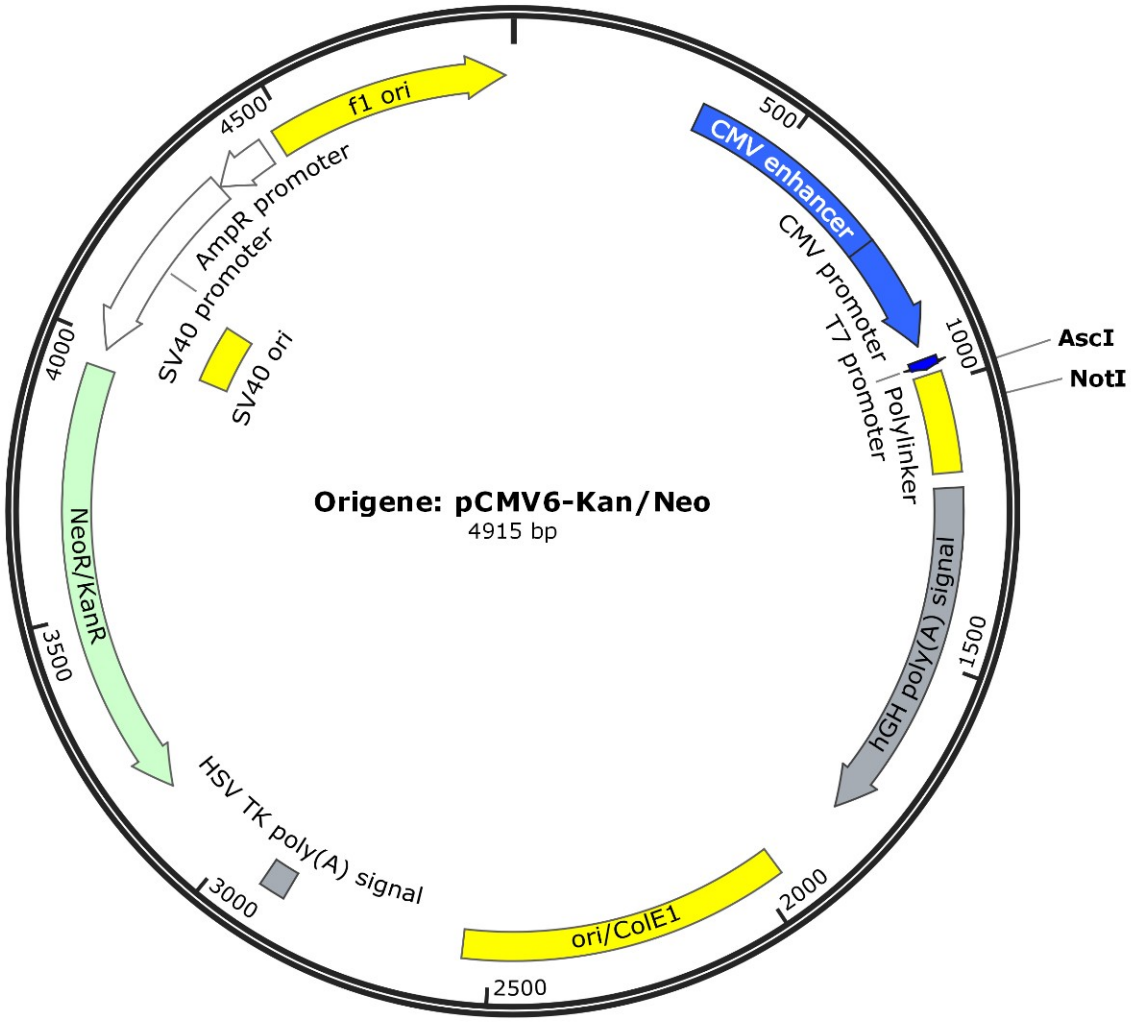

Figure 29. Original vector from Origene: BACE1 ORF inserted between AscI and NotI restriction sites.

2. We overexpressed BACE1 using the mouse stem cell virus (MSCV) retroviral system, for which the development and protocols were optimized by the Vignali and Delgoffe labs. App. Fig. 2 shows the map for the empty retroviral vector kindly provided by the Vignali lab. In order to excise the BACE1 insert from the Origene pCMV6 lentiviral vector and clone it into the pMIC II-Thy1.1 retroviral vector, BACE1 ORF was amplified using primers targeting the 5' and 3' of the BACE1 ORF in the original vector. These primers

contained additional restriction sites for two unique single cutter restriction enzymes that did not target the BACE1 coding sequence: BamHI and XhoI (Figure 31). Figure 32 shows the final BACE1 construct that was inserted (insert size: 2001bp) in the empty pMIC II-Thy1.1 empty vector (Figure 30. The resulting final vector is shown in Figure 33.

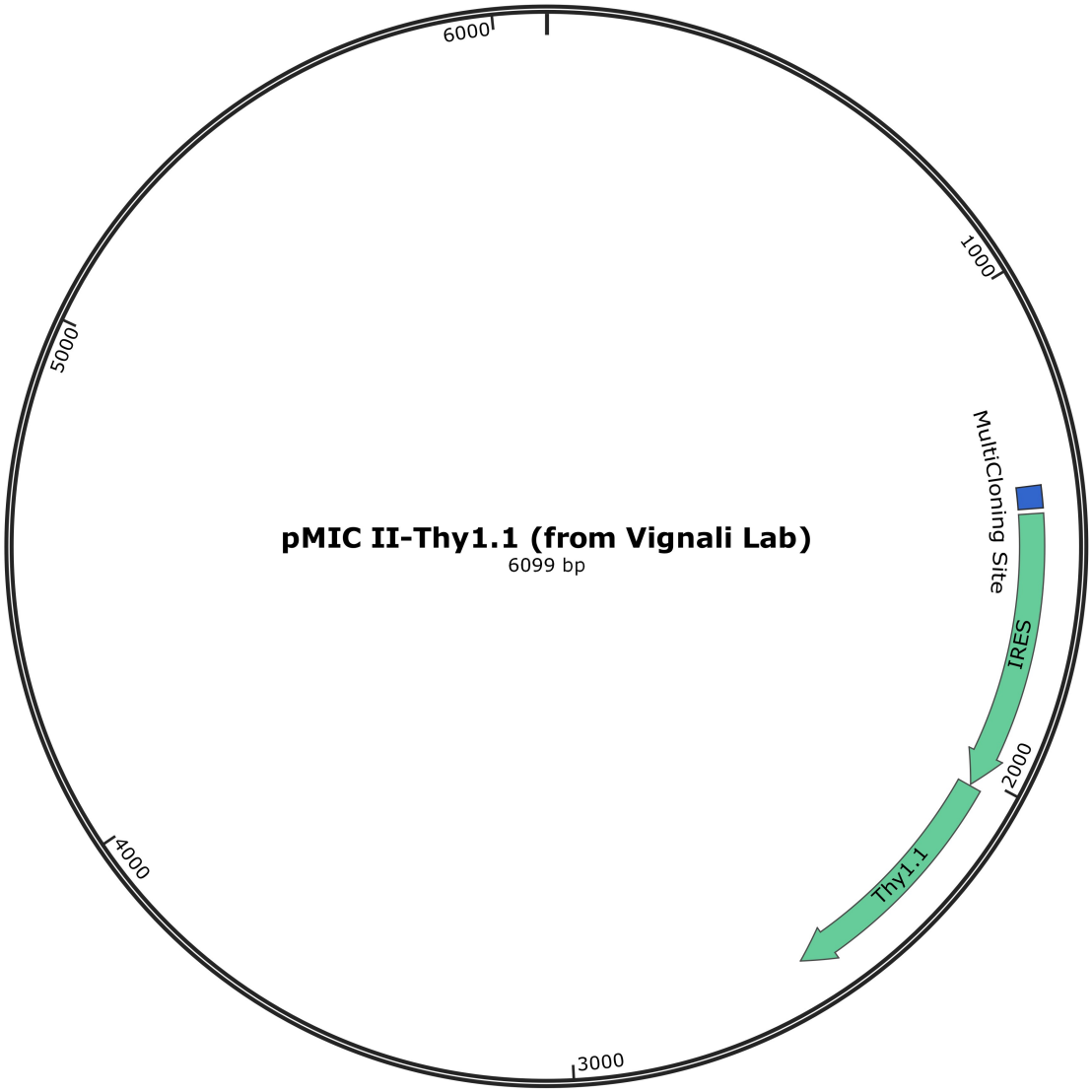

Figure 30. Empty pMIC-Thy1.1 vector (courtesy of Vignali Lab) in which BACE1 ORF was finally inserted.

Restriction enzyme	Primer sequence	Restriction site + cut pattern
BamHI	5’ CAT TAG GGA TCC GTA ATA CGA CTC ACT ATA GG 3’	G|GATCC CCTAG|G
XhoI	5’ CAT TAG CTC GAG TTA CTT GAG CAG GGA GAT GTC 3’	C|TCGAG GAGCT|C

Figure 31. BamHI and XhoI primer sequences and restriction site sequence and cutting pattern.

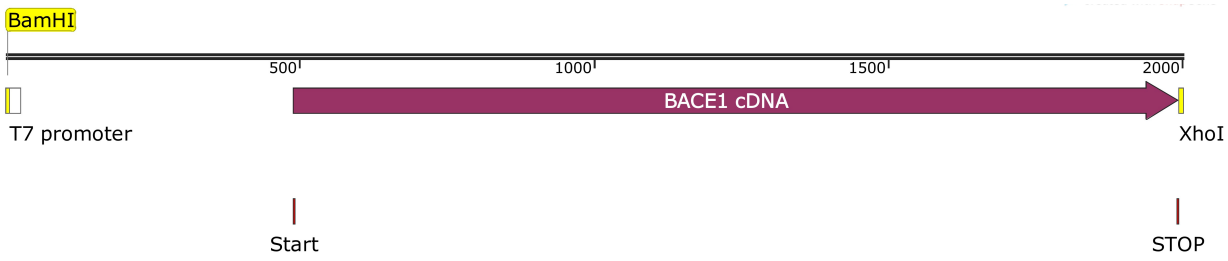

Figure 32. BACE1 final insert (BamHI - XhoI). Insert size: 2001bp

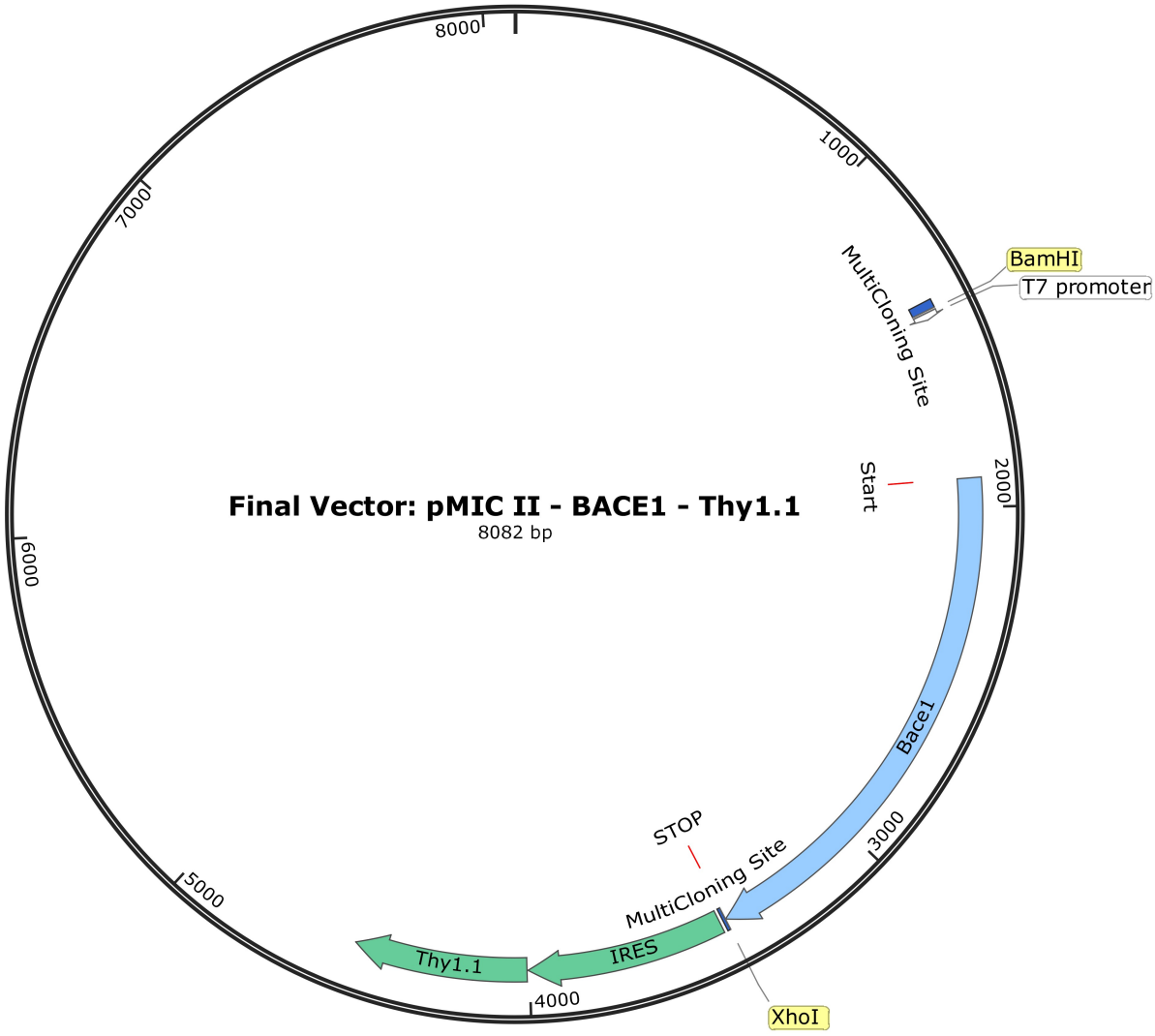

Figure 33. Final expression vector pMIC II – BACE1 – Thy1.1.

3. After cloning and expansion in E. coli, the final vector was concentrated and transiently transfected into human 293T cells. The resulting virions were used to transduce mouse GP+E-86 packaging cell lines. This multi-step process was made to ensure a switch from amphotropic to ecotropic virus and increase virus efficiency and safety. In addition, it served to generate a stably transduced cell line that constitutively produces BACE1-Thy1.1 coding viral particles.

2.2 PROTEASE-DEFICIENT BACE1 RETROVIRAL VECTOR.

Our final plasmid pMIC II – BACE1-Thy1.1 was sent to Genscript where, a single nucleotide mutation (**G**AC→**A**AC) was introduced, to change Asp 289 into Asn. This modification does not induce major structural changes in BACE1 structure but inhibits its proteolytic activity (Aspartyl proteases need two functional Asp residues in their active site). This retroviral vector is ready to transfect into 293T cells to generate viral particles.

2.3 MYC-DDK TAGGED BACE1 (LENTIVIRAL) VECTOR.

In order to better localize BACE1 expression in time, to define its subcellular localization, and to facilitate BACE1 immunoprecipitation, we purchased a lentiviral vector encoding BACE1 ORF with two tags: Myc and FLAG (DDK).

The lentiviral vector was purchased from Origene (NM_011792) and its full map is shown in Figure 34. BACE1 needs to be excised and ligated into the pMIG II-Thy1.1 vector, as previously described, for use in a retroviral system.

Bace1 (Myc-DDK-tagged) - Mouse beta-site APP cleaving enzyme 1 (Bace1), transcript variant 1, 10µg (Catalog #: MR208042)

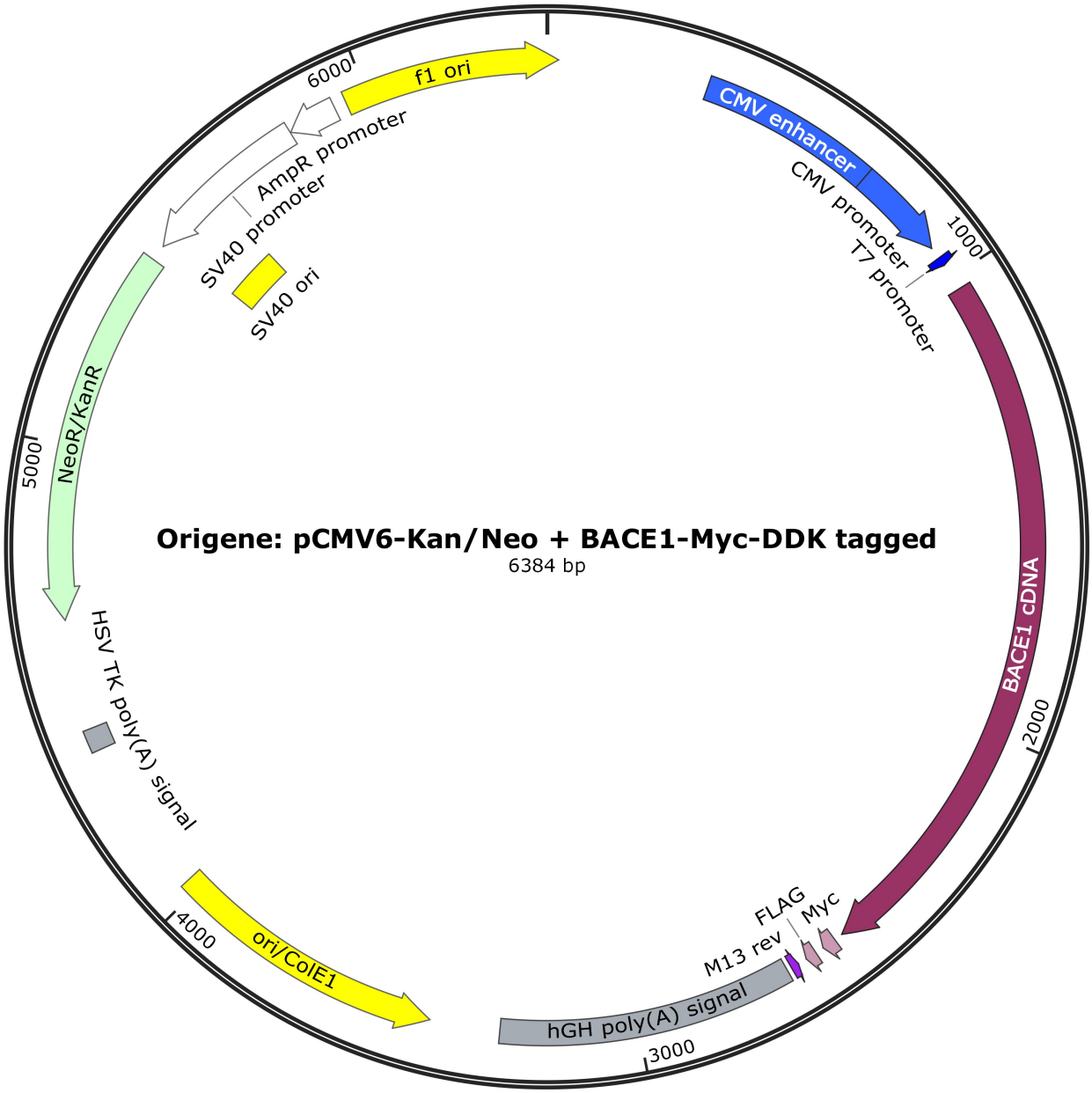

Figure 34. Origene vector encoding for BACE1 ORF with 3' Myc and FLAG tags.

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