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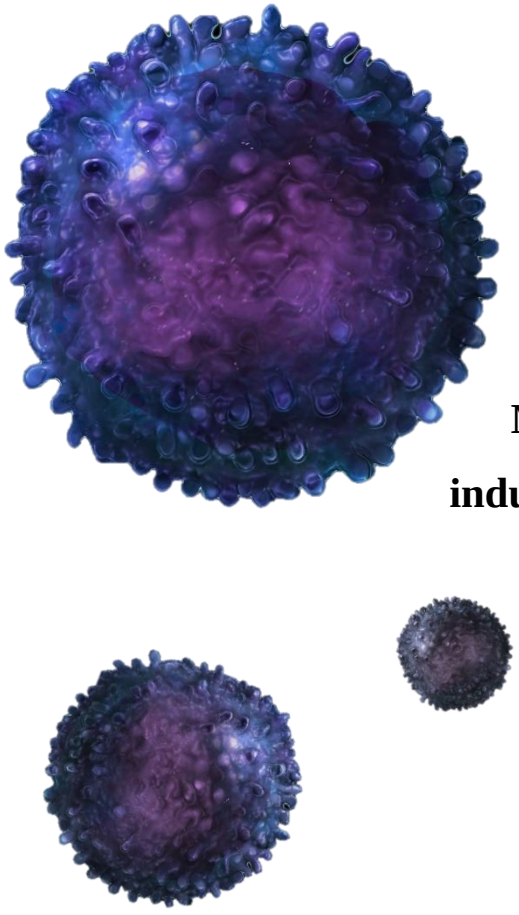

Investigation of the Molecular Mechanisms Involved in Activin-A-induced regulation of Th17 mediated CNS autoimmune inflammation

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Abstract

Introduction: Activin-A is a pleiotropic cytokine that belongs to the TGF-β superfamily. The majority of the immune cells can secrete and respond to Activin-A and its immunological relevance has been recognized in different disease contexts, where it has been associated with both pro-inflammatory and anti-inflammatory effects. Our previous studies have shown that Activin-A reduces disease severity in a mouse model of multiple sclerosis, wherein mice showed improved clinical features and decreased inflammatory immune cell infiltration in the central nervous system (CNS) upon systemic administration of Activin-A in vivo.

Hypothesis- Aims: Our hypothesis was that Activin-A can suppress Th17 cell pathogenicity through skewing their pro-inflammatory phenotype towards an anti-inflammatory one. Aim of this study was to delineate the molecular mechanisms through which Activin-A modulates Th17 cell pathogenicity.

Methods: Naïve $CD4^+$ T cells were isolated from mouse lymph nodes and spleen, skewed under Th17 pathogenic conditions in the presence or absence of Activin-A. The transcriptional and phenotypic profile of Th17 cells treated with Activin-A, were analysed by RNA-seq analysis, qPCR, Flow Cytometry, ELISA, Immunofluorescence and Western Blot. Molecular mechanisms involved in Activin-A mediated effect on Th17 cells, were analysed by bioinformatics and chromatin immunoprecipitation followed by qPCR analysis.

Results: Our studies revealed that Activin-A alters the transcriptomic profile of Th17 cells. More specifically, Activin-A signalling through its canonical pathways decreased the expression of GM-CSF, IFN-γ, IL-1β and TNF-α production, as well as, T-bet and HIF-1a transcription factors at the gene and protein level. In contrast Activin-A upregulated the expression of IL-10 immunossuppressive cytokine along with c-Maf and Ahr transcription factors. Notably, we discovered that Activin-A signaling upregulated the expression of CD39 and CD73 ectonucleotidases which mediate eATP depletion and contributed to Activin-A induced suppression of the Th17 pathogenic profile. We further analyzed the molecular pathways through which Activin-A induces these alterations in the pathogenic Th17 population wherein we observed that IL-10 upregulation occurs through the enhanced Ahr and c-Maf binding in the gene promoter region in Activin-A treated Th17 cells. Also, we discovered enhanced binding of STAT3 along with Ahr in the *entpd1 locus* (encoding CD39) and STAT3, Ahr and c-Maf increased enrichment

in the *NT5e locus* (encoding CD73) in Activin-A treated Th17 cells. Finally, we observed that Activin-A signaling decreased the expression of HIF-1a in Th17 cells through a mechanism involving Ahr-mediated upregulation of HIF-1a regulating, PHD proteins.

Conclusions: Collectively, our studies revealed that Activin-A represses the pathogenic Th17 cell phenotype by upregulating the eATP depletion pathway and the immunosuppressive cytokine IL-10, through mechanisms that involve STAT3, Ahr and c-Maf regulation. Moreover, Activin-A downregulates HIF-1a expression in Th17 cells, a key metabolic checkpoint involved in Th17 pathogenic functions. Our studies uncovered Activin-A as a negative regulator of the pathogenic profile of Th17 cells, highlighting it as a therapeutic target for MS.

1. Introduction

1.1 Multiple Sclerosis and immunopathogenesis.

Multiple sclerosis (MS) is a chronic neuroinflammatory disease of the central nervous system (CNS) that affects approximately 2.5 million people worldwide [6]. It is characterized by young age onset and a prolonged progression period that lasts about 25 years, which leads to serious and permanent clinical manifestations, such as, motor, visual and cognitive dysfunction and finally to mortality, decreasing patients' life expectancy by 5-10 years [1]. Depending on the clinical symptoms, MS is categorized into primary progressive or relapsing remitting, leading to secondary progressive multiple sclerosis. Nevertheless, it is not clear whether primary progressive MS represents a distinct form of the disease or a secondary progressive one, where the relapsing remitting phase did not manifest evident clinical symptoms [2]. During the relapse phase of the disease, the pathology includes breakdown of the blood brain barrier, peripheral immune cell infiltration, gliosis, oligodendrocyte loss, neuroaxonal demyelination and degeneration in the white matter, features that gradually expand to the grey matter [3,4]. In contrast to the grey matter lesions, rapid remyelination is triggered at lesions of the white matter after relapse in most of the patients. This is called the remission period and is accompanied with limited inflammation which helps restore functionality; however, its efficiency declines as the disease progresses, leading to permanent disability [5].

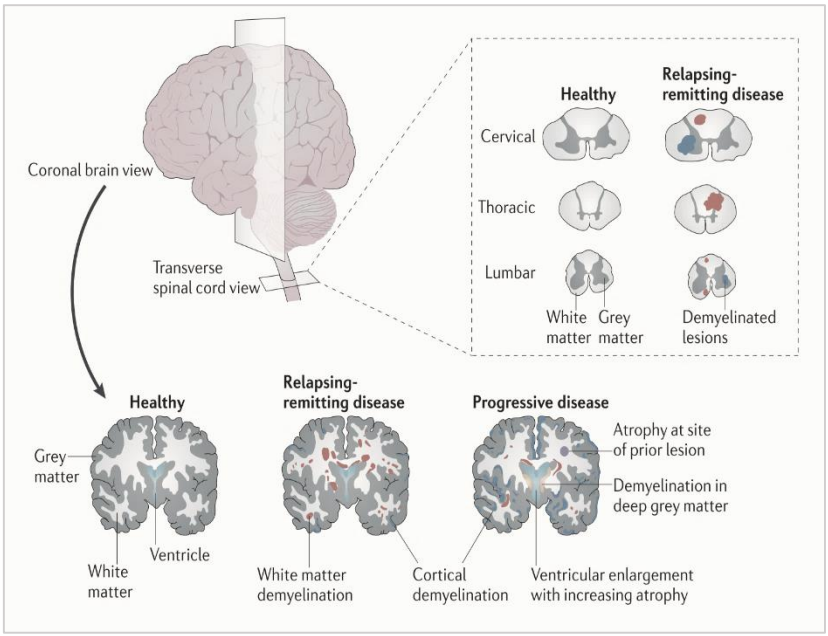

Figure 1: The pathology of Multiple Sclerosis.

Relapsing remitting multiple sclerosis is characterized by decreased myelin density at the lesions sites, where immune cell infiltration leads to oligodendrocyte loss and neuroaxonal degeneration in the white matter of brain and spinal cord. In the spinal cord, the majority of the plaques are formed at the upper and lower cervical parts whereas, at the thoracic and lumbar parts the destruction is more confined. During progressive disease, demyelination expands to the grey matter and ventricular enlargement appears as a consistence of the atrophy at the prior formed lesions.

Both the innate and the adaptive compartments of the immune system exert important roles in MS pathogenesis. Monocyte-derived dendritic cells are recruited to the central nervous system (CNS), produce pro-inflammatory cytokines, activate autoreactive $CD4^+$ and $CD8^+$ T cells and contribute to epitope spreading. Microglial cells also contribute to T cell activation, as well as, myelin destruction through phagocytosis. Depending on the cytokine milieu, microglia can also acquire an anti-inflammatory phenotype, promoting the resolution of inflammation and neuronal maintenance. Natural killer (NK), mast cells and γδ-T cells, accumulate in inflamed CNS regions and play significant roles in MS pathology [39, 3]. Nevertheless, adaptive immune responses mediated by **autoreactive $CD4^+$ T cells, $CD8^+$ T cells and B cells are the main drivers of MS pathogenesis**. More specifically, autoantigen-primed T cells, enter the CNS in the choroid plexus, meninges and the perivascular space within the parenchyma, where they acquire effector function upon local reactivation. $CD4^+$ T cells, which predominantly belong to the Th1 and Th17 lineages, mediate tissue damage through the production of pro-inflammatory cytokines, with the most important ones, being IFN-γ and GM-CSF. $CD8^+$ T cells contribute to pathology by exerting their cytotoxic effects against glial cells. B cells promote myelin destruction through the production of autoreactive antibodies and further stimulation of $CD4^+$ T cell responses [40, 3].

Regulatory $CD4^+$ T cell subsets play a crucial role in the control of autoimmune responses and impact MS progression. The first T cell lineage described to suppress effector functions from a wide variety of innate and adaptive immune cells, was the Treg lineage [79]. This T regulatory cell type is characterized as $CD4^+$ $CD25^+$, expresses the Forhead box P3 transcription factor (FOXP3) and exerts suppressive functions mainly through the reduction of proliferation and pro-inflammatory cytokine production by T effector cells and antigen presenting cells (APCs) [79]. Importantly, studies have shown that Tregs may contribute to blood brain barrier integrity maintenance [83]. Treg cells function depends on the activity of their master regulator transcription factor FOXP3, as it controls the expression of the cytotoxic T-lymphocyte antigen 4 (CTLA4) and glucocorticoid-induced TNF receptor-related protein (GITR), which are correlated with Treg-mediated immune suppression [80]. Inside the CNS, microglia have been shown to produce CCL22 and recruit Tregs. Moreover, neurons and astrocytes are capable to induce Treg cell polarization [79]. Nevertheless, during MS, the function of this T cell subset was shown to be dysregulated, as Tregs isolated from patients showed impairment in their suppressive function, most possibly, because of reduced FOXP3 expression [79, 82]. CSF isolated Tregs were shown to

be enriched in an apoptosis sensitive population characterized as $CD45RO^{hi}CD95^{hi}$, suggesting that these cells have a limited lifespan [81]. On the other hand, during remission in RRMS patients, the protein levels of FOXP3 in $CD4^+$ $CD25^+$ T cells were shown to increase again, correlated with patients' clinical improvement [79].

Another, regulatory T cell lineage with remarkable immunosuppressive capacity is the **Type 1 regulatory T cell population**. This population is characterized by high IL-10, moderate TGF-β and IFN-γ production and the surface markers $CD49b^+$, $LAG\text{-}3^+$, $CD226^+$ [84]. The lineage signature transcription factors include the avian virus oncogene musculoaponeurotic fibrosarcoma (c-Maf), the aryl hydrocarbon receptor (Ahr), the interferon regulatory factor 4 (IRF4), the repressor of GATA3 (ROG) and the early growth response protein 2 (Egr-2), which modulate the induction of key immunosuppressive molecules, such as, IL-10 and granzyme-b [84]. The IL-10 signaling pathway inhibits dendritic cell maturation and induces T cell tolerance, whereas granzyme-b provokes APC death [84]. Tr1 cells also play important role in the inhibition of excessive inflammatory responses inside the CNS, as studies have shown that, in vitro generated Tr1 cells, when transferred to mice with autoimmune CNS inflammation were capable to restrain immune responses in an IL-10 dependent manner [86]. Moreover, in the same context, it was shown that Tr1 cells restrict the function of Th17 cells, resident microglia and astrocytes [85]. As in the case of the $FOXP3^+$ Treg cell population, Tr1 cell differentiation and function was shown to be impaired in patients with multiple sclerosis with loss of IL-10 secretion and signaling has been connected to increased disease severity [87, 88, 89].

Many epidemiological and genome-wide association studies (GWAS) have identified genetic factors affecting MS risk. The epidemiological factors include microbial and viral infections associated with molecular mimicry events, the microbiome composition, vitamin D levels and dietary habits, such as, salt intake which has been shown to induce pathogenic Th17 cells [1-4, 7,8]. GWAS associated the HLA class II alleles DRB1*1501, DRB1*0301 and DRB1*1303 with increased MS risk when expressed on the surface of innate immune cells [38]. In contrast, the class I allele A2 was associated with decreased MS risk [6]. Functionally, it is considered that disease-associated MHC alleles, give rise to MHC complexes with high affinity for MS autoantigens and thus, higher presenting ability of MS autoepitopes. Importantly, the *HLA class II locus* showed the most significant association with MS. Other singe nucleotide

polymorphisms (SNPs) linked to increased MS susceptibility have been identified in several immunity-related genes, such as, the high-affinity interleukin-2 receptor complex (IL-2RA), the interleukin-7 receptor (IL7R) and the tumor necrosis factor (TNF-α) receptor [6, 38]. **Nevertheless, it is noteworthy that none of them is sufficient to induce MS alone but a combination of genetic and environmental factors is necessary for the development of clinical symptoms.**

Some of the common therapeutic strategies used to treat multiple sclerosis include chemical and biological compounds, such as, recombinant IFN-β, synthetic peptides representing myelin basic protein sequences and monoclonal neutralizing antibodies against pathogenic molecules, such as, anti-IL-23 and anti-IL-17. Most of these compounds aim to suppress lymphocytic cell activation and proliferation, promote T cell lineage skewing away from Th1/Th17 and towards Treg cells, repress inflammation through the generation of tissue-protective myelin basic peptide-specific Th2 cells, inhibit lymphocyte chemotaxis and CNS entrance and decrease the abundance of remyelination inhibitory proteins inside the lesions [2,5,9,10]. Nevertheless, these strategies only delay disease progression and are effective mostly during the RRMS stage [10]. Moreover, all of them are related with serious and hard tolerable side-effects, such as, infections sensitivity, fatigue, pain, nausea, dyspnoea, cardiac rhythm disorders and reduced numbers of blood leucocytes [2,10]. Another interesting observation is that in MS patients, characterized by more dominant Th1 responses, IFN-β therapy is beneficial while in patients with Th17-mediated pathology, its administration leads to exacerbated symptoms [11]. **Thus, there is a need to discover new immunotherapeutic strategies that can effectively suppress CNS reactive Th1 and Th17 cell responses while sparing beneficial protective immunity and thus, avoid the adverse effects of current immunosuppressive therapies.**

1.2 Th17 cell Biology and Contribution to MS Pathology.

1.2.1 Th17 cell Physiological and Pathogenic Functions.

As noted above, **Th17 cells** are considered to play a crucial role in MS pathology. An important event for a relapse initiation is the invasion of T cells inside the CNS. Brain T cell infiltration takes place when autoreactive Th17 cells expand, as they mediate in the disruption of Blood-Brain-Barrier [9, 41]. Many studies have contributed to the elucidation of the role of Th1 and Th17-cell mediated responses in MS, through the use of **autoimmune experimental**

encephalomyelitis (EAE), which is an inducible mouse model that shows the closest clinical and pathophysiological characteristics to the human disease [42]. The initial concept was that Th1 cell responses contribute to the generation and propagation of MS pathology, nevertheless, several studies later, showed that depletion of IFN-γ or IL-12 led to more severe EAE. Additionally, it was shown that only knock out mice lacking the IL-23 receptor were resistant to EAE, although the numbers of CNS immune infiltrating cells remained unaffected, suggesting that this effect was correlated with different cell type composition [42-44]. Moreover, they identified that this was due to the lack of pathogenic Th17 cells which are capable to expand in the presence of IL-23 and exert pro-inflammatory functions [42]. Interestingly, monocyte-derived dendritic cells in MS lesions produce high levels of IL-23 and enhance Th17 cell generation [45]. Studies using a Th17 cell adoptive transfer model, as well as, IL-17 blocking experiments showed EAE worsening or protection respectively, highlighting the importance of Th17 cells in MS pathology [42, 44, 46, 47]. More detailed analysis, revealed that the encephalitogenicity of Th17 depends on IL-1β and IL-23 signalling pathways which promote the production of several pro-inflammatory molecules such as, GM-CSF and TNF-α [49, 55]. Importantly, IL-17 was shown to promote the generation of ectopic tertiary lymphoid follicle-like structures inside the CNS, an event that plays a crucial role in MS pathology, as autoreactive B cells localize in these structures and expand leading to demyelination [12]. Moreover, it was shown that T-bet inhibition ameliorated EAE through the reduction of Th1 as well as pathogenic Th17 cell populations [46].

In MS patients, studies showed that IL-17 protein levels are increased in the serum, brain and cerebrospinal fluid (CSF) compared to healthy controls and correlate with disease severity and relapse rate [45, 46, 48]. Transcriptional profiling of pathogenic Th17 cell populations isolated from MS patients showed that these cells have an extended cytokine profile, as they secrete IFN-γ and other Th1 signature molecules along with increased CXCR3 that is thought to participate in Th17 cell infiltration inside the CNS and reduced production of IL-10 [46, 50, 51]. Also, it is important to note that not only Th17 cells produce IL-17 in active MS lesions but also $CD8^+$ T cells, astrocytes and oligodendrocytes highlighting the role of this cytokine in disease pathology [52]. **Thus, it is crucial to further understand Th17 biology and find ways to restrain Th17 cell pathogenicity in the context of CNS autoimmunity.**

1.2.2 Molecular Mechanisms underlying Th17 cell Differentiation.

Th17 is a distinct lineage of T helper cells, characterized by the expression of the signature cytokines IL-17A, IL-17F, IL-21 and IL-22, as well as, the chemokine CCL20, and its receptor CCR6 [13]. Th17 cells localize mostly at the mucosal barriers and their generation is thought to take place in the intestine [14]. **Many studies have unravelled the physiological role of Th17 cells and recognized them as key mediators of host defence against extracellular bacteria and fungi, a role consistent with their signature cytokine profile** [12,13]. More specifically, the majority of cell types in the gastrointestinal tract are capable to respond to IL-17, in a revolutionary-conserved way, wherein, they secrete tight-junction proteins to avoid pathogen spreading, as well as, chemokines and cytokines that lead to immune cell recruitment and pathogen clearance [14, 15]. IL-22 acts in synergy with IL-17 and promotes the production of chemokines and matrix metalloproteinases, mediating tissue repair and maintenance [13-15]. IL-21 also has an important role in generating a positive autocrine feedback loop, leading to Th17 cell lineage stabilization and commitment. Furthermore, CCL20 which is secreted in response to pathogens, recruits Th17 cells through its interaction with CCR6, and CCL20 production by Th17 cells, further boosts the Th17 cell response [13]. Interestingly, the CCL20/CCR6 axis is proposed to play another important role during inflammation by recruiting a big proportion of circulating Th17 cells to the small intestine, helping to avoid exacerbated inflammatory responses [16].

The generation of the Th17 cell lineage was shown initially to depend on TGF-β and IL-6 signalling, with IL-6 playing an important role in the stabilization of the Th17 cell differentiation program through the inhibition of the Treg cell polarization pathways [53]. Later studies showed that TGF-β signalling is dispensable for Th17 cell commitment, which can be polarized through alternative protocols that included the combination of IL-6, IL-1β and IL-23 or IL-6, TGF-β3 and IL-23 stimulation [29, 54]. Importantly, adoptive transfer experiments revealed that Th17 cells differentiated in the presence of TGF-β and IL-6 were not sufficient to promote EAE, in contrast to Th17 cells polarized under the alternative protocols, which were shown to be highly pathogenic, promoting increased EAE severity and mortality [29, 54]. Subsequent studies aiming to elucidate the phenotypic differences of these Th17 cell subsets revealed that Th17 cells polarized with IL-6, IL-23 and IL-1β or TGF-β3, were characterized by the induction of the T-bet transcription factor and a decrease of Ahr and c-Maf transcription factors, features that led to high levels of GM-CSF and IFN-γ production accompanied by IL-10 downregulation [29, 49, 54, 56]. Further evidence

about the pathogenic potential of Th17 cells came from the observation that cells isolated from draining lymph nodes of pathogenic Th17 cell adoptively-transfered mice were enriched in IL-17^{+}, IFN-γ^{+} and also double IL-17^{+} IFN-γ^{+} populations [54].

In human studies, the necessity of TGF-β in Th17 cell lineage generation is controversial [18,57,58]. Furthermore, interesting results emerged from a comparative study of the human and mouse Th17 cell transcriptome. This study revealed that cis-type of gene regulation and also the general mRNA profile is more conserved between human and mouse Th17 cells, compared to trans-regulation and long non-coding RNAs (lncRNAs) expression which was shown to be important mainly for the human Th17 cell lineage [19].

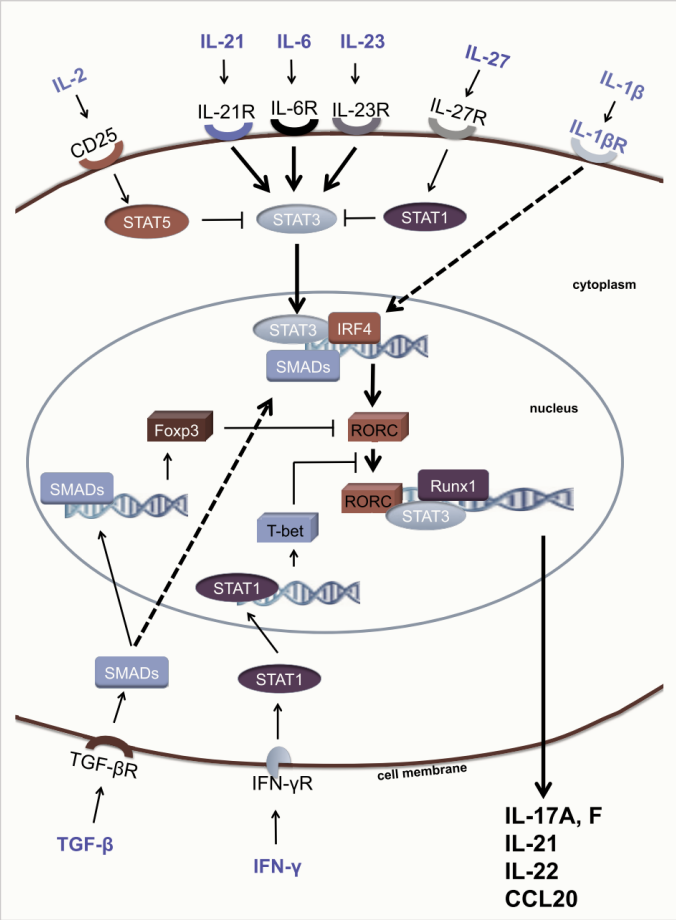

Figure 2: Mechanisms of Th17 cell Differentiation.

Several cytokines contribute to Th17 differentiation. TGF-b through the activation of the secondary messengers SMAD2/3 can drive the expression either of FOXP3 or RORc. In the presence of IL-6 and IL-1b, the Treg program is suppressed as these factors, through STAT3 induction, upregulate HIF-1a which in turn targets FOXP3 for proteasomal degradation [66]. So, during Th17 polarization, phosphorylated SMAD2/3 and STAT3, together with IRF4 transactivate the promoter of RORc. Then, the master regulator of the Th17 lineage drives the expression of the Th17 signature molecules, IL-17, IL-21, IL-22 and CCL20. The secreted IL-21, generates an autocrine loop through which maintains the Th17 program via preservation of STAT3 activity. IL-23 is also expressed at a later stage of Th17 differentiation and has an important role in Th17 lineage maintenance. Factors that are known to attenuate Th17 program include IL-2 and IL-27 through the activation of STAT5 and STAT1 respectively. That leads to the inhibition of STAT3 activity. Moreover, despite the fact that Th17 cells express in a small proportion T-bet, increased amounts of extracellular IFN-g can increase further T-bet levels which binds Runx1, disrupts its association with RORc and restrains Th17 differentiation.

1.2.3 Th17 cell Molecular Profile.

Several recent studies have contributed to the elucidation of the molecular pathways and the transcription factors that control Th17 cell lineage commitment. The core of the transcription factors, participating in Th17 cell specification consist of IRF4, BATF, STAT3, RORc and c-Maf

[20]. Among these, IRF4, BATF and STAT3 are shown to be induced early during Th17 cell differentiation (up to 4h) and participate in almost all Th17 cell lineage-specific gene expression. In contrast, RORc mRNA is induced at an intermediate phase (between 4h and 20h) with its protein levels showing an increase after 20h, something unexpected considering its significant role in Th17 cell commitment. During this late stage, the expression of Ahr starts. Finally, in the later stage of Th17 cell differentiation (between 20h and 72h) the upregulation of Th17 cell signature cytokines is observed. Interestingly, IL-23 is induced after 48h of cell differentiation validating its role in late Th17 cell fate commitment [22].

IRF4 and BATF, along with p300, were shown to be the pioneer transcription factors, promoting chromatin remodelling changes in specific DNA regions of Th17 cells, in order to make them accessible to the rest of the core transcription factors, such as, STAT3, RORc and c-Maf and generate an active transcriptional complex. These five TFs show high degree of colocalization in many Th17 cell gene loci with BATF and IRF4 physically interacting with each other. Importantly, RORc-dependent loci have been shown to require the recruitment of the total core transcription factors complex for gene regulation. In conclusion, it is believed that as the IRF4/BATF complex occupies a variety of chromatin regions in resting T cells, the recruitment of STAT3 and RORc during Th17 cell polarization to some of these loci, promotes Th17 cell specification versus other T helper cell lineages [20].

Exploring the role of cytokines in the Th17 cell lineage commitment, studies have revealed that TGF-β promotes Th17 cell specification by silencing Th1/Th2 cell alternative fates **[**21]. IL-6 and IL-1β, via STAT3 activation, promote RORc, IRF4, BATF, c-Maf, Ahr, HIF1a and IL23r expression and lead to the expression of Th17 cell signature genes. Finally, IL-23 acts at a later stage to maintain STAT3 levels and Th17 cell identity [17, 23, 54]. Studies have shown that Th17 cell intrinsic IL-1β production, through inflammasome activation, is important for Th17 cell generation, viability and effector functions [24]. On the other hand, IL-23 signalling is associated with the induction of many factors that affect the phenotype and pathogenicity of the cells. It is shown that IL-23-induced STAT3, in coordination with Ahr, binds and transactivates Aiolos gene promoter, an important transcription factor for Th17 cell lineage specificity [25]. Furthermore, the transcription factor RBPJ was identified to form a complex with RORc and activate IL-23 promoter [69]. The transcription factor Blimp-1, also a downstream effector of IL-23 signalling,

colocalizes with STAT3, RORc and p300 leading to the upregulation of IL-23, IFN-γ and CSF2. Thus, the signalling of these transcription factors establishes a pathogenic signature while additionally, it represses IL-2 expression in Th17 cells, which is known to inhibit Th17 cell generation through downregulation of IL-6R [26,27]. Importantly, Th17 cell differentiation in the presence of IL-23 activates JunB in a STAT3 dependent manner, which shows enrichment in Blimp-1 and RORc promoters, along with BATF and IRF4 [28]. Consequently, JunB transcription factor drives Th17 cells towards a pathogenic phenotype. IL-23 signalling upregulates TGF-β3 which also promotes a pathogenic Th17 cell signature through downregulation of c-Maf, Ahr and IL-10 expression along with upregulation of CSF2 and IFN-γ [29]. It is important to note that IL-23 also activates STAT4 and T-bet, something that explains the production of IFN-γ by Th17 cells [28,29]. **The expression of Th1 cell signature genes by pathogenic Th17 cells leads to the assumption that the molecular profile of Th17 cells is sensitive to environmental changes that could shift the cell phenotype of the cells towards another Th cell lineage.**

Apart from the transcription factors referred above as key modulators of Th17 cell specification with some of them driving Th17 cells to acquire pathogenic characteristics, there are several regulatory factors that suppress the establishment of the pathogenic gene signature on Th17 cells. Among these, c-Maf acts as a Th17 cell repressor through negative regulation of BATF expression and upregulation of IL-10 in synergism with Ahr [20]. Moreover, the presence of the phosphatase DUSP in Th17 cells, decreases the propagation of STAT3 signalling by dephosphorylating the protein. The endoribonuclease MCPIP1 inhibits IL-17 signalling by degrading IL-6 mRNA [30,31]. FOXP1 restrains Th17 cell signature through downregulation of IL-21 and FOXO1 inhibits the Th17 cell program via physical interaction with RORc and blockage of its transcriptional activity [67,68]. IRF8 interacts with RORc and mediates suppression of IL-17 transcription and IRF1 negatively regulates the core Th17 cell network through the promotion of alternative chromatin remodelling as it is shown to participate along with BATF, in Tr1 cell polarization [32,33]. **Therefore, the balance of several transcription factors in the Th17 cells can modulate cell fate decision and lead to the generation of beneficial or pathogenic Th17 cell populations.**

1.2.4 Th17 cell Plasticity.

As mentioned above, the Th17 cell lineage is sensitive to environmental changes and is characterized by high plasticity. Methylome analysis revealed that Th17 cells cluster closer to $CD4^+$ T naive cells than Th1 cells and also exhibit more demethylated regions, something that supports the ability of these cells to change their transcriptional profile rapidly, in response to changing environmental stimuli [34]. Studies by using fate reporter mice to trace Th17 cells in vivo identified the ability of these cells to transdifferentiate into pathogenic Th1-like or suppressive Tr1-like cells depending on the immune context [36, 37]. Notably, histone modification analysis on the IFN-γ promoter showed that in Th17 cells it is highly poised for transition to transcription but lacks remodelling marks in crucial cis-regulatory elements. Moreover, they demonstrated that in the presence of IL-12, STAT4 and T-bet are elevated in Th17 cells and promote epigenetic changes that lead to IFN-γ production [35].

Pertinent to the transdifferentiation of Th17 cells to Tr1-like cells, the authors showed that Ahr activation, played a critical role in this process, as it increased IL-10 expression [37]. Indeed, it was shown that Ahr, which is a ligand activated transcription factor that gathers cell intrinsic and extrinsic signals and impacts T cell function, has an important role in the generation of Tr1 cell lineage. Ahr modulates IL-10 production and controls metabolically these cells, by inducing the expression of key metabolic enzymes [64, 65]. Moreover, the same study highlighted the antagonistic roles of Ahr and HIF-1a transcription factors as it was shown that the one downregulates the other at the protein level [64]. HIF-1a is another well characterized transcription factor which participates in Th17 cell differentiation and in several pro-inflammatory gene induction [66].

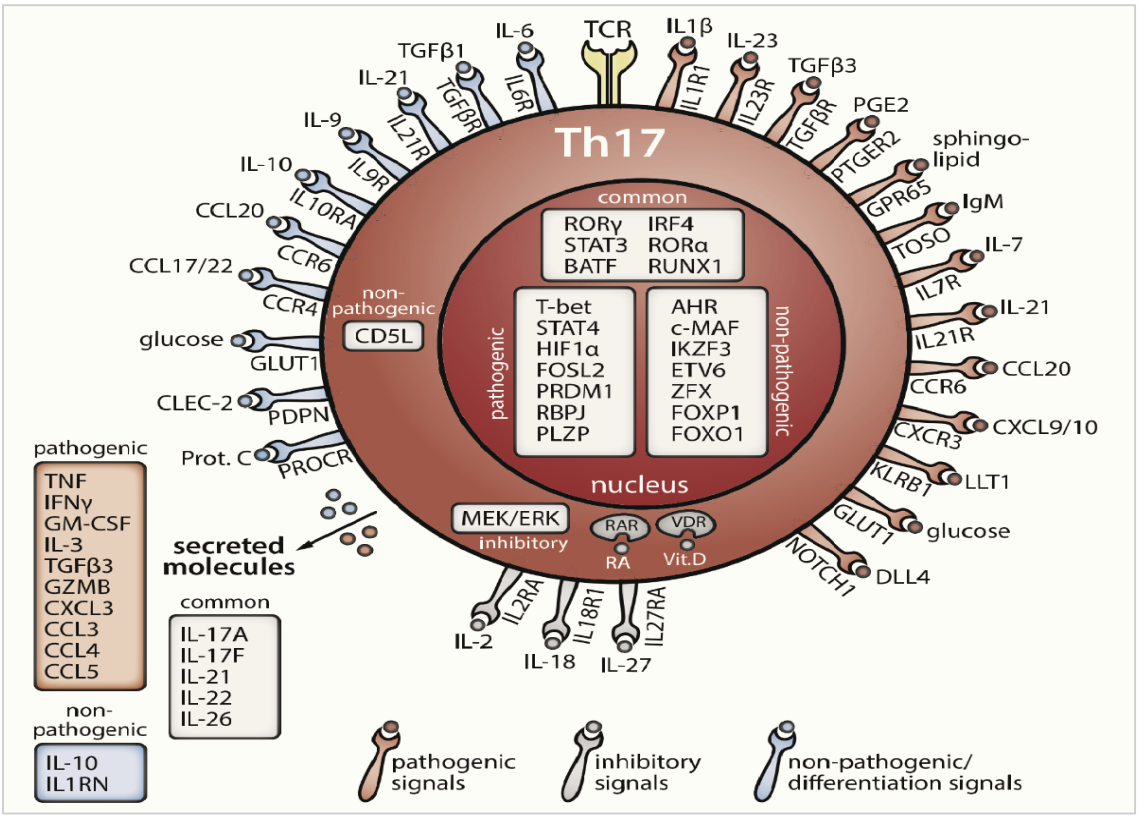

Figure 3: Th17 cell plasticity.

Th17 cells show an impressive capacity to respond to environmental stimuli and change their phenotype and inflammatory potential. Specifically, in addition to the core transcription factors that are required for Th17 lineage commitment and maintenance, a diverse of extracellular molecules can alter the expression of secondary lineage transcription factors that can lead to the establishment of either a pro-inflammatory Th1-like or anti-inflammatory Tr1-like profile. As it was referred above, the transcription factors T-bet and STAT4 establish a pathogenic profile through the induction of IFN-g. HIF-1a establishes a highly glycolytic profile necessary for Th17 pathogenicity, as it upregulates further glucose uptake via induction of GLUT1 and participates in IL-23R expression along with RBPJ and RORc [69, 70]. FOSL2 maintains the expression of Th17 survival genes, BLIMP-1 participates in the production of the pathogenic cytokines IFN-g and GM-CSF and PLZP contributes to the expression of other pro-inflammatory molecules such as CCL20 and TNF-a [20, 26, 67]. On the other hand, Ahr and c-Maf drive a Tr1-like anti-inflammatory phenotype through the induction of IL-10. ETV6 and ZFX drive a more benign Th17 phenotype with self-renewing properties in the lymph nodes, and FOXP1 as well as FOXO1 inhibit the Th17 cell intrinsic differentiation program [67]. Additionally, retinoic acid and vitamin D restrain Th17 program and promote an anti-inflammatory phenotype, as they contribute to the induction of iTreg and Tr1 cells respectively. Importantly, CD5L intrinsic expression, generates non-pathogenic Th17 cells, as it alters the intracellular lipidome, through the restriction of the lipogenesis and cholesterol biosynthesis pathways that lead to decreased RORc ligand availability [71].

1.2.5 Aryl Hydrocarbon Receptor, Hypoxia Inducible Factor and their Impact on Th17 cell Specification.

The transcription factor Ahr, which was initially considered to be a modulator of xenobiotic metabolism, was shown to play a critical role in immunity, T cell differentiation and function and its biological implications have been under extensive investigation the past years. Ahr belongs to the family of bHLH PAS (basic helix-loop-helix PER-ARNT-SIM) domain

transcription factors and can be bound and activated by a wide range of ligands derived from endogenous metabolic pathways, commensal flora, pollutants and dietary substrates [91]. The HLH domain contributes to DNA binding and the PAS domain is involved in protein-protein interactions and ligand binding. The inactivated form of the transcription factor forms a complex in the cytoplasm with the 90 kDa, heat sock protein (HSP90), the Ahr interacting protein (AIP) and the cochaperone p23. HSP90 binds to Ahr in two distinct regions in the HLH and PAS domains, inhibiting constitutive DNA binding and keeping Ahr in a conformation with high ligand binding affinity respectively. AIP protects Ahr from proteasomal degradation and p23 interacts with HSP90 and Ahr, preventing abnormal nuclear translocation and partner interaction [92]. Upon ligand binding, conformational changes are induced which expose the nuclear localization signal, allowing nuclear entrance. Once in the nucleus, Ahr forms heterodimers with other co-factors, with most the common one, the aryl hydrocarbon receptor nuclear translocator (ARNT), which is a constitutive nuclear protein. ARNT binding leads to HSP90 and p23 displacement and generates the transcriptionally active complex. This complex binds to specific DNA sequences called xenobiotic response elements (XRE) and promotes neighbouring gene expression [91,92]. Nevertheless, alternative partners of Ahr have been identified, such as the NF-Kb subunits RelA and RelB, as well as, members of the kruppel like factors family, which were shown to bind in non XRE sequences and regulate alternative gene loci [91,93].

Moreover, Ahr can affect cell function non-canonically, with the most characteristic feature, the interaction with estrogen receptor (ER) pathway and modulation of its protein levels [91-93]. Apart from these direct effects on transcription, Ahr was shown to form complexes with other protein partners affecting chromatin remodeling and local histone modifications [91,92]. Moreover, Ahr can form complex with the cullin 4B ubiquitin ligase and target proteins for degradation [92]. The Ahr signaling was shown to be activated transiently with some variations depending on the ligand type [93]. Importantly, protein kinase C was shown to inhibit nuclear translocation and gene transactivation by Ahr through phosphorylation of its nuclear localization signal [90]. Thus, Ahr signaling initiation, maintenance and inhibition, as well as alternative gene regulation are complex and still poorly understood aspects of Ahr biology.

Concerning its role in T cell differentiation and function, Ahr was shown to promote either pro-inflammatory or anti-inflammatory gene expression depending on the cell type as well as the

activating ligand, which impacts co-activators interactions. It is expressed significantly in Th17 cells, and in Treg and Tr1 cells [92]. In $FOXP3^+$ Treg cells, Ahr activation by endogenous and dietary ligands activates a signaling pathway which is shown to involve the transcription factors SMAD1 and Aiolos, leading to FOXP3 expression and Treg cell program establishment [93]. In Tr1 cells, Ahr was shown to interact with c-Maf and transactivate the IL-10 promoter [94]. Importantly, Ahr was also shown to participate in IL-21 expression which is a crucial cytokine for Tr1 cell induction and maintenance [94]. Moreover, as referred above, Ahr controls metabolically Tr1 cells through regulation of metabolic gene expression and inhibition of a HIF1a transcription program which blocks Tr1 cell differentiation [65]. IL-17 producing cells also express high levels of Ahr and several studies have shown that it controls the expression of IL-21 and IL-22, two cytokines with an important role in Th17 cell differentiation and maintenance. Moreover, it is believed that, during Th17 cell polarizing conditions, STAT3 promotes Ahr expression and together contribute to Aiolos induction, that leads to IL-2 silencing and promotion of the Th17 cell program [93, 25]. Additionally, Ahr is thought to promote Th17 cell lineage through the inhibition of STAT5 and STAT1 phosphorylation and thus, maintaining STAT3 activation [95, 96]. Nevertheless, it is known that Ahr expression in Th17 is correlated with the non-pathogenic population. Also, higher levels of Ahr in combination with c-Maf contribute to Tr1 cell lineage skewing through the inhibition of HIF-1a and the induction of IL-10, IL-21 and the immunosuppressive molecule CD39 which is involved in the extracellular ATP depletion pathway [91].

Hypoxia inducible factor is a basic loop-helix-loop transcription factor containing a DNA recognition domain and a transactivation domain through which it binds coactivators, such as CBP and p300 [97]. The regulation of HIF1a function occurs mainly through the action of iron-dependent enzymes prolyl-hydroxylases (PHDs). There are three PHD proteins which are located in the cytoplasm and their function is controlled by oxygen concentration. When oxygen availability is high, PHDs are activated and hydroxylate HIF1a at specific proline residues, making it recognizable by the E3 ubiquitin ligase, von Hippel-Lindau (VHL) and thus, promoting its proteasomal degradation [98]. Another factor modulating HIF1a activity is the factor inhibiting HIF (FIH) which hydroxylases HIF on an asparagine residue of the transactivation domain and blocks HIF1a interaction with its cofactors [98]. Thus, HIF1a normally is found at the cytoplasm and hydroxylases activity control its protein levels. Conditions of hypoxia deactivate these

enzymes, thereby stabilizing HIF1a protein which translocates to the nucleus, interacts with its partners and modulates gene transcription [97,98]. As in the case of Ahr, inside the nucleus, HIF1a forms a heterodimer with ARNT (or HIF1b) and activates transcription in collaboration with other cofactors through binding to specific DNA sequences called hypoxia response elements (HREs) [97]. In the context of T cell biology, HIF1a activation can occur through additional ways. TCR, stimulation is shown to upregulates HIF1a through the PI3K/mTOR pathway [99]. Moreover, STAT3 signaling activated by pro-inflammatory cytokines, such as IL-6, upregulates HIF1a [99]. Lastly, NF-κB signaling, can also upregulate HIF1a expression in T cells and reactive oxygen species produced during T cell activation are shown to stabilize HIF1a [98, 99].

HIF1a activity impacts cell metabolism and more specifically, is correlated with the induction of genes related to glycolysis, such as, glucose transporters and glucose metabolizing enzymes [100, 108]. T cell phenotype is shown to be significantly affected by the metabolic pathways that are established inside the cell. Notably, certain metabolites can be used as substrates for histone and DNA modifying enzymes that affect cell epigenome and thereby, gene expression and T cell function [101]. Moreover, T effector cells are shown to preferentially operate glycolysis. Although glycolysis is not an efficient pathway for ATP production compared to oxidative phosphorylation, it provides substrates for biosynthetic pathways, supporting fast cell growth, proliferation and cytokine production. In Th17 cells, activated STAT3 is shown to bind and activate the promoter of HIF1a [102]. The presence of HIF1a is necessary for the establishment of the glycolytic signature of the Th17 cell lineage and supports their effector function [103]. Importantly, HIF1a forms a complex with RORc, STAT3 and p300 and cooperatively induce proinflammatory cytokine expression, while, through interaction with the E3 ligase VHL targets FOXP3 for proteasomal degradation and thereby inhibits Treg cell differentiation [66]. In the Tr1 cell population, HIF1a was shown to control the metabolism and promote Tr1 cell lineage commitment, during the first 24 hours. After this timepoint, HIF-1a exerts an inhibitory role through the antagonism with Ahr for ARNT binding, promotion of Ahr degradation and inhibition of Tr1 cell metabolizing enzymes and signature immunosuppressive molecules [65]. On the other hand, in order to promote the Tr1 cell program, Ahr mediates the degradation of HIF1a through the upregulation of the PHD enzymes and blocks HIF1a partner interaction activity via induction of FIH [65].

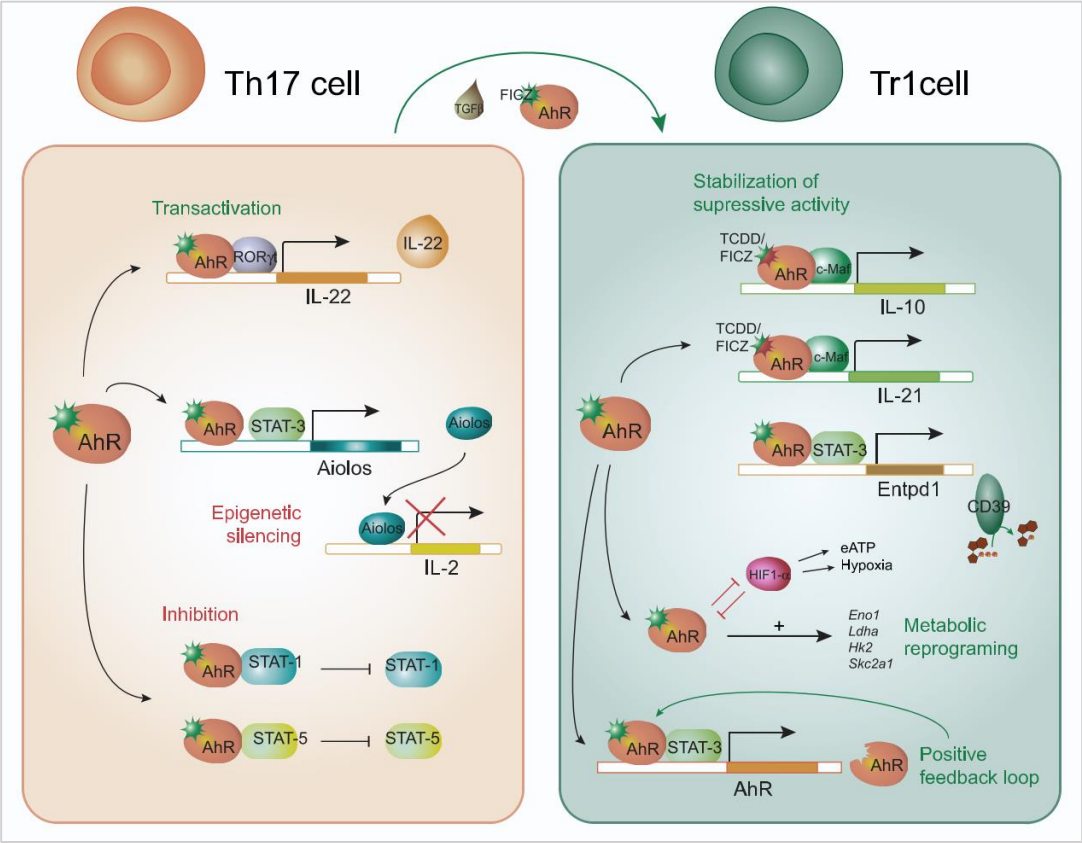

Figure 4: Ahr role in Th17 specification and Tr1 transdifferentiation.

Ahr has been shown to play an important role in the generation of the non-inflammatory population of Th17 cells. In complex with RORc, Ahr induces IL-22 production and in synergism with STAT3, upregulates Aiolos which represses the transcription of the Th17 inhibitory cytokine IL-2. Moreover, Ahr reduces the phosphorylation of STAT1 and STAT5 which interfere with STAT3 signalling and antagonize Th17 generation. Nevertheless, when these cells acquire environmental stimulus that promote Ahr and c-Maf upregulation and activation, can transdifferentiate into the immunosuppressive lineage of Tr1 cells. At this context, Ahr forms a transcriptional activation complex with c-Maf and promote the expression of IL-21, IL-10 and CD39 while binding together with STAT3 at the Ahr gene promoter, generate a positive feedback loop for its own production. More importantly, Ahr contributes to the metabolic reprogramming of the cells, as it inhibits the activation of HIF-1a either through binding to the common co-activator ARNT or by enhancing the HIF1a protein degradation pathways, and also, via participation in the transcription of key Tr1 metabolic enzymes.

1.3 Activin-A Biology.

1.3.1 Activin-A Signalling.

Activin-A is a pleiotropic cytokine that belongs to the TGF-β superfamily. It is proposed to impact several important biological processes, such as, reproduction, embryogenesis, erythropoiesis, osteogenesis, energy metabolism, neuronal function and inflammation. Activin A is formed by the homodimerization of two pro-domains, called bA. For the production of the mature protein, after dimerization, follows cleavage of the pro-domains, although the pro- and mature domains remain connected through weak interactions, protecting Activin A from proteolysis. After secretion and receptor binding, the pro-domains are getting displaced and activate signal transduction.

There are two type I and two type II Activin-A receptors that generate different combinations with each other and assembly the total receptor complex upon ligand binding. The type I receptors include ALK4 and ALK7, whereas Activin-A favours ALK4 binding and show lower affinity for ALK7. The type II receptors are the ActRII and ActRIIb. Interestingly, ActRIIb gene produces 4 alternative spliced transcripts that show different binding affinities for Activin A. Once Activin-A is bound to its type II receptor, two type I receptors are recruited and phosphorylated, an event that leads to the activation of their kinase activity. Type I receptors then, phosphorylate SMAD2 and SMAD3 proteins which along with SMAD4 translocate to the nucleus where they perform activation or silencing of gene expression. SMAD7 is the main inhibitory SMAD of Activin A signalling, preventing SMAD4 binding to SMAD2/3 complex and therefore, their transcriptional activity. Moreover, Activin-A is capable to activate, depending on the cell type, alternative, non-SMAD secondary intracellular signalling molecules, such as, p38 MAPK, ERK1/2 and JNK which impact cell migration and differentiation properties [59, 60]. Moreover, Activin A can affect the PI3K/Akt and the canonical Wnt signalling pathways, features that highlight the complexity of its signalling [72]. Extracellularly, activin signalling can be blocked by follistatin, a secreted protein, which binds to Activin-A and inhibits its receptor interaction [72].

1.3.2 Activin-A in the Regulation of Immunity.

In the context of immunity, most of the immune cell types have been shown to produce and respond to Activin-A, such as monocytes, macrophages and microglia, mast cells and dendritic cells, B and T lymphocytes. Nevertheless, Activin-A has a controversial function depending on

the cell type and the spatio-temporal context, exerting both pro- and anti-inflammatory effects [61]. Activin-A released by dendritic cells in response to microbial substrates or CD40 ligand, was shown to downregulate several cytokine and chemokine production in an autocrine/paracrine manner, contributing in this way to prevention of exacerbated immune responses [60]. Moreover, activated $CD4^+$ $CD25^-$ T cells were shown to produce Activin-A and promote alternative polarization of macrophages into the M2 immunoregulatory phenotype [60]. In macrophages, Activin-A was demonstrated to inhibit phagocytosis and MHC class II expression and reduce IFN-γ and nitric oxide production (NO). On the other hand, in dendritic cells, Activin A decreased their antigen presentation capacity, while, in Th2 cells, it inhibited proliferation and cytokine production. Activin-A was capable to enhance IgG and IgE production when acting on resting B cells. Moreover, in monocytes Activin-A stimulated migration and inflammatory features acquirement [60].

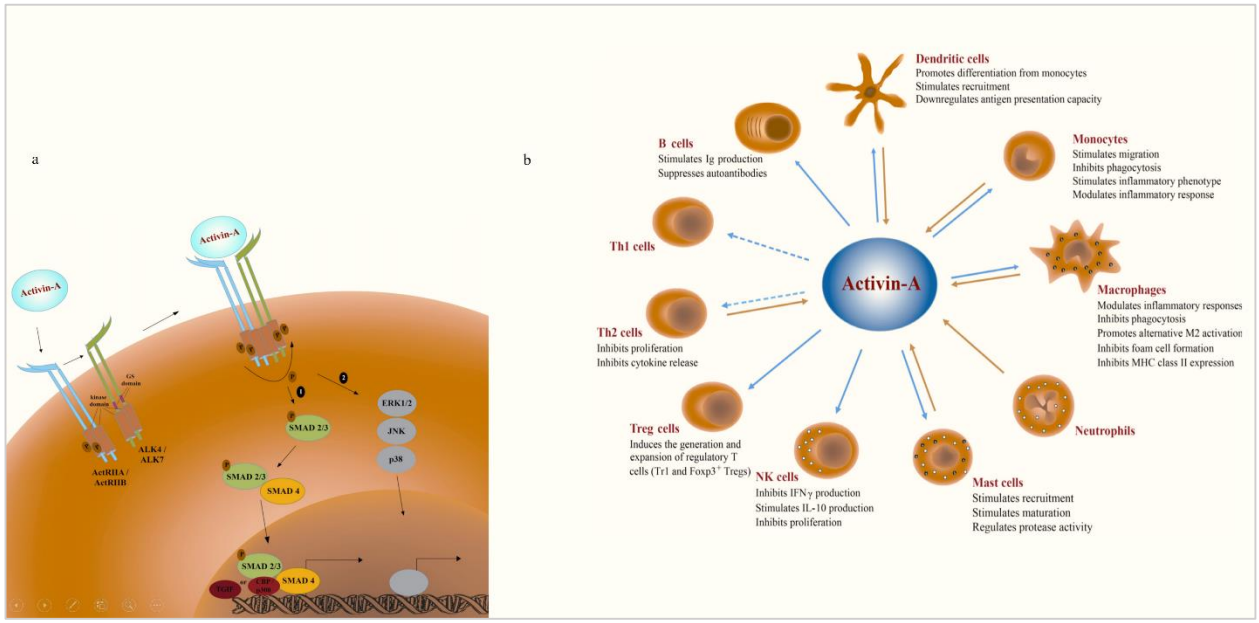

Figure 5: Activin-A signalling and immune cell function

(a) Signalling of Activin-A is mediated through a membrane heterotetrameric receptor complex. Activin-A binds to the constitutively active ActRII (ActRIIA or ActRIIB) which then recruits and phosphorylates ActRI (ALK4, ALK7 or ALK2). According to the canonical signal transduction pathway, activated ActRI phosphorylates the main intracellular mediators of activin-A signaling, Smad2/3. pSmad2/3 form a complex with Smad4 and translocate to the nucleus, where they regulate the trancription of target genes in cooperation with several transcription co-factors (e.g CBP/p300 or transforming growth interacting factor (TGIF) (1). In the non-smad-mediated signal transduction pathway, ActRI activates the ERK, p38 or JNK kinases which in turn regulate the transcription of target genes (2). **(b)** Activin-A is produced by monocytes, macrophages, dendritic cells, neutrophils, mast cells and Th2 lymphocytes (yellow arrows). The effects of activin-A on distinct immune cell subsets are also depicted (blue arrows). The role of activin-A in Th1 and Th2 cell responses remains to be established (broken arrows).

In the context of disease, Activin-A expression was correlated both with beneficial and adverse features. In rheumatoid arthritis, Activin-A promoted a pro-inflammatory macrophage polarization in the synovium. In experimental colitis models, increased Activin-A levels in the gastrointestinal tract correlated with disease severity [73, 74]. Additionally, pregnant women with systemic lupus erythematosus had increased serum Activin-A levels [75]. In contrast, in acute coronary syndrome patients, Activin-A had a potential anti-inflammatory role and in pulmonary alveolar proteinosis reduced levels of Activin-A correlated with disease complications [76, 77].

Notably, previous studies by our group in a mouse model of allergic airway inflammation showed that Activin-A suppresses Th2 cell-mediated allergic responses, while it promotes the generation of antigen-specific suppressive FOXP3 T cells, characterized by high levels of IL-10 production [62]. Furthermore, another study showed that allergen challenge during mild asthma induced the production of Activin-A by neutrophils, while Activin-A's receptors expression was increased robustly throughout the airway epithelium and submucosal cells, proposing a physiological role of Activin-A signalling pathway in the resolution of inflammation and lung tissue maintenance [63]. Finally, other studies using T cells from asthmatic patients revealed that Activin-A reduces Th2 cell proliferation and cytokine secretion, while it induces suppressive T cells with the characteristics of Tr1-like cells. Importantly, a novel role for IRF4 was demonstrated in a humanized mouse model of allergic asthma. Activin-A, induced human Tr1 cells, where IRF4 was shown to interact with the Ahr/ARNT complex and drive the expression of the immunosuppressive molecule ICOS [78]. Moreover, Ahr was shown to induce IL-10 expression, cooperatively with c-Maf, and these Activin-A-induced human Tr1 cells were able to restrain human Th2 cell mediated allergic responses [78].

Ongoing studies by our group have revealed a regulatory role for Activin A, also in the context of autoimmune CNS inflammation. More specifically, experiments using the MOG_{35-55}/CFA EAE model of multiple sclerosis, showed that in vivo Activin-A administration restrained autoreactive Th17 cell responses in the CNS and significantly reduced disease severity compared to PBS treated mice (control). In support, histopathological analyses detected reduced immune cell infiltration and demyelination in the spinal cords of mice treated with Activin-A. Notably, EAE amelioration by Activin-A treatment was associated with significantly reduced frequencies of

CNS-infiltrating IL-17$^+$, IFN-γ$^+$, and IL-17$^+$IFN-γ$^+$ CD4$^+$ T cells. In line with this reduced inflammatory cell infiltration, we detected decreased levels of TNF-α, IL-1β and GM-CSF, key mediators of Th17 cell pathogenicity, in the spinal cords of activin-A-treated mice. Altogether, these studies suggest that Activin-A restrains encephalitogenitic T cell responses in vivo.

2. Hypothesis – Aims

Our hypothesis is that Activin-A can suppress Th17 cell pathogenicity through skewing their pro-inflammatory phenotype towards an anti-inflammatory one.

Our aim is: to delineate the molecular mechanisms through which Activin-A modulates Th17 cell pathogenicity.

The results obtained from these studies may facilitate the understanding of the molecular pathways that impact Th17 cell function.

3. Methods

3.1 Mouse CD4$^+$ T cell differentiation

CD4$^+$ CD62L$^+$ T naive T cells were isolated (CD4$^+$ CD62L$^+$ T Cell Isolation Kit II mouse, Miltenyi Biotec) from the spleens and lymph nodes of C57BL/6 mice and were activated with plate-bound anti-CD3 (1μg/ml, eBioscience) and soluble anti-CD28 (1μg/ml, eBioscience) in 96-well plates for 3-7 days. For pathogenic Th17 cell differentiation, 10^5 naive CD4$^+$ T cells were supplemented with recombinant IL-6 (20ng/ml, Peprotech), IL-1β (20ng/ml, Peprotech) and IL-23 (20ng/ml, R&D Systems) in the presence of PBS (control) or recombinant activin-A (100 ng/ml, R&D Systems). In some experiments, the AhR antagonist (5μM; CH-223191, Sigma-Aldrich), the SMAD3 antagonist (20 nM, SIS3, Sigma-Aldrich) and the CD73 antagonist (100Mm, AMPCP, Sigma-Aldrich) were also added in the culture media. Cells were harvested on days 1, 3 or 7 post differentiation for RNA extraction and intracellular cytokine staining and secretion.

3.2 Immunofluorescence microscopy

Cytospins generated from in vitro cultures of iTh17 cells and act-A-Th17 cells, were incubated overnight at 4ºC with primary antibodies against human/mouse/rat HIF1a (AF1935, R&D Systems), mouse Ahr (sc-8088, Santa Cruz), mouse c-Maf (sc-7866, Santa Cruz). Slides were then incubated with fluorescently-labeled secondary antibodies (Invitrogen). Nuclear staining and mounting of the slides were carried out using To-pro-3 (ThermoFischer Scientific T3605). Vectashield (H-1000, Vector Laboratories) was used as mounting buffer. Image acquisition was performed by a confocal laser scanning microscope (Leica TCS SP5) and images were acquired by Differential Interference Contrast (DIC) optics. Immunofluorescence data analysis was performed with the ImageJ software. AHR+, c-Maf+ and HIF1-α+ T cells were counted in each cytospin and expressed as the percentage of total cells.

3.3 Cytokine Analysis

Cytokines were measured in iTh17 and act-A-Th17 culture supernatants using commercially available ELISA kits for IL-17, IFN-γ, IL-10, TNF-α and GM-CSF (R&D Systems).

3.4 Quantitative real-time PCR

Total RNA was isolated using the RNA-easy Mini Kit (Qiagen) and reverse-transcribed using Superscript II (Invitrogen), according to the manufacturer's recommendations. Gene expression was analyzed using SYBR Green Master mix and selected primers (Table). The relative expression of genes over the expression of *Gapdh* and *Polr2a* was calculated using the 2-ΔΔCt analysis method.

Gene		**Sequence** 5' → 3'
IL17A	FW:	TCATCCCTCAAAGCTCAGCG
	REV:	TTCATTGCGGTGGAGAGTCC
TNF-α	FW:	CCAGACCCTCACACTCACAA
	REV:	ATAGCAAATCGGCTGACGGT
IFN-γ	FW:	GCGTCATTGAATCACACCTG
	REV:	TGAGCTCATTGAATGCTTGG
IL-1β	FW:	ACCTTCCAGGATGAGGACATGA
	REV:	CTAATGGGAACGTCACACACCA
GM-CSF	FW:	CTCACCCATCACTGTCACCC
	REV:	TGAAATTGCCCCGTAGACCC
IL-10	FW:	TGAATTCCCTGGGTGAGAAG
	REV:	GCTCCACTGCCTTGCTCTTA
RORc	FW:	CCCGAGATGCTGTCAAGTTT
	REV:	CTTGGCCACTTGTTCCTGTT
TBX21	FW:	GGTGTCTGGGAAGCTGAGAG
	REV:	GAAGGACAGGAATGGGAACA
c-Maf	FW:	CCCTTGACAGTTTGCTTCTA
	REV:	CCCATTCTGCTATCTTTGAC
Ahr	FW:	CTCCTTCTTGCAAATCCTGC
	REV:	GGCCAAGAGCTTCTTTGATG
FOXP3	FW:	CCCATCCCCAGGAGTCTTG
	REV:	ACCATGACTAGGGGCACTGTA
CYP1a1	FW:	TCAGTCCCTCCTTACAGCCC
	REV:	GGGTTCTTCCCCACAGTCAG
NT5e	FW:	GGAAACCTGATCTGTGATGC
	REV:	CTTCAGGGTGGACCCTTTTA
Entpd1	FW:	GGACTGACCCAGAACAAACC
	REV:	AGGTACGCACCGATTTCATC
Egln1	FW:	AGGCTATGTCCGTCACGTTG
	REV:	TGGGCTTTGCCTTCTGGAAA
Egnl2	FW:	GTAGAAGGTCACGAGCCAGG
	REV:	CGCCATGCACCTTAACATCC
Egln3	FW:	AGGCAATGGTGGCTTGCTAT
	REV:	GACCCCTCCGTGTAACTTGG

3.5 Immunoblot Analysis

For immunoblot analysis, iTh17 or act-ATh17 cells isolated as previously described, were washed once with PBS and harvested in lysis buffer supplemented with protease and phosphatase inhibitors (Invitrogen). The protein homogenate was quantified eith Bradford assay (Thermo Scientific). A total of 20μgr protein was loaded on an acrylamide gel (Biorad). The gel was transferred onto a PVDF membrane (Millipore), blocked with 5% non-fat milk, at 25 ^{o}C for 1h and probed with primary antibodies for AhR (Enzo; BML-SA210), c-Maf (Santa Cruz; sc-7866) and HIF-1a (R&D; AF1935) and TATA binding protein (TBP) (Santa Cruz; SC-204) at 4 ^{o}C overnight. The blot was then incubated with respective horseradish peroxidase linked secondary antibodies at 25 ^{o}C for 1h and developed using ECL Chemiluminescence kit (Millipore). Protein expression was normalized by dividing the densitometric units corresponding to the protein of interest with that of TBP from the same sample and plotted in a bar graph.

3.6 Flow-cytometry analysis

Cells were stained with fluorescently-labeled antibodies against CD4, CD73, CD39, (eBioscience). For intracellular cytokine staining, cells were cultured as described above and then stimulated for 4h at 37^{0}C in complete medium containing PMA (10 ng/ml, Sigma), ionomycin (250 ng/ml, Sigma-Aldrich) and Golgi-Stop (1μl/ml, BD Biosciences). Then, cells were stained with antibodies against IL-17, IFN-γ, GM-CSF, IL-10 and(eBioscience), according to the manufacturer's instructions. For transcription factor staining, cells were fluorescently-labeled with antibodies against Phospho-Stat3 (BD Biosciences). FACS acquisition was performed with the cytometer Cytomics FC500 (Beckman Coulter) and data were analyzed using the FlowJo software 8.7 (Tree Star, Inc).

3.7 Free ATP measurement assay

Act-A-Th17 or Th17 cells were differentiated as described above for 48h. Cells were then washed 2 times with phenol red-free RPMI 1640 (Gibco, USA) and serum starved overnight. ATP (500μM; Jena Bioscience) was added in the last 8h of culture. ATP concentration was measured in culture supernatants using ATPlite Luminescence Assay System (PerkinElmer).

3.8 Chromatin immunoprecipitation

iTh17 or act-ATh17 cells generated as previously described, were fixed for 10 min with 1% formaldehyde at R.T. and quenched with 0.125 M glycine. Nuclei were prepared and chromatin was fragmented by sonication. The DNA fragments were incubated overnight at 4 °C with anti-mouse AhR (Enzo BML-SA210), c-Maf (Santa Cruz sc-7866), STAT3 (Cell Signaling 79D7) and HIF-1a (RnD AF1935) or control IgG antibodies. Immunoprecipitated complexes were extracted by reverse crosslinking at 65°C and proteinase K treatment. Chromatin fragments were purified using NGS paramagnetic beads (Macherey-Nagel 744970) and proceeded to qPCR. The relative enrichment of ChIP versus IgG (relative to input DNA) was determined and calculated by quantitative real-time PCR for the regions of interest. The following primer pairs were utilized:

Gene Locus		**Sequence** 5' → 3'
Nt5e Site 1	FW:	CGGCTCCCAACAGCACTTGT
	REV:	TGCCCTCCCCTTCAGCTTCT
Nt5e Site 2	FW:	AGCAAGAGAAATAGCAGGGCG
	REV:	GTTAGAGCCGTTCTTGCATTGAG
Entpd1 Site 1	FW:	CTTACACCGTCCTCCCTGAG
	REV:	GCCAGCTGTGAAATGACAAA
Entpd1 Site 2	FW:	AAGGAGGTGGACACAACCAG
	REV:	TGAATAAATGTGTGCAGAAGGA
Entpd1 SRE 1	FW:	GCTGGGCTTTAGAGACTTGTGGGC
	REV:	ACCCATGCAAATGGTTTGGGCA
IL-10 -300 (Ahr)	FW:	ACCTGGGAGTGCGTGAATGGAATCC
	REV:	GTGACTTCCGAGTCAGCAAGAAATA
IL-10 MARE 1	FW:	GGAGAAAGTGAAAGGGATGGAG
	REV:	GGAATGGAATTGACTCAAGAACTG
IL-10 MARE 2	FW:	ACCCTCTACATGGGTCTACTT
	REV:	CAAGCAACTACTTGTCCTCCT

CYP1a1	FW:	AGGCTCTTCTCACGCAACTC
	REV:	CTGGGGCTACAAAGGGTGAT
Egln2 XRE 2	FW:	TAGATGACAGACTGGGCCAC
	REV:	TGTCTCCCTATCACCTTCCTC

4. Results

4.1 Activin-A alters the pathogenic gene expression profile of Th17 cells through its canonical signaling pathways.

Having seen the in vivo role of Activin-A on EAE, we hypothesized that Activin-A has an impact on Th17 cell differentiation. To investigate this, we stimulated naive $CD4^+$ cells under pathogenic Th17-skewing conditions (IL-1β + IL-6 + IL-23) in the absence or presence of Activin-A (iTh17 or act-A-Th17 cells, respectively) [Fig. 6].

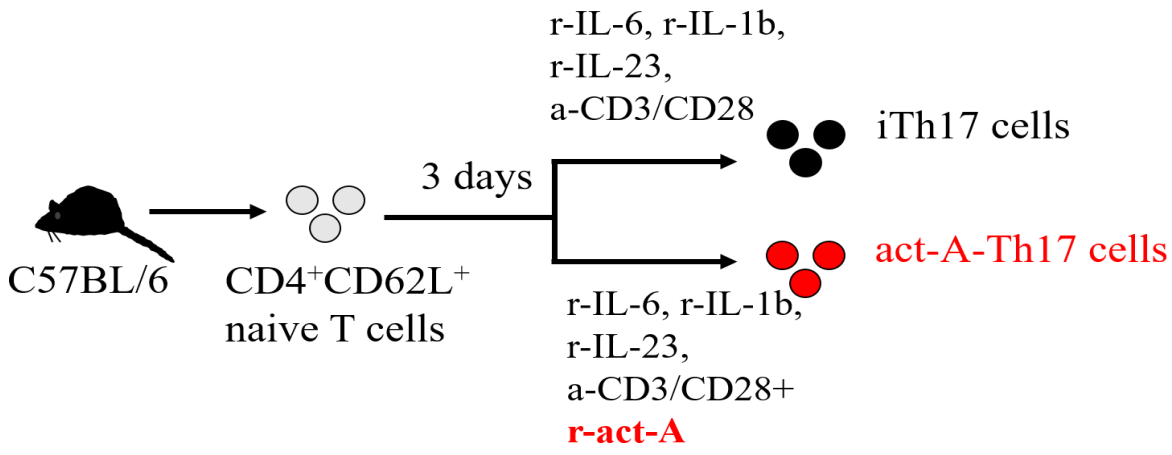

Figure 6: Experimental design.

C57BL/6 mice were sacrificed and lymph nodes and spleens were collected. Naive $CD4^+$ $CD62L^+$ T cells were isolated and cultured under pathogenic Th17-skewing conditions in the presence or absence of Activin-A. In different time points, T cells from both groups were collected and proceeded to further analyses using different experimental approaches.

In order to characterize the transcriptional profile of act-A-Th17 cells, RNA-seq analysis was performed. Our results showed a significant difference in the transcriptomic profile of act-A-Th17 cells: a total of 972 genes were found to be differentially expressed in act-A-Th17 cells compared to iTh17 cells, with 741 genes downregulated and 231 genes upregulated [Fig. 7a,b].

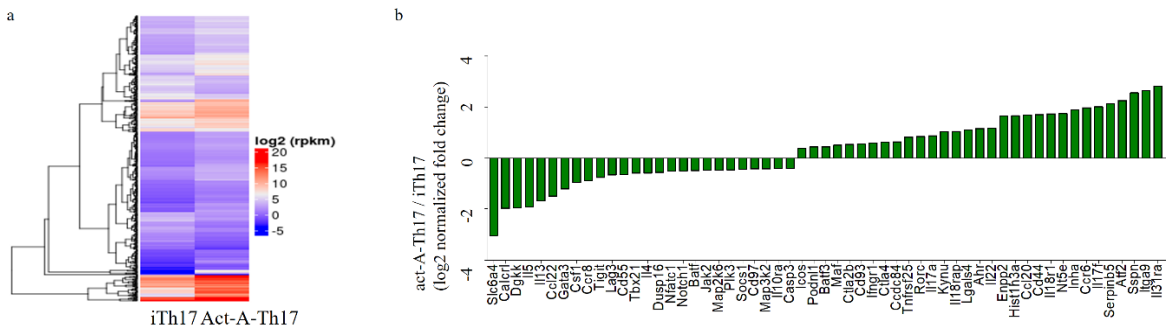

Figure 7: Activin-A promotes an altered gene expression profile in Th17 cells.

Th17 cells cultured as in figure 6 in the presence or absence of Activin-A were collected and proceeded to RNA-seq analysis. **(a)** Heatmap of logarithmized rpkm values, depicting the expression profile of 972 differentially expressed genes (log2(FC) ≥ 1) in act-A-Th17 cells relative to Th17 cells. **(b)** Demonstration of 57 differential expressed genes in act-A-Th17 relative to iTh17 cells

To validate the RNA-seq results, we proceeded to qPCR analysis [Fig. 8a]. Indeed, we identified downregulation of the pro-inflammatory cytokines *IL-1b*, *CSF2* and *IFN-γ* along with the downregulation of *T-bet* in act-A-Th17 cells compared to iTh17 cells. Importantly, we showed a significant upregulation of the immunosuppressive cytokine *IL-10*. Moreover, we identified an upregulation of *c-Maf* and *Ahr*, transcription factors that have been correlated with the non-pathogenic Th17 cell phenotype [Fig.8a]. *CYP1a1*, the most well-known target gene of Ahr was considerably upregulated, indicating increased transcriptional activity of this factor in Th17 cells in response to Activin-A stimulation [Fig. 8a]. FACS analysis showed decreased percentages of IFN-γ^+ and GM-CSF$^+$ CD4$^+$ T cells among act-A-Th17 cells, accompanied by increased IL-10$^+$CD4$^+$ T cells [Fig. 8b]. These results were also validated through ELISA measuring cytokine production in T cell culture supernatants [Fig. 8c].

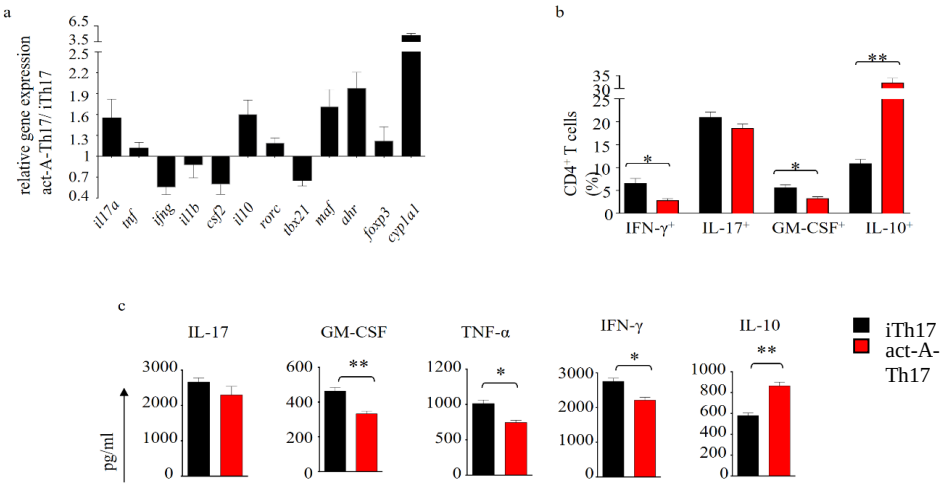

Figure 8: Activin-A restrains Th17 pathogenicity while upregulating anti-inflammatory molecules.

Th17 cells generated as in figure 6 **(a)** iTh17 and act-A-Th17 cells generated as above were collected for RNA isolation and qPCR experiments were performed. qPCR analysis showing mRNA levels of several cytokines and transcription factors involved in Th17 differentiation. Gene expression data were normalized to *gapdh* and *polr2a*. Data are pooled from n= 6-8 independent experiments. **(b)** FACS analysis in iTh17 and act-A-Th17 cells. Cumulative data showing the percentages of cytokine producing $CD4^+$ T cells subpopulations. Data are mean±SEM of *n*=2-3 independent experiments. **(c)** ELISA showing cytokine secretion in culture supernatants. Data are mean±SEM of triplicate wells from n=6-8 independent experiments.

We next investigated the molecular pathways through which Activin-A modulates Th17 cell differentiation. We initially examined Activin-A's canonical signaling pathways. In order to do that, act-A-Th17 cells were cultured in the presence of an ALK4 blocking antibody targeting the type I receptor or with SIS3, a SMAD3 chemical inhibitor, targeting canonical intracellular messenger molecules. We detected reversal in the effects of Activin-A at the gene expression level and also at the protein level, as exemplified by increased GM-CSF, IFN-γ and TNF-α and decreased IL-10 [Fig. 9a-c]. Importantly, we also observed decreased mRNA levels of *c-Maf* and *Ahr*. These results indicate that Activin-A signals through its canonical pathways in Th17 cells.

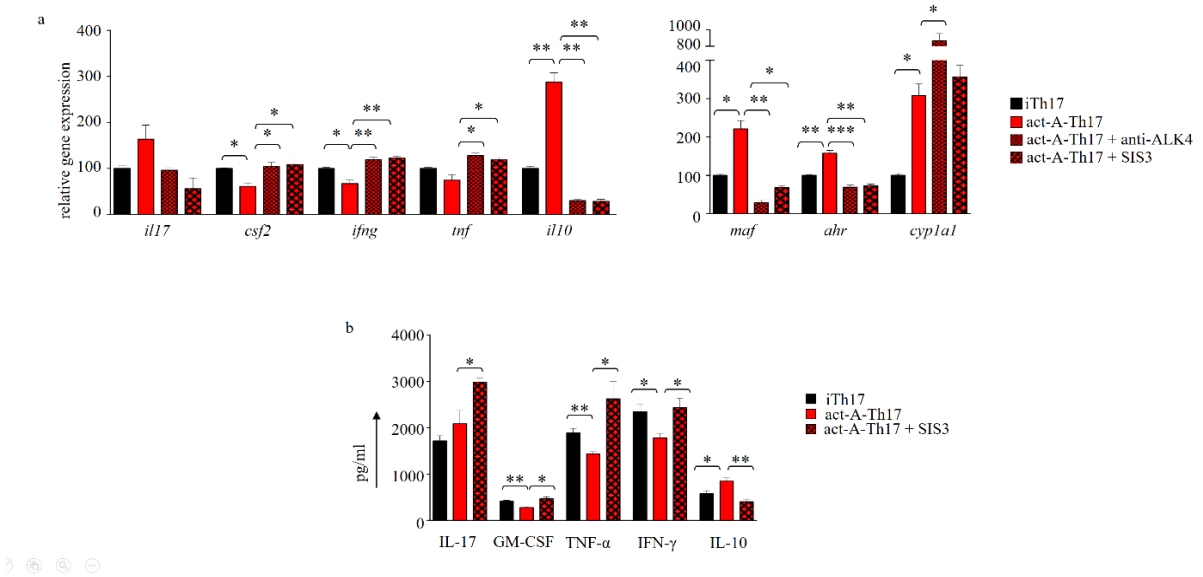

Figure 9: Activin-A effects on Th17 cells differentiation.

iTh17 or act-A-Th17 cells cultured as in figure 6 in the presence of an ALK4 blocking antibody or SIS3. **(a)** qPCR demonstrating mRNA levels of several cytokines and transcription factors related to Th17 phenotype. Gene expression data were normalized to *gapdh* and *polr2a*. Data are pooled from n= 6-8 independent experiments. **(b)** ELISA showing cytokine levels in culture supernatants. Data are mean±SEM of triplicate wells from n=6-8 independent experiments. Statistical significance was obtained by Student's t test, *p < 0.05, **p <0.01.

4.2 Activin-A modulates Th17 cell pathogenicity through the upregulation of the CD73 ectonucleotidase.

Among the molecules that were differentially expressed in the RNA-seq analysis, the immunoregulatory molecule CD73 (encoded by the *NT5e* gene) was found to be one of the most upregulated. CD73 along with CD39 (encoded by the *Entpd1* gene) are the two components of the extracellular ATP depletion pathway. CD39 catalyzes the ATP to ADP/AMP conversion and then, CD73 performs the final step in the production of adenosine, using AMP as a substrate. These molecules have been recognized as important immunoregulatory components, as they contribute to immunosuppression by depleting pro-inflammatory eATP concentration and mediate the production of the anti-inflammatory adenosine [Fig.10].

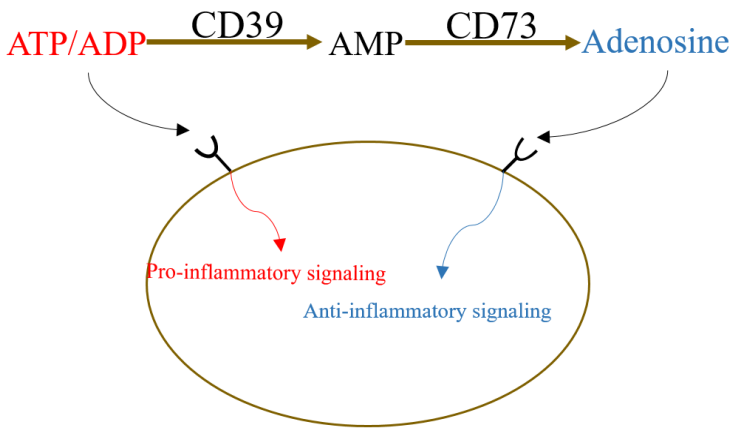

Figure 10: The mechanism of extracellular ATP depletion pathway.

The extracellular ATP depletion pathway plays an important role in immunosuppression as it removes the excess of eATP, that is released from cells in inflamed environments. CD39 initially binds ATP and hydrolyses it to ADP. Then, it acts on ADP and generates AMP. CD73 ectonucleotidase recognizes AMP and uses it as substrate to generate adenosine. Adenosine suppresses inflammation as it decreases cell proliferation rate and inhibits cytokine production. This system is one of the major mechanisms used by Tregs to suppress T effector responses.

Hence, we explored the expression of CD39/CD73 in act-A-Th17 cells by qPCR analysis. We detected increased mRNA levels of CD73 in act-A-Th17 [Fig. 11a]. Importantly, we observed increased CD39 expression, suggesting that the whole eATP depletion pathway is upregulated in act-A-Th17 cells [Fig. 11a]. Moreover, we found the same results when we examined the protein levels of these molecules by FACS extracellular staining. This is an important finding as it verifies the membrane localization of these molecules, which is the site of their function [Fig. 11b]. Again, blocking Activin-A's canonical signaling pathways abolished the induction of ectonucleotidases expression in Th17 cells [Fig. 11c].

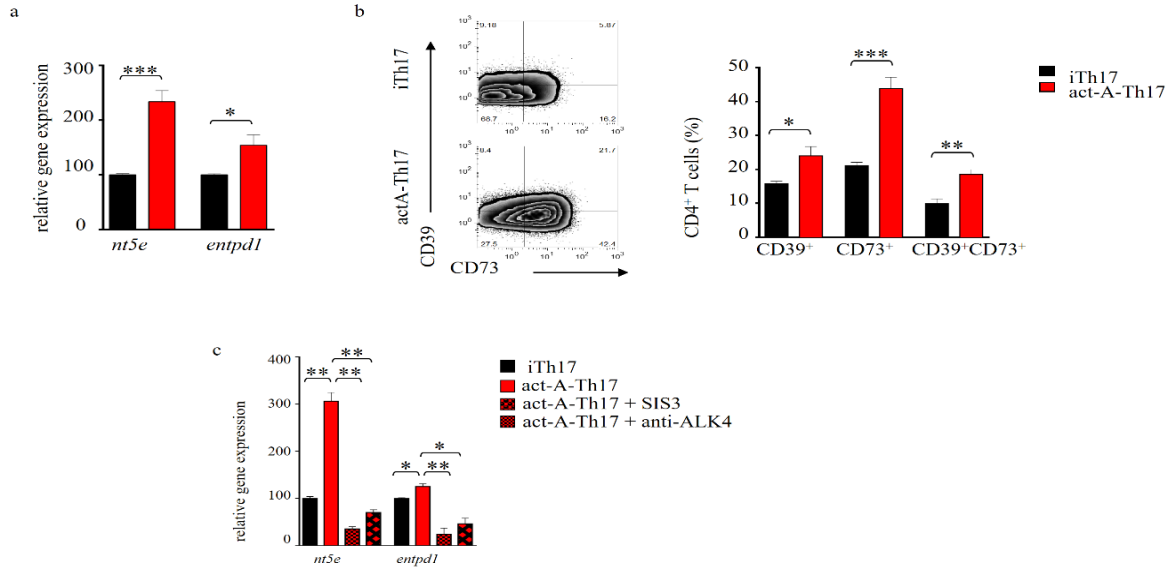

Figure 11: Activin-A upregulates the expression of eATP depletion pathway components, using its canonical signaling pathways.

(a) qPCR experiments showing CD73 and CD39 mRNA levels. Gene expression data were normalized to *gapdh* and *polr2a*. Data are pooled from n= 6-8 independent experiments. **(b)** Representative FACS plots showing the percentages of $CD39^+$ $CD73^+$ $CD4^+$ T cells in iTh17 and act-A-Th17 cells (left panel). Cumulative data showing the percentages of $CD39^+$, $CD73^+$, $CD39^+$ $CD73^+$ $CD4^+$ T cells in iTh17 and act-A-Th17 cells (right panel). Data are mean±SEM of *n*=2-3 independent experiments. **(c)** iTh17 or act-A-Th17 cells cultured as above in the presence of an ALK4 blocking antibody or SIS3. qPCR showing gene expression of *NT5e* and *Entpd1*. Gene expression data were normalized to *gapdh* and *polr2a*. Data are pooled from n= 6-8 independent experiments.

Having seen that CD39/ CD73 ectonucleotidases' expression was increased at the protein level in Th17 cells in response to Activin-A, we examined whether molecules were also functional. In order to do that, we performed ATP depletion assay where we identified decreased concentration of extracellular ATP in act-A-Th17 cells compared to the iTh17 cells, pointing to increased eATP catabolism [Fig. 12]. Using a chemical inhibitor for CD73 (AMP-CP), we observed increased eATP levels compared PBS treated act-A-Th17 cells, verifying that Activin-A's effect was dependent on CD73 function [Fig. 12].

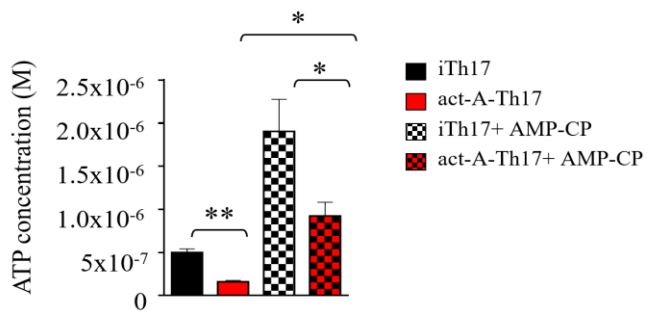

Figure 12: Activin-A promotes the generation of an active CD39/CD73 eATP depletion system in Th17 cells.

ATP depletion assay performed in in vitro cultures of iTh17 and act-A-Th17 cells cultures as mentioned above. Act-A-Th17 cells or Th17 cells were treated for the last 8 hours of culture with ATP. ATP levels in culture supernatants are shown. Data are mean±SEM of triplicate wells from two independent experiments.

To further characterize the effects of CD73 upregulation in the differentiation of act-A-Th17 cells, we blocked CD73 function. qPCR analyses showed upregulation of the pathogenic molecules *IFN-γ* and *CSF2* in CD73-inhibited, act-A-Th17 cells and also downregulation of *IL-10*, *c-Maf*, *Ahr*, *CD73* and *CD39* expression [Fig. 13a]. Moreover, the same results were observed at the protein level by ELISA in cell culture supernatants and by FACS analysis [Fig. 13b, c]. Similar results observed using CD73 KO $CD4^+$ T cells. These results indicate that CD73 function is indispensable for Activin-A's effect on the transcriptional modulation of Th17 cells.

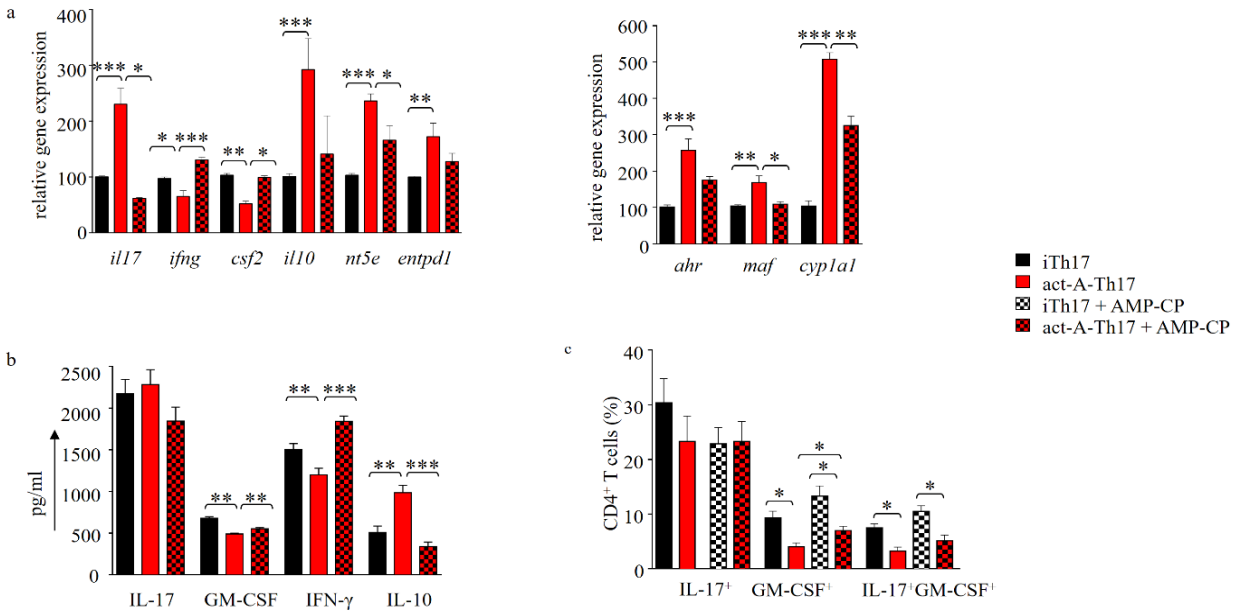

Figure 13: CD73 upregulation is essential for Activin-A's effects on Th17 cells.

iTh17 or act-A-Th17 cells were cultured as in figure 6 in the presence of AMP-CP. **(a)** qPCR analysis showing gene expression of several cytokines and transcription factors relevant to Th17 phenotype. Gene expression data were normalized to gapdh and polr2a. Data are pooled from n= 6-8 independent experiments. **(b)** ELISA on culture supernatants showing cytokines release in T cell cultures supernatants. Data are mean±SEM of triplicate wells from n=6-8 independent experiments. Statistical significance was obtained by Student's t test, *p < 0.05, **p <0.01. **(c)** Cumulative data of the percentages of $IL\text{-}17^+$, $GM\text{-}CSF^+$ and $IL\text{-}17^+$ $GM\text{-}CSF^+$ $CD4^+$ T cells analyzed by flow cytometry. Data are mean±SEM of *n*=2-3 independent experiments.

4.3 STAT3 is indispensable for Activin-A-induced CD39 upregulation in Th17 cells and controls along with c-Maf, and Ahr, CD73 expression.

Since we identified an important role of CD73 ectonucleotidase in Activin-A's mediated repression on Th17 cell differentiation, our next aim was to unravel the molecular mechanisms that drive CD73 increased expression. Previously published studies have shown that STAT3 regulates CD39 and CD73 expression in Th17 cells [104]. As it was mentioned above, STAT3 is one of the core transcription factors that regulate gene transcription in Th17 cells. Hence we first decided to examined the role of STAT3 in Activin-A-induced CD39/CD73 upregulation in Th17 cells. We observed increased phosphorylation status of STAT3 in act-A-Th17 cells, compared to iTh17 cells, indicating increased STAT3 activity [Fig. 14a]. In order to analyze further the contribution of STAT3 in CD39/CD73 expression, we blocked its activity by using STA-21, a chemical inhibitor of STAT3. FACS analysis on these cells showed a complete abolishment of CD39 expression, as well as, a partial reduction in CD73 expression in act-A-Th17 cells [Fig. 14b].

To evaluate the role of STAT3 in Activin-A-induced *NT5e* upregulation, we performed bioinformatics analysis on the *NT5e locus* and identified putative STAT3 binding sites [Fig. 14c]. We then performed chromatin immunoprecipitation followed by qPCR analysis, where we observed increased binding enrichment for STAT3 at a specific region, +1700 bp from the *NT5e* TSS on act-A-Th17 cells compared to iTh17 cells [Fig. 14c]. Moreover, we examined a previously described binding site of STAT3 on the *CD39 locus*, located in -3740 pb upstream of the TSS (Entpd1 SRE1) and we found increased STAT3 binding at that locus in act-A-Th17 cells [Fig 14c].

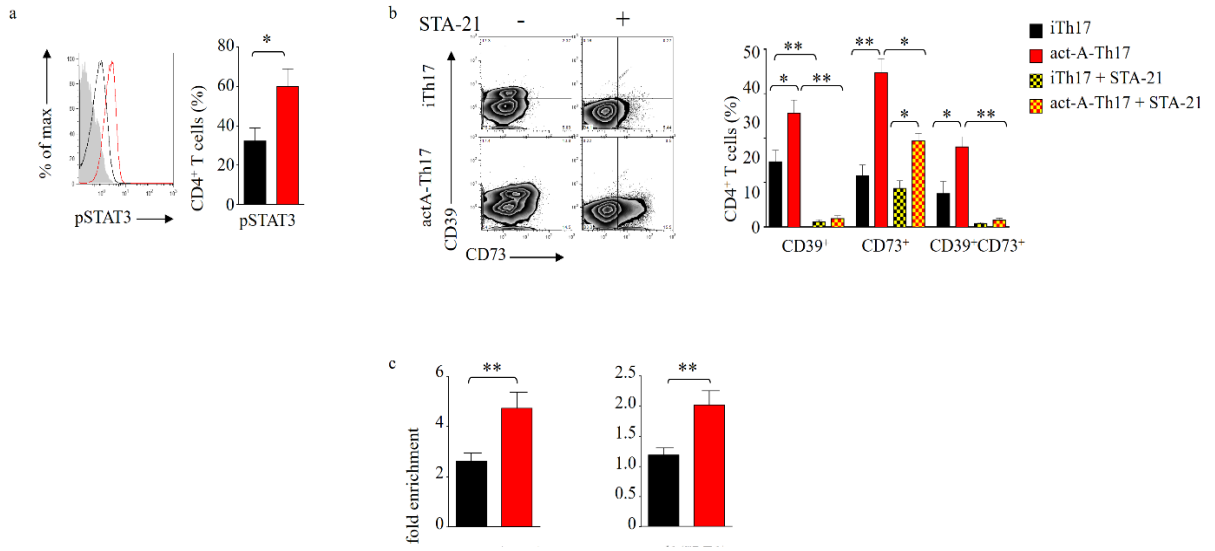

Figure 14: STAT3 is essential for CD39 expression and also plays an important role in CD73 upregulation in Activin-A treated Th17 cells.

(a) FACS representative plot showing the phosphorylation status of STAT3 analysis in iTh17 and act-A-Th17 cells. Shaded histogram represents isotype control (left). Cumulative data are shown on the right. Data are mean±SEM of *n*=2-3 independent experiments. **b)** iTh17 and act-A-Th17 cultured as in figure 6, in the presence or absence of STA-21. Representative FACS plots showing the percentages of $CD39^+$, $CD73^+$, $CD39^+$ $CD73^+$ $CD4^+$ T cell populations (left). Cumulative data are shown on the right. Data are mean±SEM of *n*=2-3 independent experiments. **(c)** Chromatin immunoprecipitation assay for STAT3 in act-A-Th17 cells and iTh17 cells followed by qPCR examining the +1700 site on *NT5e locus* and the already known SRE1 binding site on the *Entpd1 locus* (right). Results are mean±SEM (*n*=2 independent experiments).

Our results have shown increased mRNA levels of c-Maf and Ahr in Th17 cells following Activin-A stimulation. Considering that blocking STAT3 signaling did not abolish Activin-A-induced CD73 upregulation in Th17 cells, we next examined the putative role of these transcription factors in Activin-A-induced CD73 expression in Th17 cells. Hence, we performed immunofluorescence experiments that showed increased nuclear localization of these transcription factors in act-A-Th17 cells as compared to iTh17 cells [Fig. 15a]. Additionally, we identified increased c-Maf and Ahr protein levels through western blot analysis in act-A-Th17 cells [Fig.15b]. Computational analysis revealed several putative binding sites of c-Maf and Ahr on the *NT5E locus* [Fig.15c]. We then performed chromatin immunoprecipitation experiments for Ahr and c-Maf, where we identified increased binding of these transcription factors in the *NT5e locus* for two of the sites that were predicted in the analysis [Fig. 15c]. These binding sites for Ahr were located at -3992 pb upstream of the TSS (site 1) and at +1285 pb downstream of the TSS (site 2). c-Maf binding sites, were located at -3660 pb upstream of the *NT5e* TSS (site 1) and +1773 bp downstream of the TSS (site 2) [Fig. 15c]. Importantly, these enriched binding sites occurred in

the *NT5e locus* for c-Maf and Ahr, were in proximity to each other, something that supports a cooperative function of these transcription factors in gene regulation as shown in Tr1 cells [65]. In addition, we identified a significant enrichment of Ahr at two sites, located at -4503 pb upstream and at -1380 pb upstream of the TSS of the *Entpd1* TSS (site 1) [Fig. 15d]. These results indicate that Ahr and c-Maf contribute to CD73 expression in act-A-Th17 cells. Moreover, we observed that Ahr also contributes to Activin-A-induced CD39 upregulation in Th17 cells.

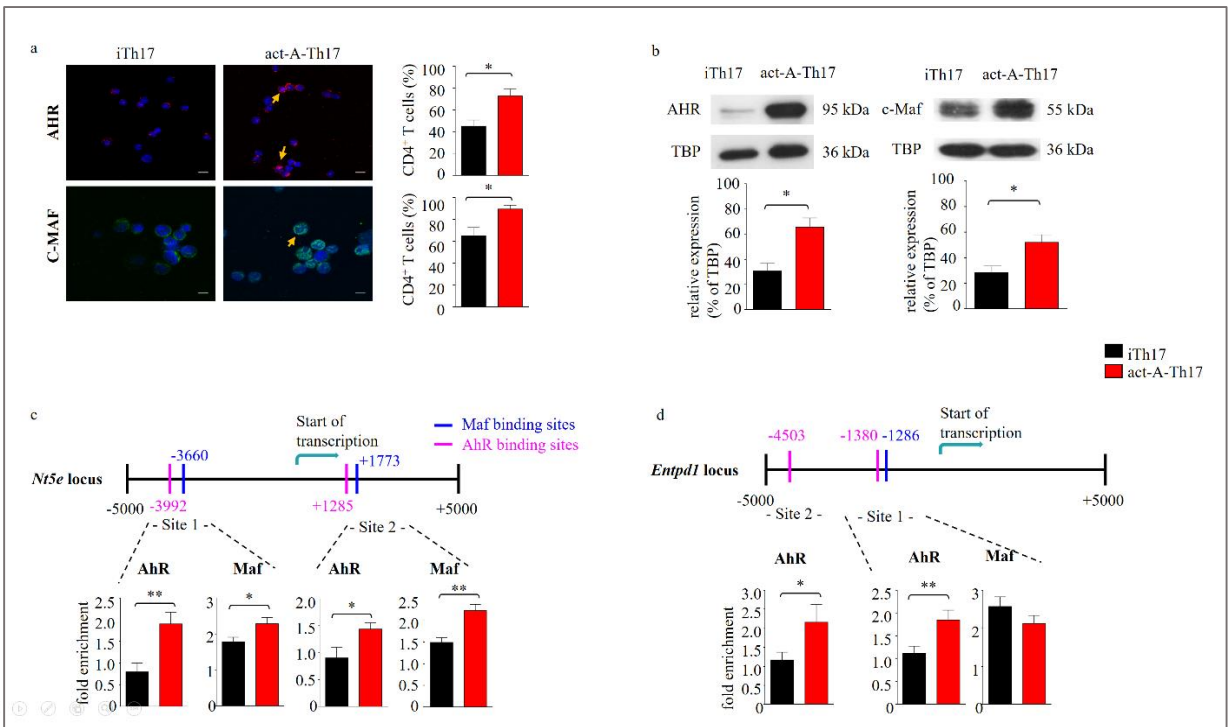

Figure 15: Ahr and c-Maf contribute to CD73 and CD39upregulation in act-A-Th17 cells.

(a) Representative confocal microscopy images of Ahr (red) and c-Maf (green) staining in iTh17 and act-A-Th17 cells. Nuclei are stained blue with DAPI (bars, 10μm). (left). Cumulative data (right). Results are pooled from *n*=2-4 independent experiments. **(b)** Western blot analysis for Ahr and c-Maf in iTh17 and act-A-Th17 cells. Protein quantification was performed using TBP for normalization. Results are mean±SEM of *n*=3 independent experiments. **(c)** qPCR analysis of Ahr and c-Maf chromatin immunoprecipitation assays showing their enrichment in the *NT5e locus*. **(d)** qPCR analysis of Ahr and c-Maf chromatin immunoprecipitation assays showing their enrichment in the

4.4 Ahr drives Activin-A mediated suppression of Th17 pathogenicity.

Studies in Tr1 cells have shown that IL-10 expression is regulated by Ahr and c-Maf [111]. Since we observed significant upregulation of IL-10, as well as, Ahr and c-Maf expression, we decided to examine their role in IL-10 induction. Through chromatin immunoprecipitation assays followed by qPCR, we identified increased binding of Ahr and c-Maf in the *IL-10 locus* in act-A-

Th17 cells compared to iTh17 cells. Specifically, binding enrichment was observed at position -300 bp upstream of the TSS for Ahr and at position -1000 bp for c-Maf (*IL-10 MARE2*) [Fig. 16a]. We did not observe significant difference in c-Maf binding in another known site at the *IL-10 locus*, -1300 bp upstream of IL-10 TSS (*IL-10 MARE1*) [Fig. 16a]. Finally, to validate the increased transcriptional activity of Ahr in act-A-Th17 cells, we performed qPCR with primers for the Ahr binding site in the *CYP1a1* promoter, at -800 bp upstream of the TSS, and as we expected, there was a strong enrichment of Ahr in the *CYP1a1 locus* in act-A-Th17 cells [Fig. 16b].

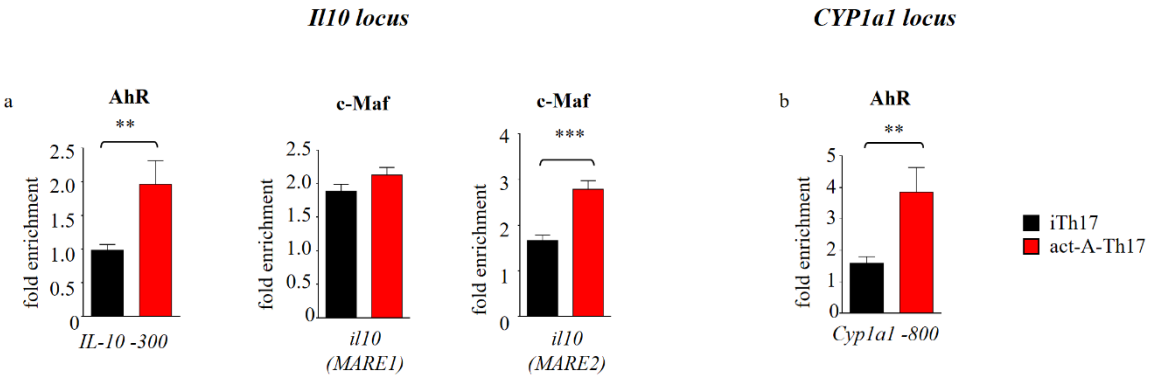

Figure 16: Ahr and c-Maf participate in IL-10 production by act-A-Th17 cells.

(a) qPCR analysis of Ahr and c-Maf chromatin immunoprecipitation assays showing their binding enrichment in the *IL-10 locus* **(b)** qPCR analysis of Ahr chromatin immunoprecipitation assays showing its binding in the *CYP1a1 locus*. Results are mean±SEM (*n*=2 independent experiments).

Since in act-A-Th17 cells, Ahr was shown to contribute in CD73 and IL-10 upregulation, we next wanted to characterize the role of Ahr in these cells. To investigate that, we cultured act-A-Th17 cells in the presence of CH-223191, a chemical inhibitor of Ahr. Gene expression data showed that blocking Ahr activity resulted in the downregulation of *IL-10*, *c-Maf*, *NT5e* and *Entpd1* and completely abolished the Ahr target gene, *CYP1a1*, in Th17 cells in response to Activin-A. In contrast, Ahr blockade increased *CSF2* and *IFN-γ* expression [Fig. 17a]. Similar findings were observed at the protein level by ELISA [Fig. 17b]. Finally, FACS analysis showed reduced $CD39^{+}CD73^{+}CD4^{+}$ T cell populations among act-A-Th17 cells treated with the Ahr inhibitor, as compared to PBS treated act-A-Th17 cells [Fig. 17c].

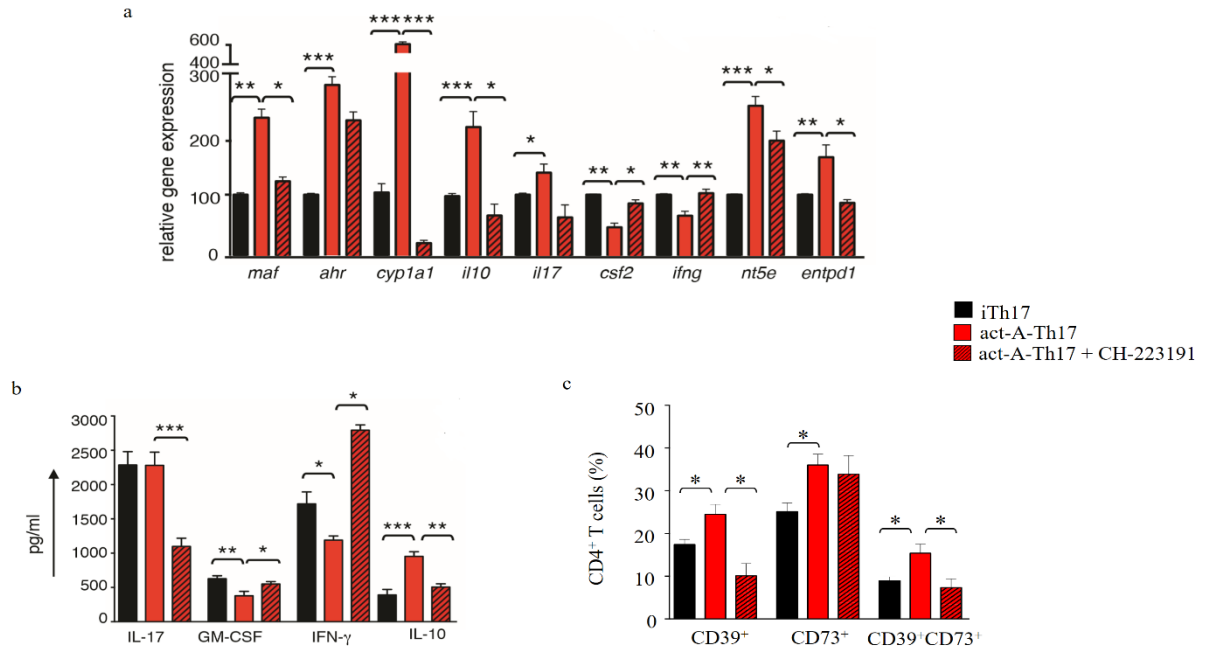

Figure 17: Ahr drives Activin-A mediated suppression of pathogenic Th17 phenotype.

iTh17 or act-A-Th17 cells were cultured as in figure 6 in the presence of CH-223191 **(a)** qPCR analysis showing gene expression of several cytokines and transcription factors relevant to the Th17 phenotype. Gene expression data were normalized to gapdh and polr2a. Data are pooled from n= 6-8 independent experiments. **(b)** ELISA on culture supernatants showing cytokine secretion. Data are mean±SEM of triplicate wells from n=6-8 independent experiments. Statistical significance was obtained by Student's t test, *p < 0.05, **p <0.01. **(c)** Cumulative data of the percentages of $CD39^+$, $CD73^+$ and $CD39^+$ $CD73^+$ $CD4^+$ T cell populations. Data are mean±SEM of *n*=2-3 independent experiments.

4.5 Negative regulation of HIF1-α is crucial for activin-A-mediated generation of non-pathogenic Th17 cells.

Several studies have shown that HIF-1a participates in the differentiation of pathogenic Th17 cells. Since we identified that Activin-A restrains the establishment of pathogenic Th17 programs, we wanted to examine whether it regulates HIF-1a expression in act-A-Th17 cells. Interestingly, confocal microscopy experiments showed that in contrast to iTh17 cells, where HIF-1a was localized mainly in the nucleus, in act-A-Th17 cells HIF-1a was localized in the cytoplasm [Fig. 18a]. Moreover, through western blot analysis we identified that HIF-1a protein levels in act-A-Th17 cells were almost diminished compared to iTh17 cells [Fig.18b]. Next we attempted to identify the mechanisms involved in Activin-A mediated HIF-1a downregulation in Th17 cells. Blimp-1 has been shown to regulate HIF-1a expression in Th17 cells. Nevertheless, qPCR experiments showed no difference in the mRNA levels of this transcription factor in Th17 cells in

response to Activin-A [Fig. 18c]. The mRNA levels of HIF1aN, which is a factor that inhibits HIF-1a dimerization and thereby its activation, were also non significantly different [Fig. 18c]. Finally, we investigated the mRNA expression of the PHD enzymes (encoded by *egln1*, *egln2* and *egln3*) that control HIF-1a through targeting it for ubiquitination and subsequent proteasomal degradation. We observed significant upregulation for *egnl1* and *egln2* genes in act-A-Th17 cells as compared to iTh17 cells [Fig. 18c]. Importantly, these two PHD proteins are not regulated from hypoxia in contrast to PHD3 [97]. Previous studies have shown that Ahr can upregulate PHDs in Tr1 cells and inhibit HIF-1a function [65]. Indeed, ChIP-qPCR analysis for a previously described binding site of Ahr in the *PHD1 locus* revealed a significant enrichment of Ahr binding in act-A-Th17 cells compared to iTh17 cells [Fig. 18d].

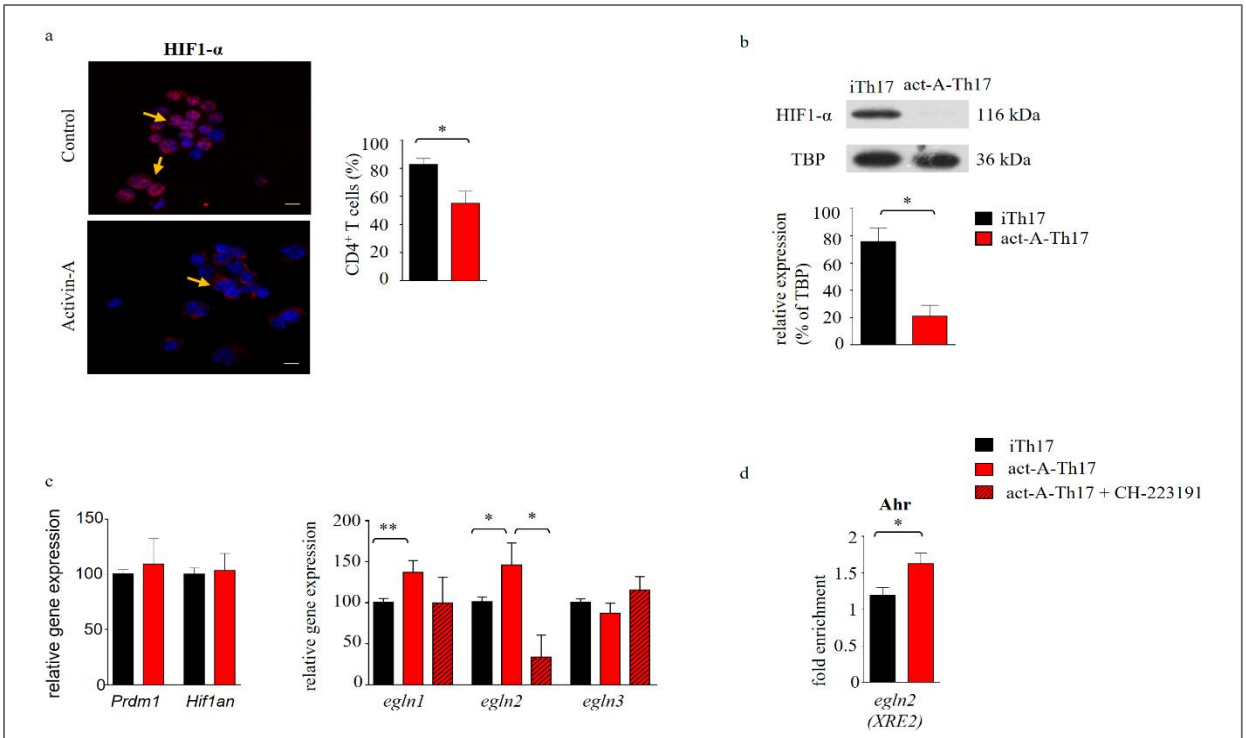

Figure 18: Ahr inhibits HIF-1a expression in act-A-Th17 cells.

(a) Representative confocal microscopy images of HIF-1a (red) staining in iTh17 and act-A-Th17 cells Nuclei are stained blue with DAPI (bars, 10μm). (left). Cumulative data (right). Results are pooled from *n*=2-4 independent experiments. **(b)** Western blot analysis for HIF-1a in iTh17 and act-A-Th17 cells. Protein quantification was performed using TBP for normalization. Results are mean±SEM of *n*=3 independent experiments. **(c)** iTh17 or act-A-Th17 cells were cultured as in figure 6 in the presence or absence of CH-223191. qPCR analysis showing gene expression of *Prdm1*, *HIF1aN*, *egln1*, *egln2* and *egln3* genes. Gene expression data were normalized to *gapdh* and *polr2a*. Data are pooled from n= 6-8 independent experiments. **(d)** qPCR analysis of Ahr chromatin immunoprecipitation assays showing its enrichment in the

5. Discussion

Activin-A is a cytokine with both pro-inflammatory and anti-inflammatory functions in immunity. In the EAE mouse model of MS, Activin-A was shown to reduce disease severity and decrease the infiltration of autoreactive IFN-γ, GM-CSF and IL-17 -producing $CD4^+$ T cells into the CNS. Since in EAE, T helper and more specifically, Th17 cell responses play important an role in disease progression, we speculated that Activin-A may modulate Th17 cell differentiation. Indeed, we discovered that Activin-A was able to alter the pathogenic Th17 cell transcriptomic profile. Importantly, we observed through qPCR, ELISA and FACS experiments that Activin-A reduces Th17 cell pathogenicity through the downregulation of GM-CSF and IFN-γ and IL-1β expression, and the upregulation of IL-10 and CD73/CD39 ectonucleotidases expression. Moreover, the T-bet transcription factor that is correlated with Th17 cell pathogenicity also was downregulated in Th17 cells in response to Activin-A. In contrast, Ahr and c-Maf transcription factors were upregulated in act-A-Th17 cells. Finally, we identified the molecular mechanisms through which Activin-A represses the pathogenic profile of Th17 cells. Thus, our studies highlight the role of Activin-A as a putative modulator of Th17 plasticity since it re-organizes the transcriptional network that controls Th17 differentiation and shifts it from a pathogenic Th17 cell phenotype to a non-pathogenic one.

The CD73 ectonucleotidase was found to be significantly upregulated at the mRNA and protein levels in act-A-Th17 cells. It was also shown that, inhibition of this molecule led to the reversal of Activin-A's effects on Th17 cell differentiation, as identified by upregulation of GM-CSF, IFN-γ and IL-1β and downregulation of IL-10, Ahr and c-Maf, compared to act-A-Th17 cells cultured without the CD73 inhibitor. Similar data were observed using *NT5e* $^{-/-}$ $CD4^+$ T cells. These findings suggest that CD73 functions are important for the generation of non-pathogenic Th17 cells in response to Activin-A. CD73 and CD39 act extracellularly and generate adenosine from ATP. ATP is released from T cells upon activation, as well as, from damaged or dying cells at sites of inflammation [105]. Physiologically, eATP is very low and small increases in its concentration can act as a pro-inflammatory signal, promoting immune cell infiltration and pro-inflammatory cytokine secretion [105]. CD39/CD73 dephosphorylate eATP and generate adenosine which acts as an immunosuppressive molecule by inhibiting cell activation, proliferation and cytokine secretion [105, 106]. ATP depletion represents one of the major mechanisms used by regulatory T

cells to control inflammation and T cell effector functions [105-107]. CD39 acts in the first step of the eATP catabolism pathway and generates AMP which can be converted back to ATP extracellularly, through the function of diphosphate kinases NDP kinase and adenylate kinase [105]. CD73 using AMP as a substrate generates adenosine which can be converted back to AMP only when it gets transported intracellularly [105]. Thus, the function of CD73 is more critical than CD39 for immune suppression, as not only it generates adenosine, the final immunosuppressive molecule that cannot be converted back to ATP extracellularly, but also, it depletes the AMP which can be used to generate ATP. Apart from this function in the modulation of inflammation, recent studies support that the CD39/CD73 mediated eATP depletion pathway can also have an impact on T cell metabolism, activation, differentiation and function [105, 106]. Consequently, our experiments showed that Activin-A can suppress the differentiation of pathogenic Th17 cells even under highly inflammatory conditions, through the upregulation of the immunosuppressive molecules CD39/CD73.

A previous study has shown that upregulation of CD39 and CD73 in Th17 cells can also be induced during differentiation with IL-6 and TGF-β. It was demonstrated that these Th17 cells mediate immune suppression in the context of cancer [104]. Mechanistic studies identified that CD39/CD73 expression in Th17 cells was dependent on TGF-β-induced downregulation of the Gfi transcription factor that acted as a repressor in *NT5e* and *Entpd1 loci*, making this region accessible for IL-6-induced STAT3 to bind and initiate gene transcription [104]. Nevertheless, CD39/CD73 upregulation was not shown when Th17 cells were differentiated in the presence of the pathogenic Th17 conditions, IL-6, IL-1β and IL-23, even when TGF-β was added in the cytokine cocktail [104]. In our experiments, we showed that CD39/CD73 upregulation in act-A-Th17 cells occurs through alternative mechanisms, as we did not identify Gfi downregulation and we also observed CD39/CD73 induction even during pathogenic Th17-skewing conditions. However, we discovered that STAT3, Ahr and c-Maf bind in the *CD39 locus* and promote gene upregulation in the presence of Activin-A, while Ahr and STAT3 induce *NT5e* gene upregulation. These findings indicate that Activin-A has stronger effects on the suppression of pathogenic Th17 cells as compared to TGF-β. Moreover, it seems that although both cytokines belong to the same superfamily, their functions are not redundant and they act through distinct mechanisms.

IL-10 is a cytokine with a strong immunosuppressive function. Tr1 cells secrete high levels of IL-10 and this is one of their major mechanisms through which they control inflammatory responses. It was demonstrated that Ahr, cooperatively with c-Maf, bind to the IL-10 promoter and activate gene transcription in Tr1 cells [91]. In Activin-A-treated Th17 cells, IL-10 was strongly upregulated both at the mRNA and the protein level. Moreover, we showed increased nuclear localization of Ahr and c-Maf in act-A-Th17 cells. Most importantly, we identified through ChIP-qPCR analysis, enhanced Ahr and c-Maf binding in the *IL-10 locus*, in Th17 cells in response to Activin-A. Remarkably, Ahr does not normally bind to the IL-10 promoter in Th17 cells. Altogether, our studies show that Activin-A can mediate the upregulation of IL-10 in Th17 cells, through similar mechanisms to those utilized by Tr1 cells. Moreover, given the enhanced expression of the CD39/CD73 eATP depletion system and the increased IL-10 levels in Th17 cells stimulated with Activin-A, it is conceivable that act-A-Th17 cells are not only less pathogenic but also exert potent immunosuppressive functions. Ongoing studies by our group are addressing this interesting and clinically useful prospect.

Ahr was shown to play an important role in Activin-A's effects on the differentiation of non-pathogenic Th17 cells. Indeed, Ahr inhibition led to an upregulation of GM-CSF and IFN-γ and downregulation of IL-10 and ectonucleotidases expression in act-A-Th17 cells. Studies have shown that Ahr contributes to the differentiation of both Th17 and Tr1 cell populations, exerting distinct transcriptional activities in each cell subset [20,65, 95,96]. Moreover, since Ahr is not considered as a pioneer transcription factor and is also characterized by transient activity, it is possible that its activation leads to alternative functions depending on the chromatin modifications and accessibility at a particular timepoint. Notably, Ahr activation is different in response to distinct ligands, while Ahr dimerization with different protein partners can also regulate alternative gene loci [91-93]. In our experimental conditions, c-Maf is also upregulated in act-A-Th17 cells and STAT3 shows increased activity. Thus, it seems that Activin-A upregulates three of the most important molecules that govern the expression of the non-pathogenic gene module in Th17 cells. In future studies, we aim to dissect whether Ahr, c-Maf and STAT3 interact with each other mediating the differential regulation of gene expression that is observed in act-A-Th17 cells.

Growing evidence has highlighted the increased reliance of Th17 cells on aerobic glycolysis for their differentiation and acquisition of effector functions [103]. HIF-1a is a

transcription factor with an important role in pathogenic Th17 lineage development, as it activates transcriptionally many components of the glycolytic pathway that support Th17 differentiation, proliferation and cytokine production [108]. Moreover, HIF-1a participates in the induction of signature Th17 molecules, such as, RORc and IL-17 [108]. In this study, we showed that in Activin-A treated Th17 cells, HIF-1a protein levels are significantly decreased. In search of the mechanisms through which Activin-A decreases HIF-1a levels in Th17 cells, we identified increased expression of the PHD1/2 proteins which are involved in HIF-1a degradation. Moreover, we detected enhanced binding of Ahr to the *egln2 locus* (encoding PHD1), concomitant with decreased PHD1 expression upon Ahr blockade, indicating that Activin-A inhibits HIF-1a expression in Th17 cells in an Ahr-dependent manner. Our findings are in accordance with the previously described antagonism between Ahr and HIF-1a in the context of Tr1 cell differentiation.

HIF-1a is considered to promote glycolysis in Th17 cells directly by upregulating glycolytic pathway proteins, such as, GLUT1 glucose transporter, hexokinase 2, glucose-6-phosphate isomerase, enolase 1, and lactate dehydrogenase but also indirectly, by upregulating pyruvate dehydrogenase kinase that inhibits PDH enzyme and thereby, prevents pyruvate from shunting into the TCA cycle [108-110]. Our ongoing proteomic studies show that the majority of these HIF-1a regulated proteins are downregulated in act-A-Th17 cells. Also, we identified a significant upregulation of several proteins participating in the assembly of the I, II, IV and V electron transport chain (ETC) complexes, indicating that act-A-Th17 cells respond to their energy demands by upregulating the oxidative phosphorylation pathway. Altogether, these data suggest a reprogramming of the metabolic profile of act-A-Th17 away from the aerobic glycolysis that is observed in iTh17 cells, towards oxidative phosphorylation, characteristic of iTreg cells. Moreover, is interesting that PHD enzymes are regulated also by cellular metabolites, such as, succinate and 2-oxoglutarate, which are metabolites belonging to the TCA cycle that is upregulated in act-A-Th17 cells, generating an additional link between Activin-A and HIF-1a associated regulation of metabolic responses [109].

Collectively, our studies revealed that Activin-A represses the pathogenic Th17 cell phenotype by upregulating the eATP depletion pathway and the immunosuppressive cytokine IL-10, through mechanisms that involve STAT3, Ahr and c-Maf regulation. Moreover, Activin-A downregulates HIF-1a expression in Th17 cells, a key metabolic checkpoint involved in Th17

pathogenic functions. In the context of autoimmunity and more specifically, in multiple sclerosis, these findings are of particular importance as it has been demonstrated that pathogenic GM-CSF and IFN-γ -producing Th17 cells drive disease progression and are correlated with disease severity. Moreover, considering that act-A-Th17 cells express immunossupressive molecules, it is conceivable that they can restrain T effector responses and mediate the resolution of CNS autoimmune inflammation.

Recent studies have highlighted the role of T cell metabolism in T cell differentiation, effector and or regulatory function. Notably, growing evidence supports the notion that immunometabolism can significantly affect chromatin regulation and gene expression in T cells. Since our data demonstrate that Activin-A interferes with the transcriptomic, as well as, the metabolic profile of Th17 cells, among our future aims is to also unravel whether and how the observed metabolic switch in Th17 cells modulates transcription factors activity, chromatin modifications and accessibility and ultimately, their gene expression profile in response to Activin-A.

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