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New inflammatory features of human T helper 17 cells in health and multiple sclerosis

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Summary

T helper (Th) 17 cells are a subpopulation of CD4 T lymphocytes characterized by the expression of interleukin (IL)-17 and the transcription factor retinoid acid receptor-related orphan receptor (ROR)γt. Pathogenic role of human Th17 cells has been demonstrated in several autoimmune diseases, including multiple sclerosis (MS), where they promote inflammatory processes. However, the mechanisms leading to the pathogenicity of Th17 cells are largely unknown.

The main objective of this thesis was to identify new mechanisms and molecules inducing inflammatory functions of human Th17 cells that could be potentially involved in the pathogenic processes of MS.

We addressed this aim by using two distinct approaches: 1) analysis of general inflammatory features of Th17 cells acquired during Th17 cell differentiation; 2) analysis of intrinsic features of Th17 cells derived from MS patients.

In order to investigate potential mechanisms responsible for pathogenic functions of human Th17 cells, we dissected their differentiation process by performing a transcriptome analysis of cells at 48 hours and 5 days of differentiation.

We uncovered three time-regulated modules: early modulation, involving exclusively 'signalling pathways' genes; late modulation, characterized by genes involved in response to infections; persistent modulation, involving effector immune functions.

To assign them an inflammatory or regulatory potential, we compared Th17 cells differentiated in presence or absence of IL-1β, respectively. We named inflammatory Th17 condition the polarizing milieu containing IL-1β, which is crucially involved in the pathology of Th17-related diseases. In contrast, Th17 regulatory condition refers to the anti-inflammatory IL-10 cytokine produced in Th17 condition lacking IL-1β.

We found that most part of the inflammatory genes belong to the persistent or late module, indicating the crucial role of these genes in the late phases of differentiation.

Thus, we elucidated the global molecular signature that characterizes the acquisition of the inflammatory profile by human Th17 cells, by analysing all genes differentially expressed in regulatory versus inflammatory Th17 conditions. Among inflammatory genes, we identified those sharing pathogenic functions with murine Th17 cells, including *IL17A*, *IFNG, TBX21, EBI3, IRF8, TNFRSF9, TNFRSF14, CCL5, CD40LG*, *BATF* and *TNF*. In addition, our analysis allowed the identification of novel effector molecules, including interferon (IFN)K, lymphotoxin (LT)-α, IL1A, platelet derived growth factor (PDGF)-A, and transcriptional regulators, such as transcriptional regulators homeodomain-only protein homeobox (HOP)X and SRY (sex determining region Y)-box 2 (SOX2), expression of which was independently validated.

In order to unveil the mechanisms underlying the acquisition of the human Th17 signature, we investigated the potential transcription factors involved in this process. In this context, we analysed the role of RORγt, known master regulator of Th17 cells, and of the two novel transcriptional regulators HOPX and SOX2, by performing RNA-interference experiments. We found that HOPX regulates IL-17A and IFN-γ, while SOX2 regulates PDGF-A and IFN-γ. As expected, RORγt regulates expression of IL-17A, IL-17F, but also IFN-γ, PDGF-A, and *IL1A*, not previously described. These results, together with the reduced expression of both *HOPX* and *SOX2* in *RORC*-interfered cells, suggest that HOPX and SOX2 are two transcriptional regulators acting downstream of RORγt signalling in human Th17 cells.

In the second approach, we studied the pathogenic features intrinsically associated to Th17 cells using Th17 cells obtained from MS patients. In particular, we compared *in-vitro* differentiated Th17 cells of MS patients and healthy donors (HD) and we systematically analysed typical features of Th17 cells, including receptors, transcription factors and soluble factors by flow cytometry, ELISA and Luminex assays. We also included in this study the expression analysis of PDGF and LT-α, two novel effector molecules that we found associated to inflammatory Th17 cells in the previous approach. Results from these analyses unveiled the increased expression of pro-inflammatory proteins IL-21, IL-2, and IL-1 receptor1 (IL-1R1) in Th17 cells derived from MS patients compared to those from HD.

Moreover, we found that Th17 cells derived from MS patients express higher levels of LT-α compared to those from HD, indicating that the pathogenic signature that we previously identified contains intrinsic inflammatory features of Th17 cells derived from patients affected by Th17-related diseases.

In conclusion, the main results of the project were: 1) the identification of the inflammatory signature of human Th17 cells, that includes novel Th17 genes, such as IFNK, IL1A, PDGF-A, and LT-α, and novel transcriptional regulators HOPX and SOX2; 2) the identification of the intrinsic Th17 features specifically overexpressed in Th17 cells of MS patients, that includes IL-21, IL-2, IL-1R1, and LT-α.

Importantly, these factors could become new biomarkers or new therapeutic targets in Th17-related autoimmune diseases.

Index

*SECTION 1

*SECTION 2

*SECTION 3

*SECTION 4

*SECTION 5

SECTION 1

GENERAL INTRODUCTION AND AIMS OF THE THESIS

1.1. Immune system

The immune system is a network composed by different cell-types and soluble molecules, which collectively defend the organism against infectious agents. The immune system exerts this function by recognizing molecules expressed by microorganisms (antigens) and activating a coordinated response.

We can distinguish two types of immune response: the innate and the adaptive, which are distinct for timing of activation, specificity against the infectious microorganism, and long-lasting memory.

The innate immunity provides an initial defence, within hours from the antigen recognition, while the adaptive immunity exerts its function after approximately one week from the infection. However, the adaptive immunity is specific for each pathogen and ensures protection against subsequent reinfection with the same pathogen (Abbas, 2012d, 2012e), while innate immunity is mainly not specific and short-lived phenomenon. However, in the last years, it is emerging the concept that also innate immunity shows a long-term adaptation, known as trained immunity, due to a previous stimulation (priming), which results in an enhanced reaction to subsequent challenges (Netea *et al.*, 2020).

Innate immune response consists in physical, chemical and biological barriers, such as epithelium, antimicrobial peptides, and mucosal microbial flora (microbiota), which prevent potential infections (Beatriz Aristizábal and Ángel González, 2013) (Figure 1). Moreover, in case of infection, other components of innate immunity, including blood factors and innate immune cells, play a crucial role.

Blood factors are cytokines, such as interferon (IFN)-γ, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, produced by innate immune cells and alerting other cells to mount a proper inflammatory response in infected anatomical sites. Blood factors also include proteins of the complement system, which contribute to fight the infection by recruiting immune cells, opsonizing and neutralizing infectious agents (Beatriz Aristizábal and Ángel González, 2013) (Figure 1).

Innate immune cells are mast cells, eosinophils, basophils, and phagocytic cells (macrophages, neutrophils, and dendritic cells) (Beatriz Aristizábal and Ángel González, 2013) (Figure 1). Other cells, such as natural killer (NK) cells, NK T cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and innate lymphoid cells (ILCs) belong to innate immunity. However, they also display typical features of the adaptive immune system, such as the specificity against a pathogen and the long-

lasting memory against the same pathogen (Vivier *et al.*, 2011; Lanier, 2013; Trottein and Paget, 2018).

Functions of innate immune cells are: phagocytosis, including ingestion and degradation of pathogen components; cytokine production, to potentiate phagocytic functions and activate other immune cells; presentation of the antigen to adaptive immune cells. These functions are crucial for the first activation of the adaptive immune response. In fact, antigen bound to the major histocompatibility complex (MHC) on the surface of innate immune cells, interacts with receptors on the surface of adaptive immune cells. This process is called antigen presentation, and innate immune cells involved in this process are defined antigen-presenting cells (APC) (Abbas, 2012c, 2012d). Moreover, cytokines produced by innate immune cells are critical for the establishment and the amplification of the proper adaptive immune response.

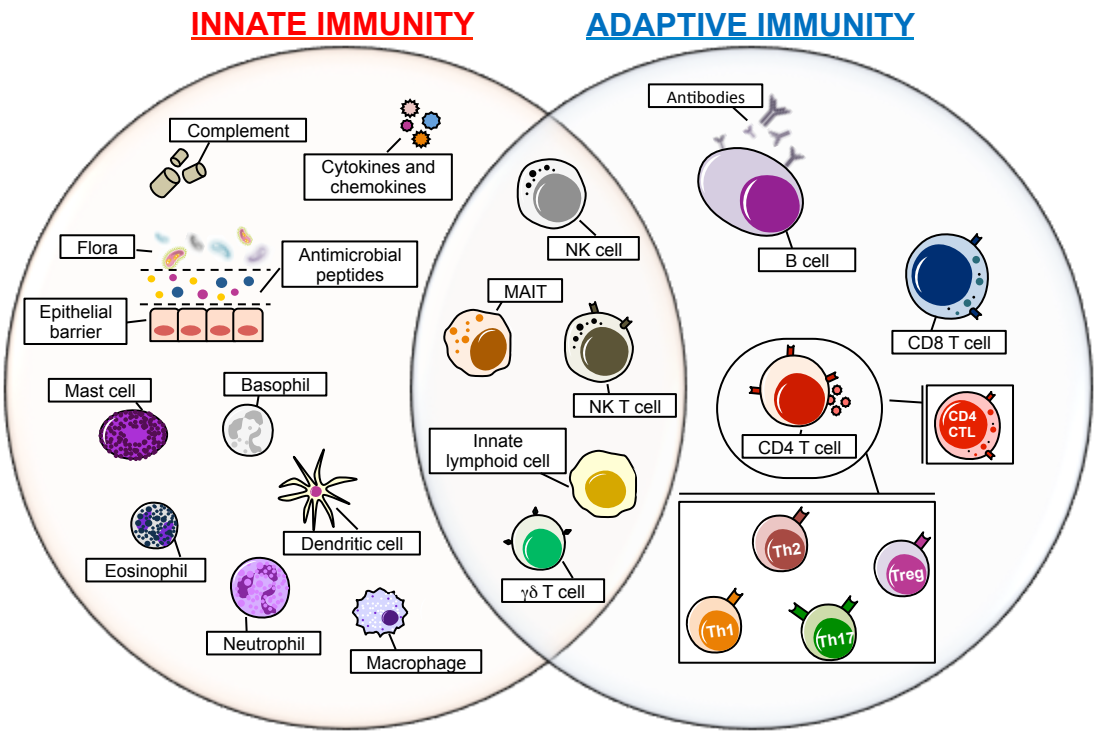


Figure 1. Components of the innate and adaptive immunity.
The innate immunity includes epithelial barrier, antimicrobial peptides, microbial flora, cytokines, chemokines, proteins of complement system and several innate immune cells. The adaptive immunity includes B lymphocytes producing antibodies, CD8 T lymphocytes and CD4 T lymphocytes (T helper subsets and cytotoxic CD4). NK cells, NK T cells, γδ cells, MAIT cells, and innate lymphoid cells share functions with both innate and adaptive immunity.

Adaptive immunity can be divided in humoral and cell-mediated response (Figure 2). The humoral immunity is mediated by B lymphocytes: naïve B cells upon antigen recognition differentiate in plasmablasts, which are the B lymphocytes producing antibodies. The antibodies neutralize microbes and toxins, induce opsonisation that favours phagocytosis by activated phagocytes, and activate the complement system (Abbas, 2012b) (Figure 2). This response is very important for extracellular microbes, such as parasitic worms.

The cell-mediated immunity is achieved by cytotoxic T lymphocytes (CTL), represented by CD8 T lymphocytes and a small part of CD4 T lymphocytes, called CD4 CTL: naïve CD8 lymphocytes and naïve CD4 T lymphocytes with the potential to differentiate into CD4 CTL, upon recognition of infected cell, release cytotoxic granules, which damage infected cells and eliminate the pathogens (Abbas, 2012a; Takeuchi and Saito, 2017) (Figure 2). This response is critical for the clearance of intracellular viruses and bacteria.

Importantly, both the humoral and the cell-mediated adaptive immune responses are regulated by CD4 T helper lymphocytes. The main function of these cells is the release of soluble factors, known as cytokines and chemokines, directed towards other immune cells (Figure 2) (Abbas, 2012a). In particular, cytokines activate or inhibit immune cells, while chemokines recruit immune cells.

In this context, we can distinguish CD4 T lymphocytes with inhibitory functions, called T regulatory (Treg) cells, and those with activating functions, called T helper (Th) cells. Treg are crucial for the suppression of the adaptive immune response after the clearance of viral, bacterial and parasitic infections. On the other side, Th cells are critical for the establishment of the proper adaptive immune response (humoral or cell-mediated) against viruses, bacteria, and parasites.

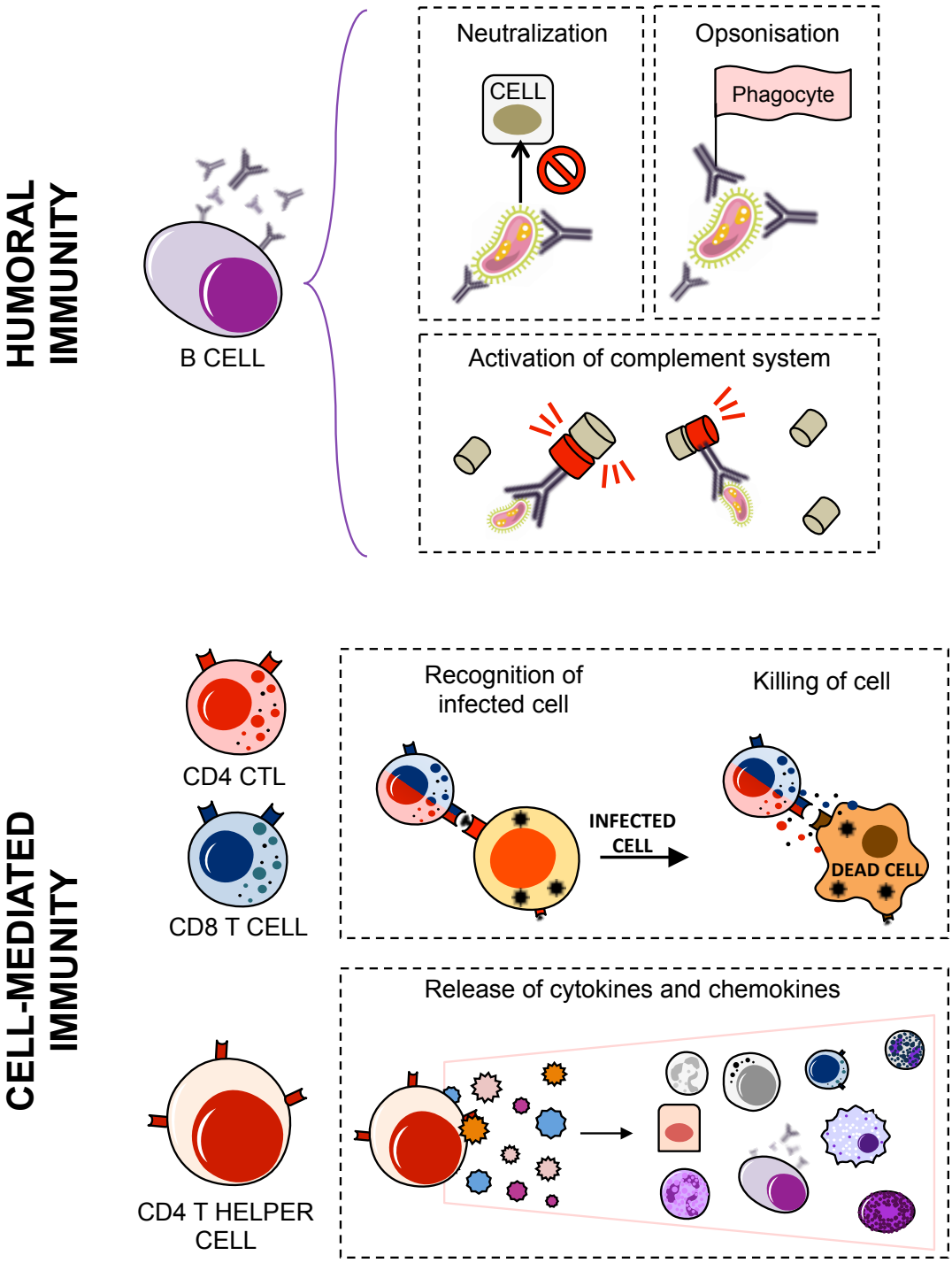


Figure 2. Adaptive immune responses.
B lymphocytes secrete antibodies and mediate the humoral response against extracellular microbes; CD8 T lymphocytes and CD4 T lymphocytes with cytotoxic activity (CD4 CTL) perform the cell-mediated response against intracellular infectious agents; CD4 T helper (CD4 Th) lymphocytes orchestrate other immune cells by producing cytokines and chemokines.

1.2. CD4 T helper (Th) lymphocytes

Similarly to B and CD8 T lymphocytes, the initial step of CD4 T cell differentiation involves activation of naïve CD4 T cells. These cells express membrane receptors, such as CD45RA, CD27, and C-C chemokine receptor type (CCR)7, which ensure their persistence into secondary lymphoid tissues (lymph nodes, spleen and mucosal-associated lymphoid tissue, such as Peyer's patches in the small intestine), where the antigen presentation by APC occurs (McLachlan and Jenkins, 2007; Ferrando-Martinez, Ruiz-Mateos and Leal, 2010; Caccamo *et al.*, 2018). The activation of naïve CD4 T cells is mediated by specialized APC, called dendritic cells, which encounter the infectious agent in periphery, migrate into secondary lymphoid tissues, and present antigen to naïve CD4 T cells. During this process, called differentiation or polarization, CD4 T cells acquire a memory phenotype ($CD45RA^-$ $CD45RO^+$) and specific effector functions. Then, differentiated CD4 T cells migrate into the site of infection and activate the proper adaptive immune response aimed to eradicate the pathogen.

The differentiation program requires three signals. The first signal is the interaction between antigen presented on MHC molecule exposed on dendritic cell surface, and T-cell receptor (TCR) on naïve CD4 T cell surface (Thomas J Kindt; Richard A Goldsby; Barbara Anne Osborne; Janis Kuby, 2007) (Figure 3). The binding between co-stimulatory molecules on T cell surface and corresponding ligands on dendritic cell surface defines the second signal (Figure 3). For instance, CD28 is the main co-stimulatory molecule constitutively expressed by T cells, whose interaction with CD80 and CD86 on mature dendritic cells leads to the proliferation and expansion of CD4 T cells (Jenkins *et al.*, 2001; Magee, Boenisch and Najafian, 2012). The third signal involves interaction of naïve CD4 T cells with cytokines released by dendritic cells through specific cytokine receptors (Figure 3). Interestingly, different sets of cytokines may activate distinct differentiation programs leading to Th1, Th2, Th17 or Treg profiles.

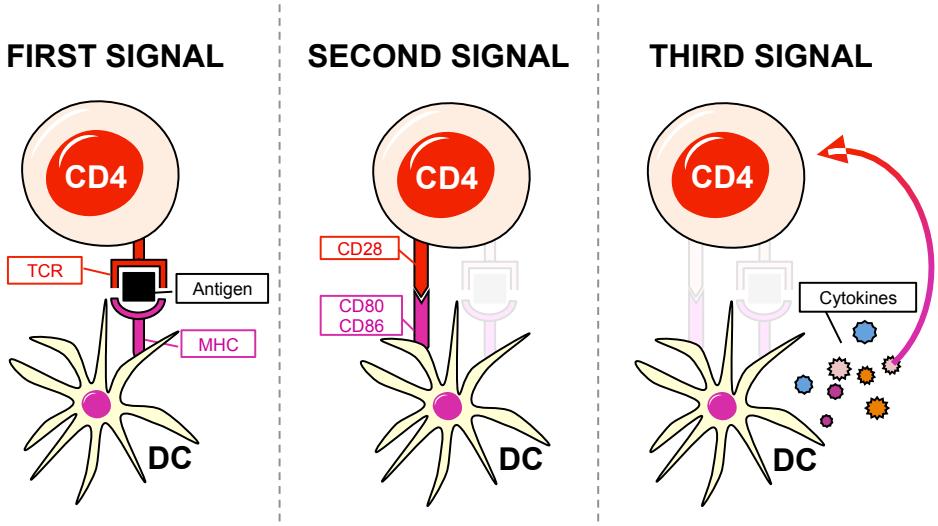


Figure 3. Three signals necessary for CD4 T lymphocyte differentiation.
The interaction between dendritic cells (DC) and CD4 T cells (CD4) required three signals. Signal 1 concerns the presentation of antigen peptide, in the context of MHC molecules on DC surface, which is recognized by TCR on T cell surface. Signal 2 involves the binding between co-stimulatory molecules on DC surface, such as CD80 and CD86, and their ligand on T cell surface, such as CD28. Signal 3 consists in the secretion of cytokines by DC, whose signalling polarizes T cells toward a specific Th profile.

Th1 and Th2 are the first two Th profiles described, and their differentiation is driven by IL-12 and IL-4, respectively. These cytokines lead to the activation of distinct differentiation programs: IL-12 activates STAT-1, STAT-4, and T-bet transcription factors (Szabo *et al.*, 2000; Lighvani *et al.*, 2001; Afkarian *et al.*, 2002), which in turn induce the production of IFN-γ (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989) (Figure 4); IL-4 activates the transcription factors GATA3 and STAT-6 (Zheng and Flavell, 1997; Horiuchi *et al.*, 2011), that induce the production of IL-4, IL-5, and IL-13 (Mosmann *et al.*, 1986) (Figure 4). Th1 cells induce cell-mediated inflammatory responses (macrophages and CD8 T cells) against intracellular bacteria, while Th2 cells activate B cells and promote a protective humoral response against helminth infection (Mosmann and Coffman, 1989; Paul and Seder, 1994).

Treg cells differentiate in presence of transforming growth factor-β (TGF-β) and IL-2 (Rodríguez-Perea *et al.*, 2016), which activate STAT5 and Foxp3 transcription factors (Fontenot, Gavin and Rudensky, 2017). They produce IL-10 and TGF-β, and are involved in suppressing humoral and cell-mediated immune responses once the pathogen has been cleared (Figure 4).

In 2005, the Th17 subset was identified as effector Th subset distinct from Th1 and Th2 (Harrington *et al.*, 2005). Th17 cells differentiate in presence of IL-1β, IL-6, IL-23 and TGF-β cytokines (Veldhoen, Hocking, Atkins, *et al.*, 2006; Volpe *et al.*, 2008), which activate several transcription factors, such as STAT3, leading to release of

IL-17A and IL-17F. However, the key transcription factor involved in the differentiation program of Th17 cells is the retinoic acid-related orphan receptor (ROR)γt (Ivanov *et al.*, 2006; Ivanov, Zhou and Littman, 2007) (Figure 4). In general, Th17 immune response induces the recruitment of neutrophils and the production of antimicrobial-peptides by epithelial cells, that improves epithelial-barrier integrity and is critical for mucosal host defence against extracellular bacteria and fungi (Weaver *et al.*, 2006; Bettelli *et al.*, 2008). However, in the last years the field of Th17 cells has been largely explored, and important progresses have been made related to the Th17 polarizing cytokines, Th17 transcriptional regulators, physiological and pathological role of the Th17 immune response.

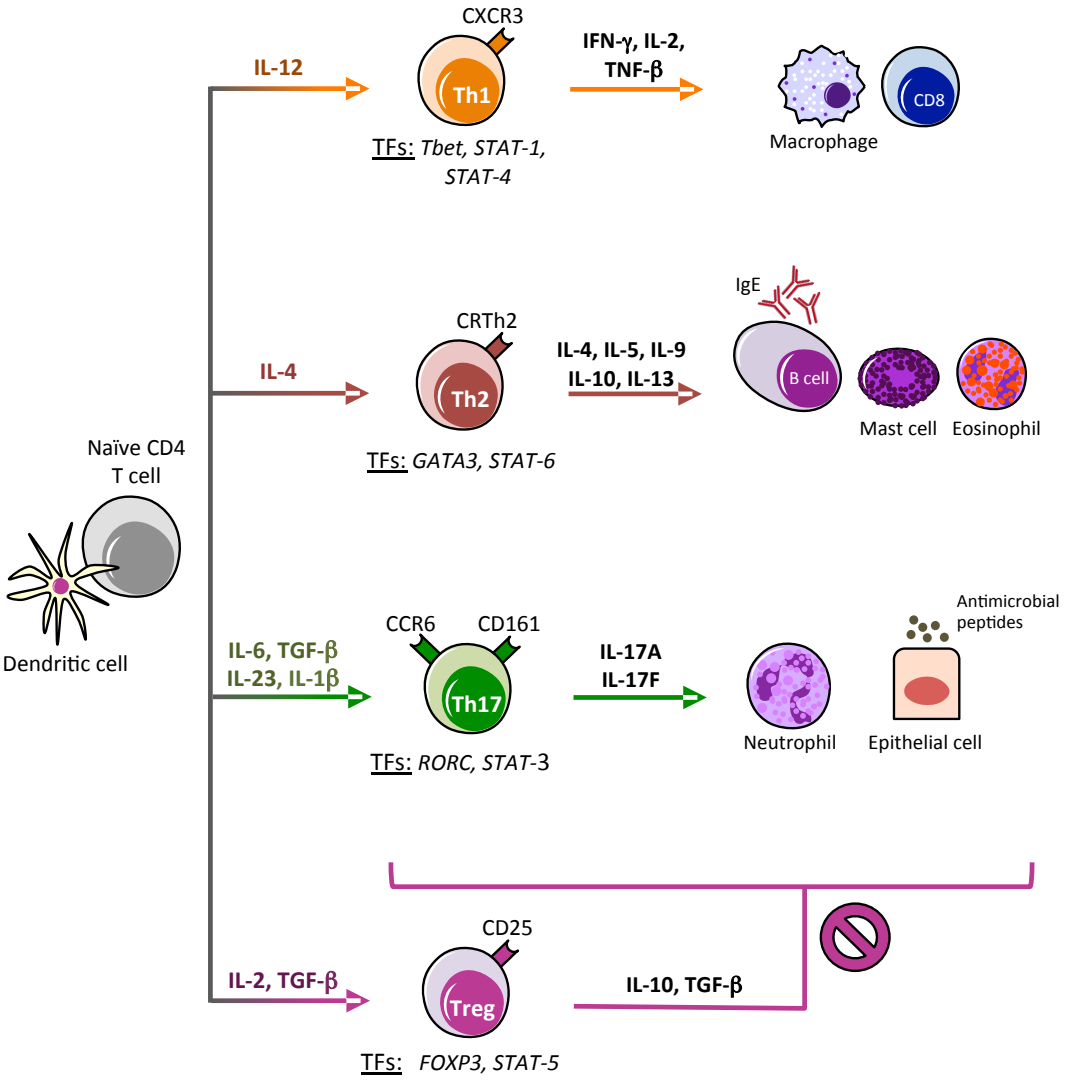


Figure 4. Differentiation and effector functions of T helper cells.
The interaction between dendritic cell and naïve CD4 T cells leads to differentiation of naïve CD4 T cells in distinct T helper (Th) profiles: Th1, Th2, Th17 and Treg cells. Each Th subpopulation requires specific polarizing cytokines, expresses specific transcription factors (TFs), cytokines, and receptors, whose synergy leads to the activation of immune responses with distinct functions.

1.3. T helper (Th) 17 cells

1.3.1. Th17 polarizing cytokines

Since the discovery of Th17 cells (Harrington *et al.*, 2005; Park *et al.*, 2005), several important aspects concerning the differentiation process have been elucidated, such as the factors promoting their differentiation (third signal of activation).

In mice, TGF-β and IL-6 have a critical role in inducing Th17 cell differentiation (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). Moreover, other pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-23, and IL-21 cooperate in this process (Figure 5) (Veldhoen, Hocking, Atkins, *et al.*, 2006; Veldhoen, Hocking, Flavell, *et al.*, 2006; Nurieva *et al.*, 2007; Bettelli *et al.*, 2008). In particular, IL-6 and TGF-β are involved in the induction, IL-21 in the amplification, and IL-23 in the stabilization phase of murine Th17 cell differentiation (Bettelli *et al.*, 2008).

Studies on human Th17 cell differentiation have reported a critical role for IL-1β, IL-6, IL-23 and TGF-β (Volpe *et al.*, 2008). Moreover, IL-21, produced by Th17 cells, synergizes with TGF-β to enhance human Th17 cell differentiation (Yang *et al.*, 2009) (Figure 5).

Although the differentiation process of human Th17 cells has never been dissected, we know that human naïve CD4 T cells require IL-6 and TGF-β to express IL-1β and IL-23 receptors on their surface (our unpublished data). Thus, we suppose that similarly to mouse, in human the differentiation process of Th17 cells is divided in induction mediated by IL-6 and TGF-β , stabilization with IL-1β and IL-23, and amplification (IL-21) steps.

Collectively, these studies reveal that similar pathways regulate both human and mouse Th17 cell differentiation (Soumelis and Volpe, 2008).

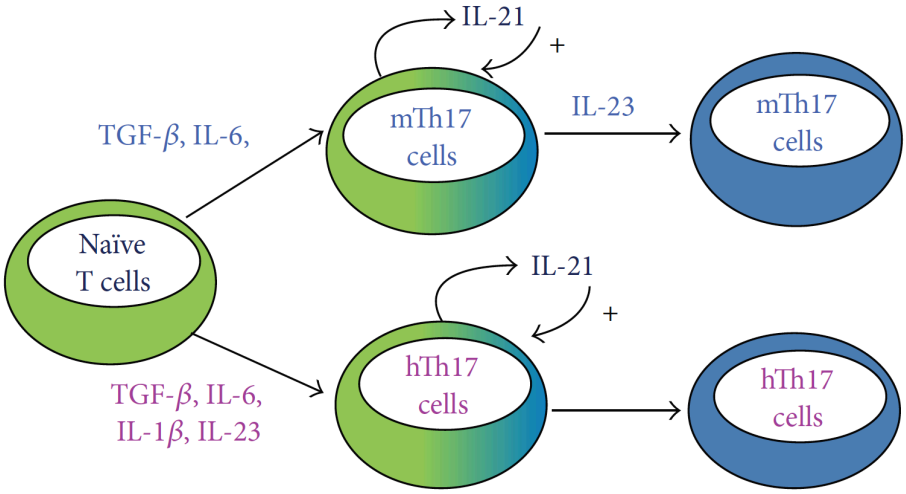


Figure 5. Th17 cell differentiation in mouse and human.
T helper (Th) 17 cell differentiation program in mice (blue labels) and humans (purple labels) shares significant similarities. In both organisms, IL-6, TGF-β, IL-23, and IL-21 are involved in complete Th17 differentiation. Human Th17 cell differentiation requires also IL-1β (Adapted by Volpe, Battistini and Borsellino, 2015)

1.3.2. Th17 transcriptional regulators

Th17 polarizing cytokines act through the binding with their specific receptors. This phenomenon activates signalling pathways, which activate nuclear transcription factors. In particular, interactions between IL-6/IL-6R, IL-23/IL-23R, and IL-21/IL-21R induce the activation and homodimerization of STAT3 that translocates into the nucleus and regulates the transcription of genes encoding for IL-17A (*Il17a*) and RORγt (*Rorc*), designated as the "lineage defining transcription factor of Th17 cells" (Figure 6) (Durant *et al.*, 2010; Bhaumik and Basu, 2017).

However, the synergy between all polarizing cytokines further activates RORγt (Ivanov *et al.*, 2006; Manel, Unutmaz and Littman, 2008), which further induces IL-17A transcription by binding the promoter and the non-coding sequences (CNS)2 of *Il17a* gene (Yang *et al.*, 2008; Zhang, Meng and Strober, 2008; Wang *et al.*, 2012). Interestingly, another member of the same family of RORγt, named ROR*a*4, activates *Il17a* transcription through the same molecular mechanisms of RORγt (Yang *et al.*, 2008) (Figure 6). Co-expression of RORα4 and RORγt causes the synergistic increase of IL-17A, indicating that RORα4 and RORγt work together to regulate Th17 cell differentiation (Sundrud and Rao, 2008; Yang *et al.*, 2008).

It has been demonstrated that other transcription factors contribute to *Il17a* expression, such as the inhibitor of NF-κB (IκB)ζ (Yamazaki, Muta and Takeshige, 2001; Muta, 2006; Okamoto et al., 2010), Kruppel-like factor (KLF)4 (Lebson *et al.*, 2010), and basic leucine zipper ATF-like transcription factor (BATF) (Durant *et al.*, 2010), which forms a complex with JunB, and regulates also *Rorc* transcription (Hasan *et al.*, 2017; Yamazaki *et al.*, 2017) (Figure 6). JunB also co-localizes in Th17 cells with another transcription factor, called interferon regulatory factor (IRF)4, involved in Th17 differentiation (Brüstle *et al.*, 2007) (Figure 6).

Moreover, p65 NF-κB subunit and NFATc2 are factors that initiate the transcription of RORγt by inducing a permissive chromatin conformation in the RORC2 regulatory regions on *Rorc* promoter (Ruan *et al.*, 2011; Yahia-Cherbal *et al.*, 2019). This allows the recruitment of other transcription factors on *Rorc* promoter, such as Runt-related transcription factor (RUNX)1 (Liu *et al.*, 2015) and hypoxia-inducible factor (HIF)-1α (Dang *et al.*, 2011), which potentiate *Il17a* expression (Figure 6) (Semenza, 2007; Zhang, Meng and Strober, 2008; Dang *et al.*, 2011).

Altogether, this information reveals a complex interconnected network of transcriptional regulators that finely regulates the generation of Th17 cells (Capone and Volpe, 2020).

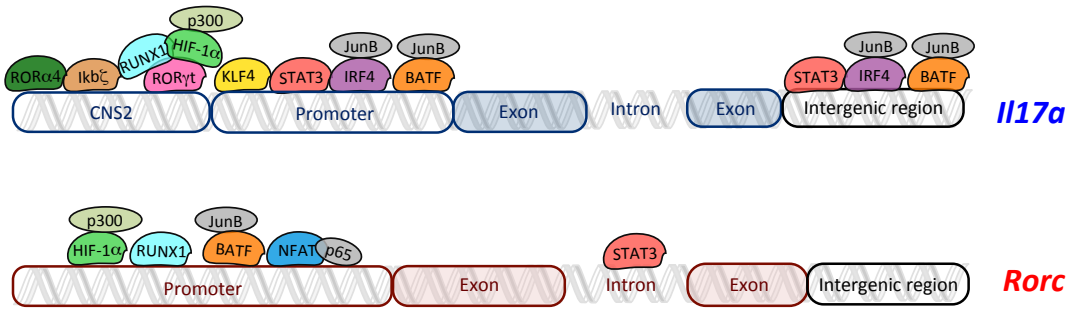


Figure 6. Overview of transcriptional regulators of Il17 and Rorc

The transcriptional regulators of Th17 cells (RORγt, RORγ4, Ikbζ, RUNX1, HIF-1α, STAT3, IRF4, NFAT, and BATF) regulate transcription of Il17a and Rorc by binding specific regions in their loci. Schema does not respect the real organization and structure of each gene locus (Capone and Volpe, 2020).

1.3.3. Physiological role of Th17 cells

The complex network of transcriptional regulators leads to production of IL-17A, IL-17F, and other cytokines, responsible of the protective functions of Th17 cells during bacterial and fungal infections. IL-17A and IL-17F are the most representative Th17 cytokines (Park *et al.*, 2005; Aujla, Dubin and Kolls, 2007; Volpe *et al.*, 2008). They exist either as homodimers or as heterodimers (Amatya, Garg and Gaffen, 2017), and both forms are able to bind the IL-17 receptor (IL-17RA) (Weaver *et al.*, 2007).
Given the ubiquitous expression of IL-17RA, several cells are targets of IL-17A and IL-17F, including haematopoietic cells, osteoblasts, fibroblasts, endothelial cells and epithelial cells (Bettelli *et al.*, 2008; Amatya, Garg and Gaffen, 2017).
The activation of IL-17RA induces the expression of several molecules: pro-inflammatory cytokines, including IL-1β, IL-6, GM-CSF, G-CSF and TNF by fibroblasts, macrophages, chondrocytes and osteocytes; chemokines by resident cells, such as CXCL9 and CXCL10, which recruit CXCR3 expressing cells, CCL2, CCL7, CXCL1, CXCL2, CXCL5 and CXCL-8, which attract neutrophils and monocytes, and CCL20, which recruits CCR6-expressing cells, including other IL-17-producing immune cells (Veldhoen, 2017); prostaglandin E2, which causes vasodilatation and favours recruitment of mast cell, neutrophils, and macrophages; several matrix-metalloproteinases (MMPs), which disrupt the extracellular matrix, thus facilitating the migration of immune cells (Bettelli et al., 2008; Veldhoen, 2017) (Figura 7).
In addition, the activation of IL-17/IL17R cascade induces tissue-specific molecules in the inflamed organ. For instance, epithelial cells express antimicrobial proteins (AMPs) that contribute to inhibit and kill invading microorganisms (Veldhoen, 2017); gut epithelia express occludin (Ocln), regenerating islet-derived protein 3g (Reg3g), and mucin 1 (Muc1) proteins, which collectively maintain the intestinal barrier integrity (Amatya, Garg and Gaffen, 2017); osteoblasts up-regulate the expression of RANKL, which promotes their differentiation and activation (Amatya, Garg and Gaffen, 2017).
Moreover, Th17 cells are producers of a broad array of other cytokines, such as IL-21, IL-22, TNF-α, IL-26, and CCL20 (Park *et al.*, 2005; Aujla, Dubin and Kolls, 2007; Wilson *et al.*, 2007; Volpe *et al.*, 2008). All these factors lead to a potent and robust Th17 effector response. In particular, IL-21 promotes the expansion and maintaining of Th17 cells (Volpe, Battistini and Borsellino, 2015), the differentiation of B cells in plasmablasts producing antibodies, and the cytotoxic activity of CD8 T and NK cells (Parrish-Novak *et al.*, 2002; Bettelli *et al.*, 2008) (Figure 7).

The cytokine IL-22 induces the proliferation of epithelial cells, known to express high levels of IL-22 receptor, and stimulates the production of AMPs and β-defensin proteins (Bettelli *et al.*, 2008; Rutz, Eidenschenk and Ouyang, 2013). IL-22 is also involved in the induction of acute-phase proteins from hepatocytes (Maddur *et al.*, 2012) (Figure 7). TNF-α promotes the expression of AMPs, CCL20, IL-1β and MMP molecules by epithelial cells, endothelial cells, fibroblasts, and macrophages (Veldhoen, 2017) (Figure 7).

The chemokine CCL20 attracts CCR6-expressing cells, including Th17 cells (Figure 7), and mediates their migration to the site of inflammation.

Finally, IL-26 exhibits a direct antimicrobial activity given its structural similarity with AMPs. Moreover, IL-26 induce type I interferon by dendritic cells, and IL-8 and TNF-α by gut epithelial cells, thus priming the anti-microbial activity of other immune cells (Meller *et al.*, 2015; Larochette *et al.*, 2019) (Figure 7).

Th17 cells are also able to produce the anti-inflammatory IL-10 and the pro-inflammatory IFN-γ cytokines. In particular, it has been demonstrated that *Staphylococcus aureus* infection promotes the differentiation of IL-10-secreting Th17 cells, while *Candida albicans* infection induces the polarization of IFN-γ-secreting Th17 cells, also called Th1/17 cells (Zielinski *et al.*, 2012). In this scenario, the ability of Th17 cells to produce IFN-γ or IL-10 is controlled by IL-1β, whose presence induces IFN-γ and suppresses IL-10 (Volpe *et al.*, 2008; Zielinski *et al.*, 2012). Interestingly, the combined expression of IL-10 or IFN-γ with IL-17A determines the regulatory or inflammatory role, respectively, of Th17 cells (Guo, 2016; Wu, Tian and Wang, 2018). In particular, IFN-γ/IL-17A double producer cells exert pathogenic functions in several autoimmune diseases, such as multiple sclerosis, psoriasis, arthritis, and inflammatory bowel disease (Sallusto, Zielinski and Lanzavecchia, 2012).

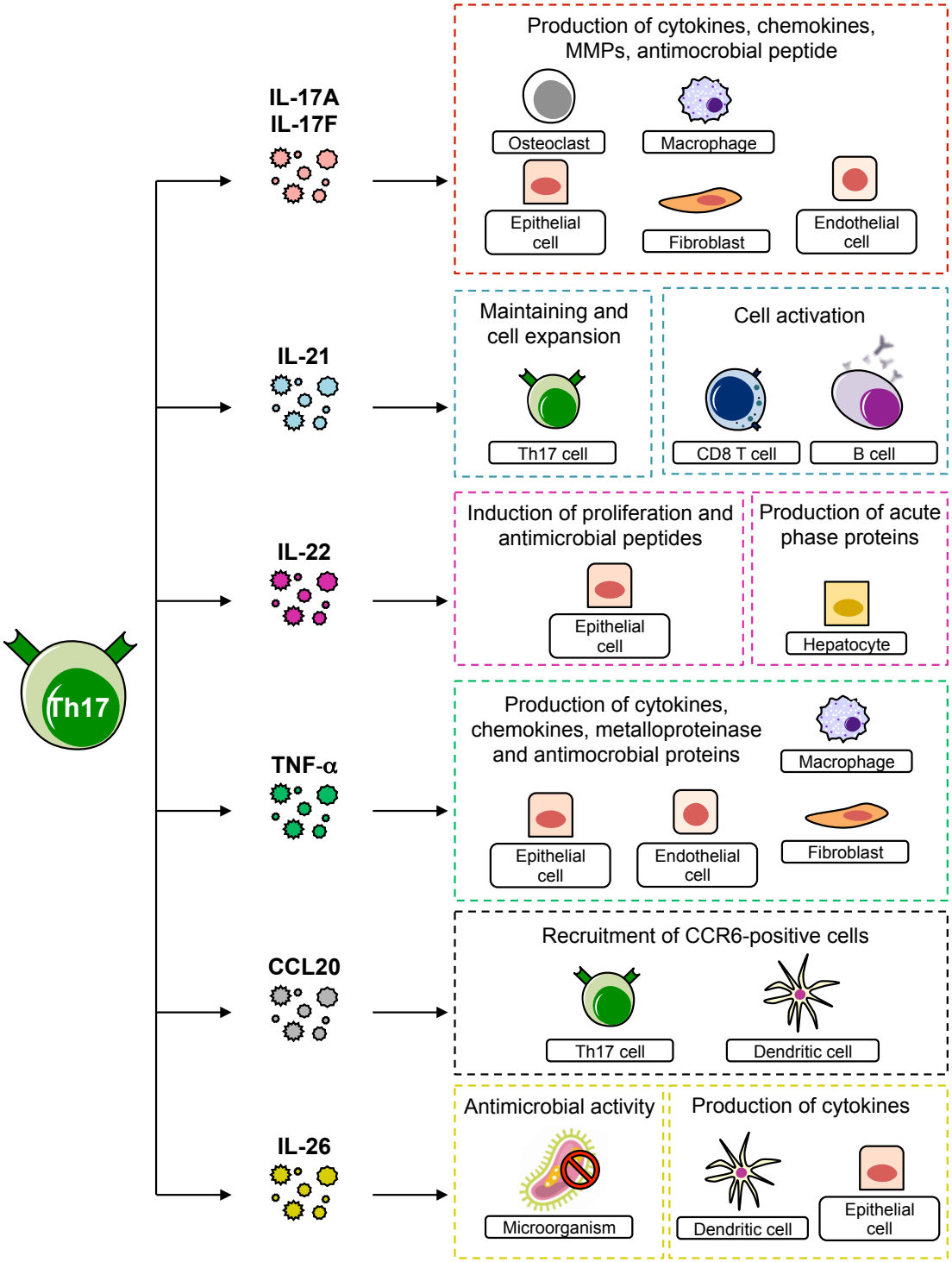


Figure 7. Functions of the main cytokines and chemokines produced by Th17 cells. *Human Th17 cells secrete several effector molecules, including IL-17A/F, IL-21, IL-22, TNF-α, CCL20, and IL-26. These soluble factors target immune and non-immune cells and mediate several functions: cell differentiation and activation; release of soluble factors; recruitment of cells to sites of inflammation; antimicrobial activity.*

1.3.4. Pathogenic role of Th17 cells and therapy targeting Th17 cells

Although Th17 cells play a protective role during infections, an abnormal Th17 cell response is involved in several autoimmune diseases. The pathogenic role of Th17 cells in autoimmune diseases is supported by both human studies and experiments performed in animal models.

Studies in murine models, such as experimental autoimmune encephalomyelitis (EAE) (Cua *et al.*, 2003), trinitrobenzene sulfuric acid (TNBS)-induced colitis (Zhang *et al.*, 2006), and antigen or collagen-induced arthritis (Lubberts *et al.*, 2004), reveal that the IL-17A pathway plays a pathogenic role in autoimmune disorders. However, the concept that Th17 cells are responsible for driving autoimmune inflammation was established when the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (MS) was shown to be induced by passive transfer of IL-17-producing myelin reactive CD4 T cells (Langrish *et al.*, 2005).

In human, IL-17A is highly expressed in the central nervous system (CNS) lesions, in blood cells, and cerebrospinal fluid (CSF) of patients with MS (Lock *et al.*, 2002; Tzartos *et al.*, 2008; Brucklacher-Waldert *et al.*, 2009; Durelli *et al.*, 2009; Camperio *et al.*, 2014), in the colonic mucosa of patients with ulcerative colitis or Crohn's disease (Fujino *et al.*, 2003), in the psoriatic skin (Teunissen *et al.*, 1998; Wilson *et al.*, 2007), and in the synovial tissues from rheumatoid arthritis patients (Kotake *et al.*, 1999; Metawi *et al.*, 2011).

Among the typical Th17 features that could confer pathogenicity to autoimmune diseases, the aberrant production of inflammatory mediators, such as IL-17A, IL-22, TNF-α, IL-21 could have a main role.

In order to block this inflammatory circuit, several pharmacological treatments directed against Th17 cells have been developed for autoimmune diseases. The main targets include molecules related to the effector functions of Th17 cells, such as IL-17A, or molecules associated to Th17 cell generation, such as IL-23 and RORγt. Interestingly, promising results have been reported: beneficial effects of antibodies against IL-23 (Tildrakizumab and Ustekinumab) have been observed in psoriasis (Leonardi, Kimball and Papp, 2008; Kopp *et al.*, 2015), and more recently, Secukinumab, a fully humanized antibody neutralizing IL-17A, has been approved as the first-line systemic treatment for moderate to severe plaque psoriasis (Garnock-Jones, 2015; McInnes *et al.*, 2015). Moreover, secukinumab, as well as the novel anti-IL17 monoclonal antibody ixekizumab, showed efficacy in rheumatoid arthritis patients not responsive to other treatments (Genovese *et al.*, 2013, 2016; Genovese, Durez, *et al.*, 2014; Genovese, Greenwald, *et al.*, 2014). Similarly, Ustekinumab is used to treat moderate to severe active Crohn's disease and moderate to severe active

ulcerative colitis in adults, when other treatments have not been effective (Sandborn *et al.*, 2012).

In the recent years, several small molecules targeting RORγt have been discovered and tested in preclinical studies or currently being tested in ongoing clinical trials for the treatment of psoriasis, rhemautoid arthritis and inflammatory bowel diseases (Sun, Guo and Wang, 2019).

In MS current therapies targeting IL-17A, IL-23, or RORγt, did not show sufficient beneficial effects on disease progression (Capone and Volpe, 2020).

Further studies are needed to better characterize the pathogenic role of Th17 cells in MS, and to identify new inflammatory molecules that could be promising therapeutic targets.

1.3.5. Insight into the pathogenic role of Th17 cells in multiple sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease affecting the central nervous system (CNS) (Keegan and Noseworthy, 2002; Hauser and Oksenberg, 2006).
Activated autoreactive Th17 cells, which recognize myelin protein-derived antigens (Dendrou, Fugger and Friese, 2015), transmigrate from periphery into the CNS, through the blood brain barrier (BBB) (Keegan and Noseworthy, 2002; Hauser and Oksenberg, 2006), where they release IL-17A and other pro-inflammatory cytokines (Hemmer, Archelos and Hartung, 2002; Brucklacher-Waldert *et al.*, 2009; Volpe, Battistini and Borsellino, 2015).
The entry and persistence of Th17 cells into CNS is ensured by the expression of surface receptors: CCR6 recognizes the CCL20 constitutively produced by epithelial cells of the choroid plexus, allowing the migration of Th17 cells into the encephalic compartment (Reboldi *et al.*, 2009); IL-1R1, IL-23R, and IL-21R interact with IL-1β, IL-23, and IL-21, and promote amplification of IL-17A production (Acosta-Rodriguez *et al.*, 2007; Volpe, Battistini and Borsellino, 2015).
IL-17A plays different pathogenic roles into CNS (Figure 8). In fact, it has been demonstrated that IL-17A inhibits the tight junctions of the endothelial cells of the human BBB (Volpe, Battistini and Borsellino, 2015), thus promoting the migration of other immune cells into CNS (Figure 8). Moreover, IL-17A interferes with remyelinating processes, by reducing survival and promoting apoptosis of oligodendrocytes, the myelin-forming cells (Volpe, Battistini and Borsellino, 2015; Kunkl *et al.*, 2020). It has been also reported a neurotoxic effect of IL-17A, by the induction of severe fluctuations of intracellular calcium levels in neurons during EAE (Siffrin *et al.*, 2010).
Recently, several studies in murine Th17 cells investigated the molecular mechanisms underlying the role of "pathogenic" Th17 cells in comparison to "non-pathogenic" Th17 cells. In particular, transcriptomic analysis of murine Th17 cells generated in presence of "pathogenic" and "non-pathogenic" Th17-promoting cocktails revealed the murine pathogenic signature of Th17 cells, that includes *Cxcl3*, *Il22*, *Il3*, *Ccl4*, *Gzmb*, and other genes (Chung *et al.*, 2009; Ghoreschi *et al.*, 2010; Lee *et al.*, 2012). Interestingly, murine pathogenic Th17 cell signature show similarities with the transcriptional profile of human Th1/17 cells, such as the expression of *Ifng*, *CCl5*, *CCl4*, *CCl3*, *Epsti1*, *Gzmb*, *Il3*, *Cxcr3*, *Rgs2*, *Csf2*, *Il23r* genes (Yosef *et al.*, 2013; Hu *et al.*, 2017).
However, important questions on the pathogenic role of human Th17 cells are still open.

In this thesis we used two approaches to identify new pathogenic mechanisms of Th17 cells that could offer new perspectives towards therapeutic targeting of MS.

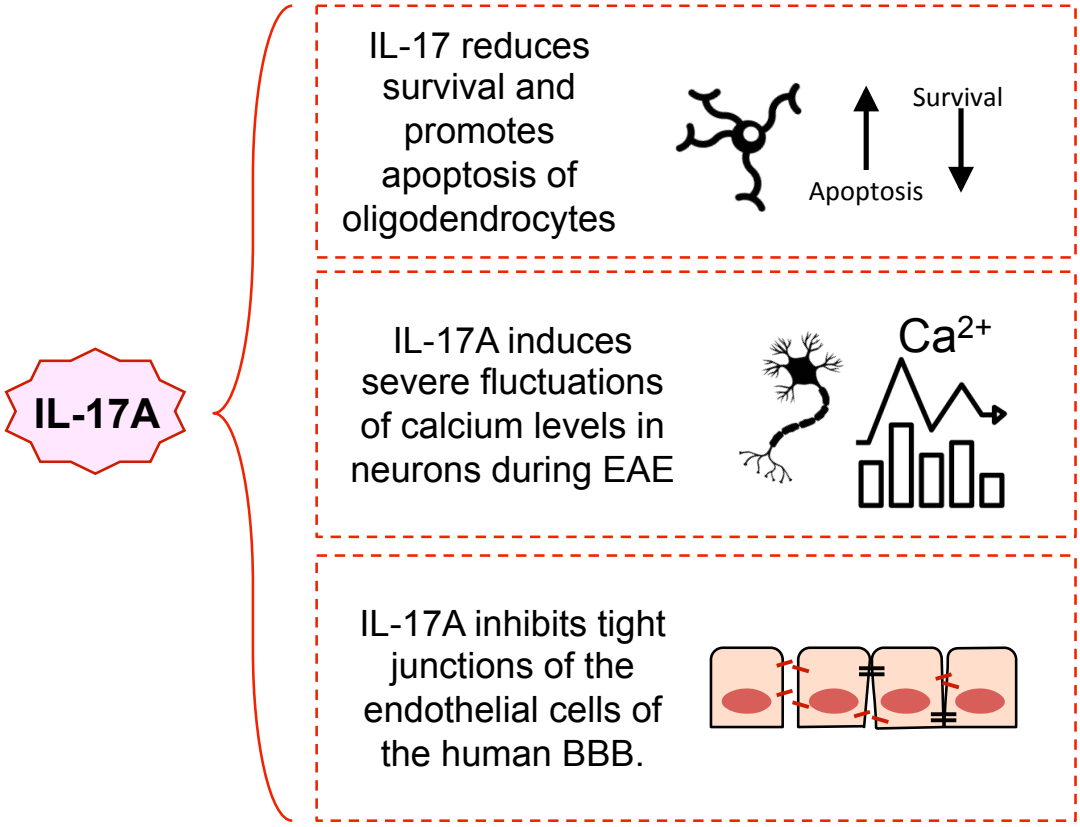


Figure 8. Role of IL-17A cytokine in the pathogenesis of MS.
Relevant functions of IL-17A in MS pathogenesis. Targets cells of IL-17A into the central nervous system (CNS) are endothelial cells of blood brain barrier (BBB), neurons, and oligodendrocytes.

1.4. Aims of the thesis

Main objective of this study was to unveil new immunological pathways and molecules of human Th17 cells that could contribute to the pathogenic role of Th17 cells in MS.

In particular, we can distinguish three specific aims:

1) Characterization of the global and stepwise transcriptional changes accompanying human Th17 cell polarization.

2) Identification of new inflammatory molecules acquired during human Th17 cell differentiation process.

3) Analysis of intrinsic Th17 features associated to Th17 cells from MS patients.

SECTION 2

GLOBAL TRANSCRIPTIONAL PROFILE OF HUMAN INFLAMMATORY AND REGULATORY TH17 CELLS

Results of Section 2 address the Aims 1 and 2, and have been included in:

Systems analysis of human Th17 cell differentiation uncovers distinct time-regulated transcriptional modules.

Alessia Capone, Chiara Naro, Manuela Bianco, Marco De Bardi, Floriane Noël, Paolo Macchi, Luca Battistini, Vassili Soumelis, Elisabetta Volpe*, and Claudio Sette* (Under revision) *(*equal contribution)*

2.1. Introduction

T helper (Th) 17 cells are mainly characterized by production of IL-17A and IL-17F and play a key role in the immune response against fungi and intracellular pathogens (Liang *et al.*, 2007). The production of Th17-specific cytokines is regulated by a unique genetic program orchestrated by transcription factors, where the RORC-encoded retinoic acid-related orphan nuclear receptor (ROR)γt is a key regulatory factor in both human and mouse Th17 cells (Ivanov *et al.*, 2006; Manel, Unutmaz and Littman, 2008; Volpe *et al.*, 2008; Capone and Volpe, 2020). Importantly, dysregulation of Th17 cell functions contributes to the pathogenesis of inflammatory and autoimmune diseases.

Since the discovery of Th17 cells as a distinct Th subset in 2005 (Park *et al.*, 2005), significant progress has been made in the field, including the identification of cytokines, transcription factors and epigenetic modifications underlying Th17 differentiation (Veldhoen, Hocking, Atkins, *et al.*, 2006; Manel, Unutmaz and Littman, 2008; Volpe *et al.*, 2008; Mukasa *et al.*, 2010; Ciofani *et al.*, 2012; Kanno *et al.*, 2012; Capone and Volpe, 2020), the discovery of typical effector cytokines regulating their functions (Annunziato *et al.*, 2007; Volpe *et al.*, 2009) and the involvement of Th17 cells in pathological conditions (Maddur *et al.*, 2012; Volpe, Battistini and Borsellino, 2015). In recent years, therapies directed against IL-17 have been developed and their efficacy has been proven. Secukinumab, a fully humanized antibody neutralizing IL-17A, is now approved as the first-line systemic treatment for moderate to severe plaque psoriasis (Garnock-Jones, 2015; McInnes *et al.*, 2015). However, important questions on human Th17 cells are still open, such as the sequence of molecular events occurring during the differentiation process, and the identification of features that synergize with IL-17 and contribute to the inflammatory properties of these cells. Elucidating the global transcriptional program of human Th17 cells is essential to answer these open questions, and to further improve therapies directed against Th17 cells.

In this study, we have characterized the transcriptional program set in motion during human Th17 cell polarization and unveiled new genes characterizing their profile, which may contribute to their inflammatory potential. This study expands our knowledge on the nature and molecular regulation of human Th17 cells and reveals new potential therapeutic targets for the treatment of Th17-related diseases.

2.2. Results

Dynamic transcriptome changes underlie stepwise acquisition of early and late features of human Th17 cells

In order to exhaustively investigate the differentiation process of human Th17 cells, we performed a genome-wide transcriptome analysis of naïve CD4 T cells differentiated under Th17 conditions (IL-1β, IL-6, TGF-β and IL-23) compared to unpolarized cells (Th0). First, we set out to determine key early and late time points of the human Th17 cell polarization process. Expression of RORC mRNA, encoding the transcription factor RORγt that is strictly required for terminal differentiation of Th17 cells (Ivanov *et al.*, 2006), was already detected at 48 hours (h) of culture under Th17 condition (Figure 9A), suggesting that the polarization program had already started. However, maximal RORC induction required 5 days (d) of culture, indicating acquisition of complete Th17 cell polarization at this later time point (Figure 9A). On this basis, we selected 48h and 5d, respectively, as early and late stages of the human Th17 polarization program (Figure 9B) for the high-throughput RNA-sequencing (RNA-seq) analysis.

To limit donor-associated variability, we analysed human T cells from 5 independent healthy donors (HD) for each condition. Bioinformatics analyses highlighted a large extent of gene expression reprogramming associated with acquisition of the Th17 profile. By setting 1.5-fold difference and $p \leq 0.05$ as filters, we identified 1130 genes differentially expressed between Th17 and Th0 cells at 48h, and 4589 genes at 5d (Figure 9C).

RNA-seq and bioinformatics analyses were validated by quantitative real time PCR (qPCR) on an independent set of human primary blood T cells. We found that 18 out of 22 tested genes (81,8% of validation) were confirmed by qPCR analysis (Figure S1A). In particular, the expression of Th17 signature genes (*IL17A*, *IL17F*, *IL23R*, *IL26*, *RORA*, *RORC*) mirrored the RNA-seq results (Figure S1B). Furthermore, analyses of other arbitrarily selected genes at either 48h or 5d of differentiation confirmed the reliability of the RNA-seq data (Figure S1C,D).

To identify genes with relevance for the acquisition of specific Th17 features, we compared three datasets: Th17 vs Th0 at 48h (early Th17 genes), Th17 vs Th0 at 5d (late Th17 genes) and Th17 at 5d vs Th17 at 48h (genes that vary over time in Th17 cells). Overlap between these comparisons identified three modules (Figure 9D). Module A (early) included genes specific of early Th17 cell differentiation (i.e. *KLRD1* and *NOTCH1*; Figure 9E), and was enriched in functional categories related to signalling pathways (Figure 9F). Module B (persistent) included genes that were constantly modulated during Th17 cell differentiation, such as the Th17 signature genes *IL23R* and *CCL20* (Figure 9E).

These genes were enriched in functional categories involved in "T cell differentiation/activation", "cytokine production", and "JAK/STAT signalling", which are all relevant for Th17 cell biology (Figure 9F). Module C (late) included genes of late differentiation, such as the Th17 effector cytokine genes *IL26* and *IL8* (Figure 9E), and was enriched in functional categories of strong relevance to the effector functions of Th17 cells, such as "response to other organisms" and "cell motility" (Figure 9F).

Th17 cell differentiation and functions are finely regulated by the expression of specific transcriptional regulators. Thus, we explored the expression of transcription factors that are known to play a key role in Th17 biology (Capone and Volpe, 2020). We found that *RORC*, *RORA*, *RUNX1*, *BATF* and *IRF4* genes belonged to the persistent module and were constantly up-regulated with respect to Th0 cells (Figure 9G), whereas *KLF4*, *HIF1A* and *STAT3* were up-regulated only at later time in Th17 cells (late module; Figure 9H). Interestingly, none of these transcription factors was up-regulated transiently at the early time point, suggesting that their expression was required for early Th17 commitment as well as for maintenance of the transcriptional program involved in human Th17 cell polarization.

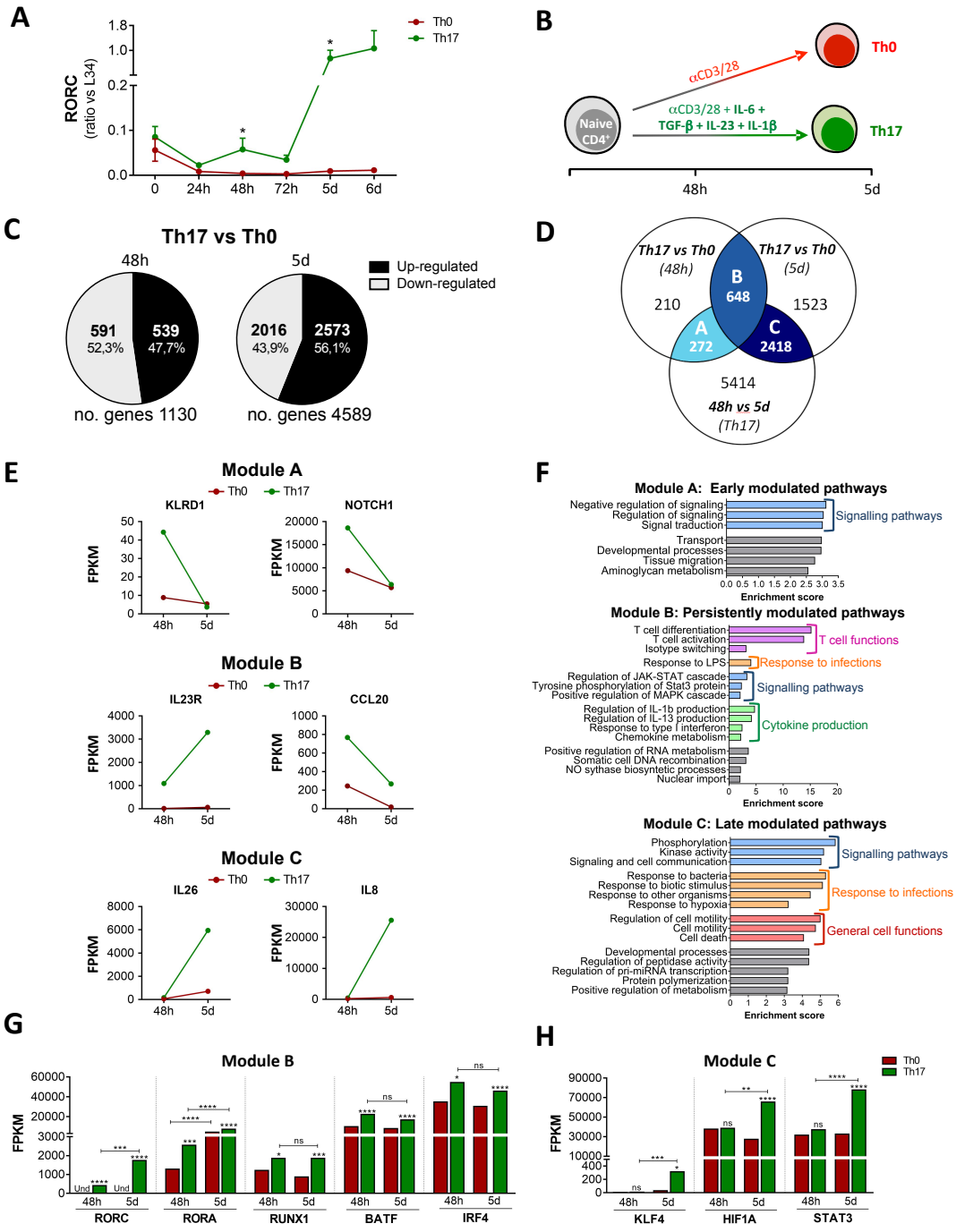


Figure 9. Early, constant and late genes acquired during human Th17 cell polarization reflect specific functions.

*Naïve CD4 T cells cultured with anti-CD3/anti-CD28 alone (Th0) or with the addition of TGF-β, IL-6, IL-23 and IL-1β (Th17), were analysed by real time PCR for the expression of RORC transcript (A). RNA sequencing of Th17 and Th0 cells at 48h and 5d of differentiation (B): number of genes differentially modulated in Th17 vs Th0 at each time point (C); Venn's diagram shows number of genes specifically modulated in Th17 profile (Th0 vs Th17) at 48 hours (early), 5 days (late), and constantly modulated (48h Th17 vs 5d Th17) (D); expression of selected Th genes included in A, B and C modules (E); bar graph representation of gene ontology for genes in A, B, C modules (F); expression of selected Th17 transcription factors (G,H). (n=5; * p≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001).*

Identification of a specific inflammatory Th17 effector program

IL-1β is crucially involved in the pathology of Th17-related diseases (Sutton *et al.*, 2006; Chung *et al.*, 2009), whereas Th17 cells differentiated in the absence of IL-1β produced the anti-inflammatory IL-10 cytokine, which is generally produced by T regulatory cells (Treg) (Volpe *et al.*, 2008; Zielinski *et al.*, 2012). Thus, in order to delineate the inflammatory or regulatory potential of early and late Th17 gene modules, we compared the transcriptome of T cells differentiated in the presence ("inflammatory" Th17 condition) or absence ("regulatory" Th17 condition) of IL-1β.

In principal component analysis (PCA), absence or presence of IL-1β did not segregate samples at 48h (Figure 10A). However, at 5d, lack of IL-1β promoted cells with an intermediate phenotype between Th0 and Th17 cells (Figure 10A). In line with the PCA profile, only 45 genes were differentially modulated in the absence of IL-1β at 48h, while more than 800 genes were affected at 5d (Figure 10B). T cells differentiated under regulatory Th17 condition displayed a profile characterized by decreased expression of some Th17 signature genes, such as low levels of *IL26* and *RORC*, but similar expression of others, like *RORA* and *IL23R* (Figure S2).

This analysis allowed to define an inflammatory Th17 program (down-regulated in absence of IL-1β) and regulatory Th17 program (up-regulated in absence of IL-1β). We found that most of IL-1β-regulated genes showed reduced expression at 48h under the regulatory condition. This observation suggested that their down-regulation may affect subsequent steps in Th17 polarization, and cause the broader gene expression differences seen at 5d (Figure 10B). Moreover, most part of the inflammatory program belongs to the persistent module at 48h and to the late module at 5d (Figure 10B). In contrast, few regulatory genes are modulated at 48h, while at 5d an equal number of genes are classified in persistent and late modules (Figure 10B). These results indicate that genes lately or persistently expressed during human Th17 cell differentiation are crucial for discriminating the commitment towards the inflammatory or the regulatory profile of these cells.

Several transcriptomic studies have highlighted the molecular signature of inflammatory mouse (Ghoreschi *et al.*, 2010; Lee *et al.*, 2012; Yosef *et al.*, 2013) and human (Hu *et al.*, 2017) Th17 cells. These studies were carried out by using *in-vitro* polarized Th17 cells and memory Th1/17 cells, respectively. Analysis of genes associated with Th17 phenotype in these previous studies (Table S1) indicated that some of them, including *IL17A*, *IFNG*, *TBX21*, *EBI3*, *IRF8*, *TNFRSF9*, *TNFRSF14*, *CCL5*, *CD40LG*, *BATF* and *TNF* (14,5%; 11/76 genes) were down-regulated in regulatory human Th17 cells a 5d of differentiation (Figure S3), while other genes were not modulated.

To elucidate the global molecular signature that characterizes the acquisition of the inflammatory profile by human Th17 cells, we analysed in more detail the genes differentially expressed in regulatory versus inflammatory Th17 conditions at 5d. Gene ontology clustering revealed that absence of IL-1β caused a significant down-regulation of functional categories related to "signalling pathways", "cytokine production" and "response to other organisms", which are all relevant for the effector functions of Th17 cells (Figure 10C). Moreover, genes involved in general cell functions, such as cell death, adhesion and motility, were also down-regulated. By contrast, genes up-regulated under regulatory conditions did not cluster in functional categories that are specific of Th17 cells (Figure 10C). Interestingly, genes encoding Th17-specific (*IL17A*, *IL17F* and *IL26*) and non-specific (*IL6*, *IFNG*, and *IL1A*) inflammatory cytokines, and other soluble proteins associated to immune functions (*IFNK*, *PDGFA* and *LTA*), were significantly down-regulated in the absence of IL-1β which may potentially alter functional features in human Th17 cells (Figure 10D,E).

Next, we explored whether the expression of transcription factors driving the Th17-specific signature was modulated in regulatory Th17 condition. Expression of *BATF*, which was described as an early determinant of the mouse Th17 cell signature (Ciofani *et al.*, 2012), was slightly down-regulated in the regulatory Th17 cell condition at both time points. However, expression of the other transcription factors, with the exception of *HIF1A* at 5d, was not significantly affected (Figure 10F,G). These results suggest that polarization of human inflammatory Th17 cells might rely on additional transcriptional regulators.

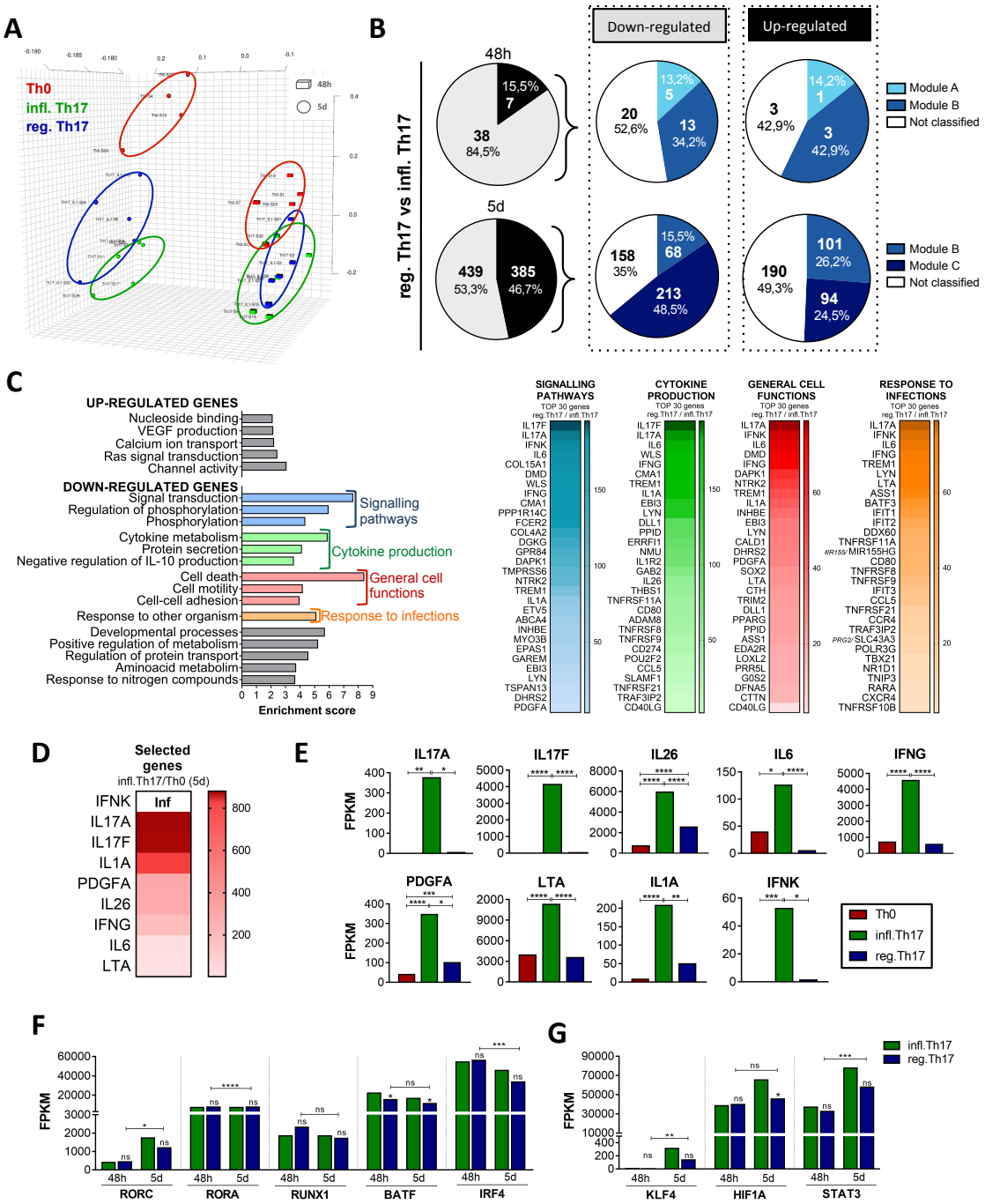


Figure 10. Transcriptional analysis of inflammatory Th17 cells reveals crucial genes for the effector functions of Th17 cells. *Naïve CD4 T cells cultured with anti-CD3/anti-CD28 alone (Th0) or with the addition of TGF-β, IL-6, IL-23 and IL-1β (infl. Th17) or in the absence of IL-1β (reg. Th17), was analysed by RNA sequencing. Global gene expression of cells is represented by principal component analysis (A). Number of genes up-regulated (black) and down-regulated (grey) in reg. Th17 vs infl. Th17 at each time point of analysis, and segregation of those genes in Module A,B, C defined in Figure 9D (B). Gene ontology of up-regulated and down-regulated genes in reg. Th17 vs infl. Th17 at 5 days, and heatmap of genes down-regulated in reg. Th17 vs infl. Th17, belonging to the most represented categories (C). Heatmap (D) and expression profile (E) of nine selected genes down-regulated in reg. Th17 vs infl. Th17 and up-regulated in infl. Th17 vs Th0. Expression of genes encoding for Th17 transcription factors in infl. Th17 and reg. Th17 profiles (F). (n=5; * p≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001). Inflammatory Th17=infl. Th17; regulatory Th17=reg. Th17.*

Experimental validation and characterization of novel transcriptional regulators of human Th17 cells

To search for novel factors possibly involved in the acquisition of an inflammatory Th17 phenotype, we further dissected the pool of genes that were lately or constantly down-regulated in the absence of IL-1β. Group Y comprised genes down-regulated in the absence of IL-1β only at 5d (late regulation), whereas Group X included genes down-regulated at both time points (constantly regulated) (Figure 11A). Next, we further selected genes that were significantly induced in Th17 versus Th0 conditions (Figure S4), and focused our attention on two genes encoding transcriptional regulators: HOPX (Group X, constantly regulated) and SOX2, (Group Y, late regulation) (Figure 11B).

HOPX is a transcriptional cofactor that has been previously involved in murine Th1 and Treg cell functions (Albrecht *et al.*, 2010; Hawiger *et al.*, 2010).

RNA-seq and qPCR analyses showed progressive and persistent up-regulation of HOPX expression during Th17 cell differentiation and confirmed its dependency on IL-1β signalling (Figure 11C). Western blot analysis at 5d of differentiation confirmed these results at the protein level (Figure 11D). Comparative analysis of human naïve T cells differentiated into different Th or Treg subsets indicated that Th17 cells expressed the highest level of HOPX, followed by Th1 cells, whereas this transcriptional regulator was expressed at barely detectable levels in the other subsets (Figure 11E). The specific expression of HOPX in human Th17 cells was even more evident at the protein level (Figure 11F), further suggesting its functional implication in these cells.

SOX2 is a transcription factor involved in stemness, a feature associated with the Th17 phenotype (Kryczek *et al.*, 2011; Muranski *et al.*, 2011). Interestingly, *in-silico* analyses of chromatin immunoprecipitation-sequencing (ChIP-seq) experiments to search for potential regulators of the genes modulated by IL-1β at early and late time points identified SOX2 as the most significantly enriched transcription factor (Figure S5). We confirmed that SOX2 expression in Th17 cells was dependent on IL-1β signalling by qPCR and Western blot analyses (Figure 11C,D). Furthermore, comparative analyses of human T cell subsets revealed that SOX2 transcript and protein were highly specific of the Th17 profile (Figure 11G,H).

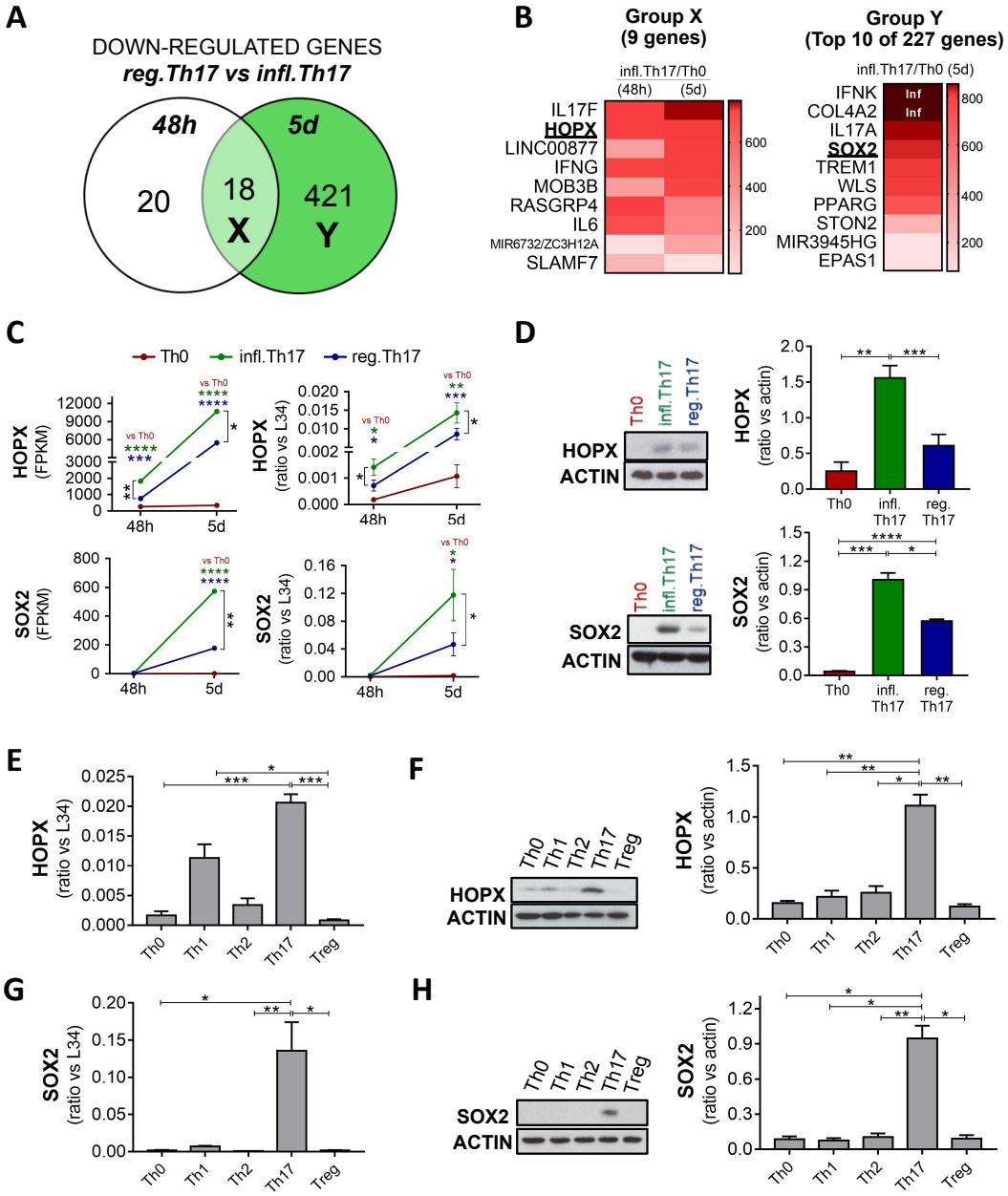


Figure 11. HOPX and SOX2 are specifically expressed in human Th17 cells.
Venn's diagram between genes down-regulated in regulatory Th17 (TGF-β, IL-6, IL-23 and IL-1β) vs inflammatory Th17 (TGF-β, IL-6 and, IL-23) at 48 hours and 5 days unveils 18 genes regulated at both time points (group X) and 421 genes specifically down-regulated at 5 days (group Y) (A). All the annotated genes included in group X, and 10 out of 421 genes in group Y are listed in panel B. HOPX and SOX2 expression obtained by RNA sequencing (n=5) and qPCR at 48 hours and 5 days (n=4) (C), and Western blot at 5 days (n=5) (D), was analysed in Th0, inflammatory Th17 and regulatory Th17 cells. Expression of HOPX transcript (n=5) (C) and protein (n=4) (D), SOX2 transcript (n=6) (G) and protein (n=4) (H) in Th0, Th1, Th2, Th17, and Treg profiles in-vitro differentiated from naïve CD4 T cells of healthy donors (p≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001). Th1:IL-12; Th2:IL-4; Th17: TGF-β, IL-6, IL-23 and IL-1β; Treg:IL-2 and TGF-b; Th0: no cytokines. reg= regulatory; infl.= inflammatory.*

HOPX and SOX2 contribute to the expression of specific soluble factors in human Th17 cells

To test whether HOPX and SOX2 expression had an impact on human Th17 differentiation, we set out to silence their expression in primary human T cells (Figure S6A) without altering the viability and proliferation potential of differentiating T cells (Figure S6B,C). Next, we analysed the expression of soluble factors previously identified as expressed in inflammatory Th17 condition and used RORC-silenced cells as reference control for a master regulator of Th17 cell profile (Figure 12A).

As expected, immunoassays by ELISA revealed that knockdown of RORC significantly reduced IL-17A and IL-17F protein expression by polarized Th17 cells (Figure 12D). RORC silencing also modulated the production of IFN-γ, PDGF-AA and PDGF-AA/BB (Figure 12D), as well as *IL1A* mRNA expression (Figure 12E), by human Th17 cells. By contrast, knockdown of RORC did not interfere with the expression of LT-α (Figure 12D), *IFNK*, *IL6* and *IL26* (Figure 12E).

Interestingly, silencing of HOPX (Figure 12B) also impaired production of IL-17A and IFN-γ (Figure 12D), albeit at lower levels than RORC, whereas expression of other soluble factors (LT-α, IL-17F, PDGF-AA, PDGF-AA/BB, *IL26*, *IL6*, *IL1A*, *IFNK*) was unaffected (Figure 12D,E). On the other hand, knockdown of SOX2 expression in human polarized Th17 cells (Figure 12C) specifically modulated IFN-γ and PDGF-AA production (Figure 12D). The common regulation of IL-17A, IFN-γ by RORγt and HOPX, and PDGF-AA and IFN-γ by RORγt and SOX2, lead us to hypothesise the involvement of RORγt in regulating HOPX and SOX2 expression. Indeed, silencing of RORC in human Th17 cells reduced the expression of both *HOPX* and *SOX2* (Figure S7). These results suggest that HOPX and SOX2 are two transcriptional regulators acting downstream of RORγt signalling in human Th17 cells.

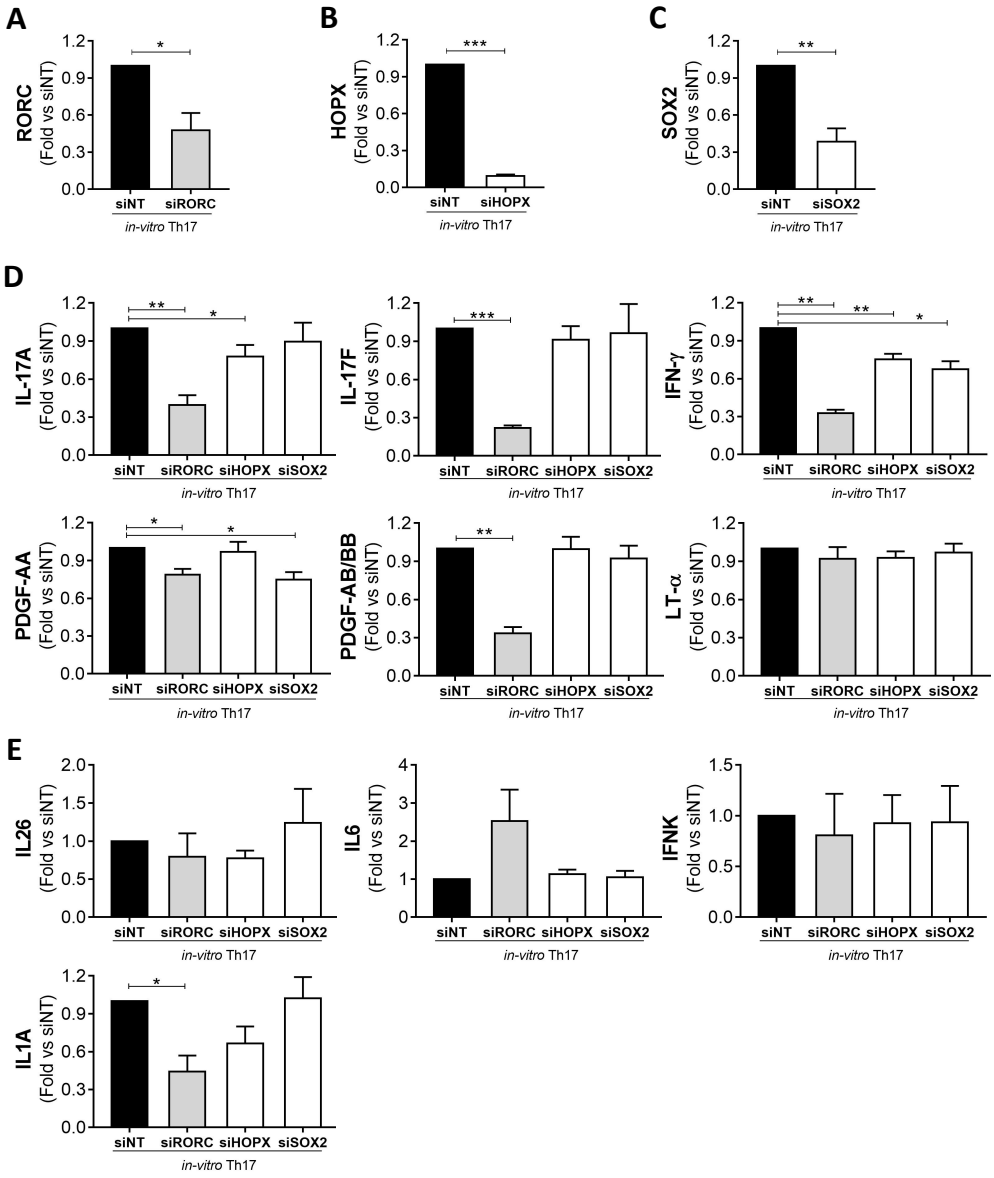


Figure 12. HOPX and SOX2 contributes to the expression of Th17 cytokines.

Expression levels of RORC (A), HOPX (B) and SOX2 (C) transcripts, IL-17A, IL-17F, IFN-γ, PDGF-AA, PDGF-AB/BB, LT-α proteins (B), and IL26, IL6, IL1A, IFNK transcripts (C), were analysed by q-PCR (transcript) or ELISA and Luminex (protein) at 5 days of cell differentiation in Th17 cells (TGF-β, IL-6, IL-23 and IL-1β) treated with non targeting (siNT), RORC (siRORC; n=4), HOPX (siHOPX; n=8), or SOX2 (siSOX2; n=6) siRNA. Transcriptional results are normalized on L34 expression and all data are presented as fold vs siNT. (p≤0.05; ** p≤0.01; *** p≤0.001).*

HOPX and SOX2 regulate features of Th1/17 cells

T cells that concomitantly produce IL-17A and IFN-γ, named Th1/17 cells, play a pathogenic role in autoimmune diseases (Kebir *et al.*, 2009; Reinert-Hartwall *et al.*, 2015). Given the role of HOPX and SOX2 in regulating IFN-γ and IL-17A production by human Th17 cells, we next investigated the role of HOPX and SOX2 in memory Th1/17 cells. In line with the effect observed in Th17 cells polarized *in-vitro*, HOPX silencing in peripheral memory CD4 T cells (Figure S8) caused a significant reduction of IL-17A expression (Figure 13A,B). On the other hand, as expected from the *in-vitro* data, silencing of SOX2 in these cells did not affect IL-17A production (Figure 13C,D). The reduction of IL-17A upon HOPX silencing was also observed by evaluating the frequency of Th17 cells, which were identified by the expression of specific receptors ($CXCR3^-$, $CD161^+$, $CCR6^+$) (Capone and Volpe, 2021) in total CD4 T cells (Figure 13E). Furthermore, in line with the effect observed *in-vitro* on IFN-γ production, HOPX depletion caused a reduced frequency of circulating Th1/17 cells, which were identified as $CXCR3^+$, $CD161^+$, $CCR6^+$ (Capone and Volpe, 2021) (Figure 13E). Notably, SOX2 was also important for the Th1/17 phenotype, as its depletion caused a reduction of their frequency, but not of the frequency of Th17 cells ($CXCR3^-$, $CD161^+$, $CCR6^+$ cells) (Figure 13F).

These results indicate that HOPX and SOX2 are two novel transcriptional regulators required for polarization of inflammatory human Th17 cells. Moreover, our results indicate that *in-vitro* differentiated Th17 cells share common mechanisms with *ex-vivo* memory Th17 cells, such as the regulation of IL-17A, and IFN-γ by HOPX, and of IFN-γ by SOX2.

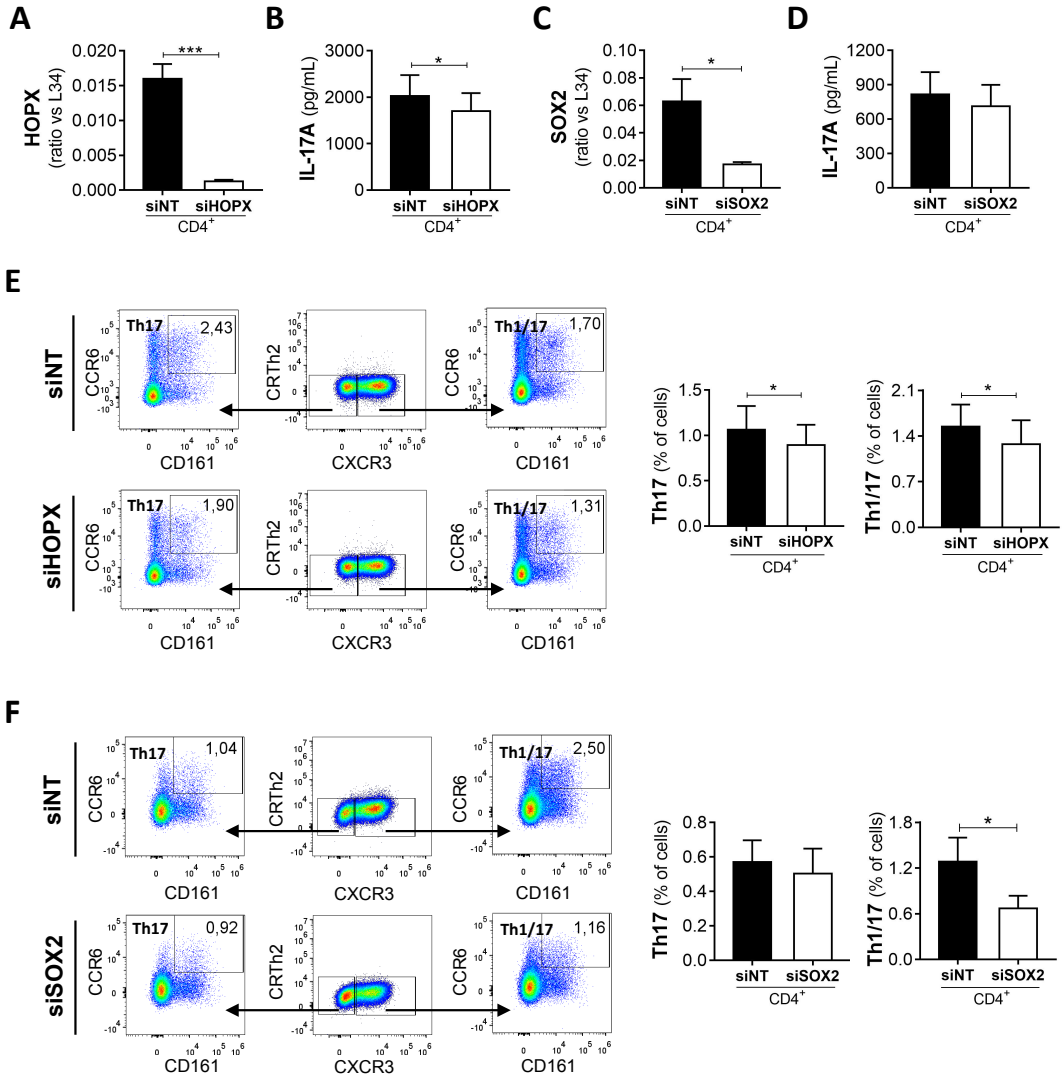


Figure 13. HOPX and SOX2 regulate features of Th1/17 cells.

Expression of HOPX transcript (A) and IL-17A protein (B) in total CD4 T cells treated with non targeting (siNT) or HOPX (siHOPX) siRNA was analysed by qPCR and ELISA, respectively (n=8). Expression of SOX2 transcript (C) and IL-17A protein (D) in total CD4 T cells treated with non targeting (siNT) or SOX2 (siSOX2) siRNA was analysed by qPCR and ELISA, respectively (n=6). Frequencies of Th17 cells ($CXCR3^-$ $CD161^+$ $CCR6^+$) and Th1/17 cells ($CXCR3^+$ $CD161^+$ $CCR6^+$) (E) were analysed by flow cytometry in total CD4 T cells treated with non targeting (siNT) or HOPX (siHOPX) siRNA (n=8). Frequencies of Th17 cells ($CXCR3^-$ $CD161^+$ $CCR6^+$) and Th1/17 cells ($CXCR3^+$ $CD161^+$ $CCR6^+$) (F) were analysed by flow cytometry in total CD4 T cells treated with non targeting (siNT) or SOX2 (siSOX2) siRNA (n=6) ($p \leq 0.05$; *** $p \leq 0.001$;).*

2.3. Discussion

To our knowledge, this is the first transcriptional study of human Th17 cells that proposes a systems level analysis followed by experimental validation of testable hypotheses. Previous transcriptomic studies did not exhaustively analyze the differentiation process of human Th17 cells (Cosmi *et al.*, 2008; Tuomela *et al.*, 2012, 2016; Äijö *et al.*, 2014; Tripathi *et al.*, 2017), because they were either performed on Th17 cells isolated from peripheral blood (Cosmi *et al.*, 2008; Ranzani *et al.*, 2015) or with Th17 cells differentiated *in-vitro* in a medium that lacked IL-23 (Tuomela *et al.*, 2012, 2016; Äijö *et al.*, 2014; Tripathi *et al.*, 2017), which plays a crucial role in human Th17 cell differentiation (Manel, Unutmaz and Littman, 2008; Volpe *et al.*, 2008) as well as in Th17-related diseases (McGeachy *et al.*, 2007). Thus, a comprehensive transcriptional analysis of human Th17 cells undergoing differentiation in optimal Th17 conditions (TGF-β, IL-6, IL-1β and IL-23) (Manel, Unutmaz and Littman, 2008; Volpe *et al.*, 2008) had not been performed to date.

In this work we dissected the gene expression program underlying early and late time points of the polarization process of human Th17 cells. Based on *RORC* expression, we found that the differentiation process requires 5d in human Th17 cells, unlike mouse cells where optimal differentiation is achieved in 3 days (Veldhoen, Hocking, Atkins, *et al.*, 2006; Gaublomme *et al.*, 2015). Moreover, our study revealed that the largest modulation of the transcriptome occurs at late stages of differentiation. In fact, while cells cultured under Th17 conditions are already distinguishable from cells cultured in absence of cytokines (Th0) at 48h, the complete segregation between Th17 cells differentiated under inflammatory or regulatory culture conditions is obtained only after 5d. The delay in differentiation of human with respect to mouse Th17 cells may rely on the different cytokine requirements: while administration of TGF-β and IL-6 is sufficient to induce murine Th17 cell polarization *in-vitro*, human cells also require the presence of IL-1β and IL-23. In fact, naïve CD4 T cells do not express IL-1β and IL-23 receptors, thus the timing requested for their up-regulation may contribute to the delay. However, also in murine Th17 cells, IL-6 and TGF-β are the early triggers of differentiation, while IL-23, IL-1β and IL-21 are involved in the late stabilization and amplification steps (Veldhoen, Hocking, Atkins, *et al.*, 2006).

We propose that the human Th17 cell differentiation process may be divided in steps. Our transcriptomic analysis highlighted genes that were modulated at either early or late time points together with others that were constantly and progressively modulated throughout the whole cell polarization process. Interestingly, we found a strong association between the timing of expression and the function of the genes undergoing modulation. Genes associated to signalling pathways were always modulated,

indicating that each time point of the polarization process requires proper response to polarizing cytokines or soluble factors released in the milieu. However, only late genes were also enriched in pathways associated to Th17 effector functions, such as response to bacteria and other organisms, that is consistent with the antimicrobial activity of these cells (Veldhoen, 2017). Persistently modulated genes were enriched in functional categories involved in specific transduction pathways, such as the phosphorylation of STAT3, which is activated by IL-23 and IL-6 (Taga and Kishimoto, 1997; Heinrich *et al.*, 2003; Yang *et al.*, 2007), and general T cell functions, such as cell activation and differentiation, but also cytokine production and response to infections. However, most part of Th17 signature genes were persistently or lately expressed in human Th17 cells, indicating that late steps are crucial for their inflammatory commitment. This result implicates that an efficient therapeutic inhibition of inflammatory Th17 cells should target persistent or late genes.

In this context, we selected the top-ranking genes induced at the late stage of differentiation in inflammatory versus regulatory Th17 conditions. Among others, we found known factors, such as *IL17A*, *IL17F*, *IL6*, *IFNG*, *IL26*, and novel effector molecules, such as *LTA*, *PDGFA*, *IL1A*, *IFNK*. *LTA*, encoding for LT-α, formerly known as TNF-β, is involved in the induction of adhesion molecules and chemokines by endothelial cells (Calmon-Hamaty *et al.*, 2011). Notably, LT-α produced by Th17 cells is involved in the formation of tertiary lymphoid tissues in the meninges of patients affected by multiple sclerosis (Grogan and Ouyang, 2012), a typical Th17-mediated disease. In turn, LT-α contributes to amplify the expression of adhesion molecules and soluble factors that potentiate the ability of the extracellular matrix-stromal-cell network to recruit, retain, and activate lymphocytes within the meninges (Pikor *et al.*, 2015). In this regard, we previously observed that Th17 cells from MS patients produce higher amount of LT-α compared to healthy donors (Capone *et al.*, 2019). IL-1α expressed by CD4 T cells (van Rietschoten *et al.*, 2006) could contribute to the amplification of the inflammatory response by binding to IL-1R1 and activating the downstream signalling pathway (Kurt-Jones *et al.*, 1985; Kaplanski *et al.*, 1994; Di Paolo and Shayakhmetov, 2016; Dinarello, 2018). Consistently with our observations, IL-1α is produced in response to a variety of stimuli, including exposure to IL-1β (Di Paolo and Shayakhmetov, 2016). Thus, IL-1α could contribute to the inflammatory environment generated by IL-1β in Th17 cells. We also observed that Th17 cells produce PDGF-AA and PDGFAB/BB. These growth factors are known to direct the differentiation of a variety of cell types (Hoch and Soriano, 2003), but their function in immune cells is not known. In addition, we found that human Th17 cells express IFNK, encoding for a member of the type I interferon family of cytokines, which play an important role in host defences against viral

infections (Nardelli *et al.*, 2002). Studies concerning the role of Th17 cells in the defence against viruses have generated conflicting results (Ma *et al.*, 2019). Thus, further investigation of the expression and function of type I interferon by Th17 cells could highlight new antiviral mechanisms of these cells.

Our study revealed that transcriptional regulators previously indicated as relevant for mouse Th17 cells, such as *RORC*, *RORA*, *HIF1A*, *BATF*, *IRF4*, *STAT3*, *RUNX1*, and *KLF4* (Capone and Volpe, 2020), were also induced in human Th17 cells. However, the expression of most of them was not differentially expressed in inflammatory and regulatory Th17 conditions, suggesting that other transcriptional regulators contribute to the acquisition of the inflammatory Th17 profile. In this context, we identified HOPX and SOX2 as transcriptional regulators that act downstream to RORγt signalling and are involved in the differentiation program of inflammatory Th17. To date, HOPX has been mainly studied for its role in cardiac and lung development (Chen *et al.*, 2002; Shin *et al.*, 2002; Yin *et al.*, 2006) and in skeletal muscle differentiation (Kee *et al.*, 2007). Nevertheless, HOPX was also previously reported to be expressed in mouse Treg and Th1 cells (Albrecht *et al.*, 2010; Hawiger *et al.*, 2010). In Treg cells, HOPX participates to anergy induction on effector T cells, while in Th1 cells it promotes cell expansion by modulating pro- and anti-apoptotic genes. However, we detected low levels of HOPX in human Th1 and Treg cell subsets, whereas it was expressed at much higher levels in Th17 cells, where HOPX contributes to IL-17A and IFN-γ expression.

SOX2 is a transcription factor associated with pluripotency of stem cells. For instance, SOX2 promotes self-renewal in embryonic stem cells (Feng and Wen, 2015) and is required for reprogramming of induced pluripotent stem cells (Takahashi and Yamanaka, 2006). Moreover, in some adult tissues, like the central nervous system, SOX2 ensures homeostasis by supporting the stem-like features of progenitor cells (Feng and Wen, 2015). Herein, we found that SOX2 contributes to the expression of PDGF-AA, a growth factor that we recently associated to the Th17 profile in healthy donors and multiple sclerosis patients (Capone *et al.*, 2019). PDGF-AA binds to PDGFRA, which is considered critical player in the disruption of the blood brain barrier (Ma *et al.*, 2011). Thus, Th17 cells could contribute to disruption of the blood brain barrier in multiple sclerosis, through production of PDGF-AA. Moreover, we found that both HOPX and SOX2 regulate production of IFN-γ by *in-vitro* differentiated human Th17 cells. Importantly, IFN-γ-secreting Th17 cells, called Th1/17 cells, are abundant in inflamed tissues of human autoimmune diseases and are considered highly pathogenic (Annunziato *et al.*, 2007; Kebir *et al.*, 2009; Nistala *et al.*, 2010; Reinert-Hartwall *et al.*, 2015). The Th1/17 cell population, which is characterized by the simultaneous production of IFN-γ and IL-17A and the

expression of the CXCR3, CCR6, and CD161 receptors (Acosta-Rodriguez *et al.*, 2007; Capone and Volpe, 2021), is significantly reduced in memory CD4 T cells depleted of HOPX or SOX2. These findings suggest that HOPX and SOX2 contribute to the acquisition of an inflammatory phenotype of human Th17 cells.

To our knowledge, this is the first study that analyzes the kinetics of the differentiation process of human Th17 cells using the optimal experimental conditions. Our findings could open new perspectives for the pharmacological modulation of Th17 responses, and could help elucidate and/or predict the outcome of specific therapeutic intervention against Th17 cells.

2.4. Materials and Methods

Purification of naïve CD4 T cells from adult blood

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation (GE Healthcare) from whole blood of healthy donors (HD). The cells were stained with the anti-CD4 FITC (Miltenyi Biotec), and CD4 T lymphocytes are purified by immunomagnetic selection, using the anti-FITC isolation kit (Miltenyi Biotec). After isolation, the cells were stained with anti-CD4 FITC (1:20) (Miltenyi Biotec), anti-CD45RA BV421 (1:20) (BD Biosciences), anti-CD45RO PE (1:20) (BD Biosciences), anti-CD27 APC (1:20) (Beckman Coulter), and CD4 naïve T cells were sorted by Astrios high-speed cell sorter (Beckman Coulter, Brea, CA, USA) as $CD4^{high}$, $CD45RA^{high}$, $CD45RO^{-}$ and $CD27^{+}$. Sorted cells had a purity of over 97%, as shown by flow cytometry (Figure S9).

Th cell differentiation assay

Naïve CD4 T cells were cultured in 96-well plates (Falcon) at a density of 50 000 per well in X-VIVO 15 serum free medium (Lonza) in presence of Dynabeads CD3/CD28 T cell expander (1 bead per cell) (Life Technologies) and Th promoting cytokines (Miltenyi Biotec) as previously described (Volpe *et al.*, 2008): IL-12 (10 ng/ml) for Th1 profile; IL-4 (25 ng/ml) for Th2 profile; IL-1β (10 ng/ml), IL-6 (20 ng/ml), TGF-β (1 ng/ml), and IL-23 (100 ng/ml) for Th17 profile; IL-2 (10 ng/ml) and TGF-β (2 ng/ml) for T regulatory cells; Th0 profile was obtained culturing cells in absence of cytokines. The cells were incubated and after 48 hours or 5 days they were harvested, extensively washed and their viability was determined by Trypan Blue exclusion for RNA extraction. The conditions of incubation were stable (temperature 37°C with 5% of CO_2).

RORC, HOPX and SOX2 silencing *in-vitro* experiments

For *in-vitro* experiments, naïve CD4 T cells were cultured in 96-well plates (Falcon) at a density of 80 000 cells per well in Accell siRNA Delivery media (Dharmacon) supplemented with 5% of X-VIVO 15 serum free medium (Lonza) for 5 days, in presence of Dynabeads CD3/CD28 T cell expander (1 bead per cell) (Life Technologies), cytokines (IL-1β 10 ng/ml, IL-6 20 ng/ml, TGF-β 1 ng/ml, IL-23 100 ng/ml) (Miltenyi Biotec) as previously described (Volpe *et al.*, 2008), and RORC, HOPX, SOX2, or Non targeting Accell siRNA (1μM) (Dharmacon). For RORC and SOX2 silencing experiments the addition of Accell siRNA was performed also at day 3 (1μM).

For *ex-vivo* experiments, memory CD4 T cells were cultured in 96-well plates (Falcon) at a density of 400 000 cells per well in Accell siRNA Delivery media (Dharmacon) supplemented with 5% of X-VIVO 15 serum free medium (Lonza) for 3 days, in presence of Dynabeads CD3/CD28 T cell expander (1 bead per cell) (Life Technologies), and HOPX, SOX2, or Non targeting Accell siRNA (1μM) (Dharmacon).
After cell cultures, the cells were harvested, extensively washed and their viability was determined by Trypan Blue exclusion for RNA extraction. The cells were incubated at temperature 37°C with 5% of CO_2.

Cytokine quantification

IL-17A and IL-17F in culture supernatant were quantified with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MS, USA). IFN-γ, PDGF-AA, and PDGF-AB/BB, and LT-α were quantified using a magnetic bead panel (Millipore, Burlington, MA, USA), following the manufacturer's protocol, acquired with Luminex 200 instrument and analysed by XPONENT software.

Flow cytometry analysis

Cells were stained with the following antibodies: anti-human CD4-FITC (Coulter) (1:100), anti- human CXCR3 Alexa 647 (1:100) (Biolegend), anti-human CRTh2 PE (1:150) (Miltenyi), anti-human CD161 PE-Dazzle 594 (1:50) (Biolegend), anti-human CCR6 PC7 (1:80) (Biolegend).
Samples were acquired using Cytoflex LX cytometer (Beckman Coulter, Brea, CA, USA) and analysed using FlowJo-10 software version 10.3.0.

RNA isolation and RNA retro-transcription

Total RNA was isolated from T cells with ReliaPrep RNA Cell-Miniprep System (Promega) following manufacturer's instructions. RNA concentration and integrity are determined using Nanodrop and a bioanalyzer (Agilent) before retro-transcription. 100 ng of RNA were retro- transcribed in a mix containing random hexamers (12,5 ng/μl) (Promega), Oligo dT15m (6,25 ng/μl) (Promega), RNasin (1 U/μl) (Promega) and Super Script II Reverse Transcriptase (5 U/μl) (Life Technologies).

Real-time quantitative PCR

The qPCR was performed using the LightCycler® 480 (Roche) with LightCycler® 480 SYBR Green I Master Mix (Roche), or Taqman Gene expression Master Mix (Life Technologies). For SYBR Green assay, the efficient annealing temperature of each couple of primers was tested and their specificity was confirmed in all assays by

single peak performances of PCR products in melt curve analysis (Table S2). The amplification protocol was 1 cycle of 5 min at 95 °C, and 45 cycles of 40 sec: 10 sec. at 95 °C, 20 sec. at specific annealing temperature reported in Table S2, and 10 sec. at 72 °C.

For Taqman, we used pre-designed TaqMan Gene Expression Assays and standard protocol (15min at 95°C, 15sec at 95°C, 60min at 60°C). The pre-designed probes were (identification number): SOX2 (Hs01053049_s1), IL17A (Hs00174383_m1), RORC (Hs01076122_m1), RORA (Hs00536545_m1), IL23R (Hs 00332759_m1); IFNK (Hs00737883_m1); IL26 (Hs00218189_m1); IL1A (Hs00174092_m1); and RPL34 (Hs00996244_g1). The gene expression was normalized to the expression of RPL34 transcript.

RNA sequencing analysis

Stranded total RNA libraries were sequenced on Illumina HiSeq2000 flow cell v3-PE100, producing 200- 300 million of uniquely mapped paired-end reads/sample, which allows reliable detection of low- expressed genes. The results were considered statistically significant for p-values ≤ 0.05 and fold-changes ≥ 1.5.

Western blot analysis

For protein extraction, Th cells were resuspended in RIPA buffer (50 mM Tris/HCl, pH 8, 200 mM NaCl, 2 mM EDTA, 1% NP-40, 0,5% sodium deoxycholate, 0.05% SDS, 1 mM Na3VO4, 1 µg/ml leupetin, 1 µg/ml aprotinin, 5 mM NaF and 1 mM PMSF freshly added). After 15 min on ice, cell lysates were centrifuged for 15 min at 15 000 g at 4°C and the supernatants were collected and used for Western blot analyses. Cell extracts were diluted in Laemmli buffer and boiled for 5 min at 95°C. Proteins were separated on SDS/PAGE (10% or 15%) gels and transferred on nitrocellulose membranes (Whatman, Sigma–Aldrich, GE Healthcare Life Science) using a wet blotting apparatus (Amersham Biosciences). Membranes were saturated for 1 hour at room temperature with 5% non-fat dry milk in PBS, containing 0.1% Tween-20. Membranes were incubated with the following antibodies overnight at 4°C: rabbit monoclonal anti-SOX2 (D6D9 clone) (Cell Signalling Technology; 1:1000 dilution) and mouse monoclonal anti-HOPX (E-1 clone) (Santa Cruz Biotechnology; 1:200). HOPX antibody was diluted in 5% BSA and SOX2 antibody in 5% non-fat dry milk in PBS, containing 0.1% Tween-20. Secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Cell Signalling Technology) were incubated with the membranes for 1 hour at room temperature at a 1:2000 dilution in PBS containing 0.1% Tween-20. Immunostained bands were detected using a

chemiluminescence method (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Fisher).

Quantification of protein expression

The quantification of protein expression was performed using the Image J 1.x version (Schneider, Rasband and Eliceiri, 2012). The software calculates for each band a two-dimensional peak, whose area represents the densitometric value. The protein expression was normalized to the expression of β-actin.

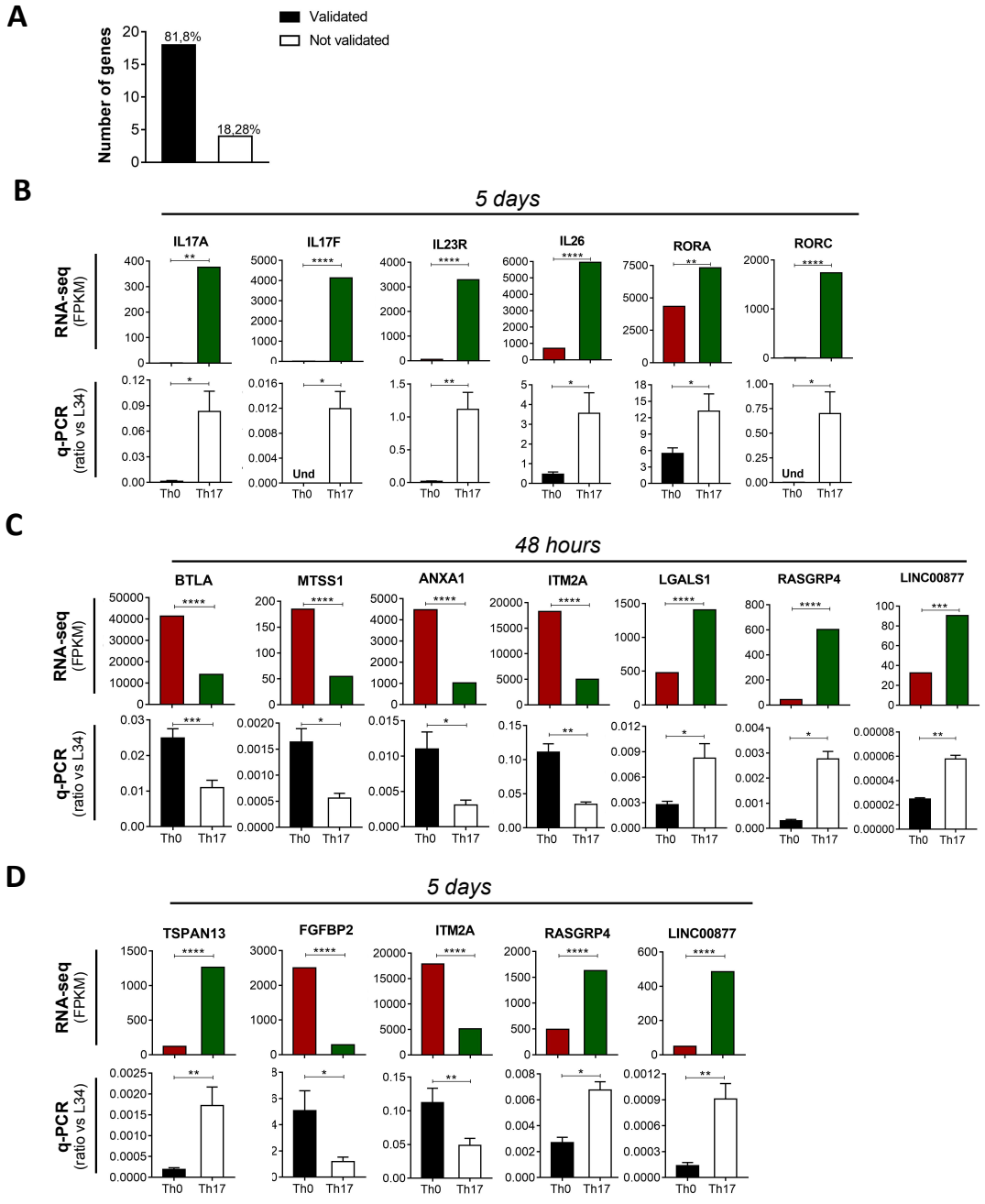
Statistical analysis

For pair-wise comparisons of different conditions from the same donors, we used a parametric two-tailed paired. One-way ANOVA was performed to analyze the main effects of two conditions on the dependent variables and their interactions. Data were presented as mean ± standard error (s.e.m). The p-values of 0.05 or less, were considered statistically significant.

Gene ontology analysis

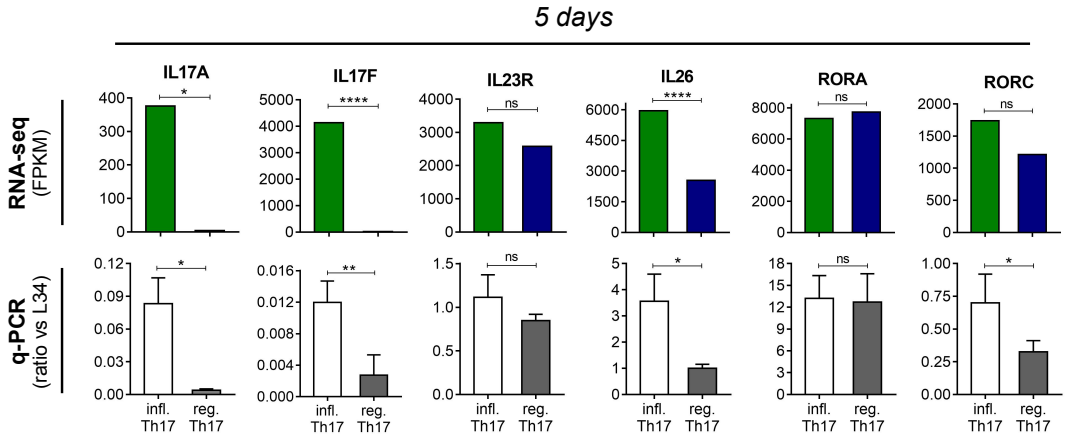
Gene ontology (GO) was performed by DAVID Functional annotation cluster Tool (v6.8) (Huang, Sherman and Lempicki, 2009), by considering all the biological process and molecular function datasets. Only the first 15 categories obtained by the analysis are reported in the figures. All analysis were considered as enriched if fold enrichment ≥ 2.0 and p-value ≤ 0.05.

2.5. Supplementary materials



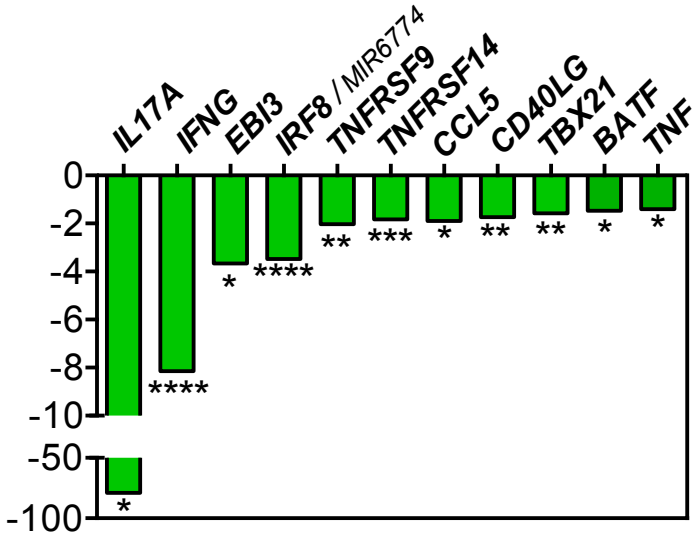
Supplementary figure 1. Validation of arbitrarily selected genes differentially expressed.

*Number and percentage of genes obtained by RNA-seq and validated in q-PCR (A). Expression of Th17 signature genes at 5d (B), and expression of random selected genes at 48 hours (C) and at 5 days (D) obtained by RNA-seq and q-PCR analysis in Th0 and Th17 cells. (n=5; * p ≤0.05; ** p ≤0.01; *** p ≤ 0.001; **** p ≤0.0001).*



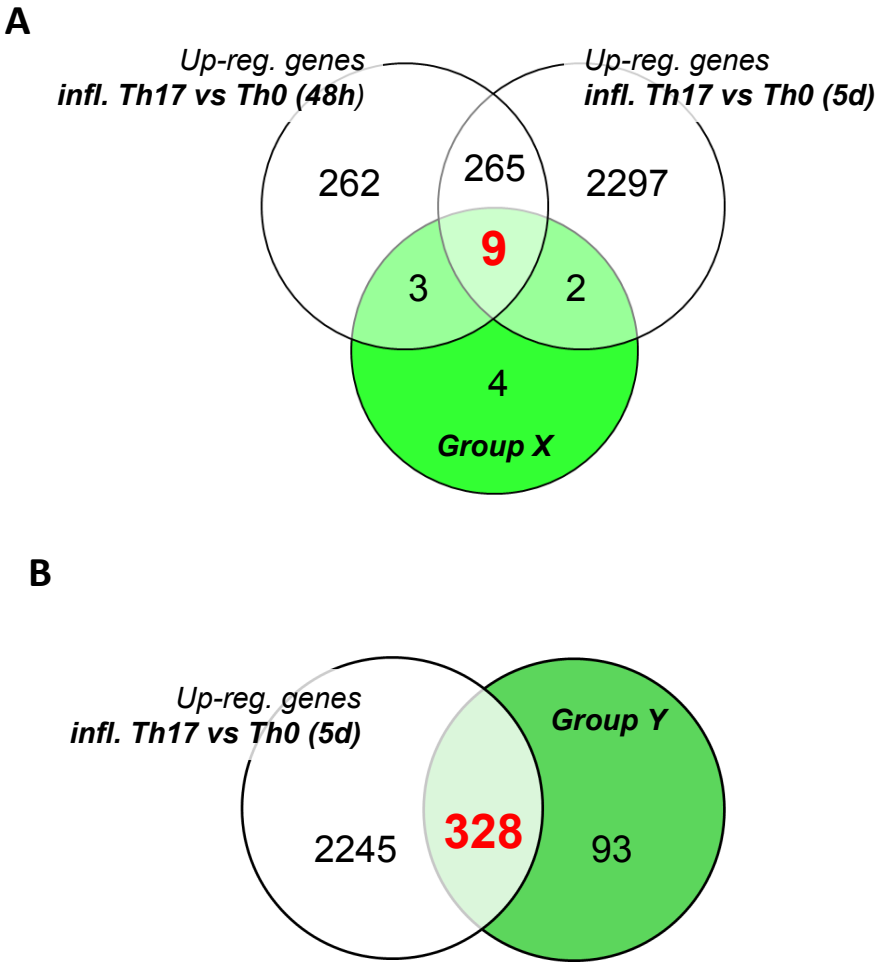
Supplementary figure 2. Regulatory Th17 polarization generates an uncompleted Th17 profile.

*Expression of Th17 signature genes at 5d, obtained by RNA-seq and q-PCR analysis in inflammatory Th17 (infl. Th17) and regulatory Th17 (reg. Th17) cells. (n=5; * p ≤0.05; ** p ≤0.01; **** p ≤0.0001)*



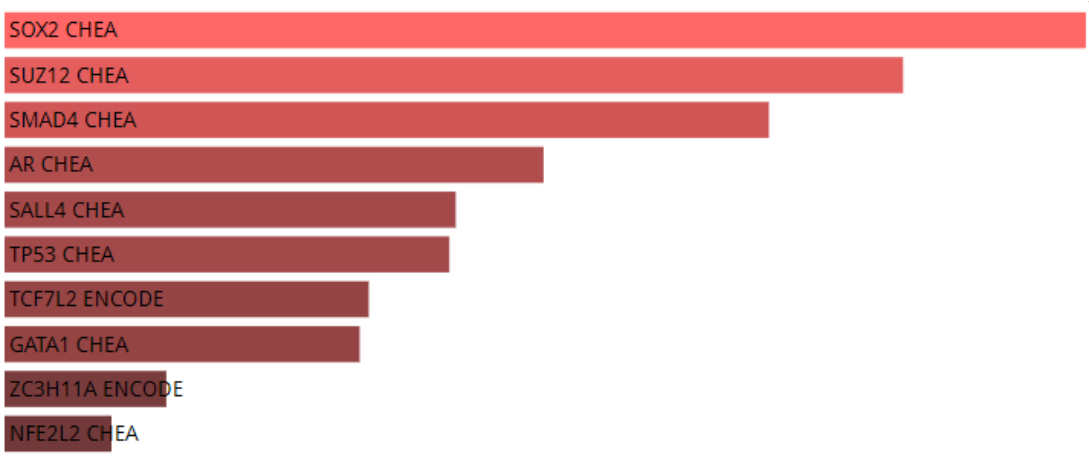
Supplementary figure 3. Murine pathogenic Th17 genes validated in human Th17 cells.

Pathogenic genes of murine Th17 cells (in bold) significantly down-regulated in human regulatory Th17 cells (reg. Th17) vs inflammatory Th17 cells (infl. Th17), analysed by RNA-sequencing.
*(n=5; * p ≤0.05; ** p ≤0.01; *** p ≤ 0.001; **** p ≤0.0001)*



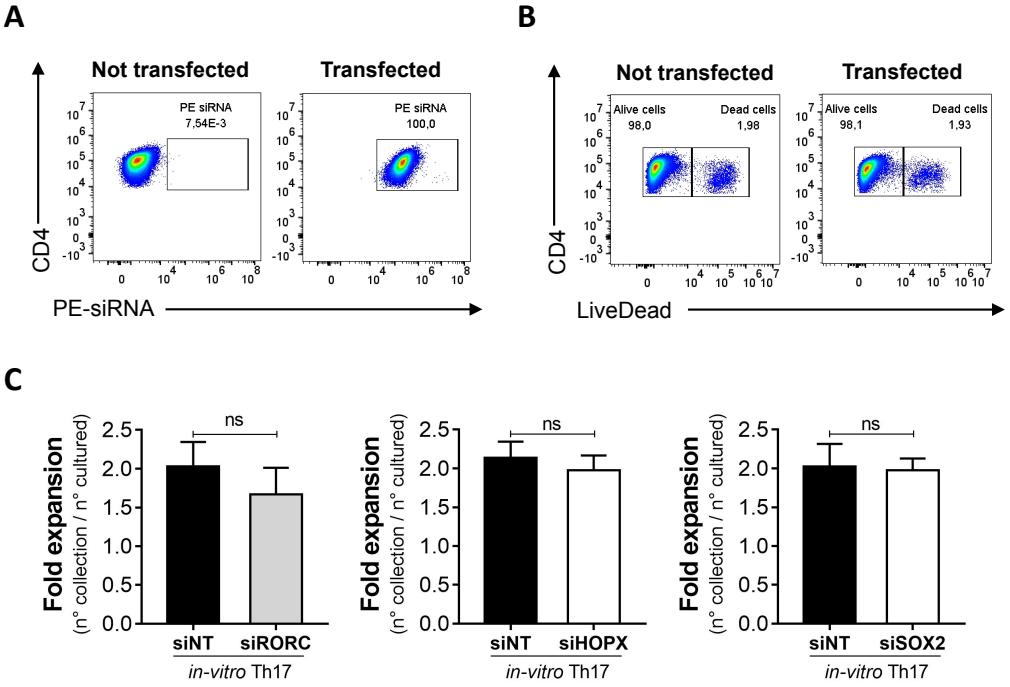
Supplementary figure 4. Selection of down-regulated genes in regulatory versus inflammatory Th17 conditions significantly induced in inflammatory Th17 vs Th0 cells.

Venn's diagram of genes up-regulated in inflammatory Th17 vs Th0 at 48h or 5d with genes of group X (Figure 15A) unveils nine genes specific of inflammatory Th17 (infl.Th17) differentiation, which are down-regulated in regulatory Th17 vs inflammatory Th17 cells at both time points (A). The Venn's diagram between up-regulated genes in inflammatory Th17 vs Th0 at 5d and group Y (Figure 15A) unveils 328 genes specific of inflammatory Th17 differentiation, which are down-regulated in regulatory Th17 vs inflammatory Th17 cells (B).



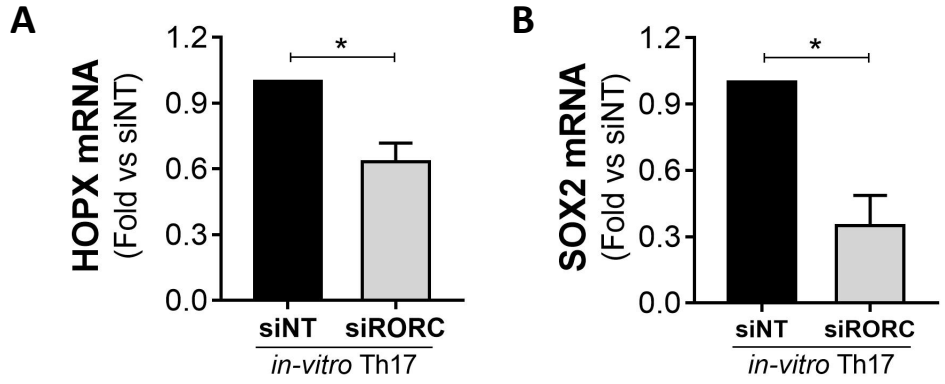
Supplementary figure 5. SOX2 is a potential transcription factors of human Th17 polarization

Data obtained by Enrichr tool (http://amp.pharm.mssm.edu/Enrichr) (p-value ≤0,05), based on public databases of chromatin immunoprecipitation-sequencing (ChIP-seq) experiments, searching for transcription factor potentially regulating genes differentially expressed in regulatory vs inflammatory Th17 cells at 48h and 5d.

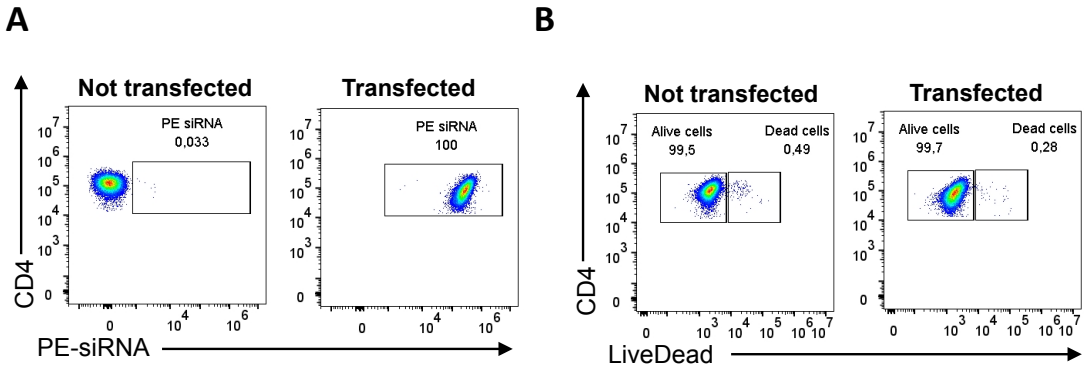


Supplementary figure 6. Transfection of in-vitro polarized Th17 cells with siRNA

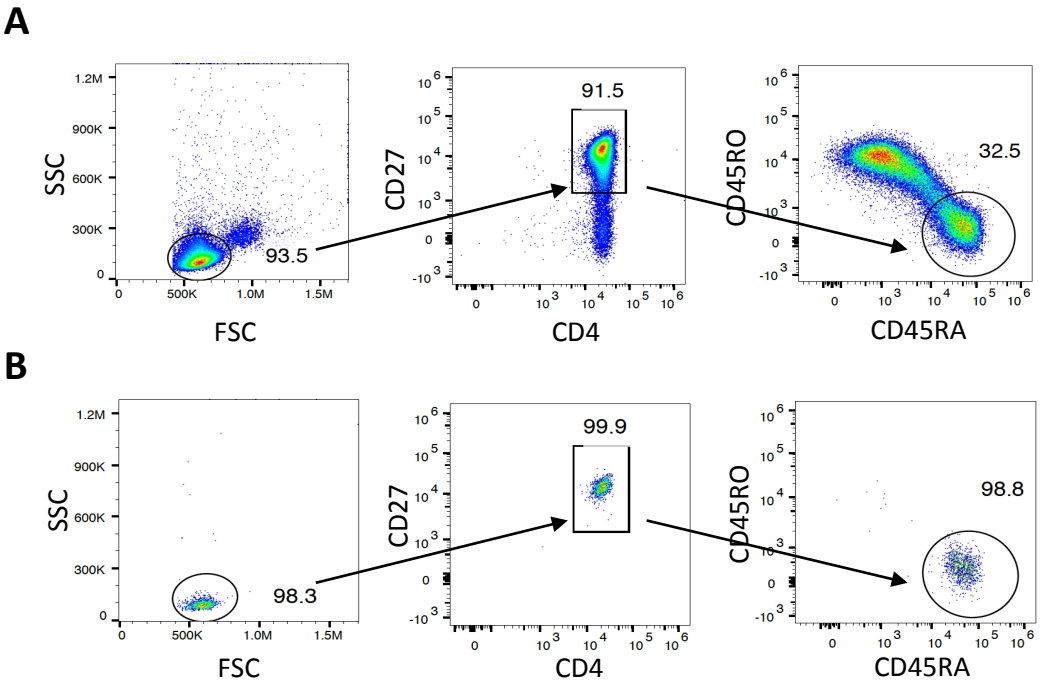
Naïve CD4 T were transfected with siRNA for 5 days during polarization into Th17 cells. Internalization of PE-siRNA upon transfection (A) and cell viability (B) were analyzed by flow cytometry. Fold expansion (represented as ratio between the number of cell harvested at 5 days and number of cells cultured at day 0) in Th17 cells treated with Non targeting (siNT), RORC (siRORC), HOPX (siHOPX), or SOX2 (siSOX2) siRNA (C).



Supplementary figure 7. RORC regulates expression of HOPX and SOX2. *Expression of HOPX (A) and SOX2 (B) transcripts were analysed by q-PCR at 5 days of cell differentiation in Th17 cells treated with Non targeting (siNT) or RORC (siRORC) siRNA. All data were normalized on L34 expression and presented as fold vs siNT. (n=3; * p<0.05;)*



Supplementary figure 8. Transfection of memory CD4 T cells with siRNA *Total CD4 T cells were transfected with siRNA for 3 days. Internalization of PE-siRNA upon transfection (A) and cell viability (B) were evaluated by flow cytometry.*



Supplementary figure 9. Gating strategy for the purification of naïve CD4 T cells *Blood CD4 T lymphocytes were labelled with specific antibodies conjugated with a fluorochrome and, by using a cell sorter, naïve CD4 T cells were isolated as* $CD4^{high}$, $CD45RA^{high}$, $CD45RO^{-}$ *and* $CD27^{+}$ *(A). The purity of isolated naïve CD4 T cells, evaluated by flow cytometry after purification, is more than 97% (B). The graphs show the data of a representative experiment.*

Supplementary table 1.

Ghoreschi et al., 2010	*Lee et al., 2012*	*Gaublomme et al., 2015*	*Hu et al., 2017*	*Wu et al., 2018*	*Yosef et al., 2013*
IL2	TBX21	IL2	IL2	RASA3	BATF
TBX21	CCL4	CXCR3	TBX21		EGR2
CXCR3	GZMB	CCL4	CXCR3		IL17RA
IL33	CCL5	ICOS	CCL4		IRF4
CCL9 (CCL23 in human)	CSF2	STAT4	GZMB		CCR6
	CCL3	RORC	CCL5		PROCR
	ICOS	CCR8	CSF2		IRF8
	STAT4	CKLF	CCL3		NOTCH1
	IL22	LFA	STAT4		MINA
	IL3	CCR6	IL22		EGR1
	LRMP	CCL20	RORC		ZEB1
	CASP1	CTLA4	IL3		NFKB1
	LGALS3	IL17A	IFNG		PML
	LAG3	CD40LG	EPSTI1		SMARCA4
	CXCL3	TNF	RSG2		POU2AF1
	IL1R	IL21	IL23R		RBPJ
	IL7R	TNFRSF9	TGFB1		Il21R
		EBI3	NFATC3		MYC
		MINA	IRF8		CCR5
		IRAK1	FASLG		ETV6
		IL1RN	AHI1		FAS
			HSPH1		IL12Rb1
			EGR2		
			TNFSF14		
			DUSP5		
			NFIL3		
			CBLB		
			LDHA		
			SOCS3		
			REL		
			NOTCH1		
			EGR3		
			MALT1		

Supplementary table 2.

Primers	Sequence	Annealing Temp°
HOPX forward	5'– CAACAAGGTCGACAAGCACC –3'	63 °C
HOPX reverse	5'– CGCTGCTTAAACCATTTCTGG –3'	
IL17F forward	5'– GAAAACCAGCGCGTTTCCAT –3'	54 °C
IL17F reverse	5'– CCTGTACAACTTCCGAGGGG –3'	
BTLA forward	5'– CTGACACAGCAGGAAGGGAAAT –3'	58 °C
BTLA reverse	5'– CTGCATCCTGAAACAAAGGTCA –3'	
MTSS1 forward	5'– GACTCGCTTCAGTGCTCCAG –3'	58 °C
MTSS1 reverse	5'– TCATAATCTGAAACTTGGGAAGGG –3'	
ANXA1 forward	5'– GGTGAGCCCCTATCCTACCT –3'	58 °C
ANXA1 reverse	5'– CTGTTGACGCTGTGCATTGT –3'	
ITM2A forward	5'– AACTGCTATCTGATGCCCCT –3'	58 °C
ITM2A reverse	5'– CATCACGAATTTCCTCCACAGC –3'	
LGALS1 forward	5'– CTCGGGTGGAGTCTTCTGAC –3'	54 °C
LGALS1 reverse	5'– GAAGGCACTCTCCAGGTTTGA –3'	
RASGRP4 forward	5'– GACTGAATCCCCACACCCTT –3'	54 °C
RASGRP4 reverse	5'– GTGAGGAAGAGAGGAGACCA –3'	
LINC00877 forward	5'– CGAGAAGGAAAGCCGGTGAT –3'	63 °C
LING00877 reverse	5'– TCCATGTGTCTGCTTTGCCT –3'	
TSPAN13 forward	5'– GCCCTCAACCTGCTTTACAC –3'	56 °C
TSPAN13 reverse	5'– ACAGCTCCAATCAGACCCAC –3'	
FGFBP2 forward	5'– CCGAGGGTGACAGGTGAAAG –3'	54 °C
FGFBP2 reverse	5'– CGTTGGATTGAAAGCGGCAT –3'	
IL6 forward	5'– CTGGCAGAAAACAACCTGAACC –3'	63 °C
IL6 reverse	5'– TGGCTTGTTCCTCACTACTCT –3'	
RPL34 forward	5'– GTCCCGAACCCCTGGTAATAGA –3'	60 °C
RPL34 reverse	5'– GGCCCTGCTGACATGTTTCTT –3'	

SECTION 3

FEATURES INTRINSICALLY ASSOCIATED TO TH17 CELLS IN MULTIPLE SCLEROSIS

Results of Section 3 address the Aim3 and have been included in:

Distinct expression of inflammatory features in T helper 17 cells from multiple sclerosis patients

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3.1. Introduction

Multiple sclerosis (MS) is a disease affecting the central nervous system (CNS), and involves inflammation and neurodegeneration (Hauser and Oksenberg, 2006; Lopez-Diego and Weiner, 2008). The symptoms of MS are due to the migration of inflammatory immune cells into the CNS and the destruction of myelin, which causes neuroinflammation and neurodegeneration (Herrera and Ebers, 2003). In MS patients, pathogenic lymphocytes express molecules that facilitate their transit through the blood-brain barrier (BBB), which is normally precluded (Hauser and Oksenberg, 2006). Once lymphocytes have penetrated the CNS, they proliferate and release cytokines, thus contributing to a complex inflammatory response that leads to myelin damage (Hemmer, Archelos and Hartung, 2002).

In particular, T helper (Th) 17 cells, a subtype of lymphocytes differentiated from naïve CD4 T cells and producing interleukin (IL)-17, are considered potent inflammatory effectors in MS (Brucklacher-Waldert *et al.*, 2009; Volpe, Battistini and Borsellino, 2015).

Importantly, the pathogenic potential of Th17 cells does not rely exclusively on the production of IL-17, and it is believed that a combination of inflammatory factors is required (Volpe, Battistini and Borsellino, 2015). For instance, Th17 cells express high levels of the C-C chemokine receptor 6 (CCR6) on the cell surface (Acosta-Rodriguez *et al.*, 2007) that binds the C-C chemokine ligand 20 (CCL20) expressed by both Th17 cells and the vascular endothelium of the BBB, thus enabling the entry of Th17 cells into the encephalic compartment through the choroid plexus (Reboldi *et al.*, 2009).

Moreover, Th17 cells produce other inflammatory cytokines, such as tumour necrosis factor (TNF)-a, IL-21, IL-22, which are likely to contribute to their pathogenicity (McGeachy *et al.*, 2007; Volpe *et al.*, 2009).

Differentiation of Th17 cells requires IL-1β, IL-6, IL-23, and transforming growth factor (TGF)-β (Volpe *et al.*, 2008), which promote the expression of the RAR-related orphan receptor (ROR)-γt transcription factor and determine the activation of the Th17

cell lineage-specific differentiation program (Ivanov *et al.*, 2006). Among the Th17 polarizing cytokines, IL-1β is considered crucial for the inflammatory properties of human Th17 cells, due to its inhibitory role in IL-10 production (Volpe *et al.*, 2008; Zielinski *et al.*, 2012). In mice, IL-1β, together with IL-23, is implicated in the enhancement of Th17 cell differentiation primed by TGF-β and IL-6 (Veldhoen, Hocking, Atkins, *et al.*, 2006), and for acquisition of a pathogenic phenotype by Th17 cells (McGeachy *et al.*, 2007; Chung *et al.*, 2009).

IL-1β binds a complex formed by its receptor (IL-1R1) and the IL-1R accessory protein (IL-1RAcP), thus triggering a signal transduction cascade involving the adaptor protein MYD88 and induction of specific inflammatory genes (Loiarro, Ruggiero and Sette, 2010; Ruggiero, 2012). This pathway plays a key role in experimental autoimmune encephalomyelitis (EAE), the mouse model of MS, as a knockout of the IL-1R1 or the MYD88 gene in mice, which significantly ameliorates disease-associated phenotypes (Sutton *et al.*, 2006). Importantly, their function in EAE seems to be related to Th17 cells, as ablation of IL-1R1 signalling strongly suppressed Th17-mediated EAE in cell transplantation experiments (Chung *et al.*, 2009). Moreover, it has been recently demonstrated that IL-1R1 expression is higher in CD4 T cells derived from MS patients in comparison to those from healthy donors (HD) (Sha and Markovic-Plese, 2016).

In this retrospective study, we analysed expression of IL-1R1 and other Th17 typical features in Th17 cells derived from MS patients and HD. In fact, a systematic study investigating the differentiation process of human Th17 cells in MS patients has never been performed. We hypothesised that Th17 cell differentiation is altered in MS patients, thus leading to the acquisition of pathogenic features that contribute to persistent inflammation in MS.

3.2. Results

IL-21 production by Th17 cells is increased in MS patients compared to HD

Given the pathogenic role played by Th17 cells in MS, we hypothesized that Th17 cells differentiated from MS patients and HD would display a differential cytokine profile. To test this hypothesis, naïve CD4 T cells from HD and relapsing-remitting (RR)MS patients were differentiated in Th17 cells under *in-vitro* culture conditions. We analysed the cytokines produced by polarized Th cells after 6 days of culture. First, we measured the production of ten Th profile-associated cytokines for the Th1, Th17 and Th2 subsets.

Interferon (IFN)-γ the prototypical Th1 cytokine, and IL-22, whose expression pattern is closely related to that of IFN-γ (Volpe *et al.*, 2009), are included in the Th1 category (Figure 14A). The Th17 class of cytokines includes IL-17A, the prototypical Th17 cytokine, IL-21, produced by Th17 cells and involved in the autocrine enhancement of their differentiation, and CCL20, the ligand of CCR6, which enables the entry of Th17 cells into the CNS (Figure 14B). A third set of cytokines produced mainly in Th2 conditions includes IL-4, IL-5, IL-13, IL-9, and IL-10 (Figure 14C). As expected, we observed the up-regulation of Th17 cytokines and IFN-γ in Th17 compared to Th0 conditions, in both HD and MS patients (Figure 14A,B). Typical Th2 cytokines (IL-4, IL-5, IL-13) were decreased in Th17 compared to Th0 conditions, while IL-9 and IL-10 were produced by Th17 cells, although not up-regulated compared to Th0 (Figure 14C). While most of these cytokines were similarly modulated in MS and HD (Figure 14A–C), we found that the expression of IL-21 was significantly higher in MS compared to HD (Figure 14B), indicating a possible role for this cytokine in Th17-related MS pathogenesis.

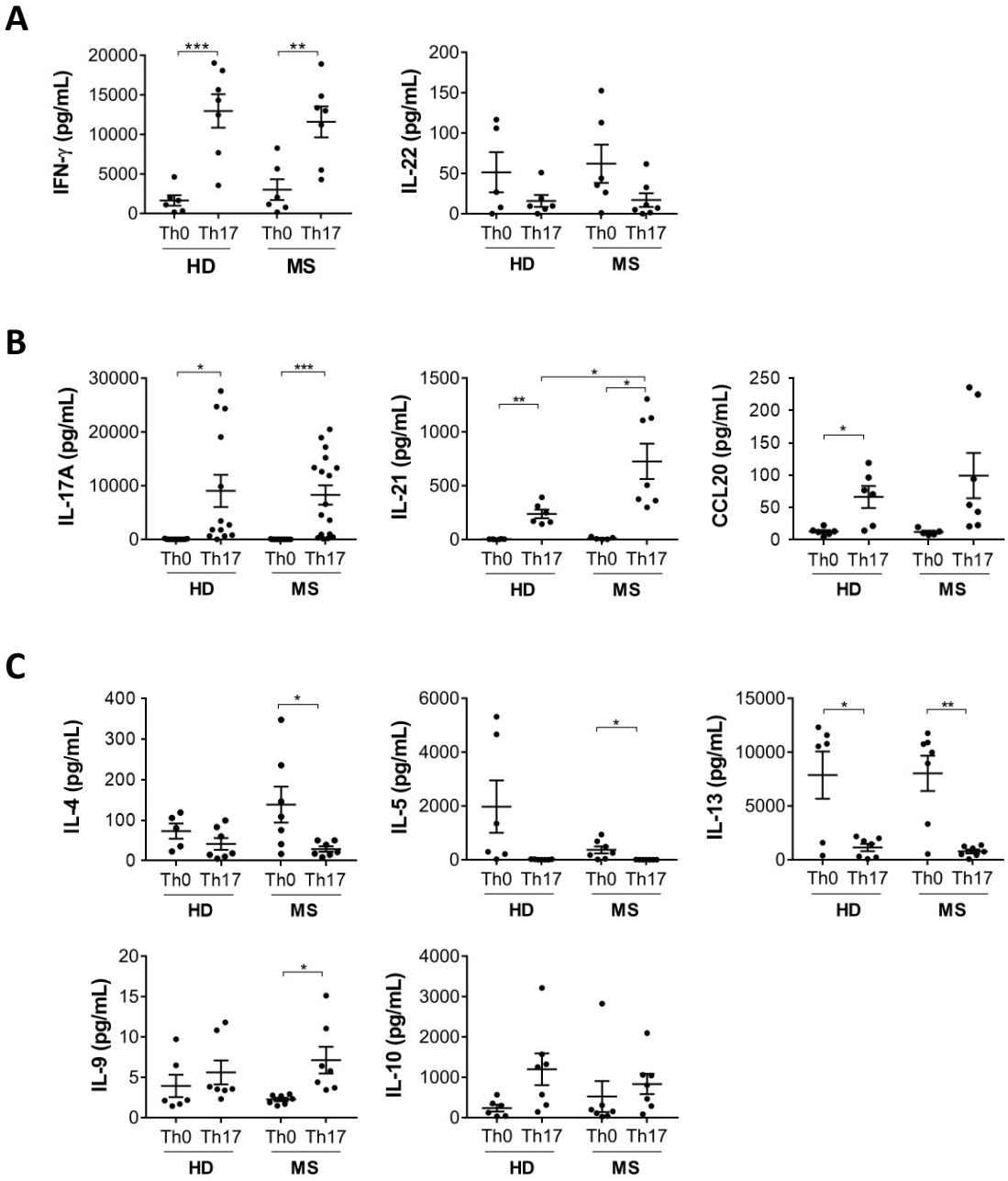


Figure 14. IL-21 production by Th17 cells is increased in multiple sclerosis patients compared to healthy donors. *Naïve CD4 T cells from healthy donors (HD) and multiple sclerosis (MS) patients were cultured with antiCD3-antiCD28 alone (Th0) or antiCD3-antiCD28, TGF-β, IL-6, IL-23 and IL-1β (Th17). At 5 days of differentiation the levels of typical cytokines of Th1 (A), Th17 (B), and Th2 (C) cells were analysed by multiplex assay (Luminex) in cell supernatants.*
($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

Production of cytokines involved in inflammation and T cell activation are increased in Th17 cells from MS patients

To investigate other features related to Th17 cells, we analysed the production of additional inflammatory cytokines that are typically produced by activated lymphocytes, including TNF-α, IL-8, and TNF-β, in Th17 cells differentiated from MS patients or HD. We found that in MS patients, the production of TNF-α and -β is significantly higher in Th0 and Th17 cells, respectively (Figure 15A). The production of IL-8, although more highly induced in Th17 compared to Th0 cells, was not differentially modulated in MS patients compared to HD.

Next, we analysed the production of three cytokines belonging to the common γ-chain family, IL-2, IL-7, and IL-15, involved in regulating the expansion and activation of all T cell subsets.

We observed that IL-2 is up-regulated in both Th0 and Th17 cells from MS patients, whereas IL-7 is up-regulated only in Th0 from MS patients and IL-15 is not modulated (Figure 15B). Given the role of IL-2 and IL-7 in T cell proliferation, these findings support the hypothesis of systemic T cell activation in MS patients.

To expand our analysis, we also tested expression of growth factors produced by T lymphocytes, such as platelet-derived growth factor (PDGF)-AA and AB/BB, and granulocyte-macrophage colony-stimulating factor (GM-CSF). We observed that PDGF, either composed by subunit AA, AB or BB, is up-regulated in Th17 cells, while an opposite trend was found for GM-CSF. However, both growth factors are not differentially modulated in MS compared to HD Th17 cells (Figure 15C).

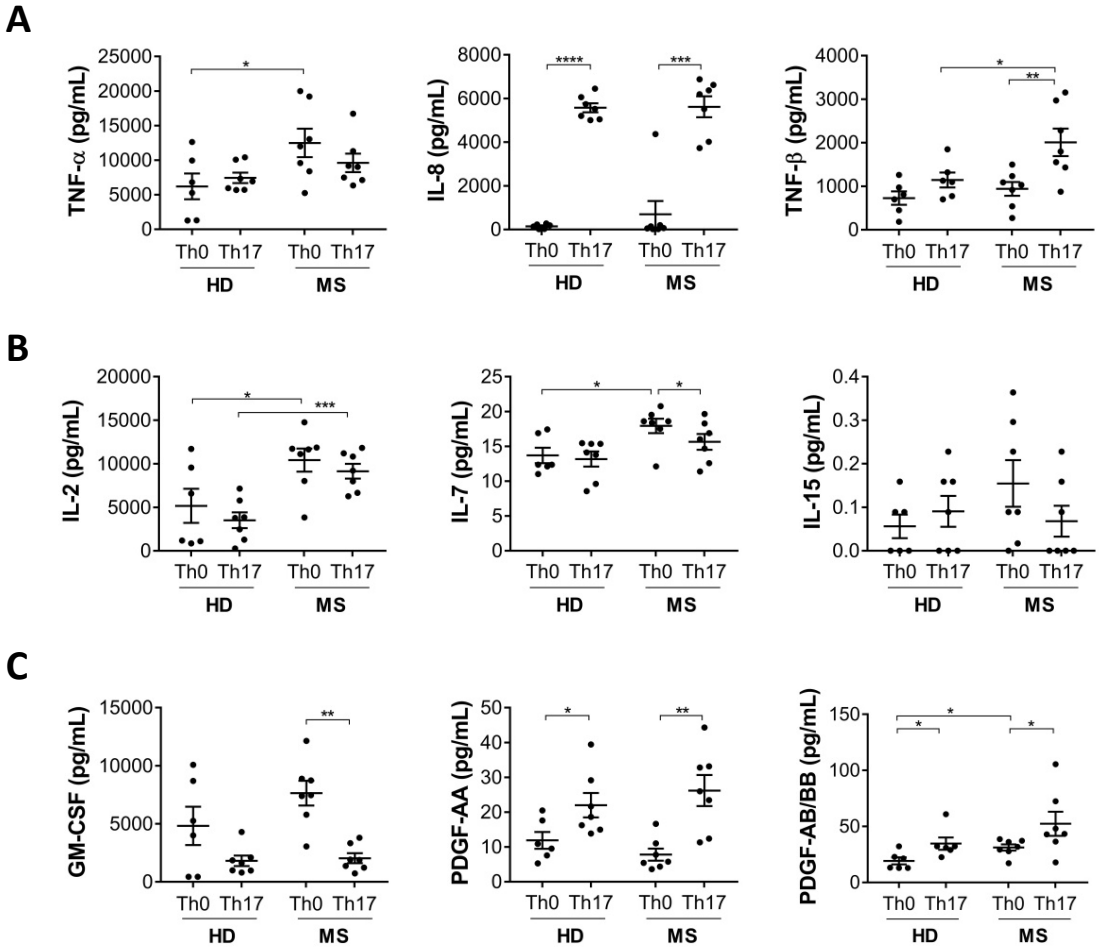


Figure 15. Production of cytokines involved in inflammation and T cell activation are increased in Th17 cells from multiple sclerosis patients.

Naïve CD4 T cells from healthy donors (HD) and multiple sclerosis (MS) patients were cultured with antiCD3-antiCD28 alone (Th0) or antiCD3-antiCD28, TGF-β, IL-6, IL-23 and IL-1β (Th17). At 5 days of differentiation the levels of inflammatory cytokines (A), cytokines involved in T cell expansion (B), and growth factors (C) were analysed by multiplex assay (Luminex) in cell supernatants
($p < 0.05$; ** $p < 0.01$; *** < **** $p < 0.0001$).*

Th17 cells differentiated from MS patients express higher IL-1R1 than those differentiated from HD

To address whether the acquisition of typical features of Th17 cells were differentially modulated in MS patients compared to HD, we analysed the expression of the transcription factor RORγt, a master regulator of both mouse (Ivanov *et al.*, 2006) and human Th17 cell differentiation (Manel, Unutmaz and Littman, 2008; Volpe *et al.*, 2008), CCR6 (Yamazaki *et al.*, 2008) and IL-1R1 (Chung *et al.*, 2009), which are not found in Th1 and Th2 cells, and are considered hallmarks of Th17 cells. This analysis revealed that RORγt and CCR6 are up-regulated in Th17 cells from all individuals with no differences between cells obtained from MS patients and HD (Figure 16A,B). In contrast, Th17 cells polarized from MS patients expressed significantly higher levels of IL-1R1 than corresponding Th17 cells polarized from HD (Figure 16C). However, no significant differences were observed in cells obtained from MS patients in either the active or inactive phase of the RR disease (Supplementary Materials, Figure S10), according to the presence ($Gadolinium^{+}$) or the absence ($Gadolinium^{-}$) of contrast-enhancing lesions detected by magnetic resonance imaging.

This suggested that IL-1R1 expression on the Th17 cell surface was not influenced by the acute stage of inflammation. To determine whether IL-1R1 expression was dependent on a specific Th17-promoting cytokine, we removed them from the polarization medium. This analysis revealed that, similar to other Th17 molecules (Volpe *et al.*, 2008), IL-1R1 expression in Th17 cells is mediated by the synergy between TGF-β and pro-inflammatory cytokines IL-1β, IL-6, and IL-23, although none of them exhibit a predominant role (Figure S11).

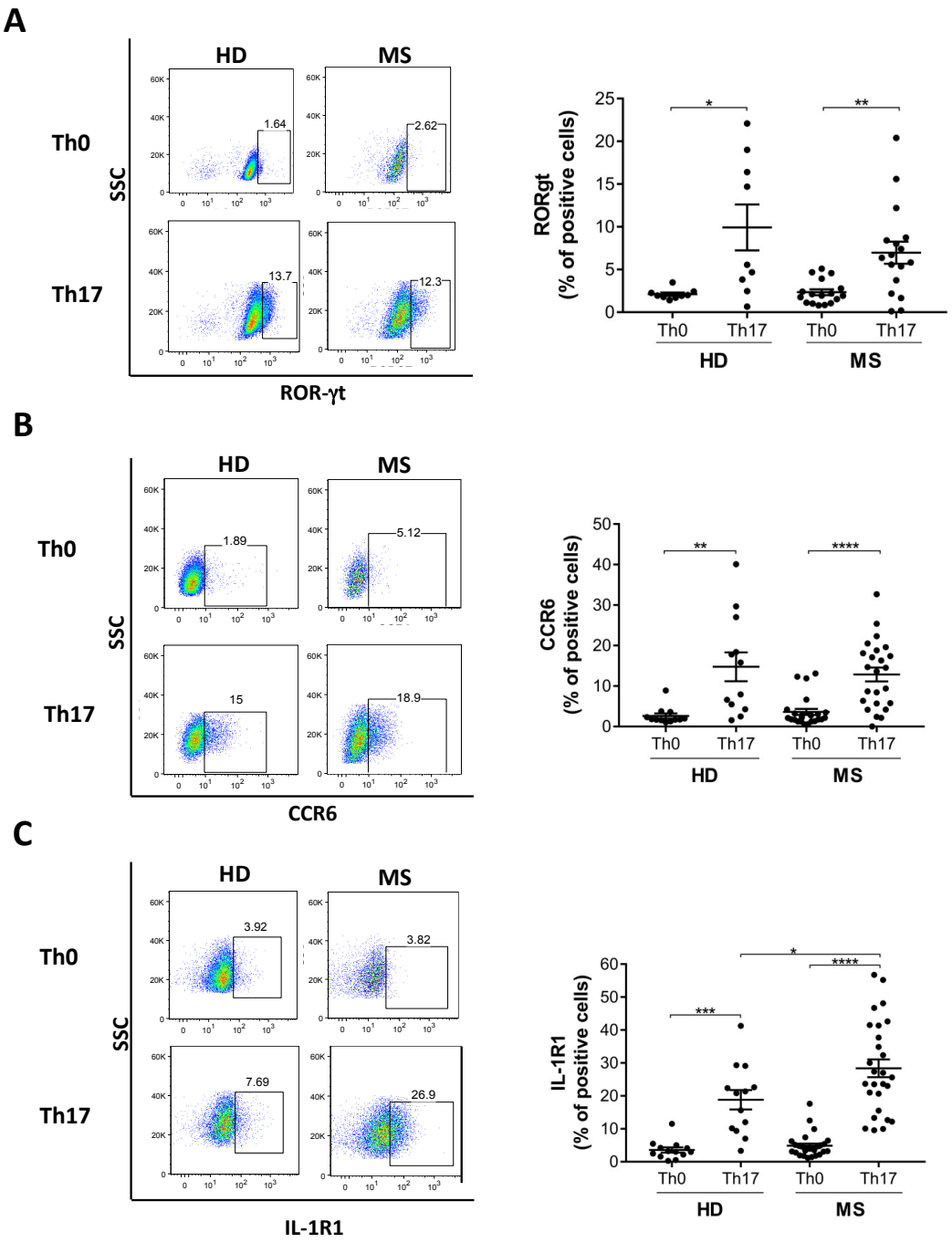


Figure 16. Th17 cells differentiated from multiple sclerosis patients express higher IL-1R1 than those differentiated from healthy donors.

Naïve CD4 T cells from healthy donors (HD) and multiple sclerosis (MS) patients were cultured with antiCD3-antiCD28 alone (Th0) or antiCD3-antiCD2, TGF-β, IL-6, IL-23 and IL-1β (Th17). At 5 days of differentiation cells were stained with specific antibodies against RORγt (A), CCR6 (B), and IL-1R1 (C) and analysed by flow cytometry. Graphs represent the results of independent experiments.
($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.)*

IL-1R1$^+$ cells in the blood are Th17 cells and are increased in MS patients compared to HD

To investigate the physiological relevance of the increased IL-1R1 expression in Th17 cells differentiated from MS patients *in-vitro*, we sorted blood memory CD4 T cells for positivity or negativity in IL-1R1 expression. We found that CD4$^+$ IL-1R1$^+$ cells produced more IL-17 than CD4 cells not expressing IL-1R1 (Figure 17A). This result indicates that IL-1R1 expression is also associated to IL-17-producing cells in vivo. In order to investigate whether Th17 cells are different in the blood of MS patients and HD, we analysed the frequency of circulating IL-1R1$^+$ cells within the CD4$^+$ CD161$^+$ CCR6$^+$ cell population, which corresponds to the population of memory Th17 cells (Annunziato *et al.*, 2007; Cosmi *et al.*, 2008). Importantly, expression of IL-1R1 in the *in-vivo* differentiated Th17 cells was higher in MS patients compared to HD (Figure 17B), indicating that mechanisms leading to overexpression of IL-1R1 in MS also occur *in-vivo*.

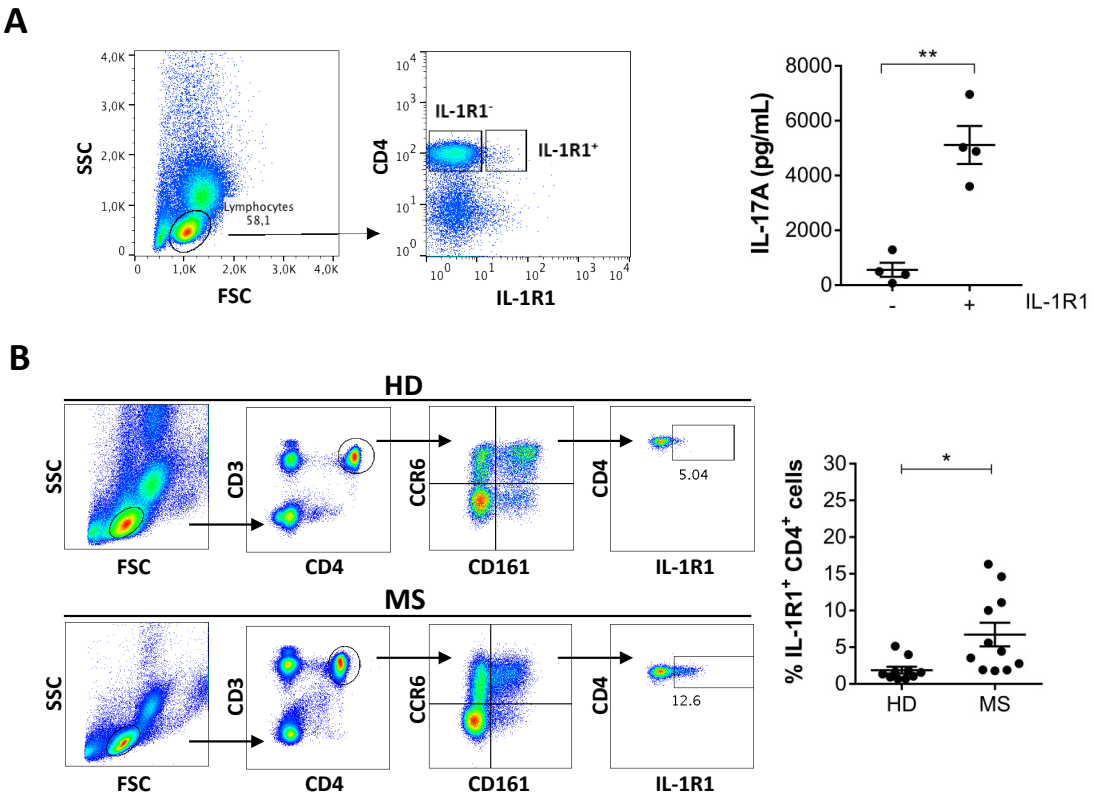


Figure 17. IL-1R1$^+$ cells in the blood are Th17 cells and are increased in multiple sclerosis patients compared to healthy donors.

Blood memory cells sorted as CD4$^+$ IL-1R1$^+$ and CD4$^+$ IL-1R1$^-$ cells (gating strategy in the left panel) were stimulated with antiCD3-antiCD28 for 24h. Supernatants were then analysed for IL-17 production by ELISA (A). Peripheral blood mononuclear cells from healthy donors (HD) and multiple sclerosis (MS) patients were stained with specific antibodies to analyse the frequency of CD3$^+$ CD4$^+$ CCR6$^+$ CD161$^+$ IL-1R1$^+$ cells by flow cytometry (B). Graphs represent the results of independent experiments. ($p < 0.05$; ** $p < 0.01$).*

3.3. Discussion

In this study, systematic analysis of the typical features of Th17 cells revealed that cells derived from MS patients display higher expression of IL-1R1 compared to Th17 cells derived from HD, and indicated that the corresponding higher expression of IL-1R1 previously observed in MS patients (Sha and Markovic-Plese, 2016) is specifically associated to Th17 cells. Furthermore, we identified other inflammatory molecules that were differentially expressed that may contribute to the pathogenicity of Th17 cells in MS.

In fact, the analysis of a broad array of cytokines produced by Th17 cells in MS patients and HD revealed that IL-21 is significantly up-regulated in MS patients compared to HD. This result is consistent with the high frequency of IL-21-producing T cells reported in both active and chronic parenchymal lesions of MS brain (Tzartos *et al.*, 2011), and increased levels of IL-21 induced by anti-CD28 in CD4 from MS patients (Camperio *et al.*, 2014).

IL-21, similarly to IL-6 and IL-23, activates a signalling pathway involving STAT3, which directly binds the *Il17* and *Il21* promoters (Wei *et al.*, 2007), thus contributing to the sustainment of Th17 lineage commitment (Nurieva *et al.*, 2007). However, IL-21 is also the most potent inducer of plasma-cell differentiation *in-vitro* (Spolski and Leonard, 2014), and provides help to B cells (Crotty, 2011). Recently it has been reported that the B-T cell interaction plays a crucial pathogenic role in MS (Jelcic *et al.*, 2018), which could be mediated by IL-21. Considering that MS involves infiltration of both T and B cells in parenchymal demyelinating lesions, and in lymphoid aggregates in the meninges (Magliozzi *et al.*, 2007; Lovato *et al.*, 2011), increased IL-21 levels produced by Th17 cells could play a critical role in MS.

Our study also revealed that TNF-β, known as lymphotoxin-alpha (LT-α), is up-regulated in Th17 cells from MS patients, confirming previous observations showing that PBMC from RR-MS patients had increased expression of LT-α (Romme Christensen *et al.*, 2012). Similar to LT-α, which is involved in the regulation of cell survival and proliferation (Bauer *et al.*, 2012), other cytokines important for these functions in T cells, such as IL-2 and IL-7, are up-regulated in MS, suggesting that they may synergize to promote T cell expansion during MS. In particular, IL-2 is significantly up-regulated in cells derived from MS patients in both Th0 and Th17 conditions. These results are in agreement with the observed increase in IL-2 levels in the serum of patients with active MS (Gallo *et al.*, 1989), suggesting that the effector Th17 cells contribute to IL-2 production in MS. In fact, T regulatory cells, known to suppress effector immune functions, from subjects with RR-MS had impaired proliferation and reduced IL-2 secretion (Carbone *et al.*, 2014).

Another interesting finding of our study is that Th17 cells produce IL-8 and PDGF. Although they were not modulated in MS versus HD, IL-8 and PDGF were not previously known to be produced by Th17 cells and are not typically considered Th cytokines. IL-8 plays an important role in inflammation due to its high neutrophil-attracting capacity (Kobayashi, 2008), and could synergize with IL-17 in exerting this function. PDGF, which can be composed of two A subunits (PDGF-AA), two B subunits (PDGF-BB), or one of each (PDGF-AB) (Hannink and Donoghue, 1989; Distler *et al.*, 2003), plays a significant role in blood vessel formation. However, whether the production of PDGF contributes or not to the pathogenic functions of Th17 cells in MS will require direct investigation.

Overall, the molecules up-regulated in Th17 cells from MS patients could be related to their high sensitivity to IL-1β, one of the most important Th17-regulating cytokine. In fact, in the same set of experiments, IL-1R1 is significantly up-regulated in MS patients, as previously demonstrated at transcriptional level in naïve and memory CD4 T cells, and *in-vitro* differentiated Th17 cells (Sha and Markovic-Plese, 2016).

We further analysed the expression of IL-1R1 on memory Th17 cells, which we found were significantly increased in MS patients. Previous comparative analysis on memory cells from MS and HD was performed in Th1/Th17 cells, and revealed a reduced transcriptional expression of inflammatory factors, such as *Ifng*, *Ccl3*, *Cll4*, and *Gzmb* (Hu *et al.*, 2017). However, a broader analysis at protein level of Th17 and Th1/17 subsets isolated from MS patients may help to identify more disease-related features.

Our results indicate that IL-1R1 is up-regulated at protein level in Th17 cells obtained from both *in-vitro* and *in-vivo* differentiated CD4 T cells of MS patients, and that IL-1R1 signalling is a critical step in the regulation of human Th17 cells in MS. In fact, it is already known that the IL-1β/IL-1R1 axis is important for differentiation of naïve CD4 T cells into Th17 cells (Veldhoen, Hocking, Atkins, *et al.*, 2006; Volpe *et al.*, 2008). Recently it has been demonstrated that IL-1β is also crucial for induction of IL-17A by memory CD4 T cells in the absence of T cell receptor (TCR) engagement, introducing a TCR-independent innate-like pathogenic role of Th17 cells mediated by IL-1β/IL1R1 signalling (Lee *et al.*, 2019).

The relevance of IL-1R1 in MS is supported by previous results reporting that IL-1R1-deficient mouse T cells developed a delayed and less severe EAE, and a lower percentage of IL-17 producing cells in the inflamed CNS compared to wild type mice (Chung *et al.*, 2009). In conclusion, our study identifies IL-21, IL-2, TNF-β, and IL-1R1, as molecules likely involved in the mechanisms conferring pathogenicity to human Th17 cells and opens another perspective on the use of these molecules as therapeutic targets or biomarkers of MS disease.

3.4. Materials and Methods

MS subjects

Patients diagnosed with relapsing–remitting (RR)-MS (n = 31) according to the revised McDonald's diagnostic criteria (Polman *et al.*, 2011) were enrolled in the study. The demographic and clinical characteristics of the RR-MS patients included in the study for blood sampling are described in Table 1. All patients included in the blood study did not take immunomodulant or immunosuppressive compounds for at least 2 months before recruitment. As controls, we used blood from age and gender matched individuals (n=28) without inflammatory or degenerative diseases of the central or peripheral nervous system. These subjects were volunteers that underwent blood testing.

Approval by the ethics committee of the IRCCS Neuromed, Pozzilli (IS), Italy and San Camillo Hospital, Rome (Italy), and written informed consent in accordance with the Declaration of Helsinki from all participants, were obtained before the study was initiated.

Number	31
Gender (male/female)	5/26
Age (years)	43 ± 9.7
EDSS	2 ± 1.35
MRI (gadolinium +/−)	7/24

Table 1. Demographic and clinical characteristics of MS subjects at the time of experiment.

Purification of naïve CD4 T lymphocytes from adult blood

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation (GE Healthcare, Little Chalfont, UK) from whole blood. For sorting of IL-1R1^{+} cells, PBMC were stained with anti-human CD4-FITC (Miltenyi, Bergisch Gladbach, Germany) (1:100) and anti-human IL-1R1-PE (RnD, Minneapolis, MN, USA) (1:20) and sorted by a MoFlo high speed cell sorter (Beckman Coulter, Atlanta, GA, USA). For sorting naïve CD4 T cells, the cells were stained with the anti-CD4-FITC (Miltenyi) (1:100), and CD4 T Lymphocytes were purified by immunomagnetic selection, using the anti-FITC isolation kit (Miltenyi). After the isolation, the cells were stained with anti-CD4 FITC (Miltenyi) (1:100), anti-CD45RA BV421 (BD Biosciences, San Jose, CA, USA) (1:60), anti-CD45RO PE (BD Biosciences) (1:30), anti-CD27 APC (Miltenyi) (1:60), and CD4 naïve T cells were sorted by MoFlo high speed cell sorter (Beckman Coulter) as CD4^{high}, CD45RA^{high}, CD45RO^{-} and CD27^{+}. Sorted cells had a purity of over 97%, measured by flow cytometry (data not shown).

Th cell differentiation assay

Naïve CD4 T cells were cultured in 96-well round-bottomed plates (Corning, New York City, NY, USA) at a density of 5 x 10^4 per well in X-VIVO 15 serum-free medium (Lonza, Walkersville, MD, USA) in the presence of Dynabeads CD3-CD28 T cell expander (one bead per cell; Life Technologies, Carlsbad, CA, USA) and indicated cytokines: IL-1β (10 ng/mL), IL-6 (20 ng/mL), TGF-β (1 ng/mL) and IL-23 (100 ng/mL) (Miltenyi) for Th17 differentiation, as previously described (Volpe *et al.*, 2008; Ruocco *et al.*, 2015). After 5–6 days, cells were harvested and stained for flow cytometry analysis, or extensively washed, counted, and re-stimulated 1 x 10^6 cells/mL with Dynabeads CD3-CD28 T cell expander (one bead per cell) for 24 h for cytokine quantification.

Flow cytometry analysis

Cells were stained with the following antibodies: anti-human RORγt BV421 (BD Biosciences) (1:20), anti-human CCR6-Alexa 647 (BD) (1:20), anti-human IL-1R1 PE (RnD) (1:20), anti-human CD4-PECy7 (Beckman Coulter) (1:100), CD161 BV421 (Biolegend, San Diego, CA, USA) (1:40), anti-human CD3 FITC (Miltenyi) (1:100). Samples were analysed using Cytoflex cytometer (Beckman Coulter, Brea, CA, USA) and analysed using FlowJo-10 software version 10.3.0.

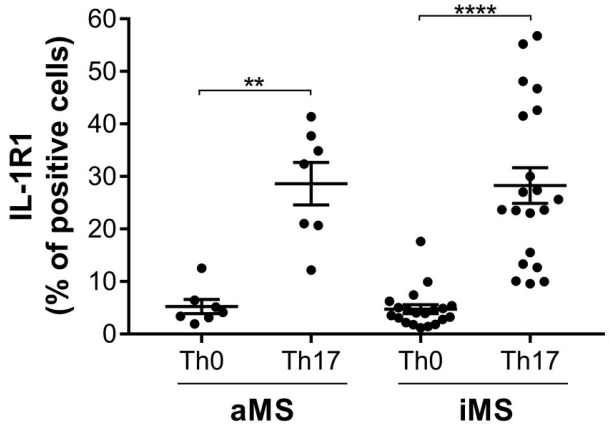
Cytokine quantification

IL-17 in culture supernatant was quantified with an enzyme-linked immunosorbent assay (ELISA) kit (RnD Systems, Minneapolis, MS, USA). Other cytokines (IL-2, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-13, IL-15, IL-21, IL-22, TNF-α, TNF-β, GM-CSF, IFN-γ, PDGF-AA, PDGF-AB/BB, CCL20) were quantified using a magnetic bead panel (Millipore, Burlington, MA, USA), following the manufacturer's protocol, and analysed by Luminex

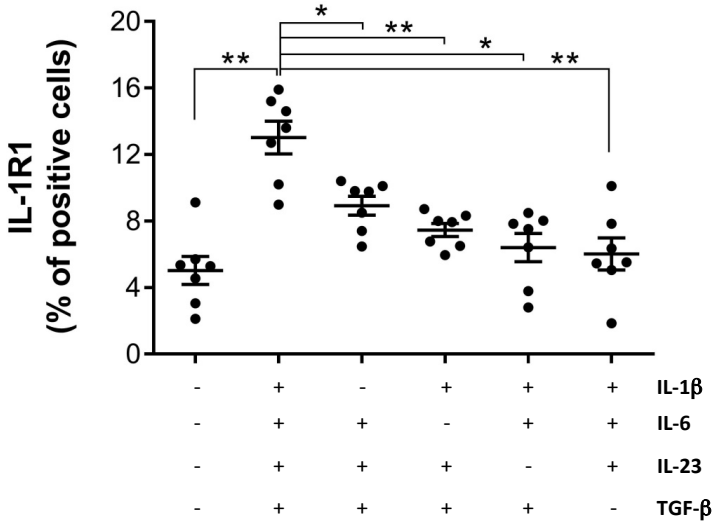
Statistical analysis

For pair-wise comparisons of different conditions from the same donors or different donors, we used a parametric two-tailed paired or unpaired t test, respectively. One-way ANOVA was performed to analyse the main effects of two conditions on the dependent variables and their interactions. Data were presented as mean ± standard error (s.e.m). The p-values (p) of 0.05 or less, were considered statistically significant.

3.5. Supplementary materials



Supplementary Figure 10. The activity of multiple sclerosis disease does not modulate IL-1R1 expression on differentiated Th17 cells.
*Naïve CD4 T cells from and multiple sclerosis (MS) patients in active (aMS) and inactive (iMs) phase were cultured with antiCD3-antiCD28 alone (Th0) or antiCD3-antiCD28, TGF-β, IL-6, IL-23 and IL-1β (Th17). At 5 days of differentiation cells were stained with specific antibodies against IL-1R1 and analysed by flow cytometry. (** p < 0.01; **** p < 0.0001.)*



Supplementary figure 11. The synergy between Th17 polarizing cytokines contributes to IL-1R1 expression on Th17 cells.
Naïve CD4 T cells from healthy donors were cultured with antiCD3-antiCD28 alone, antiCD3-antiCD28, TGF-β, IL-6, IL-23 and IL-1β, or under different suboptimal Th17 polarizing condition (cocktail of Th17 differentiation without one cytokine). At 5 days of differentiation cells were stained with specific antibodies against IL-1R1 and analysed by flow cytometry. (* p < 0.05; ** p < 0.01).

SECTION 4

CONCLUSIONS, PERSPECTIVES, AND REFERENCES

4.1. Conclusions

Results from these studies allowed to better characterize the immunological pathways underlying human Th17 cell polarization, and to identified Th17 inflammatory molecules that could have a pathogenic role in MS. In particular:

1) We characterized the global and stepwise transcriptional changes occurring during human Th17 cell differentiation. These changes can be grouped in three time-regulated modules, whose activity is related to typical Th17 cell functions. The early module, involving exclusively "signalling pathways" genes; late module, characterized by genes involved in "response to infections"; persistent module, containing genes related to "effector immune functions". We found that in regulatory versus inflammatory conditions, genes related to "signalling pathways", "cytokine production" and "response to other organisms" were significantly down-regulated, and then classified as inflammatory genes. This analysis allowed to define a global molecular inflammatory signature of human Th17 genes.

2) Among the global molecular inflammatory signature of human Th17 cells, we selected known Th17 factors, such as *IL17A*, *IL17F*, *IL6*, *IFNG*, *IL26,* and we identified novel effector molecules, such as *LTA*, *PDGFA*, *IL1A*, *IFNK*. Moreover, we identified HOPX and SOX2 as transcriptional regulators acting downstream of RORγt signalling and we demonstrated their involvement in the differentiation program of inflammatory Th17 and Th1/17 cells.

3) We identified Th17 intrinsic features associated to Th17 cells from MS: IL-21, IL-2, LT-α, and IL-1R1, whose expression is increased in MS compared to HD. Moreover, we reported that differential expression of IL-1R1 in *in-vitro* polarized Th17 cells from MS patients and HD was also confirmed in *ex-vivo*-purified Th17 cells. Thus, Th17 cells from MS patients express intrinsic features that could be related to their high inflammatory status.

Collectively, results from this thesis uncovered new inflammatory mechanisms and new molecules of human Th17 cells that could open perspectives on the use of these molecules as biomarkers or new therapeutic targets for MS disease.

4.2. Perspectives

In the recent years several therapies directed against Th17 cells have been developed and their efficacy has been proven in autoimmune diseases. In particular, Secukinumab, a fully humanized antibody neutralizing IL-17A, is now approved as the first-line systemic treatment for moderate to severe plaque psoriasis. Other therapeutic approaches, including antibodies neutralizing IL-23 p19, one of the Th17 polarizing cytokines, or compounds blocking RORγt, the transcriptional factor of Th17 cells, have been developed.

The common strategy of these therapies is the targeting of the whole compartment of Th17 cells. Results from our study propose a selection of inflammatory molecules of Th17 cells that could be blocked to specifically supress pathogenic Th17 cells. In fact, the partial suppression of pathogenic Th17 cells could be more effective, and could avoid side effects, such as fungal infectious disease.

In particular, we identified new inflammatory molecules of human Th17 cells, especially in MS.

These data could be exploited by using antibodies neutralizing these specific molecules in animal model of MS disease, such as the murine EAE. The expected reduction of disease progression in preclinical studies could lead to future therapies for MS.

Moreover, the expression of these molecules could be further investigated in Th17 cells from MS patients to identify eventual association with disease progression, or with responsiveness to the current therapies, or with sensitivity to future Th17-targeting therapies.

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SECTION 5

Ph.D. CAREER

5.1. Synopsys of the thesis

Cytokine-producing CD4 T helper (Th) cells are the main modulators of the adaptive immune response. Upon activation by the innate immune system, distinct Th lineages are generated from naïve CD4 T cells, depending on the environmental signals present during activation.

In the past, two major cell subsets, Th1 and Th2, were described as producers of interferon (IFN)-γ and interleukin (IL)-4, IL-5, IL-13, respectively, mainly involved in the response against virus and bacteria (Th1) and parasites (Th2). However, more recently, another subset of Th cells, called Th17, was described as specific for the immune response against fungi and intracellular pathogens.

In humans, differentiation of Th17 cells from naïve CD4 T cells requires the combined action of IL-1β, IL-6, IL-23 and TGF-β, and induction of RORγt, a transcription factor with a central role in Th17 differentiation. Th17 cells produce the specific cytokines IL-17A and IL-17F, but also other cytokines, such as IFN-γ, tumor necrosis factor (TNF)-α, IL-6, IL-10, IL-21, IL-22, and IL-26. Although Th17 cells play a protective role in the mucosal host defence against extracellular bacteria and fungi, they play a pathogenic role in numerous autoimmune disorders, such as multiple sclerosis (MS). Specifically, in MS Th17 cells are considered the most potent inflammatory effector CD4 Th cells. It has been demonstrated that IL-17A inhibits the expression of tight junction proteins of human blood-brain barrier (BBB) of MS patients and it plays a role in the transmigration of CD4 T cells into the central nervous system (CNS). Moreover, IL-17A induces matrix metalloproteinase expression, leading to BBB dysfunction and neuronal apoptosis.

In recent years several therapies directed against Th17 cells have been developed, and currently tested in MS. However, the pathogenic mechanisms of Th17 cells are not completely defined, and new studies on Th17 cells and MS are needed to unveil new potential therapeutic targets.

The main objective of this thesis was to identify new mechanisms and molecules inducing inflammatory functions of human Th17 cells that could be potentially involved in the pathogenic processes of MS. We addressed this aim by using two distinct approaches: 1) analysis of general inflammatory features of Th17 cells acquired during Th17 cell differentiation; 2) analysis of intrinsic features of Th17 cells derived from MS patients.

First, we investigated potential mechanisms responsible for pathogenic functions of Th17 cells, by performing a transcriptome analysis of cells at 48 hours and 5 days of differentiation. We uncovered three time-regulated modules: early modulation, involving exclusively ‘signalling pathways’ genes; late modulation, characterized by

genes involved in response to infections; persistent modulation, involving effector immune functions. To assign them an inflammatory or regulatory potential, we compared Th17 cells differentiated in presence or absence of IL-1β, respectively. We named inflammatory Th17 condition the polarizing milieu containing IL-1β, which is crucially involved in the pathology of Th17-related diseases. In contrast, regulatory Th17 condition refers to the anti-inflammatory IL-10 cytokine produced in Th17 condition lacking IL-1β.

We found that genes associated to pathogenic murine Th17 cells, such as *IL17A*, *IFNG*, *TBX21*, *EBI3*, *IRF8*, *TNFRSF9*, *TNFRSF14*, *CCL5*, *CD40LG*, *BATF* and *TNF*, were down-regulated in regulatory Th17 condition, and defined as inflammatory genes in our model. Then, we elucidated the global molecular signature of inflammatory human Th17 cells, by analysing all genes differentially expressed in regulatory versus inflammatory Th17 conditions. Among inflammatory genes we identified novel effector molecules, including interferon (IFN)K, lymphotoxin (LT)-α, IL1A, platelet derived growth factor (PDGF)-A, and transcriptional regulators, such as transcriptional regulators homeodomain-only protein homeobox (HOP)X and SRY (sex determining region Y)-box 2 (SOX2), expression of which was independently validated.

In order to unveil the mechanisms underlying the acquisition of the inflammatory human Th17 signature, we investigated the potential transcription factors involved in this process. In this context, we analysed the role of RORγt, known master regulators of Th17 cells, and of the two novel transcriptional regulators HOPX and SOX2. We found that HOPX regulates IL-17A and IFN-γ, while SOX2 regulates PDGF-A and IFN-γ expression. As expected, RORγt regulates expression of IL-17A, IL-17F, but also IFN-γ, PDGF-A, and *IL1A*.

As second approach, we analysed the pathogenic features intrinsically associated to Th17 cells in MS, by differentiating cells from naïve CD4 T cells of MS patients or healthy donors (HD) with the Th17-differentiation cocktail (IL-1β, IL-23, IL-6 and TGF-β). The immunological characterization of differentiated Th17 cells revealed intrinsic features of Th17 cells from MS patients that could lead to a more inflammatory status compared to those from HD. In particular, we observed the increased expression of pro-inflammatory proteins IL-21, IL-2, and IL-1R1 in Th17 from MS compared to Th17 from HD. Moreover, we found that Th17 cells derived from MS patients express higher levels of LT-α compared to those from HD, indicating that the pathogenic signature that we identified using the first approach contains intrinsic inflammatory features of Th17 cells derived from patients affected by Th17-related diseases.

In conclusion, results from this thesis unveiled new mechanism of inflammatory human Th17 cells that could have a role in MS.

In particular we reported: 1) the global inflammatory signature of human Th17 cells; 2) the validation of *IFNK*, *IL1A*, PDGF-A, LT-α, HOPX, and SOX2 as new inflammatory genes of human Th17 cells; 3) the overexpression of IL-21, IL-2, LT-α, and IL-1R1 in Th17 cells from MS patients versus HD.

Importantly, all these molecules could become new biomarkers or new therapeutic targets of Th17-related autoimmune diseases.

5.2. List of publications

(,# equal contribution)*

1. Sambucci M, Gargano F, De Rosa V, De Bardi M, Picozza M, Placido R, Ruggieri S, **Capone A**, Gasperini C, Matarese G, Battistini L, Borsellino G.
   FoxP3 isoforms and PD-1 expression by T regulatory cells in multiple sclerosis. Sci Rep. 2018 Feb 27;8(1):3674.

2. **Capone A**, Lo Presti A, Sernicola L, Farcomeni S, Ferrantelli F, Maggiorella MT, Mee ET, Rose NJ, Cella E, Ciccozzi M, Ensoli B, Borsetti A.
   Genetic diversity in the env V1-V2 region of proviral quasispecies from long-term controller MHC-typed cynomolgus macaques infected with SHIVSF162P4cy. J Gen Virol. 2018 Dec;99(12):1717-1728.

3. **Capone A**[*], Bianco M[*], Ruocco G, De Bardi M, Battistini L, Ruggieri S, Gasperini C, Centonze D, Sette C[#], Volpe E[#].
   Distinct Expression of Inflammatory Features in T Helper 17 Cells from Multiple Sclerosis Patients. Cells. 2019 Jun 4;8(6):533.

4. Volpe E, Cesari E, Mercatelli N, Cicconi R, De Bardi M, **Capone A**, Bonvissuto D, Fraziano M, Mattei M, Battistini L, Paronetto MP, Sette C.
   The RNA binding protein Sam68 controls T helper 1 differentiation and anti-mycobacterial response through modulation of miR-29. Cell Death Differ. 2019 Jun;26(6):1169-1180.

5. **Capone A**, Volpe E.
   Transcriptional Regulators of T Helper 17 Cell Differentiation in Health and Autoimmune Diseases. Front Immunol. 2020 Mar 12;11:348.

6. Donninelli G, Saraf-Sinik I, Mazziotti V, **Capone A**, Grasso MG, Battistini L, Reynolds R, Magliozzi R, Volpe E.
   Interleukin-9 regulates macrophage activation in the progressive multiple sclerosis brain. J Neuroinflammation. 2020 May 6;17(1):149.

7. Terracciano F, **Capone A**, Montori A, Rinzivillo M, Partelli S, Panzuto F, Pilozzi E, Arcidiacono PG, Sette C, Capurso G.
   MYC up-regulation confers resistance to everolimus and establishes vulnerability to cyclin dependent kinase inhibitors in pancreatic neuroendocrine neoplasms cells. Neuroendocrinology. 2020 Jul 2.

8. Panzeri V, Manni I, **Capone A**, Sacconi A, Di Agostino S, Pilozzi E, Piaggio G, Capurso G, Sette C. The RNA binding protein MEX3A is a prognostic factor and regulator of resistance to gemcitabinein Pancreatic Ductal Adenocarcinoma. Mol Oncol. 2020 Nov 7.

9. **Capone A**, and Volpe E. Identification and purification of human memory T helper cells from peripheral blood. In Annunziato F, Maggi L, Mazzoni A (Eds.)
   T- Helper Cells: Methods and Protocols; Methods Mol. Biol. 2021.

10. **Capone A**, Naro C, Bianco M, De Bardi M, Noël F, Macchi P, Battistini L, Soumelis V, Volpe E[*], and Sette C[*].
    Systems analysis of human Th17 cell differentiation uncovers distinct time-regulated transcriptional modules. (Under revision)

5.3. Conference oral presentations

- Giornata Romana di Immunologia (GRI) 2019 - July 12h, 2019, Rome, Italy
  Alessia Capone - New regulators of Th17 cell differentiation.

5.4. Conference communications

- Poster – "1st BraYn Congress" (Brainstorming Research Assembly for Young Neuroscientists) Congress 2018" - June 29h-30th, 2018, Genoa, Italy **Capone A**, Bianco M, Naro C, De Bardi M, Battistini L, Sette C and Volpe E - Cell activation, death and motility of human Th17 cells are finely regulated by specific transcription factors.

- Poster - "14th ISNI Congress" - August 27th-31th, 2018, Brisbane, Australia **Capone A**, Bianco M, Naro C, De Bardi M, Battistini L, Sette C and Volpe E - Cell Activation, Death And Motility Of Human Th17 Cells Are Finely Regulated By Specific Transcription Factors.

- Poster - "AISM Congress" - May 29th-31th, 2019, Rome, Italy **Capone A**, Bianco M, Naro C, De Bardi M, Battistini L, Volpe E and Sette C. Role of IL-1β in the modulation of the pathogenic response of human Th17 cells in multiple sclerosis.

5.5. Awards and grants

- Winner of Erasmus+ Unipharma Fellowship 2017/2018

- Travel grant winner for attending the 14th ISNI Congress (August 27th-31th, 2018, Brisbane, Australia

5.6. Stages

- Internship at Curie Institute / Saint Louis hospital, Paris, France
  Integrative biology of human dendritic cells and T cells laboratory/ Cancer and Immunology unit (Prof. Vassili Soumelis)
  ERASMUS+ Programme Unipharma-Graduates 2017/2018

5.7. Additional information

- Bibliographic Databases, Catalogs, Open Access - Dr. Maria Squarcione - 2018, Rome, Italy

- Regulation (EU) 2016/679 course – IRCSS Santa Lucia Foundation, Rome, Italy, 2019

- Qualification as Profession Biologist, Tuscia University, Viterbo, Italy –2020.