Systems analysis of human T helper17 cell differentiation uncovers distinct time-regulated transcriptional modules

Highlights

Human Th17 cells are driven by early, late, and persistent transcriptional modules.

Human Th17 cells express IL17A, IL17F, IL26, IL6, IFNG, IFNK, LTA, IL1A, and PDGFA.

RORC regulates the IL17A, IL17F, IFNG, PDGFA, and IL1A expression in human Th17 cells.

HOPX and SOX2 contribute to the expression of IFNG by human Th17 cells.

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Systems analysis of human T helper17 cell differentiation uncovers distinct time-regulated transcriptional modules

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SUMMARY

T helper (Th) 17 cells protect from infections and are pathogenic in autoimmunity. While human Th17 cell differentiation has been defined, the global and stepwise transcriptional changes accompanying this process remain uncharacterized. Herein, by performing transcriptome analysis of human Th17 cells, we uncovered three time-regulated modules: early, involving exclusively “signaling pathways” genes; late, characterized by response to infections; and persistent, involving effector immune functions. To assign them an inflammatory or regulatory potential, we compared Th17 cells differentiated in presence or absence of interleukin (IL)-1β, respectively. Most inflammatory genes belong to the persistent module, whereas regulatory genes are lately or persistently induced. Among inflammatory genes, we identified the effector molecules IL17A, IL17F, IL26, IL6, interferon (IFN)G, IFNK, LTA, IL1A, platelet-derived growth factor (PDGF) A and the transcriptional regulators homeodomain-only protein homeobox (HOPX) and sex-determining-region-Y-box (SOX)2, whose expression was independently validated. This study provides an integrative representation of the stepwise human Th17 differentiation program and offers new perspectives toward therapeutic targeting of Th17-related autoimmune diseases.

INTRODUCTION

T helper (Th) 17 cells are mainly characterized by production of interleukin (IL)-17A and IL-17F and play a key role in the immune response against fungi and extracellular pathogens (Liang et al., 2007). The production of Th17-specific cytokines is regulated by a unique genetic program orchestrated by transcription factors, where the retinoic-acid-receptor-related orphan receptor (ROR)γt is a key regulatory factor in both human and mouse Th17 cells (Ivanov et al., 2006; Volpe et al., 2008; Manel 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 et al., 2006; Volpe et al., 2008; Manel et al., 2008; Capone and Volpe, 2020; Ciofani et al., 2012; Mukasa et al., 2010; Kanno et al., 2012), the discovery of typical effector cytokines regulating their functions (Annuzzio et al., 2007; Volpe et al., 2009), and the involvement of Th17 cells in pathological conditions (Volpe et al., 2015; Maddur et al., 2012). 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, such as the sequence of molecular events occurring during the differentiation process and the identification of features that synergize with IL-17 and that contribute to the inflammatory properties of these cells, are still open. 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.

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

To exhaustively investigate the differentiation process of human Th17 cells, we performed a genome-wide transcriptome analysis of naïve CD4+ T cells (Figure S1) differentiated under Th17 conditions, IL-1β, IL-6, transforming growth factor (TGF)-β, and IL-23, compared with 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 induced at 48 hours (h) of culture under Th17 conditions compared with the Th0 condition (Figure 1A), suggesting that the polarization program started at 48 h. However, maximal RORC induction required 5 days of culturing, indicating acquisition of complete Th17 cell polarization at this later time point (Figure 1A). On this basis, we selected 48 h and 5 days as early and late stages, respectively, of the human Th17 polarization program (Figure 1B). To limit donor-associated variability, we performed high-throughput RNA-sequencing (RNA-seq) analyses of Th0 and Th17 cells obtained from 5 independent healthy donors (HD), at 48 h and 5 days of differentiation.

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 value (p) ≤ 0.05 as filters, we identified 1130 genes differentially expressed between Th17 and Th0 cells at 48 h and 4589 genes at 5 days (Figure 1C; Tables S1 and S2). To rule out that the differential gene expression was related to the presence of mixed subpopulations with varying degrees of activation, we evaluated the cell size and the CD25 expression in Th0 and Th17 populations (Figure S2), indicating that the differential gene expression analysis was not affected by the presence of mixed subpopulations. Next, RNA-seq and bioinformatics analyses were verified by real-time quantitative polymerase chain reaction (q-PCR) on independent sets of human Th0 and Th17 cells. We found that 18 of 22 tested genes (81.8% of validation) were validated by q-PCR analysis (Figure S3A). In particular, the expression of Th17 signature genes (IL17A, IL17F, IL23R, IL26, RORA, RORC) mirrored the RNA-seq results (Figure S3B). Furthermore, analyses of other arbitrarily selected genes at either 48 h and 5 days of differentiation confirmed the reliability of the RNA-seq data (Figures S3C and S3D).

To identify genes relevant for the acquisition of specific Th17 features at the early and late time points of differentiation, we compared three datasets: Th17 versus Th0 at 48 h (early Th17 genes), Th17 versus Th0 at 5 days (late Th17 genes), and Th17 at 5 days versus Th17 at 48 h (genes that vary over time in Th17 cells). In particular, we identified the genes upregulated or downregulated in each pair of conditions. Overlap between these comparisons identified three modules that we named A, B, and C (Figure 1D). Interestingly, while in module A and B, the percentage of genes upregulated or downregulated was similar (Figures S4A and S4B), in module C, most genes were upregulated in Th17 versus Th0 conditions (Figure S4C). Moreover, module B (persistent) included genes that were constantly modulated during Th17 cell differentiation, such as the Th17 signature genes IL23R and C-C motif chemokine ligand (CCL)20 (Figure 1E). Module B (persistent) included genes that were constantly modulated during Th17 cell differentiation, such as the Th17 signature genes IL23R and C-C motif chemokine ligand (CCL)20 (Figure 1E). These genes were enriched in functional categories involved in “T cell differentiation/activation,” “cytokine production,” and “Janus kinase/signal transducer and activator of transcription (STAT) signaling,” which are all relevant for Th17 cell biology (Figure 1F). Module C (late) included genes of terminal differentiation, such as the Th17 effector cytokine genes IL26 and IL8 (Figure 1E), and it was enriched in functional categories of strong relevance for the effector functions of Th17 cells, such as “response to other organisms” and “cell motility” (Figure 1F).

Th17 cell differentiation and functions are finely regulated by the expression of specific transcriptional regulators. To study the timing of expression of transcription factors that are known to play a key role in Th17 biology (Capone and Volpe, 2020), we explored their presence in the early (A), persistent (B), and late (C)
Figure 1. Early, persistent, and late genes acquired during human Th17 cell polarization reflect specific functions

Naive CD4⁺ T cells cultured with anti-CD3/anti-CD28 alone (Th0) or with the addition of TGF-β, IL-6, IL-1β, and IL-23 (Th17) (n = 5) were analyzed by q-PCR for the expression of RORC transcript (A). RNA sequencing of Th17 and Th0 cells (n = 5) at 48 h and 5 days of differentiation (B): number of genes differentially modulated in Th17 versus Th0 at each time point (C); Venn’s diagram shows the number of genes specifically modulated (either upregulated or downregulated) in Th17 profile (Th0 versus Th17) at 48 h (early) and 5 days (late) and persistently modulated (48 h Th17 versus 5 days Th17) (D); expression of selected Th genes included in A, B, and C modules reported as DEseq2 normalized count (E); bar graph representation of gene ontology for genes in A, B, and C modules (F); expression of selected Th17 transcription factors (G and H). Each replicate, including Th0_48h, Th17_48h, Th0_5d, Th17_5d, was performed in the same donor (paired cultures). Data are represented as mean ± SEM (Student’s T-tests; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001).
modules. We found that RORC, RORA, Runt-related transcription factor (RUNX1), basic leucine zipper ATF-like transcription factor (BATF), and interferon (IFN) regulatory factor (IRF)4 genes belonged to the persistent module and were constantly upregulated with respect to Th0 cells (Figure 1G), whereas Kruppel-like factor (KLF)4, hypoxia-inducible factor (HIF)1A, and STAT3 were upregulated only at later time in Th17 cells (C module; Figure 1H). Interestingly, none of these transcription factors was upregulated transiently only 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.

Identification of a specific inflammatory Th17 effector program
IL-1β is crucially involved in the pathology of Th17-related diseases (Chung et al., 2009; Sutton et al., 2006), whereas Th17 cells differentiated in the absence of IL-1β (IL-6, IL-23 and TGF-β) produce the anti-inflammatory IL-10 cytokine, which is generally produced by T regulatory cells (Treg) (Volpe et al., 2008; Zielinski et al., 2012). Thus, to delineate the inflammatory or regulatory potential of Th17 genes, we compared the transcriptome of T cells differentiated in presence of IL-1β, IL-6, IL-23, and TGF-β (“inflammatory” Th17 condition) or in presence of IL-6, IL-23, and TGF-β (“regulatory” Th17 condition). Principal component analysis (PCA) of RNA-seq data obtained from 5 independent donors revealed that absence or presence of IL-1β did not segregate samples at 48 h (Figure 2A). In contrast, at 5 days, inflammatory and regulatory Th17 cells segregate as two distinct profiles (Figure 2A). Importantly, the differential gene expression observed between inflammatory and regulatory Th17 cells was not related to a differential activation state (Figure S5). In line with the PCA profile, only 45 genes were differentially modulated in the absence of IL-1β at 48 h, whereas more than 800 genes were affected at 5 days (Figure 2B; Tables S3 and S4).

T cells differentiated under regulatory Th17 conditions 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, such as RORA and IL23R (Figure S6). This analysis allowed defining an inflammatory Th17 program (upregulated in presence of IL-1β) and a regulatory Th17 program (downregulated in presence of IL-1β). We found that most of IL-1β-regulated genes showed increased expression at 48 h under the inflammatory condition. This observation suggested that their upregulation may affect subsequent steps in Th17 polarization and cause the broader gene expression differences seen at 5 days (Figure 2B). Moreover, most part of the inflammatory program belongs to the persistent module B at 48 h (34.2%, Figure 2B) and to the late module C at 5 days (48.5%, Figure 2B). In contrast, few regulatory genes are induced at 48 h, while at 5 days, an equal number of genes are classified in persistent and late modules (Figure 2B). These results indicate that genes lately or persistently expressed during human Th17 cell differentiation are crucial for discriminating the commitment toward the inflammatory or regulatory profile of these cells.

Several transcriptomic studies have highlighted the molecular signature of inflammatory mouse (Lee et al., 2012; Gaublomme et al., 2015; Ghoreschi et al., 2010; 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 Th11/17 cells. The analysis of genes associated with Th17 phenotype in these previous studies (Table S5) indicated that some of them, including IL17A, IFNG, T-box transcription factor 21 (TBX21), Epstein-Barr Virus-Induced 3 (EBI3), IRF8, tumor necrosis factor (TNF) receptor superfamily (RSF) 9, TNFRSF14, CCL5, CD40 ligand, BATF, and TNF (11 of 76 genes [14.5%]) were upregulated in inflammatory human Th17 cells at 5 days of differentiation (Figure S7), 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 analyzed in more detail the genes differentially expressed in inflammatory versus
regulatory Th17 conditions at 5 days. Gene ontology clustering revealed that IL-1β caused a significant up-regulation of functional categories related to “signaling pathways,” “cytokine production,” and “response to other organisms,” which are all relevant for the effector functions of Th17 cells (Figure 2C). Moreover, genes involved in general cell functions, such as cell death, adhesion, and motility, were also upregulated. By contrast, genes downregulated under inflammatory conditions did not cluster in functional categories that are specific for Th17 cells (Figure 2C). Interestingly, top-ranking genes upregulated in Th17 versus Th0 (Figure 2D) and in the presence of IL-1β compared with the absence of IL-1β (Figure 2E) encode Th17-specific (IL17A, IL17F, and IL26) and nonspecific (IL6, IFNG, and IL1A) inflammatory cytokines, and other soluble proteins associated to immune functions, including IFNK, platelet-derived growth factor (PDGF)A, and LTA, may potentially alter functional features of human Th17 cells.

Next, we explored whether the expression of transcription factors driving the Th17-specific signature was modulated in inflammatory versus regulatory Th17 condition. The expression of BATF, which was described as an early determinant of the mouse Th17 cell signature (Ciofani et al., 2012), was slightly upregulated in the inflammatory human 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 (Figures 2F and 2G). These results suggest that polarization of human inflammatory Th17 cells might rely on additional transcriptional regulators.

**Experimental validation and characterization of homeodomain-only protein homeobox and sex-determining-region-Y-box 2 in 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 persistently upregulated in Th17 cells differentiated in the presence of IL-1β (inflammatory Th17) compared with those differentiated in the absence of IL-1β (regulatory Th17). Group Y comprised genes upregulated in the presence of IL-1β only at 5 days (late regulation), whereas group X included genes upregulated at both 48 h and 5 days (persistent regulation) (Figure 3A). Next, we further selected genes that were significantly induced in Th17 versus Th0 conditions (Figure S8) and focused our attention on two genes encoding transcriptional regulators: homeodomain-only protein homeobox (HOPX) (group X, persistent regulation) and sex-determining-region-Y-box 2 (SOX2) (group Y, late regulation) (Figure 3B).

HOPX is a transcriptional cofactor that was previously described to be involved in murine Th1 and Treg cell functions (Albrecht et al., 2010; Hawiger et al., 2010). RNA-seq and q-PCR analyses showed progressive and persistent upregulation of HOPX expression during Th17 cell differentiation and confirmed its dependency on IL-1β signaling (Figure 3C). Western blot analysis at 5 days of differentiation confirmed these results at the protein level (Figure 3D). Comparative analysis of human naive T cells differentiated into different Th or Treg subsets indicated that Th17 cells expressed the highest level of HOPX, followed by Th1 cells, whereas expression of this transcriptional regulator was barely detectable in the other subsets (Figure 3E). The specific expression of HOPX in human Th17 cells was even more evident at the protein level (Figure 3F), further suggesting its functional implication in these cells.

SOX2 is a transcription factor involved in stemness, a feature associated with the Th17 phenotype (Muranski et al., 2011; Kryczek et al., 2011). Interestingly, in silico analyses of chromatin immunoprecipitation-sequencing 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 S9). We confirmed that SOX2 expression in Th17 cells was dependent on IL-1β signalling by q-PCR and Western blot analyses (Figures 3C and 3D). Furthermore, comparative analyses of human T cell subsets revealed that SOX2 transcript and protein were highly specific of the Th17 profile (Figures 3G and 3H).

**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 human naive CD4+ T cells undergoing Th17 differentiation (Figures 4B and 4C; Figure S10A) without altering the viability and proliferation potential of differentiating T cells (Figures S10B and S10C). Next, we analyzed 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 4A). As expected, immunoassays by enzyme-linked immunosorbent assay revealed that knockdown of RORC significantly reduced the IL-17A and IL-17F protein expression by polarized
Figure 3. HOPX and SOX2 are specifically expressed in human Th17 cells

Naive CD4+ T cells cultured with anti-CD3/anti-CD28 alone (Th0) or with the addition of TGF-β, IL-6, IL-1β, and IL-23 (infl. Th17) or in the absence of IL-1β (reg. Th17) were analyzed by RNA sequencing. The Venn’s diagram between genes upregulated in inflammatory Th17 versus regulatory Th17 at 48 h and 5 days unveils 18 genes regulated at both time points (group X) and 421 genes specifically upregulated at 5 days (group Y) (A). All the annotated genes included in group X and 10 of 421 genes in group Y are listed in panel (B) HOPX and SOX2 expression obtained by RNA sequencing (reported as DEseq2 normalized count) (n = 5) and by q-PCR (n = 4) (C) at 48 h and 5 days and by Western blot (n = 5) at 5 days (D) were analyzed in Th0, inflammatory Th17 (TGF-β, IL-6, IL-1β and IL-23), and regulatory Th17 (TGF-β, IL-6, and IL-23) cells. Expression of HOPX transcript (n = 5) (E) and protein (n = 4) (F), SOX2 transcript (n = 6) (G) and protein (n = 4) (H) in Th0, Th1, Th2, Th17, and iTreg profiles differentiated in vitro from naive CD4+ T cells of HD. Data are represented as mean ± SEM (For pairwise conditions: Student’s T-tests; For three or more conditions: One-way ANOVA test; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001). Th1: IL-12; Th2: IL-4; Th17: IL-1β, IL-6, TGF-β, and IL-23; iTreg: IL-2 and TGF-β; Th0: no cytokines. Inflammatory Th17 = infl. Th17; regulatory Th17 = reg. Th17.
Figure 4. HOPX and SOX2 contribute to the expression of Th17 cytokines

Expression levels of RORC (A), HOPX (B,F), and SOX2 (C,F) transcripts; IL-17A, IL-17F, IFN-γ, PDGF-AA, PDGF-AB/BB, LT-α proteins (D), and IL26, IL6, IL1A, IFNK transcripts (E), were analyzed by q-PCR (transcript) or ELISA and Luminex (protein) at 5 days of cell differentiation in Th17 cells (TGF-β, IL-6, IL-1β and IL-23) treated with RORC (siRORC; n = 4), HOPX (siHOPX; n = 8), SOX2 (siSOX2; n = 6) siRNA. Each set was compared with Th17 cells treated with non targeting siRNA (siNT) (n = 4 for RORC experiments, n = 8 for HOPX experiments, n = 6 for SOX2 experiments). Transcriptional results were normalized on RPL34 expression. All data are presented as fold versus siNT. Data are represented as mean ± SEM. (Student’s T-tests; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).
Th17 cells (Figure 4D). RORC silencing also modulated the production of IFN-γ, PDGF-AA, and PDGF-AA/BB (Figure 4D), as well as IL1A mRNA expression (Figure 4E), by human Th17 cells. By contrast, knockdown of RORC did not interfere with the expression of LT-α (Figure 4D), IFNK, IL6, and IL26 (Figure 4E).

Interestingly, silencing of HOPX (Figure 4B) also impaired the production of IL-17A and IFN-γ (Figure 4D), albeit at lower levels than RORC, whereas the expression of other soluble factors (LT-α, IL-17F, PDGF-AA, PDGF-AA/BB, IL26, IL6, IL1A, IFNK) was not affected (Figures 4D and 4E). On the other hand, knockdown of SOX2 expression in human polarized Th17 cells (Figure 4C) specifically modulated IFN-γ and PDGF-AA production (Figure 4D). The common regulation of IL-17A and IFN-γ by RORyt and HOPX and PDGF-AA and of IFN-γ by RORyt and SOX2 lead us to hypothesize the involvement of RORyt in regulating HOPX and SOX2 expression. Indeed, silencing of RORC in human Th17 cells reduced the expression of both HOPX and SOX2 (Figure 4F). These results suggest that the transcriptional regulators HOPX and SOX2 act downstream of RORyt signaling in human Th17 cells.

**HOPX and SOX2 contribute to the regulation of Th1/17 cell features**

T cells that concomitantly produce IL-17A and IFN-γ, named Th1/17 cells, play a pathogenic role in autoimmune diseases (Kebrir et al., 2009; Reinert-Hartwall et al., 2015). Because HOPX and SOX2 contribute to regulate IFN-γ and IL-17A production by human Th17 cells, we next investigated their role in memory Th17 cells. To this end, peripheral memory CD4+ T cells were silenced for HOPX or SOX2, and their features were analyzed by flow cytometry. In line with the effect observed in Th17 cells polarized in vitro, HOPX silencing in peripheral memory CD4+ T cells (Figure 5A; Figure S11) leads to a minimal but significant reduction of the IL-17A expression (Figure 5B). This result indicates that HOPX contributes to the optimal production of IL-17A also in human memory Th17 cells. On the other hand, as expected from in vitro data, silencing of SOX2 in these cells did not affect IL-17A production (Figures 5C and 5D). 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+) in total CD4+ T cells (Figure 5E). Furthermore, in line with the effect on IFN-γ production observed in vitro, HOPX depletion caused a reduced frequency of circulating Th17 cells, which were identified as CXCR3+, CD161+, and CCR6+ (Figure 5E).

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+, and CCR6+ cells) (Figure 5F).

These results indicate that HOPX and SOX2 are two transcriptional regulators that contribute to insuring the optimal 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.

**DISCUSSION**

To our knowledge, this is the first transcriptomic study of human Th17 cells that proposes a system-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; Aijo 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; Aijo et al., 2014; Tripathi et al., 2017), which plays a crucial role in human Th17 cell differentiation (Volpe et al., 2008; Manel 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) (Volpe et al., 2008; Manel 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 5 days in human Th17 cells, unlike mouse cells where optimal differentiation is achieved in 3 days (Gaublomme et al., 2015; Veldhoen et al., 2004). 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 48 h, the complete segregation between Th17 cells differentiated under inflammatory or regulatory culture conditions is
obtained only after 5 days. 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. Because naive CD4+ T cells do not express IL-1β and IL-23 receptors, the timing requested for their upregulation may contribute to the delay. However, also in murine Th17 cells, IL-6 and TGF-β are the early triggers of differentiation, whereas IL-23, IL-1β, and IL-21 are involved in the late stabilization and amplification steps (Veldhoen et al., 2006; Bettelli et al., 2008).

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 with signaling 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 enriched in pathways associated to Th17 effector functions, such as response to bacteria and other organisms, which 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. 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 other effector molecules, such as LTA, PDGFA, IL1A, IFNK, and LTA, encoding for LT-α and formerly known as TNF-β, are 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 patients with multiple sclerosis produce higher amount of LT-α than those from HD (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-1 receptor 1 and activating the downstream signaling pathway (Kurt-Jones et al., 1985; Kaplanski et al., 1994; Di Paolo and Shayakhmetov, 2016; Dinarello, 2018). Consistent 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 PDGF-AB/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 IFN family of cytokines, which play an important role in host defense against viral infections (Nardelli et al., 2002). Studies concerning the role of Th17 cells in the defense against viruses have generated conflicting results (Ma et al., 2019). Thus, further investigation of the expression and function of type I IFN by Th17 cells could highlight new antiviral mechanisms of these cells.

Collectively, our study revealed a partial overlap between mouse and human Th17 genes, in line with the partial overlap observed for selected mouse and human Th17 proteins (Tripathi et al., 2019). Moreover, transcriptional regulators previously indicated as relevant for mouse Th17 cells, such as RORC, RORA, HIF1A, BATF, IRF4, STAT3, RUNX1, and KLF4, 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 human Th17 profile. In this context, we identified HOPX and SOX2 as transcriptional regulators that act downstream to RORγt signaling and are involved in the differentiation program of inflammatory Th17 cells. 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). Moreover, HOPX was 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 proapoptotic and antiapoptotic 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 the 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, such as 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 HD and patients with multiple sclerosis (Capone et al.,
PDGF-AA binds to PDGF receptor A, which is considered a 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 contribute to regulate the production of IFN-γ by invitro-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 (Nistala et al., 2010; Kebir et al., 2009; Reinert-Hartwall et al., 2015; Annunziato et al., 2007). 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), is significantly reduced in memory CD4+ T cells depleted of HOPX or SOX2. These findings suggest that HOPX and SOX2 participate to regulate the gene expression underlying the acquisition of an inflammatory phenotype by human Th17 cells.

In conclusion, our study 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 help elucidate and/or predict the outcome of specific therapeutic intervention against Th17 cells.

Limitations of the study
This study identified key molecules expressed by human Th17 cells and discovered that RORC, HOPX, and SOX2 are involved in the regulation of those molecules. The current data are related to invitro-differentiated Th17 cells. However, the analysis of those mechanisms in invivo-differentiated IL-17-producing cells could have provided further insight into the Th17 cell biology. Follow-up studies in this direction are required to transfer these findings in clinical research.

Ethical aspects
The usage of blood of unknown donors was approved by the Ethics Committee of the San Camillo Hospital, Rome (Italy).

Resource availability
Lead contact
Information and requests for resources should be directed to and will be fulfilled by the lead contact, Elisabetta Volpe (e.volpe@hsantalucia.it).

Materials availability
Materials used or generated in this study will be available upon reasonable request, and a material transfer agreement may be required.

Data and code availability
The RNAseq data from this study is available to the Gene Expression Omnibus (GEO) with identifier GSE172317.

METHODS
All methods can be found in the accompanying transparent methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102492.

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AUTHOR CONTRIBUTIONS
A.C. designed research, performed research, analyzed data, and drafted the paper; M.B., C.N., M.D.B., F.N. performed research and analyzed data; P.M. contributed to data analysis; L.B., V.S. contributed to research design, writing and revising manuscript; C.S., E.V. designed research, analyzed data, and wrote the article.

DECLARATION OF INTERESTS
The authors have no competing interests.

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Supplemental information

Systems analysis of human T helper17 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
Figure S1. Gating strategy for the purification of naïve CD4$^+$ T cells, related to Fig.1
Blood CD4$^+$ T lymphocytes were labeled with specific antibodies conjugated with a fluorochrome and, by using a cell sorter, naïve CD4$^+$ T cells were isolated as CD4$^{\text{high}}$, CD45RA$^{\text{high}}$, 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.
Figure S2. Activation state of Th cells at 48h and 5d of differentiation, related to Fig.1
The median fluorescence intensity (MFI) of forward side scatter (FSC) (A) and CD25 (B) in Th0 and Th17 cells, was measured by flow cytometry during the differentiation process. The graphs show data from a representative experiment.
Figure S3. Validation of arbitrarily selected genes differentially expressed, related to Fig.1
Number and percentage of genes obtained by RNA-seq (reported as DEseq2 normalized count) 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). Data are represented as mean +/- SEM. (Student’s T-tests, * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001)
Figure S4. Type of regulation of intersecting genes defined as module A,B,C, related to Fig.1
Number and percentage of genes up-regulated and down-regulated in Th17 vs Th0 at each time point of analysis, in early (A), persistent (B), and late (C) modules.
Figure S5. Activation state of Th cells at 48h and 5d of differentiation, related to Fig.2
The median fluorescence intensity (MFI) of forward side scatter (FCS) (A) and CD25 (B) in inflammatory Th17 (infl. Th17) and regulatory Th17 (reg. Th17) cells, was measured by flow cytometry during the differentiation process. The graphs show data from a representative experiment.
Figure S6. Signature genes of Th17 cells are partially reduced in regulatory Th17 cells, related to Fig. 2
Expression of Th17 signature genes at 5d, obtained by RNA-seq (reported as DEseq2 normalized count) and q-PCR analysis in inflammatory Th17 (infl. Th17) and regulatory Th17 (reg. Th17) cells (n=5). Data are represented as mean +/- SEM (Student’s T-tests; * p ≤0.05; ** p ≤0.01; **** p ≤0.0001).
Figure S7. Validation of murine pathogenic Th17 genes in human Th17 cells, related to Fig.2
Pathogenic genes of murine Th17 cells (in bold) significantly up-regulated in human inflammatory Th17 cells (infl. Th17) vs regulatory Th17 cells (reg. Th17), analysed by RNA-sequencing (n=5). Bars represent fold change of DEseq2 normalized count. (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001).
Figure S8. Selection of genes expressed by inflammatory Th17 cells and specific of Th17 cells, related to Fig.3

Venn’s diagram of genes up-regulated in inflammatory Th17 (infl. Th17) vs Th0 at 48h or 5d with genes of group X (defined in Figure 3A) unveils nine genes specific of inflammatory Th17 cells, which are up-regulated in inflammatory Th17 vs regulatory 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 (defined in Figure 3A) unveils 328 genes specific of Th17 cells, which are up-regulated in inflammatory Th17 cells vs regulatory Th17 cells (B).
Figure S9. SOX2 is a potential transcription factors of human Th17 polarization, related to Fig. 3
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.
Figure S10. Transfection of *in-vitro* polarized Th17 cells with siRNA, related to Fig.4

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). Data are represented as mean +/- SEM. (Student’s T-tests).
Figure S11. Transfection of memory CD4\(^+\) T cells with siRNA, related to Fig.5
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 table 5. Overview of pathogenic gene signature of murine Th17 cells, related to Fig.2

| 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             | TBX21                  | TBX21          | EGR2           | IL17RA           |
| CXCR3                  | GZMB             | IL2                    | IL2            | IL17RA         | IL17RA           |
| IL33                   | CCL5             | CXCR3                  | CXCR3          | GZMB           | IRF4             |
| CCL9                   | CSF2             | CCL4                   | CCL4           | CCL5           | CCR6             |
| (CCL23 in human)       | STAT4            | STAT4                  | GZMB           | PROCR          | IRF8             |
| IL22                   | IL22             | IL22                   | IL22           | IRF8           | NOTCH1           |
| IL3                    | CCR6             | CCR6                   | IL3            | MINA           | MINA             |
| LRMP                   | CCL20            | RORC                   | IL3            | EGR1           | EGR1             |
| CASP1                  | CTLA4            | IL3                    | IL3            | ZEB1           | ZEB1             |
| LGALS3                 | IL17A            | IFNG                   | IFNG           | NFKB1          | NFKB1            |
| LAG3                   | CD40LG           | EPSTI1                 | RSG2           | PML            | PML              |
| CXCL3                  | TNF              | RSG2                   | TGFBI          | SMARCA4        | SMARCA4          |
| IL1R                   | IL21             | IL23R                  | NFATC3         | POU2AF1        | POU2AF1          |
| IL7R                   | TNFRSF9          | TGFB1                  | IRF8           | RBPJ           | RBPJ             |
| EBI3                   | MINA             | NFATC3                 | FASLG          | II21R          | II21R            |
| IRAK1                  | IRAK1            | FASLG                  | AHII           | MYC            | MYC              |
| IL1RN                  | MINA             | AHII                   | HSPH1          | CCR5           | CCR5             |
|                       |                  |                        | EGR2           | ETV6           | ETV6             |
|                       |                  |                        | TNFSF14        | FAS            | FAS              |
|                       |                  |                        | DUSP5          | IL12Rb1        | IL12Rb1          |
|                       |                  |                        | NFIL3          |                |                  |
|                       |                  |                        | CBLB           |                |                  |
|                       |                  |                        | LDHA           |                |                  |
|                       |                  |                        | SOCS3          |                |                  |
|                       |                  |                        | REL            |                |                  |
|                       |                  |                        | NOTCH1         |                |                  |
|                       |                  |                        | EGR3           |                |                  |
|                       |                  |                        | MALT1          |                |                  |
Transparent methods

RESOURCES AVAILABILITY

Lead contact:

Further information and requests for resources and reagents should be directed to and will be
fulfilled by the lead contact, Elisabetta Volpe (e.volpe@hsantalucia.it).

Materials availability:

This study did not generate new unique reagents.

Data and code availability

The accession number for the RNA-seq data reported in this paper is GEO: GSE172317

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethical statement

All procedures performed in studies involving human participants were in accordance with the
ethical standards of the institutional and/or national research committee and with the 1964 Helsinki
Declaration, with San Camillo Hospital.

METHOD DETAILS

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). Human blood was obtained by an ethically approved convention, in accordance with the Declaration of Helsinki, with San Camillo Hospital. 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<sup>high</sup>, CD45RA<sup>high</sup>, CD45RO<sup>−</sup> and CD27<sup>+</sup>. Sorted cells had a purity of over 97%, as shown by flow cytometry (Fig. S1).

**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) (Volpe et al., 2008): interleukin (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), Transforming growth factor (TGF)-β (1ng/ml), and IL-23 (100 ng/ml) for Th17 profile; IL-2 (10 ng/ml) and TGF-β (2ng/ml) for T regulatory (Treg) cells; Th0 profile was obtained culturing cells in absence of cytokines. The cells were incubated and after 48 hours (h) or 5 days (d) 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 CO₂).

**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 1ng/ml, IL-23 100 ng/ml) (Miltenyi
Biotec) (Volpe et al., 2008), and the retinoic acid-receptor (RAR)-related orphan receptor (ROR)C, homeodomain-only protein homeobox (HOP)X, SRY (sex determining region Y)-box 2 (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 CO₂.

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). Interferon (IFN)-γ, platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB, and lymphotoxin (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 C-X-C chemokine receptor (CXCR)3 Alexa 647 (1:100) (Biolegend), anti-human Chemoattractant Receptor-homologous Molecule Expressed On Th2 Cells (CRTh2) PE (1:150) (Miltenyi), anti-human CD161 PE-Dazzle 594 (1:50) (Biolegend), anti-human C-C Chemokine Receptor (CCR)6 PC7 (1:80) (Biolegend), CD25 BV421 (1:60) (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 (q-PCR)**

The q-PCR was performed using the LightCycler® 480 (Roche) with LightCycler® 480 SYBR Green 1 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. 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: 63°C (HOPX: forward CAACAAGGTCGACAAGCACC; reverse CGCTGCTTTAAACCATTTCTGG), 54°C (IL17F: forward GAAAACCAGCGCGTTTCCAT; reverse CTGCATCCTGAAACAAAGGTCA), 58°C (BTLA: forward CTGACACAGCAGGAAAGGAAAT; reverse CTGCATCCTGAAACAAAGGTCA), 58°C (MTTS1: forward GACTCGCTTCAGGTCCACACAG; reverse TCATAATCTGAAACTTGGGAAGGG), 58°C (ANXA1: forward GGTGAGCCCCTATCCTACCT; reverse CTGGTGGACCTGTGCATTGT), 58°C (ITM2A: forward AACTGCTATCTGATGCCCT; reverse CATCACGAATTTCCTCCACAGC), 54°C (LGALS1: forward CTCGGGTGGAGTCTTCTGAC; reverse GAAGGCAXCTCAGGAGGAGACCA), 63°C (LINC00877: forward CGAGAAGGAAAGGAGGAGGAGGACCA), 56°C (TSPAN13: forward GCCCTCAACCTGCTTTACAC; reverse ACAGCTCCTCATCAGACCCAC), 54°C
(FGFBP2: forward CCGAGGCTGACAGGTGAAAG; reverse CGTTGGATTGAAAGCGGCAT), 63°C
(IL6: forward CTGCCAGAAAACAACCTGAACC; reverse TGGCTTGGTTCCTCACTACTCT), 60°C
(RPL34: forward GTCCCGAACCCTGGTAATAGA; reverse GGCCTGCTGACATGTGTTTCTT); 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 ribosomal protein (RP)L34 (Hs00996244_g1). The gene expression was normalized to the expression of RPL34 transcript.

RNA-sequencing (RNA-seq) analysis

Stranded total RNA libraries were sequenced on Illumina HiSeq 2000 flowcell v3 – PE100, producing 200-300 million of uniquely mapped paired-end reads/sample, which allows reliable detection of low-expressed genes. Sequencing, data quality, reads repartition (e.g., for potential ribosomal contamination), and insert size estimation were performed using FastQC, Picard-Tools, Samtools and rseqc. Reads were mapped using STARv2.4.0 (Dobin, 2013). Gene expression regulation study was performed as already described (Naro C, 2017) using Human FAST DB v2015_1 annotations and DESeq (v.1.16.0 on R v.3.1.3) (Anders S, 2010, Chiara Naro, 2019) for normalization and differential gene expression. Genes were considered as expressed if their rpkm (reads per kilobase of exon per million mapped reads) value was greater than 97.5% of the background rpkm value based on intergenic regions. 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 Na$_3$VO$_4$, 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 et al., 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.

**Gene ontology (GO)**

GO was performed by DAVID Functional annotation cluster Tool (v6.8) (Huang da et al., 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.
QUANTIFICATION AND STATISTICAL ANALYSIS

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 of the mean (s.e.m). The p-values of 0.05 or less, were considered statistically significant.

KEY RESOURCES TABLE

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit monoclonal anti SOX2 (clone D6D9) | Cell Signaling Technology | Cat#: 3579; RRID: AB_2195767 |
| Mouse monoclonal anti HOPX (clone E-1) | Santa Cruz Biotechnology | Cat#: sc-398703; RRID: AB_2687966 |
| Beta-actin Antibody – Purified Mouse Monoclonal Antibody (Mab) | Abgent | Cat#: AM1021B |
| Anti-rabbit IgG, HRP- linked Antibody | Cell Signaling Technology | Cat#: 7076S |
| Anti-mouse IgG, HRP- linked Antibody | Cell Signaling Technology | Cat#: 7074S |
| Mouse monoclonal anti CD4 (clone VIT4) Conjugate FITC | Miltenyi Biotec | Cat#: 130-092-358; RRID: AB_871682 |
| Mouse monoclonal anti CD45RA (clone HI100) Conjugate BV421 | BD Biosciences | Cat#: 562885; RRID: AB_2737864 |
| Mouse monoclonal anti CD45RO (clone UCHL1) Conjugate PE | BD Biosciences | Cat#: 555493; RRID: AB_395884 |
| Mouse monoclonal anti CD27 (clone 1A4CD27) Conjugate APC | Beckman Coulter | Cat#: B09983; |
| Mouse monoclonal anti CXCR3 (clone G025HT) Conjugate Alexa 647 | BioLegend | Cat#: 353711 |
| Mouse monoclonal anti CRTh2 Conjugate PE | Miltenyi Biotec | Cat#: 120-001-608 |
| Biological Samples | | | |
|---|---|---|---|
| Healthy adult blood buffy coats | UOC Ematologia, San Camillo Hospital, Rome, | N/A | |

| Chemicals, Peptides, and Recombinant Proteins | | | |
|---|---|---|---|
| Human IL-6 premium grade | Miltenyi Biotec | Cat#:130-095-352 | |
| Human TGF-beta1 premium grade | Miltenyi Biotec | Cat#:130-095-067 | |
| Human IL-23 premium grade | Miltenyi Biotec | Cat#:130-095-759 | |
| Human IL-1beta premium grade | Miltenyi Biotec | Cat#:130-093-563 | |
| Human IL-12 premium grade | Miltenyi Biotec | Cat#:130-096-704 | |
| Human IL-4 premium grade | Miltenyi Biotec | Cat#:130-093-920 | |
| Human IL-2 premium grade | Miltenyi Biotec | Cat#:130-097-744 | |
| Dynabeads™ Human T-Activator CD3/CD28 | Thermo Fisher Scientific | Cat#:11131D | |
| SuperScript™ II Reverse Transcriptase | Thermo Fisher Scientific | Cat#:18064014 | |
| RNasin Ribonuclease Inhibitors | Promega | Cat#:N2111 | |
| Set of dATP, dCTP, dGTP, dTTP | Promega | Cat#:U1420 | |
| Random Primers | Promega | Cat#:C1181 | |
| Oligo(dT)15 Primer | Promega | Cat#:C1101 | |
| TaqMan Gene Expression Master Mix | Thermo Fisher Scientific | Cat#: 4369016 | |
| LightCycler® 480 SYBR Green I Master | Roche | Cat#: 04887352001 | |
| Nitrocellulose blotting membranes | GE Healthcare Life Science | Cat#: A14493334 | |
| SuperSignal West Pico PLUS Chemiluminescent Substrate | Thermo Fisher Scientific | Cat#: 34580 | |

| Critical Commercial Assays | | | |
|---|---|---|---|
| Anti-FITC MicroBeads antibody | Miltenyi Biotec | Cat#: 130-048-701; RRID: AB_244371 | |
| Product Name                                    | Manufacturer   | Catalog Number                                      |
|------------------------------------------------|----------------|-----------------------------------------------------|
| ReliaPrep RNA Cell - Miniprep System           | Promega        | Cat#: Z6011                                         |
| SMARTpool: Accell HOPX siRNA                   | Dharmacon      | Cat#: FE5E013413000005                              |
| SMARTpool: Accell SOX2 siRNA                   | Dharmacon      | Cat#: FE5E011778000005                              |
| SMARTpool: Accell RORC siRNA                   | Dharmacon      | Cat#: FE5003442010010                                |
| Accell Non-targeting Pool                      | Dharmacon      | Cat#: FE5D0019101020                                |
| Human IL-17A DuoSet ELISA                      | RnD system     | Cat#: DY317-05                                      |
| Human IL-17F DuoSet ELISA                      | RnD system     | Cat#: DY1335B-05                                    |
| Milliplex Map Kit                              | Merck Milliport| N/A                                                 |

**Deposited data**

| RNA-seq data | GEO code: GSE172317 |
|--------------|---------------------|
| ChEA 2016    | Enrichr web site    | N/A

**Oligonucleotides**

| Oligonucleotide                                                                 | Manufacturer                  | Catalog Number |
|-------------------------------------------------------------------------------|------------------------------|----------------|
| SOX2 TaqMan Gene Expression Assay (FAM) Identification number: Hs01053049_s1 | Thermo Fisher Scientific     | Cat#: 4331182  |
| IL17A TaqMan Gene Expression Assay (FAM) Identification number: Hs00174383_m1 | Thermo Fisher Scientific     | Cat#: 4331182  |
| RORC TaqMan Gene Expression Assay (FAM) Identification number: Hs01076112_m1  | Thermo Fisher Scientific     | Cat#: 4331182  |
| RORA TaqMan Gene Expression Assay (FAM) Identification number: Hs00536545_m1  | Thermo Fisher Scientific     | Cat#: 4331182  |
| IL23R TaqMan Gene Expression Assay (FAM) Identification number: Hs 00332759_m1 | Thermo Fisher Scientific     | Cat#: 4331182  |
| IFNK TaqMan Gene Expression Assay (FAM) Identification number: Hs00737883_m1  | Thermo Fisher Scientific     | Cat#: 4331182  |
| IL26 TaqMan Gene Expression Assay (FAM) Identification number: Hs00218189_m1  | Thermo Fisher Scientific     | Cat#: 4331182  |
| IL1A TaqMan Gene Expression Assay (FAM) Identification number: Hs00174092_m1  | Thermo Fisher Scientific     | Cat#: 4331182  |
| RPL34 TaqMan Gene Expression Assay (FAM) Identification number: Hs00996244_g1  | Thermo Fisher Scientific     | Cat#: 4351372  |

Primer sequences used for qPCR with SYBR Green Master mix are reported in the Method section.

**Software and algorithms**

| Software                                      | Provider/Reference             |
|-----------------------------------------------|--------------------------------|
| DAVID Bioinformatics Resources 6.8           | Huang D.W. at al., 2009;        |
|                                              | https://david.ncifcrf.gov/     |
| GraphPad Prism 5.0                            | http://www.graphpad.com/       |
### Enrichr

| Kuleshov M.V. et al., 2016; Chen E.Y. et al., 2013; | http://amp.pharm.mssm.edu/Enrichr/ |
| --- | --- |
| Image J 1.x | Schneider C.A. et al., 2012 |
|  | https://imagej.net/Welcome |

## Supplementary references

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