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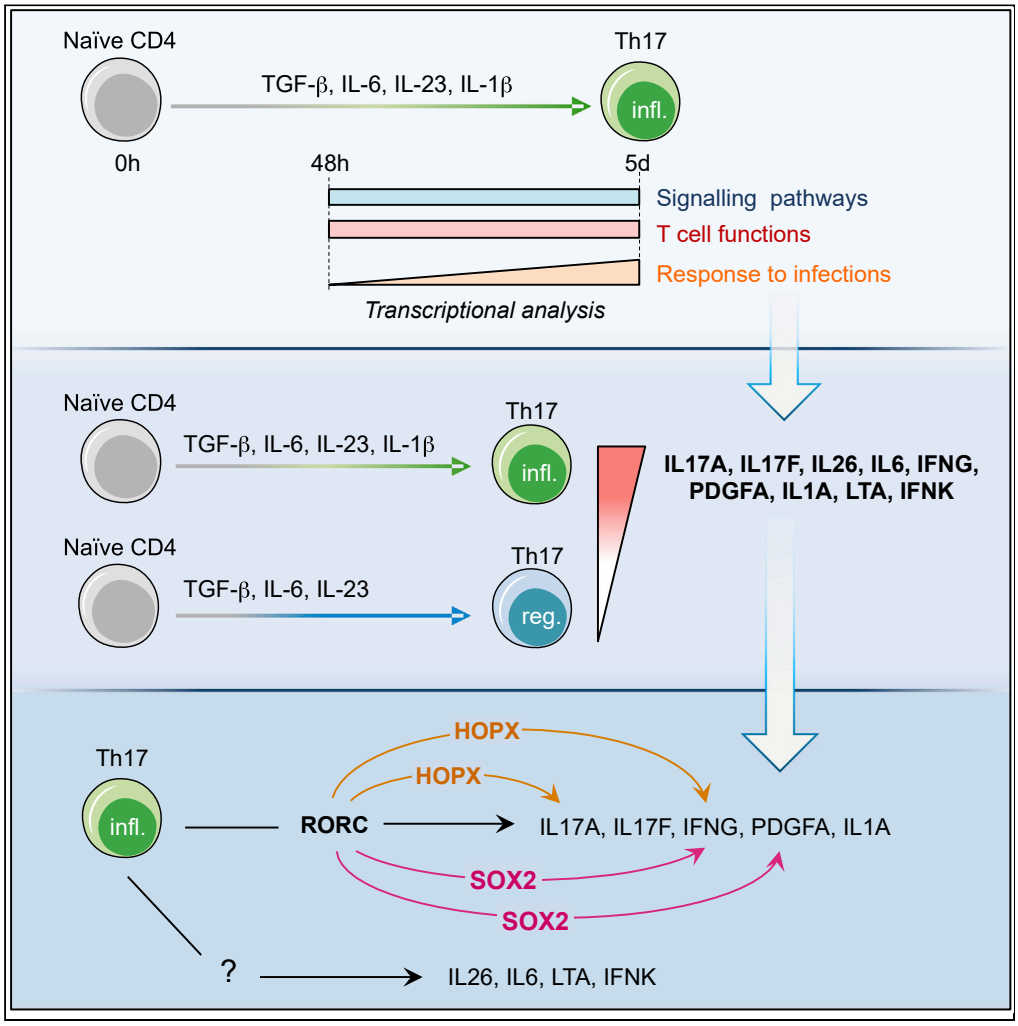

Alessia Capone, Chiara Naro, Manuela Bianco, ..., Vassili Soumelis, Elisabetta Volpe, Claudio Sette

e.volpe@hsantalucia.it (E.V.)
claudio.sette@unicatt.it (C.S.)

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

Alessia Capone,[1,2] Chiara Naro,[3,4] Manuela Bianco,[1] Marco De Bardi,[5] Floriane Noël,[6,7] Paolo Macchi,[8] Luca Battistini,[5] Vassili Soumelis,[6,7] Elisabetta Volpe,[1,10,11,*] and Claudio Sette[3,9,10,*]

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 (Annunziato 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

[1]Molecular Neuroimmunology Unit, IRCCS Santa Lucia Foundation, Via del Fosso di Fiorano 64, 00143 Rome, Italy

[2]Department of Biology and Biotechnology Charles Darwin, Sapienza University, Rome, Italy

[3]Department of Neuroscience, Section of Human Anatomy, Catholic University of the Sacred Heart, Largo Francesco Vito 1, 00168 Rome, Italy

[4]IRCCS Fondazione Policlinico Agostino Gemelli, Rome, Italy

[5]Neuroimmunology Unit, IRCCS Santa Lucia Foundation, Rome, Italy

[6]Laboratoire d'Immunologie et Histocompatibilité, AP-HP, Hôpital St Louis, Paris, France

[7]HIPI Unit, Inserm U976, Institut de Recherche Saint-Louis, Université de Paris, Paris, France

[8]Laboratory of Molecular and Cellular Neurobiology, Centre for Integrative Biology, University of Trento, Trento, Italy

[9]Neuroembriology Unit, IRCCS Santa Lucia Foundation, Rome, Italy

[10]These authors contributed equally

[11]Lead contact

*Correspondence: e.volpe@hsantalucia.it (E.V.), claudio.sette@unicatt.it (C.S.)

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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 naive $CD4^+$ 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) $\leq$ 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 populations with varying degrees of activation, we evaluated the cell size and the CD25 expression in Th0 and Th17 cells at 48 h and 5 days. Although the activation state of both Th0 and Th17 cells increased over time and Th17 expressed higher levels of CD25 than Th0 cells, each Th profile was characterized by a unique cell population (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 contains a small percentage of genes upregulated at 48 h and downregulated at 5 days or downregulated at 48 h and upregulated at 5 days (Figure S4B). Module A (early) included genes specific of initial Th17 cell differentiation (i.e. *Killer cell lectin-like receptor subfamily D* and *Notch homolog 1, translocation-associated)* (Figure 1E) and was enriched in functional categories related to signaling pathways (Figure 1F). 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)

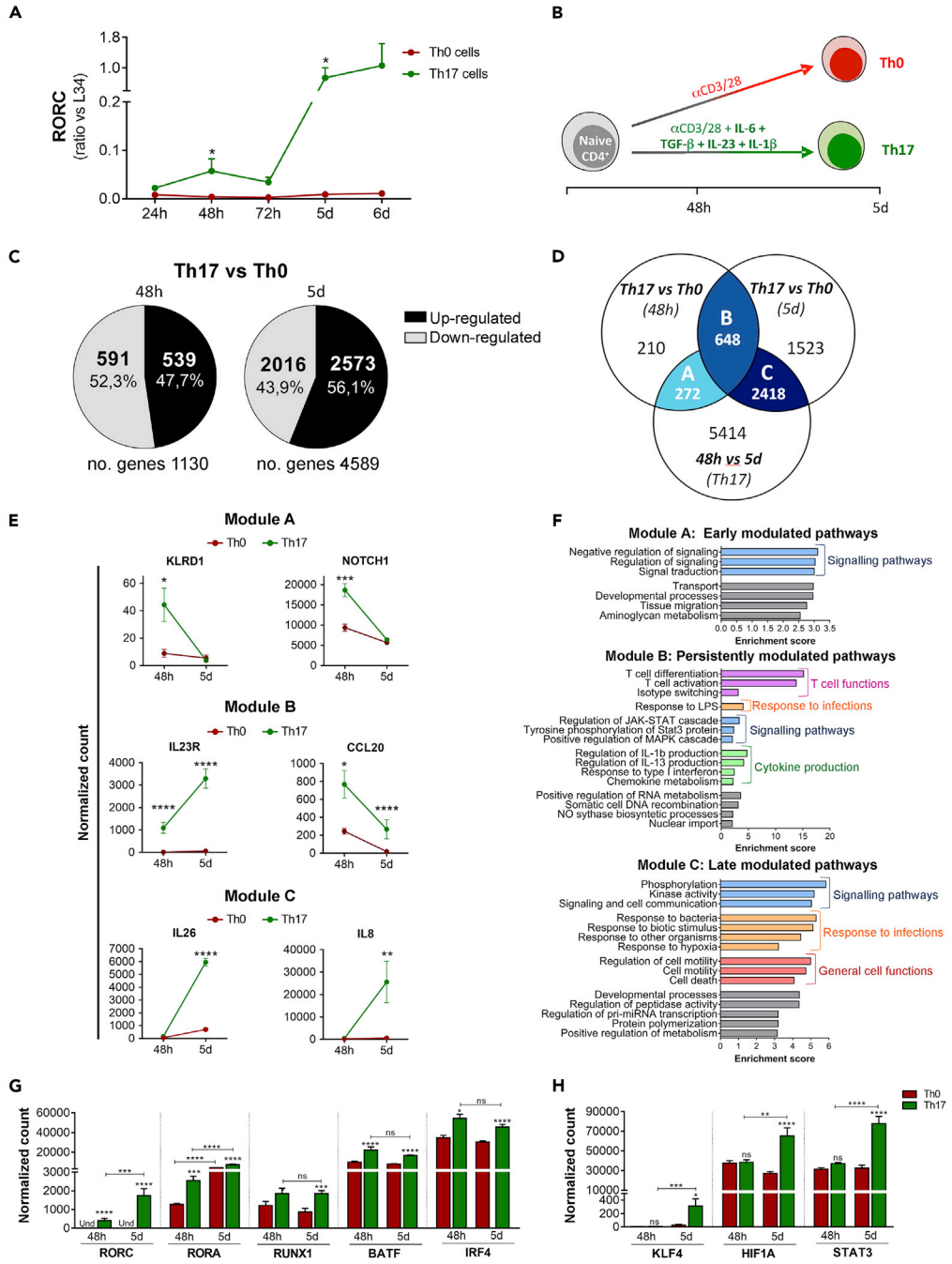

Figure 1. Early, persistent, and late genes acquired during human Th17 cell polarization reflect specific functions

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).

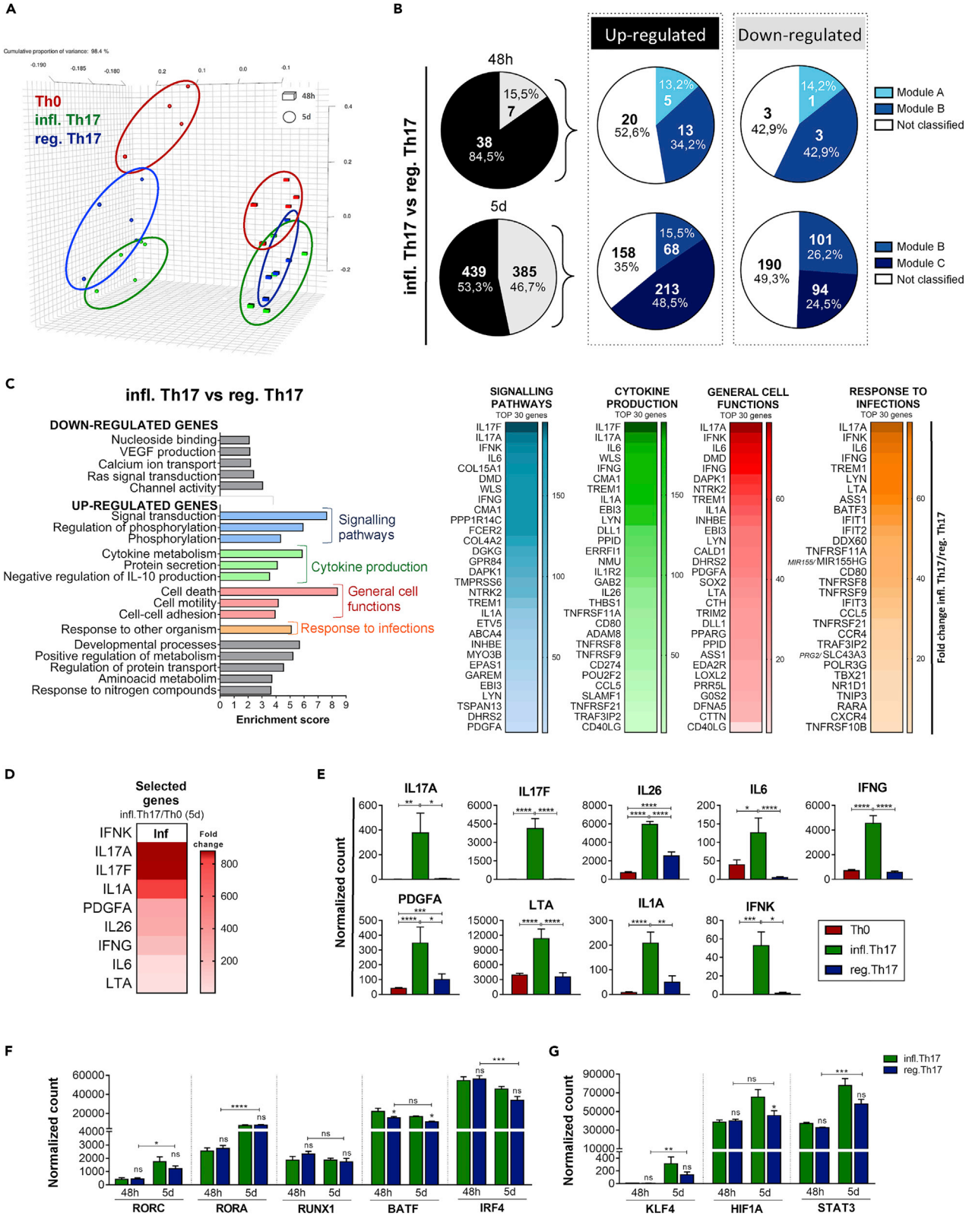
A
Cumulative proportion of variance: 98.4 %
Th0
infl. Th17
reg. Th17
48h
5d
B
Up-regulated
Down-regulated
48h
5d
infl. Th17 vs reg. Th17
15,5%
7
38
84,5%
439
53,3%
385
46,7%
20
52,6%
13,2%
5
13
34,2%
158
35%
15,5%
68
213
48,5%
3
42,9%
14,2%
1
3
42,9%
190
49,3%
101
26,2%
94
24,5%
Module A
Module B
Not classified
Module B
Module C
Not classified
C
infl. Th17 vs reg. Th17
DOWN-REGULATED GENES
Nucleoside binding
VEGF production
Calcium ion transport
Ras signal transduction
Channel activity
UP-REGULATED GENES
Signal transduction
Regulation of phosphorylation
Phosphorylation
Cytokine metabolism
Protein secretion
Negative regulation of IL-10 production
Cell death
Cell motility
Cell-cell adhesion
Response to other organism
Developmental processes
Positive regulation of metabolism
Regulation of protein transport
Aminoacid metabolism
Response to nitrogen compounds
Signalling pathways
Cytokine production
General cell functions
Response to infections
0 1 2 3 4 5 6 7 8 9
Enrichment score
SIGNALLING PATHWAYS
TOP 30 genes
CYTOKINE PRODUCTION
TOP 30 genes
GENERAL CELL FUNCTIONS
TOP 30 genes
RESPONSE TO INFECTIONS
TOP 30 genes
Fold change infl. Th17/reg. Th17
D
Selected genes
infl.Th17/Th0 (5d)
Inf
Fold change
IFNK
IL17A
IL17F
IL1A
PDGFA
IL26
IFNG
IL6
LTA
E
IL17A
IL17F
IL26
IL6
IFNG
PDGFA
LTA
IL1A
IFNK
Normalized count
Th0
infl.Th17
reg.Th17
F
Normalized count
RORC
RORA
RUNX1
BATF
IRF4
48h
5d
G
infl.Th17
reg.Th17
Normalized count
KLF4
HIF1A
STAT3

Figure 2. Transcriptional analysis of inflammatory Th17 cells reveals crucial genes for the effector functions of 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 (n = 5). Each replicate, including Th0_48hr, Th17_48hr, Th17 without IL-1β_48hr, Th0_5d, Th17_5d, Th17 without IL-1β_5d, was performed in the same donor (paired cultures). One sample Th0_48hr was omitted from analysis because it did not pass the quality control. Global gene expression of cells is represented by principal component analysis (A). Number of genes upregulated (black) and downregulated (gray) in infl. Th17 versus reg. Th17 at each time point of analysis, and segregation of those genes in modules A, B, and C is defined in Figure 1D B. Gene ontology of downregulated and upregulated genes in infl. Th17 versus reg. Th17 at 5 days, and heatmap of genes upregulated in infl. Th17 versus reg. Th17, belonging to the most represented categories (C). Heatmap (D) and expression profile (E) of nine selected genes upregulated in infl. Th17 versus reg. Th17 and in infl. Th17 versus Th0. Expression of persistent (F) and late (G) genes encoding for Th17 transcription factors in infl. Th17 and reg. Th17 profiles. Gene expression in E, F, and G are reported as DEseq2 normalized count (n = 5). Data are represented as mean ± SEM. (Student's T-tests; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001). Inflammatory Th17 = infl. Th17; regulatory Th17 = reg. Th17.

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 Th1/17 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 upregulation 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

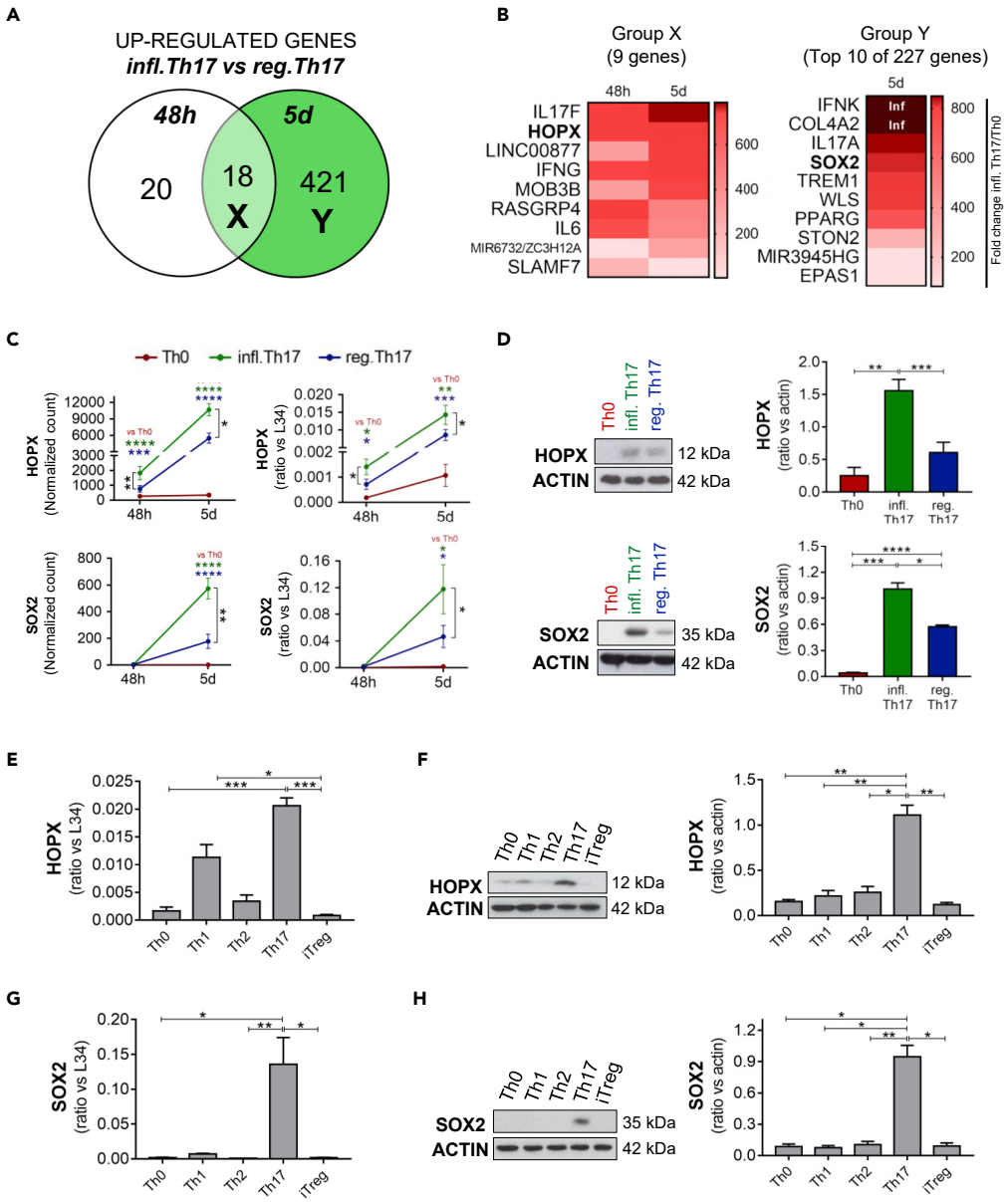

Figure 3. HOPX and SOX2 are specifically expressed in human Th17 cells

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 Treg 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.

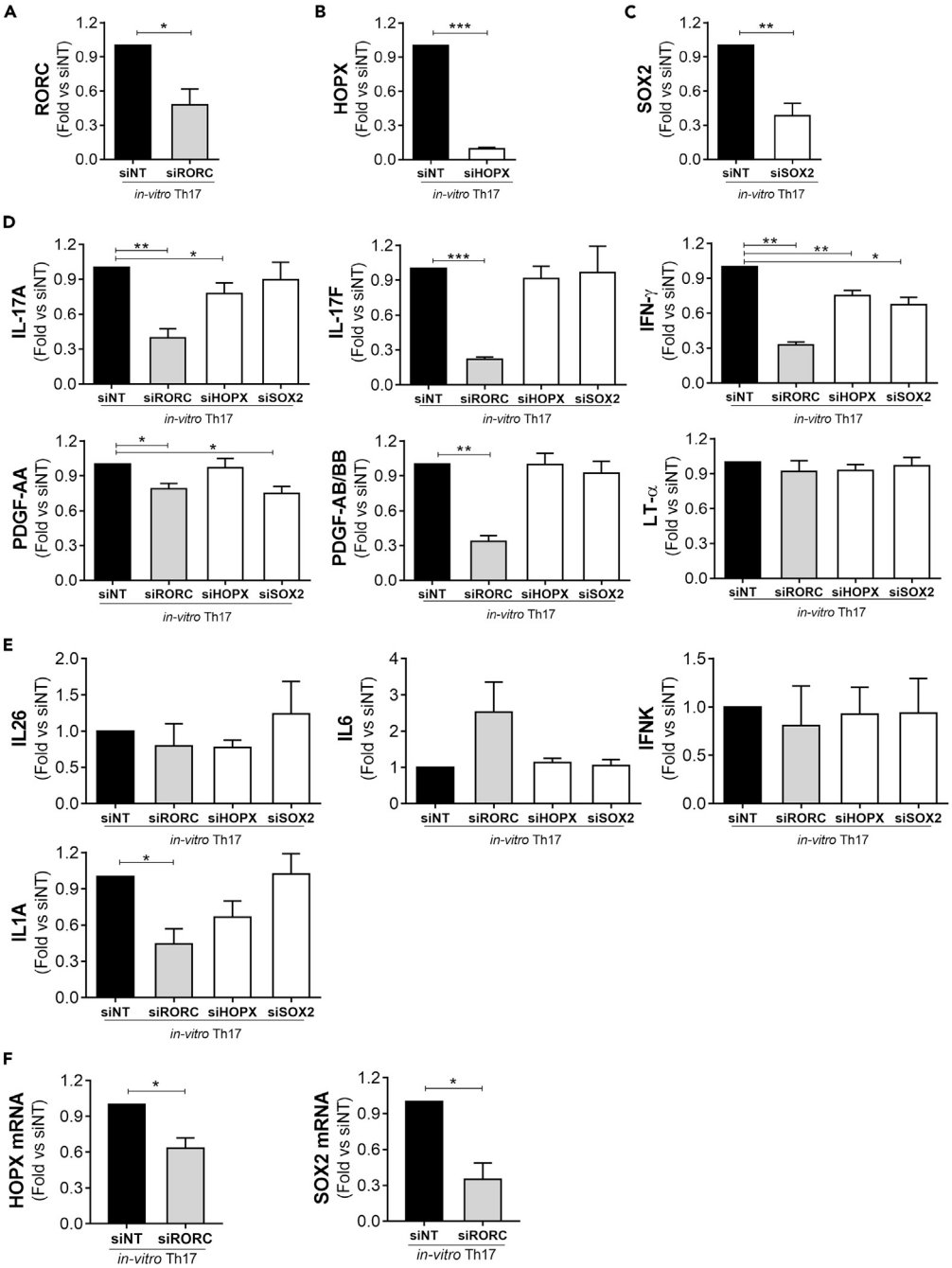

Figure 4. HOPX and SOX2 contribute to the expression of Th17 cytokines

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 RORγt and HOPX and PDGF-AA and of IFN-γ by RORγt and SOX2 lead us to hypothesize 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 4F). These results suggest that the transcriptional regulators HOPX and SOX2 act downstream of RORγt 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 (Kebir 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 Th1/17 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 Th1/17 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 Th1/17 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 insure 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 transcriptional 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., 2006). 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

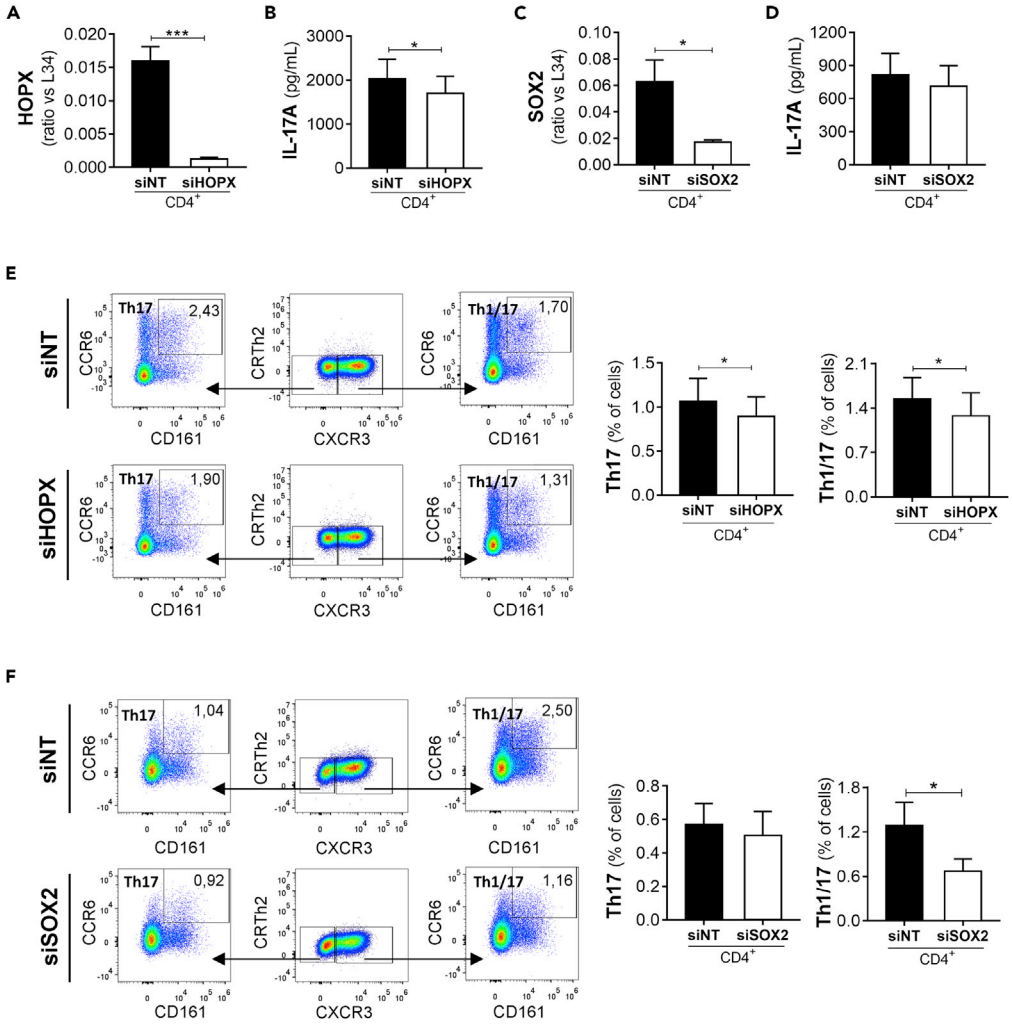

Figure 5. HOPX and SOX2 contribute to the regulation of Th1/17 cell features

Expression of HOPX transcript (A) and IL-17A protein (B) in total $CD4^+$ T cells treated with nontargeting (siNT) or HOPX (siHOPX) siRNA was analyzed by q-PCR and ELISA, respectively (n = 8). Expression of SOX2 transcript (C) and IL-17A protein (D) in total $CD4^+$ T cells treated with nontargeting (siNT) or SOX2 (siSOX2) siRNA was analyzed by q-PCR and ELISA, respectively (n = 6). Frequencies of Th17 cells ($CXCR3^-$ $CD161^+$ $CCR6^+$) and Th1/17 cells ($CXCR3^+$ $CD161^+$ $CCR6^+$) (E) were analyzed by flow cytometry in total $CD4^+$ T cells treated with non targeting (siNT) (n = 8) or HOPX (siHOPX) siRNA (n = 8). Frequencies of Th17 cells ($CXCR3^-$ $CD161^+$ $CCR6^+$) and Th1/17 cells ($CXCR3^+$ $CD161^+$ $CCR6^+$) (F) were analyzed by flow cytometry in total $CD4^+$ T cells treated with non targeting (siNT) (n = 6) or SOX2 (siSOX2) siRNA (n = 6). Data are represented as mean ± SEM (Student's T-tests; *p ≤ 0.05; ***p ≤ 0.001).

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.,

2019). PDGF-AA binds to PDGF receptor A, 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 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

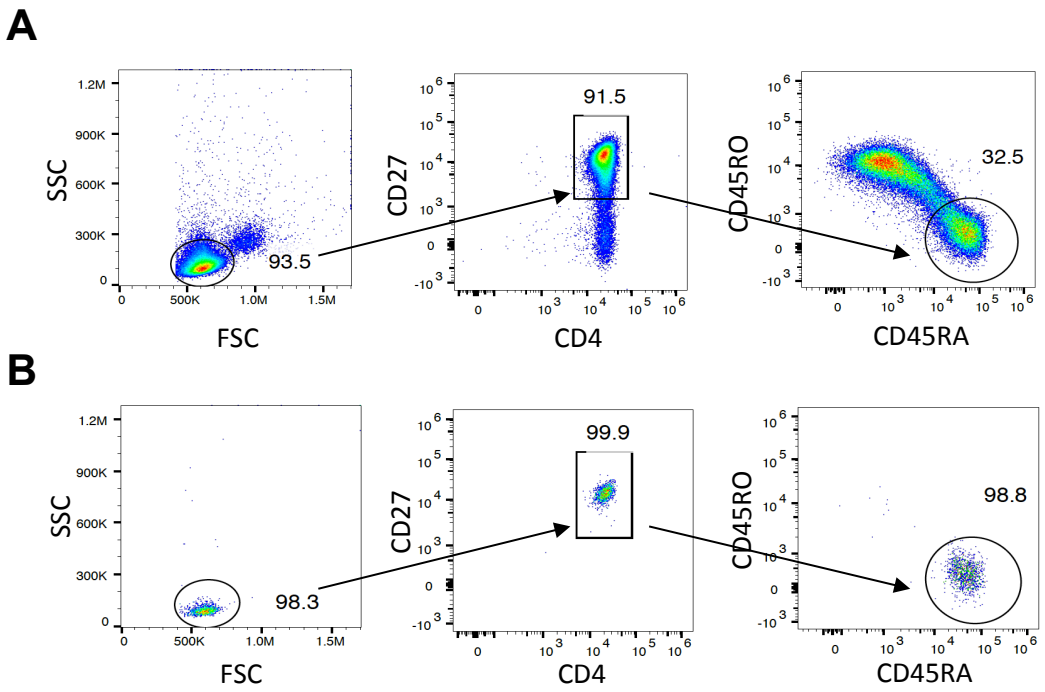

Figure S1. Gating strategy for the purification of naïve CD4$^+$ T cells, related to Fig.1
Blood CD4$^+$ T lymphocytes were labeled with specific antibodies conjugated with a fluorochrome and, by using a cell sorter, naïve CD4$^+$ T cells were isolated as CD4^{high}, CD45RA^{high}, CD45RO$^-$ and CD27$^+$ (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.

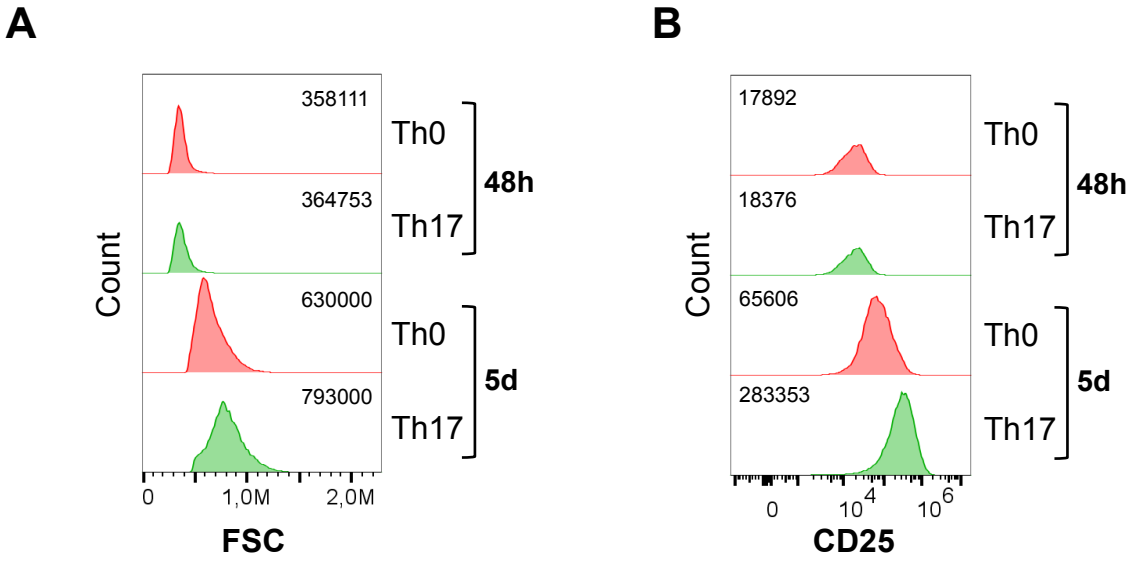

Figure S2. Activation state of Th cells at 48h and 5d of differentiation, related to Fig.1
The median fluorescence intensity (MFI) of forward side scatter (FCS) (A) and CD25 (B) in Th0 and Th17 cells, was measured by flow cytometry during the differentiation process. The graphs show data from a representative experiment.

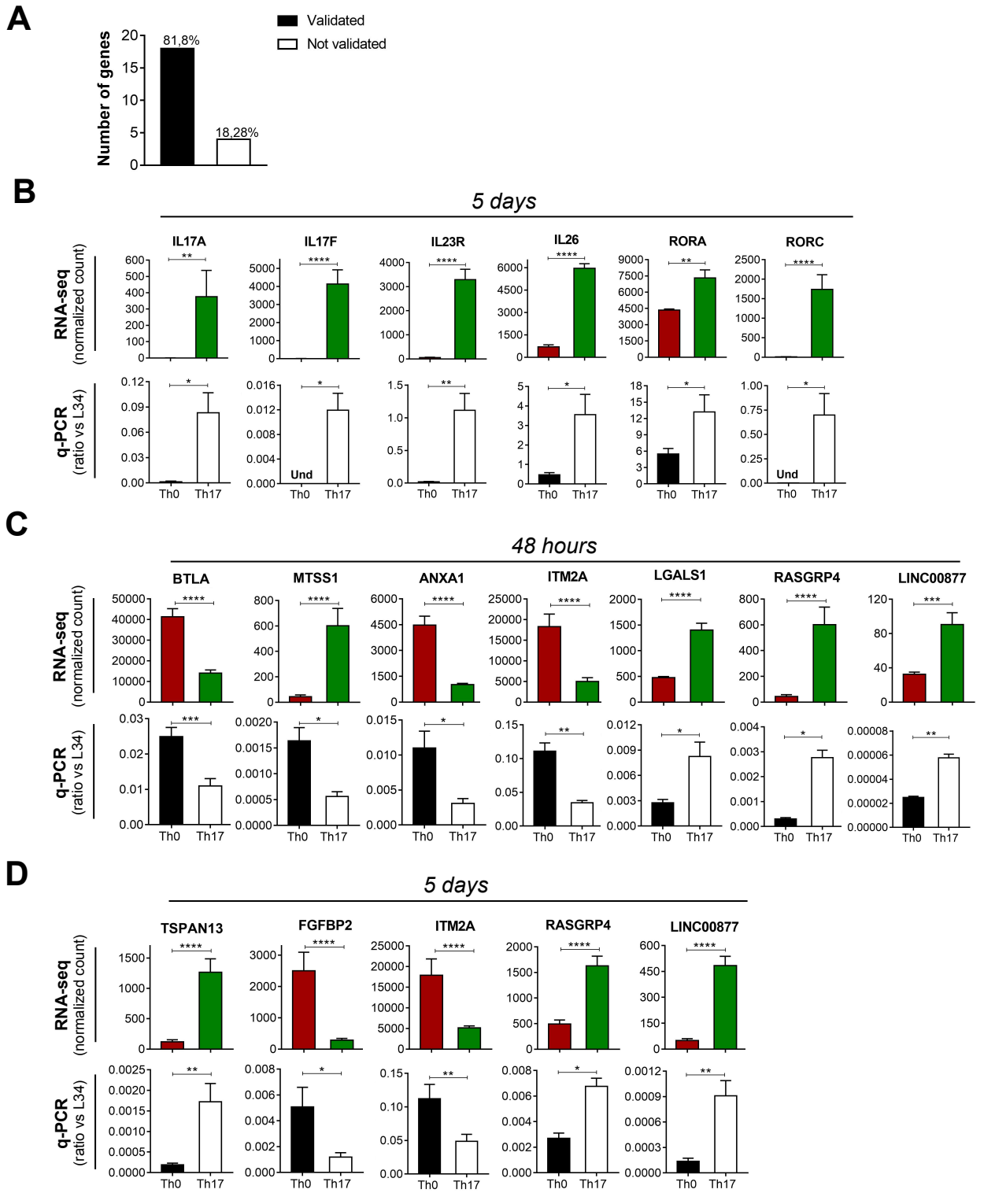

Figure S3. Validation of arbitrarily selected genes differentially expressed, related to Fig.1
Number and percentage of genes obtained by RNA-seq (reported as DEseq2 normalized count) and validated in q-PCR (A). Expression of Th17 signature genes at 5d (B) and expression of random selected genes at 48 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; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$)

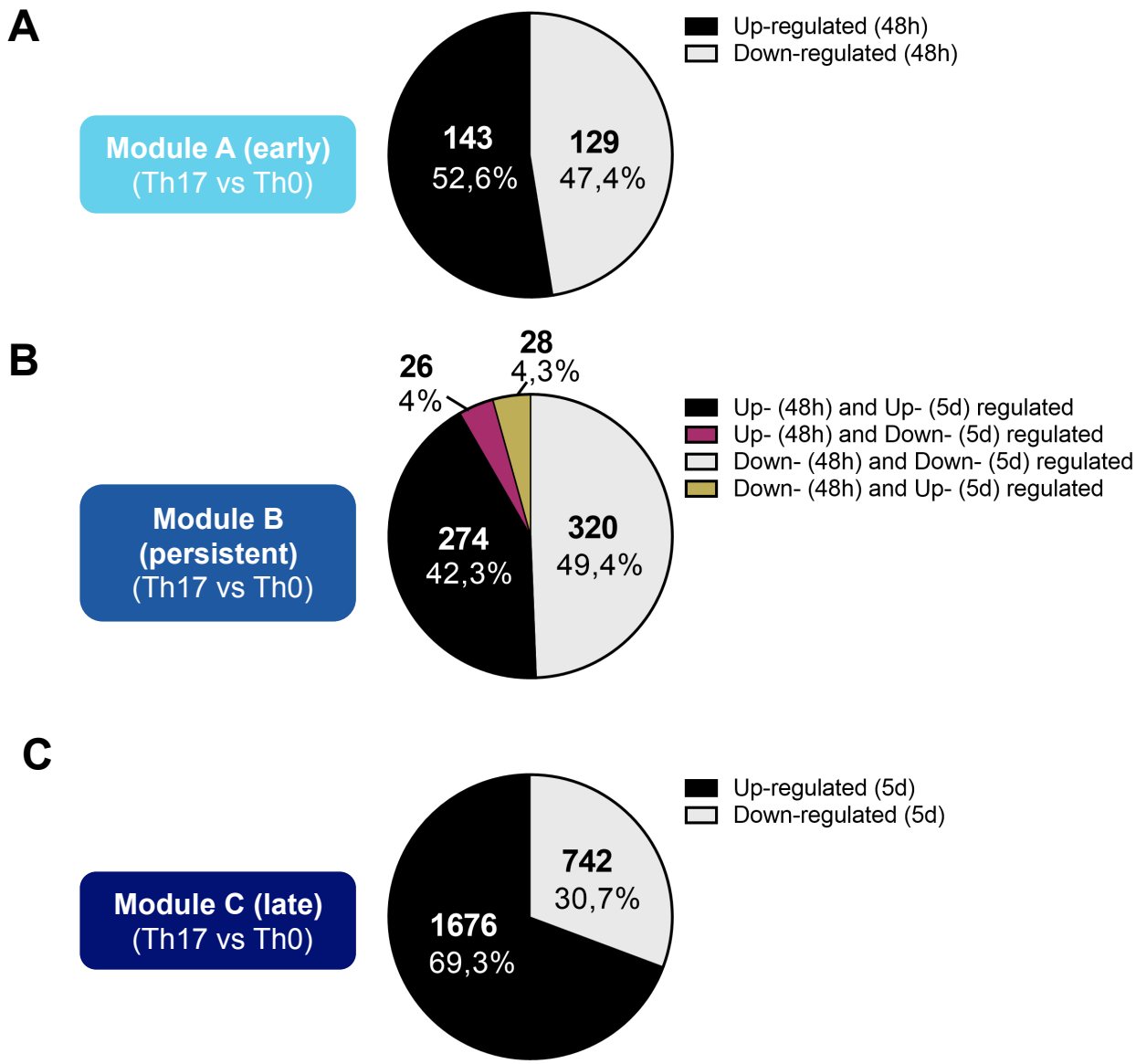

Figure S4. Type of regulation of intersecting genes defined as module A,B,C, related to Fig.1
Number and percentage of genes up-regulated and down-regulated in Th17 vs Th0 at each time point of analysis, in early (A), persistent (B), and late (C) modules.

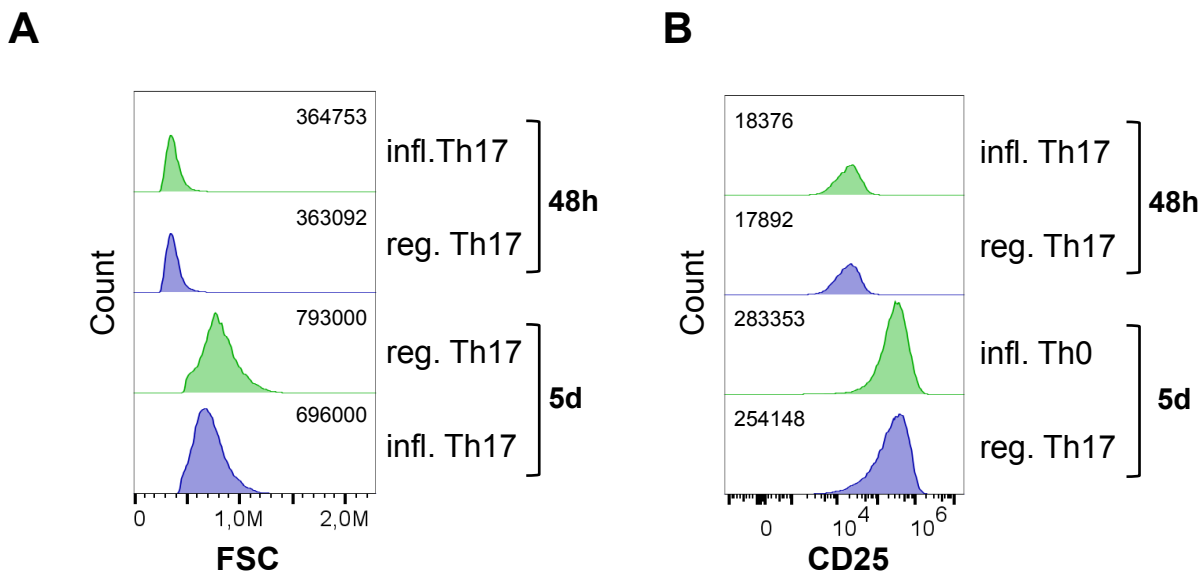

Figure S5. Activation state of Th cells at 48h and 5d of differentiation, related to Fig.2
The median fluorescence intensity (MFI) of forward side scatter (FCS) (A) and CD25 (B) in inflammatory Th17 (infl. Th17) and regulatory Th17 (reg. Th17) cells, was measured by flow cytometry during the differentiation process. The graphs show data from a representative experiment.

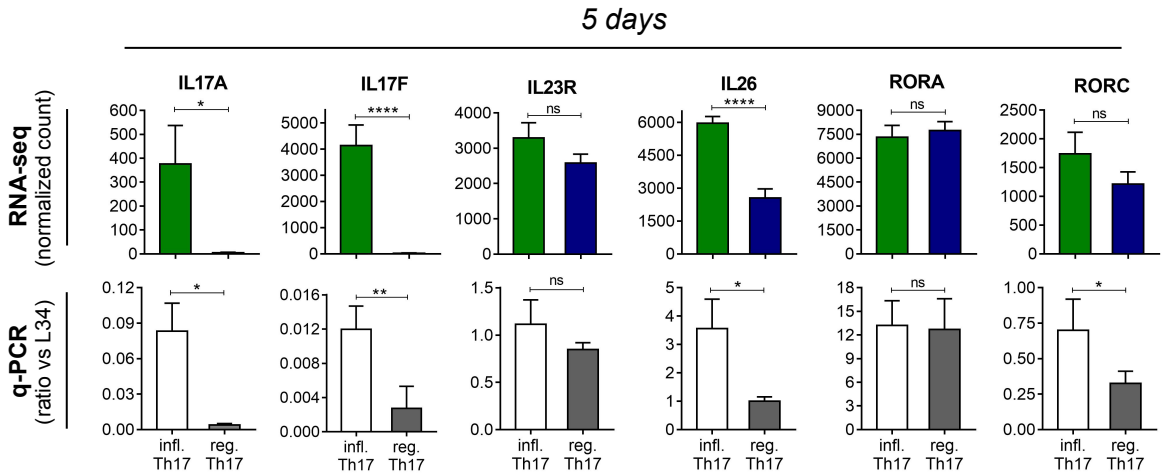

Figure S6. Signature genes of Th17 cells are partially reduced in regulatory Th17 cells, related to Fig.2

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; * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$).

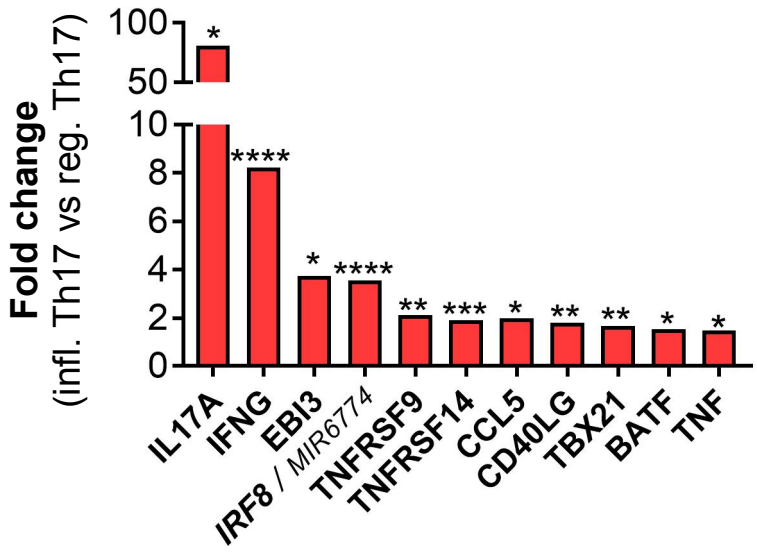

Figure S7. Validation of murine pathogenic Th17 genes in human Th17 cells, related to Fig.2
Pathogenic genes of murine Th17 cells (in bold) significantly up-regulated in human inflammatory Th17 cells (infl. Th17) vs regulatory Th17 cells (reg.Th17), analysed by RNA-sequencing (n=5). Bars represent fold change of DEseq2 normalized count. (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

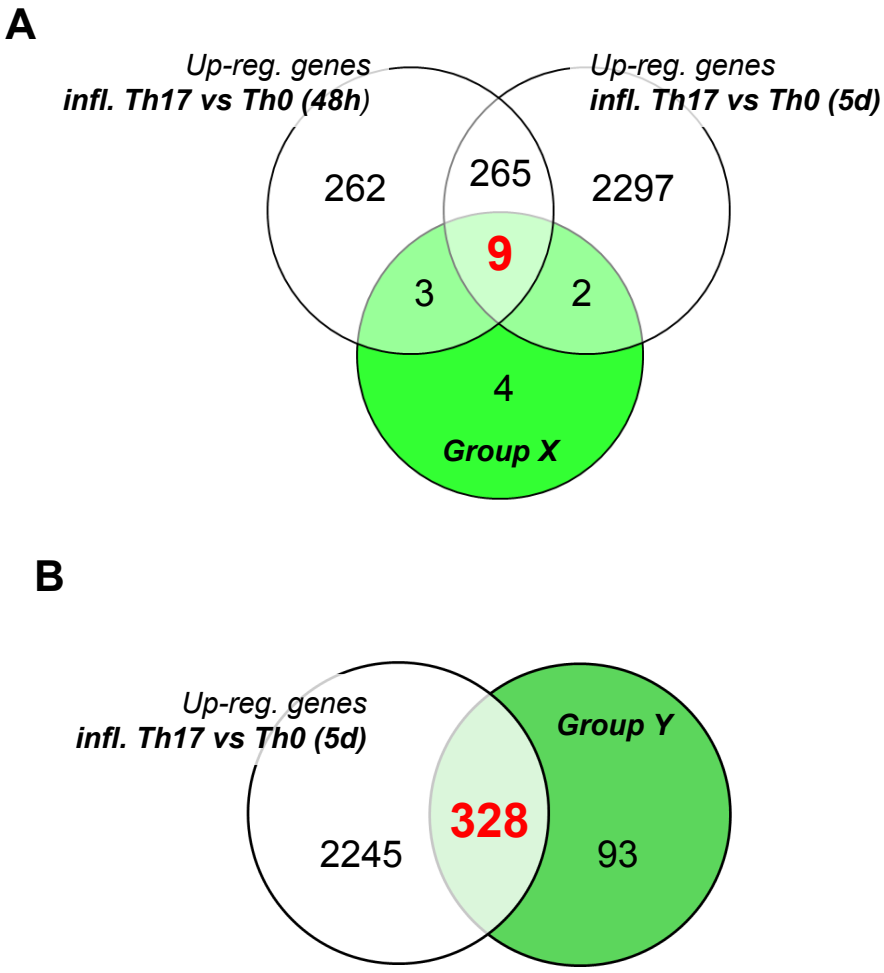

Figure S8. Selection of genes expressed by inflammatory Th17 cells and specific of Th17 cells, related to Fig.3

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).

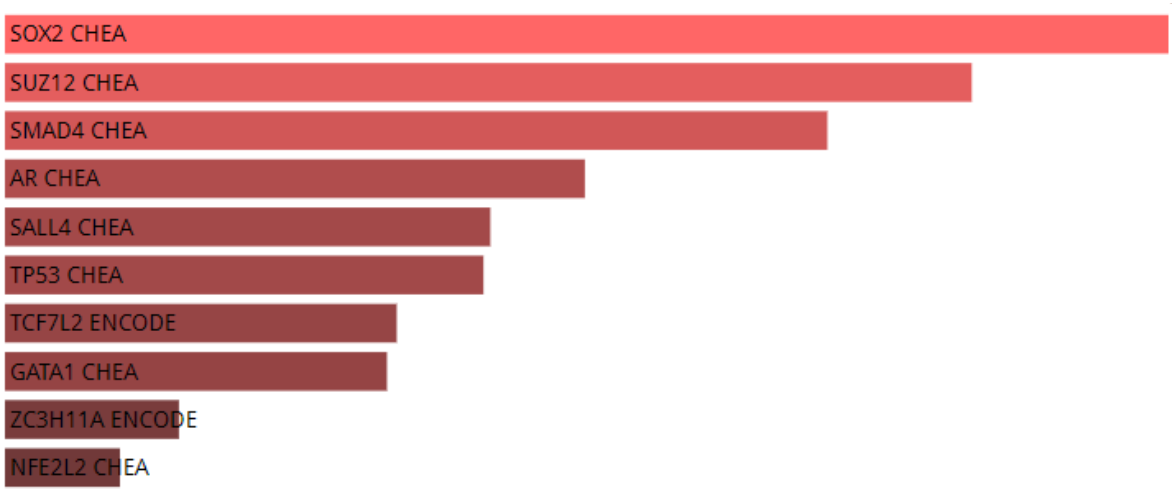

Figure S9. SOX2 is a potential transcription factors of human Th17 polarization, related to Fig. 3

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.

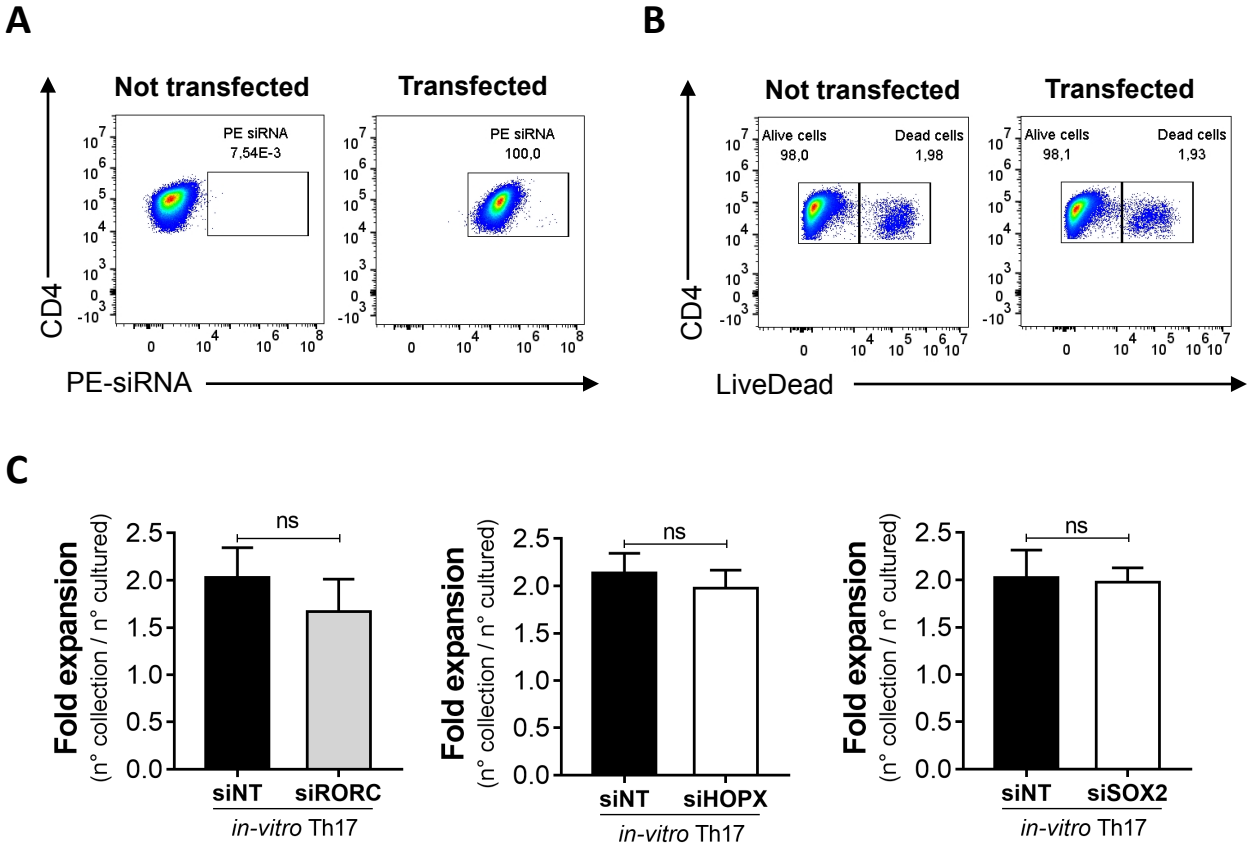

Figure S10. Transfection of *in-vitro* polarized Th17 cells with siRNA, related to Fig.4
Naïve $CD4^+$ T were transfected with siRNA for 5 days during polarization into Th17 cells. Internalization of PE-siRNA upon transfection (A) and cell viability (B) were analyzed by flow cytometry. Fold expansion (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).

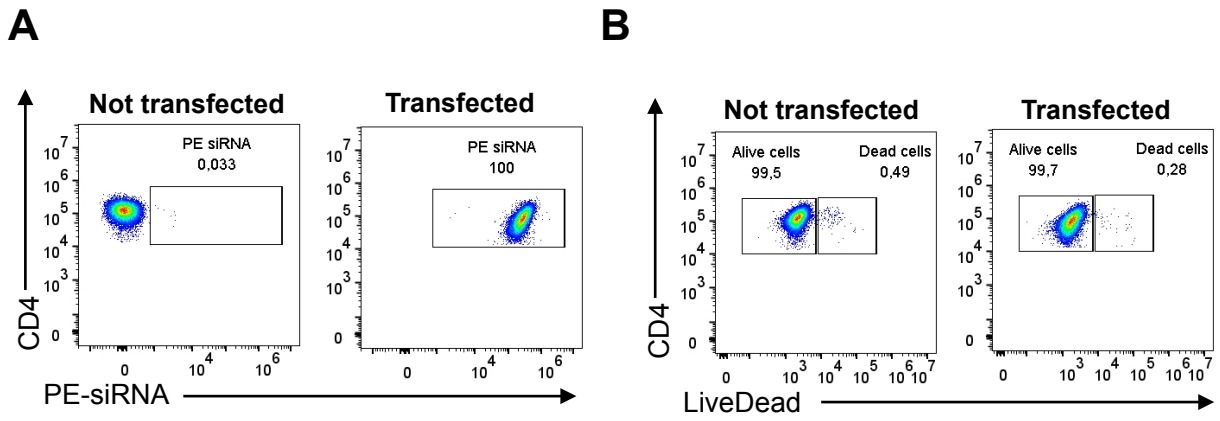

Figure S11. Transfection of memory CD4$^+$ T cells with siRNA, related to Fig.5
Total CD4$^+$ T cells were transfected with siRNA for 3 days. Internalization of PE-siRNA upon transfection (A) and cell viability (B) were evaluated by flow cytometry.

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	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		

Transparent methods

RESOURCE 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 $CD4^{high}$, $CD45RA^{high}$, $CD45RO^-$ and $CD27^+$. 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 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 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 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). 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 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. 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 CGCTGCTTAAACCATTTCTGG), 54°C (IL17F: forward GAAAACCAGCGCGTTTCCAT; reverse CTGCATCCTGAAACAAAGGTCA), 58°C (BTLA: forward CTGACACAGCAGGAAGGGAAAT; reverse CTGCATCCTGAAACAAAGGTCA), 58°C (MTTS1: forward GACTCGCTTCAGTGCTCCAG; reverse TCATAATCTGAAACTTGGGAAGGG), 58°C (ANXA1: forward GGTGAGCCCCTATCCTACCT; reverse CTGTTGACGCTGTGCATTGT), 58°C (ITM2A: forward AACTGCTATCTGATGCCCCT; reverse CATCACGAATTTCCTCCACAGC), 54°C (LGALS1: forward CTCGGGTGGAGTCTTCTGAC; reverse GAAGGCACTCTCCAGGTTTGA), 54°C (RASGRP4: forward GACTGAATCCCCACACCCTT; reverse GTGAGGAAGAGAGGAGACCA), 63°C (LINC00877: forward CGAGAAGGAAAGCCGGTGAT; reverse TCCATGTGTCTGCTTTGCCT), 56°C (TSPAN13: forward GCCCTCAACCTGCTTTACAC; reverse ACAGCTCCAATCAGACCCAC), 54°C

(FGFBP2: forward CCGAGGGTGACAGGTGAAAG; reverse CGTTGGATTGAAAGCGGCAT), 63°C (IL6: forward CTGGCAGAAAACAACCTGAACC; reverse TGGCTTGTTCCTCACTACTCT), 60°C (RPL34: forward GTCCCGAACCCCTGGTAATAGA; reverse GGCCCTGCTGACATGTTTCTT); 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_3VO_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

Mouse monoclonal anti CD161 (clone HP-3G10) Conjugate PE-Dazzle 594	BioLegend	Cat#: 339939
Mouse monoclonal anti CCR6 (clone G034E3) Conjugate PC7	BioLegend	Cat#: 353401
Mouse monoclonal anti CD25 (clone 2A3) Conjugate Brilliant Violet 421	BioLegend	Cat#: 564033
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

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		
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	This paper	N/A
Software and algorithms		
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

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