Research Article

Eomes controls the development of Th17-derived (non-classic) Th1 cells during chronic inflammation

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It is well accepted that Th17 cells are a highly plastic cell subset that can be easily directed toward the Th1 phenotype in vitro and also in vivo during inflammation. However, there is an ongoing debate regarding the reverse plasticity (conversion from Th1 to Th17). We show here that ectopic ROR-γt expression can restore or initiate IL-17 expression by non-classic or classic Th1 cells, respectively, while common pro-Th17 cytokine cocktails are ineffective. This stability of the Th1 phenotype is at least partially due to the presence of a molecular machinery governed by the transcription factor Eomes, which promotes IFN-γ secretion while inhibiting the expression of ROR-γt and IL-17. By using a mouse model of T cell-dependent colitis we demonstrate that Eomes controls non-classic Th1 cell development also in vivo and promotes their pathogenic potential. Eomes expression associates to a highly inflammatory phenotype also in patients with juvenile idiopathic arthritis. Indeed, it favors the acquisition of a cytotoxic signature, and promotes the development of IFN-γ^+GM-CSF$^+$ cells that have been described to be pathogenic in chronic inflammatory disorders.

Keywords: Colitis · Eomes · Juvenile idiopathic arthritis · Th1 · Th17

See accompanying Commentary by Dejean et al.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Upon antigen encounter, naïve CD4^{+} T helper (Th) cells acquire specific effector functions to promote pathogen clearance. Three main Th effector subsets have been described so far, which are characterized by the expression of distinct transcription factors, cytokine production and immunological function. Th1 cells express the transcription factor Tbet, produce IFN-γ and protect from intracellular pathogens; Th2 cells express GATA-3, produce IL-4, IL-5, IL-13 and protect from extracellular parasites; Th17 cells express ROR-γt, produce IL-17A and IL-17F and protect from extracellular bacteria and fungi [1–3]. Although original studies considered these phenotypes as mutually exclusive, it has been described that competing effector programs can coexist within the same cell [4–7]. Indeed, cells with a mixed phenotype can originate directly from a naïve precursor [8], as well as from the polarization of mature cells which retain a certain degree of plasticity, if exposed to appropriate cytokine stimulation. With this regard, we and others have shown the existence of cells simultaneously producing both IL-17 and IFN-γ, named Th17/Th1, as well as both IL-17 and IL-4, named Th17/Th2 [6, 9, 10]. Moreover, we have shown that both Th17 and Th17/Th1 cells can rapidly acquire a full Th1 phenotype when exposed to the activity of proinflammatory cytokines IL-12 or TNF-α [11, 12]. Human Th1 cells originated from the phenotype shift of Th17 and Th17/Th1 cells were defined as non-classic Th1, to distinguish them from classic Th1 that instead were generated directly from the polarization of naïve cells [13]. Similar findings confirming the plasticity of the Th17 phenotype were reported also in mouse [14, 15]. Epigenetic analysis has further reinforced the idea that non-classic Th1 and classic Th1 cells are distinct lineages, since the former show complete demethylation of *RORC2* (encoding ROR-γt) and hypomethylation of *IL17A*. Instead, both gene loci are hypermethylated in the latter cell subset [16]. Human Th17 cells originate from a subset of CD4^{+}CD161^{+} umbilical cord blood (UCB) naïve cells and are comprised within the CD4^{+}CD161^{+}CCR6^{+} subset of both circulating and infiltrating inflammatory cells in adults [17]. Moreover, non-classic Th1 cells retain the expression of both CD161 and CCR6, as well as the Th17 cells' master transcription factor ROR-γt [13]. Non-classic Th1 cells are enriched at sites of inflammation [18], and it has been shown that such a Th17 to Th1 phenotype transition is fundamental for disease establishment in mouse models of diabetes [19] and inflammatory bowel diseases [20]. Although the route of polarization from Th17 to Th1 is widely accepted, it is not resolved whether Th1 can be polarized to Th17 cells. Moreover, to our knowledge, no data have been published regarding the ability of the two subsets of Th1 cell, classic and non-classic, to acquire IL-17 production. In general, Th1 cells are thought to be phenotypically stable, and less capable to acquire features of other cell subsets [21].

In this study we analyzed whether non-classic Th1 cells are terminally differentiated, and thus phenotypically stable, or instead they might be reverted toward Th17 in the appropriate microenvironment. Our findings show that non-classic Th1 cells are refractory toward common cytokine cocktails known to favor Th17 cell development. By transcriptome analysis, we found that the transcription factor Eomesodermin (Eomes) is expressed by classic and non-classic Th1 cells in humans. Eomes expression has been reported in NK and CD8 T cells, where it promotes IFN-γ, perforin and granzyme production [22, 23]. However, there is less information regarding its role in CD4 T cells, mostly confined to mouse experiments. Eomes expression seems to be dependent on TCR and IL-2 signaling [24, 25]. Moreover, murine naïve cells express Eomes to favor the Th1 rather than the Th17 polarization route [26, 27]. It has been reported that human memory CD4^{+} T cells exhibit higher Eomes expression than naïve cells [28], however the role of this transcription factor in humans is yet to be defined. Our data show that Eomes is expressed by human non-classic Th1 cells and it is involved in their phenotype development from Th17 cells, favoring IFN-γ secretion, and their phenotypic stability, preventing the re-expression of *RORC2* and *IL17A*. These in vitro data were confirmed also in vivo in a mouse model of colitis. Moreover, we found that Eomes favors the acquisition of pathogenic potential by T cells. Indeed, Eomes exacerbated signs of inflammation during colitis in mice, and favored the development of a cytotoxic signature in humans. Finally, we found that Eomes drives the development of human IFN-γ^{+}GM-CSF^{+} cells in vitro. Moreover, IFN-γ^{+}GM-CSF^{+} cells were found to accumulate in the synovial fluid of inflamed joints of patients affected by juvenile idiopathic arthritis (JIA).

Results

Th17-derived (non-classic) Th1 cells are phenotypically stable

We performed transcriptome analysis on a group of Th17, non-classic Th1 and classic Th1 cell clones. Th17 and non-classic Th1 cell clones were generated from the CD161^{+}CCR6^{+} fraction of circulating CD4^{+} T cells obtained from healthy subjects. Classic Th1 cell clones instead were obtained from the CD161^{-}CCR6^{-} counterpart as already described [13]. The phenotype of all the clones used in this study is reported in Supporting Information Fig. 1. Cells were analyzed either resting or after activation with anti-CD2 -CD3 -CD28-coated beads. Clustering-assisted heatmap analysis showed that non-classic Th1 cells exhibit an intermediate gene expression profile, when compared to Th17 and classic Th1 cells, sharing the expression pattern of several genes with Th17 cells and of several others with classic Th1 cells. This intermediate behavior was more evident upon cell activation (Fig. 1A). This observation was confirmed also by Principal Component Analysis of the same transcriptome data, which showed that both resting and activated non-classic Th1 cells cluster between Th17 and classic Th1 cells (Fig. 1B). To investigate whether non-classic Th1 cells might revert toward the Th17 phenotype under Th17-promoting conditions, we activated in vitro for one or two weeks pure non-classic Th1 clones with anti-CD2 -CD3 -CD28 coated beads in the presence of IL-1β and IL-23, able to drive Th17 polarization in humans [17], or of IL-1β, IL-23, IL-6 and TGF-β [29]. As shown

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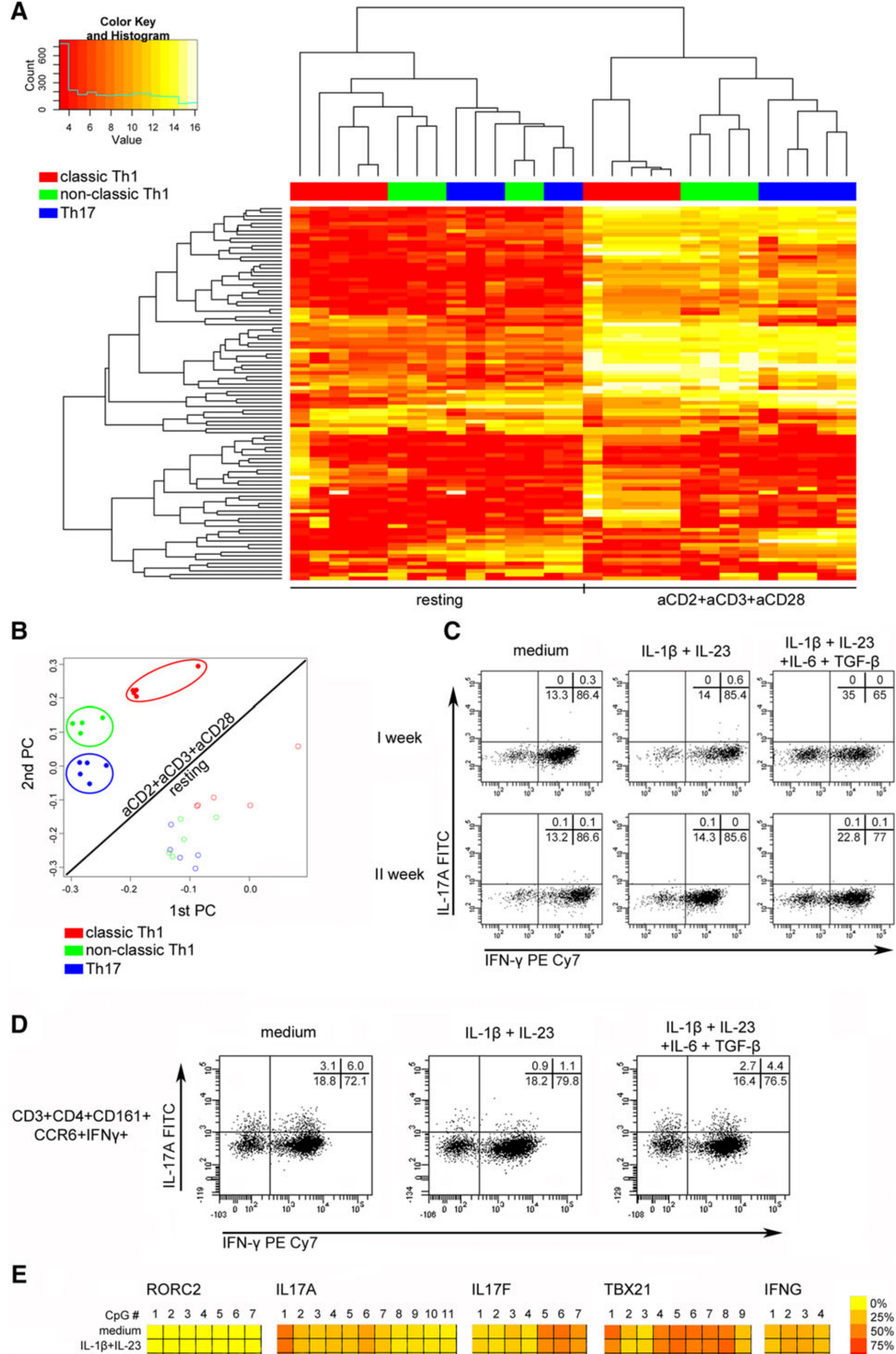

Figure 1. Non-classic Th1 cells are phenotypically stable and cannot be reverted toward Th17 in vitro (A) Hierarchical clustering-assisted heatmap performed on the top 100 most variable probes obtained from resting (left) or anti-CD2, anti-CD3, anti-CD28 stimulated (right) Th17 (blue), non-classic Th1 (green) and classic Th1 (red) cell clones. 5 distinct T cell clones for each phenotype were analyzed either resting or antiCD2/3/28 stimulated. 5 independent experiments were performed. Expression levels vary from red (low) to white (high). (B) Principal component analysis performed on transcriptome data from 5 Th17, 5 classic Th1 and 4 non-classic Th1 cell clones as in A. Empty dots refer to resting cells, filled dots to activated cells. (C) Non-classic Th1 cell clones were stimulated with anti-CD2, anti-CD3, anti-CD28 coated beads for one or two weeks in the presence of the indicated cytokine cocktails and then restimulated with PMA plus Ionomycin for flow cytometric cytokine expression evaluation. 3 independent experiments were performed on 3 different T cell clones. One representative experiment is shown. (D) Ex vivo sorted non-classic Th1 cells were cultured for 1 week and then reassessed for cytokine production as in (C). One representative experiment out of two independent experiments performed is shown. (E) DNA methylation analysis was performed at *RORC2*, *IL17A*, *IL17F*, *IFNG*, *TBX21* gene loci at the indicated CpG positions. DNA from 3 non-classic Th1 cell clones cultured in vitro in the presence of the indicated cytokine cocktails was pooled and then analyzed by pyrosequencing. Methylation levels are represented with a colorimetric representation ranging from yellow (0% of methylation) to red (100% of methylation).

in Fig. 1C, non-classic Th1 cells did not acquire the ability to produce IL-17 in any experimental condition. Moreover, ROR-γt mRNA levels were only slightly increased (data not shown). Since T cell cloning requires repetitive rounds of cell stimulation for long periods, this may lead to excessive cell senescence and terminal differentiation, thus reducing the cell plasticity potential. Thus, we performed the same experiment also on freshly ex vivo derived non-classic Th1 cells from peripheral blood of healthy donors. IFN-γ producing cells were isolated by using a combination of surface markers ($CD4^+CD161^+CCR6^+$) and cytokine secretion assay. Also directly ex vivo isolated non-classic Th1 cells were completely refractory to Th17-polarizing cytokines (Fig. 1D). To analyze whether the treatment with Th17-promoting cytokines induced any change in non-classic Th1 cells at epigenetic level, we investigated the DNA methylation pattern at cytokine and transcription factor gene loci. Non-classic Th1 cells exhibited full demethylation of *RORC2*, hypomethylation of *IL17A* and *IL17F*, reduced methylation levels of *TBX21* (encoding Tbet) and low

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methylation at the *IFNG* locus (Fig. 1E). Following cytokine treatment (i.e. IL-1β plus IL-23 or IL-1β plus IL-23 plus IL-6 plus TGF-β) no changes in the methylation pattern at these loci were observed (Fig. 1E). These data show that non-classic Th1 cells have a stable phenotype that is not affected by external cytokine stimulation.

Forced expression of ROR-γt partially restores IL-17 production by non-classic Th1 cells

ROR-γt is the master transcription factor of Th17 cells, and it is fundamental for the expression of the typical Th17 transcriptional signature [30]. Quantifying *RORC2* mRNA levels on Th17, non-classic Th1 and classic Th1 cell clones (Fig. 2A), as well as on ex vivo sorted T-cell subsets (Fig. 2B), non-classic Th1 cells expressed significantly reduced levels compared to Th17, but significantly higher than classic Th1 cells. Thus, we hypothesized that residual ROR-γt levels in non-classic Th1 cells may be insufficient to trigger *IL17A* expression. Using lentiviral vectors, we ectopically overexpressed *RORC2* in non-classic Th1 and, as a control, in classic Th1 cell clones, characterized by IFN-γ expression by virtually all cells and absence of IL-17 expressing cells. *RORC2* levels were comparable between the two populations upon transduction (data not shown). *RORC2* overexpression alone, in the absence of any exogenous cytokines, led to a significant increase in the percentage of IL-17 producing cells only in the non-classic Th1 cell subset (Fig. 2C and D). We repeated the same experiment also on freshly ex vivo-recovered non-classic Th1 and classic Th1 cells, sorted according to $CD3^{+}CD4^{+}CD161^{+}CCR6^{+}CXCR3^{+}$ and $CD3^{+}CD4^{+}CD161^{-}CCR6^{-}CXCR3^{+}$, respectively [31], to avoid the strong ex vivo restimulation required for the cytokine secretion assay. Although by this sorting strategy the enriched non-classic Th1 cell population included IL-17 expressing Th cells, ectopic *RORC2* overexpression resulted in a major increase in IL-17 producing cells in the non-classic Th1 compared to the classic Th1 subset (Fig. 2E). The increased production of IL-17 by non-classic Th1 cells was confirmed also on mRNA level (data not shown). Collectively, these data support the concept that upon proper ROR-γt expression both classic and non-classic Th1 cells can start producing IL-17, but non-classic Th1 can be more easily phenotypically redirected. However, the finding that IL-17 production can be observed only upon ectopic expression of ROR- γt, while common pro-Th17 cytokine cocktails are ineffective, suggests that a molecular machinery aimed to guarantee cell phenotype stability is active in Th1 cells.

Both non-classic and classic Th1 cells express high levels of Eomes

To identify the molecular machinery possibly involved in the inhibition of the Th17 program in Th1 cells, we analyzed the transcriptomes of Th17, non-classic and classic Th1 cell clones. Bioinformatic analysis allowed to compare differentially expressed genes within the three T cell subsets (Fig. 3A). As expected, *RORC2*, *CCR6*, *KLRB1*, *IL23R*, *IL1R1*, *RORA* were among the genes selectively expressed at lower levels by classic Th1 when compared to both non-classic Th1 and Th17 cells. Instead, classic Th1 displayed significantly higher *IFNG* expression when compared to Th17 cells. The transcription factor Eomes was among the 153 genes highly expressed by both non-classic Th1 and classic Th1 compared to Th17 cells. Quantitative Real-Time PCR on Th cell clones confirmed the selective higher *Eomes* mRNA levels in both non-classic Th1 and classic Th1 compared to Th17 and Th17/Th1 cells (Supporting Information Fig. 2). TCR activation did not affect Eomes mRNA expression (Supporting Information Fig. 2). Also freshly ex vivo sorted classic and non-classic Th1 cells expressed higher *Eomes* mRNA compared to Th17 cells (Fig. 3B). Similar findings were obtained also on protein level by confocal microscopy (Fig. 3C).

IL-2 and IL-12 cooperate to initiate *Eomes* transcription

IL-12 is the most potent cytokine able to polarize human Th17 cells toward the non-classic Th1 phenotype [11]. To analyze whether IL-12 can regulate Eomes expression, we activated Th17 cell clones in vitro with allogenic feeder cells and IL-2, in the presence or in the absence of exogenous IL-12. As shown in Fig. 4A, the addition in culture of IL-12 significantly upregulated *Eomes* levels.

It has also been proposed that *Eomes* transcription may be favored by IL-2 signaling itself [25] via a STAT5B-dependent pathway [32]. We have shown that IL-2 signaling is significantly impaired in Th17 cells, due to the activity of the transcription factor Musculin, which targets the STAT5B pathway [31]. Thus, we hypothesized that Musculin may be indirectly involved in preventing *Eomes* expression in Th17 cells. Indeed, Musculin is commonly downregulated during the non-classic Th1 cell development, thus restoring proper IL-2 sensitiveness [31]. In concordance, our transcriptome data show that *MSC* (encoding for Musculin) is one of the genes that are expressed, from high to low levels, in Th17, non-classic Th1 and finally classic Th1 cells (Fig. 3A). In line with this finding, we observed a negative correlation between *Eomes* and *MSC* mRNA expression levels in Th cell clones (Fig. 4B). To provide additional proof of the importance of the IL-2 signaling in controlling *Eomes* transcription, we ectopically overexpressed Musculin via lentiviral vector in non-classic Th1 cell lines and monitored *Eomes* mRNA levels. No significant changes in *Eomes* expression were observed (data not shown), suggesting that IL-2 signaling is not involved in the maintenance, but rather in the *de novo* induction of *Eomes* expression. To test this, we decided to reproduce in vitro the non-classic Th1 cell development by exposing Th17 cells to exogenous IL-12. To prevent the physiological downregulation of Musculin during the phenotype shift from Th17 to non-classic Th1, we decided to overexpress Musculin in Th17 cells via lentiviral approach. Cells were then stimulated in vitro with IL-12 to favor their phenotype shift toward Th1. The data showed that Th17 cells overexpressing Musculin, thus exhibiting reduced IL-2 sensitiveness, displayed a significant reduction in *Eomes* mRNA transcription (Fig. 4C).

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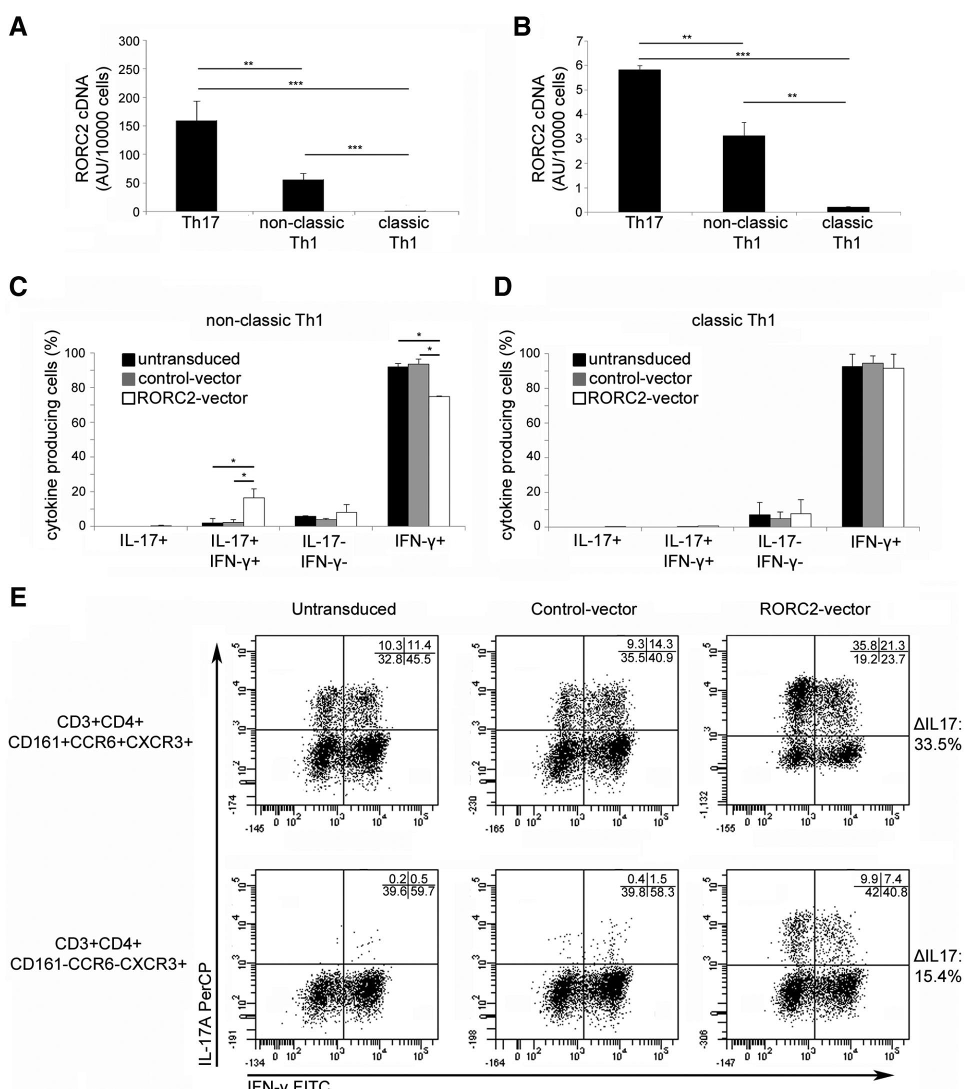

Figure 2. ROR-γt levels are critical to allow IL-17 re-expression by non-classic Th1 cells (A) *RORC2* mRNA levels were evaluated by qPCR on 20 T cell clones for each of the indicated phenotypes and normalized on cell number. T cell clones of all the phenotypes were obtained from three different donors in different experiments and frozen for mRNA expression studies. Data are presented as mean+SE and are pooled from three independent experiments. $^{**}p<0.01$; $^{***}p<0.001$ using Student's *t*-test. (B). *RORC2* mRNA levels were evaluated by qPCR on the indicated T cell subsets freshly sorted ex vivo from PB of three different donors in three independent experiments and normalized on cell number. Data are presented as mean +SE and and are pooled from three independent experiments$^{**}p<0.01$; $^{***}p<0.001$ using Student's *t*-test. (C) Non-classic Th1 and (D) classic Th1 cell clones were transduced with control- or RORC2-expressing lentiviral vectors, cultured for two weeks and then assessed by flow cytometry for cytokine production upon PMA plus Ionomycin stimulation. Three independent experiments were performed on 3 distinct non-classic and 3 distinct classic-Th1 clones. Mean percentages of cytokine producing cells from three independent experiments +SE are depicted and are pooled from three independent experiments. $^{*}p < 0.05$ using Student's *t*-test. (E) Freshly ex vivo sorted non-classic ($CD3^+CD4^+CD161^+CCR6^+CXCR3^+$) and classic ($CD3^+CD4^+CD161^-CCR6^-CXCR3^+$) Th1 cells obtained from PB of one donor were transduced in vitro in one single experiment with control- or RORC2-expressing lentivirus, cultured for 2 weeks and then assessed by flow cytometry for cytokine production upon PMA plus Ionomycin stimulation. The difference in the percentage of IL-17 producing cells between RORC2- and control-vector transduced cells (ΔIL17) for both cell subsets is indicated.

Altogether, these data support the concept that IL-2 and IL-12 combined activity is critical to ensure proper *Eomes* transcription.

Eomes can repress *RORC2* and *IL17A* in Th17 cells

By quantitative Real-Time PCR on several Th cell clones, we observed a negative correlation between *RORC2* and *Eomes* (Supporting Information Fig. 3A) as well as between *IL17A* and *Eomes* (Supporting Information Fig. 3B). Moreover, single cell analysis of expression of Eomes, IFN-γ and IL-17 by flow cytometry by peripheral blood $CD4^+$ T cells from healthy donors (Supporting Information Fig. 3C) as well as $CD4^+$ T cells from the synovial fluid of JIA patients (Supporting Information Fig. 3D) confirmed that Eomes positive cells were contained within the IFN-γ^+ cell subset but not within IL-17^+ cells.

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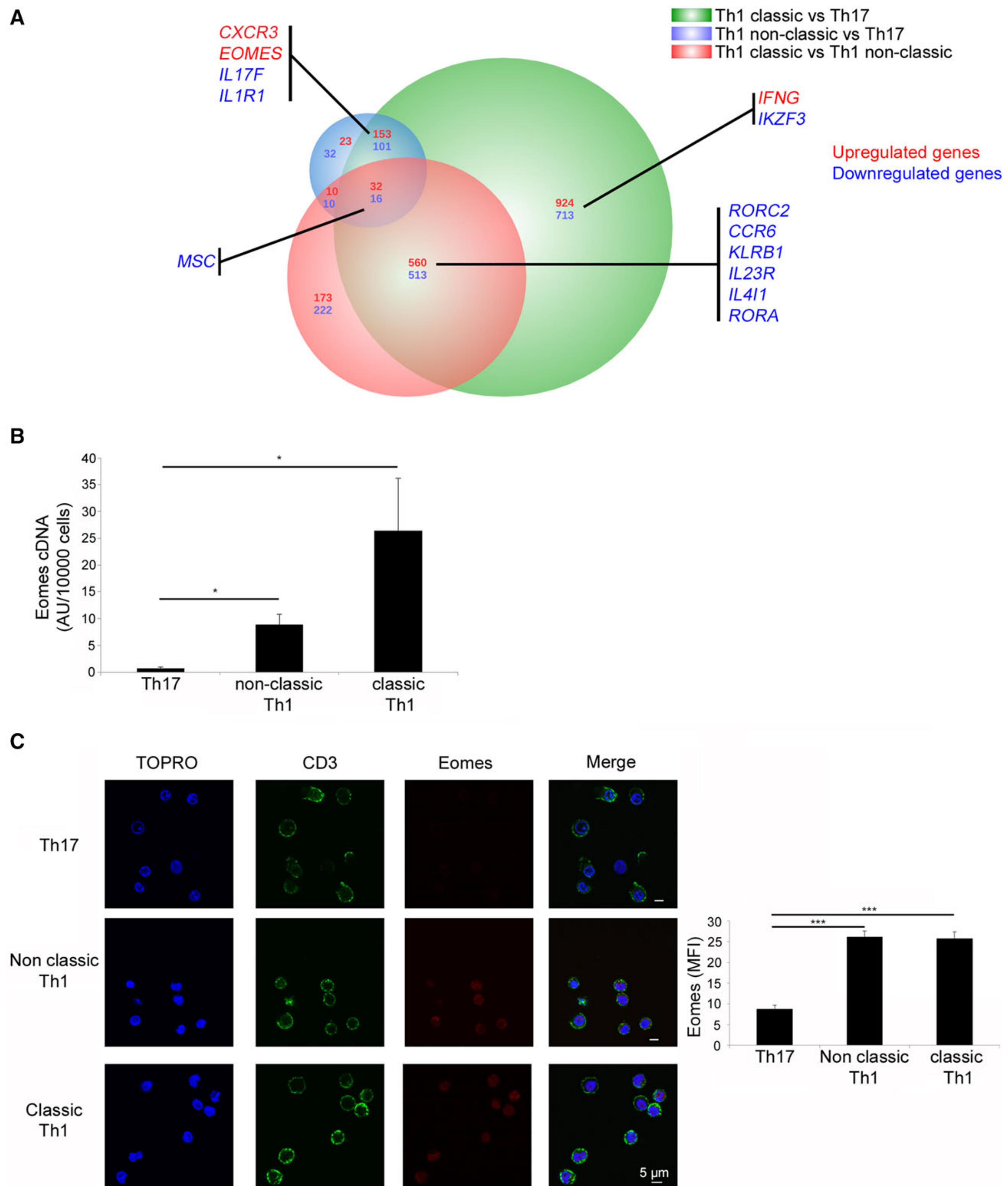

Figure 3. Eomes is selectively expressed by both subsets of Th1 cells (A) Comparison of differential expression analysis of transcriptome data from Th17, non-classic and classic-Th1 clones. The green circle contains differentially expressed genes between classic Th1 and Th17; the blue circle contains differentially expressed genes between non-classic and Th17; the red circle contains differentially expressed genes between non-classic and classic Th1. Size of the circle is proportional to the total amount of differentially expressed genes. Red numbers and names represent upregulated genes; blue numbers and names represent downregulated genes. (B) *Eomes* mRNA levels were evaluated by qPCR on the indicated T cell subsets freshly sorted from six different donors in six independent experiments and normalized on cell number. Data are presented as mean +SE and are pooled from six independent experiments. $^{*}p<0.05$ using Student's *t*-test. (C) Confocal microscopy analysis of Eomes expression (red) by Th17, non-classic and classic Th1 cell clones. Cell membranes were identified by CD3 staining (green) while nuclei were stained with TOPRO dye (blue). Image of one representative clone out of 3 evaluated for each phenotype in 3 independent experiments is shown. Scale bar, 5 μm. On the right, mean fluorescence intensity of Eomes expression pooled from three different experiments +SE $^{***}p<0.001$ using Student's *t*-test.

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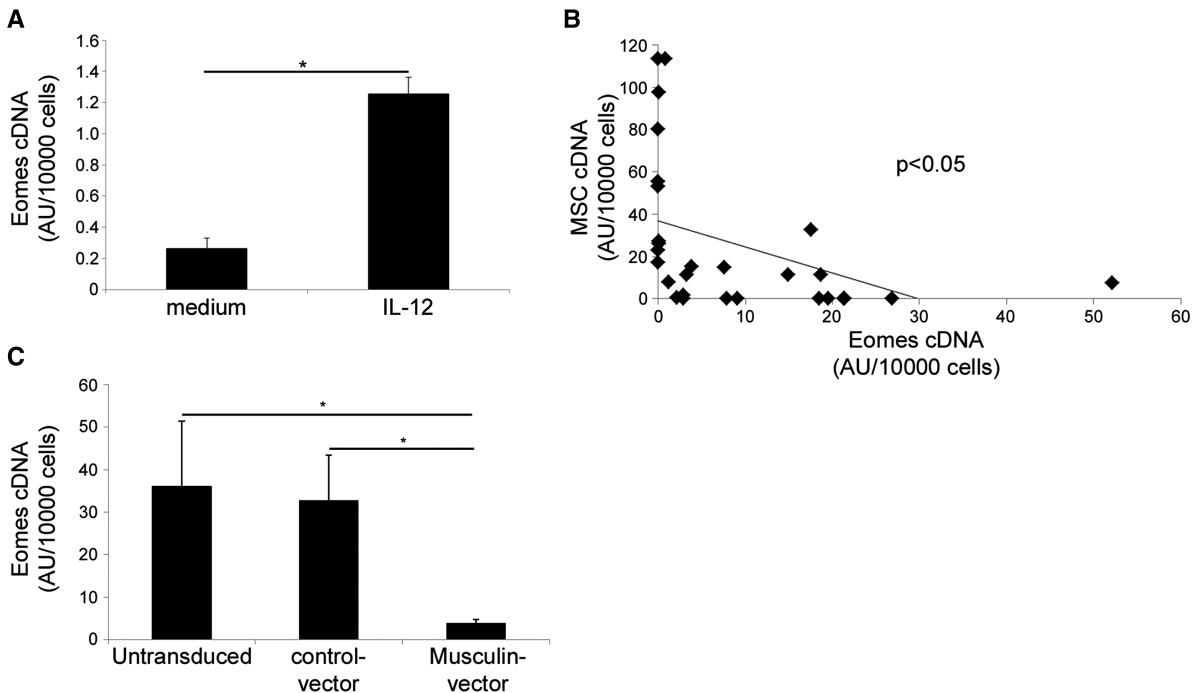

Figure 4. IL-2 and IL-12 cooperate for *Eomes* transcription initiation (A) *Eomes* mRNA levels were evaluated by qPCR in Th17 cell clones stimulated in vitro with allogenic feeder cells and IL-2, with or without exogenous IL-12 as indicated. 4 independent experiments on 4 different clones were performed. Normalization was performed on cell number. Data are presented as mean +SE and are pooled from four independent experiments. *$p<0.05$. (B) Pearson's correlation between *Eomes* and *MSC* mRNA levels evaluated by qPCR in 28 T helper cell clones calculated. T cell clones from three different donors were frozen for mRNA evaluation in different experiments. (C) PB-sorted Th17 cell lines untransduced or transduced with control- or Musculin-expressing lentiviral vector were stimulated with exogenous IL-2 and IL-12 for two weeks and then evaluated for *Eomes* mRNA expression by qPCR. Cells were collected from three donors and transduced in three independent experiments. Normalization was performed on cell number. Pooled data are presented as mean +SE. *$p<0.05$ using Student's *t*-test.

To understand the biological role of Eomes in differentiated human Th cells, we analyzed the transcriptomes in Th17 cells ectopically overexpressing Eomes. As shown in Supporting Information Fig. 3, forced expression of Eomes induced a significant alteration in the gene expression profile, reminiscent of a Th1 signature. Of note, genes encoding for cytotoxic molecules such as perforin and granzymes were upregulated in Eomes-overexpressing cells (Fig. 5A and Supporting Information Fig. 4). In parallel, genes associated to the Th17 phenotype were repressed (Fig. 5A). By Real-Time qPCR we confirmed significant *Eomes* mRNA levels only in Eomes-vector transduced cells, if compared to untransduced or control-vector transduced cells (Fig. 5B). In parallel, we noticed in Eomes-overexpressing cells a significant reduction in *RORC2* (Fig. 5C) and *IL17A* (Fig. 5D) mRNA levels, together with an increase in those of *IFNG* (Fig. 5E). Flow cytometric analysis revealed that Eomes-vector transduced subset exhibited a significant reduction in the percentage of IL-17 single producing cells. In parallel, we observed an increase in the percentage of cells producing IFN-γ alone and of cells producing neither IL-17, nor IFN-γ (Fig. 5F). After one month of in vitro culture, Eomes vector-transduced cells had lost almost entirely the capacity to secrete IL-17, while untransduced cells or control-vector transduced cells had maintained the original Th17 phenotype (Fig. 5G). Altogether these data confirm that Eomes can selectively repress the expression of Th17 associated genes, and favor that of *IFNG*, thus stabilizing the Th1 phenotype. Finally, chromatin immunoprecipitation experiments performed with an anti-Eomes antibody on Eomes-overexpressing Th17 cells, showed that Eomes binds directly to *RORC2* and *IL17A* to regulate their expression (Fig. 5H).

Eomes controls the expression of *RORC2* and *IL17* also in an inflammatory setting in vivo

To confirm the role of Eomes in controlling the expression of Th17 associated genes in mature effector T cells also in vivo, we isolated naïve T cells from Eomes$^{fl/fl}$xRosa26:CreERT2 mice and we activated them in vitro in the presence of IL-6, IL-23 and TGF-β to obtain Th17 cells (Supporting Information Fig. 6A). Despite a subset of cells were not producing IL-17 upon PMA/Ionomycin restimulation, Th17 skewing was supported by homogenous ROR-γt expression and contemporary absence of IFN-γ and Tbet (Supporting Information Fig. 6B). Polarized cells were then transferred into recipient Rag1$^{-/-}$ mice to induce T cell-dependent colitis. Previous studies have already demonstrated that transferred Th17 cells polarize toward Th1 in vivo, and only after this conversion they acquire full pathogenicity [20]. Directly after Th17 cell transfer and for the following 4 days, recipient mice were treated with vehicle or Tamoxifen to induce *Eomes* knockdown. With this

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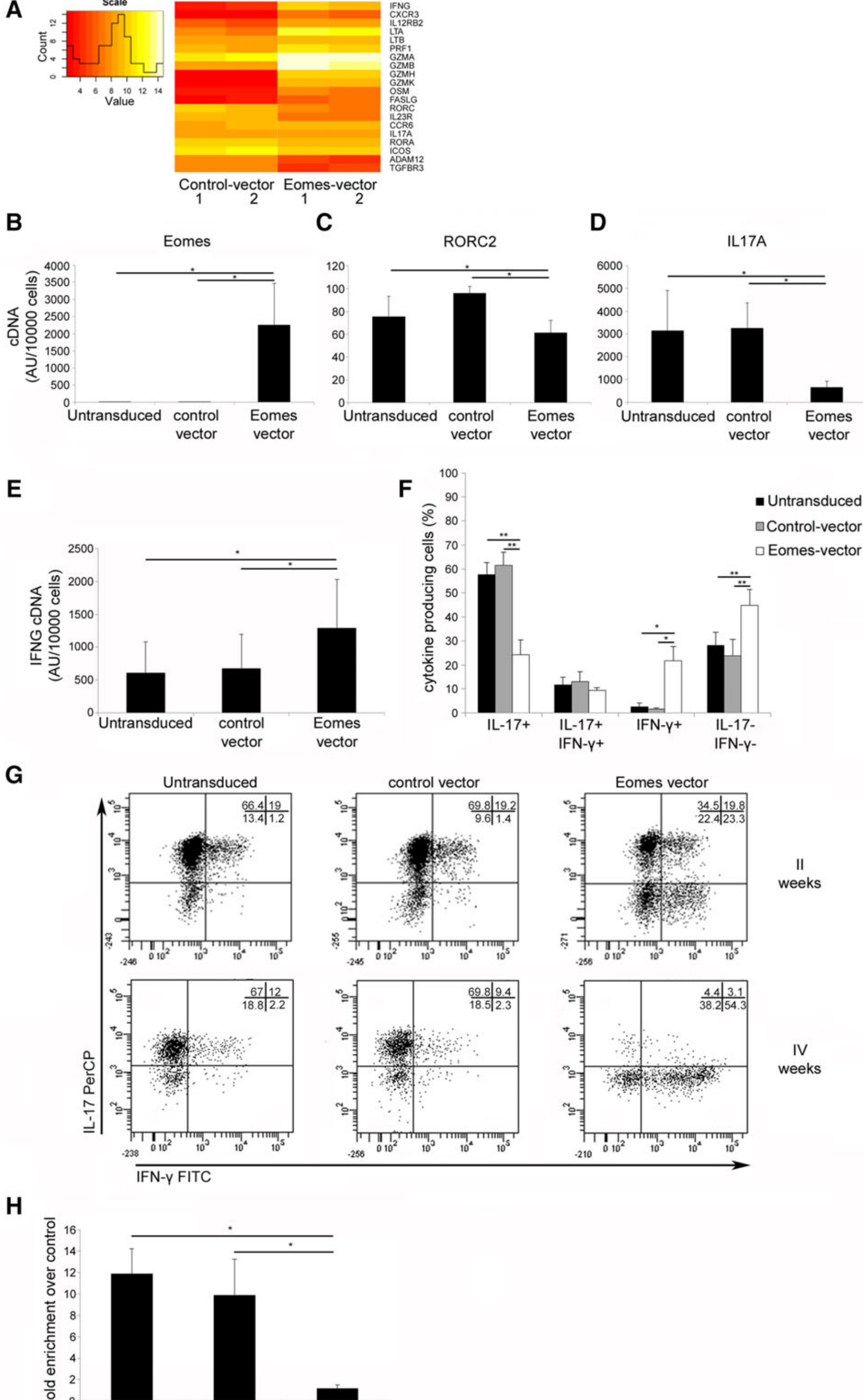

Figure 5. Eomes contributes to non-classic Th1 cell phenotype maintenance by inhibiting the expression of *RORC2* and *IL17A* and favoring that of *IFNG* (A) Hierarchical clustering-assisted heatmap analysis on selected genes from transcriptome data of control-vector or Eomes-vector transduced Th17 cells. One Th17 cell line was transduced either with control- or Eomes-vector. Two technical replicates for each subset are shown. Cells were collected in a single experiment. Expression levels vary from red (low) to white (high). Untransduced, control- or Eomes-vector transduced Th17 cell lines were evaluated for *Eomes* (B), *RORC2* (C), *IL17A* (D) and *IFNG* (E) mRNA expression by qPCR after anti-CD2+anti-CD3+anti-CD28 stimulation. Normalization was performed on cell number. Six independent experiments on cells collected from six different donors were performed. Pooled data are presented as mean +SE. *$p<0.05$ using Student's *t*-test. (F) Untransduced, control- or Eomes-vector transduced Th17 cell lines were evaluated by flow cytometry for IL-17 and IFN-γ production upon PMA plus Ionomycin stimulation. Six independent experiments were performed. Pooled data are presented as mean +SE. *$p < 0.05$; **$p < 0.01$ using Student's t-test. (G) Representative plot of cytokines production from one experiment in (F) 2 or 4 weeks after infection. (H) Chromatin Immunoprecipitation was performed with an anti-Eomes antibody on Eomes-vector transduced Th17 cells followed by qPCR amplification of *RORC2*, *IL17A* and *IL2* genes. Pooled data from four independent experiments are presented as fold enrichment of immunoprecipitated DNA over the negative control, +SE. *$p < 0.05$ using Student's *t*-test.

experimental setting we aimed to understand if Eomes plays a role in Th1 cell development from Th17 also in vivo, and if it has a role in the acquisition of the pathogenic potential of Th1 cells in colitis. Colitis progression was comparable over time between the two groups, as confirmed by the body weight-loss curves (Fig. 6A). However, Eomes ablation by Tamoxifen treatment significantly reduced absolute numbers of colon-infiltrating T cells, suggesting reduced inflammation (Fig. 6B). Of note, this was not due to a toxic effect of the drug, since a higher percentage of Ki-67^{+} CD4^{+} T cells was observed ex vivo in the group of Tamoxifen-treated mice when compared to the control group (Supporting Information Fig. 6E). These data were confirmed by a reduced

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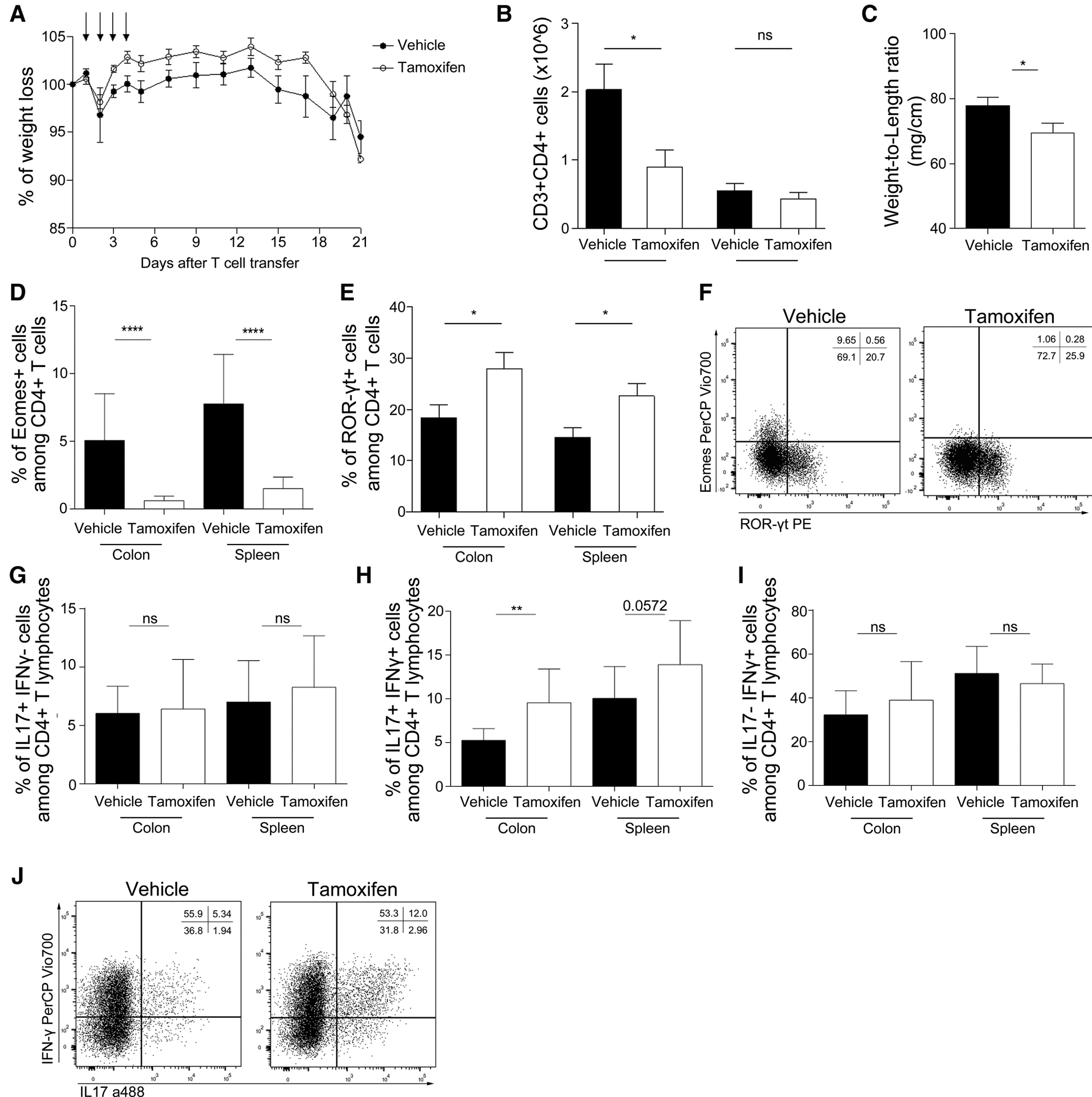

Figure 6. Eomes controls non-classic Th1 phenotype development also in an inflammatory disease setting in vivo (A) Weight loss curve of colitic RAG-1$^{-/-}$ mice after intravenous injection of in vitro differentiated Th17 Eomes$^{fl/fl}$xRosa26:CreERT2 cells. Arrows indicate tamoxifen or vehicle injections. (B) Absolute cell numbers of CD3$^+$CD4$^+$ T lymphocytes in colon and spleen of colitic RAG-1$^{-/-}$ mice that received tamoxifen or vehicle, 21 days after T cell transfer. (C) Weight-to-Length ratio expressed as mg/cm of colons isolated from colitic RAG-1$^{-/-}$ mice that received tamoxifen or vehicle, 21 days after T cell transfer. (D) Frequency of Eomes$^+$ cells among CD3$^+$CD4$^+$ T lymphocytes in colon and spleen of colitic RAG-1$^{-/-}$ mice that received tamoxifen or vehicle, 21 days after T cell transfer (E) Frequency of ROR-γt$^+$ cells among CD3$^+$CD4$^+$ T lymphocytes in colon and spleen of colitic RAG-1$^{-/-}$ mice that received tamoxifen or vehicle, 21 days after T cell transfer. (F) Representative FACS-plot of Eomes versus ROR-γt expression among CD3$^+$CD4$^+$ T lymphocytes in vehicle- or Tamoxifen-treated mice. Frequency of IL-17$^+$IFN-γ$^-$ (G), IL-17$^+$IFN-γ$^+$ (H) or IL-17$^-$IFN-γ$^+$ (I) cells among CD3$^+$CD4$^+$ T lymphocytes upon PMA/Ionomicin re-stimulation in colon and spleen of colitic RAG-1$^{-/-}$ mice that received tamoxifen or vehicle, 21 days after T cell transfer. (J) Representative FACS-plot of IL-17 vs IFN-γ expression among CD3$^+$CD4$^+$ T lymphocytes in vehicle- or Tamoxifen-treated mice. Data in (A) are representative of two independent experiments, each with 5 to 6 mice per group. Data in (B) - (J) represent two pooled experiments, each with 5 to 6 mice per group. Data are shown as mean ± SEM. * = $p \leq 0.05$, as determined by unpaired t-test with Welch´s correction or Mann–Whitney.

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weight to length ratio of colons in colitic mice upon Eomes deletion (Fig. 6C). Moreover, histological analysis showed a reduced submucosal infiltration, despite all the other parameters were not different among the two groups (Supporting Information Figs. 6C and 6D). Colon- and spleen-infiltrating lymphocytes of Tamoxifen-treated mice exhibited a marked reduction of Eomes expression (6D). Strikingly, we observed a significant enrichment of ROR-γt$^+$ cells, both in the colon and the spleen of Tamoxifen-treated mice as compared to vehicle-treated group (Fig. 6E). In line with this, the frequency of total IL-17 producing cells in Tamoxifen-treated group was increased in the colon, with a similar trend also in the spleen, compared to the control group. Of note, this difference was not due to an enrichment of IL-17$^+$IFN- γ^- but of IL-17$^+$IFN- γ^+ cells in Eomes-ablated mice (Fig. 6G-H). The IL-17$^-$IFN-γ^+ subset was not affected by Eomes deficiency (Fig. 6I). All toghether these data suggest that Eomes is primarily involved in the inhibition of the expression of Th17 associated-genes rather than in the initial IFN-γ production. In agreement, previous observations have shown that several redundant molecular pathways which are involved in *IFNG* gene expression do exist [24]. Finally, myeloid cell compartment was not affected by Eomes deletion during colitis (Supporting Information Figs. 6F, 6G, 6H, 6I). Altogether, these data confirm that Eomes represses IL-17 and ROR-γt expression also in vivo and confers proinflammatory features to Th1 cells.

Eomes drives the generation GM-CSF$^+$IFN-γ^+ cells that are enriched in the joints of JIA patients

It has been reported that Eomes may favor the development of pathogenic GM-CSF$^+$IFN-γ^+ cells in the mouse model of multiple sclerosis [33]. Indeed, we observed a positive correlation between Eomes and GM-CSF mRNA levels in Th cell lines (Fig. 7A). Accordingly, we found significantly higher GM-CSF levels in both non-classic and classic Th1 cells, if compared to Th17, both at mRNA (Fig. 7B) and protein level in culture supernatants (Fig. 7C). Finally, we observed a significant increase in GM-CSF mRNA levels in Th17 cells upon Eomes transduction (Fig. 7D), thus confirming that Eomes is involved in GM-CSF secretion by Th cells. Chromatin immunoprecipitation experiments performed on Eomes-overexpressing Th17 cells failed to demonstrate direct binding of Eomes on *CSF2* gene, in agreement with published data in mouse [33], suggesting that Eomes indirectly favors GM-CSF production. To verify whether pathogenic GM-CSF$^+$IFNγ^+ cells are present at sites of inflammation in humans, we enrolled four JIA patients and we analyzed Eomes, IFNγ and GM-CSF expression in peripheral blood and synovial fluid-infiltrating cells. Simultaneous staining of Eomes and GM-CSF was not technically possible due to the harsh fixation procedure required for transcription factor analysis which presumably alters the antibody-specific epitope of the GM-CSF protein. However, we observed a significant enrichment of both Eomes$^+$IFNγ^+ and GM-CSF$^+$IFNγ^+ cells in the synovial fluid than in PB confirming the possible involvement of these cell subsets in disease induction and maintenance (Fig. 7E and F).

Discussion

T cell plasticity is a matter of debate, since the concept of “cell polarization”, previously attributed only to naïve uncommitted cells, has more recently been extended to mature effector cells as a consequence of microenvironmental changes. Th17 cells are the most meaningful example of plasticity since these cells can be easily induced to acquire the ability to produce both IFN-γ and IL-4 if exposed to appropriate stimulation [34]. This is a widely accepted concept, since there are several data, both at cellular and epigenetic level, supporting it both in humans and mice [11, 14, 15, 18, 35]. By contrast, Th1 cells are commonly thought to be more phenotypically stable. However, a report by Liu et al [36] suggests that Th1 cells from mice possessing a transgenic TCR specific for the microbiota antigen CBir1 flagellin may acquire the ability to produce IL-17 in the gut upon injection in Rag1$^{-/-}$ mice. It must be noted that all the experimental data were generated using in vitro polarized cells, thus arising some concerns regarding the phenotype stability of the cells before their transfer.

In the studies reported so far, to our knowledge, nobody has considered the existence of two distinct subsets of Th1 cells, classic and non-classic, which may exhibit different plasticity toward the Th17 phenotype. By contrast, our data support the idea that, at least in humans, these two Th1 subsets may really exhibit different plasticity. Indeed, we show here that non-classic Th1 cells display an intermediate gene expression profile between Th17 and classic Th1 cells. Moreover, our previous observation showed a similar behavior also at epigenetic level. Non-classic Th1 cells display hypomethylation at both *RORC2* and *IL17A*, that instead are fully methylated in classic Th1 cells [16]. Here we further investigated the molecular mechanisms that control the generation of non-classic Th1 from Th17 cells, and we provided more convincing evidence regarding the possibility of the opposite plasticity, i.e. the capacity of Th1 cells to acquire IL-17 production.

We demonstrated that, despite the similarities with the Th17 subset, non-classic Th1 cells cannot be induced to produce IL-17 if cultured in presence of Th17-polarizing cytokines. This is at least partially due to their reduced ROR-γt levels. Indeed, *RORC2* forced expression allowed non-classic Th1 cells to restore IL-17 production. Of note, with this experimental approach we were able to induce *de novo* IL-17 expression also by classic Th1 cells, despite at lower levels than by non-classic Th1. These findings suggest the possibility that certain microenvironmental conditions capable of promoting high ROR-γt expression may be able to drive the polarization of Th1 cells toward Th17. This scenario is presently unknown and will require additional investigations.

We also found here that a molecular machinery dependent on the transcription factor Eomes is activated in Th1 cells and is responsible for their phenotype development and maintenance. The role of the transcription factor Eomes in CD4$^+$ T cells has been underestimated so far, mainly because of its lower expression levels compared to CD8$^+$ T cells. Moreover, most of the functional data in the literature are confined to the mouse setting, with a profound lack of information regarding its role in humans. Our present findings show that Eomes favors the phenotype shift of

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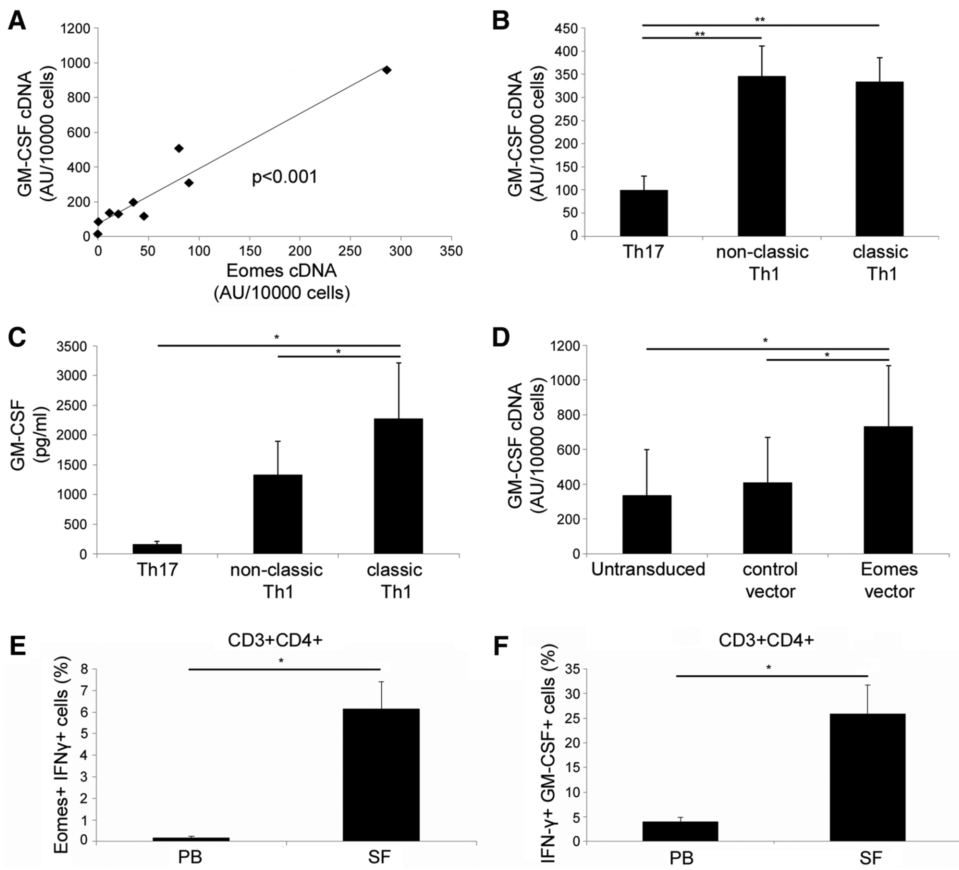

Figure 7. Eomes expressing cells display high GM-CSF levels and are enriched in the SF of JIA patients (A) Pearson's correlation between *GMCSF* and *Eomes* mRNA levels evaluated by qPCR on nine T cell lines obtained from three donors in independent experiments. (B) *GMCSF* mRNA levels were evaluated on 7 Th17, 7 non-classic and 7 classic Th1 clones in 7 independent experiments. Pooled data are presented as mean +SE. $^{**}p < 0.01$. (C) GM-CSF protein levels were evaluated in the supernatants of 6 Th17, 6 non-classic and 6 classic-Th1 cell clones in 6 independent experiments. Pooled data are presented as mean +SE. $^{*}p < 0.05$ using Student's t-test. (D) *GMCSF* mRNA levels were evaluated on untransduced, control- or Eomes-vector transduced Th17 cells. Data are pooled from four independent experiments (same as in 5B-E) +SE. $^{*}p < 0.05$ using Student's t-test. (E) The frequency of $Eomes^{+}IFN\text{-}\gamma^{+}$ CD4 T cells in the PB and SF of JIA patients was evaluated by flow cytometry. Data are presented as mean +SE pooled from four patients. $^{*}p < 0.05$ using Student's *t*-test. (F) The frequency of $GM\text{-}CSF^{+}IFN\text{-}\gamma^{+}$ CD4 T cells in the PB and SF of JIA patients was evaluated by flow cytometry. Data are presented as mean +SE pooled from four patients. $^{*}p < 0.05$ using Student's *t*-test.

Th17 cells toward non-classic Th1 by both reinforcing IFN-γ production and inhibiting the expression of *RORC2* and *IL17A* genes. In agreement with these data non-classic Th1 cells, expressing high levels of Eomes, display significantly reduced ROR-γt levels than Th17 cells and complete absence of IL-17 secretion.

It has previously been reported that inhibition of *Eomes* gene expression via a TGF-β-mediated mechanism during murine naïve T cell polarization toward Th17 is fundamental to achieve proper ROR-γt and IL-17 expression [27]. Our data show that Eomes is critical to inhibit the Th17 program also in mature effector cells. This is of importance because Tbet, the master Th1 transcription factor, seems to lack such an inhibitory effect on *RORC2* and *IL17A* since it is expressed at discrete levels by both human Th17 and Th17/Th1 cells [11]. Moreover, Th17/Th1 cells display absence of Eomes expression, which suggests that several other mechanisms are involved in the first wave of IFN-γ production, while its activity seems to be crucial for the inhibition of typical Th17 genes. Of note, in the accompanying paper by Gruarin et al., the authors show that also in human Tr1 cells Eomes is critical to inhibit the expression of RORC and Th17-associated cytokines, while promoting IFN-γ.

In agreement with findings on human cells, we also show that even in mice Eomes can regulate the development of Th1 from Th17 cells. Indeed, upon transfer of Th17 cells in recipient $Rag1^{-/-}$ mice, which leads to T cell phenotype shift toward Th1 and colitis development, we observed higher frequencies of IL-17 and ROR-γt positive cells both in the colon and the spleen in the *Eomes*–deficient group. Although a subset of the cells was not producing IL-17 upon PMA-Ionomycin stimulation prior transfer, the entire population exhibited ROR-γt expression, thus suggesting their Th17-skewing. In this experiment we also noted reduced signs of colon inflammation, as suggested by reduced weight to length ratio when compared to control mice. In agreement with this, we observed a significant reduction of total $CD4^{+}$ T cells than the control group, despite comparable numbers in the spleen. This effect was not related to a drug-related toxic effect, since $CD4^{+}$ cells from Tamoxifen-trated mice displayed even a higher percentage of Ki-67 expression than the control group. This data was

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confirmed also by histological analysis, which showed a reduced submucosal infiltrate in mice with *Eomes* deficient T cells. Thus, Eomes is involved in the acquisition of a proinflammatory potential by Th cells in colitis. These data open new possibilities for further investigation, since Eomes may control not only the cell phenotype but also cell proliferation, survival and/or recruitment to inflamed tissues. Indeed, it is already known that Th1 cells display higher proliferation rate upon TCR stimulation if compared to Th17 cells [37].

Another interesting observation of this study was that IL-2 and IL-12 cooperate for *Eomes* expression in Th17 cells. We have shown already that IL-2 signaling is impaired in Th17 cells due to the activity of the transcription factor Musculin, which selectively dampens the STAT5B pathway. Moreover, Musculin is downregulated by non-classic Th1 cells [31]. Here we found that Musculin forced expression during non-classic Th1 development from Th17 cells significantly affects the induction of *Eomes* gene transcription. In agreement with these observations, it has been demonstrated that *Eomes* is a selective target of STAT5B downstream of IL-2 stimulation [32]. We may speculate that the reduced IL-2 sensitiveness of Th17 cells is important for a mechanism of cell phenotype protection and maintenance, aimed to keep high IL-17 levels via the impairment of *Eomes* expression. Thus, proper IL-2 sensitiveness must be achieved to shut down *IL17A* transcription. A negative role for IL-2 signaling on IL-17 expression has already been proposed, due to the STAT5 occupancy of the *IL17A* promoter, that is no longer available for binding of the transcription-promoting factor STAT3 [38]. Our finding that *Eomes* expression is prevented in Th17 cells via the inhibition of the IL-2 pathway provides an additional rationale for the documented inhibitory activity of IL-2 on the Th17 phenotype.

It has been proposed that Eomes may drive the development of pathogenic Th1 cells in a mouse model of multiple sclerosis by favoring the contemporary secretion of IFN-γ and GM-CSF [33]. Our data on human cells show that both classic and non-classic Th1 cells secrete higher GM-CSF levels than Th17, and Eomes is involved in this capacity as shown by its overexpression performed in Th17 cells. In this study, we had also the possibility to analyze cells from a human inflammatory setting, i.e. the synovial fluid of JIA patients. A significant enrichment of both Eomes$^+$IFN-γ^+ and IFN-γ^+GM-CSF$^+$ cells in the synovial fluid compared to the peripheral blood was observed, thus confirming the possible involvement of Eomes$^+$-classic and Eomes$^+$-non-classic Th1 cells in disease initiation and progression. Thus, our findings are consistent with previous data suggesting that Eomes can drive the development of pathogenic cells [39]. In line with this, and with our colitis experiments, our transcriptome data show that Th17 cells ectopically overexpressing Eomes acquire a cytotoxic potential via upregulating perforin and granzymes levels.

In conclusion, our findings indicate that the phenotype shift from Th17 to Th1 is tightly controlled at molecular level. Eomes plays a role in this scenario, by reinforcing IFN-γ production and selectively inhibiting the expression of *RORC2* and *IL17A*. This concept is of particular importance for chronic inflammatory disorders and autoimmunity. Indeed, there are now accumulating evidences in the literature suggesting that Th17 cells acquire higher pathogenicity after conversion to Th1 [19, 20, 40]. For this reason, new therapeutic strategies for such chronic inflammatory disorders may be directed to prevent the conversion of Th17 cells into Th1. In this view, some biological drugs acting on factors known to favor this phenotype shift are already available. Indeed, the anti-p40 mAb Ustekinumab that contemporary neutralizes both IL-12 and IL-23, may work preventing the Th1 conversion of Th17 cells [41]. On the other side, finding a way to promote the reversion of non-classic Th1 cells toward Th17 may also be beneficial. The results of the present study suggest that this route is complex, and difficult to achieve, due to the existence of a molecular machinery which includes the transcription factor Eomes and that creates a lock to cell phenotype changes. In this view, a recent study has shown the possibility to attenuate the asthmatic response after allergen exposure in asthmatic patients via using a new gene targeting approach which allowed to specifically inhibit *GATA3* expression [42]. For this reason, the identification of all the actors that collectively favor generation and stabilization of Th1 cells from Th17 is crucial. Understanding the molecular networks that stabilize the Th1 phenotype may suggest the development of drugs to specifically target those molecules.

Materials and methods

Subjects

Peripheral Blood (PB) samples were obtained from healthy donors. Synovial Fluid (SF) and paired PB samples were collected from JIA patients. The procedures followed in the study were in accordance with the ethical standards of the Regional Committee on Human Experimentation.

Mice

RAG1$^{-/-}$ and Eomes$^{fl/fl}$xRosa26:CreERT2 mice were housed and bred under SPF conditions. Before Th17 cell transfer and colitis induction, RAG1$^{-/-}$ mice were tested for positivity to SFB and Helicobacter species (H. spp) via PCR on fecal DNA with the following primer pairs: H. spp [Forward: 5'-ctatgacgggtatccggc-3', Reverse: 5'-attccacctacctctccca-3′]; SFB [Forward: 5′-gacgctgaggcatgagagcat-3', Reverse: 5'-gacggcacggattgttattca-3']. Experiments were performed according to institutional guidelines and German Federal laws on animal protection.

Reagents

The medium used was RPMI 1640 (Seromed, Berlin, Germany), supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 2 × 10–5 M 2-mercaptoethanol (2-ME) (all

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from Gibco Laboratories, Grand Island, NY), and 10% FCS (Euroclone). Unlabelled or flurochrome-conjugated anti-CD3, CD4, CD8, CD161, CCR6, CD271, IFN-γ, GM-CSF, isotype-matched control mAbs were from BD Biosciences (Mountain View, CA). Flurochrome-conjugated anti-IL-17 and anti-Eomes mAbs were from eBiosciences (San Diego, CA). PE-Cy7-conjugated CD161 was from (Miltenyi Biotec, Bergisch Gladbach). Flurochrome-conjugated anti-CXCR3 mAbs were from R&D System. Phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and saponin were from Sigma Aldrich Co. (St. Louis, MO). Recombinant human IL-1β, IL-6, IL-12, IL-23, TGF-β were purchased from R&D Systems.

T cell recovery and expansion

Mononuclear cell (MNC) suspensions were obtained from PB and SF by centrifugation on Ficoll-Hypaque gradient. PB CD4$^+$ T cells were negatively selected by high-gradient magnetic cell sorting (Miltenyi Biotec), as previously described [17] and were then further divided into CD161$^+$ and CD161$^-$ by immunomagnetic cell sorting. PB CD4$^+$CD161$^+$ and CD4$^+$CD161$^-$ T cell population were further subdivided in CCR6$^+$ and CCR6$^-$ cell fractions by FACSAria (BD Biosciences). PB CD4$^+$CD161$^+$CCR6$^+$ and CD4$^+$CD161$^-$CCR6$^-$ cell lines were then cultured under limiting dilution (0.3 cell/well) in the presence of 10^5 irradiated (9000 rad) allogeneic PBMNCs as feeder cells, 1% PHA (vol/vol), and 50 U/mL rhIL-2 (Proleukin, Prometheus, Inc., San Diego, USA) in order to obtain Th17, non-classic Th1 and classic Th1 clones respectively. Recovered clones were classified by flow cytometry for intracellular staining of IL-17 or IFN-γ cytokines. Production of cytokines by each clone was arbitrarily considered as noteworthy when the proportion of producing T cell blasts was $< 20\%$. [11, 13]. PB CD4$^+$CD161$^-$CCR6$^-$ were also FACS enriched for CXCR3 expression to obtain classic Th1 cell polyclonal population. PB CD4$^+$CD161$^+$CCR6$^+$ were further divided in CXCR3$^+$ and CXCR3$^-$ by FACS to obtain non-classic Th1 and Th17 polyclonal cell lines, respectively.

Cytokine secretion assay

PB CD4$^+$ T cells were negatively selected by high-gradient magnetic cell sorting and further divided into CCR6$^+$ and CCR6$^-$ cells by immunomagnetic cell sorting, then were stained with anti-CD161 PE-Cy7, stimulated with PMA-ionomycin, recovered after 3 and half hours, washed and then stained with IFN-γ and IL-17 catch reagents (Miltenyi Biotec), following manufacturer's instructions. Following additional 45 min of incubation (37°C, 5% CO2) cells were stained with anti-CD3-Pacific Blue, -IL-17-APC and -IFN-γ-FITC, analyzed and sorted by FACSAria in order to collect CD4$^+$CCR6$^+$CD161$^+$ IL-17$^-$IFN-γ$^+$ T cells.

In vitro T cell stimulation with exogenous cytokines

Non-classic Th1 cells, either T cell clones or freshly ex vivo sorted, were stimulated with anti-CD2, anti-CD3, anti-CD28 coated beads (Miltenyi) in the absence or in presence of recombinant IL-1β (10 ng/mL), IL-6 (2 ng/mL), IL-23 (20 ng/mL), TGF-β (5 ng/mL) (all from R&D Systems) for one or two weeks. Following, cells were harvested and restimulated in vitro with PMA (10 ng/mL) and Ionomycin (1 μM) for 5 h total, the last 3 h in the presence of Brefeldin A (5 μg/mL). Cells were finally fixed with 2% formaldehyde, stained and analyzed by flow cytometry on BD LSR II machine. Th17 cells, either untransduced or viral vector transduced, were cultured with allogenic feeder cells in a 1:2 ratio in the absence or in presence of exogenous recombinant IL-12 (2,5 ng/mL, R&D Systems). Following two weeks in culture cells were harvested and frozen for subsequent mRNA expression investigation.

DNA methylation analysis

Genomic DNA was extracted by using DNA Blood Mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA methylation analysis was performed by Varionostic GmbH (Ulm, Germany). Briefly, genomic DNA was bisulfite converted by using EZ DNA Methylation-Gold^{TM} Kit (Zymo Research, Irvine, CA, USA) following manufacturer's instructions. Bisulfite converted DNA was then PCR amplified at the different regions of interest of *IL17A*, *IL17F*, *IFNG*, *RORC2* and *TBX21* genes by using bisulfite-DNA specific primers. PCR products were then subjected to pyrosequencing. Primer sequences for bisulfite-PCR and sequencing have already been described [16]. Methylation levels at CpG sites were obtained by using PyroQ-CpG software as previously described [43].

RNA isolation, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted by using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and treated with DNase I to eliminate possible genomic DNA contamination. RNA reverse transcription was performed with Taqman Gold kit (Thermo Fisher Scientific). Briefly, for the analysis of each gene expression, total mRNA recovered from 10^4 cells was reverse transcribed and then subjected to RT-PCR amplification. Ct values were then transformed into arbitrary units (AU) using a standard curve generated with a reference sample. Primers and probes used were purchased from Thermo Fisher.

Confocal microscopy

Th17, non-classic and classic Th1 clones were resuspended in complete medium (RPMI 1640) (2×10^5 /mL) and seeded on polylysine-coated glass slides. Cells were then incubated for 40 min at 37 °C and 5% CO_2, followed by 30 min incubation with biotin-CD3 (eBiosciences) or control isotype. Following, cells were washed in PBS pH 7.2 and incubated for 30 min with Streptavidin 488 conjugated (1:200). Cells were then washed,

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fixed in formaldehyde (4% in PBS pH 7.2), permeabilized with 0.1% Triton (Sigma) for 10 min, and incubated for 20 min at room temperature with goat serum (1 mg/mL). Cells were then incubated with an anti-Eomes (Abcam 23345, 1:100) or control isotype overnight at 4 °C. Following incubation with ab23345, cells were washed and then incubated at room temperature with anti-rabbit IgG Alexa Fluor 546 conjugated Ab for 30 min (2 μg/mL), in buffer containing TOPRO-3 dye (0.2 μM) for the nuclear counter staining. Cells were then washed in PBS for 5 min, and the slides mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlingame CA). Microscopic images were taken by a LSM 510 META Zeiss confocal microscope system (Carl Zeiss Inc., Jena, Germany), using 40X oil immersion lens, corresponding to a 400X magnification. For image analysis Confocor 2 (Zeiss) software was used.

Chromatin immunoprecipitation

Th17 cell lines overexpressing Eomes were fixed with 1% formaldehyde, followed by 0.125 M glycine incubation. Cells were subsequently lysed with SDS Lysis Buffer to release chromatin, which was then sheared by sonication. Anti-Eomes (ab23345, Abcam) was added overnight at 4 °C. Unspecific IgG was used as negative control. DNA–protein antibody complexes were immunoprecipitated via protein A beads (Thermo Fisher). Cross-linking was reversed by incubation at 65 °C for 4 h. ChIP DNA as well as input DNA was purified with DNA extraction kit (Macherey Nagel). Finally, real-time qPCR was performed to detect the abundance of *RORC2*, *IL17A* and *IL2* genes by using the following primers: RORC2-F GGAGTCCCAGCAAGATCAGA; RORC2-R TTCAGGGCC CCTCAGTATTC; IL17A-F GTGTCACCCCTGAACCCAC; IL17A-R GAGATGGACAAAATGTAGCGCT; IL2-F CCTCAACTCCTGCCA-CAATG; IL2-R CAGTAAATGCTCCAGTTGTAGCT. Genomic location of amplicon products is reported in Supporting Information Fig. 5.

Lentivirus transduction

PB-derived $CD4^{+}CD161^{+}CCR6^{+}CXCR3^{-}$ cells were activated with irradiated APCs plus anti-CD3 mAb (1 μg/mL). After 16 h, pCCL-EF1α-NGFR, pCCL-EF1α-NGFR-RORC2, pCCL-EF1α-NGFR-MSC or pCCL-EF1α-NGFR-EOMES lentivirus were added at a multiplicity of infection (MOI) of 10 and cells were inoculated at 1200 × *g*, 26 °C for 2 h. Transduced T cells were kept in culture with IL-2 (100 U/mL, Eurocetus, Italy), purified after 5 days with anti-NGFR-PE (BD Biosciences) plus anti-PE microBeads (Miltenyi Biotec) achieving purities greater than 98%, and then maintained in culture.

Microarray

Gene expression profiles on human Th1 and Th17 clones were assessed by cDNA microarray technique using the SurePrint G3 Human GE v2 8 × 60 K Microarray (Agilent Technologies) as previously described [37]. Cells were kept in culture in RPMI 1640 medium supplemented with 10% FCS and 50 IU/mL recombinanat human IL-2. Cells were then stimulated for 6 h with anti-CD2+anti-CD3+anti-CD28 coated beads. Unstimulated control cells were also prepared in absence of the beads cocktail. Total RNA was extracted using RNeasy Micro Kit (QIAGEN) following manufacturer's recommendations, quantified using a NanoDrop-1000 spectrophotometer and quality-checked with an Agilent 2100 Bioanalyzer. Total RNA was labelled with the One Color (Cyanine 3-CTP) Low Input Quick Amp Labeling Kit (Agilent Technologies) according to manufacturer protocol and hybridized to the microarray chips according to specifications in One-Color Microarray-Based Gene Expression Analysis Protocol. Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2565CA) using one color scan setting for 8 × 60 K array slides (scan resolution 2 um/pixel). Imaging data was extracted using Agilent Feature Extraction Software version 10.7.3.1 with default parameters of protocol GE1_107_Sep09. Data preprocessing was performed using R/Bioconductor (version 3.6) Agi4 × 44PreProcess (version 1.22) package. Briefly, raw Feature Extractor data was loaded into R and further background corrected (method = "half", offset = 50), normalized (method = "quantile") and filtered (wellaboveBG = 60, isfound = 60). Raw and processed data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/GEO) under the accession number GSE110230.

Gene expression profiles on Th17 cells transformed with lentivirus to overexpress *Eomes* were assessed by cDNA microarray technique using the Human Genome U133 Plus 2.0 GeneChips (Affymetrix). RNA was prepared and quality checked as described above. Labeling and hybridization was performed as per Affymetrix's standard protocols for this chip type. R/Bioconductor (version 3.6) affy package (version 1.56) was used for quality control and preprocessing (RMA). Raw and processed data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/GEO) under the accession number GSE110545.

Differential expression (DE) analysis were performed with limma package (version 3.34.6) considering DE signals with abs(logFC)>1 and adjusted (Benjamini-Hochberg) p-value < 0.05. Intersection analysis was drawn using the venn function of the gplots package (version 3.0.1).

Evaluation of cytokines in supernatants

5×10^{5} cells from three Th17, three Th1 non-classic and three Th1 classic clones were stimulated, at final concentration of 1×10^{6}/mL, with anti-CD2, anti-CD3, anti-CD28 coated beads (Miltenyi). After 3 days of culture, GM-CSF cytokine levels in supernatants were evaluated by CBA flex set assay following the manufacturer's instructions (BD Bioscience).

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Colitis induction and tamoxifen treatment

RAG1$^{-/-}$ mice that were tested positive for SFB and Helicobacter species (*H. spp*) were intravenously injected with 0,4 × 10^6 in vitro differentiated Eomes$^{fl/fl}$xRosa26:CreERT2 Th17 cells. Starting the day after the T cell transfer and for the following 4 days, RAG1$^{-/-}$ mice were intraperitoneally administered with 1 mg/mouse of tamoxifen (Sigma-Aldrich) or vehicle (sunflower oil + 10% EtOH). As indication of disease activity, weight loss and diarrhea appearance were monitored. RAG1$^{-/-}$ mice were sacrificed before losing 10% of body weight.

In vitro murine Th17 differentiation

Eomes$^{fl/fl}$xRosa26:CreERT2 mice were used as donors to obtain CD4$^+$CD62L$^+$ naïve T cells. Briefly, cells were isolated from the spleen and single cell suspension was incubated with anti-CD4-FITC (GK1.5) for 10 min on ice, washed and subsequently incubated with anti-FITC microbeads (Miltenyi), according to manufacturer´s instructions. After washing away the bound microbeads with Multisort Release Reagent (Miltenyi), the positive fraction (purity ≥ 95%) containing CD4$^+$ T cells was incubated with anti-CD62L microbeads (Miltenyi), according to manufacturer´s instructions. CD4$^+$CD62L$^+$ naïve T cells with were cultured under Th17 polarizing conditions for a total of 10 days [IL-6 (R&D) = 20 ng/mL; IL-23 (R&D) = 20 ng/mL; TGF-β (R&D) = 2ng/mL; anti-IL-4 (11B11.2) = 10 μg/mL; anti-IFN-γ (XMG1.2) = 10 μg/mL; anti-CD3 (BD Biosciences) = 1 μg/mL; anti-CD28 (BD Biosciences) = 1 μg/mL]. In order to increase the frequency of IL-17-secreting cells, at day 5 from starting of the culture, Th17 cells were collected and re-plated under the same polarizing conditions for additional 5 days. Frequency of IL-17-secreting cells prior to T cell transfer was assessed upon in vitro re-stimulation, as described.

Histopathology

Colons were fixed in 4% PFA at room temperature (RT) overnight in the "Swiss roll" conformation. Colons were then washed in PBS, embedded in paraffin and stained with hematoxylin and eosin. Histopathology was scored according to a published disease score [44].

Isolation of colonic leukocytes

Leukocytes were isolated from the colon of colitic RAG1$^{-/-}$ mice as published elsewhere [44, 45]. Briefly, colons were cut longitudinally, and fecal material was removed via washing in PBS. In order to remove the epithelial layer, colons underwent 2 rounds of incubation, each of 20 min at 37 °C, in Ca^{2+}/Mg^{2+}-free 1x HBSS supplemented with 5 mM EDTA and 10 mM HEPES. After being cut into small pieces, colons underwent 3 additional rounds of digestion, each of 20 min at 37 °C, in 1x HBSS with Ca^{2+}/Mg^{2+} supplemented with 0.5 mg/mL Collagenase D (Roche), 0.5 mg/mL DNAseI (Sigma-Aldrich), 0.5 U/mL Dispase (BD Biosciences) and 4 % FCS.

Flow cytometry and in vitro re-stimulation assay

Single cell suspensions of spleens and colons were incubated with 10 μg/mL anti-FCγRII/III (2.4G2) in FACS buffer (PBS/0.1% BSA/2mM EDTA) for 10 min at +4°C. For surface staining, cells were stained for 15 min at +4 °C with anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD64 (X54-5/7.1), anti-Ly6C (HK1.4), anti-Ly6G (1A8), anti-SiglecF (E50-2440). For staining of transcription factors, cells were first fixed for 1 h at +4 °C with Foxp3 fixation/permeabilization working solution according to the manufacturer's instructions (eBioscience), washed with 1x permeabilization buffer (eBioscience) and stained for 45 min at RT with anti-RORγt (Q31-378). For intracellular staining of cytokines, cells were first in vitro re-stimulated for 5 h at +37 °C, 5% CO2 with PMA (10 ng/mL) and Ionomycin (1 μg/mL) in complete RPMI 1640 (ThermoFisher) supplemented with 10% FCS. Brefeldin A (5 μg/mL) was added after the first 2 h. Cells were then fixed in Cytofix/Cytoperm buffer (BD Biosciences) for 15 min at RT, washed with 1x permeabilization buffer (BD Biosciences) and intracellularly stained for 45 min at RT with anti-IFN-γ (XMG1.2) and anti-IL-17A (TC11-18H10.1). Dead cells were excluded via staining with LIVE/DEAD Fixable Dead Cell Stain prior to surface staining or fixation (ThermoFisher). Stained samples were acquired on MACSQuant (Miltenyi). Flow cytometric data were analyzed with FlowJo softwares (FlowJo LLC). Flow cytometry experiments were perfomed in agreement with flow cytometry guidelines [46].

Statistics

Student's *t*-test was used for mRNA expression analysis, confocal microscopy experiments and ChIP studies. Mann-Whitney test was used for DNA methylation. p values of 0.05 or less were considered significant. Pearson's correlation coefficients were used to calculate the correlations.

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