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Rarity of Human T Helper 17 Cells Is due to Retinoic Acid Orphan Receptor-Dependent Mechanisms that Limit Their Expansion

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SUMMARY

The reason why CD4$^+$ T helper 17 (Th17) cells, despite their well-known pathogenic role in chronic inflammatory disorders, are very rare in the inflammatory sites remains unclear. We demonstrate that human Th17 cells exhibit low ability to proliferate and to produce the T cell growth factor interleukin-2 (IL-2), in response to combined CD3 and CD28 stimulation. This was due to the upregulated expression of IL-4-induced gene 1 (*IL4I1*) mRNA, a secreted L-phenylalanine oxidase, which associated with a decrease in CD3ζ chain expression and consequent abnormalities in the molecular pathway that allows IL-2 production and cell proliferation. High *IL4I1* mRNA expression was detectable in Th17 cell precursors and was strictly dependent on Th17 cell master gene, the retinoid acid related orphan receptor (*RORC*). Th17 cells also exhibited RORC-dependent CD28 hyperexpression and the ability to produce IL-17A after CD28 stimulation without CD3 triggering. Our findings suggest that the rarity of human Th17 cells in inflamed tissues results from RORC-dependent mechanisms limiting their expansion.

INTRODUCTION

In the past years, a subset of murine CD4$^+$ T helper (Th) cells showing the ability to produce IL-17A and to express the transcription factor retinoic acid-related orphan receptor (ROR)γt has been characterized and named Th17 cells (Oppmann et al., 2000; Cua et al., 2003; Ivanov et al., 2006). Analysis of gene-targeted models has suggested that Th17 cells are pathogenic in several murine models of chronic inflammatory disorders, such as experimental autoimmune encephalomyelitis (Cua et al., 2003), collagen-induced arthritis (Murphy et al., 2003), and bowel inflammatory disorders (Dubin and Kolls, 2008).

Human Th17 cells have also been described and found to be, at least partially, different from murine Th17 cells (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007). In particular, human Th17 cells express distinctive molecules such as IL-23R and RORC but they share with Th1 cells the expression of IL-12Rβ2 and TBX21 (Annunziato et al., 2007). Moreover, a subset of human IL-17A-producing CD4$^+$ T cells were found to produce also IFN-γ (which were named Th17-Th1), and both Th17 and Th17-Th1 cells exhibited plasticity toward the Th1 cell profile when cultured in presence of IL-12 (Annunziato et al., 2007). Similar findings were then observed also in some murine models (Lee et al., 2009; Kurschus et al., 2010; Hirota et al., 2011), thus suggesting that both human and murine Th17 cells probably represent a transient phenotype (Annunziato and Romagnani, 2010; Murphy and Stockinger, 2010). Recently, we and others showed that virtually all human memory Th17 cells are contained within the CD161$^+$ fraction of both circulating and tissue-infiltrating CD4$^+$ T cells (Cosmi et al., 2008; Kleinschek et al., 2009) and originate from CD161$^+$ precursors present in umbilical cord blood (UCB) and newborn thymus (Cosmi et al., 2008; Maggi et al., 2010). The murine equivalent of CD161 (the NK1.1 molecule) has not been described in mouse Th17 cells.

In humans, Th17 cells have also been implicated in the pathogenesis of chronic inflammatory disorders such as autoimmune diseases (Crohn's disease [CD], multiple sclerosis, psoriasis, rheumatoid arthritis) and allergic disorders (bronchial asthma, atopic dermatitis, and contact dermatitis) (Hu et al., 2011). However, the assessment of IL-17A-producing CD4$^+$ T cells in both inflamed tissues and biological fluids consistently revealed a low frequency of these cells in comparison with the high numbers of Th1 cells. The same observation was also done in our previous studies performed in the intestine of patients with CD and the synovial fluids (SF) of patients with juvenile

idiopathic arthritis (JIA) (Annunziato et al., 2007; Cosmi et al., 2011). The reason of the unexpected rarity of Th17 cells in the tissues and biological fluids of patients with autoimmune and chronic inflammatory disorders, despite their suggested pathogenic role, has remained unclear. One possible explanation may be that Th17 cells show a transient phenotype, resulting from their tendency to shift into Th1 cells in the context of inflammatory microenvironments (Annunziato and Romagnani, 2010; Murphy and Stockinger, 2010). However, such a hypothesis could only partially account for this phenomenon, because it is very rare that Th17 cells can be detected even in the initial phases of inflammation.

In the present study, we demonstrated that Th17, unlike Th1, cells do not proliferate in response to stimulation with anti-CD3 plus anti-CD28 (anti-CD3-CD28), mainly because of their inability to produce IL-2 and a reduced IL-2 responsiveness. Whereas the reduced IL-2 responsiveness was explained by the lack of PI3K-AKT-mTORC1 axis activation, the defective IL-2 production by Th17 cells appeared to be related to their reduced c-Fos, c-Jun, and nuclear factor of activated T cells (NF-AT) activity. The reduced activity of these transcription factors associated with high expression of the IL-4-induced gene 1 (*IL4I1*) mRNA, which encodes for a L-phenylalanine oxidase that has been shown to downregulate CD3ζ expression in T cells (Boulland et al., 2007). *IL4I1* mRNA expression was strictly RORC dependent and was already detectable in Th17 cell precursors from UBC and newborn thymus. The production of IL-17A was ensured by the RORC-dependent hyperexpression of CD28, which, upon stimulation, was able to induce NF-κB phosphorylation even in absence of CD3 triggering via a PI3K-independent pathway. These results were also confirmed on Th17 cells ex vivo derived from inflamed sites. These findings demonstrate that the rarity of human Th17 cells in inflamed tissues mainly results from RORC-dependent self-regulation of their expansion, which is probably essential to minimize their dangerous proinflammatory activity.

RESULTS

Human Th17 Cells Neither Proliferate nor Produce IL-2 in Response to Anti-CD3-CD28 Stimulation

The ability of Th17 and Th1 cell clones to proliferate when stimulated with anti-CD3-CD28 or PMA plus ionomycin (PMA-Iono) was assessed with $CD4^+CD161^+CCR6^+$ and $CD4^+CD161^-CCR6^-$ T cell populations from PB of human healthy subjects. In vitro stimulation with anti-CD3-CD28 induced strong proliferation of Th1 cell clones, whereas it was quite ineffective on the proliferation of Th17 cell clones (Figure 1A). Th17 cell clones proliferated in response to PMA-Iono, although at lower extent than Th1 cell clones (Figure 1A). IL-2 expression was then measured in the supernatants of the above-mentioned cell cultures. Th1 cell clones produced IL-2 upon either anti-CD3-CD28 or PMA-Iono stimulation. In contrast, Th17 cell clones did not produce IL-2 upon anti-CD3-CD28, but did upon PMA-Iono stimulation, at lower extent than Th1 cell clones (Figure 1B). Similar results were obtained by evaluating *IL2* mRNA expression in Th17 and Th1 cell clones (Figure 1C).

However, the addition of IL-2 in culture only partially restored the proliferation of Th17 cell clones (Figure 1D), indicating that the lack of Th17 cell proliferation in response to anti-CD3-CD28 stimulation was not only due to their reduced ability to produce this cytokine. Furthermore, the addition in culture of IL-7, IL-15, and IL-21 increased the proliferative response of anti-CD3-CD28-stimulated Th17 cell clones, but none of these cytokines was able to induce a proliferation of Th17 cells comparable to that of Th1 cells (data not shown). The reduced Th17 cell proliferation in response to anti-CD3-CD28 plus IL-2 was not due to a lower mRNA expression of *IL2RA, IL2RB*, or *IL2RG* in Th17 cell clones, either (Figure S1 available online). At this point, we investigated whether the reduced IL-2 response of Th17 cells was due to impaired signal transduction via IL-2 receptor (IL-2R). To this aim, we stimulated Th17 and Th1 cell clones with IL-2 and then assessed by flow cytometry S6 ribosomal protein (S6ribo) phosphorylation as indicator of PI3K-AKT-mTORC1 axis activation and phosphorylation of STAT5.

IL-2 stimulation induced STAT5 phosphorylation in both Th1 and Th17 cell clones, whereas S6ribo phosphorylation was found to be substantially higher in Th1 than in Th17 cell clones (Figure 1E).

Also, ex-vivo-derived PB Th17 cells, unlike Th1 cells, were unable to proliferate after anti-CD3-CD28 stimulation and the addition in culture of IL-2 restored their proliferation (Figure 1F) only partially.

Thus, Th17 cells do not produce IL-2 and do not proliferate in response to the triggering of both CD3 and CD28. The stimulation of Th17 cells with PMA-Iono (which bypasses cell surface receptors), as well as the addition of exogenous IL-2 or other T cell growth factors to anti-CD3-CD28-stimulated cells, only partially restore Th17 cell proliferation.

Human Th17 Cells Exhibit Lower *JUN*, *FOS*, and *NFATC1* mRNA Expression than Do Th1 Cells

mRNA expression of *NFKB1, RELA* (gene of NF-κBp65), *FOS, JUN*, *NFATC2,* and *NFATC1* was then analyzed in both Th1 and Th17 cell clones before and after their anti-CD3-CD28 stimulation. *NFKB1, RELA, JUN, FOS, NFATC2,* and *NFATC1* mRNA expression was comparable in unstimulated Th1 and Th17 cell clones, whereas *JUN, FOS,* and *NFATC1* mRNA expression appeared to be substantially lower in Th17 than Th1 cell clones after stimulation (Figure 2A). In addition, stimulation with anti-CD3-CD28 substantially upregulated *NFKB1, JUN, FOS*, and *NFATC1* mRNA expression in Th1 cell clones, whereas only *NFKB1* and *NFATC1* mRNA expression was found increased in Th17 cell clones (Figure 2A).

It is well known that in order to activate the *IL2* promoter, the above-mentioned transcription factors need to be either phosphorylated by active kinases (NF-κBp65, c-Jun, and c-Fos) or dephosphorylated by phosphatases (NF-AT). As shown in Figure 2B, anti-CD3-CD28 stimulation induced comparable NF-κBp65 phosphorylation in both Th1 and Th17 cell clones in a time-dependent manner. By contrast, anti-CD3-CD28 stimulation induced a significant phosphorylation of both c-Fos and c-Jun in Th1 but not in Th17 cell clones (Figure 2B). Moreover, 40 min after their anti-CD3-CD28 stimulation, Th17 cells had substantially lower NF-ATc2 nuclear localization than did Th1 cells (Figure 2C). Finally, the Lenti NF-AT Reporter assay clearly revealed reduced NF-AT binding to the transcriptional regulatory element (TRE) of *IL2* promoter in Th17 cell clones

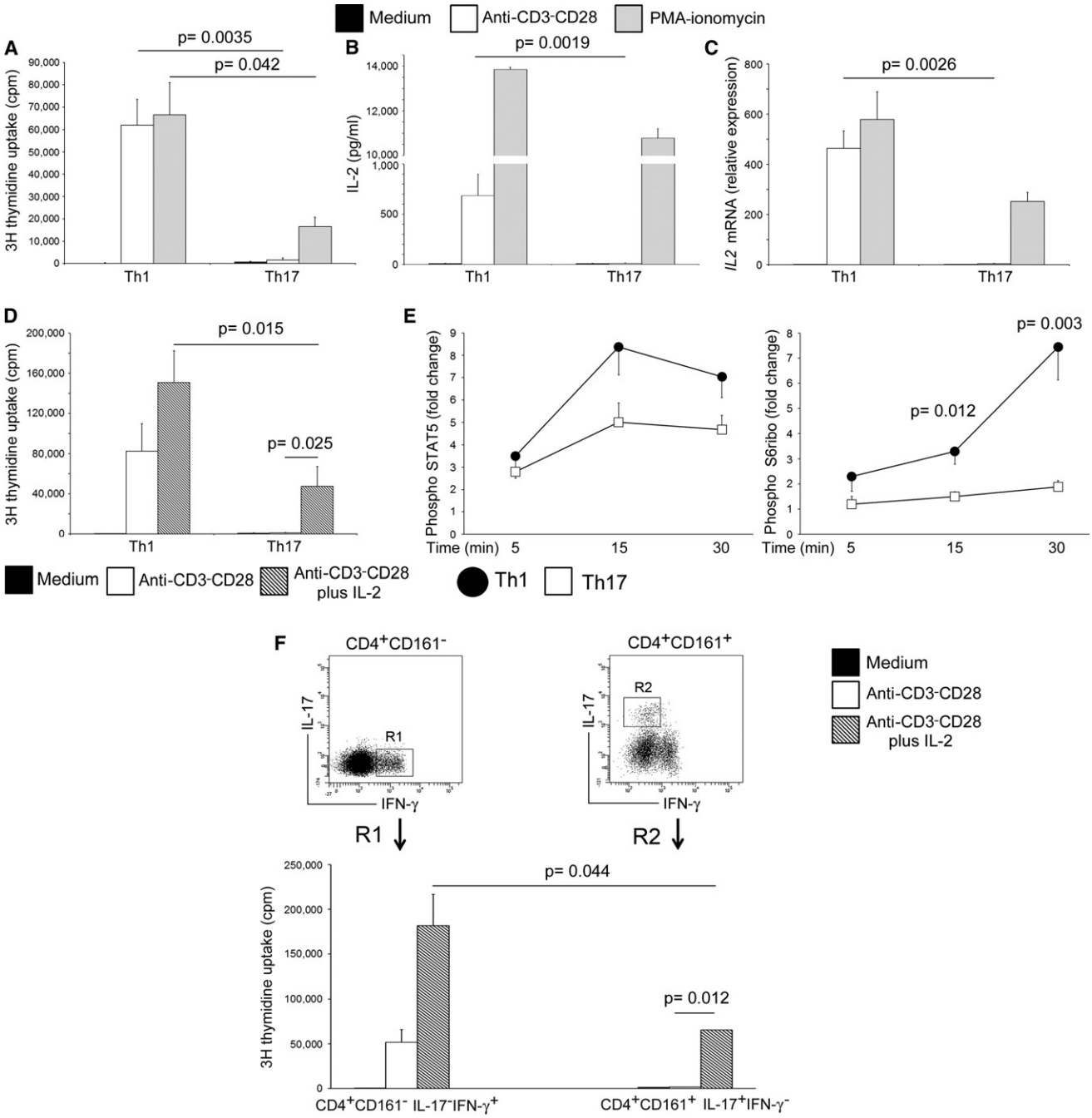

Figure 1. Impaired Th17 Cell Proliferation after Anti-CD3-CD28 Stimulation Is due to Their Inability to Produce and to Respond to IL-2

(A and B) Th1 (n = 10) and Th17 (n = 10) cell clones were stimulated with anti-CD3-CD28 or PMA-iono and then assessed at day 5 for (A) proliferation by ^{3}H-TdR uptake and (B) IL-2 production as evaluated in supernatants.

(C) Th1 (n = 10) and Th17 (n = 10) cell clones were stimulated with anti-CD3-CD28 (6 hr) or PMA-iono (3 hr) and then assessed for *IL2* mRNA expression by real-time quantitative RT-PCR. Results were normalized to *GAPDH* mRNA.

(D) Th1 (n = 10) and Th17 (n = 10) cell clones were stimulated with anti-CD3-CD28, in the absence or presence of IL-2, and then assessed at day 5 for proliferation by ^{3}H-TdR uptake.

(E) STAT5 and S6ribo phosphorylation in Th1 (n = 7) and Th17 (n = 7) cell clones induced by IL-2 stimulation was evaluated by flow cytometry. Results are expressed as fold change of mean fluorescence intensity (MFI) in stimulated versus unstimulated (time 0) cultures. See also Figure S1.

(F) Gating strategy for FACS sorting of freshly derived PB CD4^{+}CD161^{-}IL-17^{-}IFN-γ^{+} (Th1) and CD4^{+}CD161^{+}IL-17^{+}IFN-γ^{-} (Th17) cells. One representative plot is shown for each cell population. Freshly derived PB Th1 (n = 3) and Th17 (n = 3) cells were stimulated with anti-CD3-CD28 in the absence or presence of IL-2 and then assessed at day 5 for proliferation by ^{3}H-TdR uptake.

Results are expressed as mean values (±SE) of the indicated number of experiments.

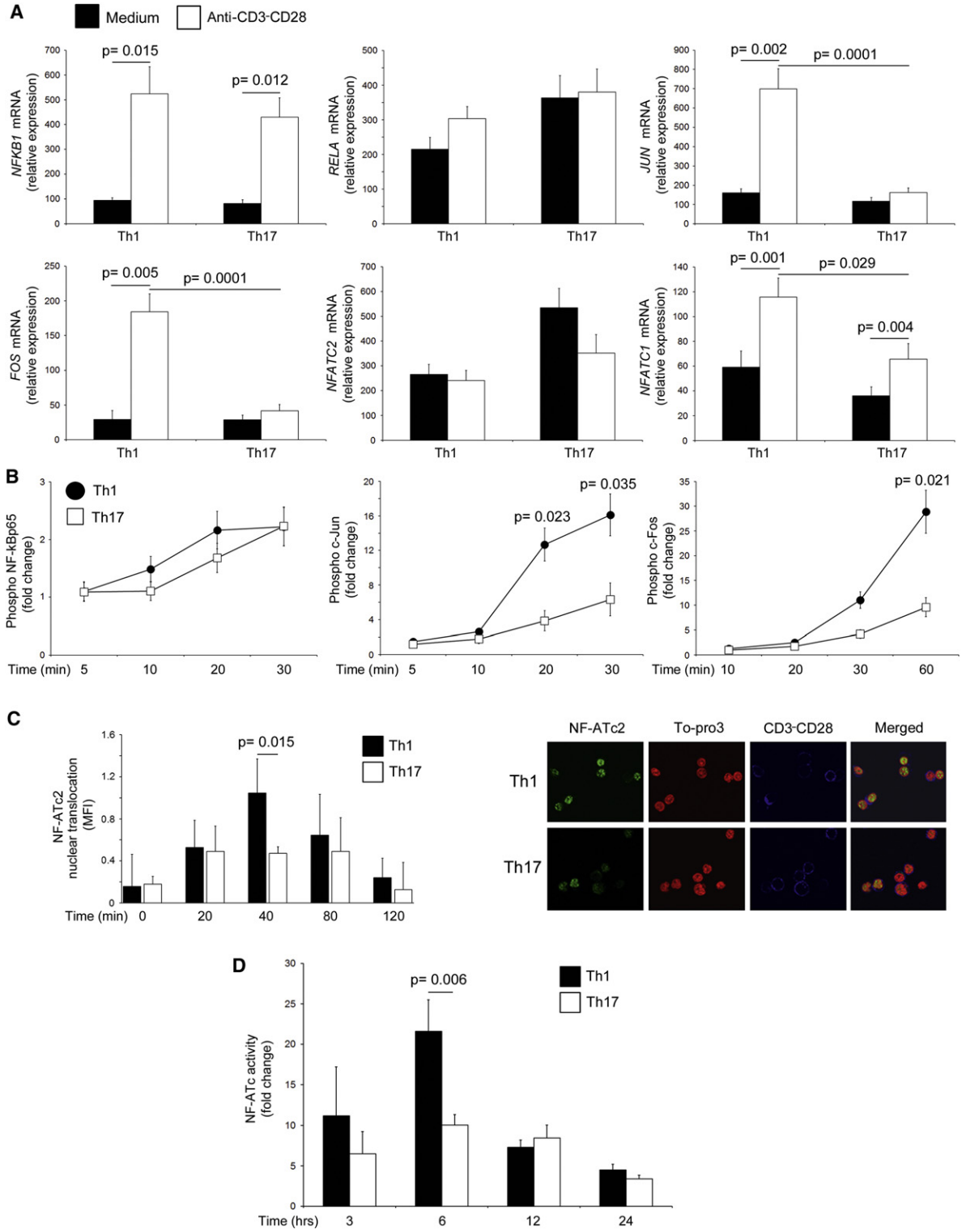

Figure 2. Reduced c-Jun, c-Fos, and NF-AT Activities in Th17 Cells

(A) *NFKB1, RELA, JUN, FOS, NFATC2*, and *NFATC1* mRNA expression in Th1 (n = 10) and Th17 (n = 10) cell clones, under the indicated culture conditions (6 hr stimulation), was evaluated by real-time quantitative RT-PCR. Results were normalized to *GAPDH* mRNA.

(B) Phosphorylation of NF-κBp65, c-Jun, and c-Fos in Th1 (n = 4) and Th17 (n = 4) cell clones induced by anti-CD3-CD28 stimulation was evaluated by flow cytometry. Results are expressed as fold change of MFI in stimulated versus unstimulated (time 0) cultures.

(C) NF-ATc2 nuclear translocation in Th1 (n = 3) and Th17 (n = 3) cell clones induced by anti-CD3-CD28 was evaluated by confocal microscopy. One representative experiment, 40 min after anti-CD3-CD28 stimulation, is also shown. NF-ATc2 (green), To-pro3 (red), CD3 and CD28 (blue).

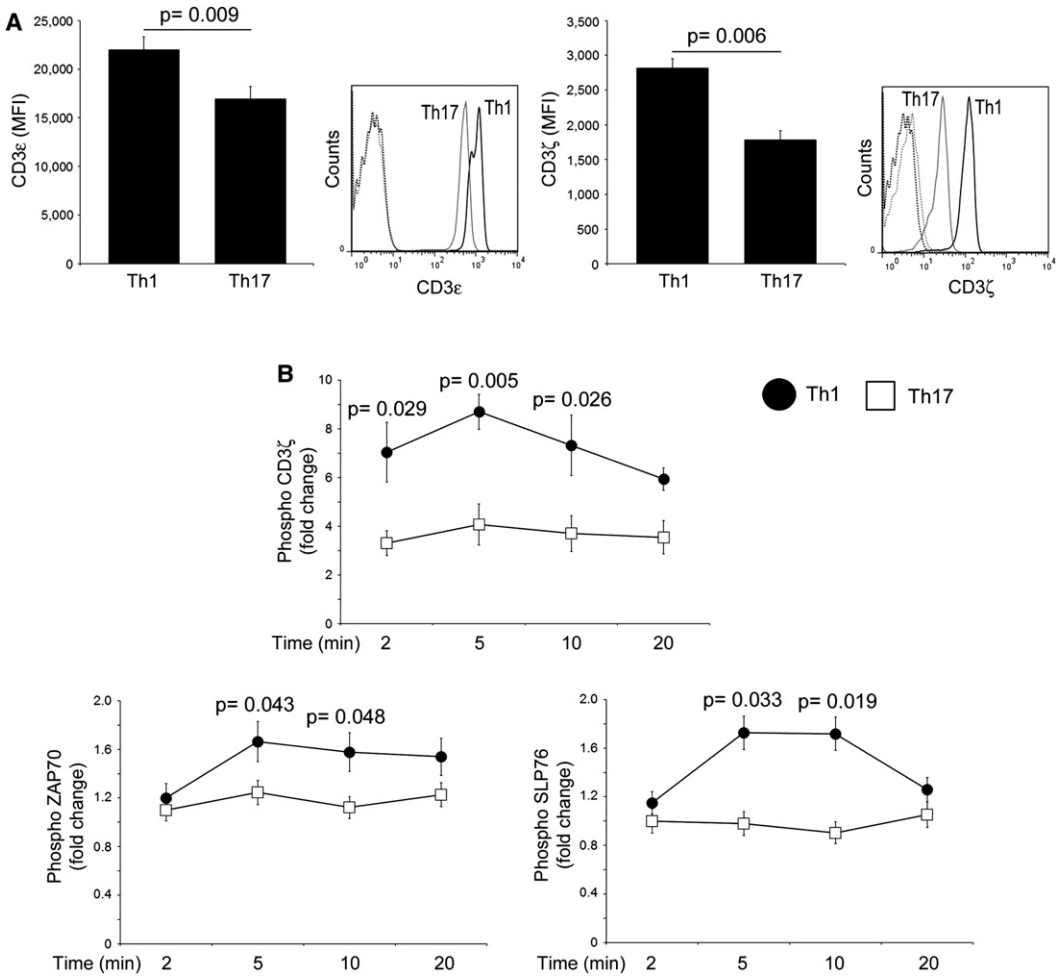

Figure 3. Reduced CD3 Expression and Activity in Th17 Cells

(A) CD3ε and CD3ζ expression by resting Th1 (n = 5) and Th17 (n = 5) cell clones was evaluated by flow cytometry. Results are expressed as difference between CD3ε or CD3ζ MFI and their appropriate isotype control MFI. One representative plot is also shown for both CD3ε and CD3ζ. Dotted lines represent the staining of isotype controls.

(B) Phosphorylation of CD3ζ, ZAP70, and SLP76 in Th1 (n = 4) and Th17 (n = 4) cell clones induced by anti-CD3-CD28 stimulation was evaluated by flow cytometry. Results are expressed as fold change of MFI in stimulated versus unstimulated (time 0) cultures.

Results are expressed as mean values (±SE) of the indicated number of experiments.

when compared to Th1 cell clones at both 3 and 6 hr after anti-CD3-CD28 stimulation (Figure 2D).

Taken together, these results indicate that the intracellular pathway responsible for *IL2* promoter activation is altered in Th17 cells.

Impaired CD3 Signaling in Th17 Cells

These findings demonstrated that several molecules involved in IL-2 production downstream of the anti-CD3-CD28 stimulation are downregulated in Th17 cells, and therefore the possible level(s) of impairment was investigated. Th17 cells showed a substantial reduced CD3ε and CD3ζ chain expression when compared to Th1 cells (Figure 3A). Along with the reduced CD3ζ protein expression, stimulation of Th17 cells with anti-CD3-CD28 resulted in a substantial reduced phosphorylation of the CD3ζ chain, ZAP-70, and SLP-76, the immediate targets of CD3ζ chain kinase activity (Figure 3B). In contrast, Th1 cell clones exhibited a clear time-dependent phosphorylation of CD3ζ, ZAP-70, and SLP-76 (Figure 3B).

These results indicate that the key membrane molecules devoted to the transduction of TCR signaling exhibit both reduced expression and activity in Th17 cells.

(D) Th1 (n = 8) and Th17 (n = 10) cell clones were transduced with Cignal Lenti NF-AT reporter. Luciferase activity, in unstimulated or anti-CD3-CD28-stimulated cells, was then evaluated and normalized on viable cell numbers as assessed by flow cytometry. Results are expressed as fold change of luciferase activity (NF-AT activity) of stimulated versus unstimulated cultures.

Results are expressed as mean values (±SE) of the indicated number of experiments.

Upregulation of *IL4I1* Expression in Th17 Cells and in Their Precursors

Gene expression profile of Th1 versus Th17 cell clones by a microarray analysis showed that *IL4I1* mRNA was substantially upregulated in Th17 compared to Th1 cells (Figure S2A). *IL4I1* encodes a secreted L-amino-acid oxidase that inhibits human T lymphocyte proliferation in vitro by inducing a temporary decrease in CD3ζ chain expression resulting from the enzymatic production of H_2O_2 (Boulland et al., 2007). We found that *IL4I1* mRNA expression was virtually undetectable on either resting or activated Th1 cell clones, but it was highly expressed on resting Th17 cell clones and further increased by anti-CD3-CD28 stimulation (Figure 4A). *IL4I1* mRNA expression was also higher in ex-vivo-derived PB Th17 than Th1 cells (Figure 4B). Of note, *IL4I1* mRNA was already expressed in Th17 cell naive precursor, both in UCB (Figure 4C) and in thymus of newborns (Figure 4D). More importantly, *IL4I1* as well as *RORC, RORA, IL17A*, and *IL17F* mRNAs were substantially increased in UCB-derived $CD4^+CD161^+$ cells when cultured in the presence of IL-1β and IL-23 (Figures 4E and S2B). Finally, *IL4I1* silencing in Th17 cell clones, by using *IL4I1*-specific siRNA, induced a 50% reduction of *IL4I1* mRNA expression and substantially increased cell proliferation, *IL2* mRNA expression, and IL-2 production by Th17 cells (Figures 4F and S2C).

Taken together, these results demonstrate that *IL4I1* mRNA is highly expressed in Th17 cells and that its activity is responsible, at least in part, for the impairment of cell proliferation and of IL-2 production by Th17 cells.

Upregulation of *IL4I1* mRNA in Th17 Cells Is Strictly RORC Dependent

We then asked whether the increased *IL4I1* mRNA expression, as well as the reduced ability of human Th17 cells to produce IL-2 and to proliferate in response to anti-CD3-CD28 stimulation, were RORC dependent. Th1 cell clones were transduced with RORC2 or control lentivirus and then assessed for *RORC, IL17A, IL17F, IL21, IL4I1*, and *IL2* mRNA expression, as well as for their ability to proliferate in response to anti-CD3-CD28 stimulation. As expected, all Th1 cell clones transduced with RORC2 showed higher expression of *RORC, IL17A*, and *IL17F* mRNA compared to cells transduced with empty vector. The expression of *IL21* mRNA was comparable (Figures 5A and S3). Moreover, RORC2-transduced cells had higher *IL4I1* mRNA expression in both unstimulated and anti-CD3-CD28-stimulated cells in comparison with their empty vector-transduced counterparts (data not shown and Figure 5A). Finally, upon anti-CD3-CD28 stimulation, RORC2-transduced Th1 cell clones showed substantially lower *IL2* mRNA expression (Figure 5A) and inability to proliferate (Figure 5B) when compared to the empty vector-transduced cells. On the other hand, gene targeting of RORC2 expression in Th17 cell clones, via RORC2-specific small interfering (si)RNA, resulted in a marked decrease of both *RORC* and *IL4I1* mRNA expression (Figure 5C) and in a significant increase in the proliferative response (Figure 5D). Finally, real-time quantitative RT-PCR, performed on DNA from Th17 cell clones precipitated with a RORC Ab and performed with *IL4I1*-specific promoter primers, indicated the direct interaction between RORC and *IL4I1* (Figure 5E).

These results indicate that *IL4I1* mRNA expression is regulated by the Th17 cell master gene *RORC,* inasmuch as its product directly binds to the *IL4I1* promoter.

CD28 Stimulation Triggers Th17 Cells to Produce IL-17A and *IL4I1*

We asked then how anti-CD3-CD28 stimulation of Th17 cells could induce NF-κBp65 phosphorylation (Figure 2B) despite the abnormalities in the pathway of CD3 signaling. Intriguingly, among genes in the microarray analysis, we found that *CD28* was upregulated in Th17 compared to Th1 cell clones. This finding was confirmed by both quantitative RT-PCR and flow cytometry analysis in Th17 and Th1 cell clones (Figure S4A and Figure 6A). A similar difference was also observed by assessing CD28 expression in ex vivo PB-derived Th17 and Th1 cells (Figure S4B). The higher expression of CD28 in Th17 cells was found to be RORC dependent, inasmuch as *RORC* viral transduction increased its expression in Th1 cells (Figure 6B), whereas *RORC* silencing in Th17 cells decreased CD28 expression (Figure 6C). Furthermore, ChIP experiments on Th17 cell clones with an anti-human RORC provided direct evidence on the linking between RORC and *CD28* (Figure 6D).

Next, we investigated whether RORC-dependent CD28 hyperexpression could account for NF-κBp65 phosphorylation in Th17 cells. CD28 triggering in the absence of CD3 stimulation induced NF-κBp65 phosphorylation only in Th17 (not in Th1) cell clones (Figure 6E). In order to understand whether CD28-mediated NF-κB activation in Th17 cells was dependent on PI3K activation, we evaluated the phosphorylation status of PI3K downstream targets PDK1 and AKT (Thr308 and Ser473).

PDK1 and AKT (Thr308 and Ser473) were phosphorylated by anti-CD3 or anti-CD3-CD28 crosslinking, but not by anti-CD28 alone, in Th1 cells. Alternatively, none of the above-mentioned conditions was able to induce PDK1 and AKT (Thr308 and Ser473) phosphorylation in Th17 cells (Figures 6F, S4C and S4D).

Finally, Th1 cell clones were unable to produce IFN-γ in the absence of CD3 stimulation, whereas Th17 cell clones produced IL-17A and CXCL8 upon CD28 triggering alone (Figures 6G and 6H and data not shown). The ability of CD28 to induce NF-κB phosphorylation in Th17 cells could also account for the upregulation of *IL4I1* induced by anti-CD3-CD28 stimulation (see Figure 4A). Indeed, stimulation of Th17 cell clones by anti-CD28 in the absence of CD3 triggering was able to substantially upregulate *IL4I1* mRNA expression (Figure 6I).

Taken all together, these findings indicate that the high CD28 expression in Th17 cells is strictly related to RORC; moreover, triggering of CD28, even in the absence of CD3 stimulation, is sufficient to induce both IL-17 production and *IL4I1* mRNA upregulation.

Th17 Cells in SF of JIA Patients Express Higher *IL4I1* and *CD28* mRNA Expression than Th1 Cells

Similar results were also obtained by testing T cells freshly derived from the SF of JIA patients. Indeed, *IL4I1* and *CD28* mRNA expression was found to be substantially higher in Th17 than in Th1 cells in these patients (Figure 7A), and activation with anti-CD3-CD28 resulted in a substantial lower proliferation of Th17 in comparison to Th1 cells (Figure 7B).

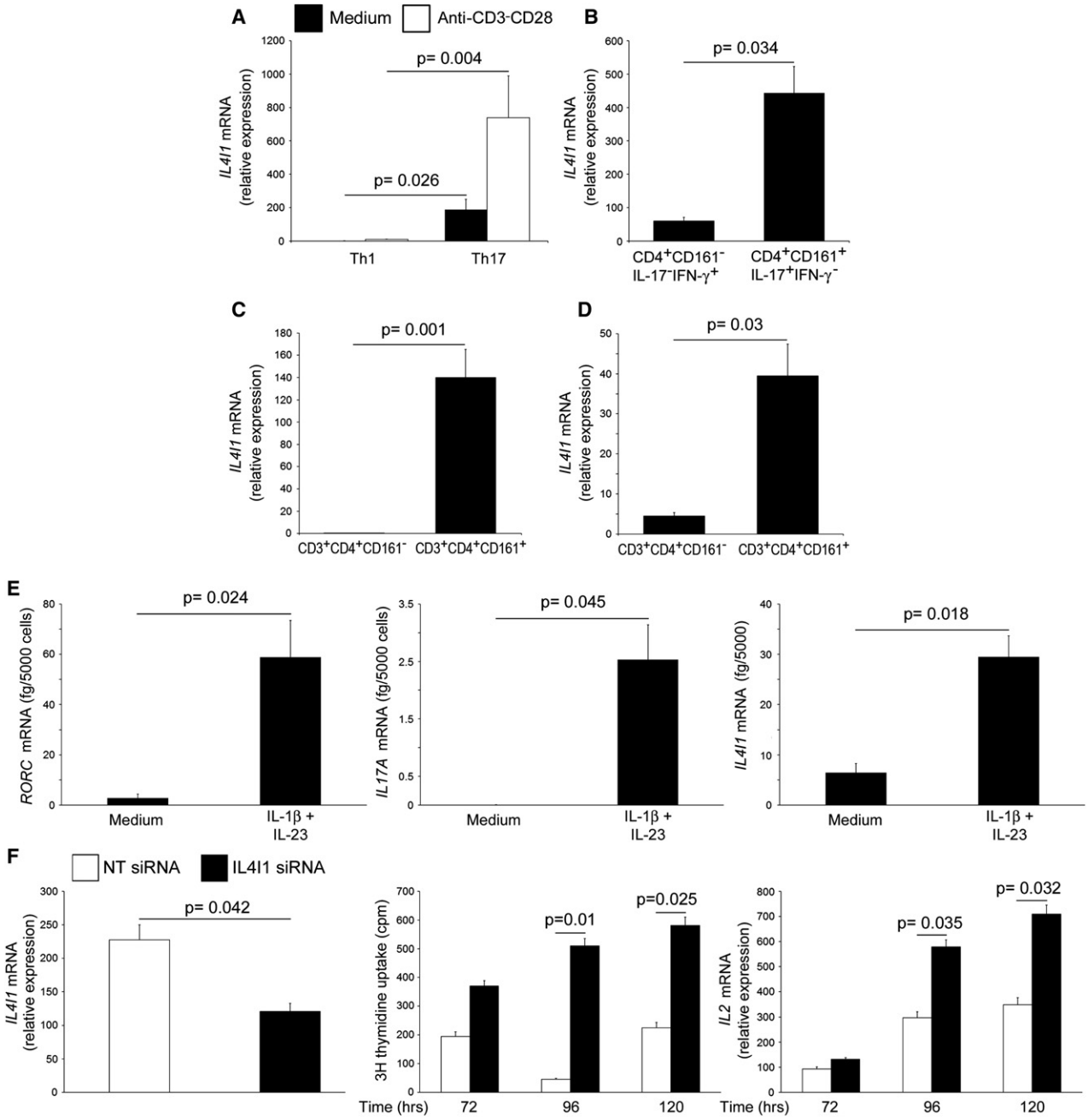

Figure 4. Upregulation of *IL4I1* in Th17 Cells

(A) *IL4I1* mRNA expression by Th1 (n = 10) and Th17 (n = 10) cell clones induced by anti-CD3-CD28 stimulation, was evaluated by real-time quantitative RT-PCR. Results were normalized to *GAPDH* mRNA.

(B) *IL4I1* mRNA expression by ex-vivo-derived PB Th1 (n = 3) and Th17 (n = 3) cells was evaluated by real-time quantitative RT-PCR. Results were normalized to *GAPDH* mRNA.

(C and D) *IL4I1* mRNA expression by $CD3^+CD4^+CD161^+$ and $CD3^+CD4^+CD161^-$ freshly derived UCB cells (n = 3) (C) and newborn thymocytes (n = 3) (D) were evaluated by real-time quantitative RT-PCR. Results were normalized to *GAPDH* mRNA.

(E) *RORC*, *IL17A*, and *IL4I1* mRNA expression by UCB $CD4^+CD161^+$ cells (n = 6) cultured in vitro for 2 weeks in the presence or in the absence of IL-1β plus IL-23 was evaluated by real-time quantitative RT-PCR. Results are expressed as mRNA concentration (fg/5,000 cells).

Results are expressed as mean values (±SE) of the indicated number of experiments.

(F) Expression of *IL4I1* mRNA was evaluated by real-time quantitative RT-PCR in *IL4I1* and NT (nontargeting) siRNA nucleofected Th17 cell clones (n = 3) 96 hr after nucleofection. Results were normalized to *GAPDH* mRNA. Proliferation of *IL4I1* and NT siRNA nucleofected Th17 cell clones (n = 3) induced by anti-CD3-CD28 was evaluated by ^{3}H-TdR uptake at the indicated time points. *IL2* mRNA expresssion in anti-CD3-CD28-stimulated *IL4I1* and NT siRNA nucleofected Th17 cell clones (n = 3) was evaluated by RT-PCR at the indicated time points. Results were normalized to *GAPDH* mRNA.

See also Figure S2. Results are expressed as mean values of three replicates (±SE).

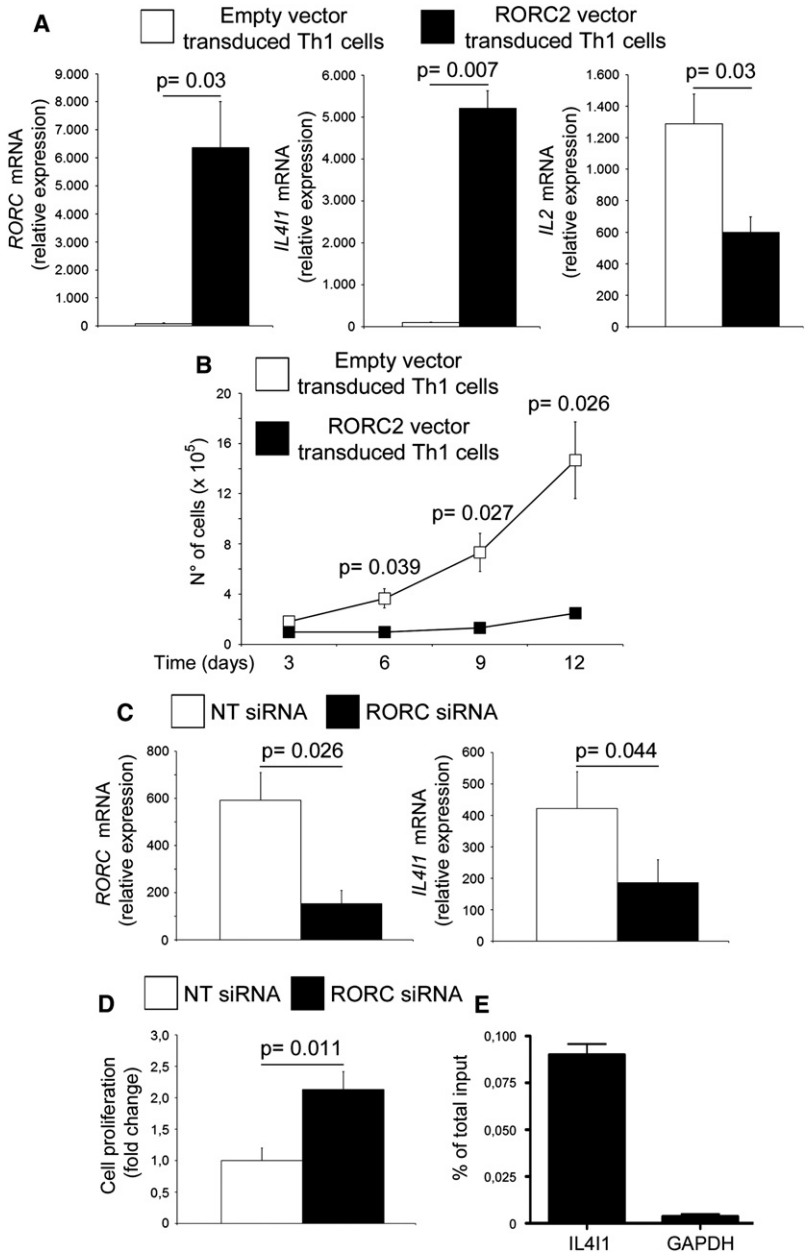

Figure 5. *IL4I1* Expression in Th17 Cells Is RORC Dependent

(A) Expression of *RORC, IL4I1*, and *IL2* mRNA was evaluated by real-time quantitative RT-PCR in empty vector- and RORC2-transduced, anti-CD3-CD28-stimulated Th1 cells (n = 3). Results were normalized to *GAPDH* mRNA.

(B) Numbers of viable cells of empty vector- and RORC2-transduced Th1 cells (n = 3) were evaluated at different time points of in vitro cultures.

(C) Expression of *RORC* and *IL4I1* mRNA was evaluated by real-time quantitative RT-PCR in *RORC* and NT siRNA nucleofected, Th17 cell clones (n = 3) 96 hr after nucleofection. Results were normalized to *GAPDH* mRNA.

(D) Proliferation of RORC and NT siRNA nucleofected Th17 cell clones (n = 3), induced by anti-CD3-CD28 stimulation for 4 days, was evaluated by ^{3}H-TdR uptake. Results are expressed as fold change of RORC versus NT siRNA nucleofected Th17 cell clones.

(E) ChIP was performed with anti-human RORC on a pool of Th17 cell clones stimulated with anti-CD3-CD28. The immunoprecipitated DNA was amplified in real-time RT-PCR with primers specific for *IL4I1* and *GAPDH* promoters, and the results obtained with the ΔΔCt method were then plotted as percentage of total input. Three independent experiments have been performed.

See also Figure S3. Results are expressed as mean values (±SE) of the indicated number of experiments.

Th17 cells obtained from SF of JIA patients exhibit the same characteristics of Th17 cell clones and circulating Th17 cells from healthy subjects, i.e., high *IL4I1* and *CD28* mRNA expression and impaired proliferation in response to anti-CD3-CD28 stimulation.

DISCUSSION

In this study, we wanted to test the hypothesis that the low number of Th17 cells found in the inflamed tissue of subjects affected by Th17 cell-mediated disorders could be due to the inability of these cells to proliferate and to expand in response to antigen stimulation.

First, we showed that in response to anti-CD3-CD28 stimulation, Th1 cells exhibited a strong proliferation and IL-2 production, whereas Th17 cells did not. Furthermore, Th17 cell clones also exhibited a reduced ability to respond to IL-2 in comparison with Th1 cell clones; in fact, IL-2 addition in culture of anti-CD3-CD28-stimulated Th17 cell clones only partially restored their proliferation. These abnormalities were also shared by ex-vivo-derived circulating Th17 cells, suggesting that we were not facing an artifact resulting from the use of long-term cultured Th17 cells.

The reduced ability of Th17 cell clones to respond to IL-2 was not due to a lower expression of *IL2RA*, *IL2RB*, and *IL2RG* mRNA than Th1 cell clones but, rather, was related to an impaired signal transduction via the IL-2R. Indeed, stimulation of Th17 cells with IL-2 resulted in a significant impairment of PI3K-AKT-mTORC1 axis activation, as revealed by the reduced S6ribo phosphorylation. Taken together, these data provide clear evidence that Th17, unlike Th1, cells exhibit poor or no proliferation because of at least two reasons: (1) an impairment to produce IL-2 in response to anti-CD3-CD28 stimulation and (2) a reduced ability to respond to IL-2.

It has to be noted that a similar behavior, i.e., reduced IL-2 response, is shown by CD4^{+}CD25^{+}Foxp3^{+} T regulatory (Treg) cells. Indeed, IL-2R signaling distinctively regulates Treg and T effector (Teff) cells, inducing activation of PI3K-AKT and STAT5 in Teff cells and of STAT5 only in Treg cells (Cheng et al., 2011). However, these findings were not investigated further and they remain presently unclear.

In this study, we focused on the mechanisms accounting for impaired IL-2 expression and production in Th17 cells. The

analysis of molecules involved in the complex network that leads to *IL2* promoter activation revealed that Th17 cells stimulated with anti-CD3-CD28 had a substantially lower mRNA expression of several binding factors for the promoter of *IL2* gene (*JUN, FOS*, and *NFATC1*) compared to Th1 cells. In addition, whereas anti-CD3-CD28 stimulation induced NF-κBp65 phosphorylation in either Th17 or Th1 cells, phosphorylation of both c-Fos and c-Jun, as well as NF-ATc2 nuclear localization and NF-AT binding to TRE of *IL2* gene promoter, were substantially lower in Th17 than in Th1 cells.

Thus, it appears that, among all transcription factors involved in *IL2* gene promoter activation, only NF-κBp65 is activated by anti-CD3-CD28 stimulation in Th17 cells.

Going back to the chain of events occurring between TCR triggering and the *IL2* gene promoter activation, it is noteworthy that Th17 cells showed substantial reduced CD3ζ and CD3ε chain expression in comparison with Th1 cells. Accordingly, CD3ζ, as well as ZAP-70 and SLP-76, showed a substantial lower phosphorylation in Th17 than in Th1 cells, in response to anti-CD3-CD28 stimulation. To better understand these findings, we took advantage of microarray data looking at the expression of genes possibly involved in the control of the initial phases after CD3 signaling in both Th1 and Th17 cells. One possible candidate gene, *IL4I1*, was substantially upregulated in Th17 cells. *IL4I1*, which was first described in B cells (Chu and Paul, 1997), was then detected even on DCs in both mice and humans, where it encodes a secreted L-phenylalanine oxidase, which associates with a temporary decrease in CD3ζ chain and inhibition of T lymphocyte proliferation, as a consequence of the enzymatic production of H_2O_2 (Boulland et al., 2007). The data reported in this study show that *IL4I1* mRNA was highly expressed in unstimulated Th17 cells and is further upregulated after their activation, whereas its expression was virtually undetectable in either resting or stimulated Th1 cells. The *IL4I1* expression in Th17 cells can explain why these cells show reduced CD3ζ and CD3ε chain expression and accounts for their impairment in CD3ζ, ZAP70, SLP76, c-Jun, and c-Fos phosphorylation and, at least in part, impaired NF-AT activation and its nuclear translocation after anti-CD3-CD28 stimulation. Accordingly, silencing of *IL4I1* in human Th17 cells substantially increased their proliferation and their ability to produce IL-2.

This study also demonstrates that *IL4I1* mRNA expression in human Th17 cells is strictly RORC dependent. Indeed, *RORC* transduction in Th1 cells not only induced high *IL17A, IL17F*, and *IL4I1* expression, but it also made these cells unable to proliferate and to produce IL-2 in response to anti-CD3-CD28 stimulation. Furthermore, *RORC* silencing in Th17 cells markedly decreased *IL4I1* mRNA expression, further supporting the concept of a direct relationship between the presence of RORC and the inability of Th17 cells to produce IL-2 and to proliferate. Finally and most importantly, ChIP experiments on Th17 cell clones by the use of a RORC Ab clearly demonstrated the direct binding of RORC to putative *IL4I1* regulatory elements.

IL4I1 mRNA was already detectable in $RORC^+CD4^+CD161^+$ UCB and thymic Th17 cell precursors, supporting the concept that *RORC*, *CD161*, and *IL4I1* expression represents a common feature of the human Th17 cell lineage. It is also of note that *IL4I1* mRNA expression was strongly increased, together with those of *RORC, RORA, IL17A*, and *IL17F*, when $CD4^+CD161^+$ human Th17 cell precursors were cultured in vitro in the presence of IL-1β and IL-23, a condition that allows human Th17 cell polarization (Cosmi et al., 2008).

The inability of Th17 cells to respond to TCR stimulation, which is due to the RORC-dependent *IL4I1* mRNA upregulation, is apparently in contrast with the observation that these cells upregulate *IL17A* and *IL4I1* expression upon anti-CD3-CD28 activation. However, both data in the literature and the findings reported in this study may provide an explanation for this apparent discrepancy. Although many aspects of the molecular pathway involved in the IL-17A production are still unknown, it has been shown that the activation of NF-κB family members RelA and RelB is essential for the regulation of IL-17A expression in mice γδ T cells (Powolny-Budnicka et al., 2011) and that IKKα selectively associates with *IL17A* locus, inducing its transcriptional activation (Li et al., 2011). In addition, it has been shown that *IL4I1* mRNA expression is regulated by NF-κB pathway (Julià et al., 2007; Marquet et al., 2010). These findings are in keeping with the observation reported in the present study showing that *NFKB1* and *RELA* mRNA expression and NF-κBp65 phosphorylation were not altered in Th17 cells when compared to Th1 cells. In addition it is known that, in the absence of TCR triggering, CD28 signaling can selectively regulate the expression of cytokines-chemokines and prosurvival proteins via the activation of an alternative—like IKKα-dependent NF-κB pathway (Marinari et al., 2004; Tuosto, 2011).

In this study, we found that *CD28* expression is strongly upregulated in Th17 cells, as shown by the microarray analysis and confirmed by quantitative PCR. Similar findings have recently been reported by Turtle et al. (2011), who found high CD28 expression in a subset of $TCR\alpha\beta^+CD8\alpha^+CD161^+$ memory adult cells showing also high expression of type 17-associated genes and downregulation of TCR signaling. However, the mechanisms responsible for downregulation of TCR signaling, as well as the role of RORC in such downregulation and in CD28 hyperexpression, were not investigated. Of note, indeed, in our study the higher CD28 expression in Th17 cells than in Th1 cells, like the upregulation of *IL4I1* responsible for downregulation of TCR signaling, was RORC dependent, inasmuch as *RORC2* viral transduction increased its expression in Th1 cells, whereas *RORC* silencing decreased its expression in Th17 cells. Moreover, ChIP experiments on Th17 cell clones by the use of a RORC Ab clearly demonstrated the existence of a direct link of RORC with *IL4I1* and *CD28* promoter. We also found that CD28 stimulation in the absence of CD3 triggering was able to induce NF-κBp65 phosphorylation, IL-17A production, and *IL4I1* upregulation in Th17 cells, whereas it had no effect on NF-κBp65 phosphorylation and IFN-γ production in Th1 cells. The NF-κB phosphorylation induced by CD28 triggering alone could be due to a PI3K-dependent or -independent mechanism (Tuosto, 2011). Data reported here demonstrate that both PI3K downstream targets PDK1 and AKT were phosphorylated by triggering of CD3 or CD3 plus CD28, but not by triggering CD28 alone, in Th1 cells and in none of these conditions in Th17 cells. These data suggest that CD28-mediated NF-κBp65 phosphorylation in Th17 cells acted via a PI3K-independent pathway. It is of note that the lack of PI3K-AKT pathway activation in Th17 cells may further explain the recent data, showing that Th17 cells have impaired IL-2 production and proliferation

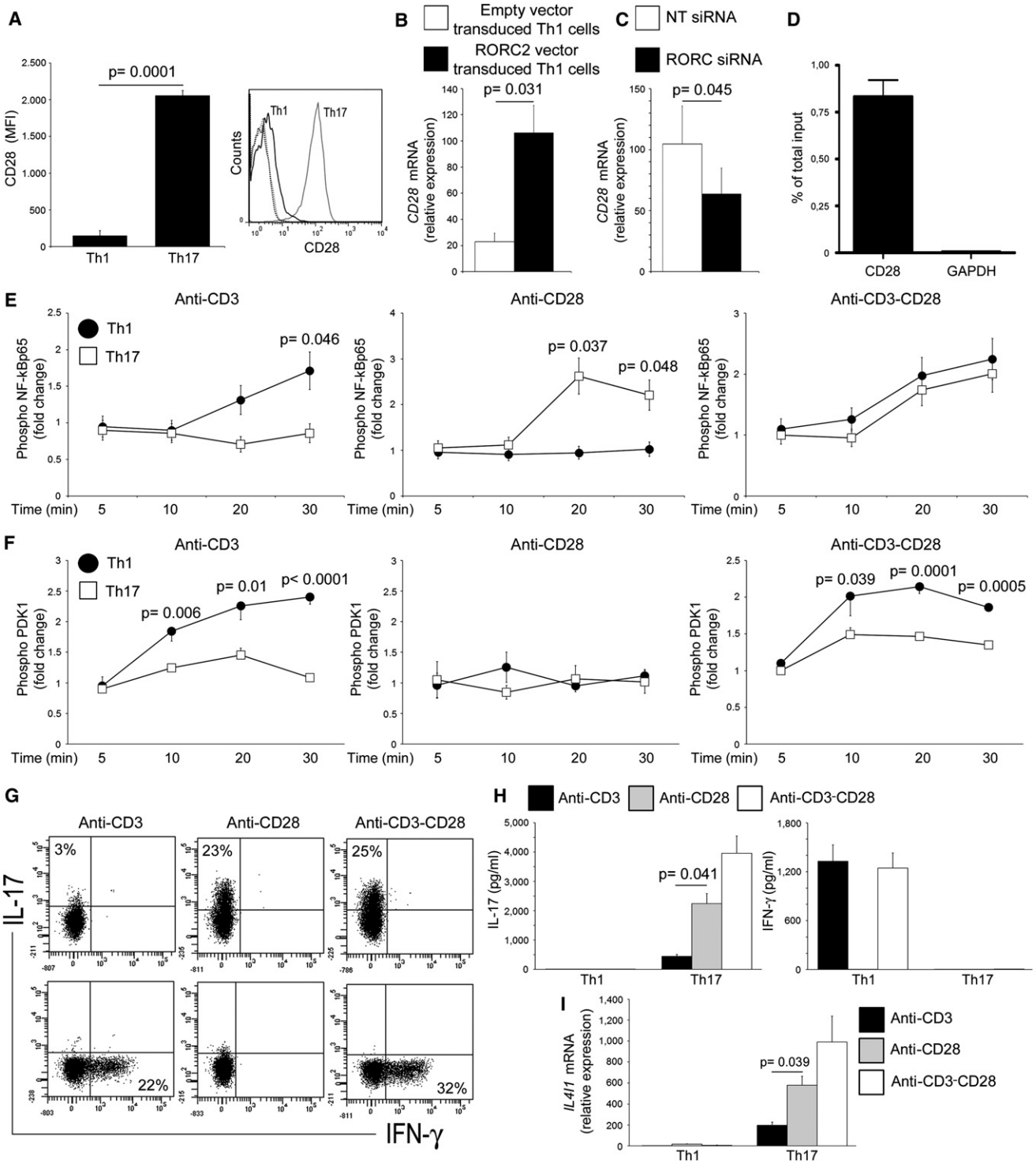

Figure 6. CD28 Is RORC-Dependently Upregulated in Th17 Cells and Its Stimulation Is Sufficient to Induce Both NF-κB Activation and IL-17A Production Independently of PI3K Activation

(A) CD28 protein expression by resting Th1 (n = 5) and Th17 (n = 5) cell clones was evaluated by flow cytometry. Results are expressed as difference between CD28 MFI and its appropriate isotype control MFI. One representative histogram is also shown. Dotted lines represent the staining of isotype controls.

(B) Expression of *CD28* mRNA was evaluated by real-time quantitative RT-PCR in empty vector-transduced and RORC2-transduced Th1 cells (n = 3). Results were normalized to *GAPDH* mRNA.

(C) Expression of *CD28* mRNA was evaluated by real-time quantitative RT-PCR in RORC and NT siRNA nucleofected Th17 cell clones (n = 3) 96 hr after nucleofection. Results were normalized to *GAPDH* mRNA.

because of a STAT3-mediated upregulation of class O Forkhead trascription factor 1, which is a well-known target of AKT activity (Oh et al., 2011). At present, we may speculate that human Th17 cells differ from other subsets of $CD4^+$ T cell effectors because, upon stimulation, they possess a RORC-dependent block of IL-2 production and their subsequent proliferation but maintain the ability to produce IL-17A.

Th17 cells have strong protective and proinflammatory properties, resulting from their ability to produce cytokines acting on several immune and nonimmune cell types. The RORC-dependent impairment of cell proliferation, based on the block of the chain of events leading from TCR stimulation to the production of the autocrine T cell growth factor, IL-2 may therefore represent an important mechanism of self-regulation of human Th17 cell expansion at level of inflamed tissues. This has been confirmed, although indirectly, by the observation that Th17 cells present in the SF of JIA patients (Cosmi et al., 2011) also exhibited the same abnormalities in IL-2 production and in the ability to proliferate as Th17 cell clones and ex-vivo-derived circulating Th17 cells from healthy subjects. The hypothesis that the impairment in Th17 cell expansion reflects a mechanism for attenuating their potentially high dangerousness is also consistent with the observation that, once activated, Th17 cells tend to shift to the Th1 cell phenotype in both humans and mice.

In this study we provide an explanation of why Th17 cells, despite their well-known pathogenic role in some inflammatory disorders, are very rare in the inflammatory sites. This phenomenon is due, at least in part, to the inability of this cell subset to expand because they have abnormalities in the molecular pathway that allows IL-2 production and, consequently, cell proliferation in response to TCR stimulation. These abnormalities are related to the upregulation of *IL4I1* mRNA, which is strictly dependent on the activity of the Th17 cell master gene *RORC*. *RORC* is also responsible for the upregulation in Th17 cells of CD28, whose stimulation allows IL-17A production even in absence of TCR triggering. Thus, the rarity of human Th17 cells in inflamed tissues is the result of RORC-dependent mechanisms that limit their expansion and, therefore, reduce their potential dangerousness.

EXPERIMENTAL PROCEDURES

Cell Sources

PB samples were obtained from eight adult healthy volunteers. UCB samples were obtained from nine donors. Normal postnatal thymus mononuclear cells were obtained from three children who underwent corrective cardiac surgery. SF samples were obtained from six JIA patients defined according to the ILAR classification. The procedures followed in the study were in accordance with the ethical standards of the Regional Committee on Human Experimentation.

T Cell Recovery and Expansion

Mononuclear cell (MNC) suspensions were obtained from PB, UCB, and thymuses by centrifugation on Ficoll-Hypaque gradient. $CD4^+$ single-positive thymocytes and $CD4^+$ T cells from PB or UCB were negatively selected by high-gradient magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach), as described elsewhere (Santarlasci et al., 2009). $CD4^+$ T cells from the different tissues were then further divided into $CD161^+$ and $CD161^-$ by immunomagnetic cell sorting (Miltenyi Biotec). In an additional experiment, PB $CD4^+CD161^+$ and $CD4^+CD161^-$ T cell population were further subdivided in $CCR6^+$ and $CCR6^-$ cell fraction by FACSAria (BD Biosciences) and then cultured under limiting dilution in order to obtain Th17 and Th1 cell clones, respectively (Cosmi et al., 2010). Purified UCB $CD4^+CD161^+$ T cells were stimulated for 7 days with anti-CD3-CD28 (5 μg/ml each), in the absence or presence of IL-1β (10 ng/ml) plus IL-23 (20 ng/ml). On day 7, T cells were restimulated under their primary culture condition. On day 14, T cells were collected and analyzed.

Intracellular Cytokine Production Assay

T cells were analyzed for intracellular IL-17A and IFN-γ production upon 6 hr PMA-ionomycin stimulation, the last 4 hr of which were in the presence of brefeldin A. In some experiments, T cell clones were stimulated with anti-CD3 (5 μg/ml), anti-CD28 (5 μg/ml), or anti-CD3-CD28, followed by cross-linking of the primary antibodies with an anti-isotype, for 6 hr, the last 4 hr of which were in the presence of brefeldin A. The cells were then fixed in formaldehyde and analyzed on a BDLSR II flow cytometry (BD Biosciences).

T Cell Proliferation

1×10^5 T cells from Th17 and Th1 cell clones or $CD3^+CD4^+$ $CD161^+IL\text{-}17^+IFN\text{-}\gamma^-$ and $CD161^-IL\text{-}17^-IFN\text{-}\gamma^+$ cells were cultured for 3 days with anti-CD3-CD28 (5 μg/ml each), in the presence or absence of IL-2 (50 IU/ml) or with PMA-ionomycin. On day 3, the T cell culture supernatants were recovered in order to evaluate cytokine production, whereas cells were pulsed for 8 hr with 0.5 μCi of ^{3}H-TdR (Perkin Elmer) and harvested, and radionuclide uptake was measured by scintillation counting.

Cytokine Secretion Assay and Cell Culture

$CD4^+CD161^+$ and $CD4^+CD161^-$ cells from PB of six healthy donors and SF of six JIA patients were stimulated with PMA-ionomycin, recovered after 3.5 hr, washed, and then stained with IFN-γ and IL-17 catch reagents (Miltenyi Biotec), according to manufacturer instructions. After an additional 45 min of incubation (37°C, 5% CO_2), cells were stained with anti-CD3-Pacific Blue, -CD161-PE, -IL-17-APC, and -IFN-γ-FITC, analyzed, and sorted by FACSAria into $CD3^+CD161^+IL\text{-}17^+IFN\text{-}\gamma^-$ and $CD3^+CD161^-IL\text{-}17^-IFN\text{-}\gamma^+$ cells.

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative RT-PCR

Total RNA was extracted by with the RNeasy Micro kit (QIAGEN) and treated with DNase I to eliminate possible genomic DNA contamination. Taq-Man RT-PCR was performed, as described elsewhere (Santarlasci et al., 2009). Primers and probes used were purchased from Applied Biosystems.

(D) ChIP was performed with anti-human RORC on a pool of Th17 cell clones stimulated with anti-CD3-CD28. The immunoprecipitated DNA was amplified in real-time RT-PCR with primers specific for *CD28* and *GAPDHP* promoters, and the results obtained with the ΔΔCt method were plotted as percentage of total input. Three independent experiments have been performed.

(E and F) Phosphorylation of NF-κBp65 (E) and PDK1 (F) in Th1 (n = 4) and Th17 (n = 4) cell clones induced by anti-CD3, anti-CD28, or anti-CD3-CD28 stimulation was evaluated by flow cytometry. Results are expressed as fold change of MFI in stimulated versus unstimulated (time 0) cultures.

(G) Intracellular IFN-γ and IL-17 production by Th1 (n = 3) and Th17 (n = 3) cell clones induced by anti-CD3, anti-CD28, or anti-CD3-CD28 stimulation was evaluated by flow cytometry. One representative experiment is shown.

(H) IFN-γ and IL-17 production by Th1 (n = 3) and Th17 (n = 3) cell clones induced by anti-CD3, anti-CD28, or anti-CD3-CD28 stimulation was evaluated in supernatants by the CBA assay. Results are expressed as cytokine concentration (pg/ml).

(I) *IL4I1* mRNA expression by Th1 (n = 3) and Th17 (n = 3) cell clones induced by anti-CD3, anti-CD28, or anti-CD3-CD28 stimulation was evaluated by real-time quantitative RT-PCR. Results were normalized to *GAPDH* mRNA.

See also Figure S4. Results are expressed as mean values (±SE) of the indicated number of experiments.

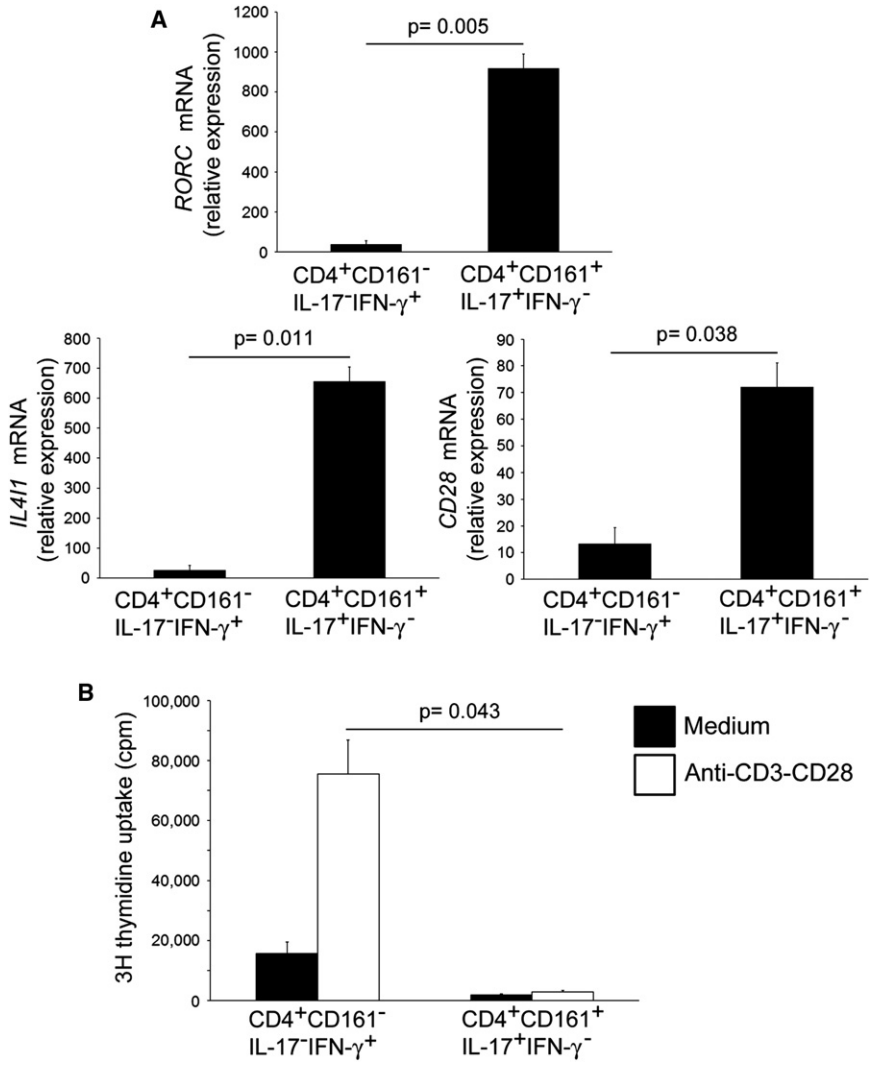

Figure 7. Th17 Cells Present in Synovial Fluid of Juvenile Idiopathic Arthritis-Affected Children Express Higher *IL4I1* and *CD28* mRNA Expression than Th1 Cells

(A) Expression of *RORC*, *IL4I1*, and *CD28* mRNA was evaluated by real-time quantitative RT-PCR in $CD4^+CD161^-IL\text{-}17^-IFN\text{-}\gamma^+$ (Th1) and $CD4^+CD161^+IL\text{-}17^+IFN\text{-}\gamma^-$ (Th17) cells derived from the SF of JIA children (n = 6). Results were normalized to *GAPDH* mRNA.

(B) Proliferation of $CD4^+CD161^-IL\text{-}17^-IFN\text{-}\gamma^+$ (Th1) and $CD4^+CD161^+IL\text{-}17^+IFN\text{-}\gamma^-$ (Th17) cells derived from the SF of JIA children (n = 4) induced by anti-CD3-CD28 was evaluated by ^{3}H-TdR uptake.

Results are expressed as mean values (±SE) of the indicated number of experiments.

Quantification was performed on cell number and results were normalized on *GAPDH* expression.

Cytokine Quantification

IL-2, IL-17A, and IFN-γ protein concentration in cell culture supernatants was assessed by Cytometric Bead Array (CBA) human cytokine kit (BD Biosciences) according to manufacturer instructions.

Phospho-Protein Assays

Phospho-c-Jun (S63), ZAP-70 (Y319), and SLP76 (Y145) were evaluated by BD Cytometric Bead Array (CBA) Cell Signaling Flex Set (BD Biosciences) according to manufacturer instructions. Phospho-PDK1 (Abcam), STAT5, NF-κBp65, CD3ς (BD Biosciences), and c-Fos, AKT, S6ribo (Cell Signaling, Danvers, MA) were evaluated by PhosFlow (BD Biosciences) intracellular cell staining according to manufacturer instructions. Detailed report of the procedure is available in Supplemental Information.

RORC2 Lentivirus Transduction

Th1 cell clones (n = 3) were activated with irradiated (9,000 rads) APCs (1:2) plus anti-CD3 (1 μg/ml) and IL-2 (50 U/mL, Eurocetus, Italy). After 16 hr, pCCL EF1α NGFR or pCCL EF1α NGFR hRORC2 lentivirus (Crome et al., 2009) were added at a multiplicity of infection (MOI) of 10 and cells were spinoculated at 1,200 × g, 26°C for 2 hr. Transduced T cells were kept in culture with IL-2 (100 U/ml), purified after 10 days with anti-NGFR-PE (BD Biosciences) plus anti-PE microBeads (Miltenyi Biotec); purities greater than 98% being consistently achieved, and then expanded with irradiated APCs, anti-CD3 plus IL-2 for other 2 weeks. The proliferation of transduced T cell clones was evaluated by cell counting, excluding the death cells by evaluating the Trypan blue-positive cells.

siRNA Delivery and Gene Silencing

10^7 cells of Th17 cell clones (n = 3) were transiently nucleofected with 3 μM of hIL4I1-siRNA (ON-TARGET plus SMART pool, Thermo Fisher Scientific, Lafayette, CO) or hRORC-siRNA (siGENOME SMARTpool, Thermo Fisher Scientific), by electroporation with the Amaxa Nucleofector (program T-020; Amaxa Biosystems) with the Human T Cell Nucleofector Kit (Lonza Basel, Switzerland). Nontargeting ON-TARGET plus or siGENOME, siRNA Pool (Thermo Fisher Scientific), were used as negative control of hIL4I1-or hRORC-siRNA, respectively. Cells were harvested at 72, 96, and 120 hr for mRNA evaluation by RT-PCR, and cell proliferation induced by anti-CD3-CD28 stimulation was evaluated after an additional 72 hr.

NF-AT Activity Evaluation

Th1 (n = 8) and Th17 (n = 10) cell clones were activated with irradiated (9,000 rads) APCs (1:2) plus anti-CD3 (1 μg/ml) and IL-2 (50 U/mL, Eurocetus, Italy). After 16 hr, cells were transduced with lentivirus particles, expressing an inducible NF-AT-responsive reporter gene (firefly luciferase) under the control of NF-AT's specific Transcriptional Response Element (TRE), Cignal Lenti NF-AT Reporter (luc) (SABiosciences, Frederick, MD). Cells were transduced at an MOI of 50 in the presence of polybreen (8 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA), spinoculated at 1,200 × g, 26°C for 2 hr, and then kept in culture with IL-2 (50 U/ml). Before NF-AT activity evaluation, cells were starved overnight in medium alone; the day after, cells were stimulated (or not) with anti-CD3-CD28 for 3, 6, 12, and 24 hr; luciferase activity was then evaluated with Luciferase Assay System (Promega Corporation, Madison, WI, USA). Normalization was performed on cell number evaluated by flow cytometry with Trucount Tubes (BD Biosciences).

Chromatin Immunoprecipitation

ChIP was performed essentially as described (Nebbioso et al., 2005) with anti-human RORC, clone ab41941 (Abcam; Cambridge, UK) on Th17 cell clones stimulated with anti-CD3-CD28 for 6 hr. The quantitative PCR, performed in an abi 7500 thermal Cycler, was carried out with different sets of primer after

analyzing in silico published sequences of *CD28* and *IL4I1* looking for putative promoter regions with TRED Database (Jiang et al., 2007) and published sequences (Wiemann et al., 2005). Primers used will be available on request. After amplification, data were analyzed according a standard calculation (ΔΔCt method) and controlled against a no-Ab input. Finally, RORC-immunoprecipitated DNA values obtained in quantitative PCR for *CD28* and *IL4I1* promoters were plotted as percentage of total chromatin input.

Microarray

Gene expression profiles on human Th1 and Th17 cell clones were assessed by cDNA microarray technique with One Color Microarray Quick Labeling kit (Agilent Technologies, Cernusco s/N, MI, Italy), as detailed in Supplemental Information.

Statistics

A standard two-tailed paired or unpaired t test was used for statistical analysis; p values of 0.05 or less were considered significant.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE30664.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.immuni.2011.12.013.

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