Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment

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In several murine models of autoimmune arthritis, Th17 cells are the dominant initiators of inflammation. In human arthritis the majority of IL-17–secreting cells within the joint express a cytokine phenotype intermediate between Th17 and Th1. Here we show that Th17/1 cells from the joints of children with inflammatory arthritis express high levels of both Th17 and Th1 lineage-specific transcription factors, RORC2 and T-bet. Modeling the generation of Th17/1 in vitro, we show that Th17 cells "convert" to Th17/1 under conditions that mimic the disease site, namely low TGFβ and high IL-12 levels, whereas Th1 cells cannot convert to Th17. Th17/1 cells from the inflamed joint share T-cell receptor (TCR) clonality with Th17 cells, suggesting a shared clonal origin between Th17 and Th17/1 cells in arthritis. Using CD161, a lectin-like receptor that is a marker of human Th17, we show synovial Th17 and Th17/1 cells, and unexpectedly, a large proportion of Th1 cells express CD161. We provide evidence to support a Th17 origin for Th1 cells expressing CD161. *In vitro,* Th17 cells that convert to a Th1 phenotype maintain CD161 expression. In the joint CD161+ Th1 cells share features with Th17 cells, with shared TCR clonality, expression of RORC2 and CCR6 and response to IL-23, although they are IL-17 negative. We propose that the Th17 phenotype may be unstable and that Th17 cells may convert to Th17/1 and Th1 cells in human arthritis. Therefore therapies targeting the induction of Th17 cells could also attenuate Th17/1 and Th1 effector populations within the inflamed joint.

juvenile | CD161 | RORC2

Th17 cells are a recently identified CD4$^+$ subset with proinflammatory actions (1) thought to be critical to the pathogenesis of collagen-induced arthritis (CIA), a murine model of autoimmune arthritis (2). Differentiation and stabilization of the Th17 program is dependent on a range of cytokines, including IL-23, a member of the IL-12 family (3, 4). Genetic ablation of IL-23 confines Th17 numbers in vivo and prevents the induction of CIA (2). In contrast, ablation of IL-12, which is central to Th1 differentiation, aggravates disease. Although Th17 cells are the dominant pathogenic population in several arthritis models (5), in human arthritis the role of Th17 is less clear. Childhood autoimmune arthritis, known as juvenile idiopathic arthritis (JIA), has provided valuable insights into, and serves as a powerful in vivo model of, the immunopathogenesis of human arthritis. We have demonstrated a role for regulatory T cells in determining JIA disease phenotype (6). We have also shown IL-17 to be highly expressed in JIA synovial membrane, IL-17–secreting cells to be enriched in the joint compared with blood, and that the frequency of synovial Th17 cells correlates with disease severity, suggesting a pathological role for Th17 in JIA (7). Additionally, Th17 responses also show a strong association with ankylosing spondylitis and psoriatic arthritis (8), but their link with rheumatoid arthritis is less clear (9).

In contrast to early reports of Th17 cells as a pure IL-17–secreting lineage (10), cells recovered from the inflamed joint produce IFN-γ and express chemokine receptors that are intermediate in phenotype between Th1 and Th17 cells (7, 11). This result raises questions about the ancestry and transcriptional control of IL-17–secreting T cells in human arthritis. Recent studies have suggested that Th17 cells may up-regulate IFN-γ and also extinguish IL-17 in response to IL-12 or IL-23 in the absence of TGF-β in vitro (12, 13), leading to a Th17/1 (IL-17+IFN-γ+) or Th1 phenotype. Plasticity of Th17 cells has been demonstrated in vivo in murine models, such that an adoptively transferred Th17 population gives rise to Th1 cells detectable at the inflammatory site (13, 14). In human autoimmune disease it remains to be determined if Th17/1 cells or indeed Th1 cells found in the inflamed organ show evidence for a Th17 origin. The local factors leading to a predominance of Th17/1 over Th17 in human autoimmune disease are uncertain.

Human studies have been limited by the difficulty in isolating viable Th17 cells. Initial studies enriched human Th17 cells on the basis of chemokine receptor expression and more recently, the lectin-like receptor CD161, which identifies the Th17 precursor pool in umbilical cord blood (15). In adults with inflammatory bowel disease (IBD), CD161 detects gut-resident Th17 but is not exclusive to Th17 cells, as it also marks Th17/1 and Th1 cells (16). In the present study, Th17 cells from arthritic joints were analyzed directly ex vivo using cytokine capture technology. We confirm that Th17/1 cells from the joint share RORC2 and T-bet expression and can be generated in vitro under conditions that mimic the disease site, namely low TGF-β and high IL-12 levels. To test the hypothesis that Th17 converts to Th1 in human arthritis, we analyzed T-cell receptor (TCR) clonality of synovial T cells and demonstrate shared TCR sequence identity between Th17 and Th1 cells, in particular Th1 cells expressing CD161.

To our knowledge this study of human arthritis is unique in directly analyzing the transcriptional, functional, and clonal properties of Th17 and Th17/1 cells from the joint. Our results have important implications for future therapeutic strategies such as blockade of the Th17 population.

Results

Synovial Th17 Cells Coexpress IFN-γ and Account for the CCR4lo Phenotype Found Within the Joint. Original reports detailing Th17 as a unique T-cell lineage emphasized their distinction from Th1 cells in terms of phenotype and function (10, 17). However, a small proportion of Th17 cells coexpress the Th1 cytokine

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IFN-γ in peripheral blood of healthy adults (18). Within the inflamed joints of JIA patients, this proportion is greatly increased, with 50% of all IL-17+ CD4$^+$ T cells coexpressing IFN-γ (here called Th17/1 cells) (Fig. 1 *A* and *B*). As Th17/1 cells have an intermediate cytokine profile between Th17 and Th1 cells, we investigated whether their transcriptional programming shared elements with Th17 and Th1 cells. To enrich for Th17 and Th17/1, we adapted a sorting strategy previously used for peripheral blood mononuclear cells (PBMC) based on chemokine receptor expression (18). After sorting synovial fluid (SF) CD4$^+$ T cells into CCR4−CCR6−, CCR4−CCR6+, and CCR4+CCR6+ populations, enrichment of Th17/1 is most marked in the CCR4−CCR6+ population (Fig. 1*C*), which accounts for the CCR4lo phenotype of synovial IL-17+ cells previously documented (7). We confirmed that "classical" Th17 cells (IL-17+IFN-γ−) from SF are most abundant in the CCR4+CCR6+ sample (18). After in vitro stimulation of sorted SF populations, cytokines released correlate with intracellular cytokine detected by flow cytometry (Fig. 1 *C* and *D*). Transcription factor analysis showed that RORC2 mRNA expression is elevated in both Th17 and Th17/1 enriched populations compared with Th1 cells, suggesting overlapping transcriptional control in both Th17 subsets (Fig. 1*E*). There is no difference in expression of the aryl hydrocarbon receptor (AHR) between sorted synovial populations (Fig. 1*E*).

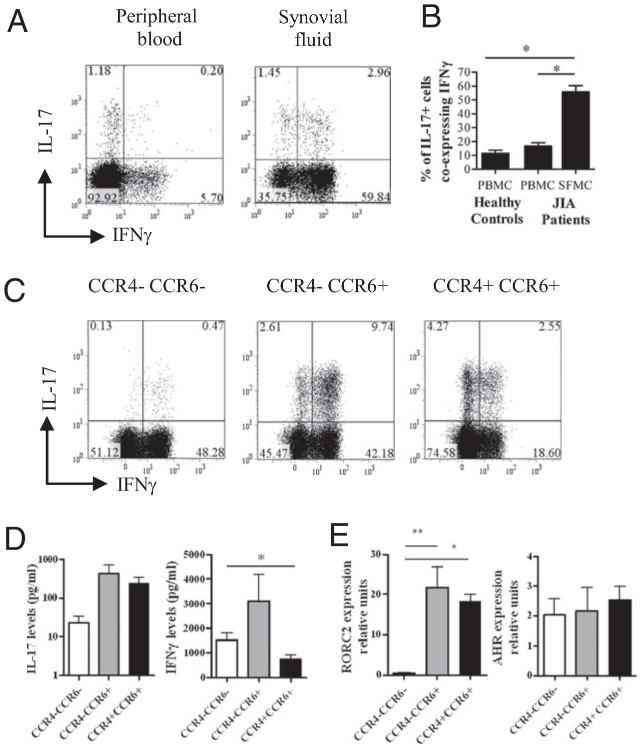

Fig. 1. IL-17+ CD4$^+$ T cells from the synovial fluid of juvenile idiopathic arthritis (JIA) patients coexpress IFN-γ. (*A*) Representative dot plots from paired PBMC and SFMC from a JIA patient showing flow cytometric analysis of IL-17 and IFN-γ production after stimulation with PMA and ionomycin in the presence of Brefeldin A. Numbers in plots indicate percentages of cytokine-producing cells gated on lymphocytes and CD4$^+$ T cells. (*B*) Coexpression of IFN-γ in IL-17+ CD4$^+$ T cells taken from healthy control PBMC (n = 9), JIA PBMC (n = 17), and SFMC (n = 21). *$P < 0.001$. Bars represent mean values (±SEM). (*C*) Synovial CD4$^+$ T cells were sorted according to expression of CCR4 and CCR6. Shown is IL-17 and IFN-γ production in CCR4−CCR6−, CCR4−CCR6+, and CCR4+CCR6+ sorted populations, one representative experiment of four. (*D*) IL-17 and IFN-γ protein detected in supernatants from sorted populations as in *C*, after stimulation with PMA and ionomycin for 5 h (n = 4). *$P < 0.05$. (*E*) mRNA expression of RORC2 and AHR in sorted populations as in *C*, normalized to β2M levels, n = 6 and 4, respectively. *$P < 0.05$, **$P < 0.005$. Bars represent mean values (± SEM).

Synovial Th17/1 Cells Express Both Th1 and Th17 Transcription Factors. We used IL-17 and IFN-γ-specific capture assays to allow clear separation of cytokine-expressing cells (Fig. S1). This assay was used to detect Th17, Th17/1, and Th1 cells from synovial fluid mononuclear cells (SFMC), on the basis of surface-captured cytokines and cells sorted into distinct populations (Fig. 2*A*). Transcription factor expression analyses of these cell populations showed greater expression of RORC2 mRNA in both Th17 and Th17/1 than in Th1 cells, but no differences in IFN regulatory factor 4 (IRF4), which has also been linked to Th17 differentiation (19) (Fig. 2*B*). Interestingly, pure Th17/1 cells show a trend for intermediate expression of T-bet compared with Th17 and Th1 (Fig. 2*B*). We next confirmed that RORC2 is detectable at the protein level by flow cytometry in Th17 and Th17/1 cells, from blood and joint, closely mirroring mRNA expression in SFMC (Fig. 2*C*).

Cytokine Microenvironment Found Within the Joint Promotes Th17 Plasticity. Our results indicate that synovial Th17/1 cells are intermediate between Th17 and Th1 cells in terms of cytokine production, chemokine receptor, and transcription factor expression (Figs. 1 and 2). Some reports suggest that the Th17/1 population arises from Th17 but not Th1 cells, in response to IL-12 or IL-23

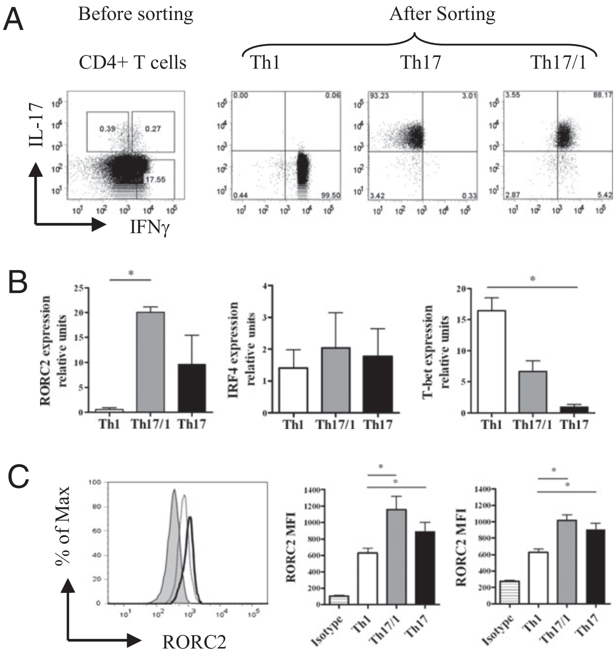

Fig. 2. Synovial T cells secreting IL-17 and IFN-γ express both Th1 and Th17 transcription factors. (*A*) IFN-γ– and IL-17–secreting CD4$^+$ SFMC were detected by flow cytometry using cytokine capture assay. Shown are representative dot plots of unsorted SFMC (*Left*) gated on CD4$^+$ T cells demonstrating surface capture of IL-17 and IFNγ and sorted by flow cytometry into Th1, Th17, and Th17/1 populations (*Right* three plots). Numbers in plots indicate percentages of cells secreting cytokines. (*B*) RORC2, IRF4, and T-bet mRNA expression in synovial CD4$^+$ T-cell populations sorted as above. *$P < 0.05$. Bars represent mean values (±SEM, n = 3, 4, and 4, respectively). (*C*) Histogram of RORC2 protein expression analyzed by flow cytometry in healthy control CD4$^+$ T cells (*Left*); isotype control (gray histogram), Th1 cells (dotted line), and Th17 cells (thick solid line), representative of n = 3 are shown. Summary is shown of RORC2 protein expression (MFI) in cytokine-expressing subpopulations from JIA PBMC (*Center*) and JIA SFMC CD4$^+$ T cells (*Right*). *$P < 0.05$. Bars represent mean values (±SEM, n = 5).

in the absence of TGF-β (12, 13). To test if these cytokines are relevant to the enrichment of Th17/1 in the joint we compared levels in the joint with levels in patients' blood. The synovial compartment has a distinct balance compared with plasma, with significantly higher levels of IL-12 and a relatively low abundance of TGF-β (Fig. 3*A*). IL-23 is not detectable in either synovial fluid or plasma. To model the effects of the synovial microenvironment on Th17 plasticity we cultured purified Th17 (mean purity 92.8%) from healthy control PBMC, in serum-free medium, in the presence of IL-12 to reflect the synovial compartment, or TGF-β to mimic plasma, or both cytokines. Th17 cells rapidly up-regulate IFN-γ production and show a significant increase in the frequency of Th17/1 cells when cultured with IL-12 (Fig. 3 *B* and *C*). In contrast, the presence of TGF-β stabilizes the Th17 phenotype, but fails to overcome the effects of IL-12 (Fig. 3 *B* and *C*). IL-12 also promotes a proportion of cells to develop a Th1 phenotype (5.2 ± 1.9%). In murine studies IL-6 has been shown to stabilize the Th17 phenotype, partly through *trans* signaling by engagement of IL-6 and sIL-6R (20). Although IL-6 was enriched within the joint (Fig. S2*A*), it failed to attenuate the effects of IL-12 on Th17 plasticity in vitro (Fig. S2*B*). Purified Th1 cells harvested directly ex vivo fail to revert to a pure Th17 or Th17/1 phenotype in response to cytokines known to induce Th17 in vitro (4): IL-1β, IL-23, IL-6, or IL-21 (Fig. S2*C*). IL-21 was detectable in synovial fluid, but was not enriched relative to plasma (Fig. S2*D*).

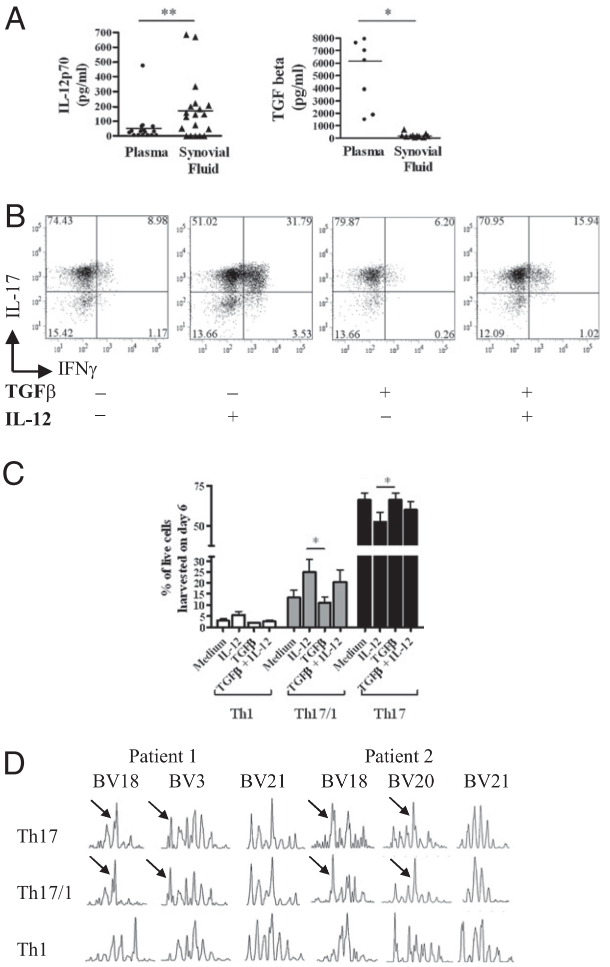

Fig. 3. Th17 cells share clonal ancestry with Th17/1 cells and Th1 cells within the joint and demonstrate plasticity in vitro. (*A*) IL-12 and TGF-β in JIA plasma and synovial fluid (IL-12, $n = 16$ and 19; and TGFβ, $n = 7$ and 11, respectively). $^{*}P < 0.01$, $^{**}P < 0.05$. (*B*) Th17 cells were captured from healthy control PBMC and cultured in serum-free medium in the presence of IL-2 or additionally with recombinant IL-12, TGF-β or both as shown. Dot plots of cytokine production in Th17 cells after culture for 6 d are shown. Numbers in plots indicate percentages of cells secreting cytokines. (*C*) Summary of three independent experiments culturing purified Th17 cells as in *B*. Bars represent mean number (±SEM) of cells as a percentage of live cells harvested with resultant Th1 cells (open bars), Th17/1 cells (shaded bars), or Th17 cells (solid bars). $^{*}P < 0.05$. (*D*) Th17, Th17/1, and Th1 cells were sorted from SFMC using cytokine capture assay and T-cell receptor BV (TRBV) spectratyping was performed ($n = 3$). Three representative TRBV families are shown, from two separate SFMC samples. Arrows indicate clonal peaks shared between Th17 and Th17/1 populations.

Th17 Cells Share Clonal Ancestry with Th17/1 Cells and Th1 Cells Within the Joint. Our results demonstrating plasticity of Th17 cells in vitro led us to hypothesize that at the inflammatory site, Th17/1 cells may originate from a Th17 but not a Th1 pool. If so, the clonal distribution within the Th17/1 population would be more similar to Th17 than to Th1 cells. To test this hypothesis we separated synovial T cells into the three populations (Th17, Th17/1, and Th1) directly ex vivo and performed analysis of the TCR-β variable chain (TRBV) across the CDR3 junction using spectratyping (Fig. 3*D*). As we have previously observed, synovial T cells exhibit oligoclonal TCR repertoires (21). Comparing oligoclonality patterns in the three populations showed that typically half or more of TRBV families share oligoclonal patterns between Th17 and Th17/1 cells, which are distinct from the Th1 oligoclonal patterns. Data from two representative patients are shown in Fig. 3*D*.

CD161 Expression May Identify Th1 Cells with a Th17 Ancestry. We next investigated whether Th17 cells that may have converted in vivo to Th1 can be identified by the surface marker CD161, the human equivalent of murine NKR-P1A (22). CD161 has emerged as a potential lineage marker for Th17 cells in humans. Overexpression of RORC2 induces CD161 expression (23), and only CD161+ cells taken from cord blood can differentiate into Th17 (15). Within the inflamed joint, we found that the majority of synovial Th17 and Th17/1 are CD161+ve; however, Th1 cells showed distinct CD161+ and CD161− populations (Fig. 4 *A* and *B*). If CD161 predicts Th17 commitment in naive cord blood T cells before IL-17 expression, we hypothesized that CD161 expression might be maintained following switch of Th17 cells to a Th1 phenotype. To test this hypothesis, sorted CD161+ Th17 cells were cultured in the presence of IL-12 to promote plasticity toward Th17/1 and Th1 cells (Fig. 4*C*). After conversion to a Th17/1 phenotype, cells maintained CD161 expression, as did the majority of converted Th1 cells (82 ± 5%, Fig. 4 *C* and *D*). CD161− $CD4^{+}$ T cells cultured under the same conditions failed to up-regulate CD161 (Fig. 4*D*). Consistent with these in vitro data suggesting that CD161 may identify cells within the Th1 population that have a Th17 ancestry, we found that CD161+ Th1 cells sorted from the inflamed joint had significantly higher RORC2 expression than CD161− cells despite both populations having very low levels of IL-17 mRNA (Fig. 4*E*). CCR6 and IL-23R, downstream targets of RORC2 (4), were also analyzed in the Th1 subpopulations. CD161+ Th1 cells are enriched for CCR6 protein expression (Fig. 4*F*) and IL-23R mRNA and protein (Fig. 4*G*) compared with CD161− Th1 cells. To test if CD161+ Th1 cells showed a functional response to IL-23, SFMC Th1 cells were enriched without the capture assay, avoiding stimulation with PMA and ionomycin (Fig. S3*A*). Having depleted the CCR6+ population to exclude Th17 cells, CD161+ cells were still enriched for IL-23R message (Fig. S3*B*) and showed a trend for increased secretion of IFN-γ and TNF-α in response to IL-23 compared with CD161− cells (Fig. S3*C*).

Finally, to test the clonal relationships between Th1 CD161+ and Th17 populations in arthritis patients, we sorted synovial

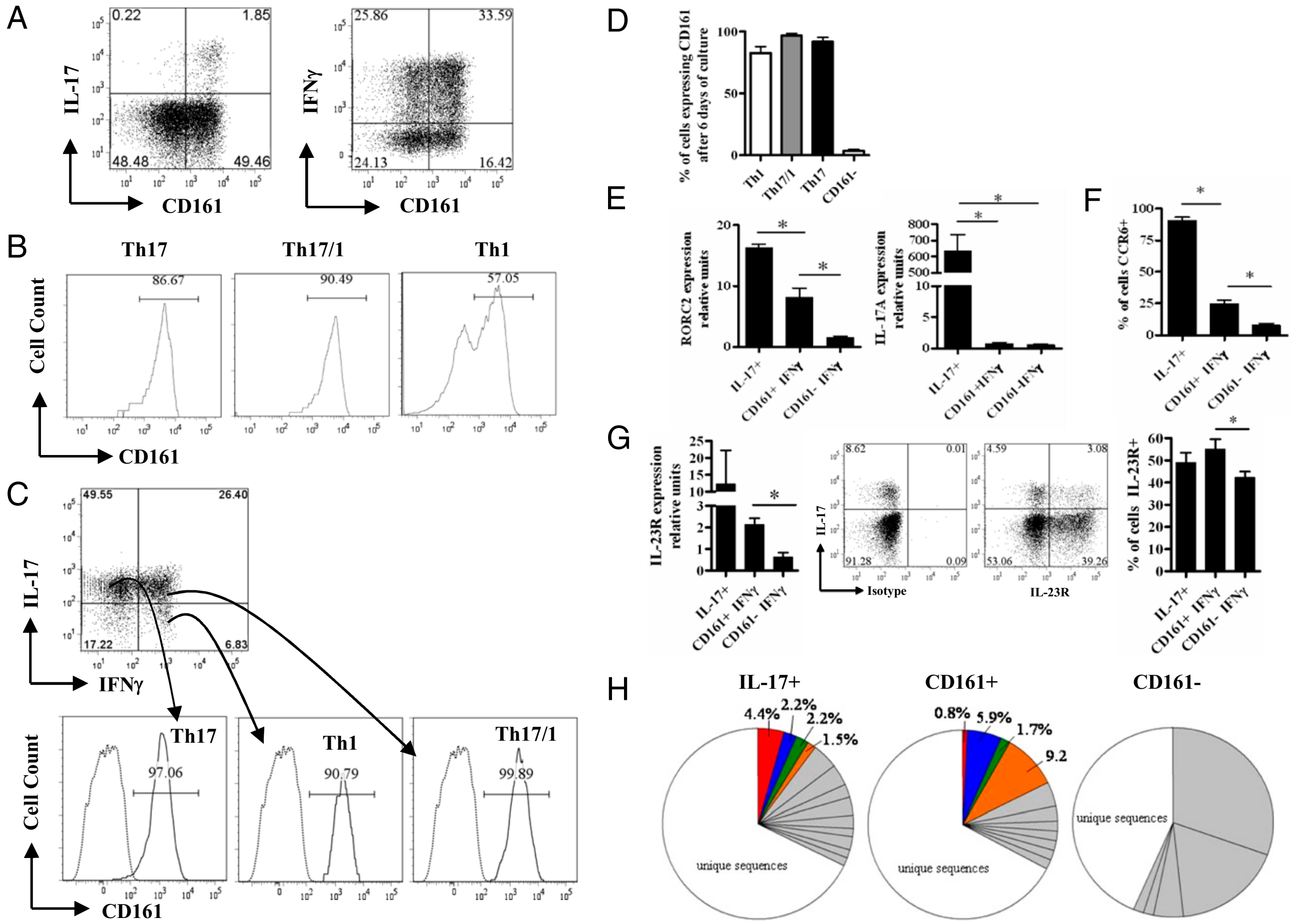

Fig. 4. CD161 expression may identify Th1 cells with a Th17 ancestry. (*A*) Representative flow cytometric analysis of SFMC comparing CD161 expression with IL-17 and IFN-γ production, gated on CD4$^+$ T cells. Numbers in plots indicate percentage of parent population. (*B*) CD161 expression in gated synovial Th17 cells, Th17/1 cells, and Th1 cells (n = 4). (*C*) IL-17+ cells expressing CD161 from healthy controls were sorted and cultured in the presence of IL-12. (*Upper Left*) Dot plot of intracellular IL-17 and IFN-γ detected on day 6 of culture and CD161 expression in the same cells (indicated by arrows) gated on Th17, Th1, and Th17/1 subpopulations (n = 3). The dotted line represents isotype control and numbers in histograms indicated percentage of cells expressing CD161. (*D*) Summary of three independent experiments, showing CD161 expression on gated subpopulations as in *C* and (last column) percentage of control CD161− population expressing CD161 after culture in the same conditions as *C*. Control data for CD161+ Th17 cells stimulated without IL-12 were equivalent to results seen in Fig. 3*B*. (*E*) Cytokine-expressing synovial CD4$^+$ T cells were sorted into IL-17+, CD161+IFN-γ+, and CD161-IFN-γ+ populations. RORC2 and IL-17 mRNA expression in sorted synovial populations is shown. *$P < 0.05$. Bars represent mean values (±SEM, n = 3). (*F*) Mean percentage (±SEM, n = 6) of above populations expressing CCR6 protein detected by flow cytometry. (*G*) (*Left*) Mean (±SEM, n = 4) IL-23R mRNA expression in sorted populations as in *E*: Representative flow cytometric analysis of SFMC IL-23R expression (or isotype) and IL-17 production, with plots gated on CD4$^+$ T cells. (*Right*) Mean percentage (±SEM, n = 6) of IL-17+, CD161+IFN-γ+, and CD161-IFN-γ+ populations expressing IL-23R protein, detected by flow cytometry. (*H*) Three hundred eighty-three PCR products for TRBV18 from patient 1 were cloned and sequenced. Sequence results are illustrated as pie charts with colored segments to indicate clones that overlap between populations (limited to IL-17+ and CD161+ Th1 populations). Numbers indicate clone size as a percentage of total number sequenced for that cell population. Nonoverlapping clones (gray) and unique sequences (white) for all three populations are shown. Full TCR sequences across the CDR3 junction are listed in Table S1.

Th1 cells by CD161 expression and compared their TRBV with those of IL-17+ synovial T cells (Fig. S3*D*). PCR products from one BV subfamily (patient 1, BV18) were cloned and sequenced. In this sample, the unique TCR sequences of CD161+ and CD161− cells within the IFN-γ+ population are distinct (Fig. 4*H* and Table S1). However, interestingly specific TRBV CDR3 sequences were identified that are shared between IL-17+ cells and the CD161+IFNγ population, but these clones were not detected in the CD161− cells. These data suggest that at least a proportion of T cells within the CD161+ IFN-γ population share a common ancestral clonality with Th17 cells.

Discussion

Following the identification of Th17 cells, evidence from several models of autoimmune arthritis led to a shift in assigning disease pathogenesis from Th1 to Th17 cells (2, 5). However, in the inflamed joints of patients with childhood arthritis, we show here that the majority of IL-17+ cells are polyfunctional, coexpressing IFN-γ. We examined the relationship between Th17/1 and "pure" Th17 and Th1 cells from the joint and show links in terms of transcriptional control, plasticity in vitro, and evidence that supports the concept of shared ancestry between Th17 and Th1 cells expressing CD161.

In the inflamed site, both Th17 and Th17/1 cells are restricted to the CCR6 compartment, which may reflect the dominant role of CCL20, a CCR6 ligand, in the recruitment of IL-17+ cells to the inflammatory site, as demonstrated in models of arthritis and multiple sclerosis (24, 25). RORC2 expression is also limited to CCR6+ populations enriched for Th17 and Th17/1 cells. To clearly distinguish viable Th17 and Th1 cells ex vivo we used a cytokine capture technique, avoiding the potential for epigenetic modification or phenotype plasticity that may accrue during long-term in vitro culture (26). Purified synovial Th1 cells have significantly higher T-bet mRNA expression than Th17 cells, whereas Th17/1 cells are intermediate between Th17 and Th1. T-bet expression has been linked to autoimmune pathology, independent of IFN-γ, and may confer a greater pathogenicity to synovial Th17/1 compared with Th17 cells (27). Interestingly, clones derived from the gut of patients with IBD did not show these differences, T-bet expression being equal in all three subsets (12). With respect to Th17 transcription factors, our results show that RORC2 expression was more specifically linked to Th17 and Th17/1 cells than either IRF4 or AHR. This finding may reflect a role for IRF4 and AHR that is permissive but not critical to Th17 differentiation (19, 28).

Our study is supported by others of human autoimmune disease where Th17/1 cells are found to be enriched at the disease site (7, 11, 12), more commonly than their murine counterparts (2, 10, 14). The mechanism(s) that lead to Th17/1 enrichment are still unknown. Some reports implicate antigen-presenting cells in promoting Th17/1 responses, through cell contact-dependent mechanism(s) (29, 30). We explored the role of soluble mediators in promoting Th17/1 cells at the inflammatory site. We show that synovial fluid is distinct from the plasma of arthritis patients, with synovial fluid having high concentrations of IL-12 but low levels of total TGF-β, of which the majority detected may be latent TGF-β rather that its biologically active form.

When recapitulated in vitro, the high IL-12, low TGF-β environment promotes the conversion of Th17 toward a Th17/1 phenotype. Although IL-23 may contribute to this plasticity in murine studies (13), we and others do not detect IL-23 within the joint (31) or its secretion by resident monocytes (29). To our knowledge evidence for Th17 plasticity has thus far been limited to in vitro studies in humans or murine models (12, 14). We have extended these findings to patients with autoimmune arthritis, showing significant overlap in TCR clonality between Th17 and Th17/1 cells taken directly from the inflamed joint. We propose that Th17 cells recruited to this chronic inflammatory site can convert to Th17/1 in response to local IL-12. Given the dominance of memory T cells (32) in the joint, it is unlikely that Th17 and Th17/1 clones arise from a common naive T-cell precursor in situ. Conversion may occur within local lymph nodes, but would require concomitant migration of both Th17 and Th17/1 cells to the joint to be detected as shared clones. Th17 cells in culture may lose IL-17 expression, possibly through a Th17/1 intermediate, to gain a Th1 phenotype (13, 14). CD161 appears to track this conversion, marking Th17 cells that switch to a Th1 phenotype in vitro. This process, if consistent in vivo, would explain our finding of elevated RORC2, CCR6, and IL-23R expression in CD161+ Th1 cells from the joint compared with CD161− Th1. Furthermore, all of the T-cell clones (defined by nucleotide sequence across TRBV CDR3), which we demonstrated were shared between IL-17+ and Th1 cells, are restricted to the CD161+ population. The frequency of overlap between the Th17 and Th1 repertoires is relatively low. Thus this result may reflect an infrequent conversion of Th17 to CD161+ Th1 cells within the synovial compartment. Alternatively, CD161+ Th1 cells may arise outside of the Th17 pathway and CD161 expression may relate to pathological events independent of IL-17. One ligand of CD161, PILAR, is highly enriched within the joint (33) and stabilizes effector T cells, through increased expression of the anti-apoptotic Bcl-xL. Binding of PILAR to CD161 leads to secretion of inflammatory chemokines and IFN-γ. Finally it is of note that none of the CD161− clones in our study overlap with either CD161+ or IL-17+ cells, suggesting separate origins for the CD161− Th1 population.

One limitation of the in vitro data in our study is that contaminating Th17/1 or Th1 cells may expand in response to IL-12 and account for some of the Th17 plasticity seen (Fig. 3*B*). However, taken together with the expression of Th17-signature genes (IL-23, CCR6, RORC2) in CD161+ Th1 synovial cells ex vivo and the overlap in TCR sequence between Th17 and CD161+ Th1 cells, this result strongly suggests that conversion of human Th17 to Th1 is a real phenomenon.

The implications of Th17 conversion to Th17/1 or Th1 and the consequent colocalization of IL-17 and IFN-γ in the inflamed joint are intriguing. The relationship between IL-17 and IFN-γ is a complex one, IFN-γ being protective in some models by regulating Th17 differentiation and pathogenic in others (34). Trials of recombinant human IFN-γ have failed to provide significant clinical benefit in rheumatoid arthritis, questioning a role for IFN-γ as a regulator of Th17-mediated disease in human arthritis (35). In psoriasis, IFN-γ from Th1 cells acts on resident APC to promote the induction of Th17 as well as their recruitment to the target site, through the production of CCL20 (36). In the context of our findings, we propose that there may be a cycle of positive feedback whereby Th17 are recruited to the joint and convert to Th17/1 or Th1 in response to local IL-12, and the resulting IFN-γ secreted promotes further recruitment of Th17 by virtue of the secreted CCL20.

Our study provides unique insights into the biology and regulation of Th17/1 cells, with evidence for Th17 plasticity toward both Th17/1 and Th1 cells in the joints of patients with arthritis. It shows that Th17/1 enrichment in the chronically inflamed site may be driven by a permissive environment that promotes Th17 plasticity. It is possible that this process of conversion varies in different subtypes of arthritis, and if found to be accelerated in rheumatoid arthritis it may explain the enrichment of Th1 but not Th17 cells seen in the joints of patients with this disease (9). If Th17 plasticity accounts, at least in part, for the sizeable population of CD161+ Th1 cells found at the inflamed site, we predict that biologic treatments targeting the generation of Th17 may have the additional benefit of also attenuating effector Th17/1 and Th1 populations.

Materials and Methods

Patients and Samples. Samples from 59 children with JIA (37) and 17 healthy controls were included in this study. The study had approval from the local ethical review committee and full informed consent was obtained from patients/parents. PBMC were isolated by density centrifugation. For preparation of SFMC, samples were first treated with hyaluronidase (Sigma-Aldrich) at 10 units/mL for 30 min at 37 °C, before density gradient isolation.

Cell Sorting and Flow Cytometry. Cell sorting was performed on the BD FACSAria (BD PharMingen). Antibodies used are listed in *SI Materials and Methods*. For sorting of SFMC by chemokine receptor expression of CCR4 and CCR6, cells were first gated on the $CD4^{+}CD25-$ lymphocytes. For analysis of cytokine production by T cells, SFMC or PBMC were cultured for 5 h in the presence of 50 ng/mL PMA and 500 ng/mL ionomycin and cell supernatants were harvested or for 3 h in the presence of 5 μg/mL Brefeldin A (all from Sigma-Aldrich) before intracellular cytokine detection by flow cytometry (7). To capture cytokine-expressing cells, PBMC or SFMC were enriched for $CD4^{+}$ T cells using negative selection magnetic beads (Stemcell Technologies) and stimulated for 2 h with PMA (10 ng/mL) and ionomycin (1 μg/mL) (38). IL-17– and IFN-γ–secreting $CD4^{+}$ T cells were detected according to manufacturer's instructions (Miltenyi Biotec) and sorted by flow cytometer. Purity was assessed by detecting intracellular cytokines after overnight incubation in Brefeldin A. Flow cytometric data were collected on a LSRII (BD PharMingen); 1×10^{5} events were collected for each condition. Data were analyzed using FlowJo (Treestar).

Cell Culture. Sorted cells were cultured in IMDM, 10% FCS (Invitrogen) in the presence of IL-12 (10 ng/mL; R&D Systems) to track CD161 expression or, in some experiments, serum-free "Ex-vivo15" medium (Lonza) in the presence of IL-2 (50 IU/mL; Roche) and combinations of TGF-β (5 ng/mL; R&D Systems), IL-6 (BD PharMingen), and IL-12. On day 6 intracellular IL-17 and IFN-γ expression was detected by flow cytometry after restimulation with PMA and ionomycin in the presence of Brefeldin A. A live/dead discriminant dye was used in accordance with manufacturer's instructions (Invitrogen).

Multiplex Immunoassay. Cell supernatants were analyzed for cytokines using a multiplex immunoassay as described (39). TGF-β and IL-21 were analyzed by ELISA (R&D Systems) in platelet-depleted synovial supernatants and plasma from JIA patients.

PCR. Generation of cDNA and RT-PCR was performed as described (40). See *SI Materials and Methods* for details.

T-Cell Receptor Analysis by Spectratyping and Sequencing. Complementarity determining region-3 (CDR3) TCR spectratyping was performed as previously described (41). TRBV PCR products were cloned using a TOPO-TA cloning strategy (Invitrogen) and amplified using M13 primers. Sequencing was performed on a 3730xl capillary sequencer and analyzed using Sequencher (Gene Codes).

Statistical Analysis. Data were analyzed using SPSS v16.0 and Graphpad Prism.

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1. Korn T, Bettelli E, Oukka M, Kuchroo VK (2009) IL-17 and Th17 Cells. *Annu Rev Immunol* 27:485–517.
2. Murphy CA, et al. (2003) Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 198:1951–1957.
3. Oppmann B, et al. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715–725.
4. Manel N, Unutmaz D, Littman DR (2008) The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* 9:641–649.
5. Nakae S, et al. (2003) IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc Natl Acad Sci USA* 100:5986–5990.
6. de Kleer IM, et al. (2004) CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J Immunol* 172:6435–6443.
7. Nistala K, et al. (2008) Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum* 58:875–887.
8. Jandus C, et al. (2008) Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. *Arthritis Rheum* 58:2307–2317.
9. Yamada H, et al. (2008) Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. *Ann Rheum Dis* 67:1299–1304.
10. Park H, et al. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133–1141.
11. Aarvak T, Chabaud M, Miossec P, Natvig JB (1999) IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. *J Immunol* 162:1246–1251.
12. Annunziato F, et al. (2007) Phenotypic and functional features of human Th17 cells. *J Exp Med* 204:1849–1861.
13. Lee YK, et al. (2009) Late developmental plasticity in the T helper 17 lineage. *Immunity* 30:92–107.
14. Bending D, et al. (2009) Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J Clin Invest* 119:565–572.
15. Cosmi L, et al. (2008) Human interleukin 17-producing cells originate from a CD161+ CD4+ T cell precursor. *J Exp Med* 205:1903–1916.
16. Kleinschek MA, et al. (2009) Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *J Exp Med* 206:525–534.
17. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179–189.
18. Acosta-Rodriguez EV, et al. (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8:639–646.
19. Brüstle A, et al. (2007) The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* 8:958–966.
20. Jones GW, et al. (2010) Loss of CD4+ T cell IL-6R expression during inflammation underlines a role for IL-6 trans signaling in the local maintenance of Th17 cells. *J Immunol* 184:2130–2139.
21. Wedderburn LR, Maini MK, Patel A, Beverley PC, Woo P (1999) Molecular fingerprinting reveals non-overlapping T cell oligoclonality between an inflamed site and peripheral blood. *Int Immunol* 11:535–543.
22. Giorda R, et al. (1990) NKR-P1, a signal transduction molecule on natural killer cells. *Science* 249:1298–1300.
23. Crome SQ, Wang AY, Kang CY, Levings MK (2009) The role of retinoic acid-related orphan receptor variant 2 and IL-17 in the development and function of human CD4+ T cells. *Eur J Immunol* 39:1480–1493.
24. Hirota K, et al. (2007) Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* 204: 2803–2812.
25. Reboldi A, et al. (2009) C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 10:514–523.
26. Wei G, et al. (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30:155–167.
27. Yang Y, et al. (2009) T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J Exp Med* 206:1549–1564.
28. Veldhoen M, et al. (2008) The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:106–109.
29. Evans HG, et al. (2009) In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proc Natl Acad Sci USA* 106:6232–6237.
30. Dhodapkar KM, et al. (2008) Dendritic cells mediate the induction of polyfunctional human IL17-producing cells (Th17-1 cells) enriched in the bone marrow of patients with myeloma. *Blood* 112:2878–2885.
31. Brentano F, et al. (2009) Abundant expression of the interleukin (IL)23 subunit p19, but low levels of bioactive IL23 in the rheumatoid synovium: Differential expression and Toll-like receptor-(TLR) dependent regulation of the IL23 subunits, p19 and p40, in rheumatoid arthritis. *Ann Rheum Dis* 68:143–150.
32. Wedderburn LR, Robinson N, Patel A, Varsani H, Woo P (2000) Selective recruitment of polarized T cells expressing CCR5 and CXCR3 to the inflamed joints of children with juvenile idiopathic arthritis. *Arthritis Rheum* 43:765–774.
33. Huarte E, et al. (2008) PILAR is a novel modulator of human T-cell expansion. *Blood* 112:1259–1268.
34. Palmer MT, Weaver CT (2010) Autoimmunity: Increasing suspects in the CD4+ T cell lineup. *Nat Immunol* 11:36–40.
35. Cannon GW, et al. (1989) Double-blind trial of recombinant gamma-interferon versus placebo in the treatment of rheumatoid arthritis. *Arthritis Rheum* 32:964–973.
36. Kryczek I, et al. (2008) Induction of IL-17+ T cell trafficking and development by IFN-gamma: Mechanism and pathological relevance in psoriasis. *J Immunol* 181:4733–4741.
37. Petty RE, et al., International League of Associations for Rheumatology (2004) International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: Second revision, Edmonton, 2001. *J Rheumatol* 31:390–392.
38. Streeck H, et al. (2008) Rapid ex vivo isolation and long-term culture of human Th17 cells. *J Immunol Methods* 333:115–125.
39. de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT (2005) Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *J Immunol Methods* 300:124–135.
40. Diss JK, et al. (2006) Brn-3a neuronal transcription factor functional expression in human prostate cancer. *Prostate Cancer Prostatic Dis* 9:83–91.
41. King DJ, Larsson-Sciard EL (2001) Clonal evolution of CD8+ T-cell expansions in HIV-infected patients on long-term HAART. *Clin Exp Immunol* 126:280–286.