IL-1β Promotes the Differentiation of Polyfunctional Human CCR6+CXCR3+ Th1/17 Cells That Are Specific for Pathogenic and Commensal Microbes

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IL-1β Promotes the Differentiation of Polyfunctional Human CCR6\(^+\)CXCR3\(^+\) Th1/17 Cells That Are Specific for Pathogenic and Commensal Microbes

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In humans, Th1/17 cells, identified by coexpression of the chemokine receptors CCR6 and CXCR3, are proposed to be highly pathogenic in several autoimmune disorders due to their ability to produce proinflammatory cytokines IL-17, IFN-γ, and GM-CSF. However, their developmental requirements, relationship with “classic” Th17 and Th1 cells, and physiological role in normal immune responses are not well understood. In this study, we examined CCR6\(^+\)CXCR3\(^+\) Th1/17 cells from healthy individuals and found that ex vivo these cells produced the effector cytokines IL-17, IL-22, and IFN-γ in all possible combinations and were highly responsive to both IL-12 and IL-23. Moreover, although the Ag specificity of CCR6\(^+\)CXCR3\(^+\) Th1/17 cells showed substantial overlap with that of Th1 and Th17 cells, this population was enriched in cells recognizing certain extracellular bacteria and expressing the intestinal homing receptor integrin β7. Finally, we identified IL-1β as a key cytokine that renders Th17 cells sensitive to IL-12, and both cytokines together potently induced the differentiation of cells that produce IL-17, IFN-γ, and GM-CSF. Therefore, interfering with IL-1β and IL-12 signaling in Th17 cells during inflammation may be a promising therapeutic approach to reduce their differentiation into “pathogenic” CCR6\(^+\)CXCR3\(^+\) Th1/17 cells in patients with autoimmune diseases.

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Upon activation, naive CD4\(^+\) T cells differentiate into different Th cell subsets depending on the nature of the Ag, the type of APC, the cytokines present in the microenvironment, and the location where the APC/T cell encounter takes place (1). During this differentiation, T cells acquire specific functional characteristics such as the production of effector cytokines, and the upregulation of adhesion molecules and chemokine receptors whose expression are governed by so-called “master” transcription factors. As a result, specialized Th cell subsets migrate to distinct anatomical locations, and this ensures that Th cells with the appropriate effector functions are mobilized during infection with different types of pathogens.

The association of specific chemokine receptors with distinct Th cell subsets has been used to identify Th17, Th1, Th2, and Th22 cells directly ex vivo in human peripheral blood (2–5). In addition to these Th subsets, Th1/17 cells are characterized by their ability to coproduce IL-17 and IFN-γ together with coexpression of the Th1 and Th17 lineage–specifying transcription factors ROR\(\gamma\)T and T-bet (6). Accordingly, in humans, Th1/17 cells have been identified by the coexpression of T-bet and ROR\(\gamma\)T target genes CXCR3 and CCR6 (2, 7), which allow them to migrate to sites of both Th1- and Th17-mediated inflammation. Although Th1/17 cells are found in healthy donors, interest in these cells has peaked because of their presence in cellular infiltrates observed in inflammatory bowel disease (IBD), multiple sclerosis, and juvenile idiopathic arthritis, where they are thought to contribute to disease pathogenesis (8–10). Recently, their pathogenicity was associated with the production of GM-CSF in addition to IL-17 and IFN-γ. Moreover, GM-CSF production by T cells has been linked to several autoimmune diseases, including multiple sclerosis, myocarditis, and rheumatoid arthritis (11–14).

The mixed character of Th1/17 cells raises important questions regarding their differentiation, specificity, and functional stability. Recent studies have shown that Th1/17 cells can differentiate from Th17 cells when stimulated via their TCR in the presence of IL-12, leading to cells producing only IFN-γ, the so-called ex-Th17 cells (8, 15, 16). However, in contrast to in vitro–differentiated Th17 cells, in vivo–generated mouse and human Th17 cells are largely unresponsive to IL-12 because of their lack of expression of the IL-12R–component IL-12Rβ2 (17). A more recent study reported that IL-23, signaling via the IL-23R and phosphorylation of STAT3 and STAT4, was required for the differentiation of Th17 cells into IL-17+IFN-γ+ Th cells in experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis (18), but the mechanisms of Th1/17 cell development in other settings are still poorly understood. In addition, although Th17 cells and Th1 cells show differential specificity for commonly encountered infectious agents such as Candida albicans, Staphylococcus aureus, and influenza virus (2, 19), little is known about how the Ag specificity of Th1/17 cells relates to that of Th1 and Th17 cells in healthy donors.

In this study, we examined the functional characteristics, specificity, and development of ex vivo–purified CCR6\(^+\)CXCR3\(^+\) “Th1/17” cells in healthy donors. We show that although sharing many features with Th1 and Th17 cells, this population has unique phenotypic and functional properties and are broadly reactive with a variety of commonly encountered microorganisms. In addition, we show that IL-1β, together with TCR stimulation, renders Th17 cells responsive to IL-12 and thereby helps promote their differ-
entiation into IL-17+IFN-γ+ Th cells. These data provide new insights into the development and function of this important T cell population and will help in determining how Th1/17 responses are dysregulated during development of autoimmune and inflammatory diseases.

Materials and Methods

Cell purification and sorting

Samples were obtained from healthy donors participating in the Benaroya Research Institute Immune Mediated Disease Registry. Informed consent was obtained from all subjects according to Institutional Review Board-approved protocols at Benaroya Research Institute. CD4+CD25− cells were enriched from PBMCs by positive selection with CD4-specific microbeads (Miltenyi Biotec). Memory cell subsets were sorted to >97% purity as CD4+CD45RA−CD45RO+CD25− (all from BD Biosciences) or IκBα (Th1) and IκBβ (Th17), including pSTAT4 (Y693) and pSTAT3 (pY705) (both from BD Biosciences), according to the manufacturer’s instructions. Cells were stained with anti–IL-17 (eBioscience), anti–IL-22 (eBioscience), and are enriched in IL-4–producing Th2 cells (Fig. 1A; data not shown). In most of our experiments, we further enriched in IL-17–producing T cells by selecting CCR6+CXCR3+ Th1/17 cells share functional characteristics with Th1 and Th1 cells.

Sorted cells (5 × 10^5) we cocultured in flat-bottom 96-well plates with irradiated autologous monocytes (1.5 × 10^5) pulsed with 5 μCi/ml [3H]thymidine. In some experiments, sorted cells were labeled with CFSE (Sigma-Aldrich), stained with anti–IL-17 (eBioscience), and are enriched in IL-4–producing Th2 cells (Fig. 1A; data not shown).

Statistics

Statistical tests were performed using Prism software (GraphPad, San Diego, CA). Significance was determined by paired two-tailed Student t test or one-way ANOVA analysis with Tukey correction, as noted in the figure legends.

Results

CCR6+CXCR3+ Th1/17 cells share functional characteristics with Th1 and Th1 cells.

As we and others have previously reported (2, 7, 20), expression of the chemokine receptors CCR6 and CXCR3 defines four subsets of CD4^+CD45RO^−CD25^− memory Th cells in the peripheral blood of healthy donors: CCR6^+CXCR3^− IL-17–producing Th17 cells, CCR6^+CXCR3^+ cells containing Th1/17 cells, CCR6^−CXCR3^+ Th1 cells, and CCR6^−CXCR3^− cells that express CCR4 and are enriched in IL-4–producing Th2 cells (Fig. 1A; data not shown). In most of our experiments, we further enriched in IL-17–producing T cells by selecting CCR6^+CXCR3^− T cells that were also CCR4^+CXCR10^−, which distinguishes them from CCR6^+CXCR3^− CCR4^+CXCR11^− Th22 cells (3). For clarity, we will refer to CCR6^+CXCR3^− T cells as “Th1/17 cells” throughout the paper. Among total memory CD4^+ T cells in peripheral blood, Th17 cells were present at the lowest frequency, Th1 cells were the most abundant, and the frequency of Th1/17 cells was intermediate between the two other subsets. Th1/17 cells did not merely represent an activated subset of Th1 or Th17 cells, because expression of markers associated with T cell activation/proliferation such as ICOS and Ki-67 was low and did not substantially differ between the Th17, Th1, and Th1/17 cell populations (data not shown).

CXCR3 and CCR6 expression by Th cells is controlled by the transcription factors T-bet and RORγt, respectively (21, 22). Consistent with this, the Th1/17 cell population homogeneously coexpressed these transcription factors in amounts similar to that observed in conventional Th1 and Th17 cells (Fig. 1B). However, despite their uniform expression of T-bet and RORγt, Th1/17 cells were highly polyfunctional and produced the cytokines associated with Th1 or Th17 cells, IFN-γ, IL-17, and IL-22, in all possible combinations (Fig. 1C). Notably, like “conventional” Th1 cells, the majority of Th1/17 cells produced IFN-γ in the absence of either IL-17 or IL-22. Consistent with IFN-γ being the dominant cytokine produced by the Th1/17 cell population, most of the IL-22–producing 6^+3^ Th17 cells coproduced IFN-γ but not IL-17. Th1/17 cells also contained the highest frequency of GM-CSF–producing cells as compared with Th17 and Th1 cells, and within the Th1/17 subset, production of GM-CSF was produced in association with IFN-γ, IL-17, and IL-22 (Supplemental Fig. 1).

Sorted cells (5 × 10^5) we cocultured in flat-bottom 96-well plates with irradiated autologous monocytes (1.5 × 10^5) pulsed for 3 h with C. albicans (Greer Laboratories), S. aureus, Streptococcus pneumoniae, Escherichia coli, Lactobacillus rhamnosus (all from InvivoGen), or influenza virus (H3N2 Brisbane 1007; Prospeco) Ags. Proliferation was measured on day 4 after 16-h incubation with 5 μCi/ml [3H]thymidine. In some experiments, sorted cells were labeled with CFSE (Sigma-Aldrich), stained with anti–IL-17 (eBioscience), and are enriched in IL-4–producing Th2 cells (Fig. 1A; data not shown). In most of our experiments, we further enriched in IL-17–producing T cells by selecting CCR6^+CXCR3^− T cells that were also CCR4^+CXCR10^−, which distinguishes them from CCR6^+CXCR3^− CCR4^+CXCR11^− Th22 cells (3). For clarity, we will refer to CCR6^+CXCR3^− T cells as “Th1/17 cells” throughout the paper. Among total memory CD4^+ T cells in peripheral blood, Th17 cells were present at the lowest frequency, Th1 cells were the most abundant, and the frequency of Th1/17 cells was intermediate between the two other subsets. Th1/17 cells did not merely represent an activated subset of Th1 or Th17 cells, because expression of markers associated with T cell activation/proliferation such as ICOS and Ki-67 was low and did not substantially differ between the Th17, Th1, and Th1/17 cell populations (data not shown). CCR6 expression by Th cells is controlled by the transcription factors T-bet and RORγt, respectively (21, 22). Consistent with this, the Th1/17 cell population homogeneously coexpressed these transcription factors in amounts similar to that observed in conventional Th1 and Th17 cells (Fig. 1B). However, despite their uniform expression of T-bet and RORγt, Th1/17 cells were highly polyfunctional and produced the cytokines associated with Th1 or Th17 cells, IFN-γ, IL-17, and IL-22, in all possible combinations (Fig. 1C). Notably, like “conventional” Th1 cells, the majority of Th1/17 cells produced IFN-γ in the absence of either IL-17 or IL-22. Consistent with IFN-γ being the dominant cytokine produced by the Th1/17 cell population, most of the IL-22–producing 6^+3^ Th17 cells coproduced IFN-γ but not IL-17. Th1/17 cells also contained the highest frequency of GM-CSF–producing cells as compared with Th17 and Th1 cells, and within the Th1/17 subset, production of GM-CSF was produced in association with IFN-γ, IL-17, and IL-22 (Supplemental Fig. 1).

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FIGURE 1. Phenotype, gene expression and cytokine production by human CCR6+CXCR3+ Th1/17 cells. (A) Expression of CCR6, CXCR3, CCR4, and CCR10 by gated CD4+CD45RO+CD25+CD127+ Th cells from peripheral blood (left panels). Summary of several different donors with 6+3+ Th17 cells, 6+3+ Th1/17 cells, and 6+3+ Th1 cells (right panel). ***p < 0.001 (ANOVA). Each symbol represents one donor; horizontal bars indicate mean. Data are from 12 donors. (B) Quantitative RT-PCR analysis of RORC and TBX21 gene expression by the indicated Th cell subsets (top panel). AU, arbitrary unit. Data are mean ± SEM of seven donors. Expression of ROR-γt and T-bet by sorted 6+3+ Th17 cells, 6+3+ Th1/17 cells, and 6+3+ Th1 cells directly ex vivo (lower panel). Data are representative of four independent experiments. (C) Production of IL-17, IL-22, and IFN-γ by sorted Th cell subsets stimulated for 5 h with PMA/ionomycin. (D) Boolean gating analysis showing each possible combination of IL-17, IL-22, and IFN-γ production representative of six different donors.
CCR6+CXCR3+ Th1/17 cells are highly responsive to IL-12 and IL-23

Given their expression of both T-bet and RORγt, we examined the ability of Th1/17 cells to respond to IL-12 and IL-23, which are associated with Th1 and Th17 cell differentiation, respectively.

Surprisingly, Th1/17 cells expressed both IL12RB2 (IL-12Rβ2) and IL23R (IL-23R) cytokine receptors at levels significantly higher than either Th1 or Th17 cells, and there was also a trend toward higher expression of IL12RB1 (IL12Rβ1), which is a shared component of the IL-12 and IL-23 receptors (Fig. 2A).

**FIGURE 2.** CCR6+CXCR3+ Th1/17 cells respond to both IL-12 and IL-23. (A) Quantitative RT-PCR analysis of IL12RB2, IL23R, and IL12RB1 gene expression by the indicated Th cell subsets. AU, arbitrary unit. Data are mean ± SEM of six donors. (B) Phosphorylation of STAT4 and STAT3 by the indicated Th cell subsets in response to IL-12 and IL-23, respectively; untreated cells (filled histograms) and cytokine-treated cells (solid line). (C) Proportion of cells that phosphorylate STAT4 or STAT3 in response to IL-12 and IL-23. Data are mean ± SEM of seven donors. ns, p > 0.05; *p < 0.05; ***p < 0.001 (ANOVA). (D) Phosphorylation of STAT4 and STAT3 by the indicated Th cell subsets in response to IL-12 + IL-23; cells treated with IL-12 (upper panels) or IL-23 (lower panels) (filled histograms) and cells treated with both cytokines together (solid line). Plots are gated on pSTAT4+ (top) and pSTAT3+ (bottom) cells. Data are representative of four independent experiments. (E) Production of IL-17 and IFN-γ by 6+3+ Th1/17 cells activated with CD3/CD28 beads and cultivated for 13 d with either medium, IL-12 or IL-23. Data are representative of six donors analyzed.
Consistent with the expression results, Th1/17 cells displayed robust functional responses to both IL-12 and IL-23 as measured by phosphorylation of STAT4 and STAT3, respectively (Fig. 2B, 2C). Interestingly, Th1/17 cells that respond to IL-12 or IL-23 expressed the highest levels of T-bet or RORγt, respectively (data not shown). In addition, ∼30% of the cells that phosphorylated STAT4 in response to IL-12 also responded to IL-23 by phosphorylating STAT3 in Th1/17 cells, whereas <5% of Th1 cells could respond to both cytokines. Similarly, ∼40% of the cells that phosphorylated STAT3 in response to IL-23 also responded to IL-12 by phosphorylating STAT4 in Th1/17 cells, whereas only 5% or less of Th17 cells phosphorylating both STATs (Fig. 2D). Thus, a significant fraction of Th1/17 cells can respond to IL-12 and IL-23, indicating that these cytokines may help modify the survival, proliferation, and/or functional characteristics of these cells depending on the inflammatory environment they encounter. To address this, we expanded Th1/17 cells with CD3/CD28 beads in the presence of IL-12 or IL-23 and analyzed their cytokine profile after 13 d (Fig. 2E). Whereas IL-12 increased the proportion of IFN-γ-only–producing cells while slightly reducing the number of cells producing IL-17, addition of IL-23 substantially increased the frequency of IL-17/IFN-γ–producing T cells without affecting the number of cells producing IL-17 alone. In addition, IL-23, but not IL-12, slightly augmented the proliferation of anti-CD3/CD28–activated Th1/17 cells (data not shown). Thus, by their coexpression of IL-12Rβ2 and IL-23R, Th1/17 cells can adjust their functional characteristics in different inflammatory environments.

**CCCR6+ CCR3+ Th1/17 cells are broadly reactive**

To further examine the functional relationship of Th1/17 cells with Th17 and Th1 cells, we compared their Ag specificities directly ex vivo. For this, we measured T cell proliferation in response to Ags from commonly encountered microorganisms. We focused first on two Th17-associated Ags (C. albicans and S. aureus) and one Th1-associated Ag (influenza) (Fig. 3A). Consistent with their mixed phenotype, Th1/17 cells proliferated in response to all three Ags but at a lower magnitude than Th17 or Th1 cells. However, T cells responding to extracellular bacteria associated with either the upper respiratory tract (S. pneumoniae) or the gut (E. coli and L. rhamnosus) were consistently enriched in the Th1/17 cell subset (Fig. 3B), with little or no response observed in the Th17 or Th1 cells. Given their polyfunctionality, we next assessed the cytokine production potential of Th1/17 cells specific for these different Ags (Supplemental Fig. 2). In contrast to Th17 and Th1 cells, Ag-specific Th1/17 cells displayed a highly polyfunctional cytokine response dominated by production of IFN-γ in conjunction with IL-17 and/or IL-22 as we observed in total Th1/17 cells. Therefore, our results show that even though Th1/17 cells shared Ag specificities with Th17 and Th1 cells, these T cells were enriched in cells recognizing certain extracellular bacteria and present a very broad cytokine response capable of mobilizing multiple effector pathways that may contribute to pathogen clearance and control.

*Integrin αβ7 expression in Th1/17 cells and CCCR6+ CCR3+ Th1/17 cells discriminate between C. albicans and E. coli responses*

Because Th1/17 cells were enriched in cells directed toward bacteria associated with mucosal surfaces such as *E. coli* and *L. rhamnosus*, we asked whether this was related to the presence of a higher frequency of cells expressing integrin β7, which in conjunction with the α4 and α6 integrin subunits helps mediate T cell migration to the intestinal lamina propria and epithelium. Indeed, compared with Th1 and Th17 cells, Th1/17 cells contained a higher proportion of integrin β7-expressing cells (Fig. 4A, 4B). Moreover, integrin β7 expression segregated Ag-specific responses to *S. aureus*, a bacterium associated with the human respiratory tract and the skin, and *E. coli*, which generally colonizes the intestines, in both Th17 cells and Th1/17 cells (Fig. 4C, 4D). Thus, Th1/17 cells are enriched in cells capable of migrating to the intestines and reactive for gut-tropic microorganisms, suggesting an association between Ag recognition in the intestinal tissues and acquisition of the unique Th1/17 cell phenotype.

**TCR stimulation and IL-1β render Th17 cells responsive to IL-12 and favor differentiation into Th1/17 cells**

Recent fate-mapping studies in murine systems have demonstrated that Th1/17 cells can be derived from Th17 cells (16). However, the cytokines that promote their differentiation in different circumstances remain unclear. Although IL-12 can potently induce expression of genes associated with Th1/17 cells such as T-bet, IFN-γ, and CXC, we and others have shown that Th17 cells are not responsive to IL-12 directly ex vivo (17). Notably, a subset of Th17 cells expressed the IL-1R (IL-1R1) (Fig. 5A, Supplemental Fig. 3A), and responsiveness to IL-1β can be measured by assessing IL-1β–dependent IKKα degradation (Fig. 5B, Supplemental Fig. 3A). Moreover, IL-12 and IL-1β are both produced upon LPS stimulation as a result of TLR4 and intracellular inflammasomes, respectively, raising the possibility that these cytokines may be coproduced following infection or tissue injury (23, 24). Therefore, we evaluated the effect of IL-1β treatment on the capacity of Th17 cells to respond to IL-12 and subsequently to differentiate into IL-17/IFN-γ+ Th cells.

Interestingly, overnight TCR stimulation itself induced Th17 cell responses to IL-12 as illustrated by STAT4 phosphorylation. However, the addition of IL-1β to the culture dramatically increased this response (Fig. 5C). Furthermore, Th17 cells activated in the presence of IL-1β + IL-12 strongly upregulated T-bet expression and displayed an enhanced capacity to respond to IL-12 (Fig. 5D). These data correlated with the upregulation of TBOX21 protein.
T-bet and IL12RB2 (IL-12Rβ2) at the gene expression level while slightly increasing RORC (RORγ) (Supplemental Fig. 3B, 3C). To determine whether this led to induction of IFN-γ expression, we expanded Th17 cells with IL-1β, IL-12, or both and analyzed cytokine production after 13 d. Because IL-1R1 is not homogeneously expressed on Th17 cells (Supplemental Fig. 3A), we analyzed IL-1R1–negative and –positive Th17 cells separately. As previously published, IL-1R1–positive Th17 cells produced the highest levels of IL-17 (25). Addition of IL-1β alone to the culture increased the percentage of IL-17–producing cells without inducing IFN-γ, whereas IL-12 had only a small impact on the proportion of IFN-γ–producing cells. However, IL-1β and IL-12 together displayed a strong synergistic effect, inducing a large proportion of Th17 cells to express IFN-γ in conjunction with IL-17, and this synergistic effect was observed with amounts of IL-1β that were ~10-fold lower than that conventionally used in T cell differentiation cultures (25). Addition of IL-1β alone to the culture increased the percentage of IL-17–producing cells without inducing IFN-γ, whereas IL-12 had only a small impact on the proportion of IFN-γ–producing cells. However, IL-1β and IL-12 together displayed a strong synergistic effect, inducing a large proportion of Th17 cells to express IFN-γ in conjunction with IL-17, and this synergistic effect was observed with amounts of IL-1β that were ~10-fold lower than that conventionally used in T cell differentiation cultures (Fig. 5G). Collectively, these data indicate that IL-1β is a key cytokine that, together with TCR stimulation, renders in vivo generated Th17 cells sensitive to IL-12, and both cytokines potently induce the differentiation of IFN-γ+IL-17+ Th1/17 and IFN-γ+IL-172 “ex-Th17 cells” that also produce GM-CSF.

**Discussion**

To adequately respond to biologically diverse pathogens, CD4 T cells undergo functional specialization into distinct Th cell subsets. However, recent data indicate that there is substantial plasticity among these populations and that cells with “blended” phenotypes can be identified (26). In this study, we performed an in depth characterization of the function, Ag specificity and developmental requirements of ex vivo–purified CCR6+CXCR3+ Th1/17 cells that have been associated with multiple inflammatory and autoimmune diseases and which display unique functional and gene expression profiles (27). By focusing on ex vivo–sorted T cells, our study brings critical insights into the phenotypic and functional characteristics of Th1/17 cells and avoids bias that can be introduced by working on in vitro–expanded T cells. This is illustrated by the lack of detectable levels of IL-12RB2 on freshly isolated peripheral blood Th17 cells as compared with previous work where human Th17 clones express T-bet and IL-12RB2 at levels similar to Th1 and Th1/17 clones (8). In addition, the notion that Th1/17 cells express higher levels of IL-12RB2 than Th1 cells was not previously appreciated. Moreover, by comparing the Ag specificities of Th17, Th1, and Th1/17 cells directly ex vivo, we observed in conventional Th17 cells (Fig. 5G). Collectively, these data indicate that IL-1β is a key cytokine that, together with TCR stimulation, renders in vivo generated Th17 cells sensitive to IL-12, and both cytokines potently induce the differentiation of IFN-γ+IL-17+ Th1/17 and IFN-γ+IL-172 “ex-Th17 cells” that also produce GM-CSF.
FIGURE 5. TCR stimulation and IL-1β render Th17 cells responsive to IL-12 and favor the differentiation into IFN-γ+IL-17+ Th cells. (A) Quantitative RT-PCR analysis of IL1R1 gene expression by the indicated Th cell subsets. AU, arbitrary unit. Data are mean ± SEM of nine donors. (B) STAT4 phosphorylation and Ikbα degradation in response to IL-12 and IL-1β by the indicated Th cell subsets directly ex vivo. (C) STAT4 phosphorylation in response to IL-12 by 6+3 Th17 cells activated for 24 h with CD3/CD28 beads in the presence or absence of IL-1β; untreated cells (filled histograms) and cytokine-treated cells (solid line). (D) STAT4 phosphorylation and T-bet expression by 6+3 Th17 cells activated for 24 h with CD3/CD28 beads in the presence of absence of IL-1β and/or IL-12. (E) Production of IL-17 and IFN-γ by IL-1R1− and + 6+3 Th17 cells expanded for 13 d with the indicated cytokines and stimulated for 5 h with PMA/ionomycin. (F) Proportion of IL-1R1− and + 6+3 Th17 cells producing IL-17, IFN-γ, and GM-CSF. (Figure legend continues)
showed that Th1/17 cells, in addition to sharing Ag specificities with Th17 and Th1 cells, have increased frequencies of cells recognizing certain types of bacteria, and this was linked to their higher integrin β7 expression. Finally, we report that IL-1β can potentially synergize with IL-12 to induce human IL-1Rα Th17 cells to upregulate IFN-γ and GM-CSF expression, therefore generating cells with a proinflammatory and potentially pathogenic cytokine profile.

Phenotypically, IL-17– and IFN-γ–coping Th cells were previously shown to coexpress CCR6 and CXCR3 (2, 7). However, we found that despite homogenous expression of the transcription factors T-bet and RORγt, Th1/17 cells displayed a highly polyfunctional cytokine profile. This degree of functional heterogeneity was not observed in classical Th1 or Th17 cells and cautions against using the CCR6+CXCR3+ phenotype to definitely polyfunctional cytokine profile. This degree of functional heterogeneity was not observed in classical Th1 or Th17 cells and cautions against using the CCR6+CXCR3+ phenotype to definitely identify IFN-γ+IL-17–coproducing cells. Although this may reflect an inherent functional instability or flexibility in the Th1/17 cells, their cytokine profiles were relatively stable during in vitro activation and expansion in the absence of added polarizing cytokines, suggesting that they are not innately less stable than those of Th1 or Th17 cells. Alternatively, recent epigenetic data have demonstrated that the “enhancer landscape” available to T-bet and RORγt is dictated by TCR- and cytokine-dependent activation of AP-1 and STAT transcription factors (28). Thus, heterogeneity in availability of specific promoter/enhancer elements to T-bet and RORγt caused by differences in TCR/cytokine signals the cells have been exposed to during differentiation may underlie the various patterns of cytokine secretion present in the Th1/17 cell population. The relatively high frequency of Th1/17 cells observed in peripheral blood of healthy donors and the presence of Th17 cells specific for commonly encountered microorganisms such as C. albicans and S. aureus and influenza virus indicate that the differentiation of cells into this “hybrid” Th cell population is a fairly common mechanism happening most likely during an ongoing immune response. However, the imbalance between these different T cell populations might be perturbed during an autoimmune disease.

Interestingly, despite the strong association of IL-22 with Th17 responses, most IL-22–producing cells in the Th1/17 cell population coproduced IFN-γ and not IL-17. However, Th1 cells were reported to express IL-22 (29), and IL-22 production in human CD4 T cells is more strongly associated with production of IFN-γ than IL-17 (3). Coproduction of IL-17 and IL-22 is beneficial in coordinating innate epithelial cell responses against extracellular pathogens. Whereas IL-17 is largely proinflammatory and causes tissue destruction, IL-22 has a regenerative and protective effect on epithelial cells (30). Similarly, IL-22 may help balance the proinflammatory functions of IFN-γ with important wound healing and tissue repair functions. For instance, in the intestine IFN-γ and IL-22 can have opposing effects in maintaining epithelial barrier function during inflammation caused by food sensitivities, toxins, or infection (31, 32). Furthermore, the genomic proximity of the IFNG and IL22 gene loci suggest that shared regulatory regions may contribute to the coexpression of IFN-γ and IL-22 in Th1/17 cells (31). It will therefore be interesting to examine the epigenetic status of the IL-22 locus to define how expression of this important cytokine is controlled in different IL-22–producing Th cell populations.

Cells specific for both Th17-associated Ags C. albicans and S. aureus and the Th1–associated Ag influenza were contained in the Th1/17 cell population, suggesting that some of these cells may originate from classical Th17 cells that upregulate T-bet and CXCR3, whereas others may be derived from Th1 cells that turn on expression of RORγt and CCR6. Whereas a firm developmental link between Th1 and Th17 cells has not been established, several studies have demonstrated that Th1/17 cells can differentiate from Th17 cells. For instance, TCR sequence analysis demonstrated that at least a fraction of the CD161+ Th1 cells (which are predominantly found in the Th1/17 cell population) were clonally related to Th17 cells (33). In addition, several reports indicated that Th17 cells are plastic and can differentiate into cells producing both IL-17 and IFN-γ or IFN-γ only (8, 15, 17, 18). Further analysis, including comprehensive TCR sequence analysis on Ag-specific T cells, is needed to help determine the developmental relationships between classical Th1 or Th17 cells and different populations of Th17 cells.

Although IL-12 is capable of promoting Th1/17 cell development from in vitro–generated Th17 cells, Th17 sorted directly from human blood do not efficiently respond to IL-12 because of low expression of IL-12Rβ2. We found that IL-1β, together with TCR stimulation renders Th17 cells responsive to IL-12, and that there is a strong synergy between IL-1β and IL-12 in promoting the differentiation of IL-1Rα+Th17 cells into cells that coproduce IL-17 and IFN-γ without impacting RORγt expression. Moreover, most of the IL-17(IFN-γ)+ cells also coproduced GM-CSF, and production of these three cytokines together has been associated with pathogenicity in autoimmune diseases. Thus, we hypothesize that IL-1β might help stabilize T-bet expression and thereby potentiate IL-12R signaling on a subset of Th17 cells, rendering them more susceptible to functional reprogramming. As a result, coproduction of IL-1β and IL-12 during bacterial or fungal infection (19, 34, 35), or during inflammation in patients with active autoimmune diseases (36, 37), could participate in the differentiation of Th17 cells into highly proinflammatory Th1/17 cells in vivo. Furthermore, inhibition of Th1/17 cell differentiation may partially underlie the therapeutic efficacy of treatments such as Anakinra that target IL-1β activity. In addition to IL-1β plus IL-12, other pathways have been identified that induce IFN-γ production by Th17 cells. Indeed, similar to IL-1β, IFN-γ itself can render Th17 cells sensitive to IL-12 (17). Moreover, although IL-23 failed to synergize with IL-1β to promote Th17 differentiation, repeated stimulation of Th17 cells in the presence of IL-23 can directly upregulate IFN-γ independently of T-bet (18). Therefore, Th17 cell plasticity may be governed in vivo by multiple pathways depending on the cytokine microenvironment and interfering with this mechanism might prove more challenging than previously anticipated.

In addition to their shared antigenic specificities with Th1 and Th17 cells, we found that Th1/17 cells present a unique reactivity profile. Indeed, this T cell population was enriched in cells responding to E. coli, L. rhamnosus, and S. pneumoniae, and a previous study demonstrated that responses to Mycobacterium tuberculosis purified protein derivative are found almost exclusively in the Th1/17 cell population (2). The unique properties of Th1/17 cells were also illustrated by the high frequency of integrin αβ7+ cells in this population and by the enrichment of T cell specific for E. coli in the integrin αβ7+ cell fraction.

IFN-γ, GM-CSF, or all three cytokines together after expansion as indicated above. Data are mean ± SEM of four donors. *p < 0.05; **p < 0.01; ***p < 0.001 (ANOVA). (G) Production of IL-10 by expanded 63+ Th17 and 63+ Th1/17 cells. Data are mean ± SEM of six donors. *p < 0.001 (two-tailed paired t test).
This suggests that the intestinal tissue may be a particularly potent site of Th1/17 cell differentiation. Consistent with this, compared with peripheral blood, the intestinal mucosa of IBD patients contains a high frequency of Th1/17 cells (8). Indeed, several studies have reported high levels of IL-1β secretion by colonic lamina propria monocytes from patients with active IBD (38, 39), and although IL-12 production is tightly controlled in the intestines it can be induced during infection with certain enteric pathogens (40). Thus, Th17 cells present at sites of intestinal inflammation can be exposed to both IL-1β and IL-12, triggering their differentiation into Th1/17 cells. Although this may be beneficial in combating enteric pathogens and maintaining proper intestinal homeostasis, when dysregulated, it also may contribute to development of inflammatory and autoimmune diseases. The unique reactivity profile of Th1/17 cells supports the idea that these cells are distinct from Th1 and Th7 cells. This was further enforced by a recent study showing that Th1/17 cells in human peripheral blood displayed a unique gene expression signature. This is illustrated by the expression of the gene ABCB1 specifically in Th1/17 cells (27) (data not shown).

The identification of new Th cell subsets such as Th17 and Th22 cells has provided important insights into the high degree of T cell specialization required to efficiently respond to a microbial insult. However, recent evidence that Th subsets are more plastic than initially believed adds considerable complexity to this issue and highlights the need to better characterize “hybrid” Th cell populations such as Th17 cells that have been implicated in autoimmune disease pathogenesis. Our characterization of the properties, specificity, and development of CCRC6 CXCR3 Th17 cells has revealed that these cells are highly polyfunctional, that they have a unique specificity profile and surface phenotype associated with activation in mucosal tissue sites, and that the cytokine IL-1β may play a key role together with IL-12 in their differentiation from IL-1R1-positive Th17 cell precursors in vivo. Given their polyfunctionality and broad Ag reactivity, it will be interesting in future studies to examine the differentiation and functional behavior of different Th17/17 populations in settings of acute and chronic inflammation. In particular, analyses of inﬂamed tissue samples will help determine whether certain functionalities and specificities are enriched during different types of inflammatory responses. This information will be useful in understanding the development and function of these cells in the contexts of normal and pathogenic immune responses and for the identification of new therapeutic strategies to manipulate Th17/17 cells in immune-mediated diseases.

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References


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