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Inflammatory Effects of Ex Vivo Human Th17 Cells Are Suppressed by Regulatory T Cells

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Th17 cells are proinflammatory cells associated with many immune-mediated diseases. Major factors limiting the study of human Th17 cells are the lack of an accepted method for their in vitro differentiation or for isolation of a homogenous population of Th17 cells that do not cosecrete IFN-γ. To overcome these hurdles, we established a novel method to isolate in vivo differentiated Th17 cells from peripheral blood by sorting CD161+CCR4+CCR6+CXCR3−CD4+ T cells. The resulting cells produce high levels of IL-17 but not IFN-γ, express high levels of retinoic acid-related orphan receptor variant 2, and maintain this phenotype upon expansion. Ex vivo Th17 cells exhibit a low cytotoxic potential and are hypersensitive to polyclonal anti-CD3/anti-CD28 stimulation. Importantly, ex vivo Th17 cells were susceptible to suppression by both naïve and memory regulatory T cells (Tregs), which inhibited production of IL-17, IL-22, and CXCL8. Moreover, Tregs suppressed the antifibrotic effects of Th17 cells in a wound-healing model. These findings provide new tools for the study of normal and pathological functions of bona fide Th17 cells in humans. They also provide new insight into the cross-talk between Th17 cells and immune and nonimmune cells, and they establish the paradigm that adoptive Treg-based therapies may effectively limit Th17-mediated inflammation. The Journal of Immunology, 2010, 185: 3199–3208.

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Abbreviations used in this paper: PI, proliferation index; RORC2, retinoic acid-related orphan receptor variant 2; Treg, regulatory T cell.

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Tregs suppressed IL-17 from CD4^+CD25^- T cells and that CD39^+ Tregs produced IL-17 themselves (25) support this notion.

To date, no study has examined interactions between pure populations of ex vivo Th17 cells and Tregs, and the true capacity of Tregs to suppress the effector function of bona fide Th17 cells therefore remains an outstanding question. To better define the phenotype of human Th17 cells and study their interactions with Tregs, we developed a flow cytometry-based sorting method to isolate a homogeneous population of in vivo-derived Th17 cells and conducted comprehensive studies to define their function and regulation.

Materials and Methods

Isolation of T cell subsets

All human tissue and peripheral blood were obtained from healthy volunteers following approval by the University of British Columbia Clinical Research Ethics Board, after obtaining written informed consent. PBMCs were isolated by Ficoll separation, and CD4^+ T cells were purified by negative selection or by RosetteSep (StemCell Technologies, Vancouver, British Columbia, Canada). For Treg sorting, a pre-enrichment was performed by incubation with CD25 beads and passing over an LS column (Milteny Biotec, Auburn, CA). CD25^-cells then were stained for CD4, CD25, and CD45RA and sorted using a FACSAria (BD Biosciences, San Jose, CA) into either CD4^+CD25^- (RA^- Treg) or CD4^+CD25^+ (RA^+ Treg) populations, achieving purities of >98% on the basis of CD25 and CD45RA expression. For isolation of Th1 and Th17 cells, either total CD4^+ or CD25^- cells were stained for CD4, CXCR3,CCR4,CCR6, and CD161 (all BD Pharmingen, San Diego, CA) and sorted using a into either CD4^+CXCR3^-CCR4^-CCR6^-CD161^- (Th1), CD4^+CXCR3^-CCR6^-CCR4^-CCR14^- (Th17), CD4^+CXCR3^-CCR6^-CCR4^-CCR14^-CD161^- (Th17), or CCR4^-CCR6^-CCR4^-CCR14^-CD161^- (CD161^+Th17) cells with purities of >97%. Sorting gates were set on the basis of fluorescence minus one controls. CXCR3^+ Th1, CD161^+Th17, and CD161^+ Th17 cells displayed a memory phenotype based on CD45RA expression (data not shown). CD161^+ Th17 cells were assayed in all functional studies, with no significant differences compared with CD161^-Th17 cells.

T cell culture, expansion, and cloning

To expand sorted T cells, they were activated with soluble anti-CD3 Abs (1 µg/ml OKT3) and autologous irradiated APCs at a 1:5 ratio of T cells to APCs in complete medium (X-VIVO 15 [Cambrex, East Rutherford, NJ] with 5% pooled AB human serum [Cambrex], penicillin/streptomycin [Invitrogen, Carlsbad, CA], and GlutaMAX [Invitrogen]) with IL-2 (100 U/ml; Chiron) for 14 d, with media replenished every 2–3 d. In some cases, T cells were cloned at 0.3 cells per well in 96-well round-bottom plates in the presence of an allogeneic feeder-cell mixture as previously described (26). T cell clones were stimulated at 5 x 10^5 cells per milliliter with mitogens and then apoptosis or anti-CD3/anti-CD28–coated T cell expander beads (Invitrogen) for 72 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added halfway through the activation. After proliferation for 96 h was assessed by flow cytometry. The proliferation index (PI), defined as the average number of cells that an initial cell became, was calculated using FCS Express software, version 3 (Los Angeles, CA). To assess Treg suppression of proliferation and cytokine production, CFSE-labeled target cells were stimulated with irradiated APCs (one T cell to five APCs) for 24 or 96 h in the absence or presence of the indicated ratio of CD45RA^- or CD45RA^+ Tregs. Cells were stimulated with PMA and Ca^2+ ionophore for the final 6 h as described above to measure cytokine production by intracellular staining.

Fibroblast scratch migration assay

An in vitro wound-healing assay was modified from Liang et al. (27). A total of 75,000 primary foreskin fibroblasts were plated overnight in DMEM (StemCell Technologies) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin, and 1% glucose in a 6-well plate. After 24 h, when the cells became confluent, the cell monolayer was scratched using a 200 µm pipette tip, and the media was aspirated. Debris was removed, and the medium was replaced with medium without FBS. In parallel, T cell lines were activated with anti-CD3/anti-CD28 T cell expander beads in the presence or absence of CD25^-CD45RA^- or CD25^-CD45RA^- CD4^+ Tregs, and supernatants were collected after 48 h. Cell-free supernatants were added to the scratched fibroblasts, which were cultured for an additional 18 h. Migratory activity was measured by a Fibroblast scratch migration assay, as previously described (28), with the cell monolayer scratched with a 200-µl pipette tip and the media aspirated. The media was replaced with complete medium without FBS, and the plates were incubated for 16 h. Photographs of the scratch were taken using an Axiovert 200M (Zeiss, Oberkochen, Germany) at the matched reference point at 0 h and after 16 h of incubation. Migration was analyzed by counting the number of fibroblasts that migrated into the scratched areas in triplicate wells by an investigator blind to the culture conditions. Similar results were observed when activated T cells were added directly to fibroblast cultures.

Statistical analysis

All of the analyses for statistically significant differences were performed with one-tailed Student t test unless otherwise indicated. The p values <0.05 were considered significant. All of the error bars represent standard deviations.

Results

Development of a method for isolation of in vivo differentiated Th17 cells

A major caveat of all of the previous studies of human Th17 cells is the heterogeneity of the cell populations that were studied. For example, the vast majority of Th17 cells that were differentiated from adult naive T cells typically only contained 1–9% IL-17–producing cells on the basis of intracellular staining (9, 12, 18, 28), and sorting cells on the basis of CCR4 and CCR6, or CD161 only resulted in 2–9% IL-17^+ cells with significant contamination from IFN-γ–producing cells (11–13). Therefore, to study the biology and regulation of Th17 cells, we sought to develop a better way to isolate homogenous populations of IL-17–producing cells from peripheral blood. To conduct functional studies, it was important that the method yield live, nonactivated cells in numbers sufficient for direct ex vivo assays.

First, in an attempt to improve the homogeneity of populations of CCR4^-CCR6^ T cells (11), we depleted CXCR3^-expressing cells, which produce IFN-γ (29) (Supplemental Fig. 1A). When sorted CXCR3^-CCR6^-CCR4^-CD4^- T cells were activated with anti-CD3/anti-CD28–coated beads, they produced high amounts of IL-17 and low amounts of IFN-γ, whereas CXCR3^+ or total CD4^+ T cells did not produce IL-17 but produced large amounts of IFN-γ (Supplemental Fig. 1B). We also examined the expression of T-box expressed in T cells and RORC2, the lineage-defining transcription factors for Th1 and Th17 cells, respectively. Interestingly, both populations expressed high levels of T-box expressed in T cells, whereas RORC2 was only expressed by CXCR3^-CCR6^-CCR4^- CD4^- T cells (Supplemental Fig. 1C). Therefore, isolation of CXCR3^-CCR6^-CCR4^-cells significantly enriches for IL-17^- and...
RORC+ cells, whereas CXCR3+CD4+ T cells have a classical Th1 phenotype. CXCR3+ Th1 cells were included in all of the subsequent experiments as a comparator population for studies with Th17 cells.

Although this sorting strategy enriched for IL-17 IFN-γ CD4+ T cells, when we performed a single-cell cloning, we noted that clones varied in the production of other cytokines, including IFN-γ, IL-6, TNF-α, IL-4, and IL-10 (Supplemental Fig. 1D). We investigated whether inclusion of the IL-23R (8) as a sorting parameter could enhance the homogeneity of the population but found that the expression of this protein was too low and variable to be suitable for this purpose. We therefore examined whether the inclusion of CD161, a molecule recently associated with Th17 cells (12, 13), as a sorting parameter would improve the homogeneity of the population. CXCR3−CCR6−CCR4−CD161+CD4+ T cells (hereafter CD161+ Th17 cells) or CXCR3−CCR6−CCR4+CD161−CD4+ T cells (hereafter CD161− Th17 cells) were sorted and compared with CXCR3− Th1 and total CD4+ T cells (Fig. 1A). Because optimal expression of IL-17 requires prior TCR stimulation (30), intracellular cytokine staining was performed after the sorted cells were stimulated with anti-CD3/anti-CD28−coated beads for 3 d in the absence of exogenous cytokine or APCs. As shown in Fig. 1B, CXCR3+ Th1 cells produced IFN-γ but not IL-17, whereas both CD161+ and CD161− Th17 cells contained significant proportions of IL-17+ cells with negligible numbers of IFN-γ+ cells. CD161+ Th17 cells contained on average 3-fold more IL-17+ cells than CD161− Th17 cells. Among the CD161+ Th17 cells, only 3.66 ± 0.22% of IL-17+ cells coexpressed IFN-γ, and in CD161− Th17 cells, 3.69 ± 0.29% of the IL-17+ cells were also IFN-γ+. Prestimulation with anti-CD3 plus APCs rather than anti-CD3/anti-CD28−coated beads resulted in higher cytokine production: 22.29 ± 2.81% of CD161+ Th17 cells expressed IL-17, and 50.14 ± 28.45% of CXCR3+ Th1 cells expressed IFN-γ (Fig. 4 and data not shown).

In addition to IL-17 and IFN-γ, the expression of IL-2 and IL-22 was assessed (Fig. 1B). Although IL-22 was expressed by a significant proportion of cells in all of the subsets, the percentage of IL-22+ cells within CD161+ Th17 cells was at least 2-fold greater than that in all of the other populations. Moreover, both CD161+ and CD161− Th17 populations contained a significant number of IL-2−secreting cells, as did the CXCR3+ Th1 cells.

Next, we assessed the amount of IL-17 secreted by the sorted populations by stimulating cells with anti-CD3/anti-CD28−coated beads and analyzing the amounts of IL-17 and IFN-γ in supernatants (Fig. 1C). In agreement with the flow cytometry data, CXCR3+ Th1 cells secreted high amounts of IFN-γ. Although both CD161+ and CD161− Th17 cells secreted high levels of IL-17 and low levels of IFN-γ, the CD161+ Th17 cells consistently produced significantly higher amounts of IL-17 than the CD161− Th17 cells. Neither total CD4+ nor CXCR3+ Th1 cells produced significant amounts of IL-17.

FIGURE 1. Optimized method for isolating ex vivo Th17 cells on the basis of CD161 expression. A, Outline of sorting and gating strategy. On average, CXCR3+ Th1 cells were 12.45 ± 6.01% (n = 6), CD161+ Th17 cells were 1.93 ± 0.49%, and CD161− Th17 cells were 2.93 ± 1.16% of CD4+ T cells (n = 4–6). B, Sorted T cells were stimulated with anti-CD3/anti-CD28−coated beads for 3 d prior to intracellular cytokine staining. For CXCR3+ Th1 cells, on average 1.53 ± 0.90% were IL-17+, 47.86 ± 22.14% IFN-γ+, 8.47 ± 5.67% IL-22+, and 18.06 ± 2.19% IL-2+(n = 4). For CD161+ Th17 cells, on average 10.27 ± 2.89% were IL-17+, 5.20 ± 4.00 IFN-γ+, 15.17 ± 9.97% IL-22+, and 17.68 ± 5.05% IL-2+(n = 4). For CD161− Th17 cells, on average 3.47 ± 0.99% were IL-17+, 4.90 ± 2.90% IFN-γ+, 12.53 ± 7.37% IL-22+, and 29.5 ± 3.32% IL-2+(n = 4). C, Sorted T cells were activated with anti-CD3/anti-CD28−coated beads, and supernatants were collected at the indicated time points to quantify IFN-γ and IL-17 production by ELISA. CXCR3+ Th1 cells produced 8.00 ± 0.35 ng/ml IFN-γ and 0.080 ± 0.052 ng/ml IL-17 (n = 3), CD161+ Th17 cells produced 0.96 ± 0.62 ng/ml IFN-γ and 2.54 ± 1.03 ng/ml IL-17 (n = 3), and CD161− Th17 cells produced 0.67 ± 0.10 ng/ml IFN-γ and 0.99 ± 0.97 ng/ml IL-17 (n = 3) at 48 h. Data are representative of independent donors, and the SD in C indicates experimental triplicates.
amounts of IL-17. Thus, although CD161− sorted cells display a Th17-like phenotype, the population sorted as CD161+ contains a higher proportion of cells that can make IL-22 and secrete higher amounts of IL-17. Therefore, inclusion of CD161 as a sorting parameter substantially enhances the homogeneity of the ex vivo isolated cells.

**CD161+ and CD161− Th17 cells maintain their phenotype upon expansion, and single-cell cloning confirms the homogeneity of the population**

To define whether the phenotypes of CD161+ and CD161− Th17 cells were stable in vitro, we expanded sorted populations in the presence of IL-2 without the addition of polarizing cytokines. Typical expansion yields from 200,000 cells were as follows: 4.1 × 10⁶ from total CD4+ cells, 4.6 × 10⁶ from CXCR3+ Th1 cells, 7.3 × 10⁶ from CD161+ Th17 cells, and 6.6 × 10⁶ from CD161+ Th17 cells. After 14 d, the expanded T cells were analyzed in the resting phase. Although the majority of cells that expanded from either CD161+ or CD161− Th17 cells expressed IL-17 (Fig. 2A), the expanded CD161+ Th17 cells consistently had a higher percentage of IL-17+ cells (Fig. 2A). As expected, expanded CXCR3+ Th1 cells remained IFN-γ+ IL-17−. Notably, expansion of the sorted populations appeared to enhance and/or stabilize their phenotypes: the percentage of IL-17+ or IFN-γ+ cells in expanded cultures of Th17 and Th1 cells, respectively, was significantly increased compared with analysis ex vivo (compare Fig. 1B and Fig. 2A). In contrast, IL-22 expression levels remained approximately equivalent (Figs. 1B, 2A).

To determine whether CD161+ and CD161− Th17 cells maintained the expected expression of RORC2, expanded T cells in the resting phase were stimulated with anti-CD3/anti-CD28–coated beads and RORC expression was assessed by quantitative RT-PCR. In Fig. 2B, the average RORC expression in expanded T cells from

**FIGURE 2.** Homogeneity of ex vivo Th17 cells is demonstrated by expansion and single-cell cloning. A, Sorted T cells were expanded for 14 d and then analyzed by intracellular staining. CXCR3+ Th1 cells were 5.81 ± 1.97% IL-17+, 73.54 ± 16.23% IFN-γ+, 15.48 ± 7.63% IL-22+, and 30.24 ± 10.72% IL-2+ (n = 3). CD161+ Th17 cells were 68.42 ± 10.66% IL-17+, 2.65 ± 2.54% IFN-γ+, 25.33 ± 10.28% IL-22+, and 18.17 ± 3.24% IL-2+ (n = 3). CD161− Th17 cells were 61.83 ± 20.38% IL-17+, 5.8 ± 2.23% IFN-γ+, 32.49 ± 11.53% IL-22+, and 20.98 ± 4.07% IL-2+ (n = 3). B, Expanded T cells were activated with anti-CD3/anti-CD28–coated beads for 48 h, and expression of RORC relative to that of GAPDH was analyzed by quantitative RT-PCR. Data are the average expression in four donors. C and D, Single-cell clones were derived from sorted populations, and 50 growing wells from each condition were expanded. Clones were restimulated with anti-CD3/anti-CD28–coated beads for 48 h, and supernatants were analyzed by ELISA or cytometric bead assay. Each dot represents data from an individual clone. A nonpaired t test was used to calculate significance in D. For B and D, *p < 0.05; **p < 0.02.
four independent donors is shown. Both CD161+ and CD161− Th17 cells expressed RORC mRNA, but the expression level was significantly higher \( (p = 0.047, n = 4) \) in the CD161+ Th17 cells. Therefore, although expanded CD161+ and CD161− T cells both display the Th17 phenotype, consistent with cells analyzed ex vivo, CD161+ Th17 cells express higher levels of IL-17 and RORC2 than CD161− Th17 cells.

Intracellular cytokine staining is a relatively insensitive technique that tends to underestimate the true capacity of cells to produce cytokines. To more precisely evaluate the homogeneity of the population, we generated single-cell clones and characterized the cytokine production profiles of 50 clones from each subset. As shown in Fig. 2C, 100% of the CXCR3+ Th1 clones produced IFN-γ and did not make significant levels of IL-17. In contrast, >90% of both CD161+ and CD161− Th17 clones were IL-17+ IFN-γ−, and 100% of both CD161+ and CD161− Th17 clones expressed IL-17 \( (n = 50). \) All of the clones expressed TCRαβ (data not shown), excluding the possibility that this method enriched for cells expressing TCRαβ.

At the clonal level, no significant differences in the ability of CD161+ Th17 cells versus CD161− Th17 cells to produce IL-17 or IFN-γ were observed. To extend the phenotypic analysis of these in vivo differentiated Th17 cells, we also examined the expression of other Th17-associated cytokines. Apart from IL-17, IL-6 was produced at a significantly higher frequency by Th17 cell clones compared with clones from total CD4+ or CXCR3+ Th1 cells (Fig. 2D). Although more CD161− Th17 clones tended to express IL-10, CD161+ Th17 clones tended to produce TNF-α, suggesting that expression of CD161 may discriminate between pathogenic and nonpathogenic Th17 cells as recently described in a mouse model of multiple sclerosis \( (31). \) Because CD161+ Th17 cells expressed more nonpathogenic Th17 cells as recently described in a mouse model of multiple sclerosis \( (31). \)

### Phenotype of bona fide ex vivo Th17 cells

Sorting of CCR4+CCR6+CXCR3+CD161+ T cells has allowed us for the first time to study the phenotype of a homogenous population of in vivo-derived Th17 cells. We, and others, have previously suggested that low expression of granzymes A and B is a distinguishing feature of Th17 cells \( (7, 10) \). Consistent with these reports, expression of both granzyme A and granzyme B was significantly lower in CD161+ Th17 cells than that in total CD4+ T cells and CXCR3+ Th1 cells (Fig. 3A and data not shown), confirming that Th17 cells likely have a low cytotoxic potential.

We previously found that overexpression of RORC2, in addition to decreasing granzyme A and B expression, conferred a hyporesponsive phenotype that could be overcome by exogenous IL-2 and IL-15 \( (10) \). We therefore examined the capacity of CFSE-labeled cells to proliferate in the absence or presence of accessory APCs. As shown in Fig. 3B, CD161+ Th17 cells exhibited an anergic phenotype unless they were stimulated in the presence of APCs. Evidence that the hyporesponsive phenotype of CD161+ Th17 cells could be reversed by the addition of cell-free supernatants from APCs cultured for 48 h suggested that the effect was mediated by a soluble factor \( (3C) \). A similar phenotype was confirmed in expanded Th17 cells (data not shown). These data suggest that despite their capacity to produce autocrine/paracrine IL-2 \( (1, 2) \), efficient expansion of Th17 cells relies on the presence of additional factors secreted by APCs.

### Tregs suppress the proliferation of and cytokine production by ex vivo Th17 cells

It has previously been reported that human Th17 cells are resistant to Treg-mediated suppression of proliferation and IL-17 production \( (6, 7, 17, 18, 32) \). A major caveat with these studies, however, is that it is now recognized that a significant proportion of IL-17–producing cells exists within populations of CD4+CD25hi Tregs \( (21, 24) \), and inflammatory cytokines produced by Th17 cells can stimulate de novo production of IL-17 from FOXP3+ Tregs \( (21–24) \). Therefore, to accurately assess the capacity of Tregs to suppress IL-17, it is essential to perform suppression assays with Tregs that are initially IL-17− and to monitor their capacity to secrete IL-17 throughout the assay. Following the methodology of Miyara et al. \( (24) \), we sorted Tregs on the basis of CD45RA expression into CD45RA+CD25hiCD4+ cells \( (RA+ \) Tregs) that are IL-17− or CD45RA+CD25minCD4+ cells \( (RA− \) Tregs) that contain IL-17–producing cells as well as memory Tregs. In parallel, autologous CXCR3+ Th1 or CD161+ Th17 cells were sorted, labeled with CFSE, and cocultured with RA+ or RA− Tregs, thereby permitting

**FIGURE 3.** Ex vivo Th17 cells exhibit a low cytotoxic potential and are hyporesponsive in the absence of APCs. A, Sorted T cells were analyzed ex vivo for expression of granzyme A. CXCR3+ Th1 cells were 58.29 ± 34.28% positive for granzyme A \( (n = 3; p = NS \) compared with total CD4), and CD161+ Th17 cells were 33.60 ± 23.22% positive for granzyme A \( (n = 3; p = 0.03) \). Similar results were observed with granzyme B \( (\text{data not shown}) \). B, Sorted T cells were labeled with CFSE and stimulated with anti-CD3/anti-CD28–coated beads or APCs plus anti-CD3, and the amount of CFSE dilution \( (\text{cell division}) \) was assessed by flow cytometry. CD161+ Th17 cells had 64.47 ± 27.94% divided cells with a PI of 4.21 ± 1.97 when stimulated with APCs plus anti-CD3 versus only 20.66 ± 15.55% \( (P = 1.42 ± 0.98) \) when stimulated with anti-CD3/anti-CD28–coated beads \( (n = 3; p = 0.02) \). CXCR3+ Th1 cells had 91.83 ± 10.43% divided cells when stimulated with APCs plus anti-CD3 \( (P = 3.05 ± 0.54) \) versus 38.86 ± 48.67% \( (P = 3.15 ± 2.78) \) when stimulated with anti-CD3/anti-CD28–coated beads \( (n = 3; p = NS) \). C, Sorted CFSE-labeled T cells were stimulated with anti-CD3/anti-CD28–coated beads with or without supernatants from APCs from three different donors. Under these conditions, CD161+ Th17 cells had a PI of 6.13 ± 0.64 \( (n = 3; p = 0.002 \) compared with anti-CD3/anti-CD28–coated beads). Data in A, B, and C are representative of three independent experiments with different donors.
us to study the suppression of Th17 cells by IL-17+ or IL-17− populations, respectively.

As shown in Fig. 4A, both RA+ and RA− Tregs potently suppressed proliferation and IFN-γ production from CXCR3+ Th1 cells. Moreover, both RA+ and RA− Tregs efficiently suppressed the capacity of CD161+ Th17 cells to produce IL-17 and proliferate (Fig. 4B). In agreement with Ayyoub et al. (21) and Miyara et al. (24), the RA− Tregs produced significant amounts of IL-17 themselves (RA− Tregs were 21.76 ± 9.21% IL-17−, whereas RA+ Tregs were 3.92 ± 3.87% IL-17−), highlighting the importance of measuring suppression of IL-17 by CFSE labeling coupled with intracellular cytokine staining rather than by measuring secreted cytokine production.

To confirm that the capacity of Tregs to suppress cytokine production was not simply a consequence of the suppression of proliferation, we also measured cytokine production after 20 h of coculture, a time point when the targets of suppression had yet to divide. IFN-γ production from CXCR3+ Th1 cells was suppressed by 56.06 ± 18.56% by RA+ Tregs (n = 3, p = 0.02) and 88.79 ± 8.20% by RA− Tregs (n = 3, p = 0.001) (Supplemental Fig. 2). IL-17 production from CD161+ Th17 cells was suppressed by 46.18 ± 31.69% by RA+ Tregs (n = 3, p = 0.03) and 64.80 ± 19.00% by RA− Tregs (n = 3, p = 0.03).

Tregs suppress the production of IL-22 and CXCL8 from ex vivo Th17 cells

The ability of Tregs to modulate the effector functions of human Th17 cells beyond IL-17 has not been well characterized to date. Because ex vivo CD161+ Th17 cells expressed higher levels of IL-22 than other subsets, we investigated whether Tregs could also limit production of this cytokine. As shown in Fig. 5A, both RA+ and RA− Tregs efficiently suppressed IL-22 production from total CD4+ T cells, CXCR3+ Th1 cells, and CD161+ Th17 cells. Furthermore, expression of secreted IL-6 from ex vivo Th17 cells was suppressed (data not shown), indicating that Tregs effectively regulate the production of cytokines from in vivo differentiated Th17 cells.

CXCL8 (IL-8) is one of the major mediators of the inflammatory response and is a potent chemotactant and angiogenic factor (33, 34). Pelletier et al. (35) recently found that Th17 clones secrete CXCL8, suggesting that production of this chemokine may be another mechanism by which Th17 cells induce inflammation. We confirmed that ex vivo CD161+ Th17 cells produced significantly higher levels of CXCL8 than total CD4+ T cells and CXCR3+ Th1 cells (Fig. 5B). Both CD45RA− and CD45RA+ Tregs potently suppressed CXCL8 production from CD161+ Th17, CXCR3+ Th1, and total CD4+ T cells. Therefore, Tregs efficiently limit the production of multiple inflammatory mediators by Th17 cells.

Tregs reverse Th17-mediated inhibition of wound healing

Nonimmune cells are major targets for the effector functions of Th17 cells. Specifically, cytokines produced by Th17 cells act on cells such as fibroblasts and keratinocytes (8, 36) and thereby contribute to immunity in barrier tissues such as the skin and gut (3, 4). Because the effects of Th17 cells on nonimmune cells are associated with pathology in diseases such as psoriasis and inflammatory bowel disease (3, 8, 37), it was also important to determine whether Tregs could suppress this aspect of Th17 cell function. Th1 cells are classically antifibrotic via the effect of IFN-γ (38, 39), whereas, via production of TGF-β, Tregs would be predicted to be profibrotic (38, 40, 41). Although exogenous IL-17 induces upregulation of matrix metalloproteinase-1 from human fibroblasts (42), the downstream effects of Th17 cells on the wound-healing process are unknown.

To determine the effects of Th17 cells on fibroblast migration, we used an established wound-healing assay in which a controlled scratch is made in a fibroblast monolayer and the number of fibroblasts that migrate into the scratch is determined 16 h later (27). Scratched monolayers of primary dermal fibroblasts were cultured in the absence or presence of supernatants collected from activated CD161+ Th17 cells or CXCR3+ Th1 cells. Supernatants from both CD161+ Th17 cells and CXCR3+ Th1 cells significantly inhibited the ability of fibroblasts to migrate into the scratch area (Fig. 6A, 6B), confirming the known antifibrotic activity of Th1 cells and establishing a similar paradigm for Th17 cells. To ask whether Tregs could suppress this effect of Th17 cells, supernatants were collected from CD161+ Th17 cells that were activated in the presence of RA+ or RA− Tregs. Supernatants produced in the presence of CD45RA+ Tregs, which do not contain a significant proportion of IL-17−secreting cells (Fig. 6A, 6C), partially restored the fibroblast migratory capacity. In contrast, CD161+ Th17 cell supernatants produced in the presence of RA− Tregs, which express

![FIGURE 4. Naive and memory Tregs suppress proliferation and IL-17 production by ex vivo Th17 cells. A and B, Sorted ex vivo T cells were labeled with CFSE and stimulated with APCs and anti-CD3 in the presence or absence of CD45RA−CD25+CD4+ Tregs (naive RA+ Tregs) or CD45RA−CD25−CD4+ Tregs (memory RA+ Tregs) at a 1:2 (Treg to target) ratio for 96 h. As a control, CD4+CD25− T cells were added at the same ratio, and as expected, neither suppressed proliferation nor cytokine production (data not shown). Both RA+ and RA− Tregs efficiently suppressed proliferation and cytokine production by both Th1 and Th17 cells. The average suppression of IFN-γ from CXCR3+ Th1 cells by RA− Tregs was 63.53 ± 24.90% (n = 3, p = 0.033), and 67.05 ± 23.77% by RA+ Tregs (n = 3, p = 0.032). The average suppression of IL-17 from CD161+ Th17 cells by RA− Tregs was 51.40 ± 16.90% (n = 3, p = 0.0054), and 42.07 ± 11.26% by RA+ Tregs (n = 3, p = 0.00047). The proliferation of CD161+ Th17 cells was suppressed by 72.55 ± 31.61% by RA− Tregs (n = 3; p = 0.002), and 81.04 ± 15.40% by RA+ Tregs (n = 3; p = 0.004).

![FIGURE 4]
IL-17, significantly inhibited fibroblast migration (Fig. 6A, 6C). Moreover, supernatants from activated RA− Tregs alone decreased the migratory capacity of fibroblasts (Fig. 6A, 6C). Addition of neutralizing anti–IFN-γ or anti–IL-17 mAbs confirmed that both of these cytokines contribute to the suppression of fibroblast migration. Evidence that anti–IL-17 mAbs reversed the suppressive effects of RA− but not RA+ Tregs confirmed that the effect of the former subset on fibroblast migration was due to their capacity to secrete IL-17 (Fig. 6D). Therefore, specific subsets of Tregs have differing capacities to regulate the antifibrotic effects of Th17 cells.

**Discussion**

We developed a novel method for the isolation of a homogeneous population of Th17 cells from peripheral blood and performed a comprehensive analysis of their phenotype and susceptibility to suppression by Tregs. We found that in addition to IL-17, IL-6, and IL-22 production, Th17 cells produce low levels of granzymes A and B, and established their requirement for exogenous factors to realize their full proliferative capacity. Contrary to previous reports, we found that Tregs efficiently suppress the effector functions of Th17 cells. Both RA+ and RA− Tregs suppressed the proliferation of Th17 cells and their capacity to produce IL-17, IL-22, and CXCL8. In contrast, only RA+ Tregs suppressed the antifibrotic effects of Th17 cells. These findings provide new tools that will allow for further investigation into the natural and pathological functions of bona fide Th17 cells in humans. The data also provide new insight into the cross-talk between Th17 cells and immune and nonimmune cells, and they have established the paradigm that therapeutic manipulation of Tregs can be explored as a strategy to limit Th17-mediated inflammation.

Sorting CD161+CCR6+CCR4+CXCR3−CD4+ T cells substantially enriches for IL-17highIFN-γlow cells, and at the single-cell level, 100% of these cells express IL-17 and 90% do not coexpress IFN-γ. This method significantly improves the homogeneity of the population obtained compared with previously described methods involving sorting of CCR6+, CCR4+CCR6+, and CD61+ cells or IL-23R in which 14–40% of IL-17+ cells coexpressed IFN-γ (8, 11–13, 28). Sorting Th17 cells ex vivo also obviates the need to differentiate them in vitro using controversial methods that are inefficient and likely result in unstable lineages of cells. Importantly, CD161+ Th17 cells could be expanded in vitro, and their characteristic phenotype became even more pronounced. Other methods that have been used to sort IL-17+ cells include the use of a bispecific Ab against CD45 and IL-17A to capture IL-17–secreting cells (43) or sorting cells expressing IL-17A on their surfaces (44). A major disadvantage of these methods, however, is that they require in vitro stimulation prior to isolation. Isolation methods that rely on in vitro stimulation are technically cumbersome and limit the types of analyses that can be performed immediately ex vivo. Moreover, expansion studies on cells isolated by these methods revealed that ~50% of the sorted IL-17–secrating cells coexpressed IFN-γ. The protocol described in this study is thus the most practical and robust method for isolation of pure populations of IL-17–producing cells, which do not coproduce IFN-γ, and yields sufficient numbers of unactivated Th17 cells to perform functional analyses.

We found that, beyond their capacity to produce IL-17, in vivo differentiated Th17 cells are unique in several aspects. First, Th17 cells are hyporesponsive to TCR/CD28 stimulation unless APCs are present, suggesting a requirement for an exogenous growth factors and/or costimulation beyond CD28. This finding is in agreement with Evans et al. (18), who found that activated monocytes were required for maximal Th17 induction, and our observation that overexpression of RORC2 results in T cell anergy, which was abrogated in the presence of exogenous IL-2 or IL-15 (10). Further investigation into the nature of the proliferative signals provided by APCs will be key to understanding how Th17 populations expand in vivo. Second, analyses at the single-cell level showed that Th17 cell clones produced significantly higher amounts of IL-6 than classical Th1 cells, in agreement with our previous finding from cells overexpressing RORC2 (10). We also confirmed that Th17 cells secrete high levels of CXCL8 (35), providing an additional mechanism whereby these cells mediate inflammation.

The question of whether Tregs have the capacity to suppress Th17 cells is of major importance, not only to understand how Th17 cells are naturally regulated but also to predict how they would be impacted by therapeutic manipulation of Tregs. Determining the answer to this question has been complicated by the finding that in humans subsets of Tregs themselves can make IL-17 (19–24), and no detailed study of the interactions between ex vivo human Th17 cells and IL-17− Tregs has been reported previously. Moreover, studies thus far have been limited to examination of the ability of Tregs to...
FIGURE 6. CD45RA⁺CD25⁺CD4⁺ Tregs reverse Th1 and Th17 cell-mediated inhibition of wound healing. A–C, Supernatants from sorted ex vivo T cells that had been stimulated with anti-CD3/anti-CD28 in the presence or absence of RA⁺ or RA⁻ Tregs for 48 h were added to fibroblasts in an in vitro wound-healing scratch assay. Images were captured at 0 h (prior to addition of supernatants) and at 16 h following incubation with supernatants from stimulated T cells. The rate of migration was measured by quantifying the total number of fibroblasts that migrated within the scratched areas in triplicate wells. A, Representative images are of fibroblasts migrating into scratched area (original magnification ×100). B, Average fibroblast migration in the absence of Tregs in three donors. C, Average fibroblast migration in the presence of RA⁺ or RA⁻ Tregs in three donors. On average, RA⁺ Tregs reversed 47.55% of the effect of CXCR3⁺ Th1 cells (n = 3; p = 0.04) and 43.09% of the effect of CD161⁺ Th17 cells (n = 3; p = 0.02). RA⁻ Tregs did not reverse CD161⁺ Th17 or CXCR3⁺ Th1 effects and inhibited fibroblast migration themselves: when supernatants from activated RA⁻ Tregs alone were added, fibroblast migration decreased by 49.96% (n = 3; p = 0.005). When RA⁻ Tregs were cocultured with total CD4⁺ T cells, there was a 43.17% decrease in fibroblast migration (n = 3; p = 0.004), notable considering that there was no effect of CD4⁺ T cells themselves. D, Average fibroblast migration in the presence of supernatants from stimulated sorted cells in the presence of neutralizing anti–IL-17 or anti–IFN-γ mAbs. On average, neutralizing IL-17 Abs reversed suppression of fibroblast migration by 40.34% with supernatants from CD161⁺ Th17 cells and 34.11% with supernatants from RA⁻ Tregs (n = 3). Neutralizing IFN-γ reversed effects of CXCR3⁺ supernatants by 39.04% (n = 3). *p < 0.05; **p < 0.005; ***p < 0.0005.
suppress IL-17 production and have not determined whether Tregs can suppress other Th17 cell effector functions. For the first time, we demonstrated that Tregs potently suppress the ability of Th17 cells to proliferate and produce IL-17, IL-22, and CXCL8, irrespective of their capacity to secrete IL-17. Because CD45RA⁺ Tregs are a heterogeneous population, it is possible that only the non–IL-17- secreting Tregs in this subset mediated suppression. Nevertheless, the capacity of this population to secrete IL-17 clearly does not preclude its ability to suppress the proliferation of, or cytokine production by, Th17 cells. Our data in humans are consistent with the finding that Tregs suppress Th17-mediated intestinal inflammation in mice (45). Because IL-17 can induce CXCL8 in several cell types (36, 46, 47) and human CD4⁺ T cells express the IL-17R (48), the suppression of IL-17 production from Th17 cells, only RA⁺ Tregs reversed the pro-inflammatory cytokine production of these cells.

Because many of the pathophysiological effects of Th17 cells are due to effects on nonimmune cells (3, 4), it was also important to investigate whether Tregs could modulate this aspect of Th17 cell function. We first demonstrated that, like Th1 cells, Th17 cells inhibit fibroblast migration in a wound-healing assay, whereas total CD4⁺ T cells had no effect, possibly due to the production of both pro- and anti-inflammatory cytokines from different effector cells (38). Neutralizing IL-17 and IFN-γ mAbs significantly reversed the Th17- and Th1-mediated effects, indicating that these signature cytokines are primary mediators of this function. To migrate into a wounded site, fibroblasts rearrange their cytoskeletal elements and secrete extracellular matrix components along which to travel (49). Therefore, destruction/inhibition of extracellular matrix proteins disrupts fibroblast migration. Because IFN-γ, TNF-α, and IL-17 stimulate expression of matrix metalloproteinasises (38, 39, 42), which function to break down collagen, inhibition of migration appears to be cytokine-mediated. The ability of Tregs to regulate this aspect of Th17 cells has important implications for Th17-mediated diseases that are perpetuated by collagen destruction such as rheumatoid arthritis (50).

Although both RA⁺ and RA⁻ Tregs potently suppressed cytokine production from Th17 cells, only RA⁺ Tregs reversed the inhibitory effects on fibroblast migration. The inability of RA⁻ Tregs to block this function of Th17 cells is related to the fact that a proportion of the RA⁺ cells secrete IL-17 themselves, as demonstrated by the use of neutralizing anti–IL-17 mAbs. These data demonstrate differential suppressive effects between naive and memory Tregs and illustrate that adoptive therapy with specific subsets of Tregs may be required to control disorders characterized by ulceration or impaired wound healing. An important consideration is whether IL-17⁻ Tregs may acquire the capacity to produce this cytokine in vivo in inflamed tissue, ultimately exacerbating the pathology. Indeed, we found that not only do IL-17⁻ RA⁻ Tregs fail to reverse anti-inflammatory effects of Th1 and Th17 cells, but they also increase MMP-1 expression in fibroblasts (data not shown) and inhibit fibroblast migration themselves. Despite these outstanding questions, the findings in this study provide strong evidence that Tregs can limit the actions of Th17 cells, thus providing hope that Treg-based therapies will be effective for Th17-mediated pathologies.

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Disclosures
The authors have no financial conflicts of interest.


Supplemental Figure 1. Isolation of CCR4⁺CCR6⁻CXCR3⁻ Th17 cells from peripheral blood. (A) Flow cytometry-based sorting strategy for isolation of in vivo differentiated Th17 cells on the basis of expression of CXCR3, CCR6 and CCR4. (B) Sorted cells were stimulated with αCD3/αCD28-coated beads for 24 or 72h, and the amount of IL-17 or IFN-γ in supernatants was measured. Expression of RORC and TBET in the sorted cells were assessed by flow cytometry. Data are representative of a minimum of 3 independent experiments with different donors. (C). Single cell clones were derived from sorted populations, and 9 growing wells from each condition were expanded. Clones were re-stimulated with αCD3/αCD28-coated beads for 48 hours and supernatants analyzed by ELISA for IL-17, and by cytometric bead assay (CBA) for other indicated cytokines (D). SD of experimental triplicates indicated in B and D.
**Supplemental Figure 2:** Tregs suppress IL-17 production independently from proliferation.

Sorted ex vivo T cells were labelled with CFSE and stimulated with APCs and aCD3 in the presence or absence of RA+Treg or RA- Treg at 1:2 ratio for 20 hrs. IFN-γ production from CXCR3+ Th1 cells was suppressed by 56.06%±18.56 by RA+ Tregs (n=3, p=0.02) and 88.79%±8.20 by RA- Tregs (n=3, p=0.001). IL-17 production from CD161+ Th17 cells was suppressed by 46.18%±31.69 by RA+ Tregs (n=3, p=0.03) and 64.80%±19.00 by RA- Tregs (n=3, p=0.003).