ARTHRITIS & RHEUMATISM
Vol. 58, No. 6, June 2008, pp 1619–1629
DOI 10.1002/art.23497

Cytokines Secreted in Response to Toll-like Receptor Ligand Stimulation Modulate Differentiation of Human Th17 Cells

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Objective. **Th17 cells (interleukin-17 [IL-17]–secreting T helper cells) have been implicated in the pathogenesis of rheumatoid arthritis and other autoimmune diseases, but the soluble factors that influence human Th17 differentiation have yet to be fully elucidated. This study was undertaken to investigate the hypothesis that the cytokines secreted by human peripheral blood mononuclear cells (PBMCs) in response to a subset of Toll-like receptor (TLR) ligands would influence Th17 polarization.**

Methods. **Supernatants from human PBMCs treated with a panel of TLR agonists were tested for their ability to induce de novo IL-17 production in naive T helper cells. Multiplex cytokine analysis was used to identify candidate cytokines for subsequent blocking and sufficiency experiments.**

Results. **Conditioned media from PBMCs stimulated with TLR-4 or TLR-8/7 agonists, but not from those stimulated with TLR-2/1, -3, or -9 agonists, evoked robust secretion of IL-17 by T helper cells, independent of coculture with antigen-presenting cells. Multiplex analysis of 22 cytokines and chemokines identified a 6-factor cytokine signature that significantly correlated with IL-17–inducing activity. T cell activation in the presence of recombinant IL-1β, IL-6, and IL-23 reconstituted robust IL-17 production, and this was enhanced by transforming growth factor β (TGFβ). IL-6 suppressed the expression of forkhead box P3 and reversed TGFβ-mediated inhibition of T cell proliferation, but did not trigger IL-17 secretion. IL-17 production was completely abrogated by anti–IL-1 or IL-1 receptor antagonist and partially inhibited by anti–IL-6, anti–IL-2, or exogenous retinoic acid, but not by anti–tumor necrosis factor α. IL-1β and IL-6 independently induced IL-21 secretion, but the presence of IL-21 alone was not sufficient for IL-17 production.**

Conclusion. **These results indicate that ligation of a subset of TLRs generates proinflammatory cytokines that combine to potentiate human Th17 differentiation.**

The identification of the Th17 subset (interleukin-17 [IL-17]–secreting T helper cells) as a distinct lineage of CD4+ T helper cells has opened new avenues of research for the study of microbial infections and a variety of tissue-specific autoimmune diseases (1,2). Consequently, insight into the programming that directs a naive CD4+ T cell to become a Th17 cell has become an area of great interest (3,4). In mice, transforming growth factor β (TGFβ) in combination with either IL-6 or IL-21 constitutes the minimum requirement for driving the de novo generation of IL-17–secreting T helper cells (5–9). Through a mechanism that is yet to be elucidated, these differential signals converge to up-regulate the expression of retinoic acid–related orphan receptor γt (RORγt), the master regulatory transcription factor of the Th17 lineage (10). These specialized cells can then regulate tissue inflammation through secretion of the effector cytokines IL-17 and IL-22, among others (11).

Supported by NIH grant AI-0151614, National Heart, Lung, and Blood Institute Proteomics contract N01-HV-28183, a grant from the Northern California Chapter of the Arthritis Foundation, a Collaborative MS Research Center Grant from the National Multiple Sclerosis Foundation, a gift from the Floren Family Trust, and by the Canadian Institutes of Health Research Team in Childhood Autoimmunity. Mr. Wong's work was supported by Stanford Program in Immunology training grant 5-T32-AI-07290 and a Mason Case Fellowship.

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Submitted for publication August 31, 2007; accepted in revised form February 29, 2008.

While considerable effort has been invested in the study of mouse models of the Th17 differentiation pathway, the clinical relevance of these cells in human immunity and disease remains incompletely understood. One obstacle that has hindered the investigation of human Th17 cells has been the difficulty in identifying the factor(s) that drive the de novo generation of these cells in vitro. However, two recent studies have provided evidence that IL-1β, not TGFβ and IL-6, can drive human Th17 differentiation (12,13). While IL-1β is dispensable for the generation of murine Th17 cells in vitro, it has been shown to augment Th17 polarization (7), and IL-1 receptor (IL-1R)–deficient mice exhibit defects in Th17 induction that correlate with amelioration of experimental autoimmune encephalomyelitis (14). This series of findings suggests a straightforward model in which IL-1β drives the differentiation of human Th17 cells; however, the complex biologic cues that affect the ability of IL-1β to induce human Th17 cells have yet to be determined.

In this study we demonstrated, through comprehensive screening, that a subset of Toll-like receptor (TLR) agonists induces a panel of proinflammatory cytokines that combine to promote robust secretion of IL-17 upon activation of human naive CD4+ T cells in vitro. Addition of recombinant IL-1β, IL-6, and IL-23 to cultures of naive CD4+ T cells generated a population of IL-17–producing Th17 cells, and supplementation with TGFβ and tumor necrosis factor α (TNFα) enhanced the frequency of this population. Our data support the notion that IL-1β is critical to the induction of human Th17 cells, but that additional soluble factors augment Th17 differentiation in humans.

MATERIALS AND METHODS

Cell isolation and culture. Buffy coats were obtained from healthy volunteer donors (Stanford Blood Center), with approval of the Stanford University Institutional Review Board. Peripheral blood mononuclear cells (PBMCs; 1×10^6 cells/ml) were cultured for 72 hours with palmitoyl-3-cysteine-serine-lysine-4 (1 μg/ml), poly(I-C) (10 μg/ml), lipopolysaccharide (5 μg/ml), CLO75 (5 μg/ml), or endotoxin-free bacterial DNA (10 μg/ml) (all from InvivoGen, San Diego, CA). Supernatants were filtered through a 0.22-μm syringe filter (Millipore, Bedford, MA). T cells were prepared using a RosetteSep Human CD4+ T Cell Enrichment Kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) followed by a Naive CD4+ T Cell Isolation Kit. CD25+ cells were depleted using CD25 MicroBeads (Miltenyi Biotec, Sunnyvale, CA). CD4+,CD45RA+,CD45RO−,CD25− T cells were isolated at 95–99% purity as confirmed by fluorescence-activated cell sorting (FACScan; BD Biosciences, San Jose, CA). Cells were cultured for 5–6 days in 96-well flat-bottomed plates (Falcon, Oxnard, CA) at 2.5×10^5 cells/ml in complete X-Vivo 15 media (Lonza, Basel, Switzerland) with 10% heat-inactivated fetal calf serum (Omega Scientific, Tarzana, CA), 100 units/ml penicillin/streptomycin (Invitrogen, San Diego, CA), 14.3 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and L-glutamine (Invitrogen), and activated interchangeably with anti-CD3/CD28 or anti-CD3/CD28/CD2–coated beads (T Cell Activation/Expansion Kit; Miltenyi Biotec) or Dynabeads CD3/CD28 T Cell Expander (Invitrogen) at 6.25×10^5 beads/ml. Naive T cells were cultured with TLR-conditioned media, or with 5 ng/ml TGFβ (R&D Systems, Minneapolis, MN), 10 ng/ml IL-1β, IL-6, IL-23, TNFα, IL-10 (all from eBioscience, San Diego, CA), or IL-21 (BioSource, Camarillo, CA), or 5 ng/ml IL-2 (BD PharMingen, San Diego, CA), unless otherwise indicated. All-*trans*-retinoic acid (Sigma-Aldrich) and LE540 (a kind gift from Dr. E. Butcher, Stanford University) were added as indicated. Anti–interferon-γ (anti-IFNγ) and anti–IL-4 (5 μg/ml; both from eBioscience) were added to all cultures. Rat IgG1, anti–IL-1α, anti–IL-1β, anti–IL-6, anti-p40 (IL-12/23), anti-TNFα, anti–IL-10 (10 μg/ml; all from eBioscience), anti-TGFβ, anti–IL-2 (10 μg/ml; both from R&D Systems), or IL-1R antagonist (IL-1Ra; 4 μg/ml) (R&D Systems) was added to naive T cell cultures as indicated. All culture reagents were added again after 48 hours.

Carboxyfluorescein succinimidyl ester (CFSE) dilution and intracellular staining. For CFSE staining, naive T cells were labeled with 1 μM CFSE and cultured in TGFβ (10 ng/ml), IL-6 (20 ng/ml), or IL-1β (20 ng/ml). Cells were activated with anti-CD3/CD28–coated beads (5×10^5 beads/ml), cultured for 72 hours at 1×10^6 cells/ml, and then analyzed. For forkhead box P3 (FoxP3) staining, naive CD4+ cells were cultured at 2×10^6 cells/ml in 96-well round-bottomed plates (Falcon), activated with anti-CD3/CD28/CD2–coated beads (1×10^6 beads/ml) for 96 hours, fixed, permeabilized with BD Cytofix/Cytoperm Plus according to the instructions of the manufacturer (BD Biosciences), and finally stained using a phycoerythrin-conjugated FoxP3 antibody (eBioscience). Intracellular cytokine staining for IL-17 and IFNγ was performed on cells that had been reactivated for 4 hours with phorbol myristate acetate (PMA) (25 ng/ml; Sigma-Aldrich) and ionomycin (750 ng/ml; Sigma-Aldrich) in the presence of 1× monensin and 1× brefeldin A (eBioscience), fixed, permeabilized with Cytofix/Cytoperm, and stained with anti-IFNγ and anti–IL-17 (eBioscience). Cells were analyzed with a FACScan or LSR flow cytometer (BD Biosciences) and analyzed using Flow Jo.

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA was extracted, using the RNeasy Mini Kit (Qiagen, Chatsworth, CA), from human naive CD4+ T cells that had been activated for 5 days in the presence of various cytokines, treated with DNase (Qiagen), and quantitated by calculation of the optical density at 260 nm (OD_{260}) with an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). Quantitative RT-PCR was performed on the Stratagene (La Jolla, CA) MX 3000, using SYBR Green

one-step RT-PCR (Qiagen) and gene-specific unlabeled primers for *RORC* (12) (the human ortholog of RORγt), GAPDH (Qiagen), and IL-21 (Qiagen). All samples were normalized to GAPDH, and gene-specific reactions were then expressed as relative units with respect to the neutral condition, determined using the MX 3000 software. Absence of genomic DNA contamination was confirmed by the absence of amplification in wells without reverse transcriptase.

Enzyme-linked immunospot (ELISpot) assay and enzyme-linked immunosorbent assay (ELISA). ELISpot and ELISA analyses were performed using anti–IL-17 capture antibody and biotinylated anti–IL-17 detection antibody according to the instructions of the manufacturer (eBioscience). For ELISpot analysis, T cells that had been cultured for 5 days were reactivated with PMA/ionomycin for 24 hours. OD_{450} was read on a Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA) and analyzed using Softmax Pro and GraphPad Prism software. Cytokine levels in TLR-conditioned media were assessed by multiplex bead analysis using the Beadlyte Human 22-Plex Multi-Cytokine Detection System according to the protocol recommended by the manufacturer (Millipore).

Statistical analysis. For analysis of the findings of the multiplex cytokine assay, a 2-class Significance Analysis of Microarrays (SAM) algorithm (15) was applied to the data set,

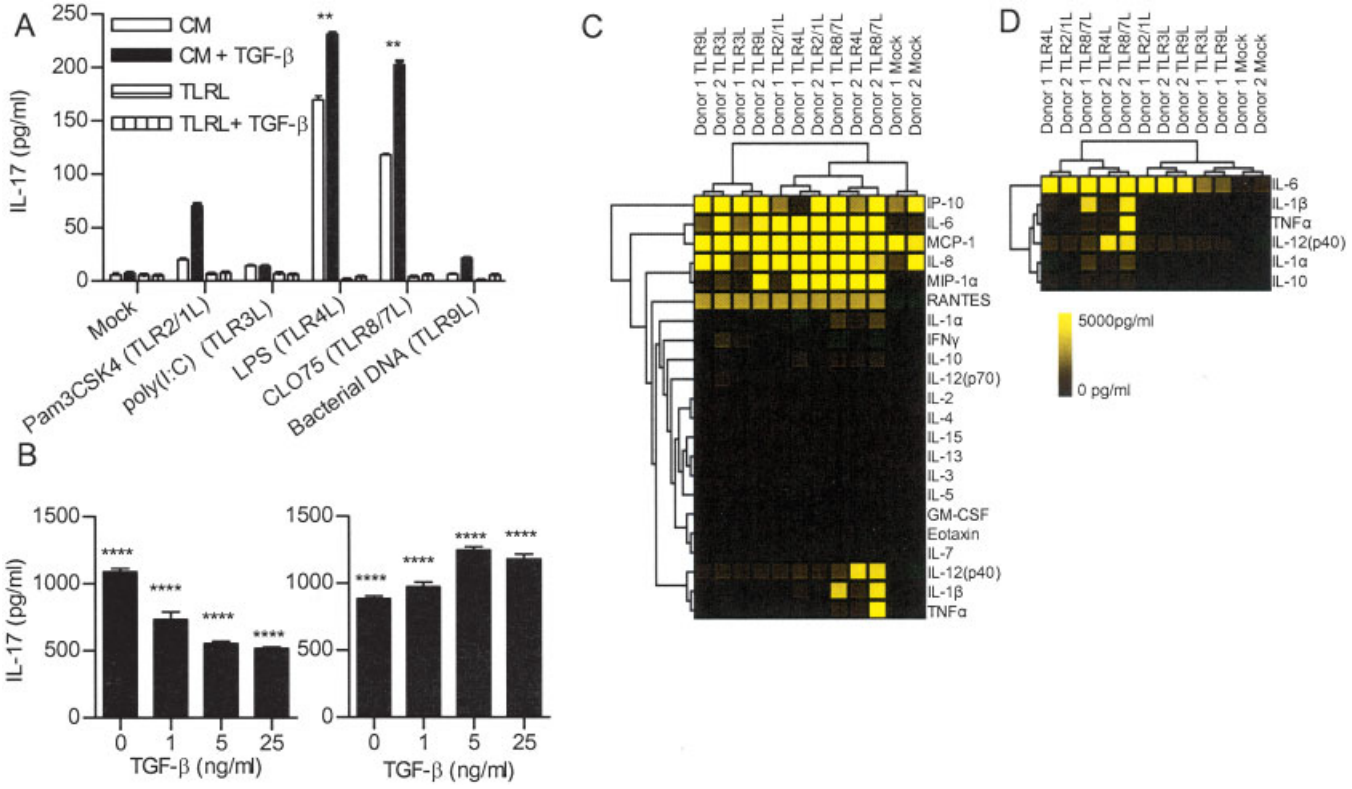

Figure 1. Toll-like receptor (TLR)–conditioned media (CM) with distinct cytokine profiles drive interleukin-17 (IL-17) production. Conditioned medium was derived from supernatants of human peripheral blood mononuclear cells cultured for 72 hours with mock ligand, palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4) (TLR-2/1 ligand [TLR-2/1L]), poly(I-C) (TLR-3 ligand), lipopolysaccharide (LPS) (TLR-4 ligand), CLO75 (TLR-8/7 ligand), or bacterial DNA (TLR-9 ligand). **A** and **B**, Results of enzyme-linked immunosorbent assay for IL-17 secretion, with supernatants from human T helper cells activated for 5 days with conditioned medium or TLR ligand in the presence or absence of transforming growth factor β (TGFβ) (**A**), or TGFβ titrated into TLR-8/7 ligand–conditioned medium (**B**; left panel) or TLR-4 ligand–conditioned medium (**B**; right panel). Values are the mean and SEM. ** = $P < 0.01$ versus mock ligand, by repeated-measures one-way analysis of variance (ANOVA) with Dunnett's post-test; **** = $P < 0.0001$ for decreasing or increasing linear trend, by repeated-measures one-way ANOVA with post-test for linear trend. **C** and **D**, Heat maps of unsupervised hierarchical clustering of cytokine concentrations and TLR ligand–conditioned media from 2 donors (**C**), and cytokines identified by Significance Analysis of Microarrays as having a statistically significant association (q value ≤6%, fold change >5.0) with TLR-4 ligand– and TLR-8 ligand–conditioned medium versus other TLR ligand–conditioned media and mock ligand (**D**). Heat maps are represented as a gradient from low (pseudo-colored black) to high (pseudo-colored yellow) cytokine levels, ranging from 0 to 5,000 pg/ml. IP-10 = interferon-γ [IFNγ]–inducible protein 10; MCP-1 = monocyte chemotactic protein 1; MIP-1α = macrophage inflammatory protein 1α; GM-CSF = granulocyte–macrophage colony-stimulating factor; TNFα = tumor necrosis factor α.

and cytokines were considered significantly up-regulated in the TLR-8 ligand– and TLR-4 ligand–conditioned media group if they had a q value of ≤6% and a fold change of >5.0 versus the other group containing the mock, TLR-2/1 ligand–, TLR-3 ligand–, and TLR-9 ligand–conditioned media. Heat map and Euclidean complete linkage hierarchical clustering images were generated using Tigr Multiexperiment Viewer (TM4: MeV) (16). One-way analysis of variance, paired *t*-tests, and generation of graphs were all performed using GraphPad Prism software.

RESULTS

In order to identify the soluble factors that polarize human naive CD4+ cells toward the Th17 lineage, we stimulated human PBMCs with a panel of TLR ligands for 72 hours, removed the cells, and harvested the supernatants. None of this conditioned medium contained detectable levels of IL-17 (data not shown). When the conditioned medium was then added to naive CD4+ T cells together with a polyclonal activation stimulus, the TLR-4 ligand– and TLR-8/7 ligand–conditioned media exhibited strong IL-17–inducing activity (Figure 1).

Since TGFβ is required for murine Th17 differentiation (5–9), it was surprising that the addition of exogenous TGFβ to the conditioned medium only modestly increased IL-17 production (Figure 1A). Interestingly, titration of TGFβ into the conditioned medium either enhanced or suppressed IL-17 production in a dose-dependent manner, and this effect was heterogeneous depending on the donor T cells and TLR ligand–conditioned medium (Figure 1B and data not shown). The ability of TGFβ to enhance IL-17 production in the setting of a proinflammatory cytokine milieu contrasts with the results of several recent studies showing that TGFβ consistently mediated suppression of human Th17 differentiation (12,13,17). Since the TLR ligands, alone or in combination with TGFβ, did not induce IL-17 production (Figure 1A), we concluded that the IL-17–promoting activity could be attributed to factors secreted by human PBMCs in response to TLR stimulation.

To identify candidate cytokines that promote IL-17 production, we profiled the TLR ligand–conditioned media for 22 cytokines and chemokines. With unsupervised hierarchical clustering, TLR-4 ligand– and TLR-8/7 ligand–conditioned medium grouped together on one branch while the TLR-3 ligand– and TLR-9 ligand–conditioned medium clustered together on another branch, based on the pattern of cytokines induced (Figure 1C). Given that the TLR-4 ligand– and TLR8/7 ligand–conditioned media exhibited the most potent IL-17–inducing activity (Figure 1A), we used a 2-class SAM approach (15), by which IL-6, IL-1α, IL-1β, IL-12 (p40), TNFα, and IL-10 were identified as a 6-factor "cytokine signature" that was significantly elevated (q value ≤6%, fold change >5.0) in the TLR-4 ligand– and TLR-8/7 ligand–conditioned medium group when compared with the group containing the other TLR ligands and mock conditioned medium (Figure 1D). All cytokines had a q value of 0 with the exception of TNFα, which had a slightly higher q value (6.1%). The fold changes between the 2 groups for IL-6, IL-1α, IL-1β, TNFα, IL-12 (p40), and IL-10 were 5.4, 45, 240, 280, 6.8, and 38, respectively. Interestingly, serum from a subset of rheumatoid arthritis patients with elevated levels of these cytokines (18) was also found to have elevated serum levels of IL-17 (data not shown).

Since the combination of TGFβ and IL-6 has previously been shown to be sufficient for development of Th17 cells in mice (5–9), we tested multiple concentrations of these 2 cytokines for the ability to induce human Th17 cells (Figure 2A). While combinations of IL-6 and TGFβ failed to induce IL-17 production, a combination of cytokines including TGFβ and those identified by the SAM algorithm (Figure 1D) induced high-level production of IL-17. Although IL-6 in combination with TGFβ was incapable of inducing IL-17 production, we tested whether IL-6 could suppress FoxP3 expression, since this phenomenon has been reported to occur in the murine system (5). Activation of human naive CD4+ T cells in the presence of TGFβ increased the percentage of FoxP3+ T cells as previously described (19), and this was inhibited by addition of IL-6 but not IL-1β (Figure 2B). Furthermore, the suppressive effect of TGFβ on proliferation was also reversed by IL-6 but not IL-1β (Figure 2B). We therefore concluded that IL-6 and TGFβ operate similarly in the murine and human systems with respect to their opposing effects on FoxP3 expression and proliferation, but not with respect to the induction of de novo IL-17 production.

To further define the cytokines that were necessary and/or sufficient for inducing IL-17 production, we performed a combinatorial analysis of cytokines identified in the IL-17–inducing cytokine signature, in combi-

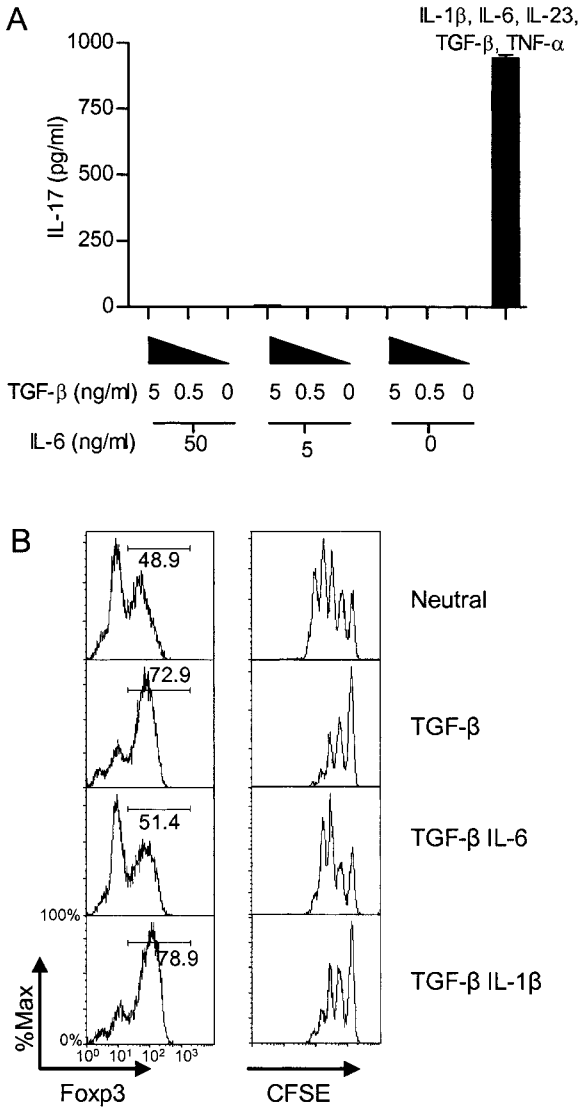

Figure 2. IL-6 in combination with TGFβ increases proliferation and reduces forkhead box P3 (FoxP3) expression but does not induce IL-17 production. **A**, Results of enzyme-linked immunosorbent assay for IL-17 secretion by naive human CD4+ T cells polyclonally activated in the presence of varying amounts of TGFβ and IL-6 or in the presence of a proinflammatory cytokine cocktail (IL-1β, IL-6, IL-23, TGFβ, and TNFα). Values are the mean and SEM. **B,** Histograms of intracellular FoxP3 staining (left panels) and carboxyfluorescein succinimidyl ester (CFSE) dilution (right panels) in the presence of no cytokine (neutral), TGFβ alone, or TGFβ with IL-6 or IL-1β. FoxP3 staining and CFSE staining were performed after polyclonal stimulation for 4 days and 3 days, respectively. See Figure 1 for other definitions.

nation with TGFβ (Figure 3A). We did not include IL-10 in this analysis since it was present at low levels (<400 pg/ml) in the TLR ligand–conditioned media (Figure 1D) and since addition of IL-10 alone or in combination with other proinflammatory cytokines did not enhance IL-17 production (data not shown). Because IL-1β and IL-1α both signal through IL-1R, we focused on IL-1β initially; studies with IL-1α yielded similar results (data not shown).

When purified naive CD4+ T cells from 5 representative donors were activated in the presence of recombinant cytokines, combinations containing IL-1β tended to induce low-level IL-17 production (Figure 3A), consistent with findings described in two recent reports (12,13). The levels induced by IL-1β alone, however, were not statistically significant. IL-17 production could be enhanced to moderate levels by the addition of IL-1β, IL-6, and IL-23 (Figure 3A), which was the minimum set of 3 cytokines necessary for consistent and robust IL-17 production. Alternatively, IL-6 and TGFβ also enhanced the efficacy of IL-1β in generating IL-17 expression (Figure 3A). Addition of the 5 cytokines (IL-1β, IL-6, IL-23, TGFβ, and TNFα), which we termed Th17-All, consistently elicited the highest levels of IL-17 production in all donors (Figure 3A). Levels of IL-17 secretion did not correlate with cell numbers (data not shown). This highlights the important role of multiple cytokines in addition to IL-1 that could drive human Th17 differentiation.

The effect of each cytokine was analyzed individually by calculating the change in IL-17 secretion when each cytokine was added to combinations of other cytokines (Table 1). Overall there were 32 cytokine combinations tested (Figure 3A). For example, we tested 16 conditions with TGFβ and 16 without TGFβ. To isolate the effect of TGFβ, each condition in which TGFβ was included was paired with the same condition in the absence of TGFβ. The paired conditions were then analyzed for the change in IL-17 production induced by addition of TGFβ. The same analysis was performed for the other 4 cytokines (Table 1). The data suggested that while IL-1β clearly enhances IL-17 secretion, IL-6, IL-23, and TGFβ could also influence production of IL-17. Exogenous TNFα did not have a discernible effect (Table 1). The analysis of TNFα is complicated by the fact that human Th17 cells have been reported to secrete TNFα (13), but in subsequent blocking experiments (see below), autocrine TNFα did not appear to be essential for Th17 differentiation. Although IL-1β clearly plays a central role, these observations demonstrated that TGFβ, IL-6, and IL-23 all have potent additive effects on IL-17 production.

In addition to their effect on IL-17 secretion as

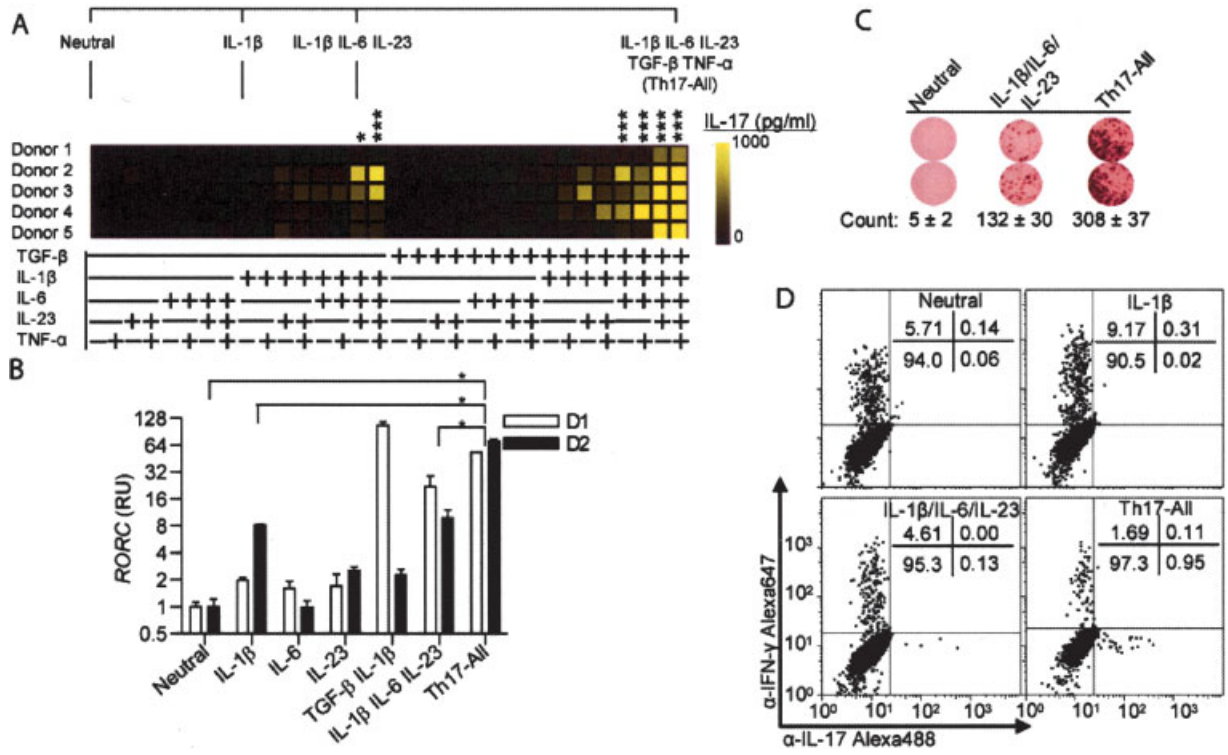

Figure 3. Combinatorial addition of TGFβ, IL-1β, IL-6, and IL-23 potentiates human Th17 differentiation. Naive human CD4+ T cells were polyclonally activated for 5 days in the presence of combinations of recombinant cytokines and analyzed for IL-17 production. **A**, Heat map of IL-17 concentrations in supernatants from 5 representative donors, activated in the presence or absence of TGFβ, IL-1β, IL-6, IL-23, and TNFα. $* = P < 0.05$; $*** = P < 0.001$ versus the neutral condition (no cytokine), by repeated-measures one-way ANOVA with Tukey's multiple comparison post-test. Th17-All refers to all 5 cytokines. **B,** Results of quantitative reverse transcriptase–polymerase chain reaction studies of transcripts encoding *RORC* (the human ortholog of murine retinoic acid–related orphan receptor γt) from 2 donors (D1 and D2), activated for 5 days in the presence of various cytokines. Values are the mean and SEM relative units (RU) in relation to the neutral condition after normalization to GAPDH. $* = P < 0.05$ by repeated-measures one-way ANOVA with Tukey's multiple comparison post-test. **C**, Enzyme-linked immunospot images and mean ± SD spot counts of 20,000 cells restimulated for 24 hours with phorbol myristate acetate (PMA)/ionomycin after 5 days of activation in the presence of the indicated cytokine combinations. **D,** Intracellular cytokine staining for IL-17 and IFNγ in cells restimulated for 4 hours with PMA/ionomycin after 5 days of activation in the presence of the indicated cytokine combinations. See Figure 1 for other definitions.

measured by ELISA, the influence of these cytokines could be linked to Th17 differentiation and frequency. The increased levels of IL-17 in the supernatant correlated with an increase in expression of the transcript encoding the human ortholog of RORγt (*RORC*), the putative murine Th17 master regulatory transcription factor (10) (Figure 3B). IL-1β alone induced RORγt to some degree, but IL-6 and IL-23 enhanced this effect, while the addition of Th17-All induced maximal RORγt expression (Figure 3B). This trend of increasing RORγt expression with additional proinflammatory cytokines was consistent with the data obtained by ELISpot (Figure 3C) and by intracellular cytokine analysis (Figure 3D). Interestingly, the frequency of IFNγ+ Th1 cells (Figure 3D, upper left quadrant of each dot plot panel)

Table 1. Change in IL-17 secretion under stimulation conditions with versus without each cytokine, in 5 donors*

Cytokine	Δ, % of maximum	P†
IL-1β	+26.3	<0.0001
TGFβ	+13.9	<0.0001
IL-6	+17.1	<0.0001
IL-23	+14.0	<0.0001
TNFα	−0.4	NS

* Interleukin-17 (IL-17) levels shown in Figure 3A were expressed as the percentage of the maximum level observed, for each individual donor. Values are the mean from the 5 donors. Positive values indicate an increase upon the addition of the cytokine; negative values indicate a decrease. TGFβ = transforming growth factor β; TNFα = tumor necrosis factor α.

† Significance (determined by paired *t*-test) of the difference between the IL-17 level observed when the given cytokine was included versus the level observed without the cytokine. NS = not significant.

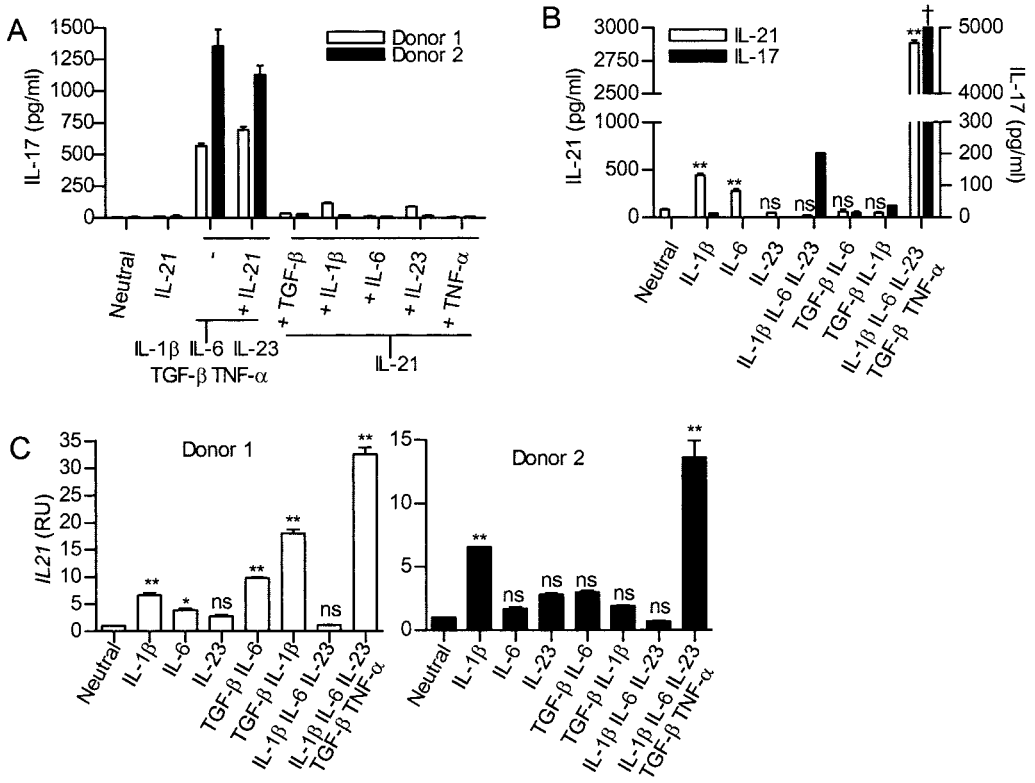

Figure 4. Exogenous IL-21 does not influence Th17 differentiation, but IL-1β and IL-6 induce human Th17 cells to secrete autocrine IL-21. Naive human CD4+ T cells were polyclonally activated for 5 days in the presence of combinations of recombinant cytokines and assayed for IL-17 and IL-21 production. **A** and **B,** Results of enzyme-linked immunosorbent assay for secretion of IL-17 (**A**) and IL-21 (**B**) in the presence of the indicated cytokine combinations. Values are the mean and SEM. † = >5,000 pg/ml. **C,** Results of quantitative reverse transcriptase–polymerase chain reaction studies of transcripts encoding *IL21* from donor 1 and donor 2 on day 5. Values are the mean and SEM relative units (RU) in relation to the neutral condition (no cytokine) after normalization to GAPDH. $* = P < 0.05$; $** = P < 0.01$ versus the neutral condition, by repeated-measures one-way ANOVA with Dunnett's post-test. NS = not significant (see Figure 1 for other definitions).

decreased reciprocally with increasing Th17 cells (Figure 3D, lower right quadrant of each dot plot panel). Maximal Th17 cell frequency was induced by Th17-All, and intermediate frequency was induced by IL-1β, IL-6, and IL-23. Taken together, these data demonstrate that IL-6, IL-23, and TGFβ augment the ability of IL-1β to polarize naive human CD4+ T cells toward the Th17 lineage.

Three recent reports have implicated IL-21 as an important autocrine factor in the induction of murine Th17 cells (8,9,20), but its role in human Th17 differentiation has yet to be determined. Exogenous IL-21 did not induce high levels of IL-17 production when tested with each cytokine separately or when added to a 3-cytokine cocktail (IL-1β, IL-6, and IL-23) or a 5-cytokine cocktail (TGFβ, IL-1β, IL-6, IL-23, and TNFα) (Figure 4A). There was some production of IL-17 when IL-21 was included with IL-1β, or when IL-21 was added together with IL-23 (Figure 4A). Despite reports that IL-21 and TGFβ together can promote IL-17 production in murine T helper cells (8,9,20), this combination did not induce human IL-17–producing T cells (Figure 4A).

We then measured autocrine IL-21 levels obtained in response to various cytokine combinations added in concert with polyclonal T cell activation (Figure 4B). As in the murine system (20), IL-6 alone or in combination with other cytokines induced production of IL-21 (Figure 4B). Unexpectedly, we also observed IL-21 secretion in response to IL-1β (Figure 4B). TGFβ has been previously reported to reduce the IL-6– or IL-21–induced up-regulation of the IL-23 receptor in

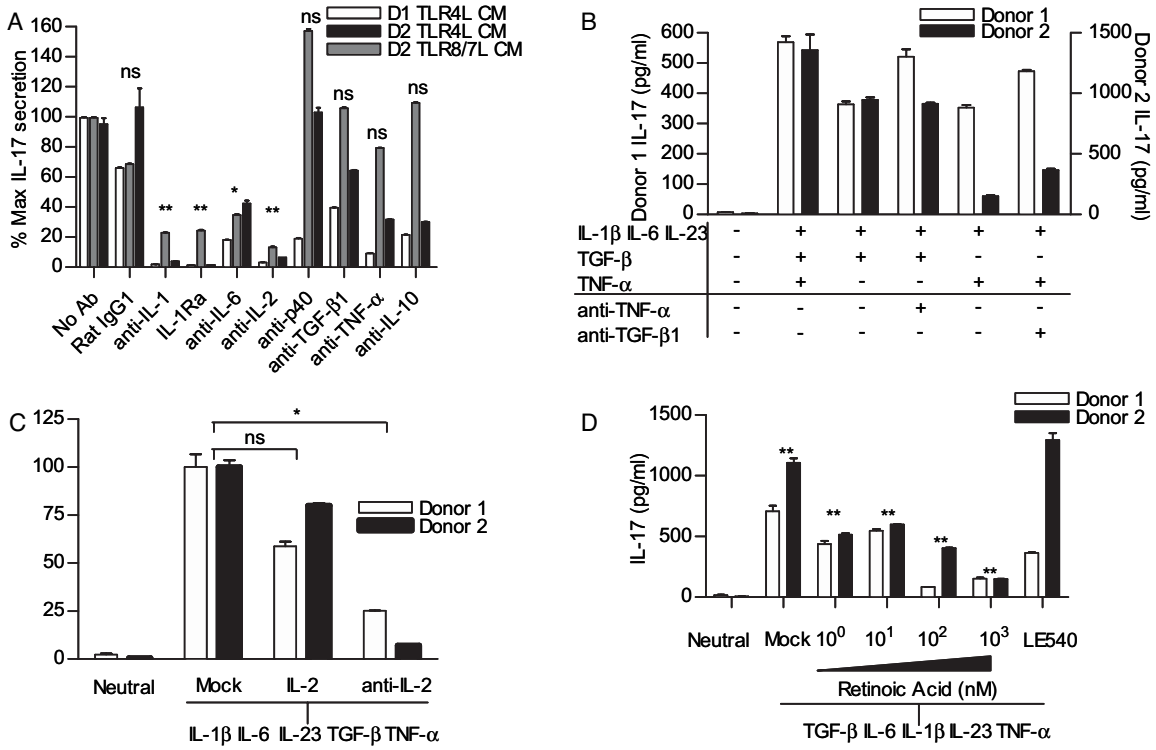

Figure 5. Suppression of IL-17 production in the presence of a proinflammatory Th17 polarizing milieu. IL-17 secretion by naive human CD4+ T cells after 5 days in culture with polyclonal activation was determined by enzyme-linked immunosorbent assay. **A**, Cells activated in the presence of TLR-4 ligand– or TLR-8/7 ligand–conditioned medium with no antibody (Ab), isotype negative control antibody (rat IgG1), neutralizing antibodies against IL-1α and IL-1β, IL-1 receptor antagonist (IL-1Ra), or other indicated neutralizing antibodies. Values are the mean and SEM percentage of maximum IL-17 secretion relative to that observed with no antibody. D1 and D2 = donor 1 and donor 2. $* = P < 0.05$; $** = P < 0.01$ versus no antibody, by repeated-measures one-way ANOVA with Dunnett's multiple comparison test. NS = not significant. **B**, Cells activated in the presence of no cytokine or with cytokine and anti-TNFα or anti-TGFβ1. Values are the mean and SEM. **C**, Cells activated with exogenous IL-2 or anti–IL-2 under Th17 polarizing conditions. Values are the mean and SEM percentage of maximum IL-17 secretion relative to mock conditions without exogenous IL-2 or anti–IL-2. $* = P < 0.05$ by repeated-measures one-way ANOVA with Tukey's post-test. **D**, Cells activated with increasing amounts of all-*trans*-retinoic acid or a retinoic acid receptor antagonist (LE540) under Th17-All (treatment with TGFβ, IL-1β, IL-6, IL-23, and TNFα) polarizing conditions. Values are the mean and SEM. $** = P < 0.01$ for decreasing linear trend with increasing retinoic acid concentration, by repeated-measures one-way ANOVA. Neutral (no cytokine) and mock conditions in **C** and **D** are from the same experiment. See Figure 1 for other definitions.

murine T cells, but was not found to have an effect on IL-21 expression (20). In human naive CD4+ T cells, however, we found that TGFβ was able to inhibit both IL-6– and IL-1β–induced IL-21 secretion (Figure 4B). It is not clear why the combination of IL-1β, IL-6, and IL-23 did not induce IL-21 when both IL-1β and IL-6 could induce this cytokine individually (Figure 4B). However, other donors exhibited IL-21 secretion in response to IL-1β, IL-6, and IL-23 (data not shown).

These trends were confirmed at the messenger RNA level by quantitative RT-PCR (Figure 4C), demonstrating IL-21 expression in response to IL-1β and IL-6. Again, the heterogeneous response to TGFβ was evidenced by increased (donor 1) and decreased (donor 2) levels of autocrine IL-21 in combination with IL-1β. Maximal IL-21 production was observed with Th17-All (Figures 4B and C). Importantly, the combination of IL-1β, IL-6, and IL-23 elicited IL-17 production, despite undetectable levels of IL-21 at either the protein or the transcript level (Figures 4B and C). Therefore, high levels of IL-21 may correlate with high levels of IL-17 production, but IL-21 does not appear to be absolutely required for human Th17 differentiation since IL-17 can be induced in the absence of measurable amounts of

IL-21. While the mechanism by which IL-1β leads to human Th17 differentiation is currently unknown, the ability of IL-1β to induce autocrine IL-21 suggests at least one potential mechanism.

Suppression of IL-17 production in a proinflammatory milieu has important biologic and clinical implications. To address this in vitro, we tested a panel of neutralizing anticytokine antibodies for the ability to suppress IL-17 production by naive human CD4+ T cells activated in the context of TLR ligand–conditioned media.

Neutralization of IL-1 by anti–IL-1α and anti–IL-1β or IL-1Ra significantly reduced IL-17 production (Figure 5A), suggesting that IL-1–dependent signals are required for Th17 differentiation. Since IL-1α and IL-1β both induced IL-17 production (data not shown), complete suppression of Th17 differentiation required neutralization of both cytokines. Despite reports that neutralization of IL-2 increases murine IL-17 production (21), anti–IL-2 blocking antibodies significantly suppressed human IL-17 production (Figures 5A and C) as well as proliferation (data not shown), while exogenous IL-2 did not suppress IL-17 production (Figure 5C). Neutralization of IL-6 also significantly reduced IL-17 production, although the efficacy is probably underestimated since the TLR-4 ligand– and TLR-8/7 ligand–conditioned media contained very high levels of IL-6 (20–30 ng/ml) that may not have been completely neutralized in our experiments. Anti-TNFα, anti-TGFβ1, and anti–IL-10 suppressed IL-17 production in some instances, but the results were not statistically significant across multiple donors (Figure 5A). We had anticipated that neutralization of the common p40 subunit of the IL-12 and IL-23 heterodimers would suppress IL-17 production, but in the context of the proinflammatory TLR ligand–conditioned medium stimuli this was not the case (Figure 5A).

Recently, human Th17 cells have been shown to secrete TNFα (13), which may explain the minimal effect of exogenous TNFα (Figure 3A and Table 1). To assess the role of autocrine TNFα in our system, anti-TNFα was added to a cytokine combination that included TGFβ, IL-1β, IL-6, and IL-23. The results revealed that autocrine TNFα is not necessary for IL-17 production in the setting of high levels of other proinflammatory cytokines (Figure 5B). Since T cells are also an important source of TGFβ1 (22), we used the same approach to determine the importance of autocrine TGFβ1. Again, the effect of exogenous TGFβ depended on the donor, but in either case, autocrine TGFβ1 was not absolutely required for IL-17 production (Figure 5B). Finally, all-*trans*-retinoic acid has been shown to suppress murine Th17 cell differentiation (23), and we observed a dose-dependent inhibition of IL-17 production by all-*trans*-retinoic acid in human T helper cells (Figure 5D).

These data suggest that neutralization of IL-1, IL-6, or IL-2 or addition of exogenous all-*trans*-retinoic acid can suppress human Th17 cell differentiation. Interestingly, targeted therapies aimed at these molecules have been approved for treatment of rheumatoid arthritis or are actively under study in ongoing clinical trials.

DISCUSSION

This study adds substantially to the findings of other published studies on human Th17 differentiation (12,13,24–26). We demonstrated that conditioned media from a subset of TLR agonist–treated PBMCs support de novo Th17 differentiation of naive CD4+ T cells independent of coculture with antigen-presenting cells. Rational selection of candidate cytokines was guided by multiplex cytokine analysis of 22 cytokines and chemokines in the conditioned media, which led to identification of a 6-cytokine signature that correlated with IL-17–inducing activity. The systematic combinatorial analysis of all possible combinations of these cytokines was more comprehensive than in previous studies (12,13,24–26). Testing of all combinations of these cytokines demonstrated that IL-1β, although central, is not the only cytokine that may influence human Th17 differentiation. The finding that TGFβ can augment Th17 differentiation when added in combination with other proinflammatory cytokines is unique to this study. In fact, TGFβ potently enhanced IL-17 production, rather than suppressing it (Figure 3A and Table 1). This heterogeneous and context-dependent response to TGFβ will be important to consider in future investigations.

Three recent studies have implicated IL-21 as a critical autocrine factor in the induction of murine Th17 cells (8,9,20), but its role in human Th17 differentiation has not been investigated (12,13,24–26). Here we report that IL-21 is induced by IL-6 and IL-1β in humans, and this effect can be suppressed or enhanced by TGFβ (Figure 4). In accordance with the findings of one study that suggested IL-21 was unable to induce IL-17 production in humans (26), IL-21, alone or in combination with other cytokines, did not enhance IL-17 production in the present study (Figure 4A). Another interesting differ-

ence between human and murine Th17 differentiation is that IL-21 does not appear to be absolutely required for IL-17 production in humans, since we observed IL-17 secretion in the absence of any detectable autocrine IL-21 at either the protein or the transcript level. While IL-21 may not be absolutely required, maximal IL-17 secretion was observed only in the presence of high levels of autocrine IL-21 (Figure 4). Currently, the mechanism by which IL-1β leads to Th17 differentiation is not known. The ability of IL-1β to induce IL-21 offers at least one potential explanation for the contribution of IL-1β to human Th17 differentiation.

The idea that cytokine combinations can act synergistically in contributing to disease has been demonstrated in mouse models of autoimmunity (27), and cytokine combinations have been shown to increase the frequency of murine Th17 cells in vitro (7). The cytokine signature induced by TLR ligand stimulation overlaps with a similar cytokine signature observed in a subset of rheumatoid arthritis patients (18), highlighting the complexity of cytokine networks in vivo. The observation, in this study and others (12,13), that IL-1β is central to Th17 differentiation in humans could offer mechanistic insight into the efficacy of IL-1Ra therapy in select adult and pediatric autoinflammatory and autoimmune diseases, such as systemic-onset juvenile arthritis and adult-onset Still's disease, and in a subset of rheumatoid arthritis patients (28–30).

On the other hand, TNFα did not seem to be a critical cytokine in the in vitro induction of human Th17 cells. The effect of TNFα on Th17 differentiation was not investigated in recent studies (12,13,24–26), but we report here that exogenous TNFα did not have a discernible effect on IL-17 production and Th17 differentiation (Table 1). This lack of effect could be explained at least in part by the ability of Th17 cells to secrete autocrine TNFα. In experiments using conditioned media with lower levels of inflammatory cytokines, neutralization with an anti-TNFα antibody reduced IL-17 production in some donors (Figure 5A). Additionally, autocrine TNFα was not required when IL-1β, IL-6, IL-23, and TGFβ were added at higher levels (Figure 5B).

If Th17 cells are indeed pathogenic in human adult rheumatoid arthritis, then these in vitro observations appear inconsistent with clinical data suggesting that anti-TNFα therapy is more efficacious than IL-1Ra in the treatment of adult rheumatoid arthritis (31,32). It remains possible that TNFα might play a more important role in vivo in Th17 biology than our in vitro data suggest. Additionally, it is plausible that IL-1 plays a role in the induction of Th17 cells during the acute phase of an immune response in vivo, but perhaps a less important role in disease maintenance during a chronic immune response. Importantly, both IL-1Ra and anti-TNF therapy have critical immunosuppressive effects on target tissues and antigen-presenting cells that operate independently of their effects on T cell differentiation. With respect to suppression of IL-17 production, it is also important to note that similar to findings in the murine system (23), retinoic acid could suppress human Th17 differentiation (Figure 5D), offering another potential avenue for suppression of pathogenic effector Th17 cells in the setting of autoimmunity.

Another question implied by results of this study and others (12,13) involves the downstream signaling pathways that control Th17 differentiation in the human and murine immune systems. If the signal transduction pathways immediately downstream of TGFβ and IL-6 are identical in humans and mice, then why does this combination not induce Th17 differentiation in humans? STAT-3 signaling is clearly important in murine Th17 differentiation (20,33), but its role in human Th17 differentiation has not been established. Furthermore, the precise molecular mechanism by which IL-1R engagement directs Th17 differentiation in humans remains to be elucidated. These data and the questions they raise further highlight the differences between the murine and human models of Th17 differentiation and have significant implications regarding the pathogenesis and treatment of rheumatoid arthritis and other autoimmune diseases.

ACKNOWLEDGMENTS

We would like to thank S. Grell for assisting with the ELISpot analysis, staff of the Stanford University Human Immune Monitoring Center for assistance with Luminex cytokine data acquisition, and staff of Dr. Utz's laboratory for helpful discussions and technical assistance. We would also like to thank W. H. Robinson, W. Hueber, and B. Tomooka for technical assistance and helpful discussions regarding rheumatoid arthritis patient serum samples.

AUTHOR CONTRIBUTIONS

Dr. Utz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Kattah, Wong, Utz.
Acquisition of data. Kattah, Wong, Yocum.
Analysis and interpretation of data. Kattah, Wong, Yocum, Utz.
Manuscript preparation. Kattah, Wong, Yocum, Utz.
Statistical analysis. Kattah, Wong.

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