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IL-1β and TGF-β Act Antagonistically in Induction and Differentially in Propagation of Human Proinflammatory Precursor CD4+ T Cells

Katrin Hebel,* Marion Rudolph,* Bernhard Kosak,* Hyun-Dong Chang,† Jana Butzmann,* and Monika C. Brunner-Weinzierl*

Cytokines are critical messengers that control the differentiation of Th cells. To evaluate their impact on the fate of human naive CD4+ T cells from cord and adult blood, early T cell differentiation was monitored after T cell activation in the presence of pro- and anti-inflammatory cytokines. Interestingly, the analysis of Th cell lineage-specific molecules revealed that IL-1β on its own mediates differentiation of Th cells that secrete a wide range of proinflammatory cytokines and stably express CD69, STAT1, IFN-γ, and IL-17. Notably, our data suggest that IL-1β induces Th17 cells independent of RORC upregulation. In contrast, TGF-β, which triggers RORC prevents Th17 cell development. This suppressive function of TGF-β is characterized by inhibition of STAT1, STAT3, and CD69. However, after repeated anti-CD3 and anti-CD28 stimulation, we observe that TGF-β provokes an increase in Th17 cells that presumably relies on reactivation of a default pathway by preferential inhibition of IFN-γ. Hence, our data extend the view that the principal cytokines for determining Th cell fate are IL-12 for the Th1 lineage, IL-4 for the Th2 lineage, and TGF-β in conjunction with IL-6 for the Th17 lineage. We propose that IL-1β induces a general proinflammatory Th cell precursor that, in the presence of the lineage-specifying cytokines, further differentiates into one of the specific Th cell sub-populations. The Journal of Immunology, 2011, 187: 000–000.

Cytokines are central mediators at the interface between the innate and adaptive immune responses. Higher-level organisms are equipped to recognize microbial threats with the complement system, TLRs, specialized receptors on NK cells, and the upregulation of coreceptor ligands and the production of proinflammatory cytokine mediators such as IL-1β, TNF-α, and IL-8. Although cytokines are regarded as key factors directing naive T cell differentiation into Th1, Th2, and Th17 cells (2–6), the role of IL-1β, a main orchestrator in chronic inflammatory diseases and autoimmune syndromes (7–9), in early human Th cell differentiation has not yet been resolved. IL-1β has been suggested to promote inflammatory diseases by inducing the expansion of differentiated Th cells (10) and has been implicated, alone or in combination with IL-6 and TGF-β, in driving murine and human Th17 priming and phenotype stabilization (11–14). Although IL-1β has been implicated in supporting the proliferation and expansion of Th cell precursors in mice (10, 15) and IFN-γ production in human naïve T cells (16), recent data suggest that it is also one of the central cytokines responsible for inducing production of IL-17A (in this study, IL-17) in both human (11, 14, 17, 18) and murine naive CD4+ T cells (19).

In this study, we show that IL-1β directs the differentiation of anti-CD3– and anti-CD28–activated naive cord blood CD4+ T cells toward a proinflammatory Th phenotype, inducing IFN-γ, IL-17, TNF-α, as well as IL-4 secretion. IL-1β induces transcription of lineage-specific mRNA such as IFNG, EOMES (Eomesodermin), IL-17, IL-22, STAT3, and GATA3 but not of RORC (retinoic acid-related orphan receptor [ROR]γt gene). In contrast, TGF-β induces RORC transcription but inhibits the formation of IL-17–secreting cells. Additionally, TGF-β inhibits IL-17 and IFN-γ induction, likely via suppression of STAT3 and STAT1. Nevertheless, after repeated stimulation with anti-CD3 and anti-CD28, TGF-β enhances the formation of IL-17–producing T cells via a default pathway mediated by suppression of IFN-γ induction and correlating with a high level of RORC expression. Hence, we propose that IL-1β is an essential cytokine for inducing expression of IL-4, IL-17, and IFN-γ. In contrast, IL-12 and TGF-β act as cross-regulating agents during Th cell phenotype stabilization (20) and ultimately dictate T cell differentiation toward the Th1 or Th17 lineage, respectively, by suppressing one phenotype while promoting the other. In contrast with Paul and Seder (20), we extended the term cross-regulation to include Th1 and Th17 cells, as these T cell subsets are subject to regulation by IL-12 and TGF-β, which are secreted by cells in the vicinity of the differentiating T cells.

Materials and Methods

**Samples**

PBMCs were obtained from leukocyte reduction filters (Sepacell RZ-2000; Asahi Kasei Medical) from the Institute of Transfusion Medicine and Immunohematology with Blood Bank at the University Clinic of Magdeburg. Cord blood samples were obtained from umbilical cord veins immediately after delivery of the placenta from the Women’s Clinic of the
University of Magdeburg. The study was approved by the Clinical Research Ethics Board of the University of Magdeburg (certificate 79/07), and all parents provided informed written consent in accordance with the Declaration of Helsinki.

PBMCs from filters and cord blood mononuclear cells were isolated from healthy donor samples on Ficoll density gradients. Briefly, naïve CD45RA+ CD4+ T cells were isolated on an autoMACS Pro using a CD4+ T Cell Isolation Kit II (Miltenyi Biotec), and for the PBMCs, this was followed by positive selection of CD45RO+CD4+ T cells and negative selection of CD45RA+CD4+ T cells. Cells were cultured at 37°C in either serum-free X-VIVO-20 (Lonza) or 10% human AB plasma-containing RPMI 1640 (Biochrom) medium, supplemented with penicillin-streptomycin. A total of 5 x 10^3 CD45RA+CD4+ or CD45RO+CD4+ T cells were cultured in U-bottom 96-well plates along with 1 x 10^5 beads coated with 100 μg/ml anti-CD3, anti-CD28, and, where indicated, anti-CD2 (Miltenyi Biotec) according to the manufacturer’s instructions. IL-6 (20 ng/ml), IL-21 (20 ng/ml), IL-23 (20 ng/ml), IL-1β, IL-12, and TGF-β (10 ng/ml or as indicated; all from eBioscience) and a neutralizing Ab to IFN-γ (10 μg/ml; 4S.B3; Deutsches Rheuma-Forschungszentrum) were added on day 0 and maintained throughout the experiment.

Cytokine quantification
Cytokine quantities from culture supernatants were determined with a Bio-Plex cytokine assay using the human 17-Plex Panel (Bio-Rad). Briefly, cytokine standards or samples were incubated with cytokine-conjugated beads, followed by incubation with a detection Ab and streptavidin-PE. Finally, samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad).

ELISPOT assay
ELISPOT plates (Millipore 96-well MultiScreen HA; Millipore) were coated with 2 μg/ml anti-human IL-17-specific mAb (eBio64CAP17; eBioscience) in PBS. Cells were cultured in the presence of PMA and ionomycin for 18 h at 37°C in 5% CO2, plated at 5 x 10^4 cells/well, and titrated 1:3 in the ELISPOT assay. Cytokines were detected with a biotinylated anti-human IL-17 Ab (eBio64DEC17; eBioscience) and developed using 0.3 μg/ml extravidin-alkaline phosphatase (Sigma-Aldrich) and an Alkaline Phosphatase-substrate Kit III Vector Blue (Vector Laboratories).

Four-color cytometric analysis of intracellular cytokines and surface markers
Cells (1 x 10^6/ml) were either stained directly on day 7 of culture or after restimulation with 10 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) for 4 h. During the final 2 h, brefeldin A (Sigma-Aldrich) was added at 5 μg/ml. The cells were then fixed with 2% formaldehyde. For intracellular staining, the cells were permeabilized with 0.5% saponin (Sigma-Aldrich) in PBS/BSA and incubated with the following Abs: PE-coupled anti-IL-17 (eBio64CAP17; eBioscience), PE-Cy7-coupled anti-IFN-γ (4S.B3; BD Pharmingen), and HorizonV450-coupled anti-CD4 (R&D Systems; BD Pharmingen). For surface staining, cells were stained with HorizonV450-coupled anti-CD4, FITC-coupled anti-CD69, FITC-coupled anti-CD45RA, PE-coupled anti-CD45RO, allopurinol-coupled anti-CD25 (all BD Pharmingen), and propidium iodide (Sigma-Aldrich) for discrimination of dead cells. Cytometric analyses were performed using FACSCanto (BD Biosciences) and FlowJo software (Tree Star).

Immunoblotting and protein quantification
First, whole-cell lysates were prepared. Next, proteins were extracted in a hypotonic buffer (20 mM HEPES, 20 mM NaF, 5 mM EDTA, 1% Nonidet P-40, 0.1 mM PMSF, 40 mM β-glycerophosphate, and 2 mM Na3VO4, protease inhibitors). Proteins were then separated on 10% SDS-polyacrylamide gels and probed with Abs directed against phospho-STAT3 (Tyr705) (D3A7), STAT3, phospho-STAT1 (Tyr701) (58D6), STAT1, and anti-tubulin (DM1A) (all from Cell Signaling Technology and NEB).

RNA isolation and real-time quantitative PCR
Total RNA was extracted using an RNaseasy kit (Qiagen). To prepare first-strand cDNA from the isolated RNA, TaqMan reverse transcription components were applied (Applied Biosystems). All primers were obtained from TIB MolBiol (Berlin, Germany). Quantification of the cDNA samples was performed by real-time quantitative PCR in triplicate (primer sets; Supplemental Table I) using FastStart DNA Master SYBR Green I (Roche) or a Maxima SYBR Green/ROX qPCR Master Mix (Fermentas), and the expression of each gene was normalized to the expression of GAPDH (Fig. 3) or ACTB (Fig. 5) using the ΔΔ threshold cycle method. Fold changes were calculated by normalization based on the sample treated with anti-CD3 and anti-CD28 beads only.

Statistical analysis
Statistical significance was calculated with the unpaired Mann–Whitney U test using Prism software (GraphPad). All p values ≤0.05 were considered significant and are referred to as such in the text.

Results
IL-1β induces a proinflammatory phenotype in human naive CD4+ T cells
To estimate the impact of IL-1β compared with other cytokines on early CD4+ T cell differentiation, we determined its capacity to induce pro- and anti-inflammatory cytokines, such as IFN-γ, IL-17, IL-4, TNF-α, IL-8, IL-2, and IL-10, by multiplex cytokine analysis. We isolated naïve CD45RA+CD4+ T cells from the cord blood of human donors and stimulated the cells with anti-CD3-, anti-CD28-, and anti-CD2–coated microbeads in human AB plasma-containing RPMI medium supplemented with no cytokines, IL-1β, IL-6, IL-12, IL-23, TGF-β, or combinations of these cytokines (Fig. 1). Analysis of the cytokine accumulation after 72 h revealed that IL-1β was the most potent cytokine of the ones tested for inducing IL-17, IFN-γ, IL-2, IL-4, TNF-α, and IL-8. When evaluating the dependency of IL-17 induction (Fig. 1A) on the absence and presence of cytokines during T cell activation, we found a significant 28-fold increase in IL-17 production by T cells that were stimulated in the presence of IL-1β compared with those primed in the absence of exogenous cytokines (Fig. 1A). In contrast, IL-6 triggered only a 5-fold increase in IL-17, and other cytokines were only able to induce a 2-fold increase (Fig. 1A). Interestingly, the addition of TGF-β did not significantly enhance IL-17 secretion. The inconsistent findings in the literature regarding the cytokine requirements for human Th17 cell differentiation might be caused by the presence of TGF-β in the serum used in cell cultures (12). To exclude this possibility, we activated the naïve CD4+ T cells under serum-free conditions (Supplemental Fig. 1) and confirmed the finding that IL-1β is the most potent cytokine among the ones tested at inducing IL-17 secretion (Supplemental Fig. 1A). Moreover, IL-1β also induced strong IFN-γ secretion, with an up to 100-fold elevation compared with anti-CD3, anti-CD28, and anti-CD2 stimulation alone (Fig. 1B), whereas IL-6–treated cells showed a 20-fold increase in the level of IFN-γ secretion. As expected, IL-12 was a potent inducer of IFN-γ production, even though a high level of variability was observed among the donors (Fig. 1B). Surprisingly, under serum-free conditions, the IFN-γ secretion was 15-fold higher in presence of IL-1β compared with IL-12 (Supplemental Fig. 1B); however, several donors need to be tested to confirm the robustness of this observation. We noted that, in cultures stimulated with IL-1β, IL-2 production was also enhanced, albeit at levels that were not substantially altered compared with cultures containing IL-6 (Fig. 1C). Although IL-4 production was very low, the levels were still significantly increased by IL-1β compared with cells treated with IL-6, IL-12, IL-23, TGF-β, or a combination of these cytokines (Fig. 1D). Furthermore, TNF-α (Fig. 1E) and IL-8 (Fig. 1F) were also elevated in the presence of IL-1β compared with the presence of the other cytokines tested, whereby the elevation of IL-8 was not significant. Notably, IL-6 and TGF-β, both alone and in combination, but not IL-1β, IL-12, or IL-23, promoted at least 15-fold induction of IL-10 in human naive CD4+ T cells (Fig. 1G).

Taken together, the pattern of cytokine secretion triggered by IL-1β in human naive CD4+ T cells from cord blood unambigu-
uously shows that IL-1β is a potent cytokine that is able to induce cytokine-secreting CD4+ T cells with a clear bias toward proinflammatory phenotypes.

Stable expression of CD69 and secretion of IL-17 induced by IL-1β is antagonized by TGF-β

To more precisely characterize the induction of proinflammatory CD4+ T cells by IL-1β and the role of TGF-β in their formation, we evaluated the expression of the activation markers CD69 and CD25. Therefore, we stimulated naive CD45RA+CD4+ T cells with anti-CD3– and anti-CD28–coated microbeads in the presence of various concentrations of IL-1β with or without the addition of TGF-β. We found that the presence of IL-1β led to a stable expression of the early activation marker CD69 on 15.2 ± 0.6% versus 1.2% (mean ± SEM) in the activated naive CD4+ T cells, which lasted until at least day 7 after stimulation (Fig. 2A, left panel). Expression of the IL-2R CD25 was only slightly elevated in the presence of IL-1β at that time point. We also determined the expression pattern of CD69 and CD25 at days 1 and 3, observing an upregulation of both surface molecules in up to 90% of all activated cells independent of IL-1β treatment (data not shown). Hence, IL-1β provoked a stable expression of the early activation marker CD69 on a subset of primary activated naive CD45RA+CD4+ T cells. Notably, addition of TGF-β reversed the IL-1β–induced maintenance of CD69 and CD25 expression (Fig. 2A, right panel). The observed ability of IL-1β to trigger a wide spectrum of proinflammatory cytokines, in particular IL-17, prompted us to evaluate whether individual IL-17–secreting cells could be identified among the newly activated human naive CD45RA+CD4+ T cells. To explore the impact of IL-1β and TGF-β on the formation of living IL-17–secreting CD4+ T cells, both cytokines were added at serial concentrations, and IL-17 secretion was triggered using PMA and ionomycin on day 7. Notably, IL-1β led to a 17-fold increase in the formation of IL-17–secreting cells (Fig. 2B, top panel). This unambiguously confirms and extends the finding that IL-1β induces the differentiation of functional IL-17–secreting cells from naive CD4+ T cells independent of other cytokines and at a very early time point. In contrast, simultaneous addition of TGF-β reversed the IL-1β–induced development of IL-17–secreting cells (Fig. 2B, top panel, 2C). This unambiguously confirms and extends the finding that IL-1β induces the differentiation of functional IL-17–secreting cells from naive CD4+ T cells independent of other cytokines and at a very early time point. In contrast, simultaneous addition of TGF-β reversed the IL-1β–induced development of IL-17–secreting cells (Fig. 2B, top panel, 2C). The effects observed for IL-1β and TGF-β were restricted to naive CD4+ T cells. IL-1β did not enhance significantly the amount of IL-17–secreting cells derived from CD45RA+ effector and memory CD4+ T cells. Also, in these cells, TGF-β did not reduce the frequency of IL-17–secreting cells (Fig. 2B, bottom panel).

In conclusion, CD4+ T cell activation in the presence of IL-1β promotes the formation of a distinguished CD4+ T cell precursor from naive CD4+ T cells only that can be triggered to secrete large amounts of IL-17 on a per-cell basis, a process that is reversed by TGF-β.
IL-1β and TGF-β regulate human proinflammatory CD4+ T cells

Because IL-1β was found to trigger the induction of proinflammatory cytokines and the formation of IL-17-secreting CD4+ T cells, and the later process was suppressed by TGF-β, we decided to clarify the role of IL-1β and TGF-β on cytokine-inducing signaling pathways, in particular those responsible for IL-17 and IFN-γ induction. Transcription of IL-17 and IFNG mRNA and the TH17-specific transcription factors RORC and STAT3, as well as that of the Th1 lineage-specifying transcription factors STAT1, TBX21 (T-bet), and EOMES, was evaluated. Additionally, expression of the regulatory T cell (Treg)-specifying FOXP3, the Th2-specifying GATA3, and several effector cytokine mRNAs such as IL-17F, IL-22, and IL-9 were evaluated. Human naive CD4+ T cells from the cord blood were activated with anti-CD3– and anti-CD28–coated microbeads in the presence of IL-1β, TGF-β, or a combination of both. The time course of mRNA expression was assessed over a 6-d period. IL-1β–treated cells showed a 100-fold induction of IL-17A (Fig. 3A) and IFNG (Fig. 3B) mRNA levels on day 3 after activation, which was entirely abrogated in the presence of TGF-β. In contrast to an earlier observation (11), an increase of RORC in the presence of TGF-β was observed as soon as on day 1 and steadily increased up to day 6, whereas IL-1β did not enhance RORC mRNA expression (Fig. 3C). Unexpectedly, the observed RORC upregulation in the presence of TGF-β did not correlate with an IL-17 induction on either a transcriptional or protein level, suggesting an IL-1β–mediated and TGF-β and possibly RORγt-independent induction of IL-17 in primary activated human naive cord blood CD4+ T cells. Surprisingly, STAT3 mRNA also showed peak expression on day 3, whereas among the Th1 transcription factors, only EOMES was upregulated on day 3 in the presence of IL-1β (Fig. 3D, 3G). STAT1 and Tbx21 did not appear to be regulated by IL-1β, as their expression, together with EOMES and RORC, increased 4 d after anti-CD3 and anti-CD28 stimulation alone (Fig. 3D–G). Again, this increase was impeded by the presence of TGF-β (Fig. 3D–G). Consistent with the multiplex analysis, the IL-4–inducing transcription factor GATA3 was upregulated on day 3 in the presence of IL-1β, whereas the Th9–specific factor FOXP3 was only slightly upregulated by TGF-β (Fig. 3H, 3I). Moreover, the mRNA expression of the Th17 effector cytokines IL-22 and IL-17F was likewise elevated in the presence of IL-1β on day 3, as well as the Th9 effector cytokine IL-9, which required the additional presence of TGF-β (Fig. 3J–L). It has been hypothesized that regulation of STAT1 and STAT3 protein phosphorylation is involved in the determination of the development of Th1 and Th17 cells (21, 22). Therefore, to determine whether the impact of IL-1β and TGF-β on IL-17 and IFN-γ expression could be explained by expression

**FIGURE 2.** IL-1β triggers stable CD69 expression and primes naive CD4+ T cells to secrete IL-17, a process antagonized by TGF-β. Naive CD45RA+CD4+ T cells were stimulated for 7 d in the presence of anti-CD3 and anti-CD28. IL-1β was added at 0.5, 5, 50, and 500 ng/ml (wedges indicate increments) and, unless otherwise noted, 1 ng/ml TGF-β. Data show representative dot plots of one out of three independent donors. A, Flow cytometry analysis of surface CD69 and CD25 expression. B, ELISPOT assay for naive CD45RA+CD4+ T cells (top panel) or memory CD45RO+CD4+ T cells (bottom panel) stimulated for 7 d as described and treated with PMA and ionomycin (18 h). Numbers on the right side indicate the number of cells plated in the ELISPOT assay. C, Number of IL-17–secreting CD4+ T cells from six independent donors. TGF-β was added at concentrations of 0.5 (open triangle), 1 (open square), and 10 ng/ml (open circle). *p ≤ 0.038, **p ≤ 0.0095; median.
and phosphorylation of the STAT1 and STAT3 proteins, Western blot analysis was performed on days 4 and 6 after start of cord blood CD4+ T cell activation in the presence and absence of IL-1β, TGF-β, and their combination. As shown in Fig. 4A, we found that expression of STAT1 and its phosphorylation was strongly regulated by IL-1β and TGF-β. Only a weak induction of STAT1 was observed without IL-1β, whereas continuous expression and phosphorylation of STAT1 appeared only in its presence (Fig. 4A, lane 3). Whether IL-1β could induce STAT1 phosphorylation directly or via IFN-γ or IL-6, which triggers STAT1 as well as STAT3 phosphorylation (23, 24), is currently under investigation. Interestingly, STAT1 expression and phosphorylation was entirely abrogated in the presence of TGF-β (Fig. 4A, lanes 2 and 4). TGF-β probably also suppressed IFN-γ–independent STAT1 expression (Fig. 4A, lanes 1 and 2), because no IFN-γ was detected in the supernatant in the anti-CD3 and anti-CD28 only stimulated naive CD4+ T cells on day 3 after activation (Fig. 1B).

Next, we addressed the regulation of STAT3 expression and phosphorylation on days 4 or 6 after the stimulation of naive CD4+ T cells. Notably, we only observed a minor increase in IL-1β–triggered STAT3 expression on day 6 (Fig. 4B, lane 3), whereas STAT3 phosphorylation was so far not detectable (data not shown). Thus, IL-1β–triggered IL-17 expression appears to occur independent of STAT3 upregulation and phosphorylation. Surprisingly, in accordance with the TGF-β–mediated suppression of STAT1, the constitutive expression of STAT3 was also suppressed by TGF-β (Fig. 4B, lanes 2 and 4).

These results collectively indicate that IL-1β triggers elevated expression of IL-17, IFN-γ, IL-17F, IL-22, EOMES, GATA3, and STAT3 on a transcriptional level and of IL-17, IFN-γ, and STAT1 on the translational level in stimulated naive cord blood CD4+ T cells. In contrast, TGF-β, although inducing the expression of RORC and FOXP3, inhibits the expression of STAT1 and STAT3 in these cells.

TGF-β biases toward a Th17 phenotype under chronic anti-CD3– and anti-CD28–induced T cell stimulation

Thus far, our findings suggested that initial differentiation of proinflammatory cytokine-producing cells was triggered by IL-1β and suppressed by TGF-β. TGF-β impaired the induction of IFN-γ and formation of IL-17–producing cells during primary T cell acti-
vation. Thus, we hypothesized that, in long-term cultures with repeated anti-CD3 and anti-CD28 stimulation, TGF-β might impede IFN-γ induction to a greater extent than it did with IL-17 induction. As a result, Th17 differentiation would be favored over Th1 differentiation. To address the question of the mode of action of TGF-β on Th cell differentiation in cultures with restimulation, CD45RA⁺CD4⁺ T cells were repeatedly stimulated with anti-CD3- and anti-CD28-coated microbeads under optimal condition for the development of Th17 cells (anti–IFN-γ, IL-1β, IL-6, IL-21, and IL-23), and increasing concentrations of TGF-β were added. Furthermore, by adding IL-12 to the cultures, the impact of TGF-β in milieus with a strong Th1 bias could be evaluated. At day 7, the cells were restimulated, the indicated cytokines renewed, and after 7 more days, the presence of IL-17⁻ and IFN-γ⁻ expressing cells was evaluated by flow cytometry. Notably, the presence of anti–IFN-γ, IL-1β, IL-6, IL-21, and IL-23 alone did not give rise to a detectable amount of IL-17⁺CD4⁺ T cells (Fig. 5A, top left panel), but instead led to a 33.45% induction of IFN-γ⁺CD4⁺ T cells. Analyzing the frequencies of IL-17⁺ and IFN-γ⁺ CD4⁺ T cells at gradual increasing TGF-β concentrations, we observed

**FIGURE 5.** TGF-β antagonizes IL-12 signals, thereby promoting Th17 versus Th1 cell differentiation. Flow cytometry of intracellular IL-17 and IFN-γ in CD45RA⁺CD4⁺ T cells stimulated with anti-CD3/anti-CD28 in serum-free X-VIVO20 medium. Naive CD4⁺ T cells were cultivated in the presence of anti–IFN-γ, IL-1β, IL-6, IL-21, and IL-23. TGF-β (0–10 ng/ml) and IL-12 (0–10 ng/ml) were added with increasing concentrations. Wedges indicate 10-fold increments. A, Intracellular staining for IL-17 and IFN-γ was performed on day 15 after activation and treatment with PMA and ionomycin (4 h). Representative data are from one out of three independently tested donors. B, Summary depicting the IL-17⁺ and IFN-γ⁺ frequencies from three independent donors. Real-time quantitative PCR analysis of RORC, TBX21, and EOMES mRNA (C–E, top panels) of CD45RA⁺CD4⁺ T cells stimulated in the presence of anti-CD3 and -CD28, anti–IFN-γ, IL-1β, IL-6, IL-21, and IL-23 9 d after beginning of the stimulation. TGF-β was added at 0, 0.1, 1, or 10 ng/ml, and IL-12 was added at 0 or 10 ng/ml. C–E, Bottom panels, Summary depicting the results of the real-time analysis from three independent donors.
a 55-fold increase of IL-17 single producers, from 0.11 to 6.06%, whereas we found a 37-fold drop of IFN-γ single producers, from 33.45 to 0.91% (Fig. 5A, top panel). As expected, the presence of IL-12 led to a reduction in IL-17/CD4+ T cells and an increase in IFN-γ/CD4+ T cells. Evaluating the reciprocal increase and decrease in the frequencies of IFN-γ and IL-17/CD4+ T cells triggered by IL-12 and TGF-β, respectively (Fig. 5B), IFN-γ+ cell formation (Fig. 5B, bottom panel) appeared to be more prone to modulation by cytokines than IL-17+ cell formation (Fig. 5B, top panel). Overall, in cultures using anti-CD3 and anti-CD28 restimulation of T cells in a proinflammatory environment, T cell differentiation had a strong tendency to differentiate into a Th1 phenotype, unless a high concentration of TGF-β dominated the milieu (Fig. 5B). To evaluate whether other IFN-γ-inducing signaling molecules bias the Th1/Th17 dichotomy further toward Th1, we amplified the IFN-γ-inducing signaling pathway by adding an anti-CD2 signal (25) during anti-CD3 and anti-CD28 stimulation. When naive CD45RA+CD4+ T cells from the same donor as above were activated under the same culture conditions in the presence of anti-CD2, the frequency of IFN-γ-producing cells was drastically enhanced and coincided with a significant impairment in formation of IL-17+CD4+ T cells (Supplemental Fig. 2). However, TGF-β was still able to impair IFN-γ induction, which allowed a few cells to differentiate toward a Th17 phenotype (<1%). Interestingly, the percentage of IL-17/IFN-γ double-producing cells in cultures receiving anti-CD2 was elevated in the presence of TGF-β. This suggests that CD2 signaling generates IL-17/IFN-γ double-producing cells, and TGF-β later shuts down IFN-γ production in these cells.

Taking into account the impact of TGF-β during early differentiation of naive CD4+ T cells in a proinflammatory milieu, we next measured how the Th17- and Th1-specific transcription factors measured on day 9 correlated with the cytokine expression pattern in the long-term cultures. Consistent with previous reports (12, 26) and our observations during early T cell differentiation, we found that RORC expression was clearly elevated in presence of TGF-β, (Fig. 5C), and this correlated well with the formation of IL-17–secreting cells in long-term stimulation conditions. In contrast to early differentiation, we observed a clear suppressive effect of TGF-β on TBX21 (Fig. 5D) and EOMES (Fig. 5E) in the presence or absence of IL-12.

Collectively, these data demonstrate that, under long-term proinflammatory conditions, TGF-β impedes the formation of IFN-γ+CD4+ T cells and the expression of the Th1 lineage-specific transcription factors Eomes and T-bet to a higher extent than during early CD4+ T cell differentiation. Simultaneously, TGF-β does not seem to suppress the formation of IL-17/CD4+ T cells and leads to an upregulation of the Th17-specifying transcription factor RORγt. In Fig. 6, we summarize our data and propose a new model for how IL-1β and TGF-β influence CD4+ T cell differentiation.

**Discussion**

In this article, we show that a single cytokine, IL-1β, in conjunction with anti-CD3 and anti-CD28 stimulation triggers naïve T cells to produce a large array of proinflammatory cytokines, such as the Th2 cytokine IL-4, the Th1 cytokine IFN-γ, and the Th17 cytokine IL-17. Soluble IL-17 can be detected as soon as 2 to 3 d after primary stimulation of naive CD4+ T cells. To date, the formation of individual IL-17–producing cells has only been described in cells from long-term cultures with at least one cycle of restimulation (11, 12, 18). To avoid any secondary effects and selection processes that might result from long-term cultures, we performed an ELISPOT assay that allowed us to detect IL-17+CD4+ T cells on day 7 in primary CD4+ T cell cultures (Fig. 2B, 2C). Several publications have discussed the importance of IL-1β for Th17 induction and Th17-mediated diseases (11, 14, 18, 27), and in both mice and humans, proinflammatory cytokines have been shown to induce and enhance the IL-2, IFN-γ, and IL-17 expression of CD4+ T cells (11, 28). In light of these studies and our findings, IL-1β appears to represent a central element of early T cell differentiation, helping to overcome the intrinsic refractoriness of naïve T cells (16). Indeed, the overall importance of inflammation in Th17 production by DCs for the immune defense has been reported recently (27, 29). Taken together, the fact that IL-1β production has been shown to be important for Th17 differentiation, whereas IL-12 and IL-23 secreted by DCs are dispensable for Th17 induction (28), supports a central role for IL-1β in the differentiation of this T cell subset. Our findings suggest that IL-1β both causes naïve T cells to develop into proinflammatory precursor Th cells that are able to produce various cytokines and appears to stabilize expression of the activation marker CD69 in a subpopulation of activated CD4+ T cells. In contrast to the present view of IL-1β as an inducer of proliferation, the observed induction of a proinflammatory Th cell precursor does not only stem from greater T cell expansion induced by IL-1β (10, 15).

The molecular mechanism by which IL-1β induces the proinflammatory cytokines is not yet resolved, but its requirement is supported by the fact that IL-1R type 1 (IL-1R1)-deficient mice fail to mount a robust Th17 response after immunization, and IL-1R1–deficient T cells cultured with IL-23 do not produce IL-17.
The general IL-1β–mediated upregulation of various T cell activation markers such as CD69, CD25, and STAT1 might point toward an IL-1β–induced signaling cascade. Future studies need to elucidate whether IL-1β–induced differentiation of IL-17–secreting cells from activated naive CD4+ T cells is triggered by a STAT-dependent (30) or -independent mechanism, possibly through activating NF-kB and the diversifying IFN regulatory factors, such as IFN regulatory factor-4 (19, 31). Alternatively, IL-1β might support triggering of NFAT, which in turn could mediate the observed induction of early IL-17 (32) and possibly also early IL-4 (33) and IFN-γ (16). Similar to IL-2, IL-4, IL-8, TNFα, and IFNγ (34), the human IL-17 gene contains two NFAT binding sites in the proximal promoter region that appear to be important in IL-17 regulation (35). Because of this, NFAT signaling downstream of IL-1β (36) would be an interesting potential mechanism for regulating primary cytokine secretion. Finally, the finding that IL-6 induces IL-10 points toward differential pro- and anti-inflammatory functions of IL-1β and IL-6.

We further propose a differential function of anti-inflammatory TGF-β during primary T cell activation and recall responses. We and others have shown that TGF-β impedes the initial Th1 (37, 38) and Th17 (11, 18) cell differentiation that, in our case, is triggered by IL-1β. Although several mechanisms have been described for TGF-β–mediated Th1 suppression beyond antiproliferative activity, such as inhibition of T-bet (37, 39) and STAT4 (38), the TGF-β–mediated Th17 suppression is unresolved yet (18). TGF-β suppresses STAT1 even in the absence of exogenous IFN-γ (Figs. 1B, 4A, lanes 1 and 2), thus it is likely that TGF-β impedes IFN-γ induction by means other than the previously described interference downstream of IFN-γ/IFN-γ receptor signaling via suppression of T-bet or Shp-1 (37, 40). We now propose that TGF-β interferes with the expression of STAT1 and STAT3 and, consequently, impedes their phosphorylation during early T cell activation in the presence or absence of proinflammatory cytokines.

The majority of studies have found TGF-β to be the essential factor for induction of IL-17 (12, 17, 28, 41, 42), and Manel et al. (12) showed that this might function in an RORγt-dependent fashion. We observe in this study that the inductive effect of TGF-β on Th17 development occurs only in long-term cultures with repeated anti-CD3 and anti-CD28 stimulation under proinflammatory conditions. Under such conditions, TGF-β likewise suppresses strongly a Th1 cell development, whereas it provokes an increase of Th17 cells (12, 43). Evidence exists that the stabilization of the Th17 phenotype is clearly dependent on RORγt (12, 44), albeit the exact mechanism of regulation is undefined (36). Nevertheless, the dual function of TGF-β with simultaneous suppression of Th1 and propagation of Th17 could also rely on a mechanism that as has been discussed earlier, suggesting that TGF-β promotes Th17 cell stabilization by a mechanism acting primarily through inhibiting other Th cell fates (45). In this study, we suggest that the development of Th17 cells is elicited by the reactivation of a default pathway that induces IL-17 independently of RORγt during initial T cell activation and that is uncovered through the inhibition of IFN-γ induction. Real-time PCR analysis revealed that IL-1β triggers IL-17 independently of RORγt (Fig. 3C), whereas others observed that the IL-1β–mediated Th17 formation is RORγt dependent (11).

Based on studies in mice, there has been speculation that TGF-β might contribute to Th17 development by limiting inhibitory actions of IFN-γ (46). But because IFN-γ−/− mice as well as IFN-γ receptor−/− mice, which are not able to mediate IFN-γ signaling, show high frequencies of IL-17–producing cells in the presence of TGF-β (47), it is more likely that this phenomenon relies on the ability of TGF-β to interfere also with the various stimuli that have been reported to promote Th1 differentiation, such as CD28 (48), CD2 (25), IL-12 (49), and IL-2 (49). Concurrently, CD28 (50), IL-12 (11, 12, 18), and IL-2 (11, 51) have been shown to antagonize Th17 formation, whereas we observed this suppressive effect also for CD2 signaling. Notably, the IL-2–mediated suppression of Th17 formation observed in mice (51) seems to vary in the human depending on dose and the state of differentiation; the reports on its impact on Th17 formation were controversial (11, 12, 18, 51). Nevertheless, for IL-12 and CD2 signaling, we found that TGF-β–mediated interference with these pathways promotes Th17 differentiation during long-term cultures with restimulation. Based on our findings, we conclude that TGF-β primarily acts by impeding the induction of STAT1 and thereby T-bet and Eomesodemin.

To summarize our findings, we propose a new model for how IL-1β and TGF-β influence CD4+ T cell differentiation (Fig. 6). We propose that IL-1β induces secretion of a vast array of proinflammatory cytokines in anti-CD3– and anti-CD28–stimulated human naïve CD4+ T cells and promotes the formation of a primed proinflammatory precursor, CD4+ T cells that develop in presence of IL-1β are able to secrete IL-17, IFN-γ, TNF-α, IL-8, and IL-4, and individual IL-17– and IFN-γ–secreting cells develop. The formation of these effector cells is inhibited by TGF-β. In long-term cultures with repeated cycles of anti-CD3 and anti-CD28 stimulation in a proinflammatory milieu, the Th cell phenotypes stabilize. In this study, IL-12, CD2, and IFN-γ signaling facilitate IFN-γ versus IL-17 induction, leading to an increase in IFN-γ/CD4+ T cell formation at the expense of IL-17/CD4+ T cell development. However, when TGF-β is added to the culture, it functions as IFN-γ inhibitor and thereby allows IL-17/CD4+ T cells to dominate.

Taken together, we hypothesize that IL-1β is part of a first line of defense driving T cells toward a Th2, Th1, and Th17 phenotype, especially as a consequence of infection or inflammation. Because IL-17 and IL-8 are evolutionary more conserved than IFN-γ (52), we believe that early secretion of IL-17 in presence of IL-1β reflects an innate mechanism of cytokine secretion, which, in the course of evolution, became inferior to the IFN-γ pathway. Under certain circumstances, such as the presence of TNFα, TGF-β is present at the site of inflammation, and the balance between the level of proinflammatory cytokines and TGF-β determines the relative Th1 versus Th17 cell differentiation. In most clinical conditions, proinflammatory cytokines such as IL-1β, IL-6, and TNF-α have an important early role during infection, inflammation, and in the onset of chronic disease states, suggesting that they are primarily involved in shaping the T cell differentiation during the first Ag encounter.

Further defining the molecular mechanism of how IL-1β induces the proinflammatory phenotype of precursor Th cells destined for Th2, Th1, and Th17 differentiation should lead to rational strategies for manipulating immune responses for prophylaxis and therapy. Our findings might explain the effectiveness of IL-1β antagonists in therapies directed against autoimmune inflammatory diseases that differ in the T cell subpopulations driving them (53). Defining the nature of the IL-1β–IL-1R–induced signaling pathways in T cells will allow the identification of new targets in the therapy of inflammatory diseases.

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