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Signal Strength and Metabolic Requirements Control Cytokine-Induced Th17 Differentiation of Uncommitted Human T Cells

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IL-17 production defines Th17 cells, which orchestrate immune responses and autoimmune diseases. Human Th17 cells can be efficiently generated with appropriate cytokines from precommitted precursors, but the requirements of uncommitted T cells are still ill defined. In standard human Th17 cultures, IL-17 production was restricted to CCR6+CD45RA+ T cells, which expressed CD95 and produced IL-17 ex vivo, identifying them as Th17 memory stem cells. Uncommitted naive CD4+ T cells upregulated CCR6, RORC2, and IL-23R expression with Th17-promoting cytokines but in addition required sustained TCR stimulation, late mammalian target of rapamycin (mTOR) activity, and HIF-1α upregulated CCR6, RORC2, and IL-23R expression with Th17-promoting cytokines but in addition required sustained TCR stimulation, late mammalian target of rapamycin (mTOR) activity, and HIF-1α expression because mice that harbor CD4+ T cells that lack STAT3 do not produce IL-17 and patients with STAT3 mutations develop hyper-IgE syndrome and also lack Th17 cells (2). Th17 cells are important to fight extracellular bacteria and fungi (3, 4), as demonstrated by hyper-IgE syndrome patients, who suffer from recurrent infections with Staphylococcus aureus and Candida albicans. Th17 cells are also believed to play a prominent pathogenic role in autoimmunity, and, in particular, polymorphisms in components of the IL-23R pathway are associated with various autoimmune diseases. Th17 cells can be generated with different combinations of cytokines, including IL-6, IL-21, and IL-23, that signal via STAT3 (1), as well as IL-1β and TGF-β1, although the role of TGF-β in human Th17 differentiation was debated (5–11). Precommitted Th17 cells are already present in the thymus of humans and mice (7, 12). Specifically, in humans it was documented by several different groups that Th17 cells could be generated from precommitted precursors with Th17-promoting cytokines, whereas uncommitted T cells failed to produce IL-17 (7, 13–16). In other reports that defined the requirements of human Th17 cell differentiation, precommitted and uncommitted naive T cells were not analyzed separately. Therefore, whether uncommitted naive T cells can differentiate into Th17 cells in humans and under what conditions is still unclear.

Th17 cells represent a recently identified T cell differentiation lineage that is defined by production of IL-17 and that expresses CCR6, IL-23R, as well as the lineage-defining transcription factor ROR-γt, in mice and RORC2 in humans (1). The generation of Th17 cells depends on the STAT3 transcription factor because mice that harbor CD4+ T cells that lack STAT3 do not produce IL-17 and patients with STAT3 mutations develop hyper-IgE syndrome and also lack Th17 cells (2). Th17 cells are important to fight extracellular bacteria and fungi (3, 4), as demonstrated by hyper-IgE syndrome patients, who suffer from recurrent infections with Staphylococcus aureus and Candida albicans. Th17 cells are also believed to play a prominent pathogenic role in autoimmunity, and, in particular, polymorphisms in components of the IL-23R pathway are associated with various autoimmune diseases. Th17 cells can be generated with different combinations of cytokines, including IL-6, IL-21, and IL-23, that signal via STAT3 (1), as well as IL-1β and TGF-β1, although the role of TGF-β in human Th17 differentiation was debated (5–11). Precommitted Th17 cells are already present in the thymus of humans and mice (7, 12). Specifically, in humans it was documented by several different groups that Th17 cells could be generated from precommitted precursors with Th17-promoting cytokines, whereas uncommitted T cells failed to produce IL-17 (7, 13–16). In other reports that defined the requirements of human Th17 cell differentiation, precommitted and uncommitted naive T cells were not analyzed separately. Therefore, whether uncommitted naive T cells can differentiate into Th17 cells in humans and under what conditions is still unclear.

Th17 cell differentiation is controlled not only by cytokines but also by the strength and duration of TCR stimulation. Naive CD4+ and CD8+ T cells differentiate poorly following short or weak TCR stimulation (17, 18). Furthermore, high concentrations of TCR agonists promote Th1 concentrations, whereas low concentrations favor Th2 differentiation and also promote IL-17 production (19). The importance of signal strength for naive human Th17 cell differentiation is, however, unknown. T cells primed with high signal strength have a high mitochondrial mass (17), suggesting that the strength of TCR stimulation enhances cellular metabolism. A crucial regulator of cellular metabolism is the mammalian target of rapamycin (mTOR) pathway (20). It integrates signals from the TCR and cytokines and also acts as a nutrient sensor that orchestrates cellular metabolism. In recent years, it has become increasingly clear that the regulation of cellular metabolism and that of differentiation are closely linked, and that the mTOR pathway is also a critical regulator of T cell differentiation (21). In particular, mTOR is important for the fate decision between Th17 cells and FOXP3+ regulatory T cells (Tregs) (20). HIF-1α, a downstream target of mTOR, not only controls glycolysis.

Abbreviations used in this article: AHR, aryl hydrocarbon receptor; mTOR, mammalian target of rapamycin; s6rp, s6-ribosomal protein; Treg, regulatory T cell; TSCM, memory T stem cell.

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but also regulates T cell differentiation and induces IL-17 transcription (22, 23). Of note, human blood T cells are in a resting state and therefore poorly active metabolically (20, 21). Upon antigenic activation they grow and become T cell blasts that enter the cell cycle and then divide rapidly many times over several days. In this expansion phase they have a very high demand for nutrients to generate energy and the building blocks for DNA and protein synthesis, and they achieve this by metabolizing glucose in the glycolytic pathway, thus switching completely their metabolic program (20).

In this article, we showed that uncommitted naive T cells have very high signal strength and metabolic requirements for cytokine-induced IL-17 production, and we identified the late, nutrient-dependent mTOR activity as the rate-limiting step for induced Th17 cell differentiation in humans.

Materials and Methods

T cell purification
Buffy-coated blood from healthy donors and cord blood were obtained from the Istituto di Ricovero e Cura a Carattere Scientifico Policlinico Ospedale Maggiore, Milan, Italy, and the Charité Hospital and the Deutsches Rotes Kreuz in Berlin, Germany. Human PBMCs were isolated by Ficoll–Hypaque (Sigma-Aldrich) gradient (Sigma-Aldrich). CD4+ T cells were enriched with magnetic beads on an autoMACS Pro Separator (Miltenyi Biotec), and T cell subsets were sorted on a FACSAria III (Becton Dickinson). Uncommitted naive T cells were sorted as CD4+CD25−CD45RA+CCR6−CXCR5+CD31+ cells; in some experiments, CXCR3 and CD161 were excluded in addition. Memory T stem cells (TSCM) were sorted as CD45RA−CD62L−CD95− cells, Th1SCM as CD45RA−CCR6+CD95− cells, and Th1 memory cells as CD4+CD25−CD45RA−CCR6+CXCR3+ cells. Anti-CD4 Ab was purchased from Miltenyi Biotec; anti-CD31, -CXCR3, -CD95, and -CD45RA Ab from Becton Dickinson; and anti-CD25 and -CCR6 from eBioscience. The purity of T cell populations was >99%.

T cell stimulation
In vitro stimulation of sorted T cells was performed with plate-bound anti-CD3 at 0.02, 0.2, or 2.0 μg/ml and at 6 μg/ml anti-CD28 Abs (Becton Dickinson). Neutralizing Abs against IL-12, IFN-γ, and IL-4 were used at 2 μg/ml (Becton Dickinson). All recombinant cytokines (R&D) were added at 10 ng/ml, with the exception of TGF-β, which was used at 1 ng/ml. Under neutral conditions, cells were stimulated with 2 μg/ml anti-CD3 Abs in the presence of anti-IL-12, –IFN-γ, and –IL-4 (Becton Dickinson). Th1 conditions were 2 μg/ml anti-CD3 Abs in the presence of IL-12 and anti-IL-4, Th1 conditions were 0.02 μg/ml anti-CD3 Abs in the presence of TGF-β1, IL-1β, IL-6, and IL-23 and neutralizing Abs. Cells were stimulated in coated 96-flat-bottom-well plates in 200 μl RPMI or DMEM medium at 50,000 cells per well in standard high-density cultures and at 5000 cells per well in low-density cultures (1:10). In some experiments, cells were also cultured at very low density (500 cells per well, 1:100). In standard short-term cultures, cells were detached after 24 h, 50% of cells were transferred to uncoated wells, 100 μl per well of fresh medium with cytokines and/or Abs was added and cells were cultured for an additional 48 h. In long-term cultures, cells were stimulated for ≤12 d before transfer to uncoated wells. In these cultures, 24–48 h half of the medium was removed, and the same volume of fresh medium containing cytokines and/or neutralizing Abs was added. Rapamycin (Sigma-Aldrich) was used at 100 nM, FICZ (Enzo Life Science) at 300 nM, the aryl hydrocarbon receptor (AhR) inhibitor CH-223191 (Sigma-Aldrich) at 3 μM, amino acids (RPML amino acid solution 50×; Sigma-Aldrich) at 1:25, and fatty acids (CD Lipid Concentrate; Life Technologies) at 1:1000, as recommended. D-Glucose was added at 25, 50, or 100 mM, L-tryptophan at 0.78 mM, and L-lysine at 8 mM, and echinocycin (Sigma-Aldrich) was added at 0.1 μM on day 5. The high-salt condition was achieved by adding 40 mM NaCl. Glucose concentrations in 10 μl cell culture medium were measured with an ALPHA-GDH blood glucose monitoring system (Alpha 1 Diagnostik, Fulda, Germany) in triplicates.

FACS analysis

For data acquisition, a FACSCanto II (Becton Dickinson) with DIVA software was used, and analysis of the data was performed using FlowJo software (TreeStar). The transcription factor FOXP3 was stained after 4 d of stimulation with aliphosphocytarin-conjugated Abs purchased from Becton Dickinson, following fixation and permeabilization with a fixation/permeabilization kit (eBioscience). For staining of the phosphorylated transcription factors STAT3 (Becton Dickinson) and STAT5 (Becton Dickinson) and Phospho-S6 Ribosomal Protein (Cell Signaling), cells were fixed after 4 d of stimulation with Cytofix Fixation Buffer (Becton Dickinson), followed by permeabilization with Perm Buffer III (Becton Dickinson). Thereafter, p-STATs were stained with aliphosphocytarin-conjugated anti–p-STAT3 or anti–p-STAT5 (Becton Dickinson). Intracellular staining for HIF-1α was performed following the manufacturer’s protocols (R&D Systems). For intracellular cytokine staining ex vivo or after in vitro activation, cells were stimulated for 5 h with 500 ng/ml PMA and 1 μg/ml ionomycin in the presence of Brefeldin A (Sigma-Aldrich). Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin. Abs specific for cytokines were purchased from eBioscience (IL-17, IL-22) or Becton Dickinson (IL-21, IFN-γ). Surface receptors were stained after 4 d of stimulation with PE-conjugated anti-CCR6 or anti-CD98 Abs (Becton Dickinson). To assess cellular proliferation, cells were stained with the CellTrace Violet Cell Proliferation Kit (Invitrogen) or CFSE, and FACS analysis was performed after 4 d of stimulation.

IL-23R and RORC expression

The expression of functional IL-23R was assessed by IL-23-induced STAT3 phosphorylation as described previously (10). To measure RORC mRNA levels, total RNA was isolated using the mirVana Kit (Ambion). m-RNA expression levels were assessed by TaqMan Gene Expression Assay (Applied Biosystems) and normalized on 18S rRNA.

Statistical analysis

Statistical analysis was performed with Prism 5 software (GraphPad), and Student t tests for paired and unpaired samples were used to evaluate differences between two variables. The one-way ANOVA test with Bonferroni correction was used for multiple parameters. Statistical significance was set at *p < 0.05, **p < 0.005, and ***p < 0.0005.

Results

Precommitted Th17SCM cells, but not uncommitted naive T cells, produce IL-17 in standard human Th17 cultures

Human Th17 cells express CCR6 and IL-17 and can be induced from precommitted T cells upon addition of cytokines, whereas Th17 differentiation of uncommitted T cells was reported to be either inefficient or impossible. To obtain uncommitted T cells from peripheral blood and exclude memory and precommitted cells, we purified naive CD4+ T cells as CD4+CD45RA+CD31+ CD25− T cells and excluded cells that expressed chemokine receptors typical for memory T cells, including CCR6. Notably, a variable fraction of bona fide naive CD45RA+ T cells expressed CCR6 in adults (Fig. 1A), a surface marker of Th17 cells (3) that is also expressed on Th17 precommitted naive T cells in neonates (7). We tried to induce Th17 differentiation of precommitted CCR6+CD45RA+ and uncommitted CCR6+CD45RA+ T cells with a mixture of Th17-promoting cytokines (6). Precommitted CCR6+CD45RA+ cells and Th17 memory cells, but not uncommitted T cells, produced IL-17 in these standard Th17 cultures (Fig. 1B). Notably, however, precommitted CCR6+CD45RA+ cells already produced some IL-17 (Fig. 1C) and IL-22 (2.9 ± 0.7%, n = 9; data not shown) ex vivo, suggesting that they contained Th1 memory cells. Consistently, CCR6+CD45RA+ cells expressed FOXP3 in adults (Fig. 1A), a transcription factor that distinguishes naive T cells among CD45RA+CD62L+ T cells in the CD8 compartment (24). Substantial fractions of CD4+ TSCM expressed CCR6 or CD161 (Fig. 1E), suggesting that some were committed to the Th17 lineage. To understand whether IL-17 production in Th17 cultures was due to TSCM, we sorted CD45RA+CD62L+ T cells into CD95+ TSCM and CD95− naive T cell subsets and cultured them with anti-CD3 and anti-CD28 Abs in the absence or presence of Th17-promoting cytokines. CD95+ TSCM, but not CD95− naive T cells, produced IL-17 and IL-22 and high levels of IFN-γ even...
in the absence of exogenous cytokines (Fig. 1F). Moreover, TSCM further upregulated IL-17 production upon addition of Th17-promoting cytokines (Fig. 1G), whereas, again, naive T cells failed to do so (data not shown). Finally, to understand whether Th17SCM coexpressed CCR6 and CD95, we sorted CD45RA+CD62L+CD95+ and CD45RA-CD62L+CD95- naive and TSCM cells under neutral conditions and IL-17 production by TSCM under neutral and Th17 conditions (n ≥ 4). CD4+ CD45RA+CD62L+CD95+ T cells were sorted according to CCR6 and CD95 expression and ex vivo IL-17 production analyzed as in (C). *p < 0.05, ***p < 0.0005.

We conclude that naive T cells fail to acquire IL-17-producing capacities in standard human Th17 cultures. In contrast, Th17SCM cells, which coexpress CCR6 and CD95 and are present at variable frequencies in bona fide naive T cell preparations from peripheral blood of adults, produce IL-17 and IL-22 ex vivo and rapidly upregulate cytokine production in culture.

Th17-promoting cytokines are sufficient to induce RORC2, CCR6, and IL-23R in uncommitted T cells

To understand why uncommitted naive T cells failed to produce IL-17 in standard Th17 cultures, we first analyzed STAT3 and STAT5 phosphorylation, which have, respectively, permissive and inhibitory effects on Th17 differentiation. Th17-promoting cytokines induced STAT3 phosphorylation (Fig. 2A) and inhibited STAT5 phosphorylation (Fig. 2B) as expected. Moreover, Th17-promoting cytokines induced m-RNA of RORC2 (Fig. 2C), the lineage-defining transcription factor of Th17 cells. We next analyzed induction of CCR6 and IL-23R, two characteristic membrane proteins of Th17 cells, in uncommitted naive T cells in response to different combinations of Th17-promoting cyto-
kines. CCR6 surface expression was induced on uncommitted T cells in response to Th17-promoting cytokines, and its expression was highest when all four Th17-promoting cytokines were added together (Fig. 2D). IL-23R expression was analyzed at the single-cell level by IL-23–induced STAT3 phosphorylation. IL-23 responsiveness was efficiently induced by Th17-promoting cytokines (Fig. 2E), and again the combination of all four cytokines was most efficient.

In summary, Th17-promoting cytokines induce a partial commitment of naive T cells to the Th17 lineage, as evidenced by the induction of Th17-associated genes like CCR6, IL-23R, and RORC2, but are insufficient to induce IL-17, the hallmark of Th17 cells.

IL-17 production by uncommitted T cells requires prolonged TCR stimulation in low-density cultures

T cell differentiation critically depends on the strength and duration of TCR signaling. We therefore wondered if optimizing signal strength might lead to IL-17 induction in uncommitted T cells and therefore varied the time of TCR stimulation, the concentration of the stimulating anti-CD3 Abs, and the cell density, which has an impact on the competition for cytokines or nutrients. In these experiments, we were able to induce robust IL-17 production in uncommitted naive T cells (Fig. 3A), but exclusively in low-density cultures (10- or 100-fold dilution) and upon sustained TCR stimulation. Similar results were obtained with uncommitted naive T cells from peripheral blood of adults and cord blood, and in naive T cell preparations in which CD161- and CXCR3-expressing cells had also been depleted (data not shown). Importantly, in our high-density condition, cells were plated at 2.5 × 10^5 cells per milliliter, that is, at the same or even at a lower concentration, as used by several previous seminal studies analyzing human Th17 differentiation [i.e., 2.5 × 10^5 − 1 × 10^6 cells per milliliter (5, 6, 8, 25, 26)]. Therefore, we will refer to this condition as “standard high-density culture” for the rest of this article.

Conflicting results on the cytokines and, in particular, on TGF-β in human Th17 cell differentiation were published (5–8, 10, 11). A possible explanation is that uncommitted and precommitted T cells, which are both present at variable frequencies in standard preparations of bona fide naive human T cells, might have different cytokine requirements. We found that the combination of TGF-β1, IL-6, IL-1β, and IL-23 was most efficient for the induction of IL-17, CCR6, and IL-23 responsiveness in uncommitted T cells, but we also detected Th17 commitment in the absence of TGF-β (Fig. 2E). Of interest, high NaCl concentrations, which were reported to enhance cytokine-driven Th17 differentiation (25), poorly induced IL-17 in standard cultures but strongly boosted IL-17 production in low-density cultures (Fig. 3C).

The striking effect of sustained TCR stimulation and cell density on cytokine production was not restricted to IL-17. In standard cultures under neutral conditions, that is, in the absence of exogenous cytokines but in the presence of neutralizing Abs to IL-4, IFN-γ, and IL-12, production of IFN-γ and IL-22 were, as expected, low and hardly detectable, respectively. IFN-γ and IL-22 production increased dramatically, however, upon sustained TCR stimulation in low-density cultures in the complete absence of exogenous cytokines (Fig. 3D). Notably, not all cytokines were similarly affected by cell density, as IL-21 production showed only a moderate and inconsistent increase in low-density cultures.

**FIGURE 2.** Uncommitted T cells upregulate RORC2, CCR6, and IL-23R expression in standard Th17 cultures. Phosphorylation of (A) STAT3 (n = 4) and (B) STAT5 (n = 8) in uncommitted T cells in neutral and Th17 conditions. Shown are representative histogram overlays and the mean of the analyzed donors. (C) RORC mRNA expression in uncommitted naive T cells in neutral and Th17 conditions and in CCR6+ memory T cells as positive control (n = 5). (D) CCR6 surface expression on uncommitted T cells in response to different combinations of Th17-promoting cytokines as indicated (n = 5). (E) IL-23R expression in response to Th17-promoting cytokines was assessed at the single-cell level by IL-23–induced STAT3 phosphorylation. No p-STAT3 was detected in the absence of IL-23 (data not shown). *p < 0.05, **p < 0.005, ***p < 0.0005.
Although IL-22 production was efficiently induced in low-density cultures in the absence of exogenous cytokines, it was strongly modulated by the cytokine environment (Fig. 3E). Thus, similar to IL-17 it was enhanced by IL-6 and inhibited by IL-4 or IL-12, but in contrast to IL-17, it was inhibited by TGF-β (Fig. 3E). Consequently, the optimal combination of cytokines to induce IL-17 strongly inhibited IL-22 production (Fig. 3E). IL-17 and IL-22 differed not only for their requirements for cytokines but also for the density of TCR agonists, as IL-17 induction was more efficient at low concentrations of anti-CD3 Abs, whereas IL-12–induced IL-22 induction was very efficient (data not shown).

We conclude that the critical factors determining the production of IL-17 in Th17 cells that develop from uncommitted precursors in response to cytokines are the duration of TCR stimulation and cell density. In contrast, IL-12–induced Th1 differentiation occurs efficiently in standard high-density cultures.

**Cell density impacts on the mTOR/HIF pathway**

Having documented that cell density is a critical parameter regulating T cell differentiation, we wanted to define the underlying molecular mechanisms. Importantly, we did not observe a significant reduction in T cell proliferation (Fig. 4A) or cell viability (data not shown) in standard as compared with low-density cultures. The fact that IL-12 efficiently induced Th1 cells in standard cultures, and that IL-22 induction in the absence of exogenous cytokines required low-density cultures, argued against a role for cytokine consumption. Consistently, addition of exogenous IL-21 (11) failed to induce IL-17 in standard high-density cultures, whereas addition of IL-2 in low-density cultures did not inhibit IL-17 production but promoted survival (data not shown). In addition, cell-to-cell contacts were unlikely to play a role because anti–LFA-1 Abs that prevent T cell clusters (27) had no effect on IL-17 production (data not shown).

T cells in low-density cultures had, however, a higher forward scatter (Fig. 4B), indicating that they were bigger and had pre-
sumably a higher metabolic activity. mTOR is a protein kinase that integrates signals from the TCR and nutrients, and activates s6-kinase that in turn phosphorylates the s6-ribosomal protein (s6rp) (20, 28). The phosphorylation of s6rp after 4 d of stimulation was hardly detectable in standard conditions, but was very high in low-density cultures (Fig. 4C), indicating a dramatic difference in mTOR activity in these two conditions. These striking differences in s6rp phosphorylation could be detected both in neutral and Th17 conditions in naive T cells, as well as Th17 memory or TSCM cells (Fig. 4C and data not shown). Surprisingly, we did not detect an enhanced expression of RORC2 in low-density cultures (black) under neutral and Th17 conditions as indicated (n ≥ 5). (D) RORC m-RNA (n = 5). (E) FOXP3 protein expression (one representative histogram overlay, grey histogram: standard condition; black histogram: low-density condition) and mean of 4 donors (right) and (F) HIF-1α protein expression (n = 10) in uncommitted T cells in standard (white bars) or low-density cultures (gray bars) under Th17 conditions. Left panel shows HIF-1α expression in one representative experiment in standard (gray histogram) or low-density cultures (black). (G) Effect of the specific HIF-1α inhibitor echinomycin on IL-17 production on day 6 (n = 7). *p < 0.05, **p < 0.0005, ***p < 0.00005.

Late mTOR activity is required for IL-17 induction and depends on low cell density and sustained TCR stimulation

The immunosuppressive drug rapamycin is a specific inhibitor of the mTOR pathway and completely abrogated s6rp phosphorylation in low-density cultures (Fig. 5A), whereas it had only a mild inhibitory effect on T cell proliferation (Fig. 5B). Rapamycin enhanced FOXP3 protein levels, as expected (Fig. 5C), but had no inhibitory effect on RORC2 m-RNA expression (data not shown). Strikingly, however, whereas rapamycin strongly inhibited IL-17 and IL-22 production by naive T cells, it had no effect on the secretion of these cytokines by Th17SCM or conventional Th17 memory cells under the same conditions (Fig. 5D). To more precisely map the signaling defect in standard high-density cultures, we performed a time course of mTOR activity by monitoring s6rp phosphorylation. mTOR activity was efficiently induced in standard cultures after 48 h but started to decline after 72 h and was low or undetectable after 96 h (Fig. 5E). Conversely, mTOR activity was sustained in low-density cultures, indicating that cell density selectively affected late mTOR activity. Consistently, s6rp was still phosphorylated at higher levels in low-density conditions at late time points, that is, after 8 d of stimulation (Fig. 5F). Of note, late mTOR activity required sustained TCR stimulation because detaching cells from anti-CD3–
coated wells after 48 h resulted in a marked reduction of phosphor-s6rp levels at day 4 (Fig. 5G). Finally, we addressed the relative importance of late mTOR activity for cytokine production by adding rapamycin at different time points. Rapamycin still efficiently inhibited IL-17 production when it was added after 3 d of stimulation (Fig. 5H), i.e., when cells had already divided two to three times (data not shown). Conversely, IL-22 was most efficiently inhibited when rapamycin was added early and became progressively less efficient when it was added at later time points. We conclude that late mTOR activity is required for IL-17 production by uncommitted naive T cells, CD45RA+CCR6+ and CCR6+CD45RA+ cells (n ≥5). (E) Time course of s6rp phosphorylation (p-s6) from 48 to 96 h, as indicated under standard and low-density conditions. One representative experiment of three. (F) S6rp phosphorylation at day 8 under Th17 conditions (n = 4). (G) S6rp phosphorylation at day 4 was measured in cells that either received sustained TCR stimulation (96 h) or were detached from anti-CD3-coated wells after 48 h and cultured for additional 48 h in uncoated wells. One representative experiment of three. (H) Effects of early and late rapamycin addition on IL-17 and IL-22 production (n = 3). Shown is the mean percentage of inhibition. *p < 0.05, **p < 0.0005.

Glucose contributes to mTOR activity and promotes IL-12–independent IFN-γ production

We observed some s6rp phosphorylation in high-density T cell cultures in DMEM (Fig. 6A), a cell culture medium that contains higher concentrations of several nutrients as compared with the standard lymphocyte culture medium RPMI, including glucose. To understand if nutrients were a limiting factor for late mTOR activity, we measured glucose concentrations after 4 d in DMEM and RPMI in standard and low-density cultures (Fig. 6B). In standard high-density cultures, glucose levels were low or hardly detectable at day 4, whereas they were still in the physiological range in DMEM or in low-density cultures in RPMI. Thus, in standard high-density T cell cultures, glucose becomes a limiting factor. Glucose is thought to indirectly affect mTOR activity, and indeed when mTOR activity was assessed in DMEM with different glucose concentrations, we observed a partial inhibition of s6rp phosphorylation in low-glucose medium (Fig. 6C). Under low-glucose conditions, we also observed a significant upregulation of FOXP3 and strongly reduced IFN-γ production, whereas IL-17 and IL-22 were less affected (Fig. 6D). Reciprocally, when we added increasing concentrations of glucose to standard high-density cultures, we observed an increase of cytokine production at high glucose concentrations under both neutral and Th17 conditions, and again IFN-γ was most strongly affected (Fig. 6E). Notably, this glucose-induced IFN-γ production was...
IL-12 independent because it occurred in the absence of exogenous cytokines and in the presence of neutralizing anti–IL-12 and anti–IFN-γ Abs (Fig. 6E).

In summary, glucose is required for optimal mTOR activity and cytokine production, and in particular promotes Th1 differentiation in the absence of IL-12.

Amino acid–induced activity of mTOR, not of AHR, promotes IL-17 production

Although glucose availability clearly affected mTOR activity and cytokine production by uncommitted T cells, per se it could not explain the complete absence of mTOR activity and IL-17 production in standard high-density cultures. Amino acid availability is also important for Th17 cell generation (29, 30), and many amino acids were present at higher concentrations in DMEM as compared with RPMI (>4-fold concentrations of threonine, valine, phenylalanine, arginine and tyrosine; ≥3-fold concentrations of lysine, tryptophan, and glycine; and >2-fold concentrations of histidine, isoleucine, and leucine). Amino acids are directly sensed by mTOR and are imported by different amino acid transporters. The mTOR pathway regulates the induction of CD98, a shared component of several amino acid transporters (21). Consistently, CD98 expression was significantly higher in low-density cultures than in standard cultures and was inhibited by rapamycin (Fig. 7A). Thus, T cells in standard high-density cultures have a limited access to amino acids. Addition of a mixture of 20 aas enhanced s6rp phosphorylation, consistent with a critical role of amino acids in mTOR activity (Fig. 7B). In contrast, addition of fatty acids or glucose alone was not sufficient to activate mTOR but had an additive effect when given together with amino acids (Fig. 7B).

Aromatic amino acids in culture medium are metabolized to ligands of the transcription factor AHR, which in turn could promote IL-17 production by CD4+ T cells (30). Of note, the AHR ligand FICZ did not induce mTOR activity (Fig. 7C), indicating that effects of AHR on cytokine production are independent of mTOR. We therefore analyzed whether the effects of amino acids on cytokine production were mediated by the mTOR or the AHR pathway. To this end, we compared the effects of the AHR ligand FICZ and the amino acid mixture on FOXP3 and cytokine expression, and blocked mTOR or AHR in amino acid–supplemented cultures with rapamycin or an AHR antagonist, respectively. Addition of amino acids inhibited FOXP3 expression (Fig. 7D) and enhanced IL-17 (Fig. 7E) and IL-22 production (Fig. 7F), as expected. In marked contrast, the AHR ligand FICZ enhanced FOXP3 expression and failed to induce IL-17, but enhanced IL-22 production. Tryptophan, an aromatic amino acid that is metabolized to AHR ligands, also enhanced IL-22 production at high concentrations (10-fold of RPMI), whereas the non–aromatic amino acid Leucin did not (data not shown). Importantly, amino acid–induced FOXP3 downregulation and IL-17 production were completely blocked by rapamycin, indicating that the positive effect of amino acids on Th17 differentiation were indeed mTOR dependent. In contrast, the AHR antagonist had no effect on amino acid–induced FOXP3 downregulation or IL-17 induction, but completely blocked IL-22 production.

We conclude that the availability of amino acids is critical for sustained mTOR activity and for the generation of AHR ligands, and consequently for both Th17 and Th22 differentiation. However, whereas AHR activity is absolutely required for IL-22 production, IL-17 induction in uncommitted human T cells requires mTOR, but not AHR activity.

Discussion

Th17 and Th22 cells are recently identified Th cell subsets that have received a lot of attention because they are important in controlling specific pathogens and are implicated in organ-specific autoimmune diseases (1). It was shown that some
Th17 cells become already committed to the Th17 lineage in the thymus, that is, before they are released to the circulation as naive T cells. These “natural” Th17 cells are autoreactive (12), whereas pathogen-specific “induced” Th17 cells differentiate from uncommitted naive T cells in the periphery. However, the conditions that lead to the generation of induced human Th17 cells, which control pathogens like *C. albicans* and *S. aureus*, are poorly defined (4).

Precommitted human Th17 cells can be identified by CCR6 or CD161 surface expression (7, 15). However, although these cells represent precommitted naive T cells in cord blood, it was unclear if they are naive or CD45RA-expressing memory T cells in adults (15, 31). Recently, a subset of CD8+ TSCM with high expansion potential and low effector functions was identified, which can be discriminated from naive T cells by CD95 expression (24). We found that CD4+ TSCM were present at variable frequencies in discriminated from naive T cells by CD95 expression (24). We potential and low effector functions was identified, which can be (15, 31). Recently, a subset of CD8+ TSCM with high expansion conditions, as indicated (n = 5). (D) FOXP3 expression (n = 4) and production of (E) IL-17 under Th17 conditions or (F) IL-22 under neutral conditions (n ≥ 6) in standard high-density cultures by FICZ or amino acids (AA) in the absence or presence of rapamycin (Rapa) or an AHR antagonist (AHR Inh). *p < 0.05, **p < 0.005, ***p < 0.0005.

![Graphs showing CD98, P-s6rp, FOXP3, IL-17, and IL-22 expression](attachment:image.png)

**FIGURE 7.** Amino acid–derived AHR ligands are required for IL-22, but not for IL-17, production. (A) CD98 surface expression in standard and low-density cultures (1:10) in the absence and presence of rapamycin (n = 5). (B) s6rp phosphorylation on day 4 in standard high-density cultures in RPMI upon addition of glucose (Glc), a mixture of 20 aa (AA, concentrations as in RPMI), or a mixture of fatty acids (FA, concentrations as RPMI), alone or in combination. Relative increase of (C) s6rp phosphorylation (P-s6) in response to FICZ or amino acids in standard high-density cultures under neutral or Th17 conditions, as indicated (n = 5). (D) FOXP3 expression (n = 4) and production of (E) IL-17 under Th17 conditions or (F) IL-22 under neutral conditions (n ≥ 6) in standard high-density cultures by FICZ or amino acids (AA) in the absence or presence of rapamycin (Rapa) or an AHR antagonist (AHR Inh). *p < 0.05, **p < 0.005, ***p < 0.0005.

The cytokine requirement of human Th17 cell differentiation is a field of recent intense research and conflicting findings (5–10). Th17 differentiation of uncommitted T cells even under optimal signal strength conditions required a combination of several cytokines, and was optimal when all Th17-promoting cytokines were added together. In marked contrast, IL-22 and IFN-γ production by uncommitted T cells, as well as IL-17 production by precommitted T cells, occurred spontaneously but were further modulated by cytokines. This different regulation of IL-17 production by cytokines in uncommitted and precommitted T cells might explain some of the conflicting findings that were published on the role of individual cytokines in human Th17 cell differentiation.

In particular, TGF-β at low doses appears to be important to induce RORC in uncommitted T cells (6) but might be redundant in precommitted cells that already express RORC2 (7), or even inhibitory at high doses that induce Foxp3 (33).

Signals from cytokine receptors are integrated with those from the TCR and other cues for cell fate decisions. In particular, we have shown previously that the duration and strength of TCR stimulation regulate human T cell differentiation (17, 18). Low anti-CD3 concentrations were reported to favor IL-17 production by total CD4+ T cells (19), but the impact of signal strength on induced Th17 differentiation is unknown. In this article, we documented that uncommitted human T cells undergo a partial Th17 differentiation in response to an optimal combination of Th17-promoting cytokines in standard cultures because they upregulate RORC2, CCR6, and IL-23 responsiveness and secrete IL-21, but fail to produce IL-17, the hallmark of Th17 cells. This was unexpected because IL-17 production is thought to be controlled by RORC2 and precede IL-23R induction in mice, which in turn induces the stabilization of developing Th17 cells (1). Conversely, acquisition of IL-17–producing capacities had very high signal strength requirements in human T cells and occurred only upon prolonged TCR stimulation and sustained availability of nutrients. The latter were required to activate mTOR and to efficiently induce HIF-1α, a pathway that regulates the balance between Tregs and Th17 cells (22). Importantly, in uncommitted human T cells
mTOR activity was required exclusively at late time points (after 3 d) for IL-17 induction, when cells had already divided several times. Because developing Th17 cells in mice already produce IL-17 after 2 d (34), the requirement for late mTOR activity for IL-17 production appears to be unique for induced Th17 cell differentiation in humans.

Cellular starvation upon efficient clonal expansion could be easily avoided by plating cells at low densities. Alternatively, culture medium containing higher concentrations of salt (25) or nutrients (30) was proposed to promote IL-17 production. Given the exponential growth of TCR-activated T cells, a nutrient-rich culture medium was, however, insufficient in our hands to induce robust IL-17 production by uncommitted human T cell cultures cultured under standard high-density conditions. This failure could be explained by the fact that the concentrations of nutrient in DMEM are only 2- to 4-fold higher than in RPMI, whereas we varied cell density and therefore nutrient availability by factors of 10 or 100. In particular, low-density cultures allow stimulation of the TCR for very long periods (≤12 d) without inducing starvation and therefore constitute the only condition that leads to robust IL-17 induction in uncommitted human T cells. Notably, Stockinger and colleagues (30) analyzed murine and total CD4+ human T cells, and attributed the Th17-promoting effect of nutrients exclusively to aromatic amino acid–derived AHR ligands. We showed in this article that although AHR ligands were absolutely required for IL-22, they were dispensable for IL-17 induction. This apparently conflicting result might reflect differences between T cell differentiation in humans and mice (35) or, alternatively, different roles for AHR in IL-17 production by uncommitted and precommitted T cells. In any case, our results indicate that high-density cultures, which are the standard condition used by most researchers (26) and are permissible for IL-12–induced Th1 differentiation, are not suited to cope with the high nutrient demands of T cells undergoing rapid polyclonal expansion. Thus, we propose that induced human Th17 differentiation should be performed at permissive cell densities, whereas medium containing higher concentrations of nutrients or salt per se are insufficient but can enhance the response under low-density conditions. In addition, our results illustrate that several nutrients, including amino acids and glucose, contribute to induced Th17 differentiation. Finally, we also documented specific roles for individual nutrients because glucose preferentially promoted IFN-γ production, whereas tryptophan selectively induced IL-22.

In summary, we identified precommitted Th17SCM cells that produce IL-17 ex vivo and rapidly upregulate IL-17 production in a rapamycin-resistant manner, indicating that they are a previously unrecognized confounding factor in T cell differentiation studies. Furthermore, we demonstrated that uncommitted T cells require sustained TCR stimulation and nutrient supply to acquire IL-17–producing capacities in response to Th17-promoting cytokines, and we identified the late, nutrient-dependent activity of the mTOR pathway as the critical checkpoint in humans. These findings have important implications for future T cell differentiation studies and for the use of mTOR inhibitors in patients.

Disclosures
The authors have no financial conflicts of interest.

References


