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TGF-β indirectly favors the development of human Th17 cells by inhibiting Th1 cells

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Human Th17 clones and circulating Th17 cells showed lower susceptibility to the antiproliferative effect of TGF-β than Th1 and Th2 clones or circulating Th1-oriented T cells, respectively. Accordingly, human Th17 cells exhibited lower expression of clusterin, and higher Bcl-2 expression and reduced apoptosis in the presence of TGF-β, in comparison with Th1 cells. Umbilical cord blood naïve CD161$^+$CD4$^+$ T cells, which contain the precursors of human Th17 cells, differentiated into IL-17A-producing cells only in response to IL-1β plus IL-23, even in serum-free cultures. TGF-β had no effect on constitutive RORγt expression by umbilical cord blood CD161$^+$ T cells but it increased the relative proportions of CD161$^+$ T cells differentiating into Th17 cells in response to IL-1β plus IL-23, whereas under the same conditions it inhibited both T-bet expression and Th1 development. These data suggest that TGF-β is not critical for the differentiation of human Th17 cells, but indirectly favors their expansion because Th17 cells are poorly susceptible to its suppressive effects.

Key words: CD161 · RORγt · TGF-β · Th17

Introduction

The role of TGF-β in the differentiation and function of human Th17 cells is still controversial. Although many authors agree that murine Th17 cells originate from naïve CD4$^+$T cells in the presence of IL-6 and TGF-β, and their development is then stabilized and/or amplified by IL-23 and IL-21 [1–4], several studies have denied the role of TGF-β in human Th17-cell differentiation [5–10]. Acosta-Rodriguez *et al.* [5] found that human Th17 cells originate in response to the combined activity of IL-1β and IL-6, whereas Wilson *et al.* [6] found that the activity of IL-1β or IL-23 alone was critical, the combined activity of the two cytokines exerting no additive or synergistic effect [6]. In another study, IL-1β and IL-6 upregulated RORγt expression, but they did not induce Th17 differentiation from human adult naïve CD4$^+$ T cells, whereas IL-23 was a powerful upregulator of its own receptor and an important inducer of IL-17A and IL-22 [7]. In some human studies, the addition of TGF-β to human naïve or memory CD4$^+$ T cells was even found to be inhibitory on the development of Th17 cells [5, 6, 8]. These differences between humans and mice and the discrepancies among different studies in humans were attributed to the difficulty to ensure a truly naïve T-cell population in these latter [9]. Accordingly, van Beelen *et al.* [10] found that the combined activity of IL-1α or IL-1β and IL-23 was required for the enhancement of IL-17A-producing human memory T cells, whereas under these or even other conditions, the differentiation of human naïve T cells from adult subjects into Th17 cells could not be achieved [10].

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Recently, however, three independent studies [11–13] showed that, in contrast to the previous observations [5–8], even the differentiation of human Th17 cells requires the activity of TGF-β. All these authors [11–13] suggested that in previous studies performed in humans [5–8], the role of TGF-β had been underscored because $CD4^+$ T cells were cultured in media comprising human or bovine serum, which usually contains TGF-β.

More recently, we provided evidence that human memory Th17 cells consistently express on their surface CD161 [14], which is the human homologue of the murine NK1.1 [15]. The human $CD161^+$ Th17 cells were not CD1-restricted NKT cells [14], but classic $CD4^+$ T cells showed a broad T-cell receptor repertoire and MHC Class-II restriction, known as NKT-like cells [16]. More importantly, we also showed that human IL-17A-producing cells exclusively originated from $CD161^+$ $CD4^+$ T-cell precursors detectable in both umbilical cord blood (UCB) and thymus, when these cells were polyclonally activated in the presence of a combination of IL-1β plus IL-23 (that could also induce the development of Th1 cells) [14]. No other cytokine or cytokine combinations (including TGF-β, IL-6 and IL-21) were able to induce IL-17A mRNA expression and IL-17A production. On the other hand, $CD161^-$ $CD4^+$ T cells from both UCB and thymus could be induced to differentiate into Th1 cells under appropriate polarizing conditions (IL-1β plus IL-23 or IL-12), but they did not differentiate into IL-17A-producing cells [14]. Because these experiments were performed by culturing UCB or thymic cells in FBS-containing medium, the possibility that TGF-β eventually present in the serum may have biased our results should be considered. However naïve $CD161^+$, but not $CD161^-$, $CD4^+$ T cells from both UCB and thymus were found to express constitutively RORγt and IL-23 receptor (IL-23R) even before their culturing [14], suggesting that RORγt expression by these cells was not dependent upon exogenously added TGF-β.

In the present study, we first evaluated the effect of TGF-β on the proliferation of human Th17 clones and of circulating IL-17A-producing memory $CD4^+$ T cells. Both Th17 clones and circulating IL-17A-producing $CD4^+$ T cells showed markedly lower susceptibility to the anti-proliferative effect of TGF-β than Th1 and Th2 clones or circulating memory $CD4^+$ T cells producing IFN-γ, respectively. The different susceptibility between Th1 and Th17 cells to the suppressive activity of TGF-β could be related to the observation that mRNA levels for clusterin (Clu), a molecule related to the TGF-β-dependent apoptosis [17], were lower, whereas mRNA levels of Bcl-2, a factor involved in the resistance to TGF-β-mediated apoptosis [18], were higher, in Th17 than in Th1 cells. Accordingly, human UCB $CD161^+$ $CD4^+$ T cells did not differentiate into IL-17A-producing cells in response to TGF-β alone and their differentiation into Th17 cells occurred only in response to IL-1β plus IL-23, even when these cells were cultured in serum-free medium. By contrast, under the same conditions, TGF-β strongly inhibited T-bet expression in $CD161^+$ $CD4^+$ UCB T cells, as well their development into Th1 cells. These data support the concept that TGF-β does not play a direct role in human Th17-cell differentiation, but only indirectly favors the expansion of human Th17 cells because these latter are poorly susceptible to the suppressive activity of TGF-β.

Results

Proliferation of human Th17 cells is less inhibited by the suppressive activity of TGF-β compared with Th1 or Th2 cells

The effect of TGF-β on the proliferation of a panel of human Th17, Th1 and Th2 clones stimulated by a combination of anti-CD3 and anti-CD28 mAb was assessed by measuring ^{3}H-thymidine uptake after 5 day-culture. As shown in Fig. 1A, the proliferation of both Th1 and Th2 clones was virtually abrogated by TGF-β, whereas the proliferation of Th17 clones was poorly affected. We then asked whether the different response to TGF-β of Th17 compared with Th1 or Th2 clones resulted from an artifact introduced by the cloning procedure or it was also shared by circulating Th cells. To this end, CFSE-labeled circulating $CD4^+$ T cells from healthy subjects were stimulated with anti-CD3/CD28 mAb and their proliferative activity, as well as their ability to produce different cytokines in the absence or presence of TGF-β, was measured. As shown in Fig. 1B, the inhibition of cell proliferation exerted by TGF-β was significantly higher on IFN-γ-producing cells than on IL-17A-producing cells accordingly, virtually all proliferating T cells in cultures containing TGF-β appeared to belong to the IL-17A-producing population, whereas IFN-γ-producing cells were strongly suppressed (Fig. 1B and C). These data suggest that the proliferation of human Th17 clones, as well as of circulating IL-17A-producing $CD4^+$ T cells, is less susceptible to the suppressive activity of TGF-β than the proliferation of Th1 or Th2 cells.

Human Th17 cells exhibit reduced expression of Clu, increased Bcl-2 expression and lower TGF-β-induced apoptosis compared with Th1 cells

To investigate the mechanisms possibly responsible for the reduced susceptibility of Th17 cells to the suppressive activity of TGF-β, we took advantage from the results of the microarray analysis performed on a panel of human Th1, Th2 and Th17 clones, as reported in our previous study [14]. Among the genes analyzed, *CLU* appeared to be downregulated, whereas *Bcl-2* was upregulated in Th17 compared with both Th1 and Th2 clones (microarray data accession number GSE11553). Therefore, mRNA levels of Clu and Bcl-2 were measured in a panel of eight Th1 and eight Th17 clones by using quantitative RT-PCR. The results of these experiments are summarized in Fig. 2A. The expression of Clu appeared to be significantly lower, whereas Bcl-2 expression was significantly higher, in Th17 than in Th1 clones. These findings may explain why Th17 cells exhibit lower susceptibility to the inhibitory activity of TGF-β, because of their reduced transduction of TGF-β signaling and/or increased

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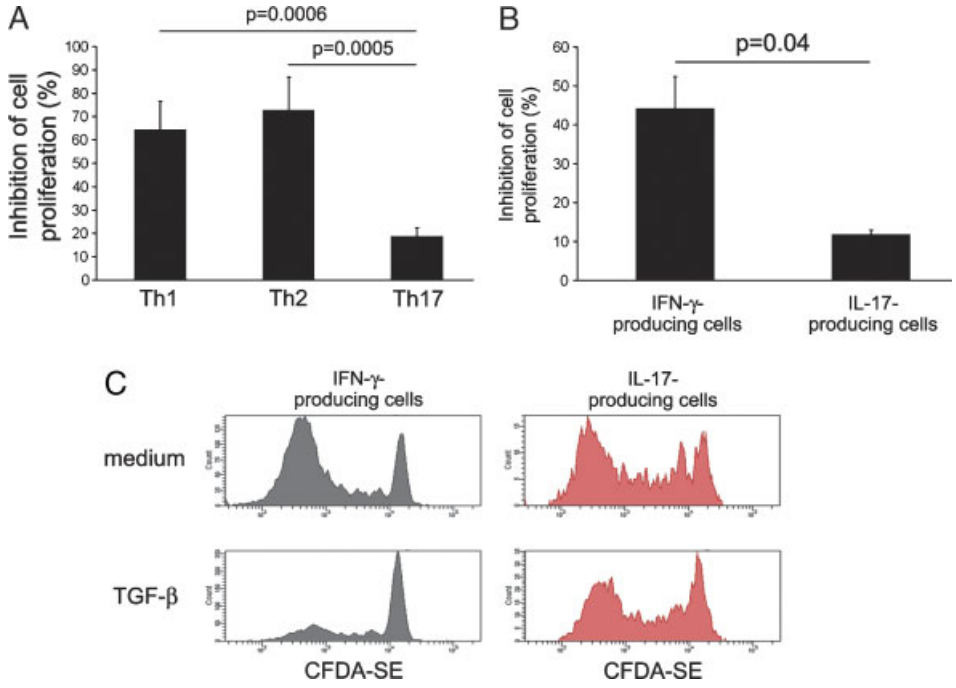

Figure 1. Poor susceptibility of Th17 clones and circulating IL-17A-producing cells to the suppressive activity of TGF-β on their proliferation. (A) Effect of TGF-β (5 ng/mL) on the proliferative response of human Th1, Th2 and Th17 clones stimulated with anti-CD3 plus anti-CD28 mAb. Results are expressed as percent inhibition of T-cell blast proliferation after 5 days, as measured by ^{3}H-thymidine incorporation. Mean values $\pm$ SE obtained in five Th1, Th2 or Th17 clones are reported. A standard two-tailed unpaired *t*-test was used for statistical analysis. (B) Effect of TGF-β (5 ng/mL) on the proliferation of circulating IFN-γ or IL-17-producing cells: CFDA-SE-labeled PB CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD28 mAb. On day 5, cells were stimulated by PMA plus ionomycin and cytokines evaluated by flow cytometry; IFN-γ- or IL-17-producing cells were selected and their CFDA-SE cell content analyzed. Results are expressed as percent inhibition of T-cell proliferation. Mean values $\pm$ SE obtained from three different healthy donors are reported. A standard two-tailed paired *t*-test was used for statistical analysis. (C) Representative histograms of results from part B.

resistance to apoptotic stimuli. In order to support the latter possibility, three Th1- and three Th17-cell clones were stimulated with anti-CD3 plus anti-CD28 mAb in the presence or in the absence of TGF-β and analyzed at different time points for annexin V and/or propidium iodide positivity by flow cytometry. As shown in Fig. 2B, Th17 clones showed lower susceptibility than Th1 clones to the TGF-β-induced apoptosis at either 48 or 72 h after *in vitro* stimulation.

TGF-β has no direct effect on the differentiation of Th17 cells from their precursors, but indirectly favors Th17 development by inhibiting Th1 cells

In order to ascertain the role of TGF-β on human Th17-cell differentiation, we first measured T-bet and RORγt expression in freshly isolated UCB CD161$^+$ CD4$^+$ T cells. In agreement with the results of our previous report [14], freshly derived CD161$^+$ T cells were found to express detectable levels of RORγt (data not shown). Therefore, the CD161$^+$ T-cell fraction was stimulated for 7 days with anti-CD3/CD28 mAb in the absence or presence of TGF-β alone, IL-1β plus IL-23, or IL-1β and IL-23 plus TGF-β, in either serum-containing or serum-free cultures. The expression of T-bet and RORγt was then measured on cells from different cultures and the ability of the same cells to produce IL-17 and IFN-γ following their stimulation with PMA plus ionomycin was assessed by flow cytometry. T-bet expression was significantly suppressed in the presence of TGF-β, whereas RORγt expression remained unchanged (Fig. 3A). The addition of IL-1β plus IL-23 significantly enhanced both T-bet and RORγt expression, but T-bet expression was significantly reduced, whereas the expression of RORγt was unchanged, when TGF-β was also added (Fig. 3A). In either serum-containing or serum-free cultures, the addition of TGF-β alone reduced the expression of IFN-γ, but it did not affect that of IL-17A (Fig. 3B). Only the addition of IL-1β plus IL-23, besides enhancing the numbers of IFN-γ-producing T cells, significantly increased the proportion of cells able to produce IL-17A and this effect was not inhibited in serum-containing cultures by the addition of an anti-TGF-β Ab (Fig. 3B). Notably, when TGF-β was added together with IL-1β and IL-23, the proportions of IFN-γ-producing cells were strongly reduced, whereas those of IL-17A-producing cells were enhanced, even if not significantly (Fig. 3B). Figure 3C shows the flow-cytometric analysis of IFN-γ and IL-17-producing cells in a representative experiment obtained in serum-free medium under the indicated *in vitro* culture conditions. Because it has been recently shown in humans that the ability of TGF-β to induce Th17 development is dependent on its concentration, high doses

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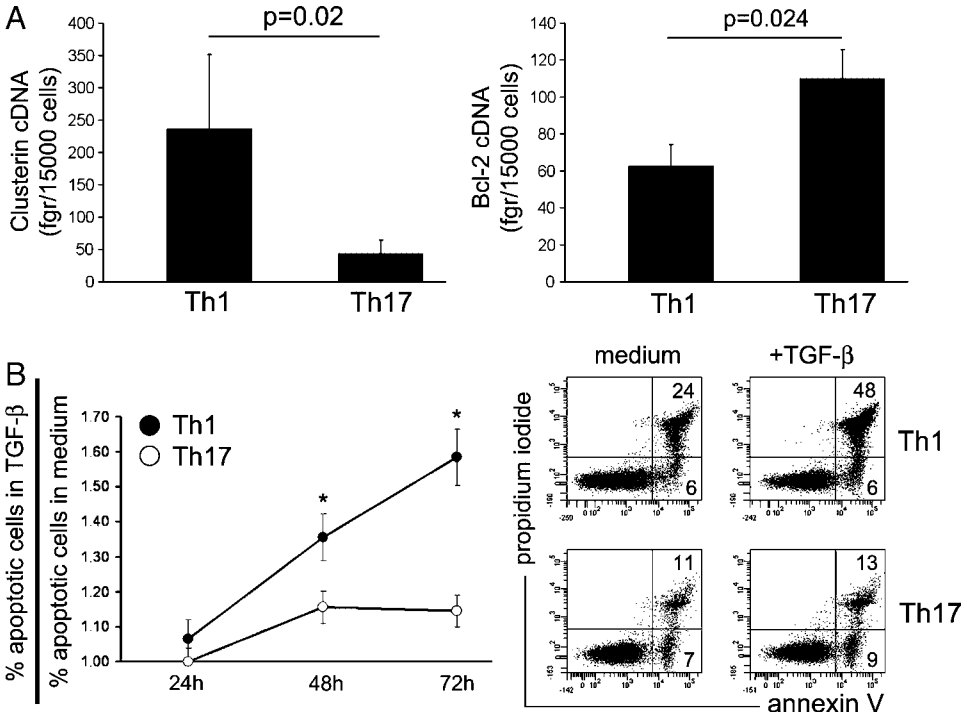

Figure 2. Human Th17 cells exhibit reduced expression of Clu, increased Bcl-2 expression and lower TGF-β-induced apoptosis compared with Th1 cells. (A) Levels of mRNA for Clu and Bcl-2 in Th17 and Th1 human clones, as measured by real-time quantitative RT-PCR. Mean values $\pm$ SE obtained on eight different Th17 and eight different Th1 clones are represented. (B) Th1 (closed circles) and Th17 (open circles) clones were stimulated with anti-CD3 plus anti-CD28 in the absence or presence of TGF-β and analyzed at different time points for annexin V binding and propidium iodide uptake. Mean values $\pm$ SE obtained in three separate experiments (three Th1 and three Th17 clones) are presented. A standard two-tailed unpaired *t*-test was used for statistical analysis. $^*p<0.05$ (Th1 *versus* Th17 clones). On the right, a representative experiment showing annexin V binding and propidium iodide uptake at 72 h by one Th1 and one Th17 clone in the absence or presence of TGF-β is shown.

of the cytokine being suppressive [11], $CD161^+$ T cells were stimulated for 7 days with anti-CD3/CD28 mAb in serum-free medium in the absence or presence of IL-1β plus IL-23, or of IL-1β and IL-23 plus scalar concentrations of TGF-β (0.1, 0.5, 1 and 5 ng/mL), or of scalar concentrations of TGF-β alone. No differences in Th17 development were observed even in the presence of different concentrations of TGF-β, whereas the lowest TGF-β concentration showed reduced inhibitory effect on the development of IFN-γ-producing cells (data not shown).

Counting of cells at the end of the culture period revealed a dramatic reduction in their number in cultures containing TGF-β (Fig. 3D). By contrast, in cultures containing IL-1β plus IL-23 the addition of TGF-β induced a lower reduction in total cell numbers (Fig. 3D), suggesting that the lack of susceptibility of IL-17A-producing cells to the suppressive activity of TGF-β at least partially counterbalanced the loss of cultured cells.

Discussion

The results of this study provide new information that may reconcile different and even contrasting results on the origin of human Th17 cells and on the mechanisms responsible for their development. Initial studies in humans provided evidence that TGF-β is not critical for human Th17-cell differentiation [5–7], as it has been demonstrated in mice [1–4]. More recently, however, the requirement for TGF-β even for the differentiation of human Th17 cells has been reported [11–13], the apparent lack of TGF-β need for human Th17-cell differentiation in the previous studies [5–7] being explained by the fact that naïve $CD4^+$ T cells were cultured in media comprising human or bovine serum, which may contain small amounts of TGF-β. Indeed, culturing of human naïve $CD4^+$ T cells in serum-free medium demonstrated the requirement of exogenously added TGF-β to get human Th17-cell differentiation [11–13]. However, more recently, we provided evidence that both human Th17 clones and circulating memory Th17 cells consistently express on their surface CD161 [14], which is the human homologue of the murine NK1.1 [15]. More importantly, we showed that human IL-17A-producing cells can exclusively originate from a small population of $CD161^+CD4^+$ T-cell precursors present in either UCB or thymus. Sorting of $CD161^+CD4^+$ T cells from UCB or thymus allowed the enrichment of a cell population that constitutively expressed RORγt and IL-23R, but not IL-17, mRNA [14]. When purified UCB $CD161^+CD4^+$ T cells were polyclonally activated in the presence of a combination of IL-1β plus IL-23 (a condition that could also induce the development of Th1 cells), remarkable numbers of IL-17A-producing cells became detectable in culture. No other

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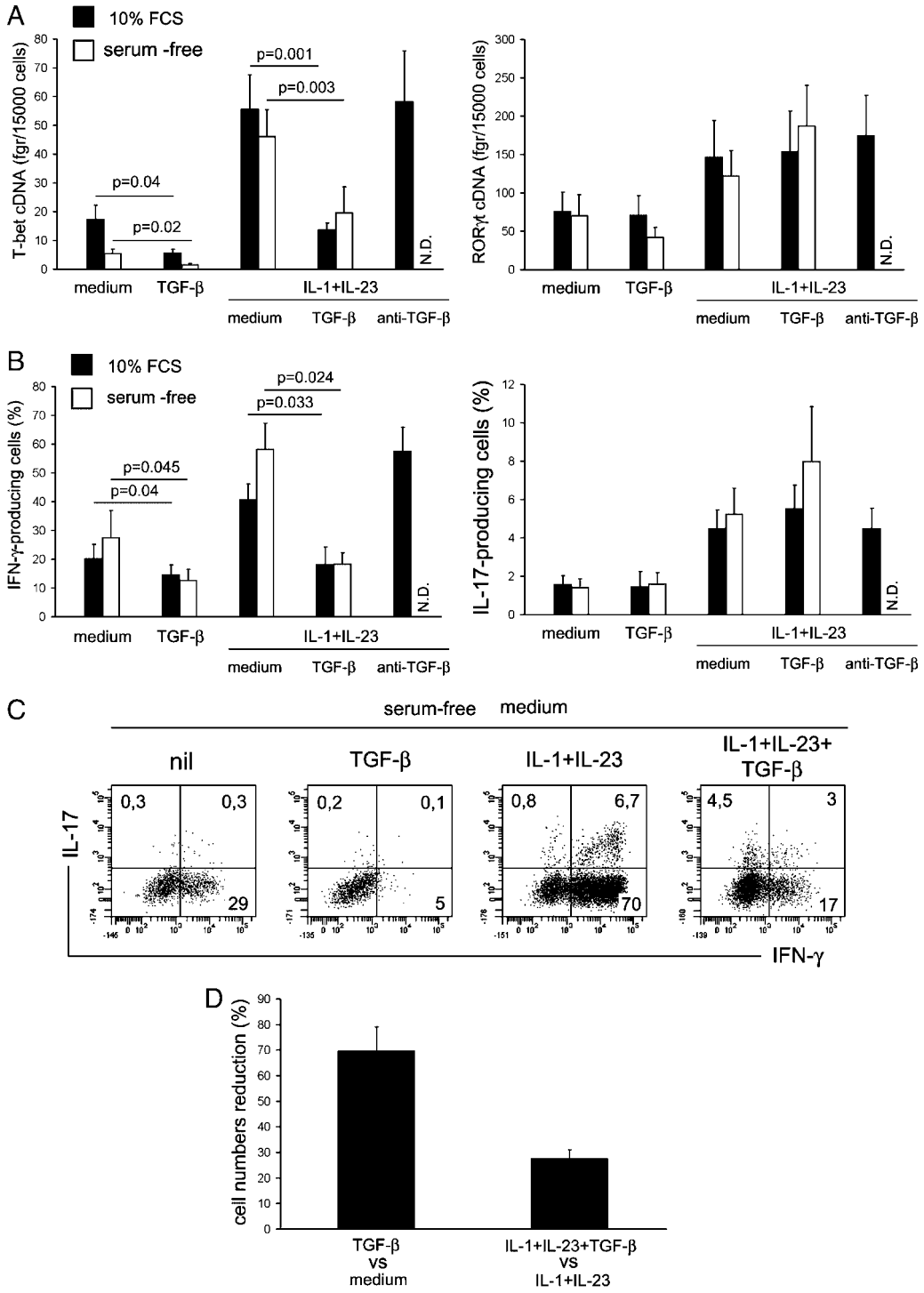

Figure 3. TGF-β is not required to induce the differentiation of UCB CD4$^+$CD161$^+$ Th17 precursors into IL-17-producing cells, but indirectly favors their development by suppressing T-bet expression and Th1 expansion (A) Levels of mRNA for T-bet and RORγt were measured on sorted naïve UCB CD161$^+$ CD4$^+$ T cells, stimulated by anti-CD3 plus anti-CD28 mAb for 7 days in the absence (medium) or in the presence of different cytokines, without or with the addition of a neutralizing polyclonal Ab against TGF-β. Mean values$\pm$SE obtained from five different healthy donors are reported. (B) The frequency of IFN-γ- and IL-17-producing cells was evaluated by flow cytometry on cells collected from the above mentioned cultures (see A), following stimulation for 5 h with PMA plus ionomycin in the presence of brefeldin A. Mean values$\pm$SE obtained from five different healthy donors are reported. (C) A representative flow-cytometric analysis of the above experiments performed in serum-free medium (see A and B) is depicted. (D) Naïve UCB CD4$^+$CD161$^+$ T cells were stimulated by anti-CD3 plus anti-CD28 mAb and cultured in the absence (medium) or in the presence of different cytokines without or with TGF-β addition. After 7 days, culture cells were harvested and counted by microscope. Values represent the percent of reduction in cell numbers in cultures containing TGF-β. Mean values$\pm$SE obtained from four different healthy donors are reported, N.D. = not done. For statistical analysis, a standard two-tailed paired *t*-test was used.

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cytokine or cytokine combinations, including TGF-β, IL-6 and IL-21, enabled CD161$^+$ cells to express IL-17A mRNA and to produce IL-17A [14].

In the present study, we could reconfirm that human UCB naïve CD161$^+$CD4$^+$ T cells express RORγt (and also IL-23R) before their culturing, suggesting that the expression of the Th17-driving transcription factor by Th17 precursors is constitutive and does not require signaling by TGF-β, possibly present in the culture serum or exogenously added *in vitro*. Obviously, we cannot exclude that RORγt expression by these cells before their culturing is due to the action exerted by TGF-β *in vivo*. However, our results are at variance with those of recent studies showing that the presence of TGF-β is needed *in vitro*, because of its ability to induce the expression of RORγt [11] or that a combination of TGF-β and IL-21 is critical for the expression of RORC2 (the human homologue of mouse RORγt) [13]. The fact that the *in vitro* presence of TGF-β is not required for the development of IL-17A-producing cells was also supported by the observation that the addition in serum-containing cultures of an anti-TGF-β Ab did not have any inhibitory effect on the IL-1β/IL-23-induced development of IL-17A-producing cells. Finally and most importantly, the development of IL-17A-producing cells induced by IL-1β plus IL-23 could be observed even in serum-free cultures in the absence of exogenously added TGF-β. The reason why TGF-β was found to be necessary in other studies using CD4$^+$CD45RA$^+$CD45RO$^-$ T cells purified from PBMC of adult subjects [11–13] or of UCB [12, 13] cultured in serum-free medium are presently unclear. One important difference between the present study and the others [11–13] is that we made use of the enriched population of CD161$^+$ Th17 precursors already expressing RORγt, that is not detectable when the whole population of naïve CD4$^+$ T cells is assessed, because it is mainly composed of CD161$^-$RORγt$^-$ cells. Indeed, neither RORγt expression, nor IL-17A-producing cells, could be detected under any condition in serum-free cultures of CD161$^-$ cells [14]. In agreement with the results previously reported [14], the addition in culture of IL-1β plus IL-23 not only induced the expansion of Th17 cells but also a significant increase in T-bet expression, as well as of the proportions of IFN-γ-producing cells in the CD161$^+$ fraction. However, when TGF-β was added in culture together with IL-1β plus IL-23, both T-bet expression and the proportions of IFN-γ-producing cells were significantly decreased. Notably, also in the studies of both Volpe *et al*. [12] and Yang *et al*. [13], the induction of IL-17A by TGF-β associated with a strong decrease in IFN-γ production. Thus, it is reasonable to suggest that the addition of TGF-β to unfractionated naïve CD4$^+$ T cells can result in an even greater positive selection of the already existing RORγt-expressing T cells, as well as in the relative expansion of IL-17A-producing cells induced by IL-1β plus IL-23.

The results of this study suggest that the effect of TGF-β on the IL-1β/IL-23-induced development of Th17 and Th1 cells can be due to the much lower susceptibility of Th17 than Th1 and Th2 cells to its suppressive activity, as shown by the poor inhibitory effect of TGF-β on the proliferation of either human Th17 clones or IL-17A-producing circulating CD4$^+$ T cells when compared with the strong inhibition exerted on the proliferation of both Th1 and Th2 clones, as well as of IFN-γ-producing circulating CD4$^+$ T cells. The reasons for this difference are presently unclear. However, in this study, we also provided evidence that Th17 cells have lower expression of Clu, and higher expression of Bcl-2, than Th1 clones. Clu is a ubiquitously expressed glycoprotein that has been implicated in a variety of physiological processes, including cell–cell interaction, lipid transport, tissue remodeling, chaperon activity and apoptosis [19, 20]. Recently, it has been shown that Clu is also a modulator of TGF-β signaling, inasmuch as it is involved in the nuclear translocation of Smad2/3 [17], which is critical for the transduction of the TGF-β signaling [21]. On the other hand, Bcl-2 is essential for cell survival [18]. These findings suggest that Th17 cells may have reduced transduction of TGF-β signaling and/or increased resistance to apoptotic stimuli when compared with Th1 cells. The latter possibility was supported by the observation that when Th17 clones were stimulated in the presence of TGF-β, they exhibited much lower susceptibility to apoptosis than Th1-cell clones. Obviously, however, our findings are not conclusive and the exact mechanisms responsible for the different susceptibility to TGF-β of human Th17 and Th1 cells still remain to be established.

In conclusion, the results of this study support the concept that TGF-β has no a direct critical role on the differentiation of human IL-17A-producing cells. This may be in keeping with a recent study on humans showing that patients with mutations of *TGFB1* or *TGFBR2* did not exhibit any difference in the numbers of IL-17A-producing T cells compared with healthy controls, whereas patients with *STAT3* and *IL-12Rβ1* mutations have impaired Th17 development [22]. Our data rather indicate that TGF-β can indirectly favor the development of IL-17A-producing cells by suppressing the expression of T-bet and selectively inhibiting the expansion of IFN-γ-producing T cells (Fig. 4). It has been suggested that if the mechanisms responsible for the differentiation of Th17 cells in mice and humans are different, this raises the serious issue that mice may be of limited use as models for the development of such cells in the human immune system [23], a statement that has been considered as unfortunate and unhelpful to the biomedical research community [24]. Although this remark is fully acceptable and has to be shared, the results of this study strongly support the possibility that the origin of IL-17A-producing cells in mice and humans is really different.

Materials and methods

Subjects

UCB samples were obtained from ten donors. Peripheral blood (PB) samples were obtained from four healthy volunteers. The procedures and all the experiments of the study were in

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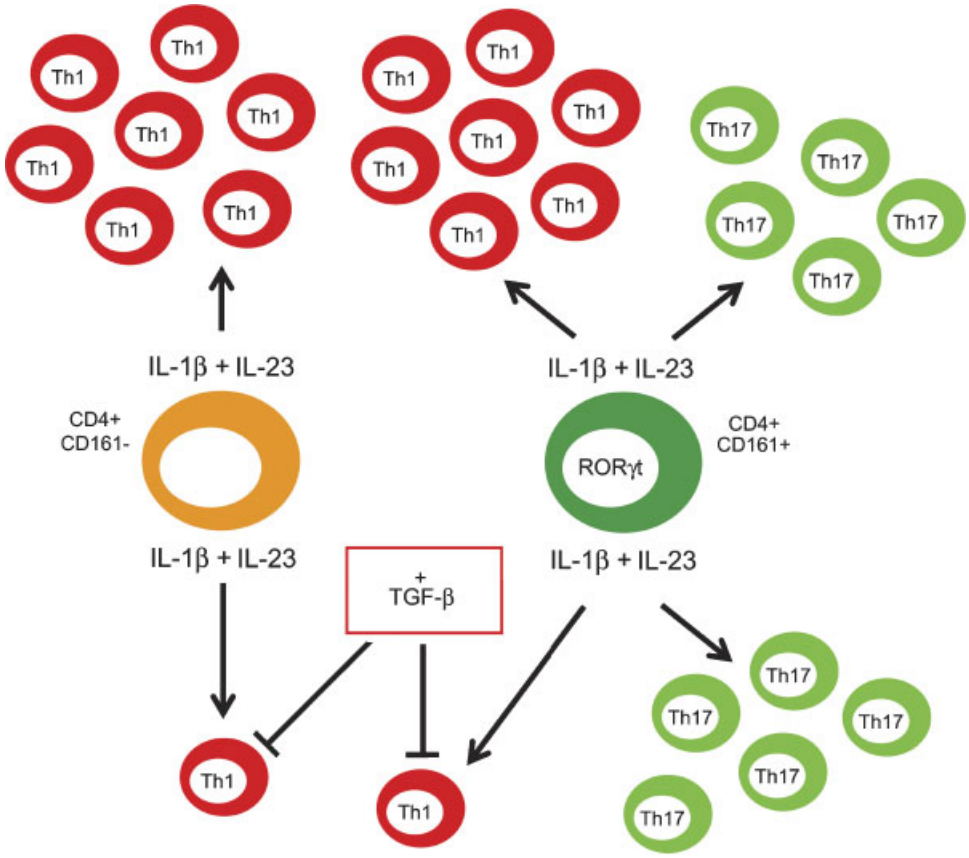

Figure 4. Hypothetical model illustrating the effect of TGF-β on human Th17-cell differentiation. UCB naïve $CD4^+CD161^+$ T cells, constitutively express RORγt, can be induced to differentiate to either Th1 or Th17 cells in response to IL-1β plus IL-23, whereas under the same conditions $CD4^+CD161^-$ T cells can only differentiate into Th1 cells. The contemporaneous presence of TGF-β inhibits the IL-1β/Il-23-induced expansion of Th1 cells without any suppressive effect on Th17 cells, thus indirectly favoring their development.

accordance to the ethical standards of and approved by the Regional Committee on Human Experimentation.

Reagents

The culture media used were (i) RPMI 1640 (Seromed) supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 2×10^{-5} M 2-ME (all from Invitrogen, Paisley, UK) and 10% FBS HyClone (Gibco Laboratories, Grand Island, NY, USA) or alternatively (ii) X VIVO-15 (Cambrex, Profarmaco Milano SRL Milan Italy). Unlabeled or fluorochrome-conjugated anti-CD3, -CD4, -CD8, -CD28, -CD161, -IFN-γ, -IL-4 and isotype-matched control mAb were purchased from BDBiosciences (San Jose, CA, USA). The fluorochrome-conjugated anti-IL-17 mAb was obtained from eBioscience (San Diego, CA, USA). The neutralizing mAb anti-TGF-β1 (10 μg/mL) was purchased from RD systems (Minneapolis, MN, USA). PMA, ionomycin and brefeldin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The following cytokines: IL-1β, IL-23 and TGF-β were purchased from R&D systems. The 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) was from Invitrogen.

$CD4^+CD161^+$ T-cell recovery and expansion

PBMC suspensions were obtained from UCB by centrifugation on Ficoll-Hypaque gradient. Isolation of $CD4^+$ T cells from UCB was performed by using the CD4 isolation kit II (Miltenyi Biotec, Bergisch Gladbach). Purified UCB-derived $CD4^+$ cells were further divided into $CD161^+$ and $CD161^-$ cells by an anti-CD161-PE (or -APC) mAb, followed by a anti-PE-(or -APC)-microbeads mAb (Miltenyi Biotec). Purified UCB $CD4^+CD161^+$ T cells were stimulated for 7 days with anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL), mAb in the absence or presence of IL-1β (10 ng/mL) plus IL-23 (20 ng/mL), TGF-β (5 ng/mL) or combinations of them. On day 7, T cells were analyzed by quantitative RT-PCR for genes expression and stimulated for intracellular cytokines detection.

T-cell cloning procedure

T-cell clones were generated from PBMC of adult healthy donors and categorized into Th1, Th2 and Th17, as reported previously [25]. Briefly, $CD4^+$ cells were seeded under limiting-dilution conditions (0.5 cell/well) in round-bottom microwell plates (Nunc), containing 10^5 irradiated (9000 rad) allogeneic PBMC

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as feeder cells, 1% PHA (Gibco), and rIL-2 (50 U/mL). Growing microcultures were then supplemented at weekly intervals with IL-2 (50 U/mL) and 10^5 irradiated allogeneic PBMC as feeder cells. Recovered $CD4^+$ T-cell clones were then classified into Th1, Th2 or Th17, on the basis of their ability to produce IFN-γ, IL-4 or IL-17, respectively, as assessed by single-cell flow cytometry following polyclonal stimulation.

Labeling and polyclonal stimulation of PB $CD4^+$ T cells with CFDA-SE

PBMC suspensions were obtained from PB by centrifugation on Ficoll-Hypaque gradient. Isolation of $CD4^+$ T cells from PBMC was performed by using the CD4 isolation kit II (Miltenyi Biotec). Labeling of PB $CD4^+$ T cells with CFDA-SE was performed as described [25]. Briefly, cells were extensively washed and resuspended at final concentration of 10^7/mL in PBS. CFDA-SE was added at a final concentration of 5 μM and incubated for 4 min at room temperature. The reaction was stopped by cells washing with RPMI 1640, containing 10% heat-inactivated FBS. CFDA-SE-labeled PB $CD4^+$ T cells were stimulated in RPMI medium containing 10% FBS serum with anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) mAb. The cells were monitored on day 5 for CFDA-SE content and cytokines production. Briefly, 1×10^6 cells were stimulated with PMA (10 ng/mL) plus ionomycin (1 μM) for 6 h, the last four of which in the presence of brefeldin A (5 μg/mL). After stimulation, cells were washed twice with PBS pH 7.2, fixed 15 min with formaldehyde (2% in PBS pH 7.2), washed twice with 0.5% BSA in PBS pH 7.2, permeabilized with PBS pH 7.2 containing 0.5% BSA and 0.5% saponin, and then incubated for 15 min at room temperature with the specific mAb. Cells were then washed and analyzed on a BDLSR II cytofluorimeter using the FACSDiva software (Becton Dickinson). The area of positivity was determined using an isotype-matched mAb, a total of 10^4 events for each sample were acquired.

Assessment of apoptosis

The assessment of apoptosis in Th1 and Th17 cell clones was performed using the annexin V kit (BD Biosciences). To this end, cells were stimulated with anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) mAb in the absence or presence of TGF-β (5 ng/mL), harvested from cultures at 24, 48 and 72 h, and then evaluated for annexin V binding and propidium iodide uptake, accordingly to the manufacturer's instructions. Samples were analyzed on a BDLSR cytofluorimeter (BD Biosciences) using the FACSDiva software.

RNA isolation, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted by using the RNeasy Micro kit (QIAGEN) and treated with DNase I to eliminate possible genomic DNA contamination. Taq-Man RT-PCR was performed, as described elsewhere [25]. Primers and probes used were purchased from Applied Biosystems.

Statistics

A standard two-tailed paired or unpaired *t*-test was used for statistical analysis; *p* values of 0.05 or less were considered significant.

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Abbreviations: **CFDA-SE:** 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester · **Clu:** clusterin · **PB:** peripheral blood · **UCB:** umbilical cord blood

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