Human RORγt$^+$ T_H17 cells preferentially differentiate from naive FOXP3$^+$Treg in the presence of lineage-specific polarizing factors

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RORγt$^+$ T_H17 cells are a proinflammatory CD4$^+$ T-cell population associated with autoimmune tissue injury. In mice, priming of T_H17 requires TGF-β, which alone directs the priming of FOXP3$^+$ regulatory T cells (Treg), in association with inflammatory cytokines. Priming of human T_H17 cells from conventional naive CD4$^+$ T cells under similar conditions, however, has proved difficult to achieve. Here, we report that differentiation of human T_H17 cells preferentially occurs from FOXP3$^+$ naive Treg (NTreg) in the presence of IL-2 and IL-1β and is increased by IL-23 and TGF-β. IL-1β–mediated differentiation correlated with IL-1RI expression in stimulated NTreg and was accompanied by induction of RORγt along with down-regulation of FOXP3. IL-17–secreting cells in NTreg cultures cosecreted TNF-α and IL-2 and contained distinct subpopulations cosecreting or not cosecreting IFN-γ and other T_H17-associated cytokines. Polarized NTreg contained significant subpopulations of CCR6-expressing cells that were highly enriched in IL-17–secreting cells. Finally, analysis of CCR6 expression with respect to that of IL-1RI identified distinct IL-17–secreting subpopulations that had maintained or lost their suppressive functions. Together our results support the concept that priming of human T_H17 from naive CD4$^+$ T cells preferentially takes place from FOXP3$^+$ Treg precursors in the presence of lineage-specific polarizing factors.

IL-1 | IL-17 | IL-23 | TGF-β | CD4 T cells

Recent studies have identified T helper 17 (T_H17) cells as a distinct lineage of CD4$^+$ effector T cells producing the proinflammatory cytokine IL-17A (hereafter IL-17), leading to chemokine production and recruitment of neutrophils to inflamed tissues (1). In mice, T_H17 cells have been shown to be involved in the pathogenesis of experimental autoimmune diseases previously attributed to unchecked T_H1 responses (1–4). In addition, assessment of patients with autoimmune diseases has suggested an involvement of T_H17 cells in some human autoimmune disorders (5, 6). In humans, CD4$^+$ T cells producing IL-17 ex vivo can be detected in circulating lymphocytes from healthy individuals, among memory CD4$^+$ T cells, mainly in the CCR6$^+$ fraction (6, 7) and contain two subpopulations cosecreting or not cosecreting IFN-γ. Both in mice and in humans, the retinoid-related orphan nuclear receptor RORγt has been identified as a lineage-specific transcription factor for T_H17 cells (6–8).

In mice, differentiation of T_H17 cells has been shown to take place in the presence of TFG-β and IL-6 (9), with IL-23 and IL-21 amplifying their expansion and/or stabilizing their phenotype (10–12). Differentiation of human T_H17 cells from naive precursors, however, has proved difficult to achieve (13), and several discrepant findings have been reported. Differentiation has been initially reported to require IL-1β and IL-6, and to be inhibited by TFG-β and IL-2 (14). Subsequent studies, however, have shown TGF-β and IL-2 to be instead required (15, 16). Yang et al. have reported differentiation of T_H17 cells from human naive conventional CD4$^+$ T cells in the presence of TGF-β and IL-21 but not TGF-β and IL-6 (17). Another study however, has suggested that several inflammatory cytokines including IL-1β, IL-6 and IL-23 are all required and act synergistically (18). Overall, these discrepancies have been attributed to the difficulty to ensure a truly naive population of human CD4$^+$ T cells (19). Finally, a recent study has proposed that human T_H17 cells originate from CD161$^+$ precursors present in thymus and cord blood but not in peripheral blood from adult individuals (20).

Opposite to T_H17 cells, a distinct subset of CD4$^+$ T cells, called T regulatory cells (Treg), actively control autoimmunity and tissue homeostasis. CD4$^+$ Treg populations in the periphery of adult individuals include thymically derived natural Treg, in charge of maintaining self-tolerance, and induced Treg that are generated in the periphery to limit exuberant immune responses to microbial or tissue antigens. We have previously shown that, in addition to memory Treg, human Treg include a naive (CD45RA$^+$/RO$^-$CCR7$^+$) CD25$^+$FOXP3$^+$ population, that we have named natural naive Treg (NTreg) present in cord blood and circulating lymphocytes (21). Similar to memory Treg, CD25$^+$FOXP3$^+$NTreg are anergic and suppressive ex vivo (21). So far, NTreg are the only identified subpopulation of naive T cells that constitutively express a lineage-specific transcription factor and are therefore precommitted to differentiation into a specific lineage. Several recent studies, including studies from our group, have documented a close relationship between FOXP3$^+$Treg and T_H17 lineages (22–25). We have shown that stimulation of human FOXP3$^+$Treg in the presence of LPS-activated monocytes and IL-2 converts them into T_H17 cells (22) and have recently identified a subpopulation of memory Treg that coexpress RORγt and secrete high levels of IL-17 ex vivo (24). Together, these findings suggested that human T_H17 cells could preferentially originate from NTreg rather than from conventional naive CD4$^+$ T-cell precursors. In this study, we show that differentiation of T_H17 cells from human circulating naive CD4$^+$ T cells is indeed predominantly obtained from NTreg, and optimally occurs following stimulation in the presence of IL-2 and of lineage-specific differentiation factors.

Results

Priming of IL-17–Producing Cells from Adult Naive Circulating CD4$^+$ T Cells Predominantly Occurs from NTreg. We initially stimulated total naive CD45RA$^+$CD4$^+$ T cells isolated from circulating lymphocytes of adult subjects with anti-CD2/CD3/CD28–coated microbeads in the presence of several previously described T_H17 polarizing cytokines, in various combinations. Under these conditions, we observed very low proportions (generally <1%) of cells secreting IL-17A (IL-17 hereafter, unless otherwise specified). To assess whether T_H17 cells could specifically differentiate from NTreg (which represent ~5% of total naive circulating

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$CD4^+$ T cells), we purified naive conventional ($CD45RA^+CD25^-CD127^{high}$) and NTreg ($CD45RA^+CD25^+CD127^{low}$) populations by flow cytometry cell sorting (Fig. 1*A*). Staining of the sorted populations with FOXP3-specific mAb confirmed the isolation of highly pure conventional ($FOXP3^-$) and regulatory ($FOXP3^+$) populations (Fig. 1*A*). We stimulated the sorted populations with anti-CD2/CD3/CD28–coated microbeads in the presence of IL-2 alone or with TGF-β, IL-1β, IL-23, IL-6, or IL-21, separately or in various combinations and, 12 d later, assessed the proportion of IL-17–secreting cells in the cultures by intracellular staining. Whereas stimulation of conventional naive $CD4^+$ T cells under these various conditions resulted in the induction of very low proportions of IL-17–producing cells, stimulation of NTreg with some of the cytokine combinations resulted in the induction of a significant fraction of IL-17–producing cells (Fig. 1 *B* and *C*). In particular, we obtained optimal induction in the presence of IL-2, IL-1β, IL-23, and TGF-β. Low proportions of IL-17–producing cells were induced from NTreg in the absence of IL-2, even in the presence of the most effective cytokine combination. Thus, priming of T_H17 cells from adult circulating naive $CD4^+$ T cells consistently occurred, and predominantly took place from NTreg in the presence of IL-2 and of lineage specific polarizing factors.

Expression of IL-1RI Is Selectively Up-Regulated in NTreg Following Stimulation and Is Increased by TGF-β. Whereas IL-23 was not active alone but increased the differentiation of T_H17 cells from NTreg in the presence of IL-1β, the latter was indispensable for differentiation to occur. To gain further insight into the differential role of these cytokines during T_H17 polarization from NTreg, we assessed the expression of their specific receptors in conventional naive $CD4^+$ T cells and NTreg. We detected no significant expression of IL-1RI ex vivo in naive $CD4^+$ T cells, either conventional or Treg (Fig. 2*A*). Following in vitro stimulation, however, IL-1RI expression was induced in a significant fraction of NTreg but not in conventional naive $CD4^+$ T cells. The proportion of IL-1RI–expressing cells induced in NTreg by stimulation in the presence of IL-2 was not significantly affected by the presence of other cytokines (including IL-1β) with the exception of TGF-β that significantly increased it. Ex vivo expression of IL-23R in naive $CD4^+$ T cells was low and was induced by stimulation in a fraction of the cells (Fig. 2*B*). At variance with IL-1RI, however, IL-23R expression was induced in both conventional naive $CD4^+$ T cells and NTreg. The presence of TGF-β, alone or in combination with inflammatory cytokines, further increased IL-23R expression in both populations. Thus, selective overexpression of IL-1RI in NTreg was in agreement with the important role of IL-1β in inducing their differentiation into T_H17 cells, whereas TGF-β synergized with IL-1β and IL-23 by increasing the expression of the corresponding receptors.

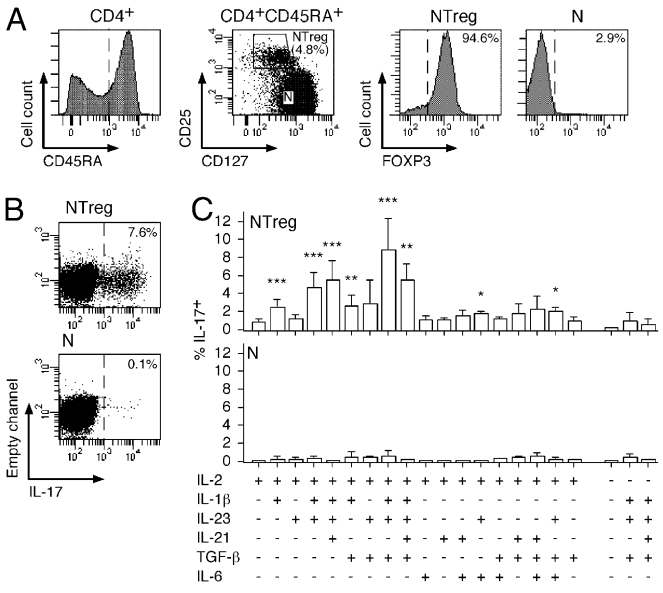

Fig. 1. In vitro differentiation of NTreg into IL-17–producing cells. (*A*) Enriched $CD4^+$ T cells were stained with anti-CD25, -CD45RA, and -CD127 mAb and $CD45RA^+$ cells (left histogram) were sorted by flow cytometry into naive conventional $CD4^+$ T cells (N, $CD25^-CD127^{high}$) and naive Treg (NTreg, $CD25^+CD127^{low}$) (dot plot). A fraction of sorted populations was stained with anti-FOXP3 mAb and analyzed by flow cytometry (right histograms). (*B* and *C*) Sorted N and NTreg were stimulated in vitro with anti-CD2/CD3/CD28 microbeads in the presence of the indicated cytokines and cultured for 12 d in the absence or presence of IL-2, as indicated. IL-17 production was assessed by intracellular cytokine staining and flow cytometry analysis following stimulation with PMA/ionomycin. Dot plots for N and NTreg from one donor stimulated in the presence of IL-2, IL-1β, IL-23, and TGF-β are shown in *B*. Results for all conditions and donors are shown in *C* (mean ± SD, $n = 6$). Statistical analyses were performed using two-tailed *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

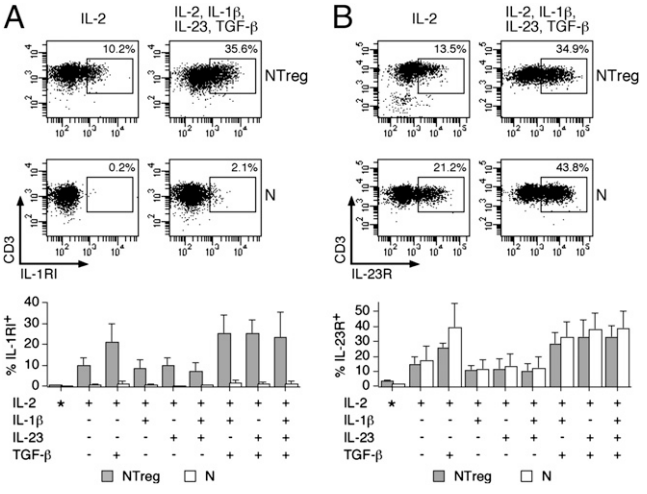

Fig. 2. Expression of IL-1RI and IL-23R in stimulated N and NTreg. Ex vivo sorted N and NTreg were stimulated in vitro with anti-CD2/CD3/CD28 microbeads in the presence of the indicated cytokines and cultured in the presence of IL-2. Ex vivo sorted N and NTreg as well as day 5 cultures were stained with antibodies specific for IL-1RI (*A*) and IL-23R (*B*) and analyzed by flow cytometry. Examples of dot plots for one donor and data for all donors (mean ± SD, $n = 5$), ex vivo (*) or after culture are shown.

T_H17 Cells Differentiating from NTreg Down-Regulate FOXP3 and Express RORγt. Expression of lineage-specific transcription factors supports the differentiation of distinct T-cell subsets by shaping their phenotypic and functional profiles. It has been proposed that the balance between the T_H17 lineage specific transcription factor RORγt, the expression of which is indispensable for IL-17 secretion, and the Treg-specific transcription factor FOXP3, which antagonizes RORγt activity, affects T_H17 cell polarization. To follow the expression of FOXP3 and RORγt during T_H17 priming from NTreg, we assessed cultures stimulated under nonpolarizing or polarizing conditions using RORγt, FOXP3, and IL-17–specific antibodies. Expression of FOXP3 in NTreg was maintained in a substantial proportion of cells after differentiation in all conditions (Fig. 3*A*). The large majority of IL-17–secreting cells derived from polarized NTreg (particularly in the absence of TGF-β), however, had lost or highly decreased FOXP3 expression (Fig. 3 *B* and *C*). Expression of RORγt, undetectable in naive $CD4^+$ T cells ex vivo (24), was induced in a low proportion of conventional naive $CD4^+$ T cells and NTreg after stimulation in the presence of IL-2 with or without inflammatory cytokines or with TGF-β alone. Following stimulation with IL-2 and inflammatory cytokines together with TGF-β, however, expression of RORγt was induced in more than

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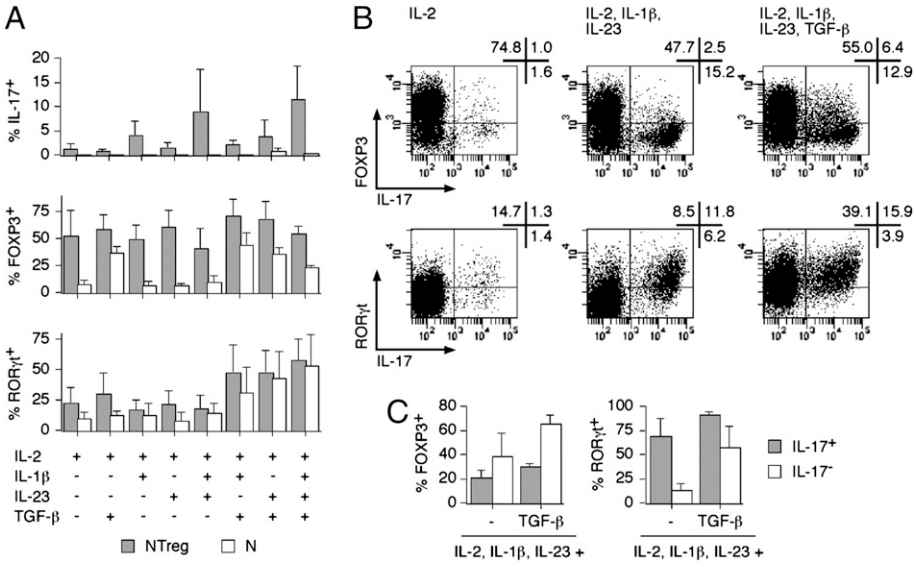

Fig. 3. Expression of FOXP3 and RORγt in T_H17 cells differentiating from NTreg. Ex vivo sorted N and NTreg were stimulated as in Fig. 2. Day 12 cultures were stimulated with PMA/ionomycin, stained with mAb specific for IL-17, FOXP3, and RORγt and analyzed by flow cytometry. Data corresponding to the percentage of IL-17–producing and FOXP3- and RORγt-expressing cells in the cultures are shown in *A* (mean ± SD, $n = 3$). Dot plots showing FOXP3 and RORγt expression among IL-17–producing cells in polarized cultures are shown for one donor in *B*, and data for all donors (mean ± SD, $n = 3$) are shown in *C*.

half of total conventional naive and NTreg populations (Fig. 3*A*). Regardless of the total proportion of RORγt-expressing cells in the cultures, the large majority of IL-17–secreting cells expressed high levels of RORγt (Fig. 3 *B* and *C*). Thus, T_H17 cells differentiating from NTreg were $FOXP3^-$ or $FOXP3^{low}$ and expressed high levels of RORγt.

NTreg-Derived T_H17 Cells Contain Distinct Subpopulations Cosecreting or Not IFN-γ, Cosecrete Other T_H17-Associated Cytokines, and Secrete IL-2. To further characterize NTreg-derived T_H17 populations, we assessed the secretion of other cytokines that have been reported as associated or not associated with the lineage. We selected cultures polarized in the presence of IL-1β and IL-23 in either the absence or presence of TGF-β (Fig. 4). NTreg-derived T_H17 cells contained both IL-17 single-secreting and IL-17/IFN-γ double-secreting populations, corresponding to the two distinct populations described in circulating human memory T_H17 cells (6). Whereas single-secreting and double-secreting cells were present in roughly similar proportions following polarization in the absence of TFG-β, IL-17 single-secreting cells were prevalent in the presence of TFG-β. Most IL-17–secreting cells cosecreted TNF-α, and about half of them cosecreted IL-22, regardless of the presence of TGF-β. In addition, ∼30% of IL-17–secreting cells cosecreted IL-21 in the presence of TFG-β. No significant cosecretion of IL-4 or IL-10 was detected. Importantly, whereas NTreg fail to secrete IL-2 ex vivo (21), T_H17 polarized NTreg populations contained significant proportions of IL-2–secreting cells that were particularly enriched in the IL-17–secreting fraction. A subpopulation of IL-17A–secreting cells in the cultures cosecreted IL-17F, another proinflammatory cytokine similar to IL-17A (26). Of note, no IL-17F single-positive cells were detected in the cultures.

IL-17–Secreting Cells in T_H17 Polarized NTreg Cultures Are Highly Enriched in $CCR6^+$ Expressing Cells. Expression of the chemokine receptor CCR6 has been described as associated with the T_H17 lineage but also to the Treg lineage (6, 7, 27, 28). Expression of CCR6 in naive $CD4^+$ T cells (both conventional and NTreg) was undetectable ex vivo (24). However, CCR6 expression was induced in a small fraction of NTreg, but not in conventional naive $CD4^+$ T cells, following stimulation in the presence of IL-2 with or without IL-1β and IL-23 (Fig. 5*A*). In contrast, the presence of TFG-β slightly inhibited expression of CCR6. To assess whether T_H17 cells in polarized NTreg were enriched within the CCR6-expressing fraction, we isolated $CCR6^+$ and $CCR6^-$ populations by cell sorting and assessed secretion of IL-17 together with expression of RORγt in the isolated fractions. As shown in Fig. 5*B*, most IL-17–secreting cells that were, as expected, homogenously $ROR\gamma t^+$ were contained in the $CCR6^+$ fraction of the cultures. In addition, regardless of their capacity to secrete IL-17, most cells in the $CCR6^+$ fraction expressed RORγt.

Expression of IL-1RI and CCR6 in T_H17 Polarized NTreg Cultures Identifies Distinct Suppressive and Nonsuppressive Subpopulations. A recent study has shown that expression of IL-1RI in in vitro cultured human memory Treg specifically identified populations of cells that have maintained suppressor functions (29). To clarify the relationship between expression of IL-1RI and CCR6, we costained polarized NTreg cultures with the corresponding specific mAb. This analysis identified several distinct populations (Fig. 6*A*). Namely, whereas a subpopulation expressing IL-1RI but not CCR6 was clearly identified, the CCR6-expressing population included both $IL\text{-}1RI^-$ and, to a lesser extent, $IL\text{-}1RI^+$ cells. To further evaluate these subpopulations, we isolated them by cell sorting, stimulated them with PMA/ionomycin, and assessed FOXP3 expression and IL-17 production. In addition, we also assessed the capacity of the isolated populations to suppress the proliferation of responder CFSE-labeled $CD4^+$ T cells in a functional suppression assay as described previously (21). As illustrated in Fig. 6*B*, IL-1RI–expressing populations, both $CCR6^-$ and $CCR6^+$, contained significantly higher proportions of $FOXP3^+$ cells, which expressed significantly higher levels of FOXP3, as compared with the $IL\text{-}1RI^-$ fraction. As expected, most IL-17–secreting cells were detected in the $CCR6^+$ populations. However, it is noteworthy that, whereas most IL-17–secreting cells in the $IL\text{-}1RI^-CCR6^+$ fraction were $FOXP3^-$, a large proportion of IL-17–secreting cells in the $IL\text{-}1RI^+CCR6^+$ population had maintained FOXP3 expression. In line with FOXP3 expression, the $IL\text{-}1RI^+$ populations (both $CCR6^-$ and $CCR6^+$) displayed suppressive functions, whereas the $IL\text{-}1RI^-CCR6^+$ failed to inhibit the growth of responder cells (Fig. 6 *C* and *D*). In conclusion, our analysis identified two distinct subpopulations of $CCR6^+$ IL-17–secreting cells: one subpopulation, less abundant, expressed IL-1RI and maintained FOXP3 expression and suppressive functions, whereas the prominent subpopulation was $IL\text{-}1RI^-$ and had lost FOXP3 expression and suppressive functions.

Discussion

In this study, we have unambiguously shown that priming of human T_H17 cells consistently takes place from adult naive circulating $CD4^+$ T cells and occurs mainly from NTreg rather than

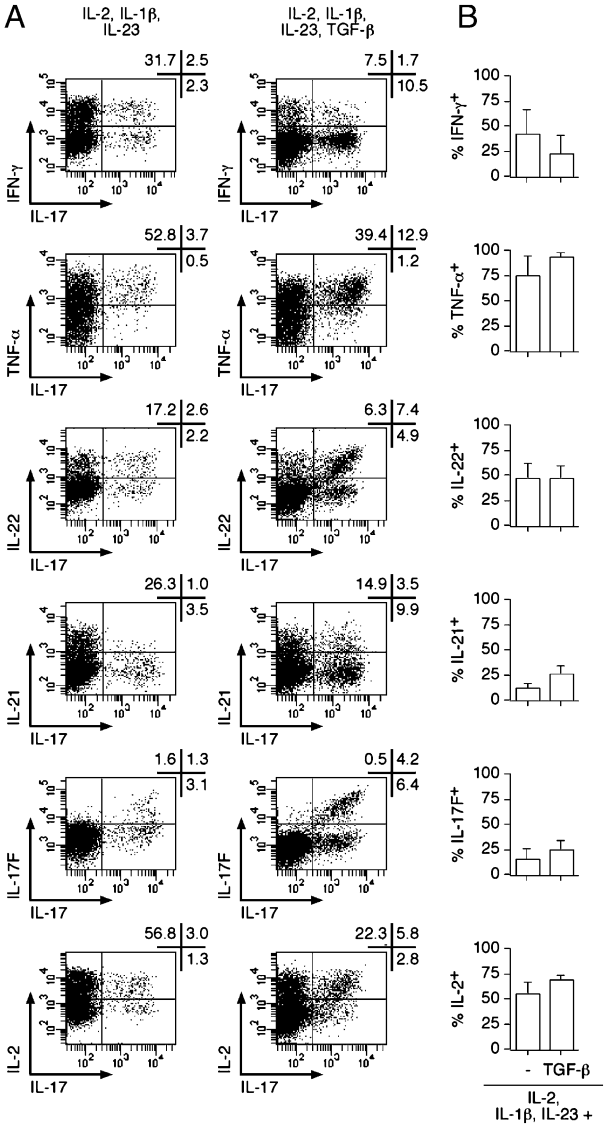

Fig. 4. Cytokine production by T_H17 cells differentiated from NTreg. Ex vivo sorted NTreg were stimulated in the presence of IL-1β and IL-23 in the absence or presence of TGF-β and cultured in the presence of IL-2 for 12 d. Cytokine production was determined by intracellular staining with specific mAb following stimulation with PMA/ionomycin. Dot plots for one donor are shown in *A*. Proportions of T_H17 cells coproducing the indicated cytokines are summarized in *B* for all donors (mean ± SD, $n = 3$).

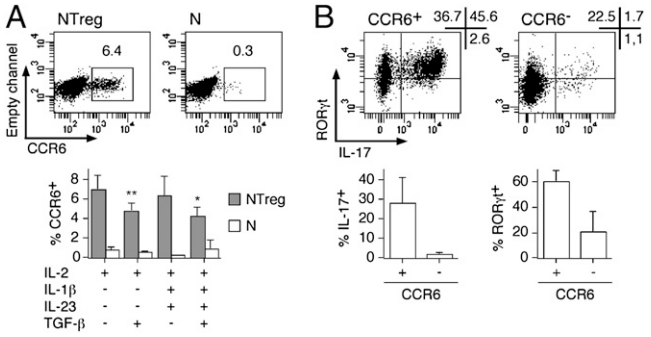

Fig. 5. CCR6-expressing fraction within polarized NTreg is enriched in T_H17 cells. (*A*) Ex vivo sorted N and NTreg were stimulated in vitro with anti-CD2/CD3/CD28 microbeads in the presence of the indicated cytokines and cultured in the presence of IL-2. Day 5 cultures were stained with anti-CCR6 mAb and analyzed by flow cytometry. Dot plots for one donor and data for all donors (mean ± SD, $n = 5$) are shown. $^*P < 0.05$; $^{**}P < 0.01$. (*B*) Day 12 cultures of NTreg, stimulated in the presence of IL-1β, IL-23, TGF-β, and IL-2, were stained with anti-CCR6 mAb and $CCR6^+$ and $CCR6^-$ populations were sorted by flow cytometry. After stimulation with PMA/ionomycin, sorted cells were stained with IL-17 and RORγt-specific mAb and analyzed by flow cytometry. Dot plots for one donor and data for all donors (mean ± SD, $n = 6$) are shown.

conventional naive $CD4^+CD25^-$ T cells. Together, our results reconcile previous reports of heterogeneous and discrepant findings likely due to the use of nonoptimally defined starting populations containing variable proportions of naive conventional $CD4^+$ T cells and NTreg populations.

Priming of T_H17 cells from NTreg was optimally achieved in the presence of IL-2, IL-1β, IL-23, and TGF-β. Based on the enhancement of T_H17 generation following genetic deletion or antibody blockade in murine models, IL-2 has been initially proposed to inhibit T_H17 differentiation (30). The exact underlying mechanism, however, has not been elucidated; and it has been suggested that IL-2, through binding of STAT5 to the IL-17 promoter, might attenuate IL-17 production in differentiated T_H17 cells rather than inhibiting T_H17 differentiation (16). In line with this hypothesis, it was later shown that IL-2 is instead required for in vitro differentiation of human T_H17 cells (15). Similarly, in our system, the presence of IL-2 highly increased differentiation of T_H17 cells from NTreg in all conditions.

TGF-β is crucial for maintenance of Treg in the periphery, although it may be dispensable for their generation in the thymus (31). TGF-β was initially reported to inhibit the in vitro differentiation of human T_H17 cells (14), but was proved later as required (15, 16). In this study, the presence of TGF-β was not indispensable, but increased the proportion of T_H17 cells differentiating from NTreg. It is noteworthy that NTreg express endogenous TGF-β (21), which likely has an impact on their differentiation into T_H17 cells even in the absence of exogenous sources.

We and others have recently shown that TCR stimulation in the presence of IL-1β (or IL-1α) and IL-2 or IL-15 induces the conversion of human memory Treg into T_H17 cells (22, 23). In this study, IL-1β was indispensable for NTreg differentiation into T_H17 cells. IL-1β signals through receptor type I (IL-1RI), homologous to Toll, the conserved region being the Toll/IL-1 receptor (TIR) domain, which defines the IL-1R/TLR superfamily (32). Interestingly, at odds with previous findings suggesting a marginal role of IL-1β in the differentiation of murine T_H17 cells in vitro, IL-1RI has recently been shown to be necessary for early differentiation of murine T_H17 cells in vivo (33). We found selective expression of IL-1R1 in stimulated NTreg in good agreement with their increased proficiency, with respect to conventional naive $CD4^+$ T-cell precursors, to differentiate into T_H17 cells. Interestingly, IL-1RI expression in NTreg was not affected by IL-1β or IL-23 but was significantly enhanced by TGF-β. Consistent with the role of TGF-β in inducing IL-1RI expression, the latter correlated with that of FOXP3 and identified suppressive populations in the cultures.

IL-23 is a heterodimeric cytokine composed of a p40 chain, common to IL-12, disulfide-linked to a specific p19 chain (34, 35). Studies using $p19^{-/-}$ murine models of autoimmunity identified IL-23 as the major factor involved in the induction of T_H17 cells in vivo (36–38). However, researchers have subsequently failed to demonstrate efficient induction of T_H17 cells from naive $CD4^+$ T cells by IL-23 in vitro (39, 40), and have concluded that the role of IL-23 was not to induce but rather to stabilize the T_H17 phenotype. In line with this hypothesis, in this study, IL-23 alone was not able to differentiate NTreg into T_H17 cells but synergized with other polarizing factors.

T_H17 cells derived from NTreg exhibited a cytokine secretion pattern similar to those described thus far for human T_H17 populations, as they contained both cells cosecreting IL-17 and IFN-γ (double-secreting) and cells secreting IL-17 in the absence

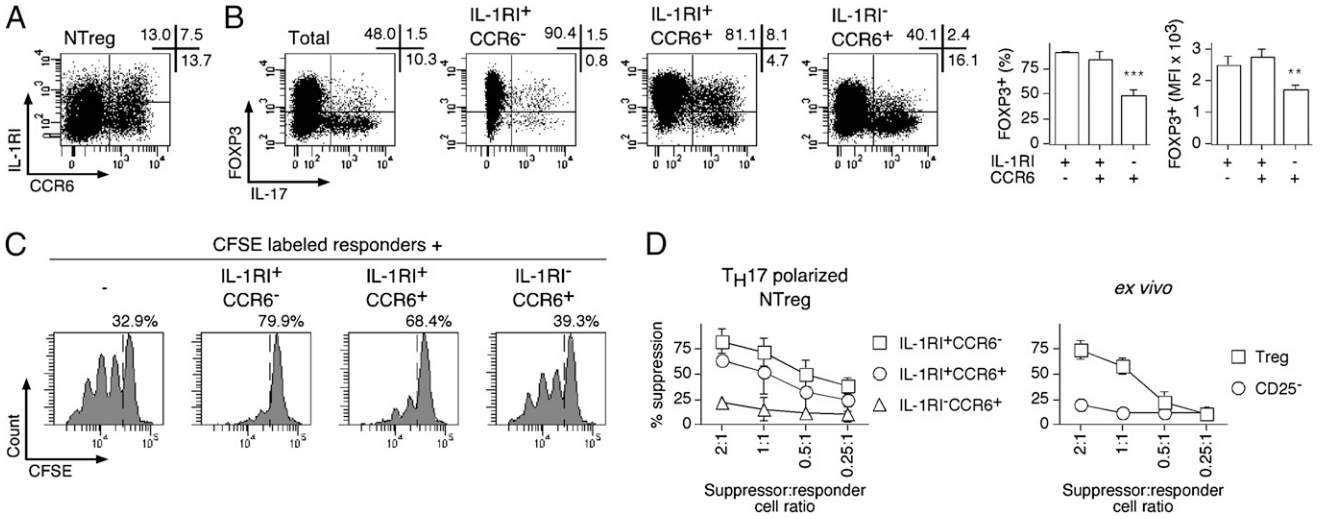

Fig. 6. T_H17 polarized NTreg cultures contain distinct suppressive and nonsuppressive subpopulations. (*A*) Ex vivo sorted NTreg were stimulated in vitro with anti-CD2/CD3/CD28 microbeads in the presence of IL-1β, IL-23, TGF-β, and IL-2. Day 12 cultures were stained with mAb specific for IL-1RI and CCR6 and analyzed by flow cytometry. (*B*) NTreg were stimulated and stained as in *A,* and IL-1RI$^+$CCR6$^-$, IL-1RI$^+$CCR6$^+$, and IL-1RI$^-$CCR6$^+$ populations were sorted by flow cytometry. Cells from the total unsorted culture as well as sorted populations were stimulated in the presence of PMA/ionomycin, stained with mAb specific for FOXP3 and IL-17, and analyzed by flow cytometry. Dot plots for one donor and data for all donors (% FOXP3$^+$ cells and mean fluorescence intensity (MFI) of FOXP3 staining in the FOXP3$^+$ populations, mean ± SD, $n = 4$) are shown. $^{**}P < 0.01$; $^{***}P < 0.001$. (*C* and *D*) CFSE-labeled conventional CD4$^+$ T cells were stimulated with PHA in the absence or presence of IL-1RI$^+$CCR6$^-$, IL-1RI$^+$CCR6$^+$, and IL-1RI$^-$CCR6$^+$ populations sorted from T_H17 polarized NTreg cultures as in *B* or in the presence of ex vivo sorted Treg and conventional CD25$^-$ cells. Growth of responder CD4$^+$ T cells was assessed by flow cytometry analysis of CFSE dilution. Examples of CFSE dilution histograms, at 1:1 suppressor-to-responder cell ratio, are shown in *C* where numbers correspond to the proportion of undivided cells. Percent suppression is shown in *D* for all tested suppressor-to-responder cell ratios, populations, and donors (mean ± SD, $n = 3$).

of IFN-γ secretion (single-secreting) (6). Interestingly, whereas double-secreting cells were prevalent following polarization in the absence of TGF-β, polarization in the presence of TGF-β prevalently induced single-secreting cells. This suggests that both single- and double-secreting T_H17 cells can originate from NTreg depending on the in vivo differentiation conditions. All IL-17–secreting cells derived from NTreg cosecreted TNF-α, also highly proinflammatory. Some of them cosecreted IL-21 and IL-22, two cytokines that have been described as T_H17 associated, although not exclusively secreted by T_H17 cells (41–44). IL-17F is a proinflammatory cytokine with functions initially reported as similar to those of IL-17A and sharing the same receptor (45). Recent studies have revealed that IL-17A and IL-17F can be expressed as homo- or heterodimers that may bind IL-17 receptors with various affinities and may play not completely redundant functions (26, 46, 47). We found expression of IL-17F in only a fraction of IL-17A–secreting cells from NTreg, indicating the existence of distinct subpopulations that differentially express these cytokines and may be functionally distinct, an observation that warrants further investigation.

Differentiation of T_H17 cells from NTreg was accompanied by induction of RORγt expression, down-regulation of FOXP3 expression and acquisition of IL-2 secretion, particularly in the IL-17–secreting fraction, suggesting that NTreg-derived T_H17 cells may have mostly lost their suppressor functions and may have instead acquired auxiliary functions. Expression of CCR6 has been reported as associated with the T_H17 but also with the Treg lineage (6, 7, 27, 28). We found increased expression of CCR6 in stimulated NTreg as compared with conventional naive CD4$^+$ T cells. In addition, by isolating CCR6-expressing cells from polarized cultures, we demonstrated that most IL-17–secreting cells in NTreg-derived T_H17 cell populations were contained in the CCR6$^+$ fraction. Interestingly, assessment of CCR6 expression in relation to that of IL-1RI revealed the existence of two distinct subpopulations of CCR6$^+$ IL-17–secreting cells: the first, less abundant, coexpressed IL-1RI and FOXP3 and retained suppressive functions, whereas the most prominent one was IL-1RI$^-$ and FOXP3$^-$ and had lost the capacity to suppress. The identification of these subpopulations is in line with our recent finding that human IL-17 secreting cells in human peripheral blood (that are all memory cells) include a population of cells that coexpress FOXP3 and exert suppressor functions (24).

Overall, the findings reported here strongly suggest that at least part of human T_H17 cells may differentiate in vivo from NTreg precursors in the presence of lineage-specific differentiation factors. NTreg are generated in the thymus as a distinct lineage of CD4$^+$ T cells that are anergic but are specific for self-antigens, and therefore potentially auto-reactive if stimulated under conditions that break their anergy (48). The finding that T_H17 cells mainly differentiate from NTreg therefore raises the possibility that some auto-reactive effectors in autoimmune pathologies associated with T_H17 might be generated through this differentiation pathway. Because some tumor-associated antigens are nonmutated self-antigens also expressed in cells of normal tissues that undergo malignant transformation (e.g., melanocyte differentiation antigens), differentiation of NTreg specific for these antigens into T_H17 would result in the generation of anti-tumor effectors. Interestingly, recent data have indeed shown a particular efficacy of T_H17 effectors in anti-tumor responses (49).

A preferential differentiation of T_H17 cells from NTreg, however, does not imply that all T_H17 effectors are specific for self-antigens or autoreactive. First, because part of Treg in adults could also be generated in the periphery through conversion of conventional CD4$^+$ T cells (50), some NTreg could represent an intermediate differentiation stage along a differentiation pathway leading from nonautoreactive, naive, conventional CD4$^+$ T cells to memory T_H17 cells. In addition, some T_H17 cells could be generated in the periphery through conversion of induced MTreg, differentiated from CD4$^+$ CD25$^-$FOXP3$^-$ T cells, under inflammatory conditions (22, 23).

Although the involvement of T_H17 cells in autoimmune pathology has been strongly suggested by studies in mice and in humans, little information is available on the antigens that they recognize (7), and their potential self-reactivity has not been thus far investigated. Based on the knowledge that T_H17 cells can derive from precursors common to autoreactive Treg, insights into

their antigen specificity, including reactivity to self-antigens, in human T_H17-associated pathologies, will undoubtedly contribute to clarify their role in inflammation-induced tissue damage.

Materials and Methods

Samples, Cell Purification, and ex Vivo Cell Sorting. Peripheral blood samples were obtained from the Etablissement Français du Sang (Nantes, France) upon informed consent and approval by institutional review board. Naive conventional $CD4^+$ T cells and NTreg were isolated by flow cytometry cell sorting, as previously described (24) and detailed in *SI Materials and Methods*.

In Vitro Differentiation of $CD4^+$ T-cell Populations and Phenotypic Assessment and Flow Cytometry Cell Sorting of Differentiated Cultures. Ex vivo sorted N and NTreg (3×10^4) were stimulated with anti-CD2/CD3/CD28–coated microbeads (Miltenyi Biotec) in the absence or presence of IL-1β (10 ng/mL, R&D Systems), IL-6 (50 ng/mL, R&D Systems), IL-21 (50 ng/mL, R&D systems), IL-23 (100 ng/mL, R&D Systems), and TGF-β (10 ng/mL, PeproTech) alone or in combination, as specified. Cells were maintained in culture in complete IMDM (Invitrogen) in the absence or presence of IL-2 (100 IU/mL, Chiron), as indicated. Day 5 or 12 cultures were stained with IL-RI-, IL-23R- and/or CCR6-specific antibodies and were analyzed and/or sorted by flow cytometry, as detailed in *SI Materials and Methods*.

Assessment of Cytokine Production, RORγt and FOXP3 Expression, and Suppressor Function. Cytokine production and expression of RORγt and FOXP3 by in vitro differentiated $CD4^+$ T cells were assessed by intracellular staining and flow cytometry analysis following stimulation with PMA and ionomycin as detailed in *SI Materials and Methods*. Their ability to suppress the proliferation of responder $CD4^+CD25^-$ T cells was assessed as previously described (21) and detailed in *SI Materials and Methods*.

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