Phenotypic and functional features of human Th17 cells

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T helper (Th) 17 cells represent a novel subset of CD4+ T cells that are protective against extracellular microbes, but are responsible for autoimmune disorders in mice. However, their properties in humans are only partially known. We demonstrate the presence of Th17 cells, some of which produce both interleukin (IL)-17 and interferon (IFN)-γ (Th17/Th1), in the gut of patients with Crohn's disease. Both Th17 and Th17/Th1 clones showed selective expression of IL-23R, CCR6, and the transcription factor RORγt, and they exhibited similar functional features, such as the ability to help B cells, low cytotoxicity, and poor susceptibility to regulation by autologous regulatory T cells. Interestingly, these subsets also expressed the Th1-transcription factor T-bet, and stimulation of these cells in the presence of IL-12 down-regulated the expression of RORγt and the production of IL-17, but induced IFN-γ. These effects were partially inhibited in presence of IL-23. Similar receptor expression and functional capabilities were observed in freshly derived IL-17–producing peripheral blood and tonsillar CD4+ T cells. The demonstration of selective markers for human Th17 cells may help us to understand their pathogenic role. Moreover, the identification of a subset of cells sharing features of both Th1 and Th17, which can arise from the modulation of Th17 cells by IL-12, may raise new issues concerning developmental and/or functional relationships between Th17 and Th1.

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Abbreviations used: Ab, antibody; CD, Crohn's disease; CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; PB, peripheral blood; RA, rheumatoid arthritis.

The adaptive effector CD4+ Th-mediated immune response is highly heterogenous, based on the development of distinct subsets that are characterized by different profiles of cytokine production. Initially, two polarized forms of Th effectors, type 1 (Th1) or type 2 (Th2), were identified in both mice and humans (1, 2). Th1 cells produce IFN-γ and are mainly devoted to protection against intracellular microbes, whereas Th2 cells produce IL-4, -5, -9, and -13 and are involved in the protection against gastrointestinal nematodes, but are also responsible for allergic disorders (3, 4). A third type of Th that is able to produce both Th1 and Th2 cytokines, type 0 (Th0), has also been detected (5). More recently, a novel subset of Th, which is distinct from Th1, Th2, and Th0 cells and is called Th17, has been described (6). Th17 cells originate under different polarizing conditions than Th1 or Th2, which have been found to be the early presence at the time of antigen presentation to the naive Th of IL-12 or -4, respectively (7). Initial findings reported that, in the presence of *Borrelia burgdorferi*, IL-17 could be produced by T cells independent of the production of Th1 or Th2 cytokines (8). However, the major breakthrough leading to discovery of Th17 lineage came from mouse models of autoimmunity. Experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), two prototypical autoimmune mouse models, have historically been associated with unchecked Th1 responses, based on studies in which disease development was ablated by treatment with neutralizing antibodies (Ab) specific for IL-12p40 or gene-targeted mice deficient in the p40 subunit of IL-12 (9, 10). However, with the discovery that a new IL-12 family member, IL-23, shares the p40 subunit with IL-12 but comprises of

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another subunit, p19, which is different from the p35 subunit of IL-12 (11), it was found that both EAE and CIA were ablated in mice deficient in IL-23, but not IL-12 (12, 13). Moreover, mice lacking the IL-12R complex also succumbed to EAE (14), suggesting that IL-23, and not IL-12, is critically linked to autoimmunity, at least in these models. Although IL-23 appeared to be required for Th17-mediated immunopathology, more recent reports indicate that it is not required for Th17 commitment, but instead appears to be important for amplifying and/or stabilizing the Th17 phenotype (15, 16). The concomitant activity of TGF-β and IL-6 appears to be necessary for initiation of Th17 differentiation (17, 18). Moreover, IL-1β and TNF-α were found to amplify the Th17 response induced by TGF-β and IL-6, but could not substitute for either of these cytokines (19). Finally, an analysis of transcription-factor expression by IL-17–producing CD4+ T cells showed that Th17 effectors lacked expression of T-bet and Hlx in comparison to Th1-polarized cells, and of GATA-3 compared with Th2-polarized populations, supporting and extending previous findings that identified Th17 as a product of an effector lineage distinct from Th1 and Th2 (20, 21). The latter finding was supported by the recent demonstration that the orphan nuclear receptor RORγt directs the differentiation program of mouse Th17 (22).

Most of the knowledge of Th17 available so far originates from studies performed in experimental animal models, whereas very little information on Th17 exists in humans. A higher number of IL-17 mRNA–expressing cells were found by using in situ hybridization in the cerebrospinal fluid than in peripheral blood (PB) from patients with multiple sclerosis (23). Moreover both IL-17 and -23p19 are present in the sera, synovial fluids, and synovial biopsies of most rheumatoid arthritis (RA) patients, whereas both are absent in osteoarthritis (24, 25). IL-17 has also been detected in the sera and diseased tissues of patients with systemic lupus erythematosus (26), in systemic sclerosis (27), and in sera and colonic biopsies of inflammatory bowel disorders (28, 29), as well as in the affected skin of subjects with nickel-induced contact dermatitis or psoriasis (30). However, all of these studies were performed by assessing the presence of mRNA for IL-17 in tissues and/or measuring IL-17 in biological fluids. Only two reports have shown the production of IL-17 by small numbers of T cell clones generated from the skin of subjects with contact dermatitis (30) or the synovial membranes and synovial fluid of subjects with RA (31); however, no additional information on the phenotypic and functional features of human IL-17–producing T cells were provided.

In this study, we demonstrate the existence of remarkable proportions of Th17 and of IFN-γ–producing Th17 (Th17/Th1) in the gut of subjects with Crohn's disease (CD). When expanded in vitro and cloned, Th17 and Th17/Th1 clones showed selective expression of IL-23R, CCR6, and the transcription factor RORγt. Both Th17 and Th17/Th1 clones showed an excellent ability to help B cells, low cytotoxic potential, and reduced susceptibility to suppression by autologous CD4+Foxp3+ regulatory T (T reg) cells. Similar receptor expression and functional capabilities were observed in IL-17–producing T cell clones obtained from normal gut, as well as in freshly derived PB and tonsillar IL-17–producing CD4+ T cells. Interestingly, both Th17 and Th17/Th1 clones also expressed the Th1-related transcription factor T-bet, and their culturing in the presence of IL-12 down-regulated the

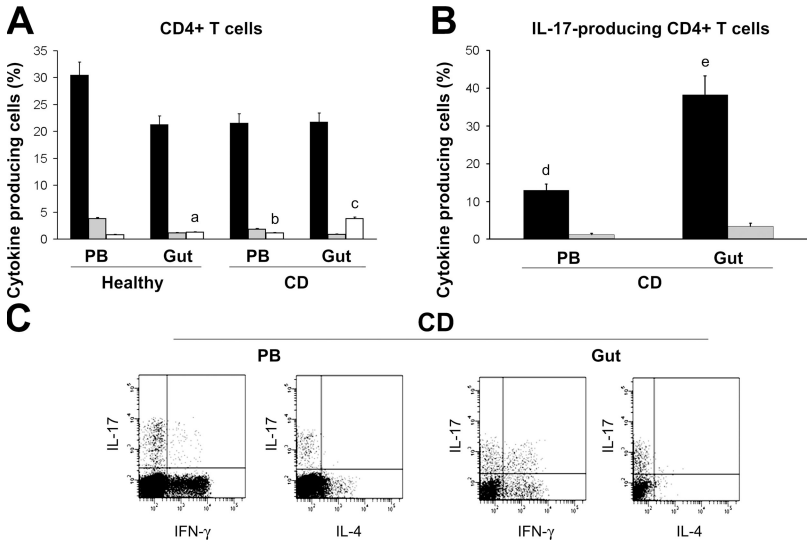

Figure 1. Ex vivo identification of human Th17 and Th17/Th1. (A) CD4+ T cells from PB of healthy subjects, healthy gut areas of subjects with colon carcinoma, and PB and disease-affected gut areas of subjects with CD were assessed by intracellular staining with flow cytometry for their ability to produce IFN-γ (black), IL-4 (gray), or IL-17 (white) after stimulation with PMA plus ionomycin. (B) Proportions of IL-17–producing cells able to also produce IFN-γ (black) or IL-4 (gray) among CD4+ T cells from PB or gut of subjects with CD. Columns in A and B represent mean values (± the SEM) obtained from 10 different subjects for each group. a versus c, $P < 0.05$; b versus c, $P < 0.05$; d versus e, $P < 0.05$. (C) Representative flow cytometric analyses in PB and gut of one subject with CD.

expression of RORγt and IL-17 and up-regulated the production of IFN-γ, suggesting that, at least in humans, a developmental and/or functional relationship between Th17 and Th1 may exist.

RESULTS

Ex vivo demonstration of IL-17–producing CD4+ T cells in subjects with CD, and their in vitro expansion and cloning

The presence of IL-17–producing cells was evaluated by flow cytometry on CD4+ T cells from PB of 10 healthy individuals, as well as from PB and disease-affected areas of 10 subjects with CD, after their stimulation with PMA and ionomycin. As an additional control, CD4+ T cells derived from apparently healthy gut areas of 10 subjects who underwent colectomy because of colon carcinoma were also assessed. Proportions of Th17 were consistently <1% in PB from healthy individuals, and only slightly higher among CD4+ T cells derived from apparently healthy gut areas of subjects with colon carcinoma or from PB of subjects with CD, whereas they were significantly higher in disease-affected gut areas of the same subjects with CD (Fig. 1 A). Comparable proportions of IL-17–producing cells were found among CD4+ T lymphocytes derived from the disease-affected gut areas of five subjects suffering from ulcerative colitis (unpublished data). A minor, but consistent, proportion of IL-17–producing CD4+ T cells derived from PB of healthy subjects or healthy gut areas (not depicted), as well as from PB or disease-affected gut areas of subjects with CD (Fig. 1, B and C), also exhibited the ability to produce IFN-γ, whereas cells producing both IL-17 and -4 were virtually undetectable. Notably, proportions of Th17 also producing IFN-γ were higher among gut T cells than PB CD4+ T cells from subjects with CD (Fig. 1, B and C). IL-17–producing CD4+ T cells were found to be contained within the CD45RO+ population, and they all appeared to be TCRαβ+ cells (unpublished data).

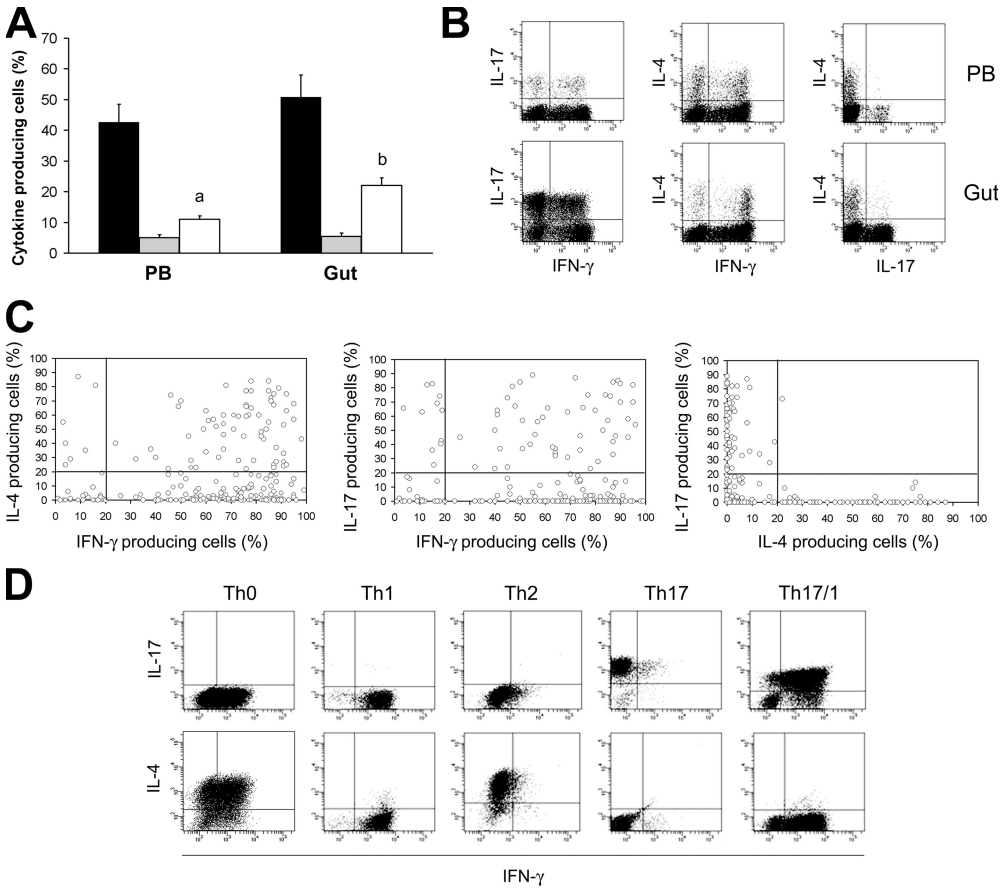

Figure 2. Cytokine production by in vitro–expanded CD4+ T cells from PB and gut and by T cell clones from gut of subjects with CD. (A) CD4+ T cells from PB or disease-affected gut areas of 10 subjects with CD were stimulated for 7 d with anti-CD3 plus anti-CD28 mAb and IL-2, and their ability to produce IFN-γ (black), IL-4 (gray), or IL-17 (white) was assessed (see Fig. 1 legend). Columns represent the mean values (± the SEM). a versus b, $P < 0.05$. (B) Representative flow cytometric analyses on expanded PB or gut CD4+ T cells from the same CD subject. (C) A total number of 217 T cell clones (circles) were obtained from CD4+ T cells of disease-affected gut areas of two subjects with CD and assessed by flow cytometry for intracellular synthesis of IFN-γ, IL-4, and IL-17 after stimulation with PMA plus ionomycin. Production of cytokines by each clone was arbitrarily considered as noteworthy when the proportion of producing T cell blasts was >20%. (D) Representative flow cytometric analyses of cytokine production for each type of clone.

To better investigate the phenotypic and functional features of IL-17–producing CD4+ T cells, those present in PB or disease-affected gut areas of the 10 subjects with CD were expanded in vitro by stimulation for 7 d with a mixture of anti-CD3 and -CD28 mAb in the presence of IL-2. Under these conditions, remarkable proportions of CD4+ T-blasts derived from both sources showed the ability to produce IL-17 after their stimulation with PMA plus ionomycin, with those derived from gut being significantly higher (Fig. 2 A). Notably, in agreement with the aforementioned ex vivo findings, some of these cells produced both IL-17 and IFN-γ. In contrast, virtually no CD4+ T-blasts producing both IL-17 and -4 were observed (Fig. 2 B). T-blasts from two randomly selected cultures derived from the disease-affected gut of subjects with CD were cloned under conditions of limiting dilution and expanded in the presence of allogeneic irradiated feeder cells and IL-2. 217 CD4+ T cell clones were obtained, and the cytokine production (IL-4, IFN-γ, and IL-17) by each clone was evaluated by flow cytometry after stimulation with PMA plus ionomycin. T cell clones producing IL-17 alone ($n = 11$), IFN-γ alone ($n = 72$), or IL-4 alone ($n = 7$) were classified as Th17, Th1, and Th2, respectively, whereas those producing both IL-4 and IFN-γ, but not IL-17 ($n = 66$), were classified as Th0, and those producing both IL-17 and IFN-γ ($n = 50$) or both IL-17 and -4 ($n = 0$) were classified as Th17/Th1 or Th17/Th2, respectively. 11 clones apparently did not produce any of the three cytokines (Fig. 2, C and D).

Th17 and Th17/Th1 clones selectively express IL-23R and CCR6

The expression of IL-12Rβ2 and IL23-R in Th17 and Th17/Th1 clones was first examined and compared with that found in the other types of T cell clones. IL-12Rβ2 was expressed by all types of clones (Fig. 3 A), whereas IL-23R appeared to be selectively expressed by Th17 and Th17/Th1 clones (Fig. 3 B). However, this receptor was apparently not involved in Th17 or Th17/Th1 cell proliferation because IL-23 did not exert any proliferative activity on both types of clones (not depicted), even after their stimulation with anti-CD3 plus anti-CD28 Ab (Fig. 3 C). In contrast, the addition of IL-2, IL-12, and mainly of IL-15, potentiated the anti-CD3/28 Ab induced proliferation of both Th17 and Th17/Th1 clones (Fig. 3 C). Among chemokine R, a high expression of CXCR4 and CXCR6 by Th17 and Th17/Th1 clones was shared with Th1 or Th2 clones (unpublished data). Significantly higher levels of CCR4 (Fig. 4 A) and CCR5 (Fig. 4 B) were found in Th17 clones in comparison with all other types of clones, whereas Th17 and Th17/Th1 apparently lacked CXCR3-A, CXCR3-B, CCR3, CCR8 and CCR9 (not depicted). In contrast, CCR6 was selectively expressed by both Th17 and Th17/Th1, but not by Th1, Th2 or Th0, clones (Fig. 4 C). CCR6 expressed by Th17 clones was functionally active inasmuch as its ligand, CCL20, induced calcium influx in Th17, but not in Th1, clones, whereas the CCR9 ligand, CCL25, did not exert any effect (Fig. 4 D).

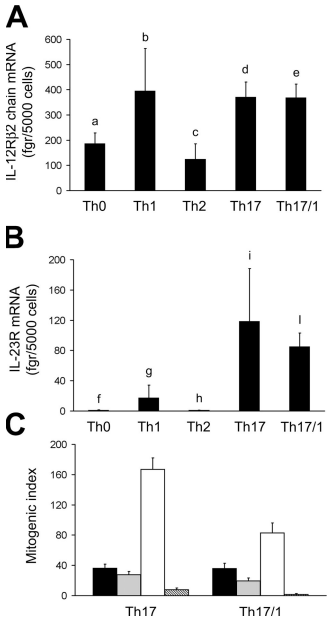

Figure 3. IL-23R and -12Rβ2 expression by Th17 and Th17/Th1 clones and their role in cell proliferation. IL-12Rβ2 (A) and -23R (B) mRNA levels were measured in Th0, Th1, Th2, Th17, and Th17/Th1 clones. Columns represent mean values (± the SEM) obtained in 10 clones from each type, except Th2, where they represent 7 clones. (A) d versus a, $P < 0.05$; d versus b, $P =$ ns; d versus c, $P < 0.05$; d versus e, $P =$ ns; e versus a, $P < 0.05$; e versus b, $P =$ ns; e versus c, $P < 0.05$. (B) i versus f, $P < 0.01$; i versus g, $P < 0.05$; i versus h, $P < 0.05$; i versus l, $P =$ ns; l versus f, $P < 0.001$; l versus g, $P < 0.05$; l versus h, $P < 0.05$. (C) Proliferative response of Th17 and Th17/Th1 clones after stimulation for 4 d with anti-CD3 plus anti-CD28 Ab without or with the addition of IL-2 (black), IL-12 (gray), IL-15 (white), or IL-23 (striped). Columns represent mean values (± the SEM) of mitogenic indexes (ratio between cpm obtained in the presence of anti-CD3/28 Ab with the indicated cytokine and cpm obtained with anti-CD3/CD28 Ab alone), obtained by testing seven clones from each type.

Th17 and Th17/Th1 clones exhibit similar functional properties

The ability of Th17 and Th17/Th1 clones to help Ab production by B cells and to display cytotoxic activity, as well as their susceptibility to the suppressive activity of an autologous CD4+CD25+Foxp3+ T cell clone were then evaluated and compared with the same activities assessed on Th1 and Th2 clones. Both Th17 and Th17/Th1 clones showed the ability to induce B cell production of IgM, IgG, and IgA, but not of IgE, with the latter appearing to be a selective property of Th2 clones (Fig. 5 A). In regard to the cytotoxic potential, Th17, Th17/Th1, and, as expected, Th2 clones exhibited granzyme A expression at levels significantly lower than those of Th1 clones, with the granzyme levels of Th17/Th1 clones higher than those present in Th17 and Th2 clones (Fig. 5 B). Moreover, the cytotoxic ability of Th17 and Th17/Th1 clones was significantly lower than that of Th1 clones (Fig. 5 C). Finally, the ability of Th17 and Th17/Th1 clones to proliferate in response to allogeneic stimulation in the presence of

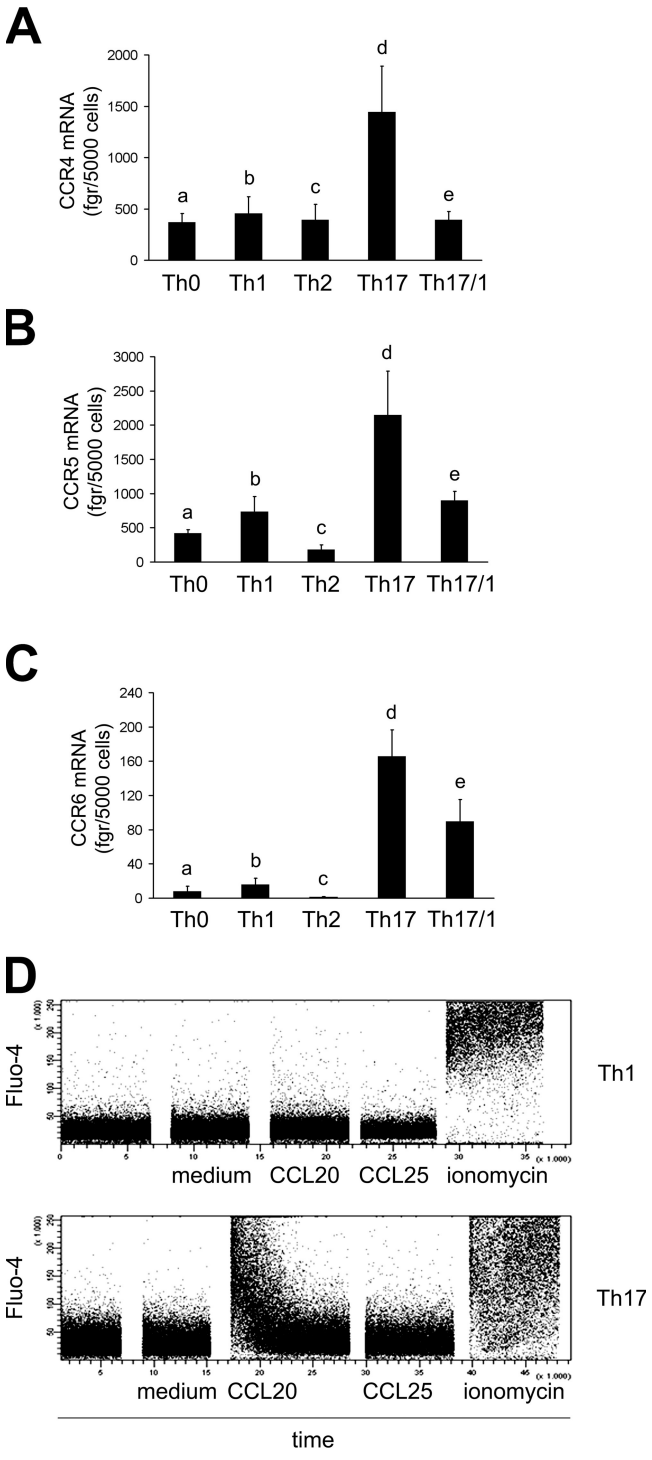

Figure 4. Selective expression of functionally active CCR6 by Th17 and Th17/Th1 clones. CCR4 (A), CCR5 (B), and CCR6 (C) mRNA levels in Th0, Th1, Th2, Th17, and Th17/Th1 clones. Columns represent mean values (± the SEM) obtained in 10 clones from each type, except Th2, where they represent 7 clones. (A) d versus a, $P < 0.005$; d versus b, $P < 0.05$; d versus c, $P < 0.05$; d versus e, $P < 0.005$; e versus a, $P =$ ns; e versus b, $P =$ ns; e versus c, $P =$ ns. (B) d versus a, $P < 0.001$; d versus b, $P < 0.05$; d versus c, $P < 0.05$; d versus e, $P < 0.01$; e versus a, $P < 0.05$; e versus b, $P =$ ns; e versus c, $P =$ ns. (C) d versus a, $P < 0.0001$; d versus b, $P < 0.001$; d versus c, $P < 0.001$; d versus e, $P =$ ns; e versus a, $P < 0.05$; e versus b, $P < 0.05$; e versus c, $P < 0.05$. (D) Cells from one Th17 and one Th1 clone were incubated with Fluo-4 and stimulated with medium alone, the CCR6 ligand, CCL20, the CCR9 ligand, CCL25, or ionomycin. Similar results were obtained with other two Th17 and three Th17/Th1 clones.

an autologous CD4+CD25+ T reg cell clone was assessed. This clone expressed Foxp3, did not produce cytokines in response to stimulation with PMA plus ionomycin (Fig. 6 A), and showed the ability to suppress the proliferative response of autologous CD25− T cells, as well as that of Th1 or Th2 clones (Fig. 6 B). In contrast, Th17 and Th17/Th1 exhibited a significantly lower susceptibility to the suppressive activity of the same autologous T reg clone (Fig. 6 B).

IL-12 down-regulates RORγt and IL-17 expression, but up-regulates T-bet and IFN-γ expression, in Th17 clones

We asked what was the relationship between Th17 and Th17/Th1, as both types of cells were already found ex vivo, and thus the latter could not result from a possible artifact caused by T cell culturing in vitro. To this end, we first assessed the expression of T-bet, which is a transcription factor that has been found to be selective for Th1 in both mice and humans (32), and of RORγt, which is a transcription factor that has been found to be selective for mouse Th17 (21). As control, the expression of GATA-3, which is a transcription factor selective for both mouse and human Th2 (32), was also assessed. As expected, Th2 clones selectively expressed GATA-3 and Th1 clones selectively expressed T-bet. In contrast, both Th17 and Th17/Th1 clones were found to express either RORγt or T-bet (Fig. 7, A–C). The presence of RORγt and T-bet proteins in Th17 clones was then assessed by confocal microscopy. Whereas RORγt was expressed in Th17, but not in Th1, clones (Fig. 7 D), T-bet protein could be detected in both Th1 and Th17 clones (Fig. 7 E).

Therefore, we asked whether Th17/Th1 could derive from each of the single Th1 or Th17 subset under particular environmental conditions. To answer this question, Th1 and Th17 clones were stimulated with anti-CD3 plus anti-CD28 Ab and IL-2 in the absence or presence of IL-12, and the expression of RORγt and T-bet, as well as the cytokine production profile, of these clones were evaluated after 7 d of culture. IL-12 down-regulated RORγt and up-regulated T-bet mRNA levels (Fig. 8 A), and induced a significant increase of IFN-γ and a reduction of IL-17, as shown by measuring cytokine mRNA levels (Fig. 8 B), cytokine concentrations into cell-free supernatants by ELISA (Fig. 8 C), or the proportions of IFN-γ– and IL-17–producing cells by flow cytometry after stimulation with PMA plus ionomycin (Fig. 8, D and E). We finally asked whether the IL-12–induced effects on cytokine production on Th17 could be influenced by the presence of IL-23. To this end, three Th17 and three Th1 clones were stimulated for 7 d with anti-CD3/28 Ab and IL-2 in presence of IL-12, -23, or -12 plus -23. The addition of IL-12 strongly increased the concentrations

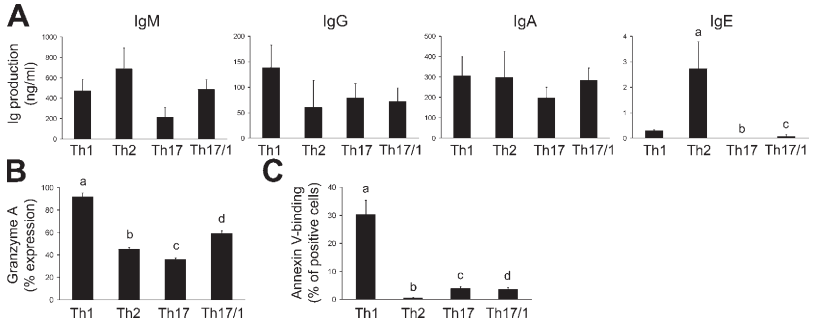

Figure 5. B cell helper ability and cytotoxic potential by Th17 and Th17/Th1 clones. (A) Measurement by ELISA of IgM, IgG, IgA, and IgE levels in supernatants of B cells cocultured for 7 d with autologous anti-CD3 Ab-stimulated Th1, Th2, Th17, or Th17/Th1 clones. Columns represent mean values (± the SEM) of five different clones from each group. b *versus* a, $P < 0.05$; c *versus* a, $P < 0.05$; b versus c, $P = ns$. (B) Granzyme A expression by different types of clones, as assessed by flow cytometry. Columns represent mean values (± the SEM) obtained with seven different clones of each type. c versus a, $P < 0.005$; c versus b, $P = ns$; c versus d, $P < 0.05$; d versus a, $P < 0.01$; d versus b, $P = ns$. (C) Cytotoxic activity by different types of clones, as assessed by detection of AnnexinV binding on target cells P815 with flow cytometry. Columns represent mean values (± the SEM) obtained with six different clones from each group. c versus a, $P < 0.01$; c versus b, $P = ns$; c versus d, $P < 0.05$; d versus a, $P < 0.05$; d versus b, $P = ns$.

of IFN-γ into cell-free supernatants of Th17 clones, and even more into those of Th1 clones (Figs. 8 C and 9 A), whereas the addition of IL-23 alone did not exhibit any effect on IFN-γ production by either Th17 or Th1 clones (Fig. 9 A). However, the addition of IL-23 partially reduced the IL-12–induced increase in the production of IFN-γ (Fig. 9 A), the proportions of Th17 clonal cells that acquired the ability to produce IFN-γ in response to IL-12, and their degree of proliferation (Fig. 9 B).

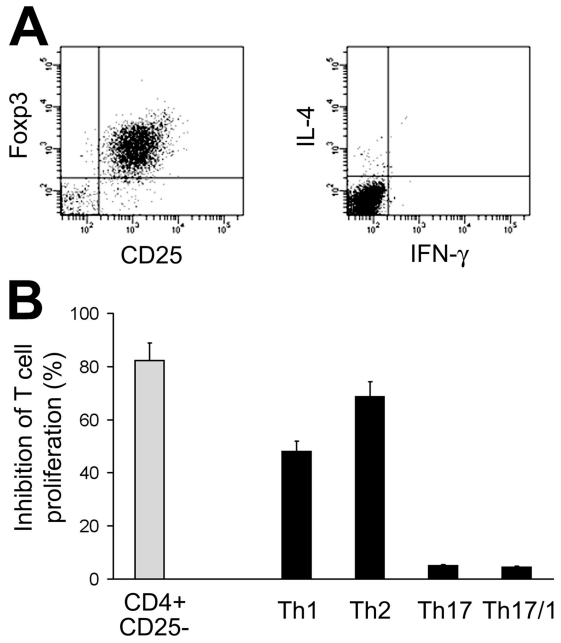

Figure 6. Low susceptibility of Th17 and Th17/Th1 clones to the suppressive activity of an autologous T reg cell clone. (A) Foxp3 and CD25 expression by the T reg cell clone (left) and its inability to produce IL-4 and IFN-γ in response to PMA plus ionomycin (right), as detected by flow cytometry. (B) Suppression by the T reg cell clone of the proliferative response of autologous CD4+CD25− T cells stimulated with allogeneic irradiated PBMC, as well as of an autologous Th1 or Th2, but not Th17 or Th17/Th1, clone. Columns represent mean values (± the SEM) of percentage of inhibition of the proliferative response in triplicate determinations.

Receptor expression and functional properties of Th17 clones derived from CD patients are shared by Th17 clones from normal gut and by freshly derived Th17 cells

Because the majority of experiments were performed on T cell clones derived from disease-affected areas of gut from patients with CD, we asked whether the observed phenotypic and functional features of Th17 cells were particular to the disease or whether they were shared by T cell clones derived from normal gut. As shown in Fig. 10 A, Th17 and Th17/Th1 clones derived under the same experimental conditions from normal gut showed nearly selective CCR6, IL-23R, and RORγt expression. Despite exhibiting IL-23R, even these clones did not proliferate in response to IL-23, both in the absence or presence of anti-CD3/CD28 Ab (unpublished data). Moreover, they displayed excellent B cell helper ability for IgM, IgG, and IgA, but not IgE, production and reduced cytolytic capacity, as Th17 clones derived from diseased areas of subjects with CD (unpublished data).

We finally asked whether the phenotypic and functional features of Th17 clones resulted from an artifact introduced by the cloning procedures or they were also shared by freshly derived Th17 cells. The ex vivo existence of T cells able to produce both IL-17 and IFN-γ, in addition to those able to produce IL-17 alone, has already been shown in Fig. 1. However, in subsequent experiments, we assessed the presence of Th17 cells in human PB and tonsil CD4+ T cell suspensions by taking advantage of the observation performed on Th17 clones of their apparently selective expression of CCR6. A small, but consistently detectable, number of CCR6+ CD4+ T cells was observed in both human PB and tonsils by flow cytometry. CCR6+ and CCR6− CD4+ T cells were then separated by immunomagnetic cell sorting and assessed for their ability to produce IL-17 and IFN-γ in response to

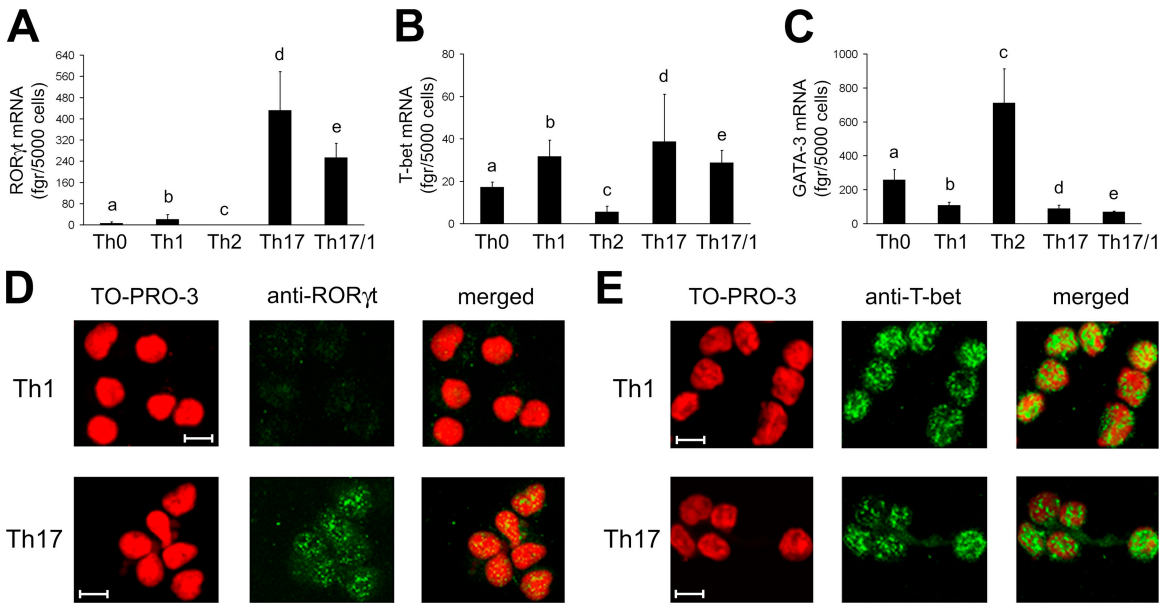

Figure 7. Expression of T-bet and RORγt by Th17 and Th17/Th1 clones. RORγt (A), T-bet (B), and GATA-3 (C) mRNA levels were measured in Th0, Th1, Th2, Th17, and Th17/Th1 clones. Columns represent mean values (± the SEM) in 10 clones from each type, except Th2, where they represent 7 clones. (A) d versus a, $P < 0.0001$; d versus b, $P < 0.005$; d versus c, $P < 0.05$; d versus e, $P =$ ns; e versus a, $P < 0.001$; e versus b, $P < 0.01$; e versus c, $P < 0.05$. (B) d versus a, $P =$ ns; d versus b, $P =$ ns; d versus c, $P < 0.05$; d versus e, $P =$ ns; e versus a, $P =$ ns; e versus b, $P =$ ns; e versus c, $P =$ ns; b versus c, $P < 0.05$. (C) d versus a, $P =$ ns; d versus b, $P =$ ns; d versus c, $P < 0.05$; e versus a, $P < 0.001$; e versus b, $P =$ ns; e versus c, $P < 0.001$; b versus c, $P < 0.001$. RORγt (D) and T-bet (E) protein expression, as assessed by confocal microscopy. Data from one Th17 and one Th1 clone are reported. Similar pictures were obtained in other two Th17 and two Th1 clones. Bars, 10 μm. TO-PRO-3 (red) was used to counterstain the nuclei.

stimulation with PMA plus ionomycin. Cell sorting enabled a strong enrichment for CCR6+ T cells (consistently >90%), whereas virtually no CCR6+ cells were detected in the CCR6-depleted CD4+ T cell population (unpublished data). Notably, all T cells producing IL-17 alone or IL-17 plus IFN-γ in response to PMA plus ionomycin were contained within the CCR6-enriched population, whereas CCR6-depleted T cells could be induced to produce IFN-γ, but not IL-17 (Fig. 10 B, top). Moreover, IL-23R and RORγt mRNA levels in CCR6-enriched and -depleted T cells were measured. Levels of both IL-23R and RORγt mRNA were found to be significantly higher in the CCR6-enriched than in the CCR6-depleted population from either PB or tonsils (Fig. 10 B, bottom). Again, despite the presence of IL-23R, even freshly derived IL-17–producing CD4+ T cells did not proliferate in response to IL-23, both in the absence or presence of anti-CD3/CD28 mAbs (unpublished data).

DISCUSSION

Several studies demonstrate the existence in experimental animal models of a novel subset of Th effectors that are distinct from the classic Th1 and Th2, and that have been named Th17 because of their ability to produce IL-17. These cells represent a distinct lineage that originates mainly in the presence of TGF-β and IL-6 and need the presence of IL-23 for their expansion and/or maintenance. IL-23 is a member of the IL-12 cytokine family, which shares the p40 subunit with IL-12 and differs in the expression of p19 instead of p35. At least in mice, Th17 cells arise as a part of mucosal host defense and their major role seems to be protection against infections sustained by extracellular bacteria (33–35), but under certain circumstances they can also be involved in the pathogenesis of chronic inflammatory disorders, including some models of autoimmune diseases (9–14). Notably, in these models, IFN-γ produced by Th1 cells, which are crucial for the protection against intracellular bacteria, does not appear to be pathogenic, but instead appears protective, as inhibition of IFN-γ signaling enhances the development of pathogenic Th17 and exacerbates autoimmunity (20). Even the neutralization of IL-4 produced by Th2 is critical in neutralizing the development of IL-17; however, neither IFN-γ nor IL-4 seem to be effective on already established Th17 (20).

Currently, very little is known about human Th17. Some studies have shown that the presence of IL-17 mRNA or IL-17 protein in tissues or biological fluids of subjects with different autoimmune disorders (23–27) or other chronic inflammatory diseases (28–30). The results of this study provide evidence of the existence of increased numbers of CD4+ T cells producing IL-17 in the disease-affected gut areas of subjects with CD, compared with either PB or apparently healthy gut areas. Based on this finding, IL-17–producing CD4+ T cells derived from the gut of CD subjects were expanded in vitro and cloned to get an amount of these cells suitable for phenotypic and functional investigations.

The first information produced by our study was that a remarkable proportion of IL-17–producing CD4+ T cells

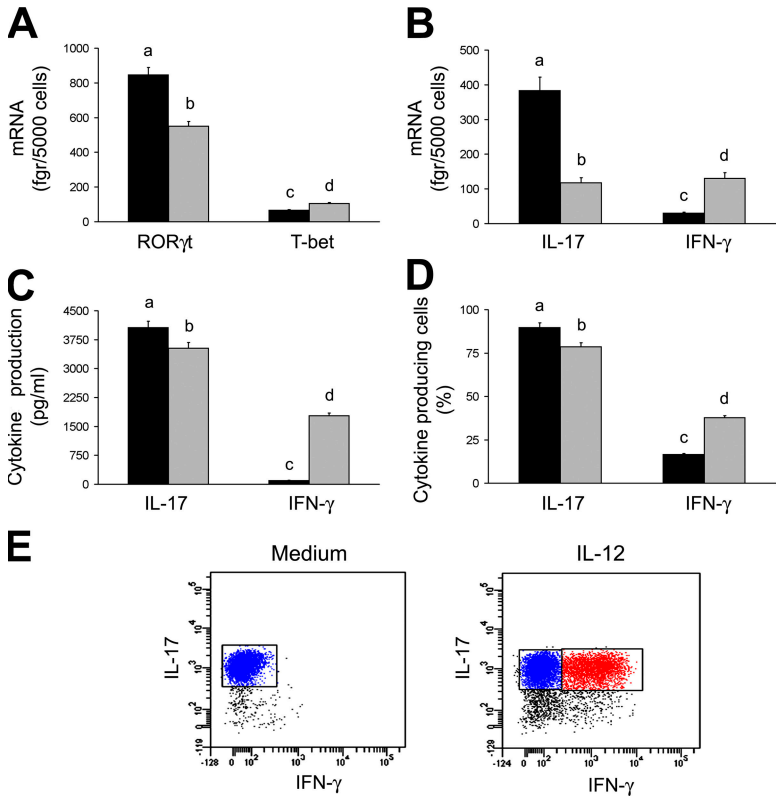

Figure 8. Effects of IL-12 on RORγt and T-bet expression and on IFN-γ and IL-17 production by Th17 clones. (A) Decreased RORγt and increased T-bet mRNA expression in five Th17 clones after stimulation for 7 d with anti-CD3 plus anti-CD28 Ab and IL-2, in the absence (black) or presence (gray) of IL-12. a versus b, $P < 0.05$; c versus d, $P < 0.05$. (B) Decreased IL-17 and increased IFN-γ mRNA levels in Th17 clones cultured under the conditions described in A. Columns represent mean values (± the SEM). a versus b, $P < 0.01$; c versus d, $P < 0.01$. (C) Reduced levels of IL-17 and increased levels of IFN-γ in supernatants of Th17 clones, as measured by ELISA. a versus b, $P < 0.05$; c versus d, $P < 0.001$. (D) Decreased proportions of IL-17–producing and increased proportions of IFN-γ–producing cells in response to PMA plus ionomycin by Th17 clones. a versus b, $P < 0.05$; c versus d, $P < 0.05$. (E) Flow cytometric analysis of IL-17 and IFN-γ production by one representative Th17 clone.

share the ability to produce IFN-γ, whereas CD4+ T cells producing both IL-17 and -4 were never observed. The cells producing both IL-17 and IFN-γ, which we named Th17/Th1, were not the result of an in vitro artifact, as about one third of ex vivo–derived IL-17–producing CD4+ T cells already showed a double IL-17+IFN-γ+ phenotype. When examined at clonal level, Th17 and Th17/Th1 shared different phenotypic properties from the other types of clones, some of which appearing to be selective. For example, only Th17 and Th17/Th1 clones exhibited the expression of IL-23R. Interestingly, the IL-23R did not apparently play any role in the expansion of these cell subsets, because they did not proliferate in response to IL-23 alone, and IL-23 did not potentiate their anti-CD3/CD28 Ab–induced proliferation. Thus, it is probable that IL-23 is not important for human Th17 expansion, but rather for their survival and/or maintenance. In contrast, under the same conditions, anti-CD3/28–stimulated Th17 and Th17/Th1 clones proliferated in response to IL-2, -12, or -15. The ability of IL-2 to promote the proliferation of human Th17, a finding that was also supported by the observation that IL-2 was the growth factor used in this study to expand Th17 from both PB or gut mucosa and to get Th17 clones, is apparently at variance with recent results showing that IL-2 constrains generation of mouse Th17 cells (36). The reason for such a discrepancy is presently not clear. Among chemokine R, both Th17 and Th17/Th1 clones apparently lacked CXCR3-A, CXCR3-B, CCR3, CCR8, and CCR9, but exhibited high levels of CXCR4 and CXCR6. Th17 clones also expressed significantly higher levels of CCR4 and CCR5 than the other types of clones, and both Th17 and Th17/Th1 selectively expressed CCR6. CCR4 expression has been associated with the ability of cells to traffic into peripheral tissues (37). CCR6 has been found to be expressed by B cells, DCs, and memory, but not naive, T cells. However, its ligand, MIP-3α/CCL20 does not exert chemotactic activity on B cells, but only on memory T cells (38). Interestingly, however, CCR6 expression by memory T cells is lost after their prolonged TCR triggering (39). Thus, the selective expression of CCR6 by Th17 and Th17/Th1, but not by Th1, Th2, or Th0, clones may mean that Th17 and Th17/Th1 are the only memory T cells that continue to express CCR6 even after prolonged antigen activation, thereby maintaining the possibility of recruitment in response to MIP-3α/CCL20. This finding may

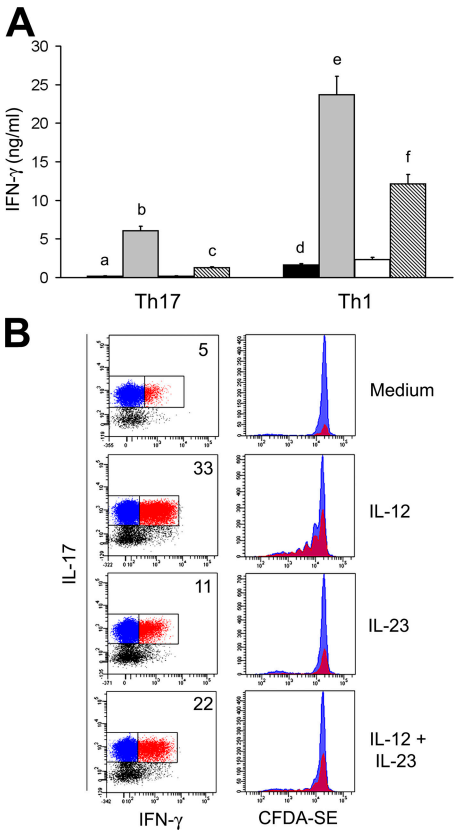

Figure 9. IL-23 partially inhibits the increase of IFN-γ production and the proliferation induced by IL-12 on Th17 clones. (A) Detection of IFN-γ into cell-free supernatants of Th17 and Th1 clones cultured for 7 d with anti-CD3/CD28 and IL-2 (black), plus IL-12 (gray), -23 (white), or -12 plus -23 (striped). Columns represent mean values (± the SEM) obtained in three different Th17 and in three different Th1 clones. a versus b, $P < 0.05$; b versus c, $P < 0.05$; d versus e, $P < 0.05$; e versus f, $P < 0.05$. (B) Flow cytometric analysis of IL-17 and IFN-γ production by one representative Th17 clone, and of its proliferation, as assessed by CFDA-SE content. Similar results were obtained in other two Th17 clones.

have important implications for the long-term maintenance of Th17 influx, supporting their important pathogenic role into the inflamed tissues.

Human Th17 and Th17/Th1 clones also shared some peculiar functional properties. We found that both types of clones were able, as Th1 clones, to provide B cell help for the production of IgM, IgG, and IgA, but not IgE, Ab. In contrast, as Th2 clones, they showed poor granzyme A expression and low cytotoxic capability, thus displaying an intermediate functional pattern in regard to B cell helper and cytotoxic activity between Th1 and Th2 clones. However, the most impressive functional feature of both Th17 and Th17/Th1 clones was their lower susceptibility, in comparison with both Th1 and Th2 clones, to the suppressive activity of an autologous T reg cell clone derived from circulating CD4+CD25highFoxp3+ cells. Although the mechanism for the higher resistance of Th17 and Th17/Th1 to the action of T reg cells is still unclear, this finding can provide additional support for an important role of IL-17–producing cells in the maintenance of the inflammatory processes in autoimmune disorders.

The receptor expression and functional capabilities observed in T cell clones derived from the affected areas of gut from subjects with CD were neither related to their pathological source nor to some in vitro artifact referable to the cloning procedure. Indeed, similar receptor expression and function were found in T cell clones derived from healthy gut areas of subjects who underwent colectomy because of colon carcinoma. More importantly, the existence of Th17 and Th17/Th1 cells, as well as their expression of CCR6, IL-23R, and RORγt was also observed in freshly derived CD4+ T cells from gut, PB, and tonsil. In this respect, it is of note that sorting of CCR6+ cells allowed to obtain populations strongly enriched in IL-17–producing cells, suggesting that this marker may be useful to get high numbers of Th17 cells and, therefore, of potentially great help in clarifying their pathophysiologic role in humans. Of note, during the revision process of this paper, a study was published (40) that also reports preferential expression of CCR6 and RORγt by human PB Th17 cells.

Another important finding emerging from this study was the apparent discrepancy with some results reported in mice in regard to the relationship between Th17 and Th1. In a first proposed mouse model, it was suggested that the early differentiation of Th1 and Th17 from naive CD4+ T cell precursors was shared, and thus Th1 and Th17 diverged contingent upon the selective availability of IL-12 or -23 acting on a common "Th1 precursor" or "pre-Th1" intermediate that coexpressed IL-12R and -23R (41). However, in a subsequently proposed mouse model, which was based on the demonstration that the differentiation into Th17 depends upon TGF-β and IL-6 rather than upon IL-23, it was suggested that Th1 and Th17 subsets were not overlapping and represented distinct lineages (19, 20). In this study, we do not provide any evidence for the mechanisms responsible for the differentiation of human Th17, as well as for the question of whether in humans, as in mice, Th17 and Th1 represent distinct lineages. However, we not only demonstrated the ex vivo existence of CD4+ T cells able to produce both IL-17 and IFN-γ but we were also able to induce Th17 clones to produce IFN-γ in addition to IL-17 after their culturing in the presence of IL-12. The possible relationship between human Th17 and Th1 was also supported by the demonstration that Th17 clones expressed not only RORγt, a finding that is in agreement with the results reported in mice (21), but also remarkable amounts of the Th1 transcription factor T-bet, and that amounts of both T-bet and RORγt were comparable in human Th17 and Th17/Th1 clones.

Interestingly, the ability of IL-12 to induce Th17 cells to produce IFN-γ, in addition to IL-17 associated with the up-regulation of T-bet and the down-regulation of both RORγt expression and IL-17 production in Th17 clones. This means that in humans, even established Th17 clones, independent of whether they have a distinct or common origin with Th1,

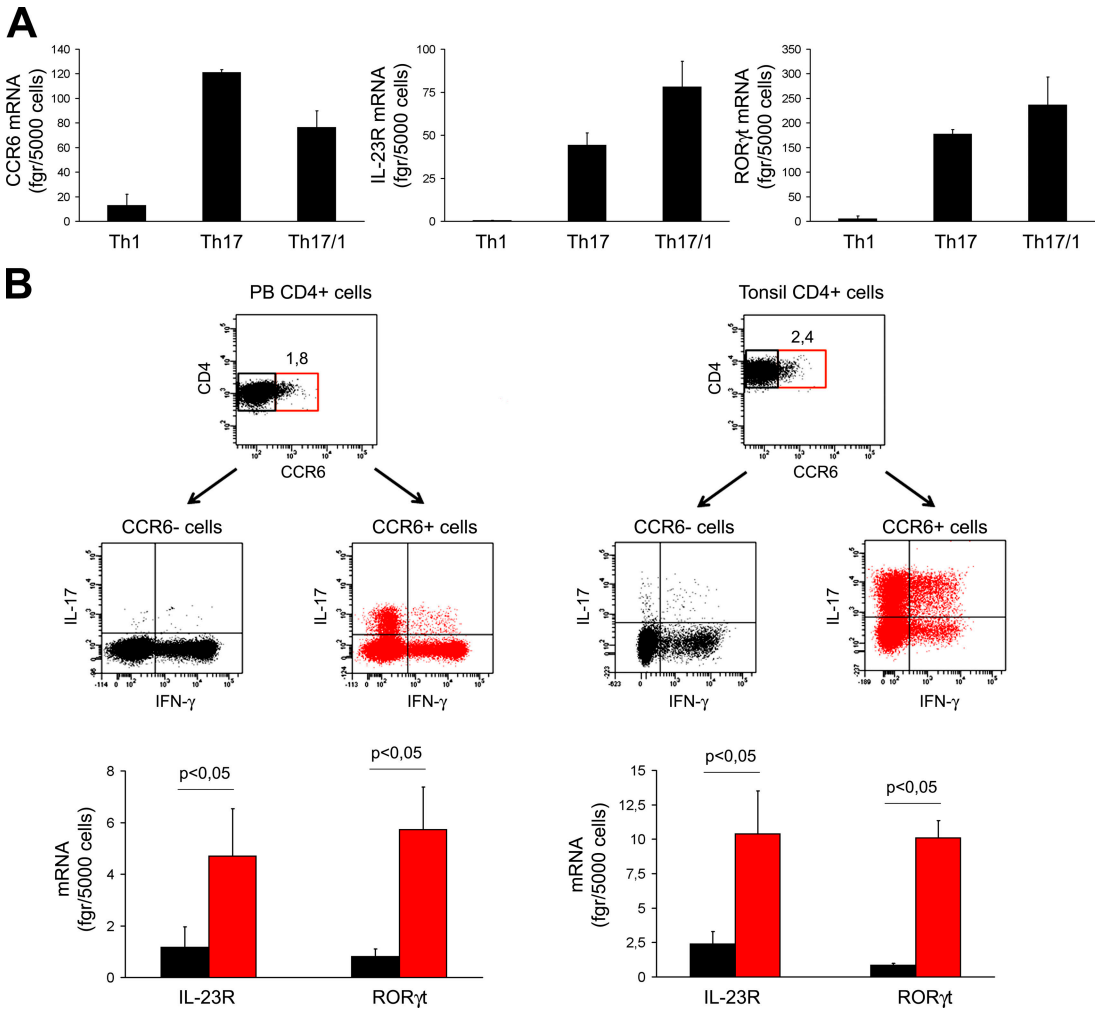

Figure 10. Th17 clones derived from normal gut, as well as freshly derived IL-17–producing CD4+ T cells, exhibit similar features to gut CD-derived Th17 clones. (A) CCR6, IL-23R, and RORγt mRNA expression by Th1, Th17, and Th17/Th1 clones derived from normal gut of subjects who underwent colectomy because of colon carcinoma. Columns represent mean values (± the SEM) obtained from five clones of each type. (B) Detection of IL-17 and IFN-γ production by CCR6-depleted and CCR6-enriched CD4+ T cell populations from PB or tonsil (top). Measurement of IL-23R and RORγt mRNA levels in CCR6-depleted (black columns) and CCR6-enriched (red columns) CD4+ T cells from PB (left) or tonsils (right). Columns represent mean values ± the SEM were obtained in three different donors of PB or tonsil.

are responsive to IL-12 and are still sufficiently flexible to acquire the ability to produce IFN-γ. These findings strongly suggest that in human Th17 both RORγt and T-bet can play an important modulatory role. The possibility that T-bet–dependent signaling was responsible for the increased production of IFN-γ by our T cell clones was not directly explored in this study, and thus remains unproved. However, it has recently been shown that ectopic T-bet expression in mouse Th17 cells can promote IFN-γ secretion and decrease IL-17 production, suggesting that even in mice the Th17 phenotype is not stable and can give rise to IFN-γ production in vitro via T-bet–mediated signaling (42). In this respect, it should be taken into account that other recent data in mice, again, raise the question of the developmental relationship between Th1 and Th17. First, the existence of a dual population of IL-17- and IFN-γ–producing cells has also been reported in studies performed in mice (42, 43). Second, T-bet has been found to be required for optimal IL-17 production in the presence of IL-23 (44). Finally, therapeutic administration of a small interfering RNA specific for T-bet significantly improved the clinical course of established EAE by limiting the differentiation of autoreactive Th1 and inhibiting pathogenic Th17 via regulation of IL-23R (45). These findings suggest that even in mouse models T-bet and RORγt may play distinct, yet complementary, roles in the development of Th17 cells. In this study, the IL-12–induced IFN-γ production by Th17 clones was partially inhibited by the contemporaneous presence of IL-23. Because a similar inhibitory effect by IL-23 on the IL-12–induced IFN-γ production could also be observed in Th1 clones, which do not express IL-23R, it is reasonable to suggest that it was mediated by the ability of IL-23 to bind both IL-23R and IL-12Rβ1

via the p40 chain shared between IL-12 and -23, which therefore display binding competition. Because IFN-γ is a well known inducer of IL-12Rβ2 (46), it can strongly influence the susceptibility of Th17 to the activity of IL-12, thus favoring the shifting of these cells toward the Th1 phenotype. This finding may also account for the reported inhibitory activity of IFN-γ on the development of mouse Th17, as well as for its protective effects (47).

In conclusion, our data provide the first detailed phenotypic and functional characterization of human Th17 isolated from the disease-affected gut mucosa of subjects with CD, as well as from normal tissues, showing that Th17 display distinct functional properties from Th1 or Th2 cells and identifying IL-23R, CCR6, and RORγt as Th17–specific markers. In addition, we describe a new subset of IFN-γ–producing Th17 sharing features with both Th1 and Th17, which has not been previously reported in mice. This novel subset exists in vivo in humans and can be induced in vitro by stimulating Th17 in presence of IL-12, thus raising new issues on the Th17 developmental and/or functional relationship with Th1.

MATERIALS AND METHODS

Subjects. Small bowel specimens were obtained from the apparently healthy areas of 10 subjects who underwent surgery because of colon cancer, the disease-affected areas of 10 subjects with CD, and the disease-affected areas of 5 subjects with ulcerative colitis. PB samples were obtained from the 10 subjects with CD and from 13 healthy donors. Tonsillar specimens were obtained from three children, who were subjected to tonsillectomy because they were affected by chronic tonsillitis. The procedures followed in the study were in accordance with the ethical standards of the Regional Committee on Human Experimentation.

Reagents. The medium used was RPMI 1640 (Seromed) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 2×10^{-5} M 2-mercaptoethanol (2-ME; all from Invitrogen), and 10% FCS. FITC-, PE-, allophycocyanin-orperidin chlorophyll protein–conjugated anti-CD3, CD4, CD8, CD25, CD45RA, CD45RO, TCRαβ, TCRγδ, IFN-γ, IL-4, and isotype-matched control mAbs were purchased from BD Biosciences. Anti–IL-17 and -Foxp3 mAbs were obtained from eBioscience. PMA, ionomycin, and brefeldin A were purchased from Sigma-Aldrich. Anti-CCR6 mAb was from R&D Systems. Anti-RORγt rabbit polyclonal Ab was purchased from Abcam. Anti–T-bet rabbit polyclonal Ab was from Santa Cruz Biotechnology.

T cell recovery. For isolation of T lymphocytes, epithelial gut layer was removed by calcium- and magnesium-free PBS, supplemented with 1 mM EDTA and 1 mM DTT (Sigma-Aldrich). Fragments were mechanically disrupted with a MEDI machine (BD Biosciences). MCs were then separated from the other cells by Ficoll-Hypaque gradient centrifugation. PBMC and tonsil mononuclear cell suspensions were also obtained by centrifugation on Ficoll-Hypaque gradient.

Flow cytometry. Analysis of cytokine synthesis at the single-cell level was performed as previously reported (48). Foxp3 flow cytometric determination was performed according to the manufacturer's instructions (eBioscience).

Confocal microscopy. Detection of RORγt and T-bet protein in Th17 and Th1 clones was performed by confocal microscopy, by using a previously described technique (49).

Immunomagnetic cell sorting. Separation of CCR6+ from CCR6− PB or tonsillar CD4+ T cells was performed by immunomagnetic cell sorting, as previously described (49).

T cell expansion and cloning. Gut-derived and PB T lymphocytes were expanded by stimulation for 10 d with 5 μg/ml anti-CD3 plus 5 μg/ml anti-CD28 mAb (BD Biosciences) and 20 UI/ml IL-2 (Eurocetus). On day 10, T cell blasts were cloned under limiting dilution (0.3 cell/well), as previously reported (50). T reg clones were generated from CD4+CD25+Foxp3+ T lymphocytes obtained from PBMC of one subject with CD. To this end, DCs were generated from CD14+ cells cultured for 5 d with IL-4 and GM-CSF. CD4+CD25− or CD25high T cells were obtained from PBMC by cell sorting. CD4+CD25high cells were cultured with autologous DCs (1:1 ratio) and 100 U/ml IL-2 for 10 d and cloned under limiting dilution (0.3 cell/well).

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 (51). Primers and probes used were purchased from Applied Biosystems.

Proliferation assay. 5×10^4 total T cell blasts from each clone were stimulated with 2 μg/ml anti-CD3 plus 2 μg/ml anti-CD28 mAb (BD Biosciences), in the absence or presence of different cytokines (rIL-2, 50 IU/ml; rIL-12, 2.5 ng/ml; rIL-15, 7 ng/ml; rIL-23, 20 ng/ml). In T cell suppression experiments, 2×10^4 T cell blasts from Th1, Th2, Th17, or Th1/Th17 clones were cultured with 4×10^4 irradiated (9,000 rad) T cell-depleted allogeneic PBMC, with or without 2×10^4 T cell blasts from a CD4+CD25^{high}Foxp3+ T clone. Cultures were pulsed for the last 8 h with 0.5 μCi (0.0185 MBq) of ^{3}H-TdR (GE Healthcare), harvested, and radionuclide uptake was measured by scintillation counting. In some experiments, cells were labeled with CFDA-SE (Invitrogen), as previously described (52).

Calcium influx. 5×10^5 cells from a Th1 and a Th17 clone were incubated in complete medium in the presence of 2.5 μM Fluo-4 AM (Invitrogen) for 30 min in the dark at room temperature. After dye loading, cells were washed twice in complete medium and incubated at 37°C in the presence of 5% CO_2 for an additional 30 min. Cells were analyzed by a BD-LSRII flow cytometer using the Diva software (Becton Dickinson). The basal fluorescence intensity was assessed before the stimulus. The stimulation was obtained in presence of medium alone or in the presence of 1 μg/ml CCL20, 1 μg/ml CCL25, or 1 μM ionomycin.

B cell helper activity. 2×10^5 human B cells, isolated as CD19+ cells by immunomagnetic cell sorting (49), were stimulated with 10^5 Th1, Th2, Th17, or Th1/Th17 blasts stimulated with 100 ng/ml anti-CD3 Ab. On day 7, supernatants from each cell culture were collected and evaluated by ELISA for IgM, IgG, IgA, and IgE content.

Flow cytometric detection of granzyme A and evaluation of cell cytotoxicity. Granzyme A expression by Th1, Th2, Th17, or Th17/Th1 clones was assessed according to the manufacturer's instructions (BD Biosciences). Th1, Th2, Th17, and Th1/Th17 cell clones were tested for their ability to kill P815 target cells upon anti-CD3 mAb activation (5 μg/ml) by evaluating the percentage of AnnexinV-binding cells with flow cytometry, as described elsewhere (52).

Statistics. Statistical comparison between groups was performed using the Mann-Whitney test or the Wilcoxon test, as appropriate. Differences were considered as statistically significant when $P < 0.05$.

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