nature immunology

Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T_H-17, T_H1 and T_H2 cells

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Interleukin 22 (IL-22) is a member of the IL-10 cytokine family that is involved in inflammatory and wound healing processes. Originally considered a T helper type 1 (T_H1)-associated cytokine, IL-22 has since been shown to be produced mainly by IL-17-producing helper T cells (T_H-17 cells). Here we describe a previously uncharacterized IL-22-producing human helper T cell population that coexpressed the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10. These cells were distinct from both T_H-17 cells and T_H1 cells. Downregulation of either the aryl hydrocarbon receptor (AHR) or the transcription factor RORC by RNA-mediated interference affected IL-22 production, whereas IL-17 production was affected only by downregulation of RORC by RNA-mediated interference. AHR agonists substantially altered the balance of IL-22- versus IL-17-producing cells. This subset of IL-22-producing cells may be important in skin homeostasis and pathology.

Interleukin 22 (IL-22), a member of the IL-10 (A001243) family[1–3], is important in regulating inflammatory responses associated with many autoimmune diseases. The receptor for IL-22 is a complex of two chains, IL-10R2 (A001245) and IL-22R, and is expressed on epithelial cells of organs in the digestive and respiratory tracts and the skin but not on immune cells, such as T cells, B cells, monocytes or dendritic cells[2–5]. Evidence is accumulating that IL-22 is important in epithelial cell homeostasis and in infection and inflammation, as well as in tissue repair and wound healing[4,6,7]. The involvement of IL-22 in infection is demonstrated by its importance in the early immune response to *Citrobacter rodentium* in the gut[7]. IL-22 seems to be important in many diseases, including hepatitis and psoriasis; however, in many cases it is still uncertain whether IL-22 ameliorates or exacerbates the disease[6,8–10]. In a mouse model of psoriasis, IL-23-mediated dermal inflammation is suppressed in *Il22*$^{-/-}$ mice[11] and in mice treated with antibody to IL-22 (anti-IL-22)[12]. Additionally, expression of IL-22 mRNA in psoriatic skin lesions, as well as serum IL-22 concentrations, are higher in patients affected by the autoimmune skin disease psoriasis than in unaffected lesions or normal subjects[6]. Whereas IL-22 is important in the initiation and exacerbation of psoriasis, its involvement in other autoimmune diseases such as inflammatory bowel disease remains to be established. Expression of IL-22 mRNA is higher in inflamed lesions from patients with Crohn's disease, and IL-22 is associated with higher expression of inflammatory mediators[8]. However, in a mouse model of ulcerative colitis, IL-22 has been shown to diminish intestinal inflammation[9]. The anti-inflammatory properties of IL-22 have also been noted in hepatitis, in that IL-22 seems to provide protection to hepatocytes during acute liver inflammation[10].

Although it was originally thought to be a T helper type 1 (T_H1)-associated cytokine[2,3,13,14], IL-22 has since been linked with IL-17 expression in IL-17-producing T helper cells (T_H-17 cells)[11,12,15–18]. IL-22 is also produced in natural killer T cells[19] and in small amounts in natural killer cells[3]. It has been shown that natural killer cell–like NKp46$^+$ cells are major IL-22 producers in the mucosa, in particular in the intestine[20–25]. These IL-22-producing cells expressed the transcription factor RORγt (called 'RORC' in humans). In addition, lymphoid tissue–inducer cells (LTi cells), which depend on RORγt[26] and are crucial in the initiation and maintenance of lymphoid structures, also produce large amounts of IL-22 both in humans[23] and in mice[27], and it is possible that IL-22 production by LTi cells is important for the tissue-regenerating ability of these cells.

In the mouse, IL-22 is produced mainly by T_H-17 cells, and the transcription factor RORγt, which controls the generation of T_H-17 cells, also seems to be critical for IL-22 production, as retrovirus-mediated transfer of RORγt into mouse T cells endows these cells with the ability to produce not only IL-17 (ref. 28) but also IL-22 (ref. 29). However, transduction of RORC into naive human cells does not lead to the production of IL-22, which suggests a difference between humans and mice in the requirement for RORC in IL-22 production[30]. A transcription factor that might be involved in IL-22 expression is the aryl hydrocarbon receptor (AHR; A000229). AHR interacts with many ligands, including environmental toxins such as 2,3,7,

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Received 20 March; accepted 10 June; published online 5 July 2009; corrected online 13 July 2009; doi:10.1038/ni.1770

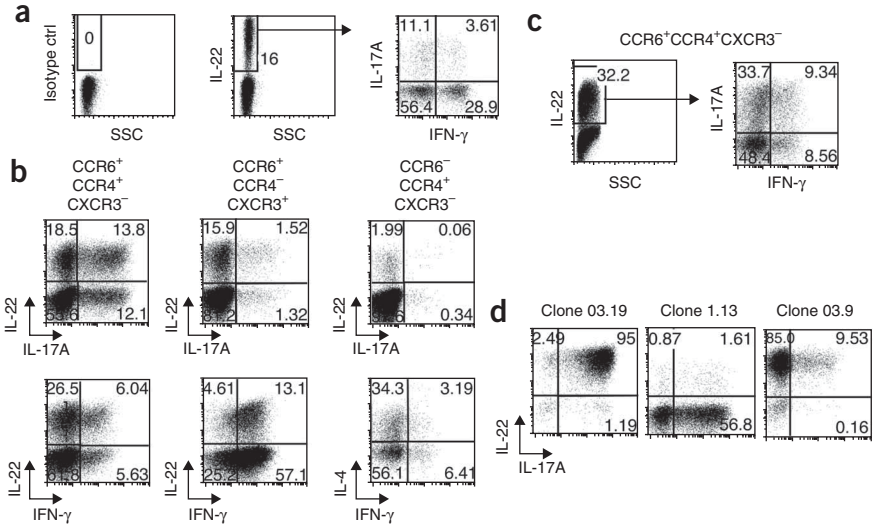

Figure 1 Production of IL-22 and IL-17 can be 'disconnected' in peripheral blood CD4$^+$ memory T cells. (**a**) Flow cytometry of the production of IL-17A and IFN-γ by a population of IL-22-producing peripheral blood CD4$^+$ memory T cells stimulated for 6 h with TPA and ionomycin. Isotype ctrl, staining with isotype-matched control antibody. SSC, side scatter. Data are representative of three experiments with a total of five different donors (one presented here). (**b**) Flow cytometry of the production of IL-17A, IFN-γ, IL-22 and IL-4 by sorted CCR6$^+$CCR4$^+$CXCR3$^-$ (left), CCR6$^+$CCR4$^-$CXCR3$^+$ (middle) and CCR6$^-$CCR4$^+$CXCR3$^-$ (right) CD4$^+$ memory T cell populations expanded for 7 d with Dynabeads coated with monoclonal anti-CD3 plus monoclonal anti-CD28, in the presence of IL-2 (20 IU/ml), then restimulated for 6 h with TPA and ionomycin. Data are representative of three experiments. (**c**) Flow cytometry of the CCR6$^+$CCR4$^+$CXCR3$^-$ subset in **b**, showing production of IL-17A and IFN-γ by IL-22$^+$ cells. Data are representative of three experiments with one donor of three presented here. (**d**) Flow cytometry of the production of IL-17A and IL-22 by clones obtained from the CCR6$^+$CCR4$^+$CXCR3$^-$ subset in **b** (n = 3 clones from one donor). Data are representative of two experiments with 20–50 clones. Numbers in or adjacent to outlined areas and in quadrants indicate percent cells in each.

8-tetrachlorodibenzo-*p*-dioxin (TCDD) and tryptophan metabolites such as 6-formylindolo[3,2-b]carbazole (FICZ), which has been proposed to be an endogenous AHR ligand present in humans[31]. AHR has been reported to be another transcriptional regulator of T_H-17 cells, as agonists of this receptor increase expression of IL-17 and IL-22 (refs. 32,33). T cells of AHR-deficient mice are unable to produce IL-22, which suggests that this receptor is essential for IL-22 production[32]. Here we describe a population of CCR6$^+$CCR4$^+$CCR10$^+$ human memory T cells that produced IL-22 as well as IL-13 but not IL-17 or interferon-γ (IFN-γ), and report that RORC and AHR regulated the production of IL-22 by these cells.

RESULTS

CD4$^+$ T cells that produce IL-22 but not IFN-γ or IL-17

We analyzed the expression of IL-22 and IL-17 in CD4$^+$ memory T cells from the blood of healthy adult humans after stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) plus ionomycin. A substantial proportion of IL-22-producing cells was negative for both IL-17 and IFN-γ (**Fig. 1a**). To characterize these IL-22-producing cells in more detail, we took advantage of published data indicating that circulating memory T_H1, T_H2 and T_H-17 cells can be distinguished on the basis of expression of the chemokine receptors CCR6, CCR4 and CXCR3 and that populations of IL-22-producing cells are enriched for CCR6$^+$ cells[34]. Therefore, we sorted CD4$^+$ memory T cells from the peripheral blood by flow cytometry into CCR6$^+$CCR4$^+$CXCR3$^-$, CCR6$^+$CCR4$^-$CXCR3$^+$ and CCR6$^-$CCR4$^+$CXCR3$^-$ subsets and stimulated these cells for 7 d with beads coated with anti-CD3 plus anti-CD28. After restimulation with TPA and ionomycin, most IL-22-producing cells were in either the CCR6$^+$CCR4$^+$CXCR3$^-$ (T_H-17-enriched) population or the CCR6$^+$CCR4$^-$CXCR3$^+$ (T_H1-enriched) population (**Fig. 1b**), whereas a few IL-22$^+$ cells were present in the CCR6$^-$CCR4$^+$CXCR3$^-$ (T_H2-enriched) population, which had abundant production of IL-4. In the T_H1 population, most IL-22$^+$ cells coexpressed IFN-γ (**Fig. 1b**); however, a substantial proportion of IL-22$^+$ cells in the enriched T_H-17 population was negative for both IL-17 and IFN-γ (**Fig. 1c**), which suggested that a separate subset of IL-22 producing cells was present in the CCR6$^+$ population. To confirm that the CCR6$^+$CCR4$^+$CXCR3$^-$ population contained separate IL-17- and IL-22-producing cells, we did clonal analysis of this subset. Analysis of intracellular cytokine production by individual clones showed clones with different phenotype in terms of production of IL-22 and IL-17, including IL-22$^+$IL-17$^+$ 'double-producers', as well as IL-17$^+$IL-22^{lo} and IL-17^{lo}IL-22$^+$ clones (**Fig. 1d**).

IL-22 is prominently expressed by T cells in skin inflammation[6,35]. Thus, we analyzed the expression of another skin-homing chemokine receptor, CCR10, on peripheral blood memory T cells and found that it was expressed on a considerable proportion of CCR6$^+$CCR4$^+$CXCR3$^-$ CD4$^+$ memory T cells but not on CCR6$^+$CCR4$^-$CXCR3$^+$ CD4$^+$ memory T cells (**Fig. 2a**). We sorted CCR10$^+$ and CCR10$^-$ cells from the CCR6$^+$CCR4$^+$CXCR3$^-$ population and analyzed the cytokine expression of these cells. As control, we also sorted a CXCR3$^+$ cell population enriched for T_H1 cells[34]. We stimulated the sorted cells with beads coated with anti-CD3 plus anti-CD28 and used a portion of the cells to analyze the secretion of cytokines and the expression of transcription factors involved in the differentiation of helper T cells. We cultured the remainder of the cells for 6 d in the absence of exogenous cytokines and then restimulated them with TPA plus ionomycin to analyze cytokine production by intracellular staining. Both CCR10$^+$ and CCR10$^-$ cells had abundant production of IL-22; however, we found that CCR10$^-$ cells produced large amounts of IL-17, but CCR10$^+$ and CXCR3$^+$ cells did not (**Fig. 2b**). IFN-γ was produced mainly by CXCR3$^+$ cells and not by CXCR3$^-$ cells. CCR10$^+$ cells had abundant secretion of tumor necrosis factor (TNF) as well as of IL-2, and secreted more IL-10 and IL-13 than did the other two subsets (**Fig. 2b**). In addition, activated CCR10$^+$ cells contained more transcripts of IL-26, which, like IL-22, also acts on epithelial cells and can be induced by overexpression of RORC in naive human CD4$^+$ T cells[30] (**Fig. 2b**).

RORC mRNA was expressed similarly in all subsets of freshly isolated CCR6$^+$ cells; however, after activation mediated by anti-CD3 plus anti-CD28, RORC was strongly upregulated in CCR10$^-$ cells but not in CCR10$^+$ or CXCR3$^+$ cells (**Supplementary Fig. 1**). AHR was also broadly expressed in the various sorted cell populations, yet there was a trend for higher AHR expression in resting CCR10$^+$ cells than in CCR10$^-$ cells or CXCR3$^+$ cells; however, this difference was not significant (**Supplementary Fig. 1**). *CYP1A1* (which encodes an inducible P450 cytochrome enzyme), a direct target of AHR, had

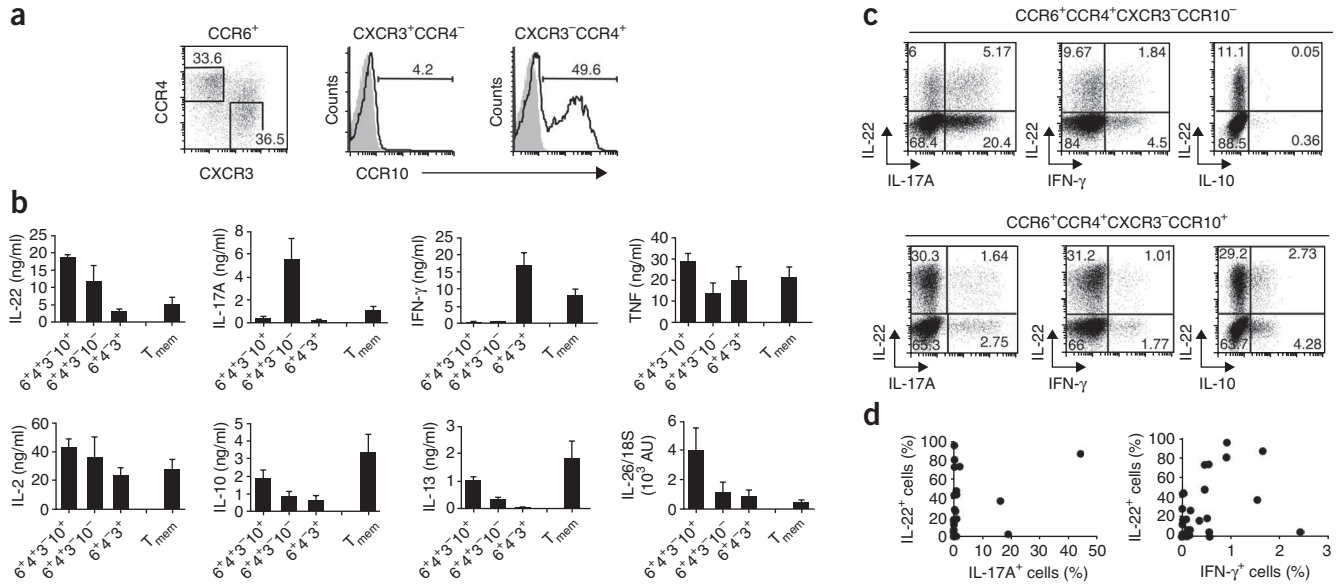

Figure 2 IL-22$^+$IL-17$^-$ memory cells express CCR10. (**a**) Flow cytometry of the expression of CCR4 and CXCR3 in the CCR6$^+$ subset of memory CD4$^+$ T cells (left), with subsequent analysis of CCR10 expression in CCR6$^+$CCR4$^-$CXCR3$^+$ subset (middle) and CCR6$^+$CCR4$^+$CXCR3$^-$ subset (right). Numbers adjacent to outlined areas (left) indicate percent CCR4$^-$CXCR3$^+$ cells (top left box) or CCR4$^+$CXCR3$^-$ cells (bottom right box); numbers above bracketed lines (middle and right) indicate percent CCR10$^+$ cells. Black lines, CCR10 staining; shaded areas, isotype-matched control antibody. Data are representative of four different experiments, with one donor of four presented here. (**b**) Luminex analysis and enzyme-linked immunosorbent assay (ELISA) of cytokine production by freshly isolated CCR6$^+$CCR4$^+$CXCR3$^-$CCR10$^+$ (6$^+$4$^+$3$^-$10$^+$), CCR6$^+$CCR4$^+$CXCR3$^-$CCR10$^-$ (6$^+$4$^+$3$^-$10$^-$) and CCR6$^+$CCR4$^-$CXCR3$^+$ (6$^+$4$^-$3$^+$) CD4$^+$ memory T cells and total CD4$^+$ memory T cells (T_{mem}) after 48 h of stimulation with anti-CD3 plus anti-CD28. Bottom right, real-time PCR of IL-26 mRNA expression, presented (in arbitrary units (AU)) relative to 18S rRNA expression ('housekeeping' gene). Data are representative of three independent experiments (mean and s.e.m.). (**c**) Flow cytometry of IL-17A, IL-22, IFN-γ and IL-10 in CCR6$^+$CCR4$^+$CXCR3$^-$ cells, sorted as CCR10$^+$ and CCR10$^-$ cells, after a 6-day *in vitro* population expansion and restimulation with TPA and ionomycin. Numbers in quadrants indicate percent cells in each. Data are representative of two independent experiments, with one donor of two presented here. (**d**) Flow cytometry of IL-22, IL-17A and IFN-γ in single-cell clones obtained from the sorted CCR10$^+$ population in **c**. Each symbol represents a single clone. Data are representative of two experiments with 30-50 clones.

low expression all cell subsets in resting state and was upregulated after activation (**Supplementary Fig. 1**). The transcription factor T-bet was highly expressed by CXCR3$^+$ cells, but little T-bet was produced by CCR10$^+$ cells (**Supplementary Fig. 1**). Flow cytometry of intracellular cytokine expression in sorted CCR10$^+$ and CCR10$^-$ cells, after a short *in vitro* population expansion, showed that the CCR10$^+$ population was enriched for IL-22$^+$IL-17$^-$ cells, whereas IL-17-producing cells were mainly present in the CCR10$^-$ population (**Fig. 2c**). In accordance with the secretion data, a fraction of the IL-22$^+$ cells in the activated CCR10$^+$ subset also expressed IL-10 protein intracellularly (**Fig. 2c**). To further analyze the phenotype of these IL-22-producing cells, we generated clones starting from CCR10$^+$ cells. Flow cytometry showed that most of the clones produced IL-22 but not IL-17, and they produced only very small amounts of IFN-γ (**Fig. 2d** and **Supplementary Fig. 2a**,**b**). We also obtained few clones that produced both IL-22 and IL-17 and clones that did not produce IL-22, IL-17 or IFN-γ (**Fig. 2d** and **Supplementary Fig. 2a**,**b**). Analysis of cytokine secretion confirmed that most of the CCR10$^+$ clones produced large amounts of IL-22 but no IL-17 and very little IFN-γ (**Supplementary Fig. 2c**). IL-13 was abundantly produced by most of the clones, with the exception of the few IL-17-producing clones (**Supplementary Fig. 2a**,**c**), which confirmed that IL-22-producing cells also produce IL-13. These data suggest that CCR10$^+$ cells constitute a stable population of IL-22$^+$IL-17$^-$IFN-γ$^-$ cells distinct from both T_H-17 cells and T_H1 cells.

RORC and AHR regulate IL-22 in CD4$^+$ memory T cells

In mice, RORγt and its related receptor RORα have been shown to be required for IL-17 expression[29]. However, although in mice *Il22* is also under the control of RORγt, in humans the dependency of *IL22* on RORC is unclear. We analyzed the respective functions of RORC and AHR in the production of IL-17 and IL-22 with a small interfering RNA (siRNA)-mediated approach. We transiently transfected CD4$^+$ memory T cells, which include IL-17$^+$ and IL-22$^+$ cells (**Fig. 1a** and data not shown), with RORC-specific or AHR-specific siRNA or with a pool of nontargeting siRNAs. In all cases, we managed to achieve 50% or more downregulation of RORC and AHR mRNA (**Fig. 3a**). In addition, expression of *CYP1A1* mRNA was also downregulated after treatment with AHR-specific siRNA but not after treatment with RORC-specific siRNA (**Fig. 3a**), which indicated that the AHR-specific siRNA was efficacious. Secretion of IL-17 was significantly inhibited (47% average inhibition) after RORC knockdown (**Fig. 3b**). RORC-specific siRNA also diminished the secretion of IL-22 at an average of 32% (**Fig. 3b**), which suggests that IL-22 is less dependent on RORC than is IL-17. AHR-specific siRNA also substantially diminished the secretion of IL-22 (40% average inhibition; **Fig. 3b**) but did not significantly affect IL-17 production (**Fig. 3b**). Linear-regression analysis of the correlation between the extent of IL-22 inhibition and the efficacy of knockdown of RORC or AHR showed no difference between the two siRNA treatments (**Fig. 3c**), which suggests that RORC and AHR are similarly involved in the regulation of IL-22 production. Other cytokines such as IFN-γ, IL-13 and IL-10 (**Fig. 3b**), as well as cell viability (data now shown), were not significantly affected by any siRNA used. Overall, these data indicate that RORC is required for the production of both IL-17 and IL-22 by CD4$^+$ memory T cells. The finding that AHR downregulation resulted in significantly less secretion of IL-22 but not of IL-17 suggests that these cytokines are regulated differently in human T cells and provides evidence that AHR is involved in IL-22 production in humans, as is also the case in mice.

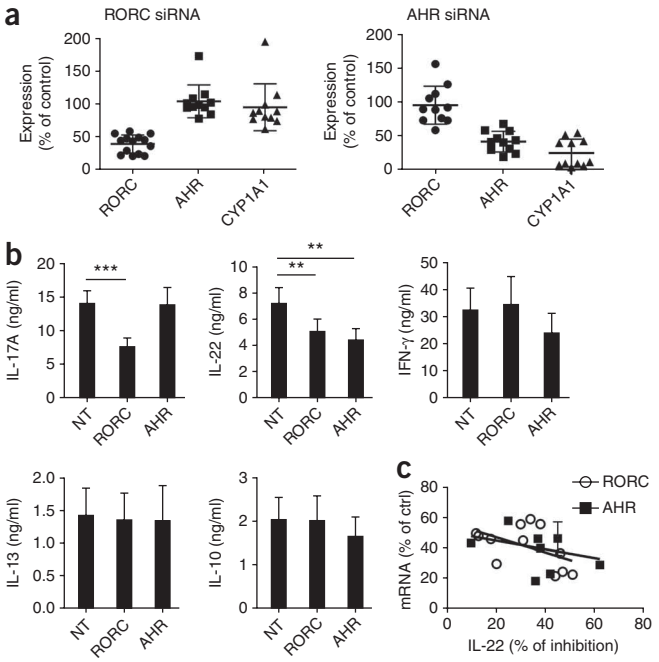

Figure 3 Knockdown of RORC or AHR in CD4$^+$ memory T cells has different effects on the production of IL-17 and IL-22. (**a**) Real-time PCR analysis of the expression of *RORC*, *AHR* and *CYP1A1* in CD4$^+$ memory T cells 48 h after transient transfection of RORC-specific or AHR-specific siRNA, presented relative to expression in cells transfected with nontargeting siRNA (control). Each symbol represents one sample; large middle horizontal lines indicate the mean (small horizontal lines at ends, s.d.). (**b**) Luminex analysis and ELISA of cytokine secretion by siRNA-transfected CD4$^+$ memory T cells, 3 d after stimulation with Dynabeads coated with anti-CD3 plus anti-CD28. NT, nontargeting siRNA. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, RORC- or AHR-specific siRNA versus nontargeting siRNA (two-tailed, paired Student's *t*-test). (**c**) Linear-regression analysis of IL-22 inhibition versus the efficiency of downregulation of RORC or AHR. RORC and AHR mRNA are presented relative to cells transfected with nontargeting siRNA (control (ctrl)); for IL-22, secretion by cells transfected with nontargeting siRNA is set as 100%. Data are representative of seven (RORC siRNA) or five (AHR siRNA) independent experiments with a total of 10–13 donors (mean and s.e.m. in **b**).

Naive CD4$^+$ T cells produce IL-22 after activation

The differences in the expression and regulation of IL-22 and IL-17 prompted us to investigate the signals needed to induce the production of these two cytokines in naive CD4$^+$ T cells. To ensure the purity of the starting population, we first enriched naive CD4$^+$ T cells from cord blood by magnetic selection, followed by flow cytometry–based sorting of the CD4$^+$CD45RA$^+$CD45RO$^-$ population. As shown before[30,36], culture of naive T cells with IL-1β and IL-23, in the presence or absence of IL-6, led to the production of IL-22 but not of IL-17, whereas the addition of transforming growth factor-β (TGF-β) to this cytokine combination was necessary to induce the differentiation of IL-17$^+$ cells (**Supplementary Fig. 3a**,**b**). We therefore used the combination of IL-1β, IL-23 and TGF-β to induce T_H-17 differentiation throughout this study. Naive T cells were able to produce small amounts of IL-22 immediately after activation (data not shown). After culture of activated naive cells for 6 d in various conditions, the highest percentage of IL-22-producing cells was obtained in the absence of exogenous cytokines (nonpolarizing conditions; **Fig. 4a**). IL-22 was also produced by *in vitro*–generated T_H-17 and T_H1 cells (**Fig. 4a**,**b**) but not by T_H2 cells. In contrast, only T_H-17 cells produced IL-17, and these cells secreted small amounts of both IL-4 and IFN-γ (**Fig. 4a**,**b**). RORC was expressed in T_H-17 cells but not in naive T cells, in T cells cultured without exogenous cytokines or in T_H2 cells, whereas T_H1 cells occasionally expressed rare RORC transcripts (**Fig. 4c**), which indicated that RORC expression in T cell subsets generated *in vitro* correlated with IL-17 production but not with IL-22 production. As expected, expression of T-bet was high in T_H1 cells and expression of the transcription factor GATA-3 was higher in T_H2 cells (**Fig. 4c**). AHR transcripts were present in freshly isolated naive CD4$^+$ T cells (**Fig. 4c**), and AHR expression was higher in T_H-17 cells than in the other cell subsets,

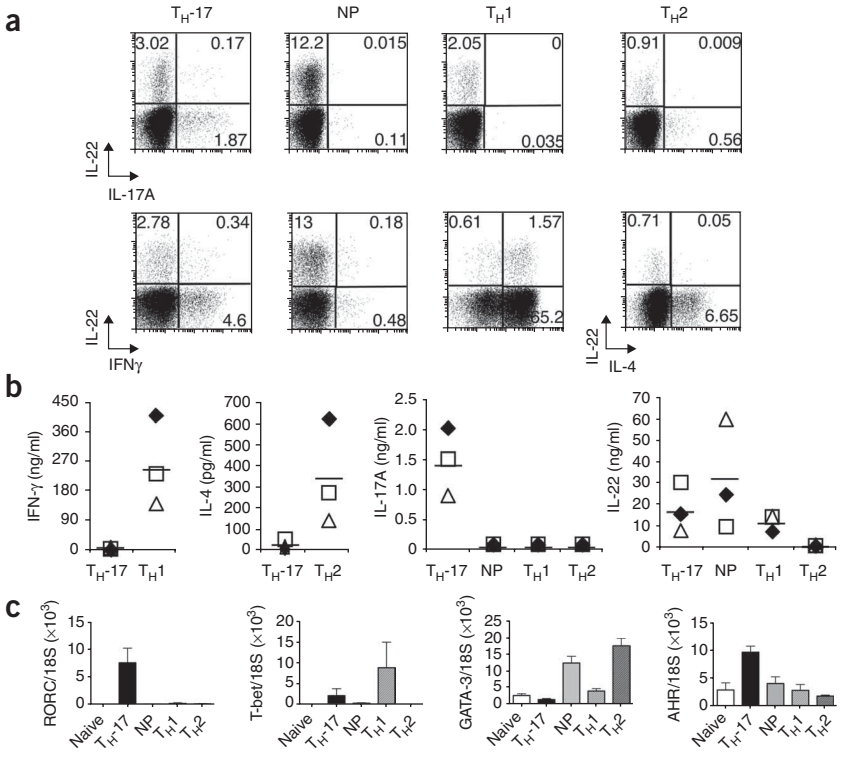

Figure 4 Abundant IL-22 production by activated naive cord blood CD4$^+$ T cells in the absence of exogenous cytokines. (**a**) Flow cytometry of the production of IL-17A, IL-22, IFN-γ and IL-4 by cord blood–derived naive CD4$^+$ T cells after two rounds of polarization in T_H-17, nonpolarizing (NP), T_H1 or T_H2 conditions, followed by restimulation for 6 h with TPA and ionomycin before intracellular staining. (**b**) Luminex analysis and ELISA of the secretion of IFN-γ, IL-4, IL-17A and IL-22 by the cells in **a** after 3 d of stimulation with Dynabeads coated with anti-CD3 plus anti-CD28. Each symbol represents an individual donor; small horizontal lines indicate the mean. (**c**) Real-time PCR analysis of the expression of RORC, T-bet, GATA-3 and AHR in unstimulated sorted naive CD4$^+$ T cells from cord blood (Naive) or from cells polarized once in either T_H-17, nonpolarizing, T_H1 or T_H2 conditions; results are presented relative to 18*S* rRNA expression. Data are representative of three independent experiments with one donor each (mean and s.e.m. in **c**).

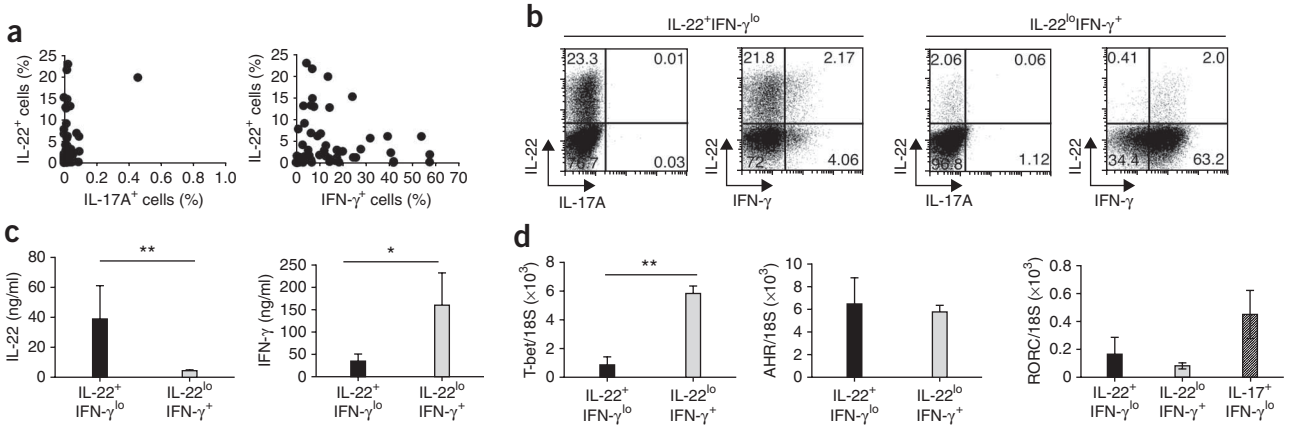

Figure 5 T cell clones that produce IL-22 but not IL-17 can be generated from *in vitro*–differentiated T_H-17 cells. (**a**) Flow cytometry of the frequency of cells positive for IL-22, IL-17A and IFN-γ by clones obtained from *in vitro*–generated T_H-17 cells, cloned in the presence of neutralizing anti-IL-12 without the addition of exogenous cytokines. Each symbol represents a single clone; clones were obtained from one donor. (**b**) Cytokine profiles of a IL-22$^+$IFN-γ^{lo} clone and a IFN-γ$^+$IL-22^{lo} clone among the clones in **a**. Numbers in quadrants indicate percent cells in each. Data in **a**,**b** are representative of two experiments with 50–70 clones. (**c**) Luminex analysis and ELISA of the secretion of IL-22 and IFN-γ by cells stimulated for 24 h with TPA and ionomycin. $*P < 0.05$ and $**P < 0.01$ (two-tailed unpaired *t*-test). Data are representative of two experiments with three to four clones per group (mean and s.e.m.). (**d**) Expression of T-bet, AHR and RORC in the clones in **c**, presented relative to 18*S* rRNA expression. $*P < 0.05$ and $**P < 0.01$ (two-tailed, unpaired *t*-test). Data are representative of two experiments (T-bet and AHR: mean and s.e.m. of three clones per group; RORC: mean and s.e.m. of three IL-17$^+$IFN-γ^{lo} clones generated by independent cloning).

as reported before[32,33]. T_H2 cells, which do not produce IL-22, had the lowest expression of AHR mRNA (**Fig. 4c**).

In vitro–derived IL-22$^+$IL-17$^-$ T cell clones

We cloned naive cord blood–derived cells, polarized twice in T_H-17 conditions, in the presence of IL-12-neutralizing antibodies. Flow cytometry of cytokine production by individual clones showed that in these conditions, we did not obtain clones that produced IL-17, which indicated that these culture conditions were nonpermissive for the maintenance of IL-17 production. However, we obtained clones that were IL-22$^+$ (**Fig. 5a**,**b**) and had low expression of IFN-γ, as determined by intracellular staining (**Fig. 5a**,**b**). We confirmed that observation at the level of cytokine secretion, showing that these clones secreted less IFN-γ but more IL-22 than did clones with T_H1-like profile (IL-22^{lo}IFN-γ$^+$ cells; **Fig. 5c**). In accordance with IFN-γ expression, T-bet expression was significantly higher in IL-22^{lo}IFN-γ$^+$ clones than in IL-22$^+$IFN-γ^{lo} clones (**Fig. 5d**), whereas expression of AHR transcripts was similar in both types of clones (**Fig. 5d**). RORC expression did not differ significantly in IL-22$^+$IFN-γ^{lo} versus IL-22^{lo}IFN-γ$^+$ clones and was generally lower in IL-22$^+$ IFN-γ^{lo} clones than in IL-17$^+$IFN-γ^{lo} clones generated in an independent cloning experiment (presented here as control; **Fig. 5d**). These data indicate that expression of RORC transcripts correlates with IL-17 production

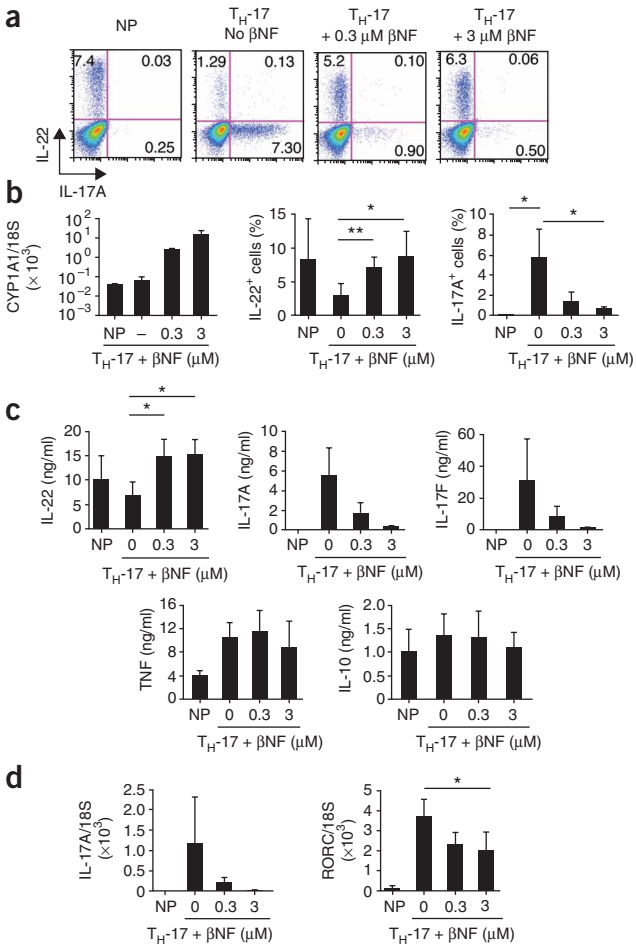

Figure 6 The AHR agonist βNF enhances IL-22 production in developing T_H-17 cells. (**a**) Flow cytometry of sorted naive CD4$^+$ T cells cultured for 6 d in nonpolarizing conditions (anti-IL-4 plus anti-IFN-γ) or in T_H-17 conditions with or without βNF (0.3 or 3 μM), then restimulated for 6 h with TPA and ionomycin before intracytoplasmic staining. Numbers in quadrants indicate percent IL-22$^+$IL-17A$^-$ cells (top left), IL-22$^+$IL-17A$^+$ cells (top right) or IL-22$^-$IL-17A$^+$ cells (bottom right). Data are representative of four independent experiments with one donor of four presented here. (**b**) Real-time PCR analysis of the expression of CYP1A1 mRNA (left) and flow cytometry of the production of IL-22 and IL-17A (middle and right) by the cells in **a**. Data are representative of three to six independent experiments with one donor each (mean and s.e.m.). (**c**) Luminex analysis and ELISA of cytokine secretion by the cells in **a** after 48 h of stimulation with anti-CD3 plus anti-CD28. Data are representative of three to six independent experiments with one donor each (mean and s.e.m.). (**d**) Real-time PCR analysis of the expression of IL-17A mRNA and RORC mRNA in the cells in **a** after 6 d of culture. Data are representative of three to five independent experiments with one donor each (mean and s.e.m.). $*P < 0.05$ and $**P < 0.01$ (two-tailed, paired *t*-test).

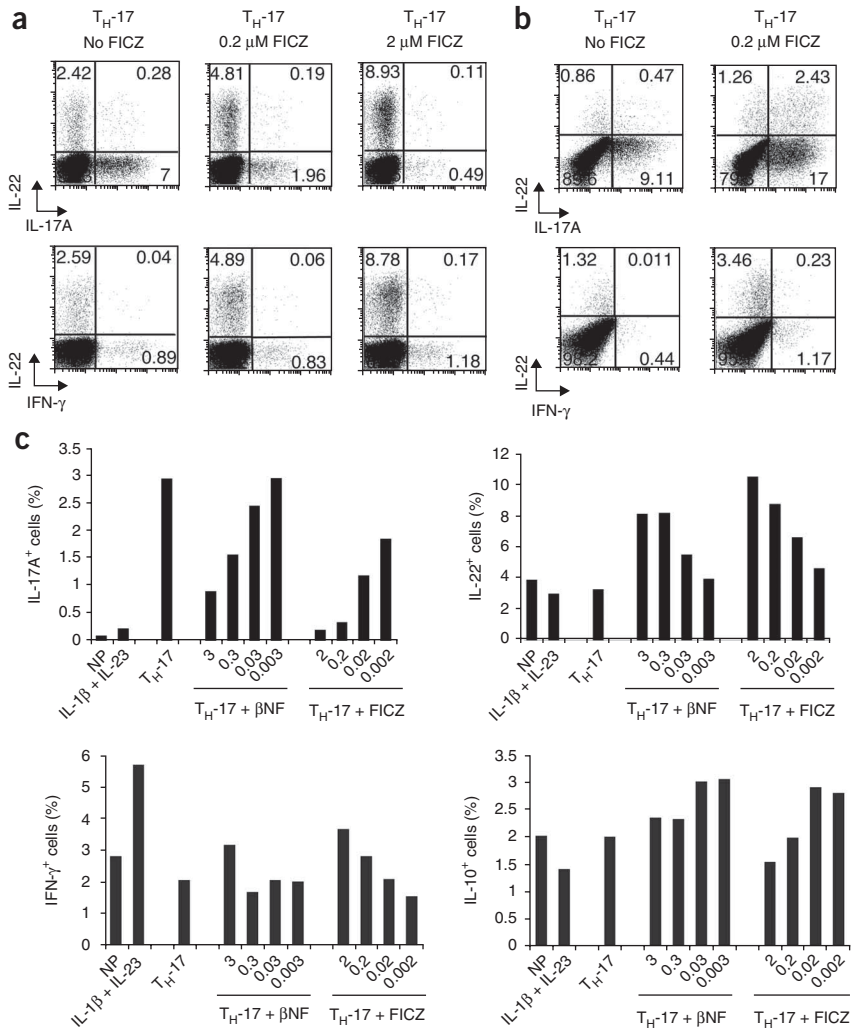

Figure 7 FICZ enhances IL-22 production in human and mouse T cells but has divergent effects on IL-17. (**a**) Flow cytometry of the production of IL-17A, IL-22 and IFN-γ by cord blood–derived sorted naive CD4$^+$ T cells cultured for 6 d in T_H-17 conditions in the presence or absence of FICZ (0.2 or 2 μM), then restimulated for 6 h with TPA and ionomycin before intracytoplasmic staining. Data are representative of three independent experiments with one donor each. (**b**) Flow cytometry of the production of IL-17A and IL-22 by sorted naive mouse CD4$^+$ T cells cultured for 7 d in T_H-17 conditions with or without the addition of 0.2 μM FICZ. Data are representative of two independent experiments with two mice each. Numbers in quadrants (**a**,**b**) indicate percent cells in each. (**c**) Flow cytometry of the production of IL-17A, IL-22, IFN-γ and IL-10 in human cord blood–derived naive CD4$^+$ T cells cultured in nonpolarizing conditions with IL-1β plus IL-23, or in T_H-17 conditions in the presence or absence of increasing concentrations of FICZ or βNF (horizontal axis, in μM).

but not with IL-22 production. We found that clones with an IL-22$^+$IFN-γ^{lo} phenotype also secreted substantial amounts of TNF and IL-13 (**Supplementary Fig. 4**), similar to the results we obtained with CCR6$^+$CCR4$^+$CCR10$^+$ memory T cells (**Fig. 2b** and **Supplementary Fig. 2c**). In addition, these data also showed that conditions that allow the maintenance of IL-22 production *in vitro* were nonpermissive for IL-17 production. Indeed, in a separate cloning experiment in which IL-1β, IL-23 and TGF-β were constantly present throughout the cloning experiment, we generated T_H-17 clones with abundant production of IL-17, little production of IL-22 and variable production of IFN-γ (IL-17$^+$IFN-γ^{lo} clones; **Fig. 5d** and **Supplementary Fig. 5a–c**). Our data indicate that for cloning that starts with cultured naive T cells, stable clones can be generated with a cytokine production profile that resembles that of CCR6$^+$CCR4$^+$CCR10$^+$ memory T cells.

AHR agonists increase IL-22 production but not IL-17 production

Our siRNA experiments indicated that AHR is involved in the production of IL-22 by memory cells. We therefore exploited the use of various AHR agonists during differentiation of naive CD4$^+$ T cells to study the effect on the differentiation of IL-22$^+$ cells. We cultured naive CD4$^+$ T cells from cord blood in nonpolarizing or T_H-17 conditions in the presence or absence of the AHR agonists β-naphthoflavone (βNF) (**Fig. 6**) and FICZ (**Fig. 7**). We found a higher percentage of IL-22$^+$ cells among T cells cultured in T_H-17 conditions in the presence of βNF than among cells cultured in the absence of βNF (**Fig. 6a**,**b**). This increase was paralleled by a lower percentage of IL-17A$^+$ cells (**Fig. 6a**,**b**), whereas the percentage of IFN-γ-producing cells was not affected (data not shown). AHR was triggered by βNF in these cultures, as βNF treatment led to considerable induction of CYP1A1 transcripts (**Fig. 6b**). CYP1A1 expression was similar in untreated, nonpolarized and T_H-17 cells, which indicated that the activity of AHR in the two cell types was similar, despite the higher expression of AHR mRNA in T_H-17 cells (**Fig. 6b** and **Fig. 4c**); this also suggests a disconnection between the abundance of AHR transcripts and the transcriptional activity of the AHR protein. In accordance with the flow cytometry data, we found that βNF-treated T_H-17 cells secreted significantly more IL-22 than did untreated T_H-17 cells, whereas the secretion of both IL-17A and IL-17F was lower than that of untreated T_H-17 cells (**Fig. 6c**). The secretion of other cytokines such as IL-10 and TNF was not significantly affected by treatment with AHR agonists (**Fig. 6c**). In addition, we found that treatment with βNF did not induce the production of IL-13 (or other T_H2 cytokines) in T_H-17 cells (data not shown). We confirmed the inhibition of IL-17A production at the mRNA level and correlated it with the diminished RORC mRNA in T_H-17 cells treated with the highest dose of βNF (**Fig. 6d**). To investigate whether AHR triggering also affected IL-17 production in already differentiated T_H-17 cells, we restimulated cells cultured in T_H-17 conditions without AHR agonist and cultured these cells in the same conditions with or without βNF. As expected, the second polarization in the absence of βNF further increased the percentage of IL-17A$^+$ cells, whereas the increase was lower in the presence of βNF (**Supplementary Fig. 6a**,**b**), which suggests that βNF prevents further new accumulation of IL-17-producing cells. It is unlikely that the effect of βNF on the differentiation of IL-17-producing cells was caused by inhibition of TGF-β signaling, as we did not find any change in the expression of mRNA encoding the signal transducer Smad7 or the receptors TGF-βRI and TGF-βRII after treatment of T_H-17 cells with βNF (**Supplementary Fig. 7**), and

increasing doses of TGF-β were not able to overcome the inhibition (data not shown). The effect of βNF on IL-22 production was not limited to cells cultured in T_H-17 conditions; indeed, we also noted more IL-22-producing cells in nonpolarized cultures treated with βNF (**Supplementary Fig. 8a**,**b**). We found very similar effects of FICZ on the development of IL-17- and IL-22-producing cells (**Fig. 7a**). As FICZ has been shown to increase the production of both IL-22 and IL-17 in mouse T cells[32], we cultured naive C57BL/6 $CD4^+$ T cells in T_H-17 conditions in the presence or absence of FICZ. We found that treatment with FICZ resulted in a higher percentage of both $IL\text{-}22^+$ cells and $IL\text{-}17A^+$ cells among mouse T cells (**Fig. 7b**), which confirmed published data[32]. We obtained similar results with βNF (data not shown). The effect of both FICZ and βNF on developing human T_H-17 cells was dose dependent, as we found more $IL\text{-}22^+$ cells and concomitantly fewer $IL\text{-}17A^+$ cells at doses as low as 20 nM FICZ and 30 nM βNF (**Fig. 7c**). We also found that FICZ treatment led to slightly more IFN-γ-producing cells and concomitantly slightly fewer in IL-10-producing cells, especially at the highest dose (**Fig. 7c**). However, given that these changes were not reflected at the level of cytokine secretion (data not shown) and that AHR-specific siRNA had no significant effect on the production of either IFN-γ or IL-10 (**Fig. 3b**), this result may be attributed to an off-target effect of FICZ. Together, our data support the idea that AHR is involved in IL-22 production and indicate possible species-specific effects of AHR agonists in terms of the differentiation or population expansion of IL-17-producing cells.

DISCUSSION

Here we have described a population of circulating memory $CD4^+$ T cells that expressed CCR6, CCR4 and CCR10. These cells had abundant production of IL-22 and IL-13 but no production of IFN-γ, IL-4 or IL-17 and were therefore distinct from T_H1, T_H2 and T_H-17 cells. The cytokine profile of the $CCR10^+$ memory cells was stable, as indicated by the finding that T cell clones with this particular pattern of cytokine production could be generated both from $CCR6^+CCR4^+CCR10^+$ cells and from *in vitro*–differentiated naive $CD4^+$ T cells. Similar findings have also been reported in a related article in this issue[37].

Our data have indicated that both RORC and AHR are involved in regulating the production of IL-17 and IL-22 by human memory T cells. In $CD4^+$ memory T cells, RORC-specific siRNA inhibited not only the production of IL-17, as reported before[30], but also the production of IL-22. It has been shown before that transduction of RORC into naive human T cells induces the production of IL-17 but not of IL-22 (ref. 30), which suggests that RORC is not essential for IL-22 production by human T cells. That idea is consistent with our observation that after activation, large amounts of IL-22 were produced by naive $CD4^+$ T cells cultured in conditions that did not induce RORC expression. Nonetheless, the observations that freshly isolated $CCR10^+$ IL-22-producing cells had relevant expression of RORC and that RORC-specific siRNA indeed affected IL-22 secretion by human memory T cells suggest that RORC influences IL-22 production by RORC-expressing T cells. We found that transfection of AHR-specific siRNA into $CD4^+$ memory T cells resulted in significantly less IL-22 production but had no effect on IL-17. In accordance with those findings, we found that the AHR agonists FICZ and βNF increased the percentage of IL-22-producing cells when added to naive $CD4^+$ T cells cultured in T_H-17 conditions. The effect on IL-22 was not limited to T_H-17 cells, as AHR agonists increased IL-22 production by nonpolarized cells as well as by T_H1 cells (data not shown). Therefore, our results show that in humans, as in mice[32,33], AHR agonists promote IL-22 production. Given the broad expression of AHR in various subsets of memory and *in-vitro*–derived helper T cells, it is possible that other factors, together with AHR, contribute to the regulation of IL-22, with RORC being one of those factors. It will be of interest to investigate whether AHR and RORC act together in the same cells to regulate IL-22 production.

We found that AHR agonists had opposite effects on the *in vitro* development of IL-17-producing cells in humans and mice. Indeed, although the treatment of developing mouse T_H-17 cells with FICZ led to a further increase in IL-17 (ref. 32), the same treatment of human cells resulted in considerable inhibition of IL-17 production, thereby changing the balance of IL-22-producing cells versus IL-17-producing cells. These results point to a species-specific regulation of IL-17 production by AHR that may partially be attributed to differences in the affinities of the human and C57BL/6 mouse AHRs for their ligand, with the affinity of the human AHR being much lower than that of the mouse AHR[38].

We found that although the generation of $IL\text{-}22^+IL\text{-}17^-$ cells did not require TGF-β, this cytokine was required for the differentiation of activated naive cord blood cells into T_H-17 cells and for the generation of T_H-17 clones from these cells. This result contrasts with some published data[15,39] but is consistent with subsequent observations[30,36]. Possible explanations for these discrepancies have been discussed[40] and may include the use of T cell populations contaminated with memory T cells versus rigorously purified naive T cells and the use of serum that might contain TGF-β. It is unlikely that the effects of AHR agonists on IL-17-producing cells that we observed were caused by interference with TGF-β signaling, because βNF, which strongly inhibited generation of T_H-17 cells, did not affect TGF-β-induced phosphorylation of Smad3 and increasing TGF-β concentrations were unable to neutralize the T_H-17-inhibitory effects of βNF (data not shown). TCDD, the prototypical AHR ligand, has been shown to induce regulatory T cells positive for the transcription factor Foxp3 and thereby counteract the development of T_H-17 cells[33]. However, we did not find more $Foxp3^+$ cells in cultures treated with βNF (data not shown). In addition, we noted a similar effect on the generation of IL-17- and IL-22-producing cells after βNF treatment of cord blood T cell samples initially depleted of $CD25^+$ cells (data not shown), which suggests that the effects of this AHR agonists are unlikely to be mediated by regulatory T cells. Although the mechanism of AHR agonist–mediated downregulation of IL-17 remains to be determined, our data reinforce the idea that that IL-17 and IL-22 are regulated differently in human T cells. The data also raise the possibility that in humans, AHR ligands alter immune reactions mediated by T_H-17 cells and $IL\text{-}22^+IL17^-$ T cells.

The expression of CCR4 and CCR10 suggested that the IL-22-producing cells described here serve a prominent function in the skin. Indeed, interactions between the CCR4 ligand CCL17 and the CCR10 ligand CCL27, which are expressed by cutaneous venules and keratinocytes, respectively, result in the recruitment of T cells to the skin[41,42]. $CCR4^+CCR10^+$ T cells constitute a population of effector T cells in various inflammatory human skin disorders, including psoriasis and allergic dermatitis[43], but not in others, such as delayed-type hypersensitivity or bacterial lesions[44]. $CCR6^+CCR4^+CCR10^+$ IL-22-producing cells might indeed be instrumental in psoriasis, as psoriatic lesions have more of the CCR10 ligand CCL27 (ref. 43) and of IL-22, as well as of IL-23, which is a potent inducer of IL-22 (refs. 6,45). Moreover, studies of genetic risk factors suggest that IL-23 is involved in psoriasis[46]. In addition, IL-22 has been found to mediate IL-23-induced dermal inflammation and acanthosis[11,12]. It has also been found that the circulating memory T cells of patients with atopic dermatitis contain many more IL-13-producing cells than

IL-4-producing cells[47] and that transgenic expression of IL-13 in the mouse skin induces an atopic dermatitis–like syndrome[48]. It might be possible that the IL-13$^+$IL-4$^-$ cells found in patients with atopic dermatitis are indeed IL-22-producing CCR4$^+$CCR10$^+$ cells. In accordance with our findings, T cells that produce IL-22 but not IL-17, IFN-γ or IL-4 have been shown to be the main cell subset in human atopic dermatitis skin lesions[49]. In conclusion, our data have provided evidence of the existence of a helper T cell population that produces IL-22, TNF and IL-13 and that could be an important participant in many skin disorders. Our data have also provided further evidence for a notable functional diversity of CD4$^+$ memory T cells in humans that is much more extensive than previously appreciated.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001243, A001245 and A000229.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank W. Ouyang for discussions; J. Wu for doing and analyzing the experiment in **Supplementary Figure 9**; J. Cupp, L. Gilmour, A. Paler Martinez, R. Neupane, C. Poon and W. Tombo from the Genentech flow cytometry lab for assistance in sorting and Luminex assays; and the Genentech Blood Donor Program for blood samples.

AUTHOR CONTRIBUTIONS

S.T. and C.D.K. designed the experiments, analyzed and interpreted the data, and wrote the paper; E.H.T. and N.K.C. provided intellectual input and did some of the experiments; H.S. conceived the project, with contributions from S.T. and C.D.K., and interpreted the data and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureimmunology/.

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ONLINE METHODS

Cell purification and sorting. $CD4^+$ cells were enriched from whole peripheral blood or buffy coats with the Rosettesep Human $CD4^+$ T Cell Enrichment kit (StemCell Technologies) and the $CD4^+$ Memory T Cell Isolation kit (Milteny Biotech). Cells were then labeled with anti-CCR6, anti-CD25, anti-CD45RA, anti-CCR4, anti-CXCR3 (all from BD Bioscience) and anti-CCR10 (R&D Systems; antibody details, **Supplementary Table 1**). CCR6 was identified by incubation with Pacific blue–conjugated streptavidin (4 μg/ml; Invitrogen). Cells were sorted on a FACSAria (Becton Dickinson) as $CD45RA^-CD25^-CCR6^+CCR4^+CXCR3^-$, $CD45RA^-CD25^-CCR6^+CCR4^-CXCR3^+$ or $CD45RA^-CD25^-CCR6^-CCR4^+CXCR3^-$. Some experiments included anti-CCR10. After being sorted, cells were activated with Dynabeads coated with anti-CD3 plus anti-CD28 (Invitrogen) at a cell/bead ratio of 1:1, and cytokine secretion and transcription factor expression were assessed after 48 h. A portion of the cells was cultured for additional 4–6 d and then intracellular cytokine production was assessed after restimulation with TPA plus ionomycin. Blood samples were obtained from the Genentech blood donors program or from the Blood Centers of the Pacific after written informed consent was provided. Ethical approval for the use of this material was obtained from the Western Institutional Review Board. Naive $CD4^+$ T cells were obtained from human cord blood (AllCells) with a Naive $CD4^+$ T cell Isolation Kit II (Miltenyi), followed by sorting of the $CD4^+CD45RA^+CD45RO^-$ population (purity, >98%).

Cell culture and differentiation. Human T cells were cultured in Yssel's medium (Gemini Bio-Products or prepared 'in house') plus 1% (vol/vol) human AB serum (Gemini Bio-Products). Naive T cells were cultured with Dynabeads coated with anti-CD3 plus anti-CD28 in the following conditions: medium only (nonpolarizing); with anti-IFN-γ (10 μg/ml), anti-IL-4 (5 μg/ml), recombinant human TGF-β1 (10 ng/ml), recombinant human IL-1β (10 ng/ml) and recombinant human IL-23 (10 ng/ml) with or without recombinant human IL-6 (10 ng/ml; T_H-17); with recombinant human IL-12 (5 ng/ml) and anti-IL-4 (T_H1); or with recombinant human IL-4 (10 ng/ml) and anti-IFN-γ (T_H2). Where indicated, anti-IFN-γ and anti-IL-4 were added to the cells cultured in nonpolarizing conditions. Anti-IL-4, IL-1β, IL-23, IL-6 were from R&D Systems; anti-IFN-γ was from BD Pharmingen; TGF-β was from eBioscience; and IL-21 was from Invitrogen. After 6 d, Dynabeads were removed and cells were analyzed or maintained in culture with recombinant human IL-2 (100 IU/ml; R&D Systems). Where indicated, βNF (Sigma Aldrich) or FICZ (BioMol–Enzo Life Science) was added at various concentrations. After 6 d, cells were extensively washed and restimulated for cytokine production.

Knockdown with siRNA. Enriched $CD4^+$ memory T cells were transiently transfected with 3 μg RORC-specific, AHR-specific or nontargeting siRNA (SmartPool; Dharmacon) with the Amaxa Nucleofector (program V-024; Amaxa Biosystems) according to the manufacturer's protocol. All siRNA sequences are commercially available. Knockdown efficiency was analyzed by real-time PCR 48 h after transfection. At 2 d after transfection, cells were stimulated with Dynabeads coated with monoclonal anti-CD3 plus monoclonal anti-CD28 and cytokine secretion was analyzed by Luminex assay or ELISA after 3 d.

Real-time PCR. Total RNA was extracted with an RNeasy Mini or Micro kit (Qiagen), then cDNA was reverse-transcribed with a High-Capacity cDNA Archive kit (Applied Biosystems). Primers and probes were all predesigned Taqman Gene Expression Assays from Applied Biosystems. A 7500 Real-Time PCR system (Applied Biosystems) was used for real-time PCR with the following probes (Applied Biosystems identification numbers in parentheses): RORC (Hs01076112), AHR (Hs00169233), CYP1A1 (Hs00153120), TBX21 (Hs00203436), GATA-3 (Hs00231122), IL-17A (Hs00174383), IL-26 (Hs00218189_m1), 18*S* (Hs99999901). All results are presented in arbitrary units relative to 18*S* rRNA expression.

Intracellular cytokine staining. Intracellular staining was done in T cells after 6 h of stimulation with TPA (10 ng/ml) and ionomycin (500 nM; Calbiochem) in the presence of brefeldin A (5 μg/ml; BD Bioscience) for the final 3 h of culture. Cells were stained with a LIVE/DEAD Viability/Cytotoxicity kit (green fluorescence; Invitrogen), then were fixed overnight at 4 °C in 3% (wt/vol) paraformaldehyde. Cell permeabilization, staining and washing were done with the BD Perm/Wash buffer (BD Biosciences). Details about antibodies used to visualize IL-17, IFN-γ, IL-22 and IL-10 are in **Supplementary Table 1**. Cells were stained for 1 h at 20–25 °C. Data were acquired on an LSR II (BD Biosciences) and were analyzed with FlowJo software (Tree Star). Monoclonal mouse anti–human IL-22 was generated 'in house' by immunization of mice with human IL-22–Fc fusion protein. Clone 3F11 was chosen for its ability to detect intracellular human and mouse IL-22 (**Supplementary Fig. 9**). Anti-IL-22 was conjugated with Alexa Fluor 647 with an Invitrogen labeling kit. During analysis, dead cells were gated out with green fluorescent dye from the LIVE/DEAD Viability/Cytotoxicity kit and doublets were eliminated on the basis of physical parameters.

Mouse cells were fixed and stained as described above for human cells. IL-22 was detected with the same 3F11 clone. IL-17A was detected with Alexa Fluor 488–conjugated anti-IL-17A (eBioscience).

Luminex and ELISA. IL-10, IL-17A, IFN-γ, TNF and IL-13 were measured with the Bio-Plex cytokine assay (Bio-Rad) and analyzed with the Luminex 100 system (Luminex).

Helper T cell cloning. Naive $CD4^+$ T cells isolated from cord blood were polarized twice in T_H-17-inducing conditions as described above. By serial dilution, T_H-17 cells were plated at a density of 1 or 0.3 cells per well and were stimulated with a feeder mixture containing irradiated allogenic peripheral blood mononuclear cells (6,000 rads), irradiated JY Epstein-Barr virus–transformed human lymphoblastoid B cells (10,000 rads) and phytohemagglutinin (0.5 μg/ml). IL-2 was added at a concentration of 40 IU/ml at day 3 and half the medium was replaced with fresh medium plus IL-2 (40 IU/ml) every 3 d. Two different cloning experiments were done starting with *in vitro*–derived T_H-17 cells. In the first, IL-12-neutralizing antibodies (5 μg/ml) were added at every round of restimulation. In the second, anti-IL-12 (5 μg/ml), anti-IL-4 (5 μg/ml), anti-IFN-γ (10 μg/ml), IL-1β (10 μg/ml), IL-23 (10 μg/ml) and TGF-β (10 μg/ml) were added at every round of stimulation. $CCR6^+CCR4^+$ and $CCR6^+CCR4^+CCR10^+$ memory T cells were cloned in the presence of anti-IL-4, anti-IL-12 and anti-IFN-γ. Cells were stimulated with the feeder mixture every 2 weeks. Experiments were done between 9 d and 13 d after the addition of the feeder mixture.

Culture of mouse T cells. Naive splenic $CD4^+$ T cells were isolated from 8- to 12-week-age female C57BL/6 mice (Jackson Laboratories) by initial enrichment with the $CD4^+$ T Cell Isolation kit (Miltenyi Biotech), followed by sorting of $CD4^+CD62L^+CD25^-$ naive cells on a FACSAria (antibody details, **Supplementary Table 1**). Cells were cultured for 7 d on immobilized anti-CD3 (2 μg/ml; eBioscience) and immobilized anti-CD28 (5 μg/ml; BD Bioscience) in DMEM high glucose (Genentech 'in-house' products based on CellGro DMEM powder), supplemented with nonessential amino acid solution (Sigma Aldrich), sodium pyruvate, penicillin-streptomycin and 30 μM β-mercaptoethanol (Sigma Aldrich). The following neutralizing antibodies and cytokines were added for T_H-17 differentiation: anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml; BD Pharmingen), recombinant mouse IL-1β (10 ng/ml; R&D Systems), recombinant mouse IL-6 (20 ng/ml; R&D Systems) and recombinant mouse IL-23 (10 ng/ml; R&D Systems), plus recombinant human TGF-β1 (1 ng/ml; Peprotech). FICZ was added at a dose of 0.2 μM and was maintained throughout the culture. Where needed, cells were split 1:2 with the addition of recombinant human IL-2 (20 IU/ml). For analysis of cytokine production, cells were washed the restimulated for 5 h with TPA (10 ng/ml) plus ionomycin (500 ng/ml). Brefeldin A (5 μg/ml) was added for the final 4 h of the stimulation and cells were stained as described above.

Statistical analysis. Statistical significance was determined with either the paired, two-tailed *t*-test (difference between two groups or conditions) or the unpaired two-tailed *t*-test.

doi:10.1038/ni.1770