Th17 cells demonstrate stable cytokine production in a pro-allergic environment

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Abstract

Th17 cells are critical for the clearance of extracellular bacteria and fungi, but also contribute to the pathology of autoimmune diseases and allergic inflammation. Following exposure to an appropriate cytokine environment, Th17 cells can acquire a Th1-like phenotype, but less is known about their ability to adopt Th2 and Th9 effector programs. To explore this in more detail, we used an IL-17F lineage tracer mouse strain that allows tracking of cells that formerly expressed IL-17F. In vitro derived Th17 cells adopted signature cytokine and transcription factor expression when cultured under Th1, Th2 or Th9-polarizing conditions. In contrast, using two models of allergic airway disease (AAD), Th17 cells from the lungs of diseased mice did not adopt Th1, Th2 or Th9 effector programs, but remained stable IL-17-secretors. Although in vitro derived Th17 cells expressed IL-4Rα, those induced in vivo during AAD did not, possibly rendering them unresponsive to IL-4-induced signals. However, in vitro derived antigen-specific Th17 cells transferred in vivo to OVA and alum-sensitized mice also maintained IL-17 secretion and did not produce alternative cytokines upon subsequent OVA challenge. Thus, although Th17 cells can adopt new phenotypes in response to some inflammatory environments, our data suggest that in allergic inflammation, Th17 cells are comparatively stable, and retain the potential to produce IL-17. This might reflect a cytokine environment that promotes Th17 stability, and allow a broader immune response at tissue barriers that are susceptible to allergic inflammation.

Introduction

Upon activation, naïve CD4^{+} T cells differentiate into specific T helper lineages depending on the cytokines in the environment. IL-12 promotes the IFN-γ-secreting Th1 phenotype, IL-4 induces the development of Th2 cells, which produce IL-4, IL-5, and IL-13 and the combination of IL-4 and TGF-β promotes the development of IL-9-secreting Th9 cells (1–9). Together, IL-6, TGF-β, IL-23 and IL-1β induce the development of IL-17-secreting Th17 cells (10–15). In addition to IL-17A and IL-17F, Th17 cells produce IL-21 and IL-22 and

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are important for immunity against extracellular bacteria and fungi, but also contribute to the pathology of autoimmune diseases and allergic inflammation (16–20). The Th17 effector program is induced by a network of transcription factors, which includes RORγt and STAT3, and is negatively regulated by the Th1 and Th2/Th9-inducing cytokines, IFN-γ and IL-4, respectively (11, 21–25).

T helper lineages were originally thought to have stable phenotypes, and once a T helper cell acquired the potential for secreting a particular cytokine, the cell was committed to this phenotype. However, experiments with Th17 cells demonstrated that they had dramatic instability, defaulting to an IFN-γ-secreting phenotype in vitro (25–28). Maintaining the Th17 phenotype in vitro requires a specific cytokine environment that includes IL-23 and IL-1 (26). The ability of a Th17 cell to acquire IFN-γ-secreting potential requires IL-12-induced STAT4, and the induction of T-bet to repress Runx1 and IRF4 (25, 27, 29, 30). Th17 plasticity, the ability to acquire other T helper cell phenotypes, is reflected by the increased expression of a stem cell signature and bivalent chromatin marks at T helper lineage transcription factors that allow responsiveness to the cytokine environment (31–34). Although other T helper subsets have some plasticity, the dramatic instability of the Th17 phenotype suggests that maintenance of IL-17-secreting cells might be detrimental to the host.

The plasticity of the Th17 lineage in vivo was first shown in a series of studies where polyclonal populations, or Th17 cells purified on the basis of reporter expression, were adoptively transferred into mice with autoimmune diseases including colitis and type I diabetes, or lymphopenic hosts (27, 35–37). These studies agreed with in vitro studies, and demonstrated the acquisition of IFN-γ-secreting potential following transfer. However, these studies did not exclude the possibility that some IL-17-negative cells could have been transferred and expanded in vivo.

The use of IL-17A and IL-17F lineage tracer mouse models allowed tracking of cells that formerly expressed IL-17, and confirmed the acquisition of a Th1-like phenotype by Th17 cells in vitro, and in vivo during the development of autoimmune disease (38, 39). In experimental autoimmune encephalomyelitis (EAE), the majority of IFN-γ-secreting cells found in the CNS are former secretors of IL-17A and IL-17F (38, 39). IL-17-secreting T cells can acquire other phenotypes as well. Th17 cells adopt a follicular helper T cell phenotype in Peyer's patches inducing the development of IgA-producing germinal center B cells and promoting gut homeostasis (40). Additionally, IL-17-secreting T cells can terminate IL-17 production without producing cytokines associated with other lineages. Upon clearance of acute cutaneous infection with *Candida albicans*, Th17 cells shut off IL-17 production, potentially dampening the immune response (39). Thus, specific inflammatory environments can induce the conversion of Th17 cells to other phenotypes in vivo, promoting homeostasis, host defense and inflammation.

In vitro addition of IL-4 to Th17 cultures results in diminished IL-17 production, and increased production of cytokines associated with the Th2 phenotype (26, 37). This suggests that Th17 cells might also acquire a pro-allergic phenotype in appropriate cytokine environments. However, the stability of Th17 cells in a Th2 or Th9-biased environment is

not well understood. In this report, we used a newly generated IL-17F lineage tracer mouse to define the stability of IL-17-secreting T cells during allergic inflammation in vivo.

Materials and Methods

Mice

A bacterial artificial chromosome spanning the *Il17f* locus was used to generate a targeting vector that replaced the 3′ end of exon 1 with an EGFP-Cre fusion protein and an *frt*-flanked neomycin resistance cassette (Vega Biolabs). The targeting vector was transfected into C57BL/6 embryonic stem cells. Neomycin-selected clones were screened for correct recombination and embryonic stem cells were injected into albino C57BL/6 blastocyts. The resulting founder mice were bred to establish germline transmission. These mice are referred to as *Il17f*[Cost] mice using 'Cost' as an acronym for 'Cre On Seventeen Transcript'. To excise the *frt*-flanked neomycin-resistance cassette, *Il17f*[Cost] mice were crossed to FLPeR mice (expressing a variant of the *FLP1* recombinase gene inserted into the *Gt(ROSA)26Sor* locus). *Il17f*[Cost]*Rs*[YFP] mice were generated by crossing *Il17f*[Cost] mice to R26-stop-EYFP reporter mice (have a *loxP*-flanked stop sequence followed by the *eYFP* gene inserted into the *Gt(ROSA)26Sor* locus). *Il17f*[Cost]*Rs*[YFP]-OT-II mice were generated by crossing *Il17f*[Cost]*Rs*[YFP] mice to OT-lI mice (express a transgenic T cell receptor specific for chicken ovalbumin 323–339). C57BL/6 mice were purchased from Harlan Laboratories and FLPeR mice, R26-stop-EFYP mice and OT-II mice were purchased from Jackson Laboratory. Mice were kept in pathogen-free conditions and all studies were approved by the Indiana University School of Medicine Animal Care and Use Committee.

Murine T helper cell differentiation

Naïve $CD4^+CD62L^+$ T cells were purified from spleens via magnetic isolation (Miltenyi Biotec) and activated with plate-bound anti-CD3 (2–4 μg/ml, 2C11) and soluble anti-CD28 (1–2 μg/ml). Cells were polarized to generate Th1 (5 ng/ml IL-12; 50 u/ml IL-2; 10 μg/ml anti-IL-4, 11B11), Th2 (10 ng/ml IL-4; 10 μg/ml anti-IFN-γ, XMG), Th9 (20 ng/ml IL-4; 2 ng/ml TGF-β; 10 μg/ml anti-IFN-γ) and Th17 (100 ng/ml IL-6; 2 ng/ml TGF-β; 10 ng/ml IL-23; 10 ng/ml IL-1β; 10 μg/ml anti-IL-4; 10 μg/ml anti-IFN-γ) cells. Cells were expanded after 3 days with fresh media alone for Th1 and Th2 cells or in the presence of additional cytokines for Th9 (20 ng/ml IL-4; 2 ng/ml TGF-β) and Th17 (50 ng/ml IL-6; 5 ng/ml IL-23; 5 ng/ml IL-1β) cells. Cells were harvested after five days in culture for analysis. For long-term Th17 cultures, cells were cultured as noted above for five days. Cells were then harvested and re-activated under long-term Th17-polarizing conditions (1 μg/ml anti-CD3; 10 ng/ml IL-23; 10 ng/ml IL-1β; 10 μg/ml anti-IL-4; 40 μg/ml anti-IFN-γ). Cells were expanded after 3 days in the presence of additional cytokines (5 ng/ml each IL-23 and IL-1β). Cells were harvested on the fifth day of the second round of culture and live YFP^+ cells were sorted by flow cytometry. Sorted cells were re-activated with plate bound anti-CD3 (1 μg/ml) and cultured for a third round under Th1, Th2, Th9 or long-term Th17-polarizing conditions. Cells were expanded after 3 days as noted above for Th1, Th2, Th9 and long-term Th17 conditions. Cells were harvested on the fifth day of the third round of culture for further analysis. For experiments using cells from *Il17f*[Cost]*Rs*[YFP]-OT-ll mice, purified naïve $CD4^+CD62L^+$ T cells were activated with soluble $OVA_{323-339}$ peptide (5

µg/ml; GenScript) and soluble anti-CD28 (1 µg/ml) in the presence of CD4^{+} depleted splenocytes (1:5) that were first treated with Mitomycin C (Calbiochem) according to the manufacturer's instructions. Cytokines and antibodies were purchased from Bio X Cell (anti-CD3, anti-IFN-γ and anti-IL-4), BD Biosciences (anti-CD28), eBioscience (IL-1β), Miltenyi Biotec (TGF-β) Peprotech (IL-2, IL-4, IL-6, IL-12) or R&D Systems (IL-23).

Intracellular staining

For cytokine analysis from in vitro differentiated T helper cells, cells were re-stimulated with PMA and ionomycin for 5 h with the addition of monensin during the last 3 h of stimulation. Cells were collected, stained with a fixable viability dye, fixed with 2% final formaldehyde, permeabilized with saponin, and stained with fluorochrome-conjugated anti-mouse IFN-γ, IL-9, IL-13, IL-17A or IL-17F. For transcription factor analysis from in vitro differentiated T helper cells, live YFP^{+} cells were sorted by flow cytometry. Unstimulated cells were fixed and permeabilized using a transcription factor staining buffer set (eBioscience) and stained with fluorochrome-conjugated anti-mouse GATA3, RORγt or T-bet. For ex-vivo analyses, splenocytes, BAL, lung or mononuclear cells from the brain were stimulated with PMA and ionomycin as described above. Cells were collected, incubated with Fc Block (BD Biosciences) and stained with fluorochrome-conjugated anti-mouse CD4. Cells were then washed, stained with a fixable viability dye and fixed, permeabilized and stained with anti-mouse cytokine antibodies as described above. Antibodies were purchased from BD Biosciences, BioLegend or eBioscience.

Quantitative RT-PCR

Quantitative PCR was performed with total or sorted YFP^{+} unstimulated cells using Taqman assays as previously described (41).

Induction of allergic airway disease

Induction of allergic airway disease using OVA and alum has been described previously (42). In brief, 8–10 week old mice were sensitized by intraperitoneal (i.p.) injection of OVA (20 µg; Sigma) adsorbed with alum (2 mg; Sigma) on days 0 and 7. Mice were challenged with OVA (100 µg) intransally (i.n.) from days 14–19 and were sacrificed 24 h after the last challenge. For some experiments, mice were sensitized on days 0 and 7 as mentioned above and in vitro derived OVA-specific YFP^{+} Th17 cells (1×10^{5}) were transferred by intravenous (i.v.) injection to the tail on day 20. Mice were challenged with OVA (100 µg) i.n. from days 21–26 and were sacrificed 24 h after the last challenge. To induce AAD using house dust mite (HDM), 8–10 week old mice were administered intranasal doses of HDM (50 µg; Greer) for 3 consecutive days each week for 5 weeks. Mice were sacrificed 24 h after the last dose was administered. For both models, mice were sacrificed by intraperitoneal injections of pentobarbital (5 mg per mouse) 24 h after the final intranasal challenge. The trachea was cannulated and the lungs were lavaged 3 times with 1 ml PBS. The cells recovered in the bronchoalveolar lavage (BAL) fluid were counted with a hemacytometer. The lungs were isolated and single cell-suspensions were prepared using a gentleMACS Dissociator (Miltenyi Biotec).

Induction of experimental autoimmune encephalomyelitis

Induction of EAE disease has been previously described (43, 44). In brief, 8–10 week old female mice were immunized subcutaneously (s.c.) on days 0 and 7 with myelin oligodendrocyte glycoprotein (MOGp35-55) antigen peptide (100 μg; Genemed Synthesis) emulsified in complete Freund's adjuvant (150 μl; Sigma). Mice were injected intraperitoneally with pertussis toxin (100 ng; Sigma) on days 0 and 2. Mice were sacrificed 19 days after induction of disease and spleen and brain were harvested. Mononuclear cells were isolated from brain using a 30%/70% Percoll gradient. Splenocytes and mononuclear cells from the brain were stimulated for 5 h with PMA and ionomycin with the addition of monensin during the final 3 h of stimulation before further staining for flow cytometry analysis.

Statistical analysis

The one-way ANOVA was used for statistical comparison. p values of 0.05 or less were considered as significant.

Results

Generation and characterization of *Il17f*^{Cost} mice

IL-17 reporter and lineage tracer mouse strains are tools to identify cells that currently produce IL-17 (reporter mouse) or which have produced IL-17 (lineage tracer mouse). Our goal was to generate a mouse strain that combined both approaches, to simultaneously identify cells that currently produce IL-17F or that previously produced IL-17F, but have since turned off the *Il17f* gene. An EGFP-Cre recombinase fusion was inserted into exon 1 of *Il17f*, termed Cost (<u>C</u>re <u>o</u>n <u>s</u>eventeen <u>t</u>ranscript) (Fig. 1A). *Il17f*^{Cost} mice express a fusion of EGFP and Cre recombinase when the *Il17f* locus is turned on. To observe Cre activity, *Il17f*^{Cost} mice were crossed with a reporter mouse strain that expresses YFP upon expression of Cre (referred to as *Rs*^{YFP} here). In theory, cells from *Il17f*^{Cost}*Rs*^{YFP} mice would express EGFP and Cre when *Il17f* is turned on, and retain Cre-induced YFP expression if *Il17f* expression were discontinued. Expression of EGFP and YFP was tested from in vitro derived Th17 cells from *Il17f*^{Cost}*Rs*^{YFP} mice and we found that while both EGFP and YFP could be detected in Th17 cells by immunofluorescence microscopy, YFP, but not EGFP, was detected by flow cytometry, possibly due to insufficient expression of the fusion protein for cytometric detection (data not shown). Thus, in subsequent experiments, *Il17f*^{Cost}*Rs*^{YFP} mice were used as an *Il17f* lineage tracer mouse strain, similar to that previously described by Croxford et al (36), using flow cytometry analyses to define YFP$^+$ cells as those that had activated the *Il17f* locus.

To validate the *Il17f*^{Cost}*Rs*^{YFP} mouse strain, naïve CD4$^+$ T cells were isolated from the spleen of *Il17f*$^{+/+}$*Rs*$^{YFP/YFP}$, *Il17f*$^{+/Cost}$*Rs*$^{YFP/YFP}$ and *Il17f*$^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for five days. As expected, Th17 cells from *Il17f*$^{+/+}$*Rs*$^{YFP/YFP}$ mice do not express YFP, but express high percentages of IL-17F$^+$ and IL-17A$^+$ cells. Th17 cells from *Il17f*$^{+/Cost}$*Rs*$^{YFP/YFP}$ mice demonstrate expression of YFP and display an expected reduction in IL-17F, as the cells have only one functioning *Il17f* allele. Th17 cells derived from *Il17f*$^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice display a further increase in

YFP expression, but do not produce IL-17F, as they are *Il17f* null (Fig. 1B). Although only the *Il17f* locus was targeted in the generation of *Il17f*[Cost] mice, it is important to note that Th17 cells derived from *Il17f*[+/Cost]*Rs*[YFP/YFP] and *Il17f*[Cost/Cost]*Rs*[YFP/YFP] mice produce less IL-17A than control mice, suggesting that there is an effect of the *Cost* allele on the expression of the adjacent *Il17* allele (Fig. 1B). Importantly, in comparison to total Th17 cells derived from *Il17f*[+/Cost]*Rs*[YFP/YFP] and *Il17f*[Cost/Cost]*Rs*[YFP/YFP] mice, the YFP[+] population of Th17 cells has a higher expression level of IL-17A and IL-17F, demonstrating an enrichment of IL-17-producing cells in the YFP^{+} population (Fig. 1B).

To confirm that in vitro differentiated Th17 cells and not other T helper subsets expressed YFP, we isolated naïve CD4^{+} T cells from the spleen of *Il17f*[+/Cost]*Rs*[YFP/YFP] mice and stimulated them under Th1, Th2, Th9 or Th17-polarizing conditions. YFP is expressed in Th17 cells, but not in other T helper lineages (Fig. 1C). To further validate the use of *Il17f*[Cost]*Rs*[YFP] mice for in vivo studies intended to track the fate of IL-17F-expressing cells, we induced EAE in *Il17f*[+/Cost]*Rs*[YFP/YFP] mice and analyzed cytokine secretion in CD4^{+} cells from the brain. Similar to what has been previously reported (38, 39), we found that CD4^{+} cells from the brain of mice at the peak of paralysis co-expressed IL-17A and IFN-γ. The cytokine-positive population was enriched in the CD4^{+}YFP^{+} population demonstrating that in EAE, some IFN-γ-secreting CD4^{+} cells co-express IL-17F or arise from an IL-17F-positive precursor (Fig. 1D). Together, these data demonstrate that *Il17f*[Cost]*Rs*[YFP] mice can be used for in vitro and in vivo studies to further explore the stability of IL-17F-secreting cells.

Altered Th17 cytokine expression upon stimulation under Th1, Th2 or Th9-polarizing conditions

Th17 cells can produce robust amounts of IFN-γ when stimulated in vitro under Th1-skewing conditions, however less is known about their cytokine profile when stimulated under Th2 or Th9-polarizing conditions. To examine this, we isolated naïve CD4^{+} T cells from the spleen of *Il17f*[+/Cost]*Rs*[YFP/YFP] mice and stimulated them under Th17-polarizing conditions for two rounds of culture to establish a more differentiated population of Th17 cells. YFP^{+} cells were sorted and maintained under Th17-polarizing conditions or stimulated under Th1, Th2 or Th9-polarizing conditions for a third round of culture. After two rounds of stimulation under Th17-polarizing conditions, YFP^{+} cells produce IL-17A and IL-17F with little to no IFN-γ, IL-13 or IL-9 production (Fig. 2A). In most experiments we observed diminished IL-17F expression after the second round of polarization. YFP^{+} cells stimulated for a third round of culture under Th17-polarizing conditions display an increase in IL-17A^{+} and IL-17A^{+}IL-17F^{+} cells (Fig. 2B, 2C). In contrast to cells maintained under Th17-skewing conditions for a third round of culture, YFP^{+} cells stimulated under Th1, Th2 or Th9-polarizing conditions display reduced IL-17A production, particularly in the IL-17A^{+}IL-17F^{+} population (Fig. 2B, 2C). YFP^{+} Th17 cells stimulated under Th1-polarizing conditions display reduced IL-17A^{+}IFN-γ^{−} cells with an increase in IL-17A^{+}IFN-γ[+] and IL-17A^{−}IFN-γ^{+} cells in comparison to cells maintained under Th17 conditions (Fig. 2B, 2D). An increase in IL-17A^{+}IL-13^{+} and IL-17A^{−}IL-13^{+} cells is observed when YFP^{+} Th17 cells are cultured under Th2-skewing conditions, and to a lesser extent following culture under Th9-polarizing conditions (Fig. 2B, 2E). Furthermore, YFP^{+} Th17 cells

cultured under Th9-polarizing conditions display an increase in IL-17A$^+$IL-9$^+$ cells along with a smaller increase in IL-17A$^-$IL-9$^+$ cells compared with cells maintained under Th17-skewing conditions (Fig. 2B, 2F). YFP$^+$ Th17 cells cultured under Th2-polarizing conditions also display an increase in IL-17A$^-$IL-9$^+$ cells with a more moderate increase in IL-17A$^+$IL-9$^+$ cells, however both populations are produced at lower levels than cells cultured under Th9-polarizing conditions (Fig. 2B, 2F). Enhanced IL-9 secretion from Th17 cells stimulated under Th2-skewing conditions could be an effect of prior TGF-β-induced signals during Th17 differentiation in combination with additional IL-4 stimulation under Th2-polarizing conditions. Taken together, these data demonstrate that Th17-associated cytokines are differentially expressed when YFP$^+$ Th17 cells are stimulated under different T helper-polarizing conditions, while the expression of IFN-γ, IL-13 or IL-9 is increased when YFP$^+$ Th17 cells are stimulated under Th1, Th2 or Th9 polarizing-conditions, respectively.

Stimulation of Th17 cells under different T helper-polarizing conditions induces the expression of the respective T helper-associated transcription factors

The changes in cytokine production observed in YFP$^+$ Th17 cells maintained under Th17-polarizing conditions or stimulated under Th1, Th2 or Th9-polarizing conditions led us to determine if there were also alterations in transcription factor expression among these cell populations. To examine this, naïve CD4$^+$ T cells from the spleen of $Il17f^{+/Cost}Rs^{YFP/YFP}$ mice were cultured as described for Figure 2 and YFP$^+$ cells were sorted after 3 days of the third round of stimulation under Th17-skewing conditions or Th1, Th2 or Th9-polarizing conditions. Although we saw a reduction in IL-17A$^+$ cells when YFP$^+$ Th17 cells were cultured under Th1, Th2 or Th9-skewing conditions in comparison to cells maintained under Th17-polarizing conditions (Fig. 2), RORγt protein was similar among YFP$^+$ Th17 cells cultured under different polarizing conditions (Fig. 3A, 3B). However, in comparison to YFP$^+$ Th17 cells maintained under Th17-skewing conditions, *Rorc* mRNA was significantly reduced in cells stimulated under Th1-polarizing conditions (Fig. 3C).

YFP$^+$ Th17 cells stimulated under Th1-polarizing conditions express increased T-bet protein and mRNA (Fig. 3A–3C), coinciding with the observed increase in IFN-γ production from these cells (Fig. 2). GATA3 is essential for Th2 and Th9 development and YFP$^+$ Th17 cells stimulated under Th2 or Th9-polarizing conditions display enhanced GATA3 levels in comparison to cells maintained under Th17-polarizing conditions (Fig. 3A–3C). Furthermore, Th17 cells stimulated under Th9-polarizing conditions express a significant increase in *Irf4* and *Maf*, both of which encode transcription factors with enriched expression in Th9 cells (Fig. 3C). BATF has been shown to play an important role in the development of a number of T helper subsets, and while *Batf* is expressed at similar levels in YFP$^+$ Th17 cells stimulated under Th2, Th9 or Th17-polarizing conditions, expression levels are significantly reduced in YFP$^+$ Th17 cells stimulated under Th1-polarizing conditions in comparison to YFP$^+$ Th17 cells maintained under Th17-skewing conditions (Fig. 3C). These data demonstrate that YFP$^+$ Th17 cells stimulated under Th1, Th2 or Th9-polarizing conditions induce the expression of transcription factors associated with the development of each T helper subset.

Th17 cell stability in acute allergic airway disease

Several reports have demonstrated that Th17 cells can adopt the IFN-γ-secreting phenotype of Th1 cells in vivo, and we have confirmed that using our reporter mouse (Fig. 1D). However it is less clear if Th17 cells can adopt a Th2 or Th9 effector program in an in vivo environment that promotes their development. To explore this possibility, we first examined the stability of Th17 cells in an acute model of AAD. $Il17f^{+/Cost}Rs^{YFP/YFP}$ mice were sensitized with OVA and alum followed by intranasal challenges with OVA. In comparison to control mice challenged with PBS, mice challenged with OVA display an increase in the total number of cells in the bronchoalveolar lavage (BAL) as well as in the number of CD4$^+$YFP$^+$ cells isolated from the BAL (Fig. 4A). While most of the CD4$^+$ T cells from the BAL are single producers of IL-17A, IFN-γ or IL-13, there is a small population of cells that co-produce IL-17A and IFN-γ or IL-17A and IL-13 (Fig 4B). CD4$^+$YFP$^+$ cells produce IL-17 and generate a proliferative response to OVA (data not shown), demonstrating that YFP$^+$ cells are antigen-specific. However, the IL-17A-producing CD4$^+$YFP$^+$ cells from the BAL and lung do not express the other T helper cell-associated cytokines analyzed (Fig. 4B, 4C, data not shown), suggesting a stable Th17 phenotype in acute AAD.

Th17 cell stability in chronic allergic airway disease

As there are differences in the allergic environment induced by different models of AAD, we also explored the stability of Th17 cells in a chronic model of AAD using house dust mite (HDM) challenge, a model that has been shown to be dependent on IL-17 and Th17 cells (45–47). $Il17f^{+/Cost}Rs^{YFP/YFP}$ mice received 3 consecutive intranasal challenges with HDM each week for five weeks. Allergic mice display an increase in the total number of cells that infiltrated the BAL along with an increase in CD4$^+$YFP$^+$ cells in the BAL in comparison to non-allergic control mice (Fig. 5A). Similar to what was observed in the acute model of AAD, CD4$^+$YFP$^+$ cells from the BAL and lung of allergic mice express IL-17A, but do not express IFN-γ, IL-13 or IL-9 (Fig. 5B, data not shown). These data demonstrate that YFP$^+$ Th17 cells remain IL-17-producers and do not express cytokines associated with other T helper subsets during the development of acute and chronic AAD.

Differential cytokine receptor expression from in vitro and in vivo derived Th17 cells

Our data show that Th17 cells derived in vitro are capable of adopting a Th2 or Th9 effector program, however in the models of AAD tested, Th17 cells remain IL-17-secretors and do not adopt alternative T helper phenotypes. It is possible that in vitro and in vivo derived Th17 cells display differences in cytokine receptor expression, affecting their responsiveness to cytokines in the environment. To explore this further, we first analyzed cytokine receptor expression from sorted YFP$^+$ Th17 cells derived from $Il17f^{+/Cost}Rs^{YFP/YFP}$ mice prior to stimulation under T helper-polarizing conditions (Th17 pre-switch). As expected, after two rounds of culture under Th17-polarizing conditions, YFP$^+$ cells display significantly increased levels of $Il23r$ expression in comparison to naïve CD4$^+$ T cells (Fig. 6A). YFP$^+$ Th17 cells display higher levels of $Il12rb2$ and $Il4ra$ expression compared to littermate control naïve CD4$^+$ T cells (Fig. 6A). YFP$^+$ Th17 cells before switching to Th2 conditions have high IL-4Rα staining on a similar percentage of cells to cells in Th2 cultures (Fig. 6B). We next assessed cytokine receptor expression from CD4$^+$ T cells in the BAL and lung of

Il17f$^{+/Cost}$*Rs*$^{YFP/YFP}$ mice that had developed OVA and alum-induced AAD. CD4$^+$YFP$^-$ T cells from the BAL and lung of allergic mice display enhanced expression of IL-4Rα in comparison to control mice, which coincides with an increase in Th2 and Th9 cell development in allergic mice (Fig. 6C, 4B, data not shown). However, CD4$^+$YFP$^+$ T cells from the BAL and lung of allergic mice do not express IL-4Rα (Fig. 6C, data not shown). Together, these data suggest that although in vitro derived Th17 cells express the cytokine receptors necessary to respond to cytokines essential for the development of other T helper effector programs, Th17 cells developed during AAD do not.

In vitro derived Ag-specific Th17 cells remain stable IL-17-secretors in an in vivo allergic environment

To understand if the differences observed in the stability of Th17 cells derived in vitro or during the development of AAD is due to differences in cytokine receptor expression or can be attributed to the cytokine environment induced during AAD, we determined if in vitro derived Ag-specific Th17 cells could adopt other T helper effector programs upon transfer to an allergic environment. Ag-specific naïve CD4$^+$ T cells from *Il17f*$^{+/Cost}$*Rs*$^{YFP/YFP}$-OT-II mice were stimulated under Th17-polarizing conditions for two rounds of culture. YFP$^+$ Th17 cells were sorted, transferred intravenously to OVA and alum-sensitized wild type mice and mice were subsequently challenged with OVA. We transferred a relatively small number of cells (10^5) to be able to track the cells without having the transferred cells dominate the in vivo response. The overall cellular infiltrate in the lung was not different between recipient mice that did or did not receive YFP$^+$ Th17 cells.. While endogenous CD4$^+$YFP$^-$ cells from the BAL and lung express IL-17A, IFN-γ or IL-13, transferred CD4$^+$YFP$^+$ cells maintain expression of IL-17A, but do not produce IFN-γ, IL-13 or IL-9 (Fig. 7, data not shown). These data demonstrate that while in vitro derived Th17 cells express IL-4Rα, respond to IL-4-induced signals and adopt pro-allergic phenotypes in vitro, Th17 cells in a Th2 and Th9-biased pro-inflammatory environment, retain an IL-17-secreting phenotype without adopting alternative T helper effector programs.

Discussion

Lineage differentiation and commitment are essential biological processes, which have been studied extensively in a number of systems, including hematopoiesis and T cell development. Recent research has demonstrated that T helper lineages display considerable plasticity and can change their pattern of expression of lineage specific transcription factors and cytokines in response to an altered cytokine environment. Th17 cells are unstable in vitro, and the development of lineage tracer mouse models has further revealed an unstable phenotype in vivo, particularly in inflammatory environments that promote Th1 development. Our results demonstrate that Th17 cells are more stable in a pro-allergic environment, not acquiring other cytokine-secreting phenotypes and maintaining an IL-17-secreting phenotype.

The mechanism of Th17 stability in allergic inflammation is at least two-fold. First, Th17 cells derived in vivo lack expression of the IL-4Rα chain that would be required for the induction of either Th2 or Th9 phenotypes. This is distinct from Th17 cells cultured in vitro,

and suggests that in vivo, there is an active process that represses expression of *Il4ra*. Second, the allergic environment in the lung was not sufficient to promote Th17 switching to Th2 or Th9 phenotypes. Even in vitro derived Th17 cells are incapable of acquiring IL-13 or IL-9 secretion when adoptively transferred into challenged mice. This could simply be the result of an insufficient concentration of IL-4 to promote a switch. Although it is difficult to determine what concentration of cytokine a Th17 cell might encounter in vivo, it is clearly sufficient in these experiments to generate Th2 cells from the endogenous T cell population. It is also possible that additional components of the allergic inflammatory milieu actively contribute to Th17 stability. Distinguishing among these possibilities will require further studies.

Allergic inflammation has a heterogeneous cellular infiltrate and is orchestrated by a large number of immune cells, including Th1, Th2, Th9 and Th17 cells (48). Two recent reports have identified a population of T effector/memory cells in humans that co-express GATA3 and RORγt, and co-produce Th2 and Th17 cytokines. This cell population was increased in asthmatic patients compared with non-asthmatics and further identified in the lungs of allergic mice, however the origin of the Th2/Th17 effector/memory population is not completely understood (49, 50). In our experiments we detected a small population of CD4^{+} cells in the lung of allergic mice that co-produced IL-17A and IL-13, although these cells did not express YFP. This would suggest that IL-17A/IL-13-secreting T cells did not previously express IL-17F or that they only recently expressed IL-17F and YFP expression was not yet established. Our results imply that IL-17A/IL-13-secreting T cells might develop from cells with a Th2 phenotype, but are unlikely to develop from Th17 cells.

Th17 cells play an important role in the development of allergic inflammation in the lung of mice by promoting neutrophilic airway inflammation and further enhancing Th2-mediated airway eosinophilia (51, 52). Th17 cells also promote steroid-resistant airway responses in mice, linking them to severe AAD (53). However, IL-17 may have a dual role in allergic inflammation because it is essential during allergic sensitization in some models of airway inflammation, but it has also been shown to repress airway responses in mice that have been sensitized to allergen (54–56). The role of IL-17 in human allergic disease is less clear. While IL-17 levels are increased in the lung of asthmatic patients, the cellular source of IL-17 and its association with disease severity are still not well defined (57–60). The association of asthma and SNPs in *IL1R1* and *RORA,* and clinical effects of trials targeting IL-17A and IL-17R in asthma, are suggestive of a role for the Th17 cell pathway in human allergic disease (20, 61–63). Collectively, these findings suggest a role for Th17 cells in the development of allergic responses in the mouse and human lung, and it remains possible that Th17 cell stability is essential for the development of allergic disease.

The stability of Th17 cells in a pro-allergic environment might be important for the development of appropriate immune responses. Hirota et al found that during the development of a skin infection with *Candida albicans*, Th17 cells repressed IL-17 expression and did not secrete alternative cytokines upon clearance of the infection, which was accompanied by diminished IL-23 expression and increased IL-10 expression in the skin, suggesting a switch to an anti-inflammatory environment (39). The role in the immune response of Th17 (YFP^{+}) cells that have turned off IL-17 expression but do not acquire the

ability to secrete other cytokines is not well defined. These cells could simply be a result of turning off a specific arm of the immune system. In contrast, these cells might perform a distinct function in vivo, or acquire distinct cytokine-secreting phenotypes at a different time point. Our studies did not address the kinetics of YFP and IL-17 expression over time in allergic mice, although the level of IL-17 produced by in vitro derived YFP+ Th17 cells remained the same before and after transfer to allergic mice. Moreover, we observed only a small number of YFP+ cells in the draining lymph node, suggesting that switched cells were not migrating to other lymphoid organs. Our results suggest a selective advantage for Th17 stability in this environment. The pro-allergic cytokine milieu might maintain the Th17 phenotype to potentiate neutrophilic inflammation that might aid in immunity to infection coincident with a flare of allergic inflammation. Thus, in contrast to immune responses promoting an evolving immune response from Th17-mediated to Th1-mediated inflammation, allergic inflammation might allow for a broader immune response to potential pathogens.

Using our newly generated *Il17f*^Cost mice, we demonstrated that although Th17 cells can adopt the effector programs of Th1, Th2 or Th9 cells in vitro, during allergic inflammatory disease, Th17 cells are comparatively stable, and retain the potential to produce IL-17. Thus, our data demonstrate that the inflammatory environment dictates the stability of Th17 cells in vivo. Future studies will define inflammatory mediators and underlying mechanisms involved in the regulation of Th17 cell stability in the allergic airway environment and other anatomic locations of allergic inflammation.

Acknowledgments

Abbreviations

AAD	Allergic airway disease
Alum	Aluminum hydroxide
BAL	Bronchoalveolar lavage
Cost	Cre On Seventeen Transcript
EAE	Experimental autoimmune encephalomyelitis
EGFP	Enhanced green fluorescent protein
GATA	GATA binding protein
HDM	House dust mite
IRF	Interferon regulatory factor
ROR	Retinoid-acid-related orphan receptor
Runx	Runt-related transcription factor

T-bet   T-box

**YFP**   yellow fluorescent protein

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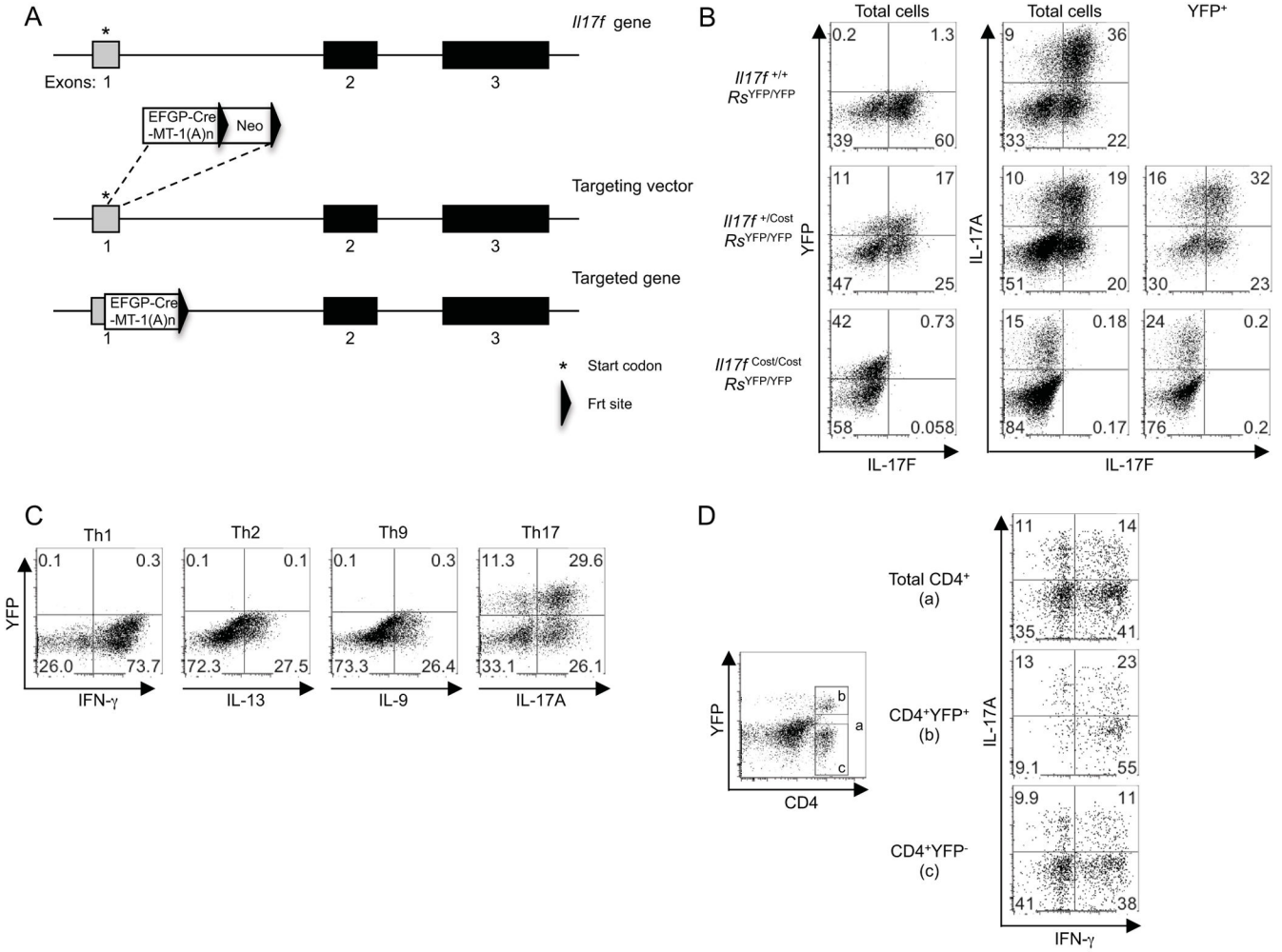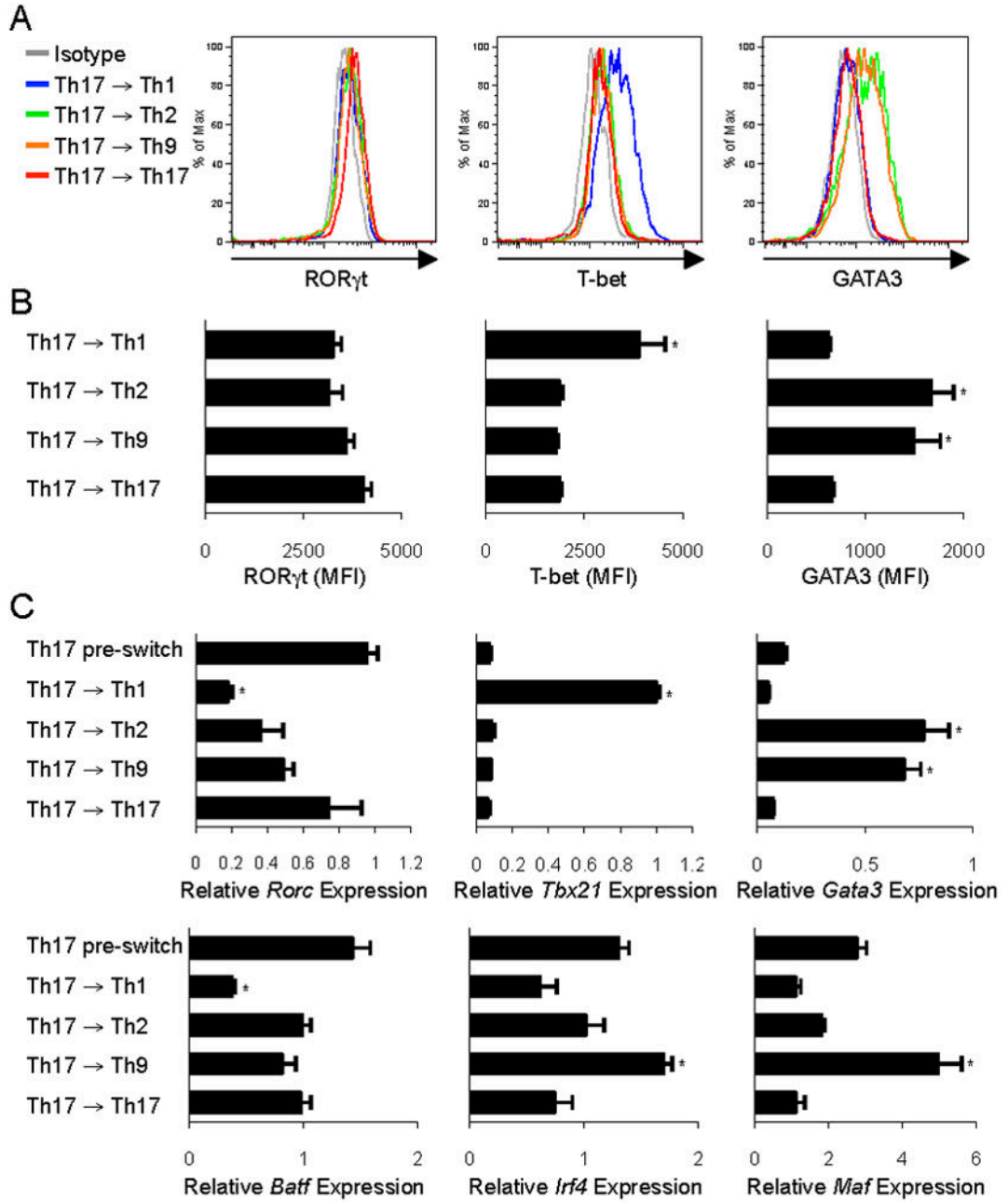
Figure 1.
Generation of $Il17f^{Cost}$ mice. *A*, Schematic of the *Il17f* locus, targeting vector and the targeted *Il17f* locus. *B*, Naïve CD4$^+$ T cells were isolated from the spleen of $Il17f^{+/+}Rs^{YFP/YFP}$, $Il17f^{+/Cost}Rs^{YFP/YFP}$ or $Il17f^{Cost/Cost}Rs^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days. Cells were re-stimulated with PMA and ionomycin and YFP and cytokine expression was analyzed by flow cytometry. Cells are gated on the live population (left and middle panels) and live YFP$^+$ population (right panel). *C*, Naïve CD4$^+$ T cells were isolated from the spleen of $Il17f^{+/Cost}Rs^{YFP/YFP}$ mice and stimulated under T helper cell polarizing conditions for 5 days. Cells were re-stimulated with PMA and ionomycin and YFP and cytokine expression was analyzed by flow cytometry in the live population. *D*, EAE was induced in $Il17f^{+/Cost}Rs^{YFP/YFP}$ mice. Mice were sacrificed 19 days after induction of disease and spleen and brain were harvested. Splenocytes (data not shown) and mononuclear cells from the brain were re-stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live total CD4$^+$ (gate a), CD4$^+$YFP$^+$ (gate b) or CD4$^+$YFP$^-$ lymphocytes (gate c). Representative dot plots are shown and data are representative of 2 or more independent experiments with 3–4 mice per group (*B–D*).
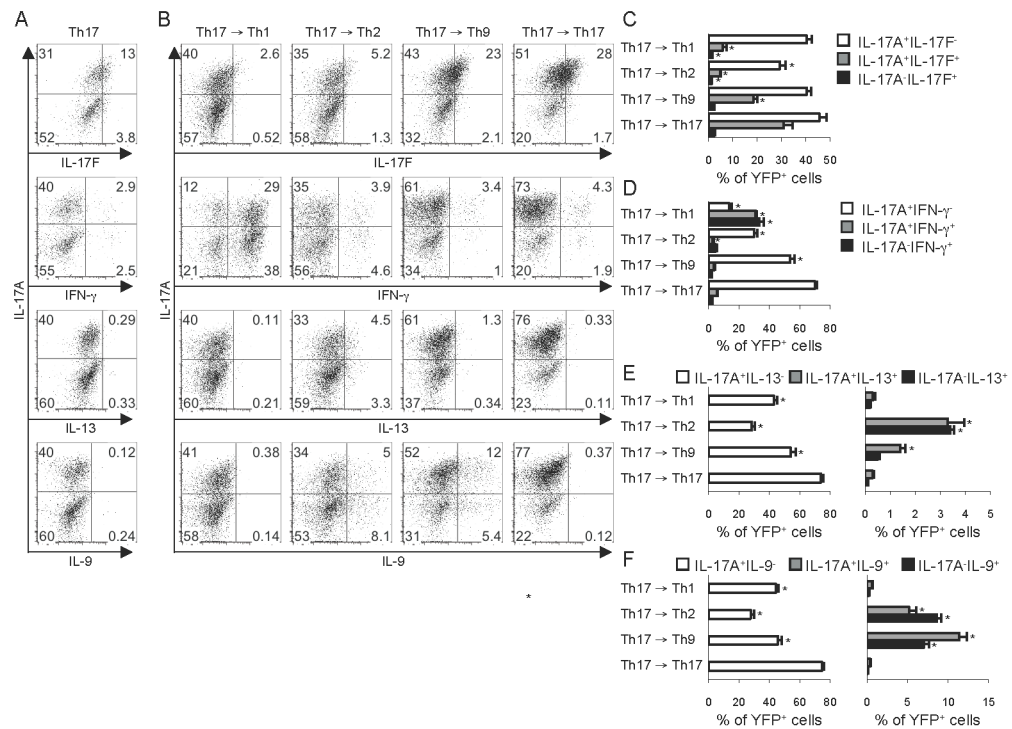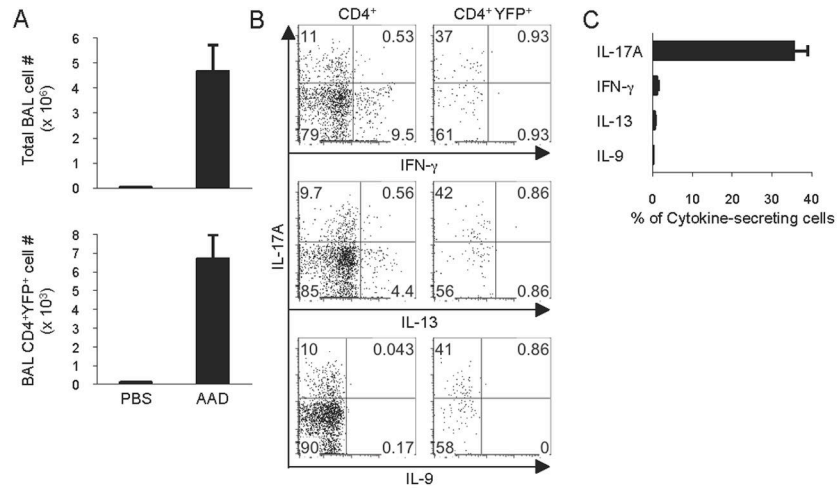
Figure 2.
Th17 cells display an altered cytokine profile when cultured under T helper cell-polarizing conditions. *A–F,* Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f*$^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17 polarizing conditions for another five days (Round 2). Live YFP$^+$ cells were sorted by flow cytometry and cultured under Th1, Th2, Th9 or long-term Th17-polarizing conditions for five days (Round 3). *A*, After 2 rounds of stimulation under Th17-polarizing conditions, cells were re-stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry. Cells are gated on the live YFP$^+$ population. Representative dot plots are shown. *B*, After culture under polarizing conditions (Round 3), cells were re-stimulated, stained and analyzed as in *A*. Cells are gated on the live YFP$^+$ population. Representative dot plots are shown. *C–F*, Graphical representation of the data displayed in *B*. The data are the mean ± SEM of 5 mice. Statistical analysis in *C–F* was performed using the one-way ANOVA. *$p < 0.05$, compared with Th17 → Th17 samples. Data are representative of three or more independent experiments (*A–F).*
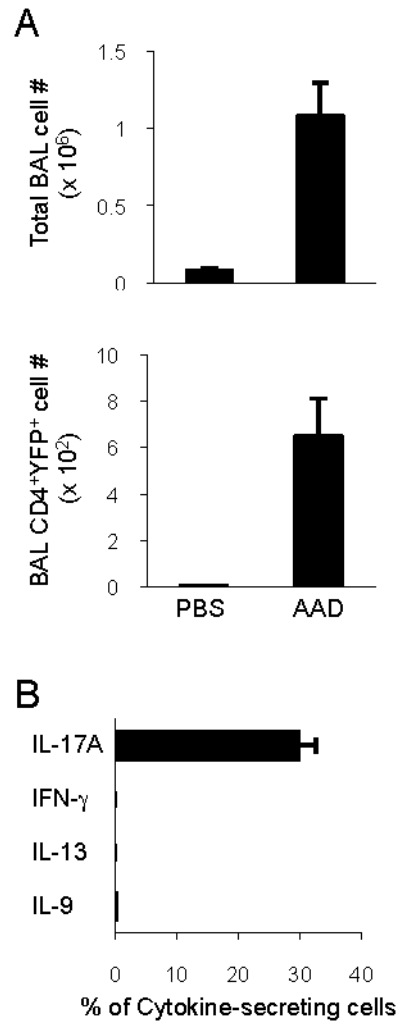
Figure 3.
Expression of transcription factors in Th17 cells cultured under Th cell-polarizing conditions. *A–C*, Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f*$^{+/\text{Cost}}$*Rs*$^{\text{YFP/YFP}}$ mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17-polarizing conditions for another five days (Round 2). Live YFP$^+$ cells were sorted by flow cytometry and cultured under Th1, Th2, Th9 or long-term Th17-polarizing conditions for three days (Round 3) and live YFP$^+$ cells were sorted for further analysis. *A*, Expression of the indicated transcription factors was assessed in unstimulated cells by flow cytometry. Representative histograms are shown. *B*,

Graphical representation of the mean fluorescence intensity (MFI) data shown in *A*. The data are the mean ± SEM of 3 mice. *C*, RNA was isolated from sorted live YFP^{+} cells on the third day of round 2 (Th17 pre-switch) or sorted live YFP^{+} cells after three days of the third round of culture. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to Th17 → Th1 cells (*Tbx21*), Th17 → Th2 cells (*Gata3*) or Th17 → Th17 cells (*Rorc*, *Batf*, *Irf4*, *Maf*). The data are the mean ± SEM of 3 mice. Statistical analysis in *B–C* was performed using the one-way ANOVA. *$p < 0.05$, compared with Th17 → Th17 samples. Data are representative of 3 or more independent experiments (*A–C*).
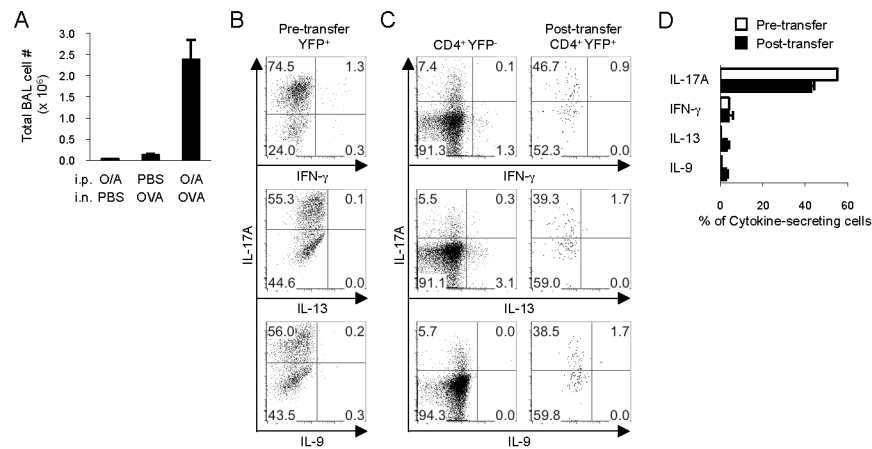
Figure 4.
Stability of Th17 cells in an acute model of AAD. *A–C*, *Il17f*[+/Cost]*Rs*[YFP/YFP] mice were sensitized i.p. with OVA and alum on days 0 and 7, challenged i.n. with PBS or OVA on days 14–19 and sacrificed 24 h after the final challenge. Cells were isolated from the BAL and lung (data not shown) for further analysis. *A*, Total cell number was calculated in the BAL of control (PBS) or allergic (AAD) mice (top panel). CD4^{+}YFP^{+} cell number was determined from the BAL of control or allergic mice using flow cytometry (bottom panel). Data are the mean ± SEM of 3–5 mice. *B*, BAL cells were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live CD4^{+} (left panel) or CD4^{+}YFP^{+} lymphocytes (right panel). Representative dot plots are shown. *C*, Graphical representation of the CD4^{+}YFP^{+} data displayed in *B*. The data are the mean ± SEM of 5 mice. Data are representative of 3 or more independent experiments (*A–C*).

Figure 5.
Stability of Th17 cells in a chronic model of AAD. *A–B*, *Il17f*[+/Cost]*Rs*[YFP/YFP] mice received i.n. doses of HDM for 3 consecutive days each week for 5 weeks and were sacrificed 24 h after the final challenge. Cells were isolated from the BAL and lung (data not shown) for further analysis. *A*, The total cell number was calculated in the BAL of control (PBS) or allergic (AAD) mice (top panel). The CD4^{+}YFP^{+} cell number was determined from the BAL of control or allergic mice using flow cytometry (bottom panel). Data are the mean ± SEM of 3–5 mice. *B*, BAL cells were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live CD4^{+}YFP^{+} lymphocytes. The data are the mean ± SEM of 3 mice.
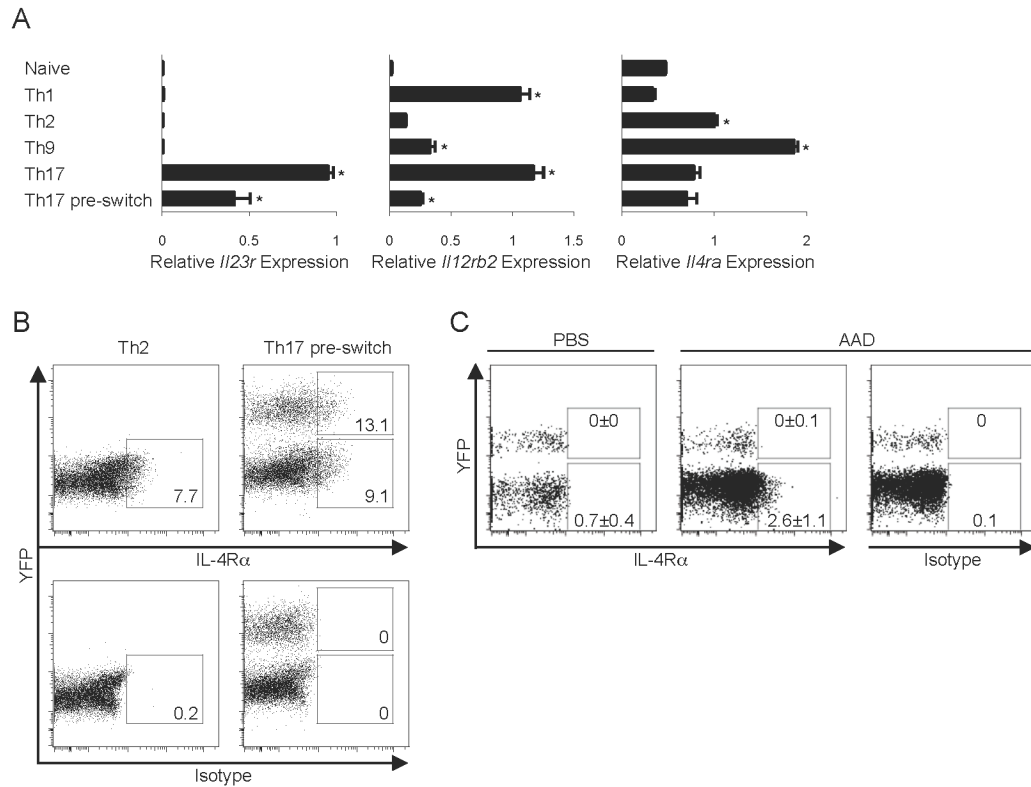
Figure 6.
Cytokine receptor expression from Th17 cells derived in vitro or in vivo during the development of AAD. *A*, For Th17 pre-switch cells, naïve CD4^{+} T cells were isolated from the spleen of *Il17f*[+/Cost]*Rs*[YFP/YFP] mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17-polarizing conditions for another five days (Round 2) and live YFP^{+} cells were sorted by flow cytometry. For other Th samples, naïve CD4^{+} T cells were isolated from the spleen of *Il17f*^{+/+}*Rs*[YFP/YFP] littermate control mice and stimulated under Th-polarizing conditions for five days before RNA was isolated. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to Th17 cells (*Il23r*), Th1 cells (*Il12rb2*) or Th2 cells (*Il4ra*). The data are the mean ± SEM of 3–5 mice. *B*, Th2 and Th17 pre-switch cells were cultured as in *A* and IL-4Rα expression was analyzed in live YFP[−] and YFP[+] populations by flow cytometry. Percentages indicated are of the respective YFP[+] or YFP[−] populations. Representative dot plots are shown. *C*, OVA and alum-induced AAD was induced in *Il17f*[+/Cost]*Rs*[YFP/YFP] mice as in Figure 4. Cells were isolated from the BAL and lung (data not shown) and IL-4Rα expression was analyzed in YFP^{−} and YFP^{+} CD4^{+} lymphocytes from control (PBS) or allergic (AAD) mice. The cells are gated on live CD4^{+} lymphocytes. Representative dot plots are shown with the mean ± SD of 2–4 mice displayed. Statistical analysis in *A* was performed using the one-way ANOVA. *$p < 0.05$, compared with Naïve samples. Data are representative of 2 independent experiments (*A, C*).

Figure 7.
Stability of Ag-specific in vitro-derived Th17 cells upon adoptive transfer to allergic mice. *A–D,* WT mice were injected i.p. with PBS or sensitized i.p. with OVA and alum (O/A) on days 0 and 7. All mice were injected i.v. with sorted YFP$^+$ Th17 cells (1×10^5) differentiated from *Il17f*$^{+/Cost}$*Rs*$^{+/YFP}$ –OT-ll mice (2 rounds under Th17-polarizing conditions) on day 20. Mice were challenged i.n. with PBS or OVA on days 21–26 and sacrificed 24 h after the final challenge. Cells were isolated from the BAL and lung for further analysis. *A*, Total cell number was calculated in the BAL of each group of mice. Data are the mean ± SEM of 7–8 mice. *B*, Sorted YFP$^+$ Th17 cells differentiated from *Il17f*$^{+/Cost}$*Rs*$^{+/YFP}$ –OT-ll mice (2 rounds under Th17-polarizing conditions) were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry (Pre-transfer YFP$^+$). *C*, BAL (data not shown) and lung cells were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live YFP$^-$ (endogenous) or YFP$^+$ (Post-transfer) CD4$^+$ lymphocytes. Representative dot plots are shown. *D*, Graphical representation of the Pre-transfer YFP$^+$ and Post-transfer CD4$^+$YFP$^+$ data displayed in *B–C*. Different IL-17A antibody clones were used in the top versus bottom two panels of dot plots in *B–C*. The data are from pooled Th17 cells (Pre-transfer) or the mean ± SEM of 4 mice (Post-transfer).