Differential Roles of Interleukin-17A and -17F in Host Defense against Mucoepithelial Bacterial Infection and Allergic Responses

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SUMMARY

Interleukin-17A (IL-17A) is a cytokine produced by T helper 17 (Th17) cells and plays important roles in the development of inflammatory diseases. Although IL-17F is highly homologous to IL-17A and binds the same receptor, the functional roles of this molecule remain largely unknown. Here, we demonstrated with $Il17a^{-/-}$, $Il17f^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice that IL-17F played only marginal roles, if at all, in the development of delayed-type and contact hypersensitivities, autoimmune encephalomyelitis, collagen-induced arthritis, and arthritis in $Il1rn^{-/-}$ mice. In contrast, both IL-17F and IL-17A were involved in host defense against mucoepithelial infection by *Staphylococcus aureus* and *Citrobacter rodentium*. IL-17A was produced mainly in T cells, whereas IL-17F was produced in T cells, innate immune cells, and epithelial cells. Although only IL-17A efficiently induced cytokines in macrophages, both cytokines activated epithelial innate immune responses. These observations indicate that IL-17A and IL-17F have overlapping yet distinct roles in host immune and defense mechanisms.

INTRODUCTION

Naive CD4^{+} T cells are categorized into several helper T (Th) cell subsets, including Th1 and Th2 cells, on the basis of their cytokine production profiles and effector functions. Recently, Th17 cells that preferentially produce interleukin-17A (IL-17A), IL-17F, IL-21, and IL-22 were identified in mice (McGeachy and Cua, 2008; Ouyang et al., 2008). Th17 cell differentiation is induced by TGF-β plus IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a) or IL-21 (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007) and accelerated by the coordinated activities of IL-1 and TNF (Veldhoen et al., 2006a). IL-23 is required for the growth, survival, and effector functions of

Th17 cells and promotes IL-17A and IL-17F production by this T cell subset (Veldhoen et al., 2006a; Zhou et al., 2007).

IL-17F and IL-17A are highly homologous members of the IL-17 protein family and are encoded by genes that are located nearby each other in both humans and mice (Kawaguchi et al., 2004; Kolls and Linden, 2004; Weaver et al., 2007). It has been reported that IL-17A and IL-17F may bind the same receptor complexes consisting of IL-17RA and IL-17RC (Toy et al., 2006; Zheng et al., 2008), suggesting that these cytokines have similar biological functions. Consistent with this notion, both IL-17A and IL-17F induce the production of antimicrobial peptides (defensins), cytokines (IL-6, G-CSF, GM-CSF), and chemokines (CXCL1, CXCL2, CXCL5), as well as enhance granulopoiesis and neutrophil recruitment (Kawaguchi et al., 2004; Kolls and Linden, 2004; Weaver et al., 2007). Overexpression of IL-17F or IL-17A in the lungs leads to increased proinflammatory cytokine and chemokine expression, resulting in inflammation associated with neutrophil infiltration (Hurst et al., 2002; Oda et al., 2005; Park et al., 2005; Yang et al., 2008).

Several lines of evidence have established that the IL-23–IL-17A signaling axis rather than the IL-12–IFN-γ signaling axis is responsible for the development of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD), as well as allergic diseases such as contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) in mice (McGeachy and Cua, 2008; Oboki et al., 2008). Recent studies suggest that Th17 cells are also involved in the host defense against infection, because antigen-presenting cells (APCs) stimulated with such microbial products as lipopolysaccharide (LPS), peptidoglycans, and zymosan produce a large amount of IL-23, resulting in the development of Th17 cells (LeibundGut-Landmann et al., 2007; van Beelen et al., 2007; Veldhoen et al., 2006b). Furthermore, $Il17ra^{-/-}$ mice and/or $Il23a^{-/-}$ mice are more susceptible to *Klebsiella pneumoniae* in the lungs (Happel et al., 2005) and *Citrobacter rodentium* in the intestines (Mangan et al., 2006; Zheng et al., 2008). However, the relative contributions of IL-17A and IL-17F to autoimmune and allergic diseases as well as host defense processes remain to be elucidated.
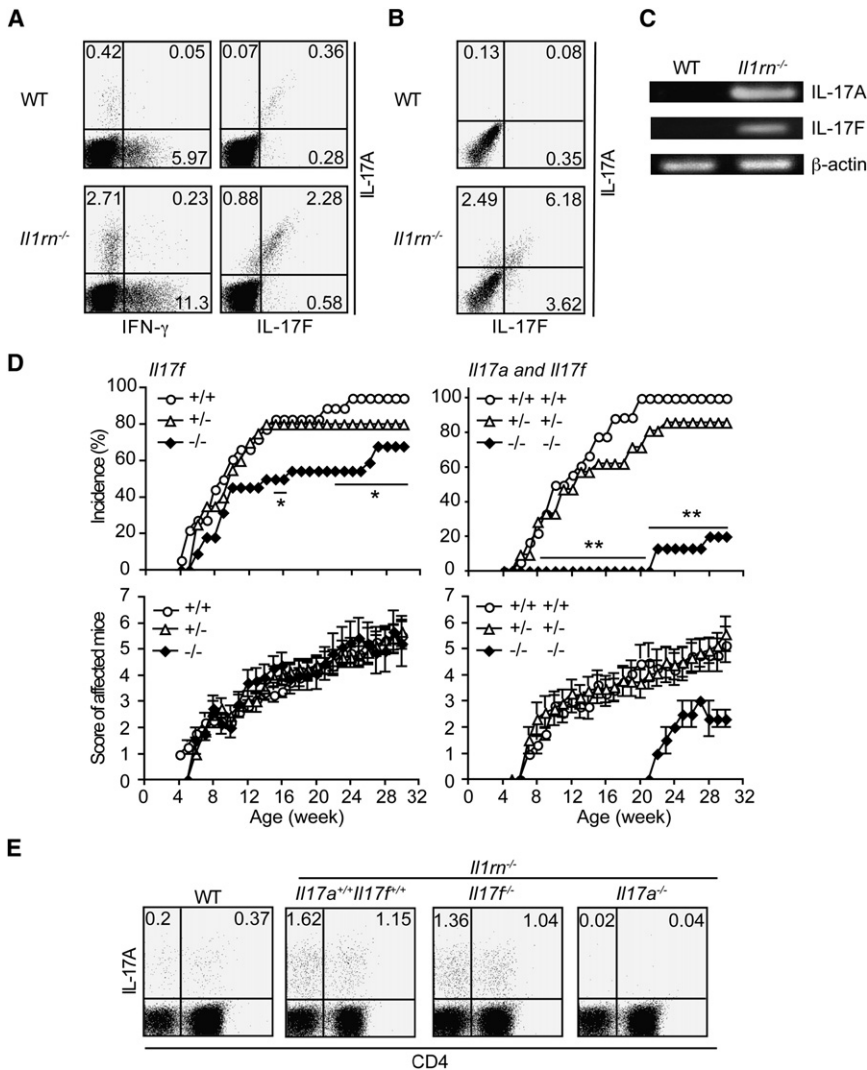
Figure 1. IL-17F Contributes to the Development of Spontaneous Autoimmune Arthritis in *Il1rn*$^{-/-}$ Mice

(A) Profiles of intracellular IL-17F, IL-17A, and IFN-γ expression in LN cells from wild-type and arthritic *Il1rn*$^{-/-}$ mice stimulated with PMA and ionomycin in vitro.

(B) Profiles of intracellular IL-17F, IL-17A, and IFN-γ expression in cells from the ankle joints of wild-type and arthritic *Il1rn*$^{-/-}$ mice stimulated with PMA and ionomycin.

(C) Expression of IL-17A and IL-17F mRNA in the joints of arthritic *Il1rn*$^{-/-}$ mice.

(D) Arthritis incidence and severity scores in *Il1rn*$^{-/-}$ mice. Left panels: open circles represent *Il17f*$^{+/+}$, open triangles represent *Il17f*$^{+/-}$, and closed diamonds represent *Il17f*$^{-/-}$ mice on an *Il1rn*$^{-/-}$ background; right panels: open circles represent *Il17a*$^{+/+}$ *Il17f*$^{+/+}$, open triangles represent *Il17a*$^{+/-}$*Il17f*$^{+/-}$, and closed diamonds represent *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice on an *Il1rn*$^{-/-}$ background (n = 15–22/group). *, p < 0.05 and **, p < 0.01 versus *Il17f*$^{+/+}$ or *Il17a*$^{+/+}$*Il17f*$^{+/+}$ mice determined with χ^2 tests.

(E) Intracellular IL-17A expression in LN cells from wild-type, *Il17a*$^{+/+}$*Il17f*$^{+/+}$*Il1rn*$^{-/-}$, *Il17f*$^{-/-}$*Il1rn*$^{-/-}$, and *Il17a*$^{-/-}$*Il1rn*$^{-/-}$ mice stimulated with PMA and ionomycin.

Data are representative of two (C and E) or three (A and B) independent experiments.

In this study, we discriminated between the functions of IL-17F and IL-17A in immune responses and host defense mechanisms directed against bacterial infection. To accomplish this, we have generated mice lacking IL-17F (*Il17f*$^{-/-}$) or both IL-17A and IL-17F (*Il17a*$^{-/-}$*Il17f*$^{-/-}$), which were used together with previously generated *Il17a*$^{-/-}$ mice (Nakae et al., 2002), and shown that IL-17A and IL-17F play distinct roles in the development of T cell-mediated inflammation and immune responses against bacterial infection.

RESULTS

IL-17F Contributes to the Development of Arthritis in IL-1 Receptor Antagonist-Deficient Mice

To elucidate the functional differences between IL-17F and IL-17A in the immune system, we generated *Il17a*$^{-/-}$, *Il17f*$^{-/-}$, and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice (Figure S1, available online). These mice were born healthy at the expected Mendelian ratio, were fertile, and showed no gross phenotypic abnormalities, including in their lymphoid cell populations (data not shown). Proliferative responses and interferon-γ (IFN-γ) production were normal in

Il17a$^{-/-}$*Il17f*$^{-/-}$ mice, IL-17A production was normal in *Il17f*$^{-/-}$ mice, and neither IL-17A nor IL-17F was required for TGF-β plus IL-6-induced Th17 cell differentiation (Figure S2).

IL-17A plays a crucial role in the spontaneous development of arthritis in IL-1 receptor antagonist-deficient (*Il1rn*$^{-/-}$) mice (Nakae et al., 2003b). There were more IL-17F-producing cells, which also produced IL-17A, among the arthritic *Il1rn*$^{-/-}$ lymph node (LN) cells than among the wild-type LN cells, which was also true of the IFN-γ-producing cells (Horai et al., 2004) (Figure 1A). The IL-17A$^+$IL-17F$^+$ cell number and IL-17A and IL-17F mRNA expression were also augmented in LN cells from arthritic *Il1rn*$^{-/-}$ mice (Figures 1B and 1C). The development of arthritis was considerably, but only partially, suppressed in *Il17f*$^{-/-}$ *Il1rn*$^{-/-}$ mice compared with littermate *Il17f*$^{+/+}$*Il1rn*$^{-/-}$ and *Il17f*$^{+/-}$*Il1rn*$^{-/-}$ controls during the 30 week observation period (Figure 1D). Compared with *Il17f*$^{-/-}$*Il1rn*$^{-/-}$ mice, arthritis development was markedly suppressed in *Il17a*$^{-/-}$*Il17f*$^{-/-}$*Il1rn*$^{-/-}$ mice (Figure 1D). The IL-17A$^+$ T cell populations in LNs from *Il17a*$^{+/+}$*Il17f*$^{+/+}$*Il1rn*$^{-/-}$ and *Il17f*$^{-/-}$*Il1rn*$^{-/-}$ mice were similar (Figure 1E).

Likewise, EAE, CIA, DTH, 2,4,6-trinitrochlorobenzene (TNCB)-induced CHS, and neutrophilic airway inflammation induced by OVA in DO11.10 mice, in which IL-17A plays an important role (Komiyama et al., 2006; Nakae et al., 2002, 2003a, 2007), also developed normally in the *Il17f*$^{-/-}$ mice (Figures S3–S7, and Table S1). These results indicate that IL-17A plays a major role in T cell-dependent autoimmune and allergic responses, but IL-17F only marginally contributes to these responses, if at all.
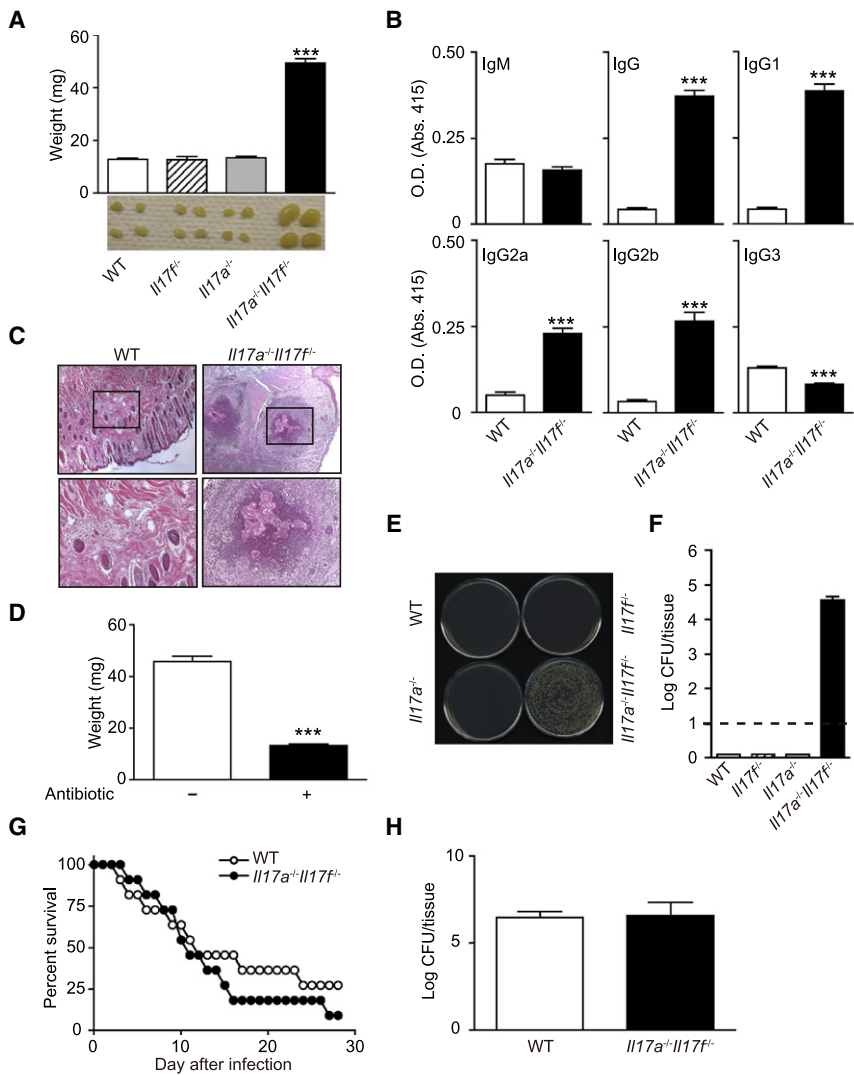
Figure 2. Increased Susceptibility of $Il17a^{-/-}$ $Il17f^{-/-}$ Mice to Opportunistic *S. aureus* Infection

(A) Weight and gross morphology of submandibular LNs from BALB/cA mice at 12–16 weeks of age (n = 3–8/group).

(B) Immunoglobulin titers in sera from mice at 8–10 weeks of age (n = 6–8/group). Similar results were also observed on a C57BL/6J background.

(C) Histopathology of $Il17a^{-/-}Il17f^{-/-}$ mucocutaneous tissues around the nose and mouth (H&E, top, 40×; bottom, 120×). Data are representative of four mice for each group.

(D) Weight of submandibular LNs from $Il17a^{-/-}$ $Il17f^{-/-}$ mice on a BALB/cA background with or without oral antibiotic treatment between 4 and 8 weeks of age (n = 10/group).

(E) Representative plates showing bacterial colonies recovered from mucocutaneous tissues of BALB/cA mice at 12–16 weeks of age.

(F) CFU of *S. aureus* in homogenates from mucocutaneous tissues in mice at 12–16 weeks of age. Data are pooled from three independent experiments.

(G) Survival rate in mice after intravenous (i.v.) injection of 1×10^7 CFU of *S. aureus* (n = 11/group). Data are representative of two independent experiments.

(H) *S. aureus* CFUs in kidney homogenates collected 3 days after i.v. injection of 1×10^7 CFU of *S. aureus* (n = 4/group). Data are representative of two independent experiments.

$*$ $p < 0.05$, $**$ $p < 0.05$, and $***$ $p < 0.05$ versus wild-type. Data represent means ± SEM in (B), (D), (F), and (H).

submandibular LNs in $Il17a^{-/-}Il17f^{-/-}$ mice (Figure 2D). Then, we tried to recover infected microorganisms from the mucocutaneous tissues around the nose and mouth of the $Il17a^{-/-}Il17f^{-/-}$ mice; the opportunistic bacterium *Staphylococcus aureus* was recovered from the affected tissues of these mice. When mucocutaneous tissue homogenates from these mice were cultured, more bacteria was observed in $Il17a^{-/-}Il17f^{-/-}$ mouse homogenate compared with samples from wild-type, $Il17f^{-/-}$, and $Il17a^{-/-}$ mice (Figures 2E and 2F), suggesting that both IL-17A and IL-17F are critically important to protect the mice against mucocutaneous *S. aureus* infections. To investigate whether IL-17A and IL-17F play a role in systemic *S. aureus* infection, we challenged mice with *S. aureus* by intravenous injection. However, no difference was observed in the survival and the number of bacteria recovered from the kidney 72 hr later between wild-type and $Il17a^{-/-}Il17f^{-/-}$ mice (Figures 2G and 2H). These results suggest that both IL-17A and IL-17F play critical roles in protecting against local, but not systemic, infection against *S. aureus*.

$Il17a^{-/-}Il17f^{-/-}$ Mice Show Increased Susceptibility to Opportunistic Infection by *S. aureus*

We found that the submandibular LNs of $Il17a^{-/-}Il17f^{-/-}$ mice, but not of wild-type, $Il17f^{-/-}$, or $Il17a^{-/-}$ mice, became enlarged as the mice aged; this effect was observed in various genetic backgrounds, including the C57BL/6J, BALB/cA, and 129/Ola × C57BL/6J strains (Figure 2A, and data not shown). At 8–10 weeks of age, IgM titers were similar between $Il17a^{-/-}Il17f^{-/-}$ and wild-type mice. Whereas total IgG, IgG1, IgG2a, and IgG2b titers were increased 2–4 fold in the $Il17a^{-/-}Il17f^{-/-}$ mice compared with wild-type mice, IgG3 titers in the $Il17a^{-/-}Il17f^{-/-}$ mice were reduced (Figure 2B). Interestingly, the $Il17a^{-/-}Il17f^{-/-}$ mice developed mucocutaneous abscesses around the nose and mouth (Figure 2C). Histological analyses revealed fibrin-encased abscesses and marked leukocyte infiltration specifically in the mucocutaneous tissues of the $Il17a^{-/-}Il17f^{-/-}$ mice.

The LN enlargement, subcutaneous abscess formation, and increased Ab production suggested that the $Il17a^{-/-}Il17f^{-/-}$ mice may have been responding to an infection. In support of this idea, antibiotic treatment suppressed the enlargement of

Both IL-17F and IL-17A Are Required for Host Defense against *C. rodentium* Infections

Because the $Il17a^{-/-}Il17f^{-/-}$ mice were susceptible to opportunistic infections by *S. aureus*, we examined the susceptibilities of $Il17f^{-/-}$, $Il17a^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice to experimental

C. rodentium infection. After oral infection with *C. rodentium*, the number of bacteria in the colons of wild-type 129/Ola × C57BL/6J mice increased until 14 days after infection, and decreased thereafter (Figure 3A). A substantially greater number of bacteria was detected in the colons of $Il17f^{-/-}$, $Il17a^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice compared with wild-type mice at each time point after infection, although the bacterial burden in the mutant mice declined by day 21 and all of the genotypes returned to the wild-type level by day 28 after infection. Notably, bacterial numbers in the colon were similar among $Il17f^{-/-}$, $Il17a^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice. Marked expansion of the bacterial population was also observed in the distal colon of $Il17f^{-/-}$, $Il17a^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice compared with that of wild-type mice 14 days after infection (Figure 3B). Furthermore, whereas remarkable hypertrophy of the colon and spleen was observed in $Il17f^{-/-}$ and $Il17a^{-/-}Il17f^{-/-}$ mice, only mild hypertrophy was detected in $Il17a^{-/-}$ mice (Figures 3C and 3D). Consistent with these observations, more severe inflammatory changes were observed in the colons of $Il17f^{-/-}$ and $Il17a^{-/-}Il17f^{-/-}$ mice compared to $Il17a^{-/-}$ mice 14 days after infection, suggesting that IL-17F plays a larger role than IL-17A in the immune response against this bacterium (Figures 3E and 3F). These observations clearly show that both IL-17A and IL-17F play important roles to protect hosts against *C. rodentium* infections.

IL-17F and IL-17A Are Required for the Expression of β-Defensin in the Colon

Then, we analyzed the antibacterial mechanisms induced by IL-17A and IL-17F. The serum amounts of *C. rodentium*-specific IgG, which is important for bacterial clearance (Mundy et al., 2005), were increased in all of the mutant mice (Figure S8), suggesting that the humoral immune response against *C. rodentium* was not responsible for delayed bacterial clearance in the $Il17f^{-/-}$, $Il17a^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice.

Both IL-17A and IL-17F regulate innate immunity by inducing neutrophil recruitment and antimicrobial peptide production (Ouyang et al., 2008). Compared to wild-type mice, however, mRNA expression of neutrophil chemoattractants, such as CXCL1 and CXCL2, and proinflammatory mediators, such as IFN-γ, IL-1β, IL-6, TNF, and iNOS, were similarly increased in the colons of $Il17f^{-/-}$, $Il17a^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice 14 days after *C. rodentium* infection (Figure 4A). However, the expression of antimicrobial peptides, such as β-defensin 1, 3, and 4 (but not β-defensin 2, lipocalin 2, S100A8, S100A9, Reg3β, and Reg3γ), was markedly impaired in the colons of $Il17f^{-/-}$, $Il17a^{-/-}$, and $Il17a^{-/-}Il17f^{-/-}$ mice on day 14 after *C. rodentium* infection (Figure 4B). These results suggest that both IL-17F and IL-17A are critical to induce the expression of β-defensins, which are important for the host defense against *C. rodentium*.

IL-17F and IL-17A Are Produced by Different Cells in the Colon

IL-17A mRNA is more highly expressed in the small intestine than in the colon (Ivanov et al., 2006). In contrast, IL-17F mRNA expression in the colon was higher than that in the small intestine (Figure 5A). During *C. rodentium* infection, the expression of both IL-17A and IL-17F mRNA was induced in the colon of wild-type mice, although a larger increase was observed for IL-17A mRNA expression (day 14: IL-17A, 29-fold; IL-17F, 14-fold) (Figure 5B). Under these conditions, IL-17A mRNA expression was not influenced by IL-17F deficiency and vice versa. Although only a few IL-17A- and IL-17F-producing cells were found among the colonic lymphocytes of uninfected wild-type mice (Figure 5C), the population of IL-17F-producing cells, which also produced IL-17A, increased in infected wild-type mice (Figure 5D). In contrast to the coordinate production of IL-17A and IL-17F by LN cells after the development of DTH, EAE, or arthritis (Figure 1A and Figure S5), however, the percentage of IL-17A+IL-17F− cells in the colonic lymphocytes was much larger than that of IL-17A−IL-17F+ cells (Figure 5D). Both IL-17A+ and IL-17F+ cells were not observed in the $Il17a^{-/-}Il17f^{-/-}$ colonic lymphocyte population, whereas the number of IFN-γ+ cells markedly increased during *C. rodentium* infection (Figure 5D).

Because the induction kinetics was different for the two molecules and IL-17F producer cells were scarcely found in colonic lymphocytes, IL-17A and IL-17F may be produced by different cells in the colon. The mRNA expression of these molecules was examined in the colons of recombination activating gene-2-deficient ($Rag2^{-/-}$) mice, in which both T and B cells are absent. The expression of IL-17A mRNA in the mesenteric LNs (MLNs) was markedly higher than that in colons on day 7 after *C. rodentium* infection in wild-type mice (Figure 5E). The amount of IL-17A mRNA, however, was markedly decreased (approximately 20% of wild-type) in $Rag2^{-/-}$ mice (Figures 5E and 5F), suggesting that Th17 cells may be the major producer of IL-17A in the MLN. In contrast, the amount of IL-17F expression was only decreased by approximately 50% in the MLNs of these mice (Figures 5E and 5F). IL-17A mRNA expression was also markedly decreased in the colons of $Rag2^{-/-}$ mice, whereas the IL-17F mRNA expression was similar between wild-type and $Rag2^{-/-}$ mice (Figures 5E and 5F). In addition, IL-17F, but not IL-17A, production in the whole-colon-culture supernatants from $Rag2^{-/-}$ mice was increased by the treatment with IL-23, whereas both IL-17A and IL-17F production were induced in those from wild-type mice, indicating that IL-17F is also produced by non-T and non-B cells (Figure 5G). We next examined which cells produce IL-17F in response to IL-23. Interestingly, IL-23 stimulation led to enhanced IL-17F production in splenocytes or MLNs from $Rag2^{-/-}$ or C.B-17 SCID mice compared to those from wild-type mice, whereas only a small amount of IL-17A was produced in these cells (Figures 5H and 5I, and Figure S9). Among innate immune cells, CD11c−Gr1−B220−F4/F80−Gr1− cells were likely to mainly produce IL-17F upon IL-23 stimulation (Figure S9).

Because IL-17F is expressed in lung epithelial cells (ECs) (Suzuki et al., 2007), we also examined whether IL-17F was expressed in colonic ECs. IL-17F mRNA, but not IL-17A mRNA, was detected in CD45− FACS-sorted colonic ECs from infected wild-type mice; this contrasted to CD45+ intraepithelial immune cells and ConA-stimulated splenocytes, in which both IL-17A and IL-17F were detected (Figure 5J). Moreover, IL-17F, but not IL-17A, mRNA was expressed in mouse colonic EC lines (Figure 5K). These results indicate that, in addition to infiltrating lymphocytes, IL-17F is produced by non-T, non-B innate immune cells and colonic ECs in response to infection with *C. rodentium*.
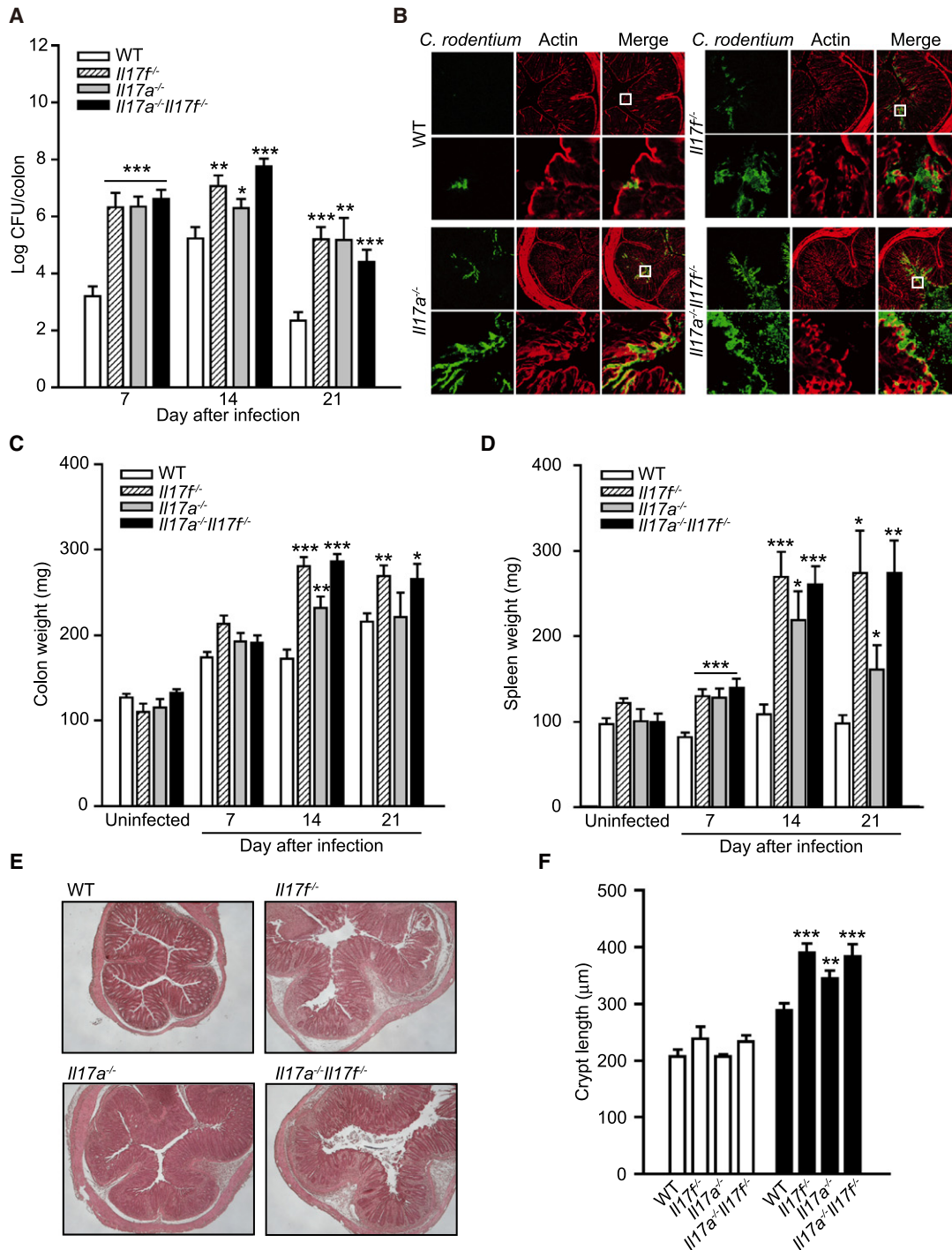
Figure 3. IL-17F and IL-17A Are Required for the Protection against *C. rodentium* Infection

Wild-type, *Il17f*^{−/−}, *Il17a*^{−/−}, and *Il17a*^{−/−}*Il17f*^{−/−} mice were orally infected with 2×10^8 CFU of *C. rodentium,* and the colons and spleens were harvested at the indicated time points after infection.

(A) *C. rodentium* CFUs in colon homogenates (n = 10–16/group). Data show pooled results from two or three independent experiments.

(B) Visualization of *C. rodentium* in the distal colon 14 days after oral infection (top, 40×; bottom, 120×). Data are representative of four to six mice for each group.

(C and D) Colon weight (C) and spleen weight (D) after oral infection as shown in (A). Data show pooled results from two or three independent experiments (n = 10–16/group).

(E and F) Histopathology (E) and crypt length (F) in the distal colon 14 days after oral infection (H&E, 40×). Data are pooled from two or three independent experiments (uninfected, n = 3/group; day 14, n = 20–23/group). In (F), white bars represent uninfected mice, and black bars represent day 14 mice.

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus wild-type mice. Data represent means ± SEM in (A) and (C)–(F).
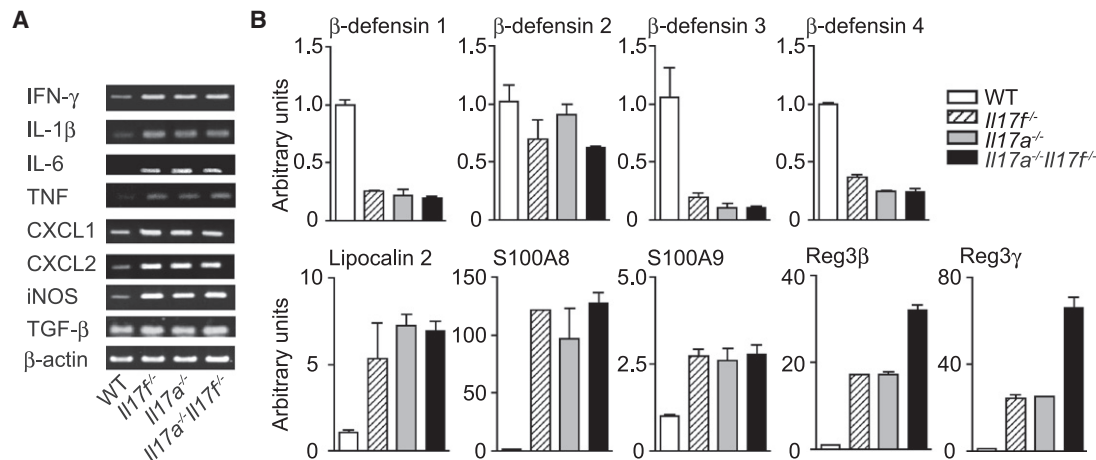
A   **B**

Figure 4. IL-17F and IL-17A Are Required for the Induction of β-Defensin Expression during *C. rodentium* Infection

(A) The expression of inflammatory mediators in the colon 14 days after infection with *C. rodentium* was determined with semiquantitative RT-PCR.

(B) The expression of antimicrobial peptide in the colon 14 days after infection with *C. rodentium* was determined with real-time RT-PCR. Data represent the means ± SEM. The RNA sample was pooled from six to eight mice for each group.

All data are representative of three independent experiments.

IL-17RC Is Highly Expressed in Colonic Epithelial Cells

Two receptor molecules, IL-17RA and IL-17RC, reportedly bind IL-17A and IL-17F (Toy et al., 2006; Zheng et al., 2008). Because the binding affinities of IL-17A and IL-17F for these receptors are different (Hymowitz et al., 2001; Kuestner et al., 2007; Wright et al., 2008), we examined the tissue distribution of these molecules. As reported previously (Kuestner et al., 2007), IL-17RA mRNA was highly expressed in such lymphoid tissues as the thymus, spleen, and LNs (Figure 6A). On the other hand, IL-17RC mRNA was expressed at high amounts in such nonhematopoietic tissues as the colon, small intestine, and lung (Figure 6A). Consistent with these observations, T and macrophage cell lines expressed higher amounts of IL-17RA mRNA than a colonic EC line, whereas the colonic EC line expressed higher amounts of IL-17RC mRNA than the T cell line (Figure 6B). We also found that IL-17RA or Act1 mRNA was constitutively expressed in Thy1.2^{+} cells, B220^{+} cells, CD11c^{+} cells, CD11b^{+} cells, peritoneal macrophages, and colonic epithelial cells (CMT93), whereas IL-17RC mRNA was detected only peritoneal macrophages and colonic ECs (Figure 6C). Thus, the tissues distributions of these receptors are strikingly different, and colonic ECs preferentially express IL-17RC.

We next examined whether IL-17F can transduce signals to T cells, peritoneal macrophages, or colonic epithelial cells. We found that IL-17A could induce IL-6 by peritoneal macrophages, CCL2 by CD4^{+} T cells, or lipocalin 2 and β-defensin 3 by colonic epithelial cells (CMT93) in a dose-dependent manner (Figures 6D–6F), and 50 ng/ml of IL-17A was sufficient to induce several cytokines and chemokines by these cells (Figures 6D–6I and Figure S10). The cytokine-inducing activity of IL-17A was not the effect of contaminated LPS, because we found that IL-6 production was observed in IL-17A-treated peritoneal macrophages from both C3H/HeN (LPS-sensitive) and C3H/HeJ (LPS-insensitive) mice (Figure 6D). However, whereas IL-6, CCL3, and G-CSF production were induced in peritoneal macrophages by the treatment with IL-17F (50 ng/ml), this cytokine could not increase other inflammatory mediators, which was

induced by IL-17A (Figures 6D and 6H and Figure S10). In contrast, similar to IL-17A, treatment of IL-17F in colonic epithelial cells induced most of inflammatory mediators examined, although the activity of IL-17F was slightly lower compared to that of IL-17A (Figures 6F and 6G and Figure S10). IL-17A, but not IL-17F, also induced several cytokine and chemokine production in CD4^{+} T cells. We could not observe any synergy between IL-17A and IL-17F (Figures 6F, 6G, and 6I and Figure S10). Thus, these observations suggest that IL-17A and IL-17F can differentially induce the expression of cytokines and antimicrobial peptides in a cell-type-specific manner.

DISCUSSION

In this report, we have demonstrated that IL-17A is critical for the development of DTH, CHS, EAE, CIA, and arthritis in *Il1rn*^{−/−} mice, whereas IL-17F is not only dispensable for the induction of these responses, but also does not have any substantial additive, synergistic, or compensatory effects to those of IL-17A in these disorders. These observations suggest that IL-17F has only low activity compared to IL-17A in these immune responses, although IL-17A and IL-17F are produced simultaneously by Th17 cells and bind the same receptors. In this regard, we found that cytokine-inducing activity of IL-17F from macrophages or T cells was much lower than IL-17A. Because IL-17A enhances immune responses by activating T cell priming (Nakae et al., 2002, 2003b), and induces inflammation by inducing cytokines from various types of cells including macrophages (Da Silva et al., 2008; Jovanovic et al., 1998) and dendritic cells (Antonysamy et al., 1999; Coury et al., 2008), this low cytokine-inducing activity of IL-17F on immune cells may be responsible for the inefficiency of this cytokine in allergic and autoimmune responses.

We showed that *Il17a*^{−/−}*Il17f*^{−/−} mice were sensitive to opportunistic infection with *S. aureus*, indicating that IL-17A and IL-17F are important for host defense against this bacterium. Consistent with this observation, increased susceptibility to *S. aureus* was
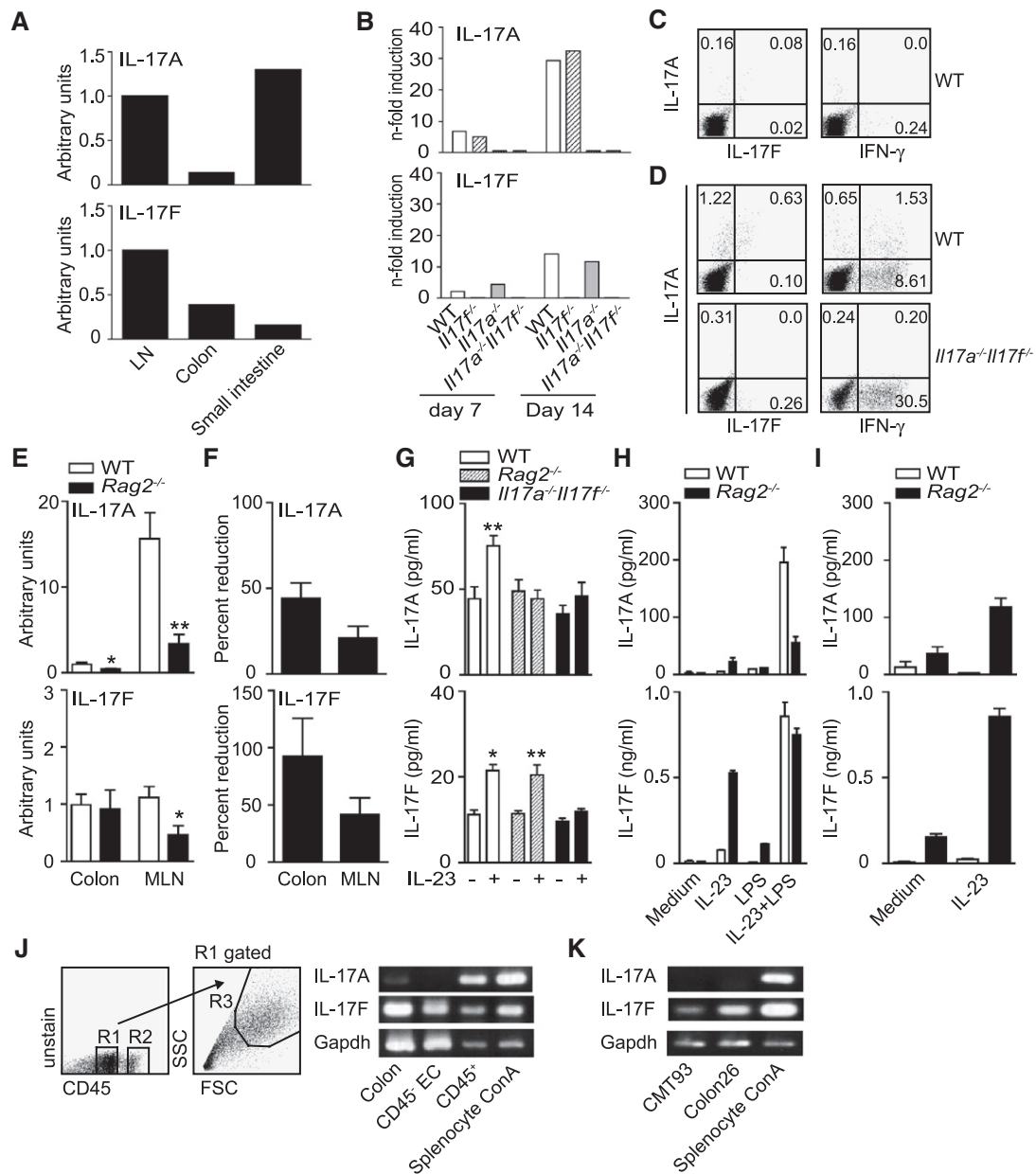
Figure 5. IL-17F and IL-17A Are Produced by Different Cells

(A) Colon, small intestine, and peripheral LNs from wild-type mice were analyzed for IL-17A and IL-17F mRNA expression with real-time RT-PCR. Expression in LN cells was defined as 1.

(B) The expression of IL-17A and IL-17F in the colons of mice 7 and 14 days after infection with *C. rodentium* was determined with real-time RT-PCR. The RNA sample was a pool of samples from four to six mice for each group. The expression in uninfected wild-type mice was defined as 1.

(C and D) Profiles of intracellular IL-17F, IL-17A, and IFN-γ expression in colonic PMA- and ionomycin-stimulated lymphocytes from uninfected mice (C) or mice 14 days after infection with *C. rodentium* (D).

(E and F) The colons and MLNs of C57BL/6J wild-type and *Rag2*$^{-/-}$ mice 7 days after infection with *C. rodentium* were analyzed for IL-17A and IL-17F mRNA expression with real-time RT-PCRs (E) (n = 5–6/group). The expression in wild-type colon was defined as 1. The expression of these cytokines in the colon and MLNs of *Rag2*$^{-/-}$ mice was determined as a percentage of the expression in wild-type mice (F).

(G) Whole colons of uninfected wild-type, *Rag2*$^{-/-}$, and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice were cultured for 24 hr in the presence or absence of 20 ng/ml IL-23. The concentrations of IL-17A or IL-17F in supernatant were determined by ELISAs and were normalized to total protein content for each sample (n = 5–8/group). Similar results were also observed in C.B-17 SCID mice.

(H and I) Splenocytes (5 × 10^5 cells) (H) or MLNs (1.5 × 10^5 cells) (I) of wild-type and *Rag2*$^{-/-}$ mice were cultured in 24- or 48-well plates, respectively, in the presence or absence of 5 μg/ml LPS and 20 ng/ml IL-23 for 72 hr, and IL-17A and IL-17F amounts in culture supernatants were determined with ELISA.

(J) Colonic epithelial (CD45$^-$ and high FSC and SSC; gates R1 and R3) cells and intraepithelial immune cells (CD45$^+$; gate R2) were isolated from the colons of uninfected wild-type mice with flow cytometry, and the expression of IL-17F and IL-17A was examined with RT-PCR.
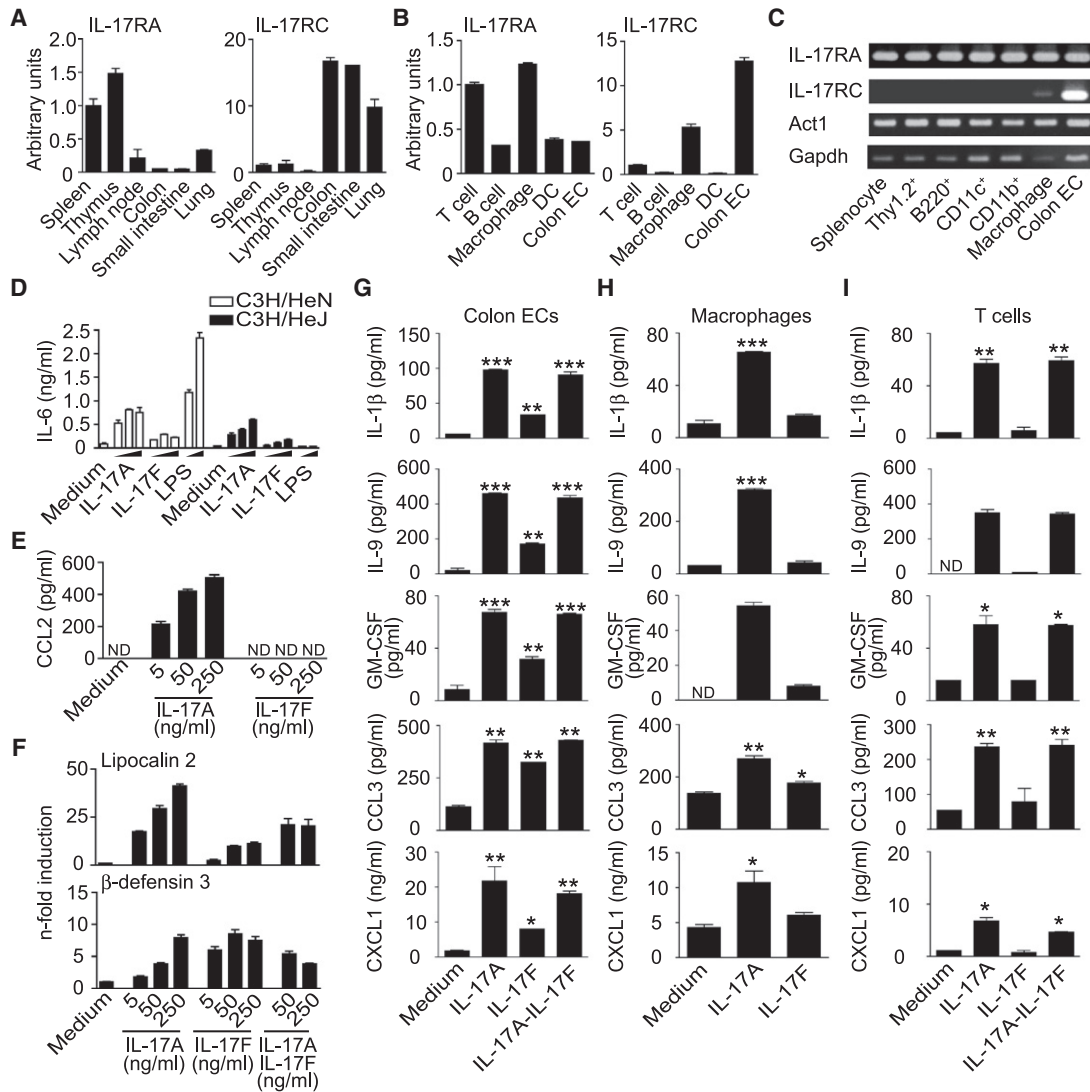
Figure 6. IL-17RA and IL-17RC Show Different Tissue Distributions

(A and B) The expression of IL-17RA and IL-17RC in tissues from 129/Ola × C57BL/6J wild-type mice (A), and different cell lines (B) were determined with real-time RT-PCR.

(C) The expression of IL-17RA, IL-17RC, and Act1 in different cell populations obtained by MACS sorting was determined with RT-PCR.

(D) Peritoneal macrophages were stimulated for 24 hr with 5–250 ng/ml IL-17A or IL-17F, or 10–100 ng/ml LPS, and IL-6 amounts in the culture supernatants were determined with ELISAs.

(E) CD4$^+$ T cells from C57BL/6J mice obtained by MACS sorting were stimulated for 48 hr with 5–250 ng/ml IL-17A or IL-17F, and CCL2 in the culture supernatants was determined with Bio-Plex suspension array system (Bio-Rad).

(F) The expression of lipocalin 2 and β-defensin 3 in colonic epithelial cell line (CMT93) stimulated for 6 hr with 5–250 ng/ml IL-17A or IL-17F individually, or with combination of 50–250 ng/ml IL-17A and IL-17F, was determined with real-time RT-PCR.

(G–I) Colonic epithelial cell line (CMT93) (G), peritoneal macrophages from C3H/HeJ mice (H), or CD4$^+$ T cells from C57BL/6J mice (I) were stimulated for 24 hr (G and H) or 48 hr (I) with 50 ng/ml IL-17A or IL-17F individually, or with a combination of 50 ng/ml IL-17A and IL-17F. IL-1β, IL-9, GM-CSF, CCL3, or CXCL1 in the culture supernatants were determined with the Bio-Plex suspension array system (Bio-Rad). ND denotes not detected. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus medium alone.

All data represent means ± SEM and are representative of three independent experiments.

reported in *Il17ra*$^{-/-}$ mice (Schwarzenberger and Kolls, 2002) and subjects with mutations in STAT3, in whom Th17 cell differentiation and function is impaired (Milner et al., 2008). Because

Il17f$^{-/-}$ and *Il17a*$^{-/-}$ mice showed normal sensitivities to *S. aureus*, IL-17A and IL-17F complement each other in this setting. Furthermore, we showed that IL-17A and IL-17F are

(K) The expression of IL-17A and IL-17F in mouse colonic epithelial cell line (CMT93 or Colon 26) was examined with RT-PCR.

Data are representative of two (A, B, and K) or three (C–J) independent experiments. Data represent means ± SEM in (E)–(I).

also involved in responses against *C. rodentium*. The bacterial burdens in the colon after infection with *C. rodentium* showed similar increases in *Il17f*$^{-/-}$, *Il17a*$^{-/-}$, and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice, indicating that deficiency of just one of the IL-17 proteins results in full susceptibility to *C. rodentium* infection. Notably, splenomegaly and colon hypertrophy, which are associated with severe colonic inflammation, were more pronounced in *Il17f*$^{-/-}$ mice than in *Il17a*$^{-/-}$ mice, suggesting that IL-17F is more important than IL-17A in protecting colonic epithelial cells from the pathogenic effects of this bacterium. The observation that both IL-17A and IL-17F are required for the protection against *C. rodentium* makes clear contrast to the case of *S. aureus*, infection in which either IL-17A or IL-17F is enough for the protection, suggesting that the defense mechanisms against *S. aureus* and *C. rodentium* infection are different.

We found that β-defensin production was impaired in the infected colons of *Il17a*$^{-/-}$ and *Il17f*$^{-/-}$ mice, suggesting both IL-17A and IL-17F are required for the induction of these molecules in vivo, although either IL-17A or IL-17F alone can promote β-defensin production from ECs (Kao et al., 2004; Liang et al., 2006). Because β-defensins play an important role in immune responses against these pathogens (LeBlanc et al., 2008; Simmons et al., 2002), it seems likely that the defect in β-defensin production contributes to the increased susceptibility of *Il17a*$^{-/-}$ and *Il17f*$^{-/-}$ mice to *C. rodentium*. Although these in vivo data may suggest possible synergistic action between IL-17A and IL-17F in the defense against *C. rodentium*, we could not observe any synergism between these molecules in vitro with a colon epithelial cell line as the target, suggesting that the response of this cell line may be different from colon defensin producer cells in vivo. We also could not observe any synergism between IL-17A and IL-17F in vivo in the protection against *S. aureus*. IL-17A and IL-17F are not required for adaptive immune responses against *C. rodentium*, because *C. rodentium*-specific Ab production in *Il17f*$^{-/-}$, *Il17a*$^{-/-}$, and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice was normal.

We found that IL-17A and IL-17F producer cells in the colon are different; IL-17F is primarily produced by colonic ECs and innate immune cells, whereas the major part of IL-17A is produced by Rag2-dependent cells that are likely to be Th17 cells. Furthermore, our data show that IL-17A production is markedly induced after bacterial infection, whereas the induction of IL-17F was not so prominent in infected colons. These observations suggest that colonic EC- and/or innate immune cell-derived IL-17F induce antimicrobial peptides in the ECs, providing protection against initial bacterial invasion and dissemination. This differential action of IL-17A and IL-17F and also the apparent synergy between these two molecules in inducing defensins may explain why these two cytokines are not complementary in the colonic *C. rodentium* infection.

It was reported that *Il17ra*$^{-/-}$ mice show ulcerative syndrome around mucous membranes of the mouth and eyes as a result of colonization of staphylococcus species (Schwarzenberger and Kolls, 2002). These phenotypes closely resemble those observed in *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice, suggesting that IL-17RA is involved in both IL-17A and IL-17F signaling. However, we found that IL-17RC is highly expressed in colonic epithelial cells, whereas IL-17RA is preferentially expressed in immune cells such as macrophages and T cells. Because the binding affinity

of IL-17F to IL-17RA is much lower than that of IL-17A (Hymowitz et al., 2001; Wright et al., 2008), and only IL-17F binds to IL-17RC in the mouse (Kuestner et al., 2007), it seemed likely that IL-17A and IL-17F differentially use these receptors. In support of this notion, we showed that the effects of IL-17A and IL-17F are different among colonic epithelial cells, macrophages, and T cells; both IL-17A and IL-17F can induce neutrophil chemoattractants and β-defensins in colonic epithelial cells, whereas only IL-17A can efficiently induce cytokines in macrophages and T cells. These observations suggest involvement of other forms of receptors than IL-17RA–IL-17RC heterodimer complex in the colon. Indeed, IL-17RA and IL-17RC may also form homodimers (Kramer et al., 2006), and IL-17RA interacts with IL-17RB for IL-25 signaling (Rickel et al., 2008). Thus, in addition to the difference of producer cells, cell-type-specific IL-17 receptor distribution and the different binding affinities of IL-17A and IL-17F for these receptors may explain why IL-17A is important in both allergic and host defense responses and IL-17F only contributes innate immune responses in epithelial cells.

A recent study of *Il22*$^{-/-}$ and *Il17rc*$^{-/-}$ mice, which do not respond to IL-17A or IL-17F, demonstrated that IL-22, but not IL-17A and IL-17F, expressed in response to IL-23 is essential for the early host response against *C. rodentium* (Zheng et al., 2008). IL-22 is produced by innate immune cells, such as dendritic cells, during the *C. rodentium* infection and induces the expression of Reg-family antimicrobial proteins in colonic ECs (Zheng et al., 2008). These observations with *Il17rc*$^{-/-}$ mice apparently contradict our results that both IL-17A and IL-17F are involved in the host defense against *C. rodentium*, although our data are compatible with the involvement of IL-22. Further studies to elucidate the relationship between ligand and receptor in the IL-17 system and relative contributions of the bacterial and mouse strains are needed to address these issues. Furthermore, it remains to be investigated whether other IL-17A- and IL-17F-producing cells than ECs and Th17 cells, such as γδ T cells (Ivanov et al., 2006; Yang et al., 2008), granulocytes (Hue et al., 2006), monocytes (Hue et al., 2006), or mast cells (Kawaguchi et al., 2004), are also involved in host defense against *C. rodentium*.

In conclusion, we have demonstrated the different contributions of IL-17F and IL-17A in allergic responses and protection against bacterial infection. Our findings provide insights into the molecular mechanisms of IL-17A- and IL-17F-mediated responses and should be useful to the development of new therapeutics for allergic diseases and bacterial infections.

EXPERIMENTAL PROCEDURES

Mice

Il17f$^{-/-}$ and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice were generated as shown in Figure S1. *Il17a*$^{-/-}$ (Nakae et al., 2002), *Il17f*$^{-/-}$, and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice on a 129/Ola × C57BL/6J background, or mice backcrossed to C57BL/6J (Nihon SLC) or BALB/cA mice (CLEA Japan) for eight or four generations, respectively, were used as described. The sexes and ages (2–3 months old) of the mice were matched in all experimental groups. *Il17f*$^{-/-}$*Il1rn*$^{-/-}$ and *Il17a*$^{-/-}$*Il17f*$^{-/-}$*Il1rn*$^{-/-}$ mice were produced by crossing *Il17f*$^{-/-}$ and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice with *Il1rn*$^{-/-}$ mice, which were backcrossed for eight generations to BALB/cA mice (Horai et al., 2004). *Rag2*$^{-/-}$ mice were obtained from the Central Institute for Experimental Animals. C3H/HeJ and C3H/HeN or C.B.-17 SCID mice were purchased from Nihon SLC or CLEA Japan, respectively. All mice were kept under specific pathogen-free conditions in an

environmentally controlled clean room at the Center for Experimental Medicine (The Institute of Medical Science, University of Tokyo). The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments.

Cell Isolation

Thy1.2$^+$, CD4$^+$, B220$^+$, CD11c$^+$, and CD11b$^+$ cells were isolated from spleens with an autoMACS (Miltenyi Biotec) after being stained with microbead-conjugated anti-mouse Thy1.2, CD4, B220, CD11c, and CD11b mAbs (Miltenyi Biotec), respectively, according to the manufacturer's instructions. For isolation of thioglycollate-elicited peritoneal macrophages, mice were injected intraperitoneally with 2 ml of 4% thioglycollate (Nissui), and peritoneal cells were collected by washing with PBS 4 days after injection.

Cell Culture

The mouse T cell line (BW5147), B cell line (X5563), macrophage cell line (RAW264), and colonic epithelial cell line (CMT93 or Colon26) were cultured with RPMI 1640 (Sigma) containing 10% FBS. The mouse dendritic cell line (DC2.4) was cultured with RPMI 1640 containing 10% FBS, HEPES, and nonessential amino acids (GIBCO). For the measurement of cytokines, chemokines, and antimicrobial peptide amounts, CMT93 cells, peritoneal macrophages, or CD4$^+$ T cells were treated with 5–250 ng/ml recombinant mouse IL-17A or IL-17F (R&D systems) for 6–48 hr.

Measurement of Cytokines, Chemokines, and Antigen-Specific Igs

Concentrations of IFN-γ, IL-6 (OptEIA kit, BD PharMingen), IL-17A, and IL-17F (DuoSet ELISA kit, R&D systems) in culture supernatants were determined with ELISAs according to the manufacturer's instructions. IL-1α, IL-1β, IL-9, IL-10, IL-12/23 p40, IL-12 p70, IL-13, G-CSF, GM-CSF, IFN-γ, CXCL1, CCL2, CCL3, CCL4, and CCL5 amounts in culture supernatants were measured by the Bio-Plex system (Bio-Rad) following the manufacturer's instructions. *C. rodentium*-specific Ig amounts in sera were measured as described previously (Bry and Brenner, 2004).

Flow Cytometry

Cells were stimulated with 50 ng/ml PMA (Sigma), 500 ng/ml ionomycin (Sigma), and 1 μM monensin (Sigma) for 5 hr. Intracellular cytokine staining was performed as described previously (Komiyama et al., 2006). Cells were treated with anti-mouse CD16 and CD32 mAbs (2.4G2) in staining buffer (HBSS containing 2% FCS and 0.1% sodium azide) to block FcR binding and then stained with APC-anti-CD4 mAbs (Gk1.5; BioLegend). After washing, the cells were fixed with 4% paraformaldehyde. After washing with permeabilization buffer (0.1% saponin [Sigma] in staining buffer), cells were incubated with PE-anti-mouse IFN-γ mAbs (XMG1.2; BD PharMingen), PE-anti-mouse IL-17A mAbs (TC11-18H10; BD PharMingen), or goat anti-mouse IL-17F polyclonal Abs (AF2057 or BAF2057; R&D systems). For secondary staining, we used Alexa Fluor 488-anti-goat IgG (A-11055; Invitrogen), PE-anti-goat IgG (Santa Cruz), or FITC-streptavidin (BD PharMingen). The cells were analyzed on a FACSCalibur system (Becton Dickinson) and data were analyzed with FlowJo software (Tree Star).

Real-Time RT-PCR

Total RNA was extracted with Sepasol reagent (Nacalai Tesque) according to the manufacturer's instructions. RNA was denatured in the presence of an oligo dT primer and then reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time RT-PCRs were performed with a SYBR Green qPCR kit (Invitrogen) and an iCycler system (Bio-Rad) with the sets of primers described in Table S2.

Clinical Assessment of Arthritis

Arthritis development in *Il1rn*$^{-/-}$ mice was monitored by macroscopic evaluation as described previously (Horai et al., 2004). Arthritis development in each paw was graded by macroscopic evaluation as follows: 0, no change; 1, mild swelling; 2, obvious joint swelling; and 3, severe joint swelling and ankylotic changes.

S. aureus Infection

Bacteria *S. aureus* 834 was prepared as described (Nakane et al., 1995). Bacteria were cultured on tryptic soy agar (Difco) for 12 hr at 37°C, inoculated into tryptic soy broth (Difco), and incubated for another 12 hr. The organisms were collected by centrifugation and resuspended in PBS. The concentration of resuspended cells was adjusted spectrophotometrically at 550 nm. Mice were infected intravenously with 200 μl of a solution containing 1 × 10^7 CFU of viable *S. aureus* cells in PBS. For determination of the bacterial burden in the infected tissues, kidneys were homogenized, and diluted in 10-fold steps in sterile PBS. Bacterial CFU was determined by plating each dilution on tryptic soy agar after culture for 12 hr at 37°C.

C. rodentium Infection

C. rodentium infections were performed as described previously (Nagai et al., 2005). In brief, 129/Ola × C57BL/6J mice were inoculated with 200 μl of a bacterial suspension (2 × 10^8 CFU/head) via an oral gavage. For the colony formation assays, colons were harvested and homogenized, and serially diluted homogenates were plated on MacConkey agar (Difco). For histological analysis, colons were fixed with 4% paraformaldehyde in PBS at 4°C overnight and frozen in tissue-freezing medium (Leica Jung). Frozen sections were prepared and stained with anti-*C. rodentium* sera as described previously (Nagai et al., 2005).

Statistics

Unless otherwise specified, all results are shown as mean and the standard error of the mean (SEM). Unpaired Student's t tests, Mann-Whitney's *U* tests, or χ^2 tests were used to statistically analyze the results. Differences were considered significant at p < 0.05.

SUPPLEMENTAL DATA

Supplemental Data include ten figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00554-2.

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