Published in final edited form as:
Immunity. 2012 December 14; 37(6): 1061–1075. doi:10.1016/j.immuni.2012.08.024.

Th22 Cells are an Important Source of IL-22 for Host Protection against Enteropathogenic Bacteria

Rajatava Basu[1,*], **Darrell B. O'Quinn**[1,*], **Daniel J. Silberger**[1], **Trenton R. Schoeb**[2], **Lynette Fouser**[3], **Wenjun Ouyang**[4], **Robin D. Hatton**[1], and **Casey T. Weaver**[1]

[1]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

[2]Department of Genetics and Genomics, University of Alabama at Birmingham, Birmingham, AL 35294, USA

[3]Inflammation and Immunology, Biotherapeutics Research and Development, Pfizer, Cambridge, MA 02140, USA

[4]Department of Immunology, Genentech, South San Francisco, California 94080, USA

SUMMARY

Interleukin-22 (IL-22) is central to host protection against bacterial infections at barrier sites. Both innate lymphoid cells (ILCs) and T cells produce IL-22. However, the specific contributions of $CD4^+$ T cells and their developmental origins are unclear. We found that the enteric pathogen *Citrobacter rodentium* induced sequential waves of IL-22 producing ILCs and $CD4^+$ T cells that were each critical to host defense during a primary infection. Whereas IL-22 production by ILCs was strictly IL-23–dependent, development of IL-22 producing $CD4^+$ T cells occurred via an IL-6–dependent mechanism that was augmented by, but not dependent on, IL-23, and was dependent on both transcription factors T-bet and AhR. Transfer of $CD4^+$ T cells differentiated with IL-6 in the absence of TGF-β ("Th22" cells) conferred protection of infected IL-22-deficient mice whereas transferred Th17 cells did not. These findings establish Th22 cells as an important component of mucosal anti-microbial host defense.

INTRODUCTION

Effective host protection is characterized by integrated responses of innate and adaptive arms of immunity. At barrier sites (eg. skin, respiratory and intestinal tracts), production of IL-22 by both innate and adaptive lymphoid cells is important in host defense through its actions on epithelial cells (Ouyang et al., 2011; Wolk et al., 2010). Because expression of IL-22 and its receptor segregate between hematopoietic and non-hematopoietic cells, respectively, the IL-22–IL-22R axis represents a critical link in the integration of immunity with barrier defense (Aujla et al., 2008; Wolk et al., 2004; Zheng et al.,2008).

Cellular sources of IL-22 are diverse. IL-22–expressing innate lymphoid cells (ILCs) have been identified in mice and humans, particularly natural killer (NK)-like cells and lymphoid

Correspondence to: C.T.W. (cweaver@uab.edu).
*These authors contributed equally to this work

tissue-inducer (LTi) cells (Colonna, 2009; Sonnenberg et al., 2011a). IL-22 producing ILCs express the transcription factors RORγt and AhR, and have a shared requirement for IL-23 to produce IL-22 (Sawa et al., 2010; Takatori et al., 2009). Mice deficient for IL-23 fail to resist infection by intestinal or pulmonary bacterial pathogens (Aujla et al., 2008; Mangan et al., 2006; Zheng et al., 2008). In the well-studied *Citrobacter rodentium* model (Higgins et al., 1999; Mundy et al., 2005), deficiency of IL-23 is fatal early in infection due to impaired induction of intestinal IL-22 (Cella et al., 2009; Sonnenberg et al., 2011b; Zheng et al., 2008). While specific contributions of different IL-22 producing ILCs remain to be defined, their role in early barrier protection appears indispensable.

$CD4^+$ T cells also produce IL-22. Originally described as a product of Th1 cells (Gurney, 2004), Th17 cells are also an important source of IL-22 (Liang et al., 2006; Zheng et al., 2007). In common with ILCs, IL-22 expression by Th17 cells is positively regulated in by the transcription factors RORγt and AhR (Schraml et al., 2009; Veldhoen et al., 2008). A subset referred to as Th22 cells has been described in humans (Duhen et al., 2009; Trifari et al., 2009). Human Th22 cells are characterized by production of IL-22 with little or no IL-17, and appear to have a counterpart in mice based on *ex vivo* studies that have shown that naive $CD4^+$ T cells differentiated with IL-6 under conditions of no addition of TGFβ express high IL-22 and negligible IL-17 compared to conventional Th17 cells differentiated in the presence of IL-6 plus TGFβ (Zheng et al., 2007). A similar reciprocal pattern of IL-22 and IL-17 expression was linked to a “pathogenic” subset of murine Th17 cells induced under similar conditions (Ghoreschi et al., 2010). However, the normal function of this cell population is not well understood.

While ILCs are a critical source of IL-22 during mucosal infection, less is known regarding specific contributions of IL-22 produced by $CD4^+$ T cells. Here, we have examined the relative contribution of ILCs and $CD4^+$ T cells in host protection against the enteric bacterial pathogen, *Citrobacter rodentium*. We find that while early contribution of ILC-derived IL-22 induced by IL-23 is critical to curb bacterial loads pending development of pathogen-induced $CD4^+$ T cells, the latter are critical for late host protection via an IL-6 dependent mechanism that is augmented by, but not strictly dependent on, IL-23. Further, $CD4^+$ T cells appear to be the major producers of IL-22 in the infected intestinal tissues, with cells differentiated in the absence of TGFβ (referred to herein as Th22 cells) providing more potent protection on a per cell basis than classical Th17 cells. The transcriptome of Th22 cells differed significantly from Th17 cells and both AhR and T-bet were required for optimal IL-22 production by Th22 cells and their host-protective function. These findings identify Th22 cells as important contributors to mucosal host defense, and suggest overlap between the functional programming of these cells with that of the recently described subset of Th17 cells proposed to be key contributors to autoimmune disease.

RESULTS

IL-23-Independent, IL-22-Dependent Protection against *C. rodentium* Infection

We previously identified an essential role for IL-23 in the protective host response against *C. rodentium* infection (Mangan et al., 2006). Challenge of IL-23-deficient mice ($Il23a^{-/-}$) with high doses of *C. rodentium* ($\geq 0.5 \times 10^9$ cfu) is uniformly fatal, whereas wild-type (WT) mice develop a transient colitis before clearing infection. In the course of titering inocula of *C. rodentium* in $Il23a^{-/-}$ mice (Fig. 1A), we found that at lower doses ($0.5-1.0 \times 10^7$ cfu) >90% of mice survived. To monitor bacterial loads, a strain of *C. rodentium* expressing luciferase was used to enable real-time whole body imaging (Wiles et al., 2006). In contrast to $Il23a^{-/-}$ mice infected with high-dose *C. rodentium*, nearly all $Il23a^{-/-}$ mice infected with low dose cleared infection with similar kinetics to WT mice. This was despite greater than a

log-fold higher bacterial load at the peak of infection (Fig. 1B and 1C). Thus, IL-23 is essential for protection at high doses of *C. rodentium*, but is dispensable at low doses.

Blockade of IL-22 in *Il23a*$^{-/-}$ mice challenged with low-dose infection was uniformly fatal, whether antibody was administered coincident with infection (day 0), or after the peak of the innate immune response (d5; Fig. 1D). In extension of a previous study (Zheng et al., 2008), we found that mice deficient for global IL-17 signaling (*Il17ra*$^{-/-}$) cleared infection to high-dose challenge with similar kinetics to WT mice, albeit with significantly higher bacterial loads over the mid-coarse of infection (Fig. S1). Thus, while IL-17 signaling appears to be important in restraining bacterial growth during infection, IL-17 cytokines, including IL-17A, IL-17F and IL-17C, are not essential for clearance. We also found that *Ifng*$^{-/-}$ mice cleared infection with comparable kinetics and bacterial loads to WT mice (Fig. S1). These results implicate an IL-23-independent, IL-22-dependent pathway that contributes to protection against *C. rodentium*.

IL-6 is Indispensable for Development of IL-22–Producing CD4$^+$ T cells

IL-23 is required for production of IL-22 by ILCs (Cella et al., 2009; Sonnenberg et al., 2011b; Zheng et al., 2008). Whereas published data are in agreement that IL-6 induces the development of IL-22 producing CD4$^+$ T cells from naive precursors in the absence of TGFβ (Duhen et al., 2009; Rutz et al., 2011; Zheng et al., 2007), disparate results concerning the role for IL-23 to induce development of IL-22 producers in mouse and human have been reported (Duhen et al., 2009; Zheng et al., 2007). In view of our findings that implicated an IL-23–independent pathway to IL-22–mediated clearance (Fig. 1), we reexamined this issue.

In agreement with previous reports (Rutz et al., 2011; Zheng et al., 2007), IL-6 alone induced the development of CD4$^+$ T cells that expressed high amounts of IL-22 mRNA and protein, but not IL-17A, and optimal development of IL-22 producers was suppressed by TGFβ in favor of Th17 development under APC-free conditions (Fig. S2A). Addition of IL-23 to T cells differentiated by IL-6 alone significantly enhanced their production of IL-22, in accord with heightened expression of the IL-23 receptor (IL-23R) induced by IL-6 or IL-6+IL-23 in the absence of TGFβ Fig S2A–C). However, IL-23 alone did not induce the development of IL-22 producing T cells from naïve precursors (Fig. S2D–F). Indeed, the reported ability of IL-23 to induce development of IL-22 producing CD4$^+$ T cells appears to be due to production of IL-6 by splenic APCs (Zheng et al., 2007), as IL-22 was induced by addition of IL-23 alone only under conditions of IFNγ and IL-4 neutralization (Figs. 2A–C), and was ablated either by use of *Il6*$^{-/-}$ APCs or addition of neutralizing anti-IL-6 (Fig. 2A–C, and S2D–F). Thus, IL-6 is essential for the differentiation of IL-22-producing CD4$^+$ T cells from naïve precursors, whereas IL-23 is not. Rather, actions of IL-23 to enhance IL-22 appear primarily limited to T cells primed by IL-6 under conditions of low or absent TGFβ co-signaling — not through its direct actions on naïve T cells. In view of similar differentiation requirements and functional properties of these cells with those of human Th22 cells (Duhen et al., 2009), we refer to them as —"Th22" cells, to distinguish them from conventional Th17 cells differentiated with TGFβ.

IL-23–Dependent Induction of IL-22 and Proinflammatory Cytokines Predominates Early in *C. rodentium* Infection

Based on the foregoing, we postulated that the innate and adaptive immune responses contributed sequential production of IL-22 from ILCs and CD4$^+$ T cells during primary infection, induced principally by IL-23 and IL-6, respectively. To characterize the relative contributions of IL-23 and IL-6 to the innate versus adaptive responses to *C. rodentium* infection, WT, *Il6*$^{-/-}$, *Il23a*$^{-/-}$ and *Il22*$^{-/-}$ mice were challenged with high-dose luminescent

C. rodentium and monitored for survival and colonization kinetics until immune-deficient mice began dying (Fig. 3 A–C). By d3 post-infection (PI), *Il23a*$^{-/-}$ mice had the greatest bacterial loads until beginning to succumb on d8. Colonization kinetics and peak bacterial loads were similar for *Il6*$^{-/-}$ and *Il22*$^{-/-}$ mice: both groups had greater bacterial loads than WT controls, but less than *Il23a*$^{-/-}$ mice. All *Il23a*$^{-/-}$ mice succumbed by d12 PI (Fig. 3C). Infected *Il6*$^{-/-}$ and *Il22*$^{-/-}$ mice had significantly improved survival compared to *Il23a*$^{-/-}$ mice, but the majority still succumbed by d14 PI. All WT mice survived and cleared the infection by 21 d PI (Fig. 1C, and data not shown).

C. rodentium infection involves the descending colon and cecal patch in WT mice (Fig. S3), sites at which the bacterium attaches to the apical surface of the epithelium. At d3 PI, increased bacteria colonized the epithelium of *Il23a*$^{-/-}$ mice, although no other differences were evident between groups (data not shown). However, by d8 PI there were severe multifocal ulcerations of the descending colon in *Il23a*$^{-/-}$ mice, with translocation of dense colonies of *C. rodentium* as well as multifocal transmural inflammation — none of which were evident in WT mice (Fig. 3D, E) (Mangan et al., 2006). Despite severe, multifocal injury in *Il23a*$^{-/-}$ mice, the degree of mucosal inflammation in non-ulcerated areas was significantly reduced (Fig. 3D, E, and data not shown), resulting in reduced recoveries of T cells and other mononuclear cells from colons of *Il23a*$^{-/-}$ mice (>40% reduction vs WT).

Compared to WT mice, colons of infected *Il6*$^{-/-}$ and *Il22*$^{-/-}$ mice had significantly increased epithelial injury and ulceration, which was comparable in both groups, although significantly reduced and more focal than in *Il23a*$^{-/-}$ mice (Fig. 3D, E). This was associated with significantly greater numbers of invasive bacterial colonies. Mucosal inflammation was increased in both *Il6*$^{-/-}$ and *Il23a*$^{-/-}$ mice compared to *Il23a*$^{-/-}$ mice, as was crypt hyperplasia (Fig. 3E). T cell recoveries were similar in WT, *Il6*$^{-/-}$ and *Il22*$^{-/-}$ mice, consistent with comparable inflammation scores. Thus, at the peak of infection (d8), when the adaptive immune response had become predominant, *Il6*$^{-/-}$ and *Il22*$^{-/-}$ mice had similarly impaired host protection against *C. rodentium*, which, although substantial, was less severe than that of *Il23*$^{-/-}$ mice. The greater severity of disease and mortality in *Il23*$^{-/-}$ mice imply that there are host-protective effects of IL-23 during the early course of infection that are not mediated by IL-6 or IL-22 alone.

To define differences in the inflammatory response of *Il23*$^{-/-}$ versus *Il6*$^{-/-}$ and *Il22*$^{-/-}$ mice that might contribute to the distinct survival and bacterial clearance, production of key cytokines and chemokines at the peak of the innate response was compared (Fig. 3F). Notably, IL-6 was highly attenuated in colon explants of *Il23a*$^{-/-}$ mice compared to both WT and *Il22*$^{-/-}$ mice, akin to the defect in IL-22 production in the absence of IL-23. Significant reductions in IL-1β and TNFα, as well IFNγ, were associated with the loss of IL-6 production in *Il23*$^{-/-}$ mice, suggesting that multiple proinflammatory signals downstream of IL-23 might contribute to reductions in mucosal IL-6. Notably, marked reductions of IL-1β and TNFα found in colons of infected *Il23a*$^{-/-}$ mice might also contribute to reductions in IL-22, as both cytokines can enhance IL-22 induction by ILCs. Conversely, normal production of IL-1β and TNFα was found in the involved colonic segments of *Il6*$^{-/-}$ and *Il22*$^{-/-}$ mice.

Decreases in chemokines (eg CCL2 and CXCL1) and proinflammatory cytokines in *Il23a*$^{-/-}$ mice likely contributed to the more limited inflammation in the colons of these mice, despite profound loss of barrier function (Fig. 3F, and data not shown). Of the additional cytokines examined, GM-CSF and G-CSF were the only ones that were significantly abrogated in both *Il23a*$^{-/-}$ and *Il6*−/− mice compared to WT, with significantly greater reductions in *Il23a*$^{-/-}$ mice. Thus, IL-23, but not IL-6, is essential for induction of key proinflammatory cytokines and IL-22 during the early course of infection, consistent with its indispensable role in

coordination of the mucosal innate immune response (Buonocore et al., 2010; Cella et al., 2009; Sonnenberg et al., 2011b; Zheng et al., 2008).

IL-6–Dependent Production of IL-22 by CD4$^+$ T cells Predominates Late in *C. rodentium* Infection

To better define the contribution of IL-6 to IL-22 expression, kinetics of IL-22 production by mice deficient for IL-23 or IL-6 were compared to WT controls. As early as d2 PI, reduced IL-22 was detected in *Il23a*$^{-/-}$ mice. This became significant on d3 and was reduced through d8 when the infected mice began to succumb (Fig. 4A). Despite a modest, progressive increase in IL-22 in *Il23a*$^{-/-}$ mice, this was insufficient to prevent bacterial dissemination and death (Fig. 3A–E). Reduction of IL-22 was also observed in *Il6*$^{-/-}$ mice (Fig. 4A), albeit delayed relative to that of *Il23a*$^{-/-}$ mice; it was not significant until d5 PI, when decreased IL-22$^+$ CD4$^+$ T cells were observed (Fig. 4B, C). Of note, significant production of IL-22 was induced by anti-CD3 as early as d3 PI (Fig. S4), indicating presence of IL-22$^+$ T cells when IL-22 production by ILCs is dominant (Sonnenberg et al., 2011b; Zheng et al., 2008).

By d8 PI, the deficit in IL-22 was significantly greater in *Il6*$^{-/-}$ than in *Il23*$^{-/-}$ mice (Fig. 4A). This correlated with a more pronounced decrease in frequencies of IL-22$^+$ T cells in the lamina propria (LP) of *Il6*$^{-/-}$ mice (Fig. 4B, C) and was consistent with a predominance of IL-22$^+$CD4$^+$ T cells relative to ILCs (CD3$^-$CD4$^-$ and CD3$^-$CD4$^+$ cells) in WT mice (Fig. S4A, B). Indeed, of the total IL-22$^+$ cells over the first 8 days of infection, CD4$^+$ T cells were significantly more abundant. Further, IL-22$^+$IL-17$^-$ (single-positive; SP) CD4$^+$ T cells were more frequent than IL-22$^+$IL-17$^+$ (DP) cells at each time point in infected WT mice (Fig. S4C), and, under conditions of ex vivo co-stimulation with IL-23, SP cells expressed the highest IL-22 per cell indicating they were likely the dominant source of IL-22 (Fig. S4A). Accordingly, IL-22 production in WT mice at d8 PI was almost a log-fold higher than at d3 (605pg/ml vs. 74pg/ml) (Fig. 4A), indicating that IL-22 contributed by CD4$^+$ T cells surpassed that contributed by ILCs earlier in the infection. This was consistent with significantly more IL-22 elicited by anti-CD3 than IL-23 stimulation of colonic lymphoid cells (Fig. 4D), and the observation that IL-22 production in *Il23a*$^{-/-}$ mice was IL-6-dependent and therefore of T-cell origin (Fig. S4D). The critical role for IL-6 in the absence of IL-23 was highlighted by uniform fatality of low-dose *C. rodentium* infection in *Il23a*$^{-/-}$ mice treated with neutralizing antibody to IL-6 (Fig. S4E). Note that although the relative deficits of IL-22 produced by *Il23a*$^{-/-}$ and *Il6*$^{-/-}$ mice at d8 (Fig. 4A) appear more modest than might be expected from the far greater decrease in IL-22$^+$ CD4$^+$ T cells in the latter (Fig. 4B), when normalized for the considerably reduced numbers of T cells recruited to the colons of *Il23a*$^{-/-}$ mice (Fig. 3E, and data not shown), the greater deficit of IL-22 in *Il6*$^{-/-}$ mice more closely mirrored the reduction in IL-22$^+$ T cells in these mice.

Because IL-21 has been identified as an autocrine factor downstream of IL-6 that promotes Th17 development and IL-22 expression by Th17 cells (Nurieva et al., 2007), we sought to determine whether IL-21 is important in host protection and the induction of CD4$^+$ T cell-derived IL-22. Despite delayed clearance and elevated bacterial loads between weeks 2–5 PI (Fig. S4F, G), and modest reductions in mucosal IL-22$^+$CD4$^+$ T cells in *Il21*$^{-/-}$ mice, there was no increased mortality compared to WT controls and treatment of these mice with anti-IL-6 resulted in significant reductions in IL-22$^+$CD4$^+$ T cells and increased mortality (Fig. S4H, I, and data not shown). Thus, although IL-21 is required for efficient clearance of *C. rodentium*, in its absence the IL-6–dependent T cell response is sufficient to control bacterial loads and prevent mortality.

The importance of T cell-derived IL-22 to host protection was reflected in the marked decline of surviving WT mice treated with anti-IL-22 after d5 of infection (Fig. 4E).

Whether administered during the onset of infection (days 0 and 3) or on days 6, 7 and 8, after the peak of the innate response, IL-22 blockade in WT animals resulted in comparable survival kinetics to that of *Il22*$^{-/-}$ and *Il6*$^{-/-}$ mice (Fig. 3C). Conversely, administration of either IL-22-Fc after the peak of the innate response rescued both *Il22*$^{-/-}$ and *Il6*$^{-/-}$ mice (Fig. 4F, G). Because *Il6*$^{-/-}$ mice retain IL-22–producing ILCs but are deficient in IL-22–producing T cells, IL-6–dependent induction of IL-22^{+}CD4^{+} T cells appears critical for host protection after the early wave of IL-22 production by ILCs has peaked.

Reciprocal Expression of T-bet, AhR and RORγt in Th22 vs Th17 Cells

To identify factors that might distinguish Th22 and Th17 developmental programs, comparative transcriptome analysis was performed (Fig. 5A, B). Among 611 genes with at least a 2-fold difference in expression between the two subsets (Fig. 5A), notable reciprocal differences were found between transcripts encoding RORγt, RORα and the aryl hydrocarbon receptor (AhR), and T-bet by Th17 and Th22 cells, respectively (Fig. 5B). This was in contrast to IRF4 and BATF (Huber et al., 2008; Schraml et al., 2009), which were not significantly different between the two populations (data not shown). Expression differences were validated by quantitative RT-PCR (Fig. 5C, and data not shown). Thus, transcripts encoding RORγt (*Rorc*) and AhR (*Ahr*) were increased in polarized Th17 cells, with increasing expression differences correlating with the dose of TGFβ, whereas T-bet (*Tbx21*) was increased in polarized Th22 cells.

Differential expression of T-bet and RORγt in Th22 and Th17 cells was verified by flow cytometry, gating on the IL-22^{+} fraction of each (Fig. 5D). Consistent with gene expression data IL-22^{+} Th22 cells expressed T-bet, which was not detected in IL-22^{+} cells Th17 cells; T-bet expression was modestly, but reproducibly increased by IL-23. Conversely, RORγt expression by IL-22^{+} cells was markedly increased in Th17 polarized cells, and was reduced in Th22 polarized cells, irrespective of IL-23 addition. Consonant with these results, IFNγ did not inhibit, but modestly enhanced, Th22 cell development (data not shown), in contrast to the potent inhibitory effects of IFNγ on the development of Th17 cells (Harrington et al., 2005). In sum, these observations establish that distinct transcription factor networks underpin Th22 versus Th17 development and suggest a T-bet-dependent mechanism for enhanced IL-22 expression by developing Th22 cells. These data further suggest that induction of IL-22 expression might be differentially regulated by T-bet and AhR in the context of the Th22 and Th17 pathways, respectively.

Th22 Cells are Protective against *C. rodentium* Infection via a T-bet- and AhR-dependent Mechanism

In view of the foregoing findings, we postulated that Th22 cells were protective against *C. rodentium* infection and required T-bet for their optimal development and function. Further, we posited that, in contrast to Th17 cells, AhR might be dispensable for IL-22 expression by Th22 cells. We therefore examined the expression of *Il22* transcripts from Th17 and Th22 cells generated in vitro from WT, *Tbx21*$^{-/-}$, or *Ahr*$^{-/-}$ mice (Fig. 6A). Notably, deficiency of T-bet significantly compromised expression of *Il22* transcripts in Th22 cells, but had no significant impact on *Il22* expression by Th17 cells (Fig. 6A). However, differentiation of Th17 cells in the presence of IL-23, which induced ~4-fold higher amounts of T-bet (Fig. S5A), augmented their IL-22 production in a T-bet-dependent manner (Fig. S5B, C).

In agreement with previous reports (Veldhoen et al., 2008), AhR was essential for *Il22* induction in Th17 cells. Thus, although *Il22* transcripts were substantially lower in Th17 cells than Th22 cells, they were reduced to background in AhR-deficient Th17 cells (Fig. 6A). Unexpectedly, despite lower expression of *Ahr* by Th22 cells (Fig. 5C), AhR deficiency also significantly reduced their expression of *Il22*. Reciprocal expression of T-bet

and AhR in Th22 and Th17 cells suggested that actions of T-bet to enhance *Il22* expression were unlikely due to increased expression of *Ahr* downstream of T-bet. Indeed, expression levels of *Ahr* were increased in T-bet-deficient Th22 cells (Fig. 6B). Conversely, *Tbx21* was increased in AhR-deficient Th22 cells. Finally, c-Maf, is a repressor of *Il22* transcription (Rutz et al., 2011), raising the possibility that T-bet might suppress c-Maf as a mechanism to enhance *Il22* transcription. However, while *Tbx21* and *maf* were reciprocally expressed by Th22 and Th17 cells (Fig. 5B), *maf* transcripts were not affected by T-bet deficiency in either Th22 or Th17 cells (Fig. 6C), indicating that IL-22 enhancing actions of T-bet were not due to suppression of *maf*.

Collectively, these results indicated that optimal *Il22* induction by Th22 cells is both T-bet- and AhR-dependent, suggesting cooperative actions of these factors in enhancing *Il22* transcription. To directly test this, WT and T-bet–deficient Th22 cells were treated with the AhR antagonist, CH-223191, to determine whether the residual expression of IL-22 found in T-bet–deficient T cells was AhR-dependent (Fig. 6D). Pharmacologic inhibition of AhR in T-bet-deficient Th22 cells further reduced IL-22 to near background levels. IL-22 produced by Th17 cells from WT and T-bet–deficient mice were also ablated by AhR antagonism, indicating that at the high levels of expression AhR found in Th17 cells, *Il22* expression is induced independently of T-bet.

To determine whether IL-22 production by T cells was T-bet and AhR dependent in vivo, WT, *Tbx21*$^{-/-}$ and *Ahr*$^{-/-}$ mice were inoculated with *C. rodentium*. In contrast to WT and *Tbx21*$^{-/-}$ mice, nearly all *Ahr*$^{-/-}$ mice rapidly succumbed to infection, consistent with this factor's role in IL-22 production by ILCs (Kiss et al., 2011). However, in the few *Ahr*$^{-/-}$ mice that survived long enough to assess the T cell response, the frequency of IL-22^{+}CD4^{+} T cells was significantly reduced, supporting the *in vitro* findings (Fig. 6A, S6, and data not shown). In contrast, the majority of *Tbx21*$^{-/-}$ mice survived infection, and when examined 8d PI were found to produce more than 2-fold less IL-22 than WT controls (Fig. 6E), and frequencies of IL-22^{+}CD4^{+} T cells in *Tbx21*$^{-/-}$ mice were reduced ~3-fold (Fig. 6F). Notably, although T-bet deficiency compromised both total IL-22^{+} T cells and IL-22/IL-17 DP T cells, frequencies of IL-17 SP cells were not affected, consistent with *in vitro* findings (Fig. 6A, D) and T-bet–independent IL-22 induction in conventional Th17 cells, but not Th22 cells.

In accord with decreased IL-22–producing CD4^{+} T cells in *Tbx21*$^{-/-}$ mice, bacterial loads were significantly greater during the adaptive phase of the response (Fig. 6G). Nevertheless, few of the *Tbx21*$^{-/-}$ mice innoculated with high dose *C. rodentium* succumbed to infection due to unimpaired ILC responses, and bacterial loads were gradually controlled due to the development of an unimpaired, protective antibody response in these mice (data not shown).

To bypass the contribution of ILC-derived IL-22 and more directly examine the protective function of Th22 and Th17 cells, transfers of different populations of polarized CD4^{+} T cells into IL-22-deficient mice were performed (Fig. 7A, B). Naïve WT CD4^{+} T cells were differentiated with IL-6 alone (Th22 polarizing conditions), IL-23 alone, or with IL-6+TGFβwithorwithoutadditionofIL23 (Th17 polarizing conditions), and then transferred into *C. rodentium*-infected *Il22*$^{-/-}$ mice after the peak of the innate response (Fig. 7A). Whereas *Il22*$^{-/-}$ recipients of Th22 cells were nearly completely protected compared to mice that received no T cells (10% vs. 85% mortality; $p<0.01$), recipients of IL-23-induced CD4^{+} T cells were not protected (100% mortality), in accord with their lack of IL-22 production (Figs. 2A and S2A, D). In contrast to recipients of Th22 cells, recipients of Th17 cells showed only modest, albeit significant, improvement in mortality and survival times whether treated with IL-23 or not.

Consistent with a requirement for T cell-derived IL-22 for protection, transfers of *Il22*$^{-/-}$ T cells polarized under Th22 conditions failed to enhance survival (Fig. 7B). Notably, recipients of Th22 cells derived from either *Tbx21*$^{-/-}$ or *Ahr*$^{-/-}$ mice had similarly impaired protection, consistent with important roles for both T-bet and AhR in expression of IL-22 by Th22 cells. Thus, Th22 cells provide greater host protection against lethal *C. rodentium* infection than Th17 cells, via an IL-22–dependent mechanism that requires T-bet and AhR for optimal anti-pathogen defense.

DISCUSSION

In this study we define an important role for Th22 cells in mucosal defense against the enteric bacterial pathogen, *Citrobacter rodentium*. Whereas Th17 cells were thought previously to be central to anti-bacterial protection at barrier sites, including the intestines (Aujla et al., 2008; Mangan et al., 2006; Ouyang et al., 2008; Sonnenberg et al., 2011a; Zheng et al., 2008), we find that Th22 cells differentiated in an IL-6–dependent, TGF-β–independent manner are more effective in host protection than Th17 cells. Programming of Th22 cells for high expression of IL-22 by IL-6 was independent of IL-23, but IL-23 enhanced production by Th22 cells primed by IL-6. Thus, although IL-23−induced expression of IL-22 by ILCs is indispensable for protection at high inoculating doses of this pathogen, we find that IL-23−deficient mice are protected against infection with lower inocula via an IL-6–dependent mechanism that is linked to development of Th22 and Th17 cells and is independent of production of IL-22 by ILCs. Importantly, whereas Th17 cells were strictly dependent on AhR for expression of IL-22, Th22 cells required co-expression of AhR and T-bet for optimal expression of IL-22, and both transcription factors were necessary for host protection by Th22 cells.

Anti-pathogenic responses are characterized by integration of the innate and adaptive arms of immunity. Recent studies have emphasized the critical role of different populations of ILCs in protection against *C. rodentium* challenge, including NK-like cells (NK-22) and CD4$^+$ lymphoid tissue inducer (LTi) cells (Colonna, 2009; Sonnenberg et al., 2011a). While ILCs provide a rapid source of IL-22 that is essential for early host protection, we find that CD4$^+$ T cells become the dominant source of IL-22 in the intestinal LP where the anti-microbial response is concentrated at the height of infection. While the current study does not define the relative contribution of IL-22–producing CD4$^+$ T cells derived from Th22 cell precursors, Th17 cell precursors, or both, it does establish a critical role for IL-22–producing CD4$^+$ T cells in epithelial barrier protection as the innate response declines. Based on greater production of IL-22 by Th22 cells derived ex vivo, the higher frequency of IL-22$^+$ CD4$^+$ T cells that produce IL-22 independently of IL-17A or IFNγ co-expression, and the superior protective function of Th22 cells transferred into infected IL-22–deficient recipients, we favor the view that Th22 cells are the principal contributors of IL-22 derived from the adaptive response, although this will require further study. Irrespective of developmental origin, however, our findings highlight a sequential model of IL-22–dependent bacterial clearance wherein early production of IL-22 by ILCs gives way to production of IL-22 by CD4$^+$ T cells that become the dominant source of IL-22 and are required for complete clearance of infection.

Because ILCs are strictly dependent on IL-23 for their production of IL-22, the discovery herein that IL-23−deficient mice survive infection by low doses of *C. rodentium* via an IL-22–dependent mechanism provoked inquiry into ILC–independent mechanisms of pathogen clearance, and led to a re-examination of mechanisms by which IL-6 deficiency confers increased mortality (Dann et al., 2008; Zheng et al., 2008). Given that IL-6 is required for Th22 and Th17 differentiation, the finding that CD4$^+$ T cells become the dominant source of IL-22 concordant with the decline in bacterial load provides a basis for

the impaired survival of IL-6–deficient mice despite a lack of significantly diminished production of IL-22 by ILCs. It was reported previously that IL-6 produced by the intestinal epithelium and macrophages conferred protection against *C. rodentium* infection largely through its anti-apoptotic effects on the epithelium (Dann et al., 2008). While direct actions of IL-6 on the intestinal epithelium no doubt contribute to host protection, our finding that delivery of IL-22-Fc conferred protection of infected IL-6–deficient mice supports a more critical role for the effects of IL-6 on induction on IL-22 producing T cells. This is consistent with the remarkably similar survival, colonization kinetics and colonic histopathology of IL-6– and IL-22–deficient mice inoculated with *C. rodentium*, and reinforces the importance of IL-22 derived from $CD4^+$ T cells as a critical component of the host's response to maintain barrier integrity.

It is notable that resistance to *C. rodentium* infection is more impaired in IL-23−deficient mice than either IL-22– or IL-6–deficient mice. This implies that IL-23 exerts host-protective effects that are independent of its actions to induce IL-22 and IL-6, production of which in the intestinal mucosa are severely compromised in the absence of IL-23. Thus, in addition to directly impairing IL-22 production by ILCs and mature Th22 and Th17 cells, and perhaps indirectly impairing development of Th22 and Th17 cells through diminished IL-6 induction, deficiency of IL-23 compromises anti-bacterial resistance through additional mechanisms. As noted above, IL-23–dependent induction of IL-6 that directly promotes epithelium sparing as well as the induction of IL-1β and TNF-α that promotes epithelial production of anti-microbial peptides via an IL-17C–dependent autocrine loop are likely contributory (Ramirez-Carrozzi et al., 2011; Song et al., 2011). Further, effects of reduced local chemokine production, as exemplified by CXCL1 (KC), among others (data not shown), impair recruitment of innate and adaptive immune cells that contribute to barrier defense and eradication of the pathogen (Aujla et al., 2008; Kolls et al., 2008; Ouyang et al., 2008). Suffice to say that in view of the range of proinflammatory cytokines and chemokines that are uniquely diminished in the intestines of infected IL-23–deficient mice, the protective effects mediated by IL-23 are likely multifactorial and will require further study.

Whereas IL-6 was essential for the host-protective development of IL-22 producing $CD4^+$ T cells, IL-21 proved dispensable. Despite a decrement in the frequency of $IL\text{-}22^+CD4^+$ T cells in IL-21–deficient mice challenged with *C. rodentium*, there was no increased mortality despite delayed pathogen clearance. We speculate that the delayed clearance is due to an impaired *C. rodentium*-specific antibody response, as systemic and mucosal antibody production is important for host protection in this model and IL-21-deficiency results in impaired IgG antibody responses (Bry and Brenner, 2004; Ozaki et al., 2002; Yoshida et al., 2006). In viral infections, IL-21 has been shown to be essential for preventing chronic infection and supporting optimal anti-viral antibody responses (Elsaesser et al., 2009; Eto et al., 2011; Yi et al., 2009), and in infection with *Listeria monocytogenes*, IL-21 deficiency was associated with increased IL-17 production and unimpaired pathogen clearance (Ertelt et al., 2010). Findings herein define a marginal role for IL-21 the development of $IL\text{-}22^+CD4^+$ T cells and suggest that its principal role in host-protective mucosal immunity might be more related to its role in providing B cell help.

Comparative transcriptome analysis of Th22 and Th17 cells revealed differential expression of several hundred genes, among which were several key transcription factors. This included reciprocal expression of transcripts encoding T-bet and RORγt, consistent with TGFβ's established actions in suppressing *Tbx21* and enhancing *Rorc* expression, and led to identification of T-bet as an important factor in driving high-level expression of IL-22 and host protection by Th22 cells. In contrast to T-bet, we found no significant difference in expression of *Eomes*, a related T-box family member expressed in T cells, suggesting that

Eomes is unlikely to contribute to Th22 development. Remarkably, despite the low levels of AhR in Th22 cells, AhR also contributed significantly to *Il22* expression in Th22 cells such that combined loss of T-bet and AhR actions resulted in nearly complete abrogation of *Il22* expression. Regardless of higher levels of AhR in Th17 cells, optimal IL-22 induction is severely compromised, possibly due to TGFβ, which might inhibit IL-22 induction by repressing T-bet and enhancing other transcription factors, including c-Maf (Rutz et al., 2011).

Because neither T-bet nor AhR positively regulated the expression of the other, their actions on *Il22* expression appear to be cooperative. This is in contrast to Th17 cells, which are strictly dependent on AhR for *Il22* expression (Veldhoen et al., 2008). Thus, while our findings confirm that Th22 cells can express *Il22* independently of AhR (Rutz et al., 2011), AhR clearly contributes to the transcriptional regulation of *Il22* in both Th17 and Th22 cells and is a dominant transcription factor regulating *Il22* transcription. It should be noted that *Il22* is closely linked to the *Ifng* gene on chromosome 10 in mice (and to *IFNG* on chromosome 12 in humans), suggesting that T-bet, originally described as a critical regulator of *Ifng* gene expression, might have more extended regional actions on this gene cluster. This could offer an explanation for the original description of IL-22 as a product of Th1 cells (Gurney, 2004), which also co-express AhR, and has implications for *Il22* expression in Th17 cells as well, in which enhanced expression of *Il22* downstream of IL-23 is contingent upon STAT1- and/or STAT4-dependent expression of T-bet (unpublished observations). In this regard, it has been shown that ectopic expression of T-bet in Th17 cells enhanced *Il22* expression while suppressing *Il17a* and *Il17f* (Lazarevic et al., 2011). Given the role of IL-23 induced T-bet in the late divergence of Th17 cells towards expression of *Ifng* (Lee et al., 2009), it would appear that this mechanism might facilitate the development of Th22 type cells from Th17 precursors, a subject of current investigation.

In agreement with a recent report (Rutz et al., 2011), we found higher expression of the AP-1 transcription factor family member *Maf* (c-Maf) by Th17 cells. In view of our finding that low expression of *maf* by Th22 cells was not reversed by T-bet deficiency, the actions of T-bet to promote *Il22* expression by Th22 cells are not due to derepression of c-Maf. In addition to their decreased expression of *Maf*, Th22 cells also expressed significantly lower levels of *Rorc* compared to Th17 cells, consistent with T-bet's actions to repress *Rorc* expression by direct binding to the *Rorc* locus and complexing with *Rorc* positive regulator, Runx1 (Lazarevic et al., 2011; Mukasa et al., 2010), expression of which was also significantly decreased in Th22 cells (data not shown). Whether RORγt is required for *Il22* expression by Th22 cells despite its relatively low expression, akin to AhR, remains to be determined. However, these data establish both AhR and T-bet as critical positive regulators of *Il22* expression in Th22 cells and suggest that the enhanced expression of *Il22* downstream of IL-23 in both Th22 and Th17 cells are linked to its induction of *Tbx21* expression by activation of STAT1, STAT4, or both.

Notably, the transcriptome of Th22 cells described herein is remarkably similar to that of the 'pathogenic' Th17 cells whose development from naïve $CD4^+$ T cells was induced by actions of IL-6, IL-23 and IL-1β in the absence of TGFβ (Ghoreschi et al., 2010). These cells, referred to as Th17(23) cells, were distinguished from conventional Th17 cells differentiated with TGFβ, termed Th17(β) cells, by their distinct gene expression profile and greater pathogenicity in a transfer model of experimental allergic encephalomyelitis (EAE). Given their similarities to Th17(23) cells, this begs the question of whether Th22 cells might under certain circumstances also contribute to pathogenicity in the intestine and other tissue sites. IL-22–producing $CD4^+$ T cells were recently reported to be pathogenic in a transfer model of colitis that used $CD45RB^{lo}$ cells depleted of Treg cells (Kamanaka et al., 2011). Given that Th22 cells were originally described in humans (Duhen et al., 2009; Trifari et al.,

2009) and that elevations of IL-22 are found in the intestines of patients with inflammatory bowel disease (IBD) as well as patients with psoriasis and rheumatoid arthritis (Andoh et al., 2005; Ikeuchi et al., 2005; Wolk et al., 2004; Wolk et al., 2007; Wolk et al., 2006), it will be important to determine whether Th22 cells are contributors to pathogenicity in at least some variants of human autoimmune disease and IBD previously thought to be linked to conventional Th17 cells.

EXPERIMENTAL PROCEDURES

Mice

Mice were purchased from the Jackson Laboratories and/or bred at our facility: C57BL/6J (B6); B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII TCR transgenic); B6.129S6-*Tbx21*^{tm1Glm}/J (*Tbx21*$^{-/-}$); B6.129S2-*Il6*^{tm1Kopf}/J (*Il6*$^{-/-}$); B6.129S7-*Ifng*^{tm1Ts}/J (*Ifng*$^{-/-}$); B6. *Il17ra*$^{-/-}$; B6.*Il23a*$^{-/-}$ (kindly provided by Schering-Plough Biopharma); B6.129S5-*Il22*^{tm1Lex} mice (*Il22*$^{-/-}$; kindly provided by Genentech); B6.129S5-*Il21*^{tm1Lex} (*Il21*$^{-/-}$) and B6.*Ahr*$^{-/-}$. All animals were bred and maintained in accordance with institutional animal care and use committee regulations.

Citrobacter rodentium Infection and Treatments

Citrobacter rodentium strain, DBS100 (ATCC), was used for all inoculations with the exception of experiments in which whole body imaging was performed. For imaging experiments, the bioluminescent *C. rodentium* strain ICC180 (derived from DBS100) was used (generously provided by Drs. Gadi Frankel and Siouxsie Wiles, Imperial College London) (Wiles et al., 2006). Mice were inoculated with 1–2 × 10^9 cfu (high dose) or 0.5 ×10^6 cfu (low dose) in a total volume of 200 μl of PBS via gastric gavage. *In vivo* neutralization studies used 150 μg per dose of anti-IL-22 mAb (Genentech, clone 8E11), anti-IL-6 mAb (clone MP5–20F3) or isotype control mAb, each injected IP. For *in vivo* delivery of IL-22, hIL-22.Fc protein (Pfizer) was given IP at 50 μg per mouse.

Bioluminescence Imaging

Mice were anesthetized with isoflurane and placed in a supine position in a custom built chamber for imaging with a IVIS-100 system and Living Image Software (Xenogen, Inc.). Baseline images were collected prior to gavage with 2 × 10^9 cfu *C. rodentium* strain ICC180 and whole body images were taken at a binning of 4 over 1 to 3 min at the indicated times during infection. Luminescence emitted from the same gate in individual mice was quantified as counts per second and pseudocolor images representing light intensity generated.

Gene Expression Analysis

Naïve OTII CD4$^+$ T cells were sorted and activated by OVAp and T-cell depleted splenocytes from *Il23a*$^{-/-}$ mice in the presence of IL-6 alone (Th22) or under IL-6 + TGFβThi7. T cells were isolated after 60 hours of culture and DNase-free RNA was isolated for microarray analysis using the Affymetrix Mouse Gene ST 1.0 Array as previously described (Lee et al., 2009) and analyzed per Supplemental Procedures.

Adoptive transfer experiments

Naïve CD4$^+$ T cells from pooled spleens and lymph nodes of WT, *Il22*$^{-/-}$ or *Tbx21*$^{-/-}$ mice were purified by FACS sorting and differentiated for 3 days under Th22 or Th17 conditions (see Supplemental Procedures for details). Recovered T cells were transferred by IP injection (5 × 10^6) cells per mouse) into recipients inoculated with *C. rodentium* 4 days prior to cell transfer and were analyzed for survival.

Statistical Analysis

Statistical analyses were performed using Student's *t*-test and $p<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to Dr. David Randolph and members of the Weaver laboratory for their helpful comments and suggestions. We thank Drs. Josh Mezrich and Chris Bradfield (Univ. Wisconsin) for provision of AhR-deficient mice, and Dr. David Crossman for assistance with analysis of gene expression data. We also thank the UAB Digestive Diseases Research Developmental Center (DDRDC), the UAB Small Animal Imaging Facility, UAB Genomics Core, UAB Center for AIDS Research Flow Cytometry Core, and UAB Epitope Recognition and Immunoreagent Core Facility for genotyping of gene-targeted mice, imaging studies, gene expression studies, flow cytometric sorting, and antibody preparations, respectively. This work was supported by grants from the NIH (C.T.W. and R.D.H.) and the Crohn's and Colitis Foundation of America (R.B. and D.B.O.).

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Highlights

- IL-23–deficient mice are protected from low-dose *Citrobacter rodentium* infection.
- $CD4^+$ T cell derived IL-22 is required for *C. rodentium* infection protection.
- Th22 cells provide more effective anti-bacterial defense than Th17 cells.
- T-bet acts cooperatively with AhR in *Il22* induction in Th22 cells.

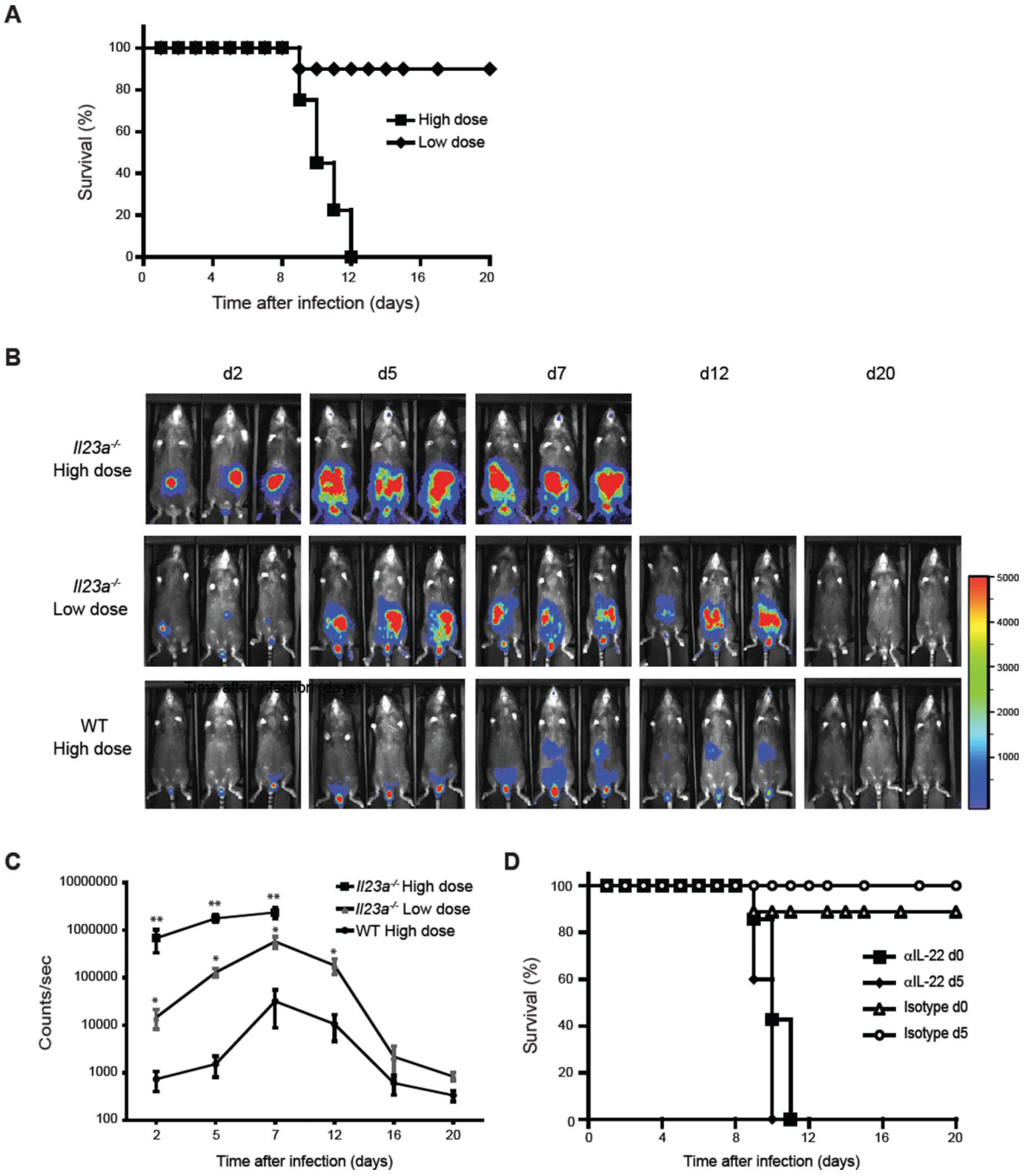

Figure 1.

IL-23-independent, IL-22-dependent pathway to protection from *C. rodentium* Infection

(A) Survival rates of *Il23a*$^{-/-}$ mice infected with high-dose (2×10^9 cfu) or low-dose (0.5×10^6 cfu) *C. rodentium* (n= 4–5 mice per group). Data are from one of three similar experiments.

(B) Serial whole-body imaging of *Il23a*$^{-/-}$ mice inoculated with high- or low-dose of the luminescent strain of *C. rodentium* (strain ICC180) by gastric gavage and imaged at the indicated days PI.

(C) Colonization kinetics of WT or *IL23a*$^{-/-}$ mice inoculated with indicated doses of *C. rodentium*. Light unit counts/sec directly correlate with the bacterial load (data not shown). Data are means +/− SEM. *p<0.01, **p<0.001.
(D) Survival rates of *IL23a*$^{-/-}$ mice infected with low-dose *C. rodentium* and dosed IP with 150 μg of neutralizing anti-IL-22 mAb or isotype control every other day starting at d0 or d5. Data are representative of two or more experiments with a minimum of 3–4 mice per group.

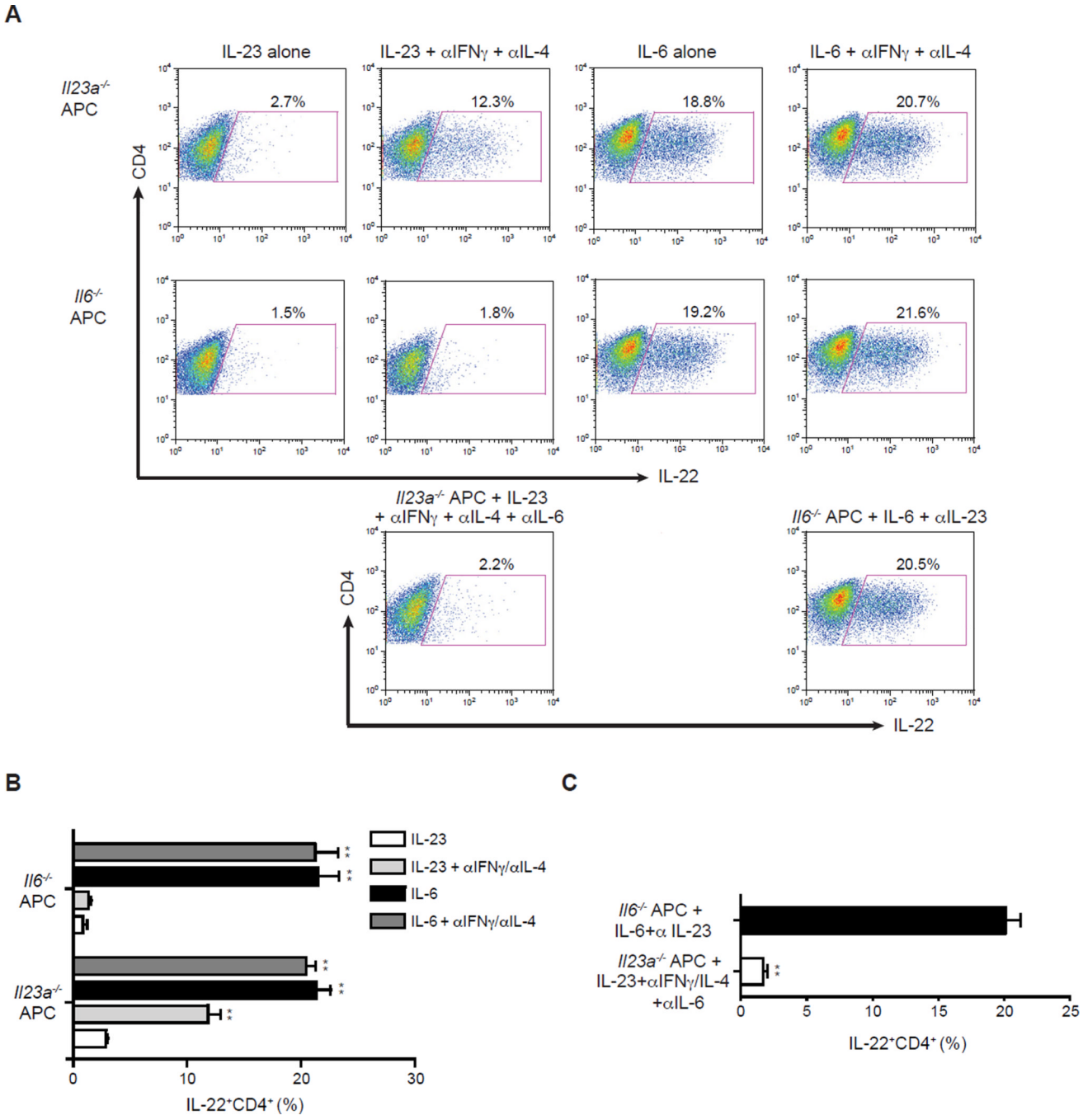

Figure 2.

IL-6 is indispensable for development of IL-22-producing CD4$^+$ T cells

(A) Naïve OTII-Tg CD4 T cells were sorted and activated by Ova peptide for 3d using splenic APCs from *Il23a*$^{-/-}$ or *Il6*$^{-/-}$ mice, with or without addition of the indicated neutralizing mAbs. Recovered cells were stained for surface CD4 and intracellular IL-22 and analyzed by flow cytometry. Numbers indicate the frequencies of IL-22$^+$ CD4$^+$ T cells. (B, C) Pooled frequency data from three or more independent experiments performed as in (A). Data are means +/− SEM (*p<0.05, **p<0.01).

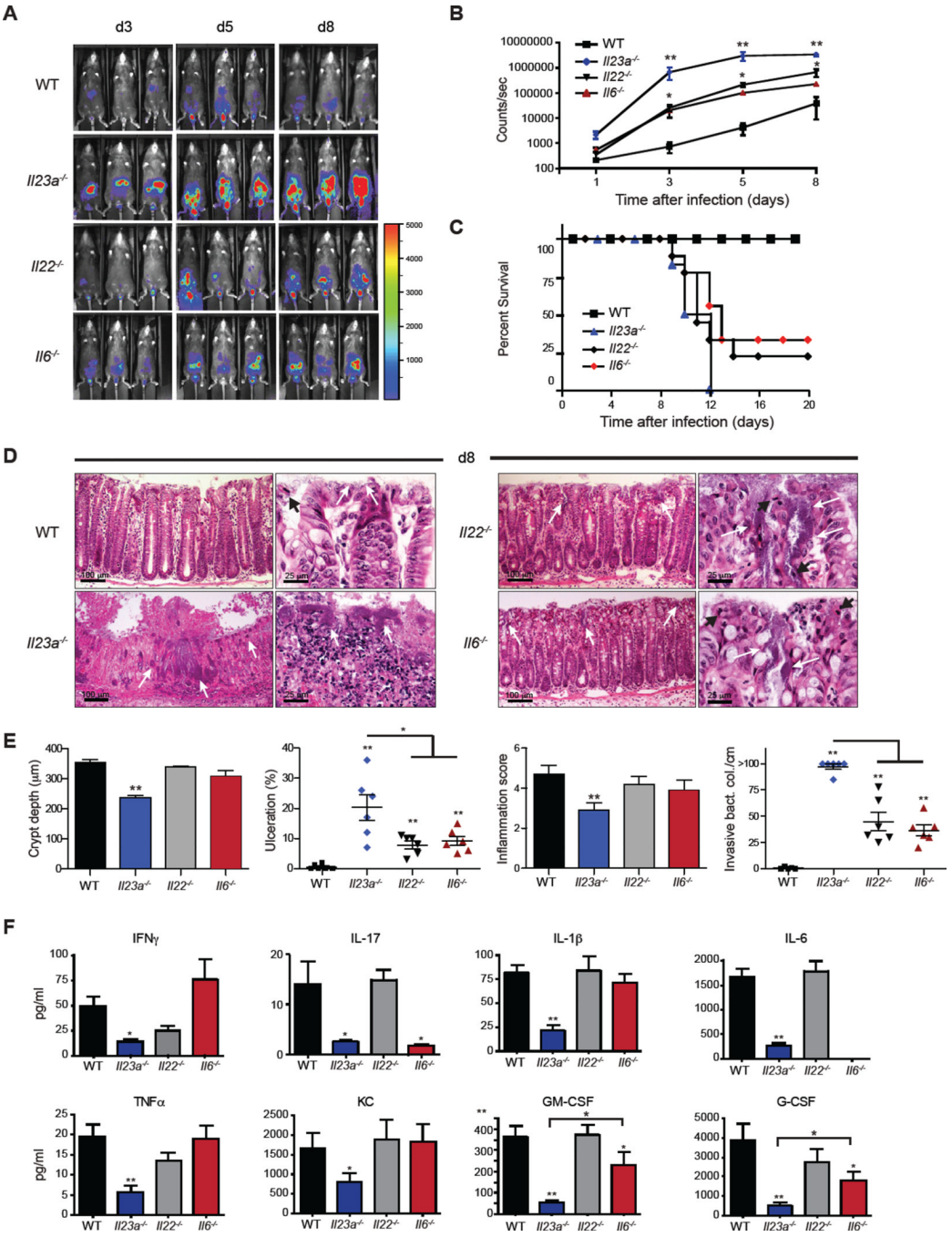

Figure 3.

IL-23 governs early protective immunity to *C. rodentium*

(A) Whole-body imaging of WT, *Il23a*$^{-/-}$, *Il22*$^{-/-}$ and *Il6*$^{-/-}$ mice inoculated with 2 × 10^9 cfu of luminescent strain of *C. rodentium* and imaged at the indicated days PI.

(B) Colonization kinetics of WT, *Il23a*$^{-/-}$, *Il22*$^{-/-}$ and *Il6*$^{-/-}$ mice inoculated with 2 × 10^9 cfu of luminescent strain of *C. rodentium*. Data are means +/− SEM. *p<0.05, **p<0.01.

(C) Survival rates of WT, *Il23a*$^{-/-}$, *Il22*$^{-/-}$ and *Il6*$^{-/-}$ mice inoculated with 2 × 10^9 cfu of *C. rodentium*.

(D) Histopathology of distal colonic tissues from WT, *Il23a*$^{-/-}$, *Il22*$^{-/-}$ and *Il6*$^{-/-}$ mice collected eight days PI. H&E-stained sections (scale bars: 100 mm, left panel; 25 mm, right

panels). White arrows denote individual clusters of *C. rodentium* attached to the apical surface of the colonic epithelium (WT) or dense colonies that extend into colonic crypts and penetrate the epithelium (*Il22*$^{-/-}$ and *Il6*$^{-/-}$) or deeply invade the necrotic mucosa (*Il23a*$^{-/-}$). Black arrows denote dying colonic epithelial cells.

(E) Histopathological scoring of colons from infected WT, *Il23a*$^{-/-}$, *Il22*$^{-/-}$ and *Il6*$^{-/-}$ mice was performed at d8 PI as per Supplemental Experimental Procedures. Data are means +/− SEM from two or more experiments (n=3–4 mice per group). *p<0.05, **p<0.01.

(F) Multiplexed cytokine ELISA of the indicated cytokines and chemokines from supernatants of colonic tissue homogenates from WT, *Il23a*$^{-/-}$, *Il6*$^{-/-}$, *Il22*$^{-/-}$ mice collected d3 PI (24h cultures). Data are means +/− SEM from two or more experiments (n=3–4 mice per group). *p<0.05, **p<0.01.

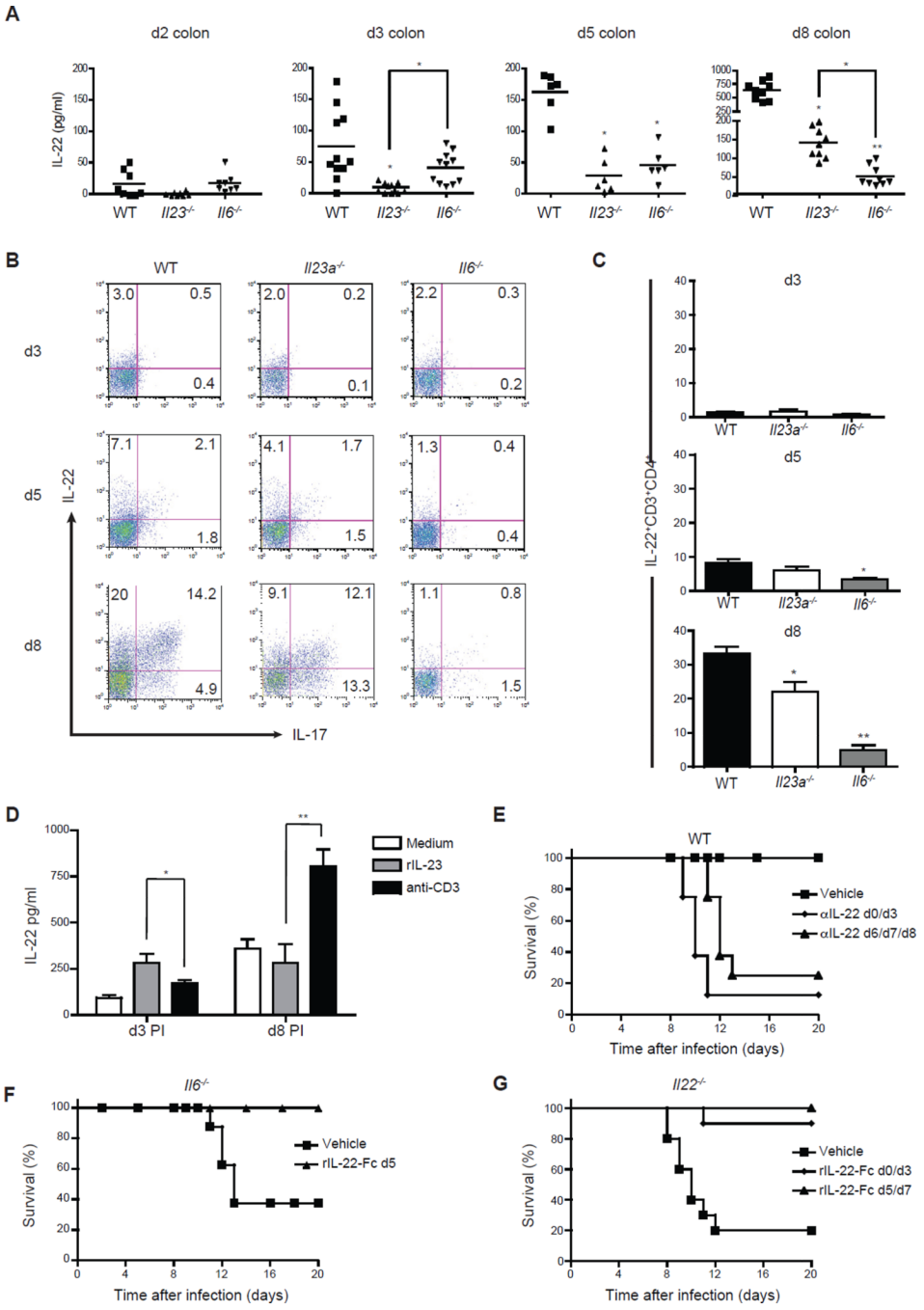

Figure 4.

IL-6–induced CD4$^+$ T cells are major source of protective IL-22 late in *C. rodentium* infection

(A) WT, *Il23a*$^{-/-}$ and *Il6*$^{-/-}$ mice were inoculated with 2 × 10^9 cfu *C. rodentium* and colonic tissues collected at the indicated days PI for assessment of production of IL-22 in homogenate supernatants cultured for 24h. Data are means +/− SEM (*p<0.05, **p<0.01).

(B) Colonic LP cells of WT, *Il23a*$^{-/-}$ and *Il6*$^{-/-}$ mice infected with *C. rodentium* were isolated on the indicated days PI and analyzed by flow cytometry for expression of intracellular IL-22 and IL-17A by CD3$^+$CD4$^+$ gated cells. Numbers indicate the frequencies of cells in each quadrant.

(C) Flow cytometric frequency data for LPL isolates of WT, *Il23a*$^{-/-}$ and *Il6*$^{-/-}$ mice generated as in (B) and pooled from three independent experiments (n=3–4 mice per group). Data are means +/− SEM (*p<0.05, **p<0.01).
(D) Survival rates of *C. rodentium* infected WT mice that were untreated or treated with neutralizing IL-22 mAb on days 0 and 3 PI, or on days 6, 7 and 8 PI.
(E) Survival rates of *Il6*$^{-/-}$ mice infected with *C. rodentium* and treated with rIL-22-Fc protein (50 μg IP) or vehicle alone at d5 PI.
(F) Survival rates of *C. rodentium* infected *Il22*$^{-/-}$ mice treated with rIL-22Fc protein (50 μg IP) or vehicle alone at days 0 and 3, or days 5 and 7 PI.
(G) Survival rates of *C. rodentium* infected *Il22*$^{-/-}$ mice treated with rIL-22Fc protein (50 μg IP) or vehicle alone at days 0 and 3, or days 5 and 7 PI.
All data are representative of three or more experiments with a minimum of 3–4 mice per group.

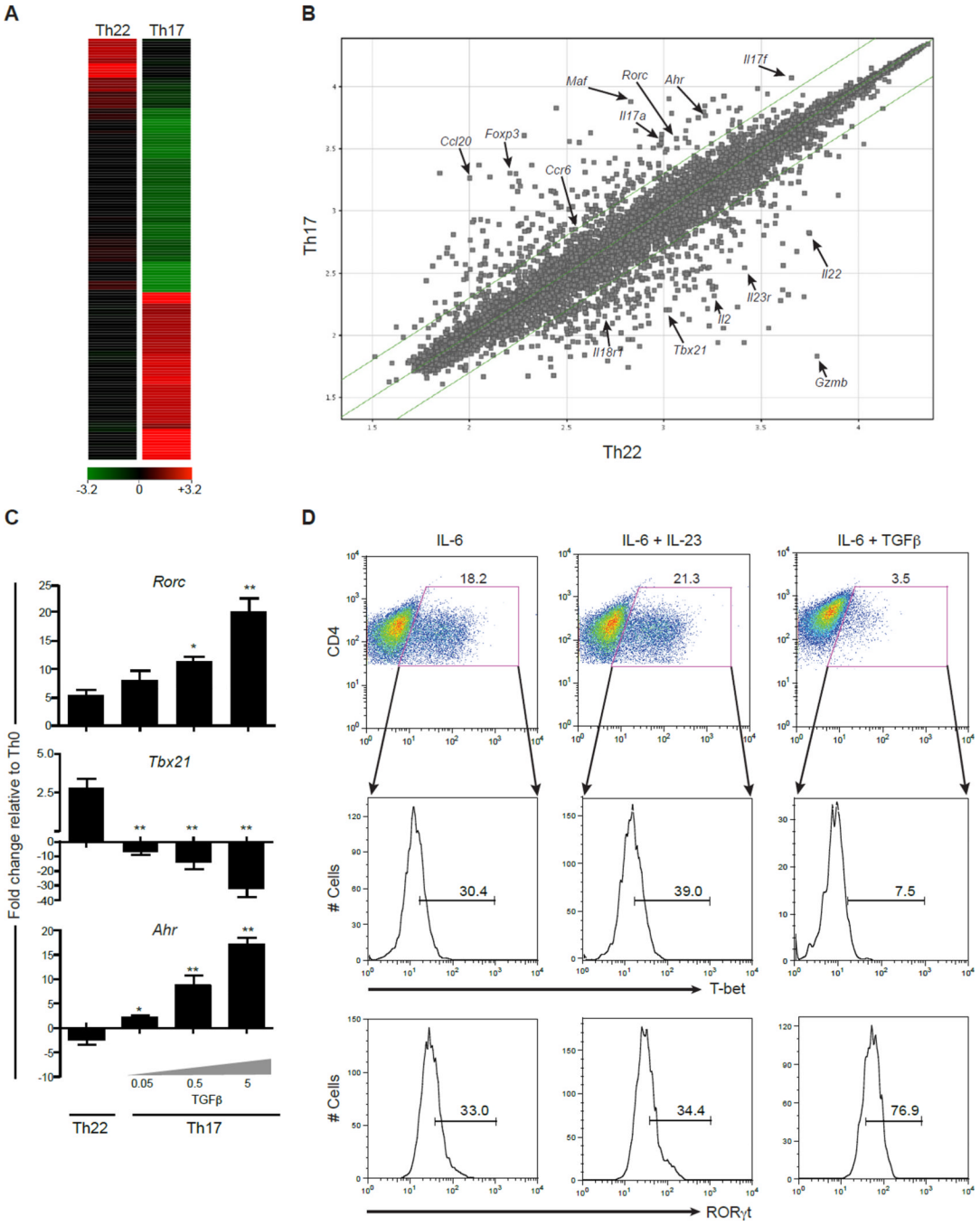

Figure 5.

Th22 and Th17 cells are distinguished by reciprocal expression of T-bet, AhR and RORγt (A, B) FACS-sorted naïve OTII-Tg CD4$^+$ T cells were activated by Ova peptide in presence of CD4$^+$ T-cell depleted splenic APCs from *Il23a*$^{-/-}$ mice in the presence of IL-6 alone (Th22 polarization) or IL-6+TGFβ Thi7 polarization. CD4$^+$ T cells were purified by magnetic sorting after 60h of culture and RNA was isolated for microarray-based gene expression analysis. Shown is a heat map of genes with more than 4-fold expression differences between the two populations (A) and transcriptome profiling of Th22- or Th17-polarized cells by scatterplot analysis (B).

(C) Naïve $CD4^+$ T cells isolated from C57BL/6 mice were activated by anti-CD3 and anti-CD28 in presence of IL-6 and IL-23 (Th22 polarization), IL-6 and the indicated concentrations of TGFβ Thi7 polarization), or absence of added cytokines (Th0). On day 3 the cells were further stimulated with PMA/ionomycin for 6 hours and analyzed by RT-PCR for *Rorc*, *Tbx21* and *Ahr* transcripts. Data are means +/− SEM (*p<0.05, **p<0.01).
(D) Naïve OTII $CD4^+$ T cells were simulated with Ova peptide in presence of IL-6, IL-6+IL-23 or IL-6+TGFβ and analyzed by flow cytometry for frequencies of $IL\text{-}22^+$ $CD4^+$ T cells on day 3 following stimulation for 4 hours with PMA/I in the presence of brefeldin A. The expression of T-bet and RORγt within the $IL\text{-}22^+$ $CD4^+$ gates was determined by intracellular staining (histograms).
All data are representative of three or more experiments with a minimum of 3 mice per group.

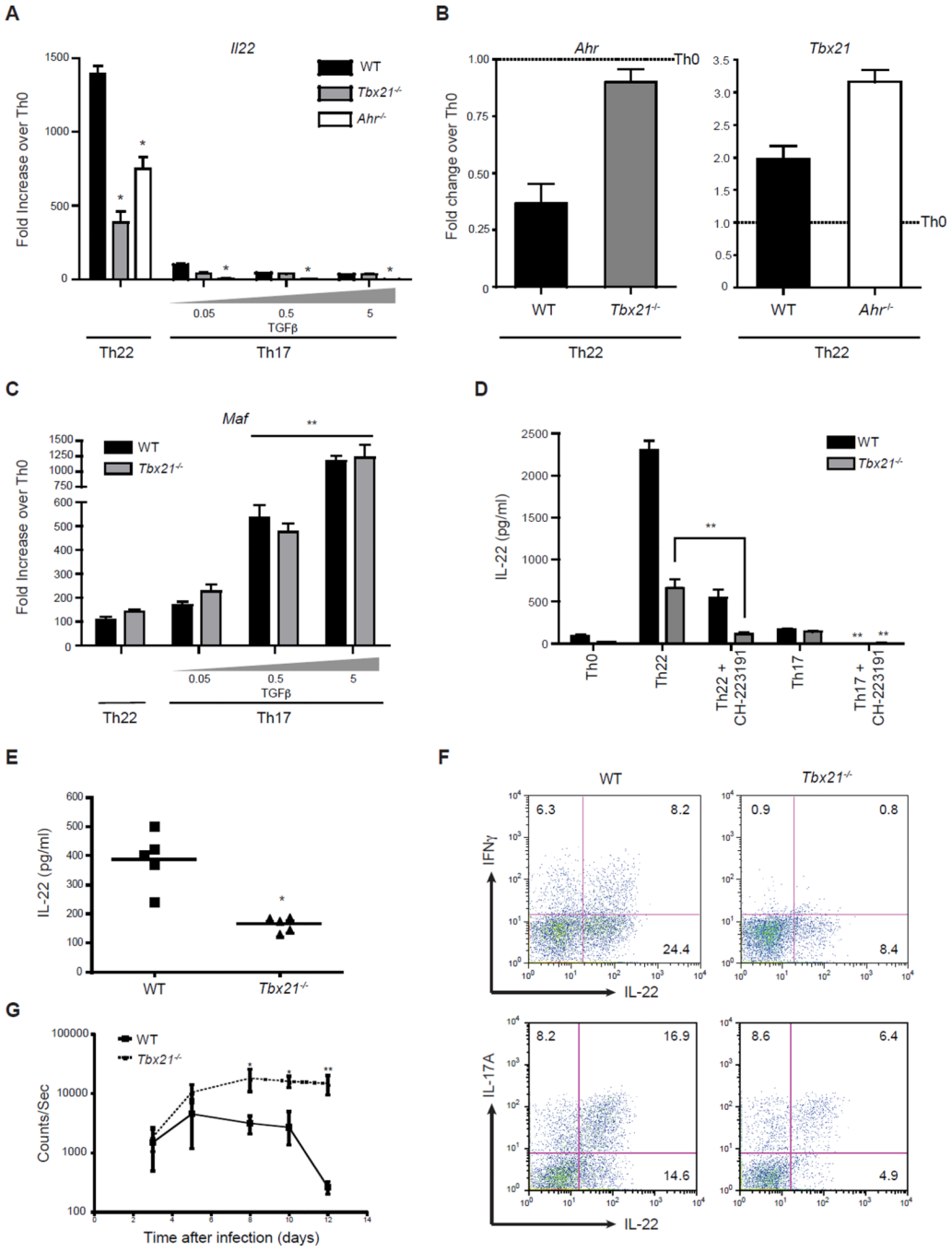

Figure 6.

IL-22 production by Th22 cells is T-bet- and AhR-dependent

(A-C) FACS-sorted naïve CD4 T cells ($CD4^+CD25\text{-}CD62L^{high}$ $CD44^{low}$) from WT, *Tbx21*$^{-/-}$ or *Ahr*$^{-/-}$ mice were activated with anti-CD3/CD28 in presence of IL-6 and IL-23 (Th22 polarization), IL-6 and different concentrations of TGFβ Thi7 polarizations, or absence of cytokines (Th0). On day 3 $CD4^+$ T cells were re-stimulated with PMA/ionomycin for 6 hours and analyzed by RT-PCR for *Il22* (A), *Ahr* and *Tbx21* (B), or *maf (C)* transcripts. Expression values are normalized to Th0 controls. Data are means +/− SEM (*p<0.05). (D) Naïve CD4 T cells from WT or *Tbx21*$^{-/-}$ mice were differentiated under Th0, Th22 or Th17 conditions. Where indicated, the AhR-antagonist, CH-223191 (3μM),

was added at the initiation of cultures. IL-22 protein was quantified by ELISA from culture supernatants of T cells treated with PMA/ionomycin for 48 hours. Data are means +/− SEM (*p<0.01). (E, F) WT and *Tbx21*$^{-/-}$ mice were inoculated with *C. rodentium* and sacrificed on d8 PI for analysis of IL-22 production from supernatants of colon homogenates cultured *ex vivo* for 24h (E; *p<0.05), or for analysis of expression of intracellular IL-22 and IFN-γ by $CD3^+CD4^+$ cells (F). Numbers in each quadrant indicate frequencies (CD3 and CD4 gated).

(G) Colonization kinetics of WT *and Tbx21*$^{-/-}$ mice infected with 2×10^9 luminescent *C. rodentium* bacterial strain and assessed by whole body imaging on the indicated days PI. Data are means +/− SEM (*p<0.05, **p<0.01).

All data are representative of two or more experiments.

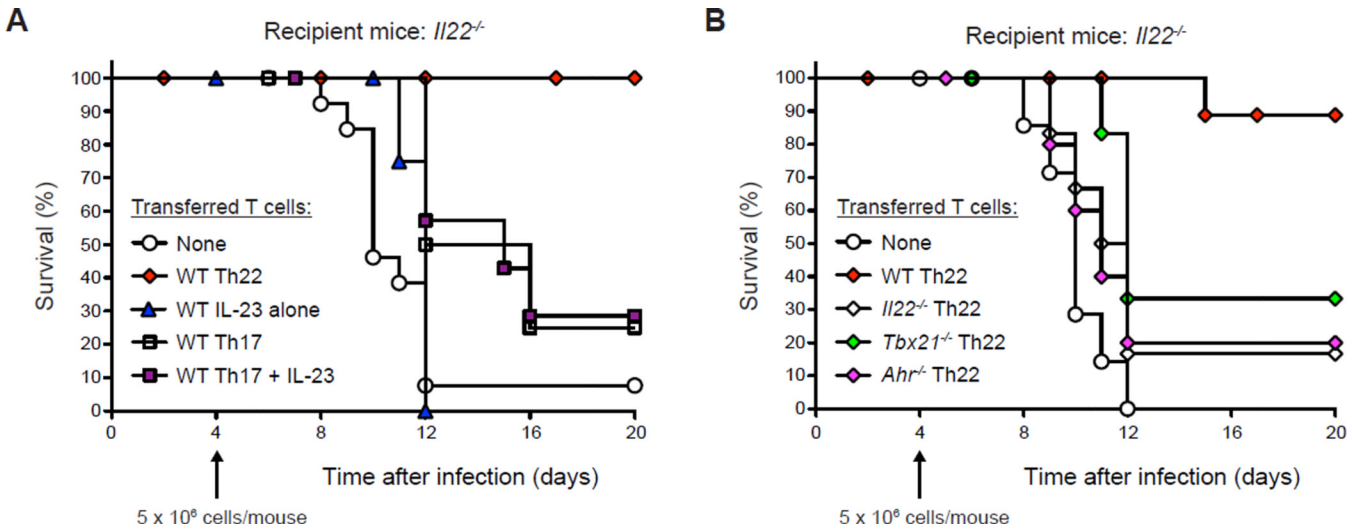

Figure 7.

Th22 cells provide superior host protection to Th17 cells

(A, B) Naïve CD4$^+$ T cells from WT mice were stimulated *in vitro* for 72h with anti-CD3+anti-CD28 with addition of IL-6 (Th22), IL-23 (IL-23 alone), or IL-6+TGFβ, without or with IL-23 addition (Th17 and Th17+IL-23, respectively), prior to isolation and transfer (A). Naïve CD4$^+$ T cells from WT, *Il22*$^{-/-}$, *Tbx21*$^{-/-}$ and *Ahr*$^{-/-}$ mice were similarly stimulated *in vitro* with addition of IL-6 (WT Th22, *Il22*$^{-/-}$ Th22, *Tbx21*$^{-/-}$ Th22 and *Ahr*$^{-/-}$ Th22, respectively), prior to isolation and transfer (B). N ≥ 5 mice per group; representative of two or more experiments.