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Innate and Adaptive Interleukin-22 Protects Mice from Inflammatory Bowel Disease

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SUMMARY

Inflammatory bowel disease (IBD) is a chronic inflammatory disease thought to be mediated by dysfunctional innate and/or adaptive immunity. This aberrant immune response leads to the secretion of harmful cytokines that destroy the epithelium of the gastrointestinal tract and thus cause further inflammation. Interleukin-22 (IL-22) is a T helper 17 (Th17) T cell-associated cytokine that is bifunctional in that it has both proinflammatory and protective effects on tissues depending on the inflammatory context. We show herein that IL-22 protected mice from IBD. Interestingly, not only was this protection mediated by $CD4^+$ T cells, but IL-22-expressing natural killer (NK) cells also conferred protection. In addition, IL-22 expression was differentially regulated between NK cell subsets. Thus, both the innate and adaptive immune responses have developed protective mechanisms to counteract the damaging effects of inflammation on tissues.

INTRODUCTION

Interleukin-22 (IL-22) is a member of the IL-10-related family of cytokines, which also include IL-19, IL-20, IL-24, and in humans, IL-26, which are expressed during chronic inflammation. These cytokines share 20%–30% amino acid identity and also have homologous secondary structures (Kotenko, 2002). IL-22 signals through a heterodimeric receptor that consists of IL-22R and IL-10Rβ, whereas IL-10 signals through IL-10Rα and IL-10Rβ (Kotenko et al., 2001). Because IL-10Rβ is ubiquitously expressed, signaling specificity is conferred by IL-10Rα and IL-22R expression; IL-10Rα is limited to cells of the immune system, whereas IL-22R expression is limited to tissue cells, such as epithelial cells (Wolk et al., 2004). Just as IL-10 protects the immune system from overwhelming itself, IL-22 is proposed to protect the tissues during inflammation via a signal transducer and activator of transcription 3 (Stat3)-mediated mechanism. IL-22 has recently been shown to be protective during acute inflammation in a hepatitis model (Radaeva et al., 2004; Zenewicz et al., 2007). In contrast, IL-22 has been shown to mediate dermal inflammation (Ma et al., 2008; Zheng et al., 2007). The dual nature of this cytokine, protective versus inflammatory, probably depends on the inflammatory context. This includes, but is not limited to, the duration and amount of IL-22 present, the overall cytokine milieu, and the involved tissues.

IL-22 is highly expressed by Th17 cells and is strongly linked to chronic inflammation (Chung et al., 2006; Liang et al., 2006; Zheng et al., 2007). Th17 cells were first defined by their expression of IL-17A but have since also been shown to preferentially express IL-22, as well as IL-17F and IL-21 (Korn et al., 2007; Liang et al., 2006; Nurieva et al., 2007; Weaver et al., 2006). In mice, IL-23 was once thought to control Th17 differentiation, but it now appears that its role is in survival and expansion of Th17 cells (Aggarwal et al., 2003; Cua et al., 2003; Veldhoen et al., 2006). Differentiation in mice appears to be directed by the presence of both transforming growth factor-beta (TGF-β) and inflammatory cytokines, such as IL-6 or IL-21, that activate Stat3 signaling pathways in the T cells (Korn et al., 2007; Nurieva et al., 2007; Veldhoen et al., 2006). However, it is becoming increasingly apparent that the IL-22 expression profile differs from that of IL-17A. Whereas TGF-β and IL-6 are both necessary for induction of IL-17A, IL-22 can be induced via IL-6 alone, and increasing amounts of TGF-β are actually inhibitory to its expression (Zheng et al., 2007 and our unpublished observations).

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract and is caused by aberrant innate and/or adaptive immune responses (Podolsky, 2002). IBD has long been described as a Th1-mediated disease because interferon-gamma (IFN-γ) is essential for disease progression (Powrie et al., 1994b). However, the recent discovery of Th17 cells has led to a re-evaluation of the role of T cells in disease. IL-23, important for the maintenance of Th17 cells, is essential for development of IBD in mouse models (Kullberg et al., 2006), and protective IL-23R polymorphisms in the human population have been identified through a genome association study (Duerr et al., 2006). However, the role of individual Th17 cytokines, such as IL-22, in IBD remains elusive. IL-22 has been shown to be highly upregulated in the sera and in lesions of patients with either Crohn's disease or ulcerative colitis (Andoh et al., 2005). IL-22 can have proinflammatory effects on colon epithelial cells and induce secretion of IL-6 and IL-8, as well as activate the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1) (Andoh et al., 2005). On the other hand, ectopic

expression of IL-22 in the gastrointestinal tract by targeted micro-injection is protective to colitis (Sugimoto et al., 2008). However, it remains to be determined whether the immune response itself has this protection mechanism during IBD.

In this study, we have investigated the role of IL-22 during IBD. Using both innate and T cell-driven colitis animal models, we have found a protective role for IL-22 during IBD. Our data suggest that IL-22 secretion by not only $CD4^+$ T cells but also, surprisingly, NK cells in the colon mediates this protection.

RESULTS

Colon Epithelial Cells Are Responsive to IL-22

The IL-10-related cytokines (IL-10, IL-19, IL-20, IL-22, and IL-24) share use of a family of heterodimeric cytokine receptors (Kotenko, 2002; Moore et al., 2001). To begin to understand which of these cytokines play a role in IBD, we compared expression of the different receptor subunits in the gastrointestinal tract to other tissues, such as the liver, spleen, and skin (Figure S1A in the Supplemental Data available online). IL-10Rα, which is only used by IL-10 and whose expression is primarily limited to immune cells and not tissues, was highly expressed in the spleen compared to other tissues. IL-10Rβ, the β chain for both the IL-10 and IL-22 receptor, was ubiquitously expressed in all tissues, as previously reported. IL-22R, the α chain for the IL-22 receptor, was highly expressed in the colon and small intestine and to a lesser extent in the other tissues we examined. We additionally examined expression of IL-20Rα and IL-20Rβ, which recognize IL-19, IL-20, and IL-24, and found that both of these chains were most highly expressed in the skin, which agrees with published data indicating that these cytokines' primary targets are keratinocytes (Blumberg et al., 2001; Boniface et al., 2005).

The expression of the IL-22 receptor in the gut suggested that the gastrointestinal system should be highly responsive to IL-22. To further examine to which IL-10 family cytokine members colon cells are responsive, and to confirm expression of the functional receptor, we stimulated the human colon epithelial cell line Caco-2 with recombinant human IL-10, IL-19, IL-20, or IL-22. Upon recognition of the appropriate receptor, each of these cytokines activates Stat3 in responsive cell types (Blumberg et al., 2001; Dumoutier et al., 2001; Dumoutier et al., 2000). IL-22, but not IL-10, IL-19, or IL-20 induced detectable phosphorylation of Stat3 (Figure S1B). Thus, colon epithelial cells are a target for IL-22 activity.

IL-22 Is Induced in the Colon during IBD

To examine which cytokines are expressed during IBD in mice, we used the $CD45RB^{hi}$ transfer model of colitis. This T cell-dependent model involves the transfer of purified naive CD4 T cells ($CD45RB^{hi}$) into $Rag1^{-/-}$ mice, which lack T cells (Morrissey et al., 1993; Powrie et al., 1994a). In the absence of both host and regulatory T cells, these cells rapidly expand and gain effector functions, such as IFN-γ secretion. In the absence of regulatory T cells, these functions are unchecked and result in massive inflammation primarily of the gut, but eventually of other organs such as the liver and skin. Five weeks after the transfer, when mice had lost considerable body mass, we examined cytokine expression in their inflamed colons. IFN-γ has long been associated with IBD, and accordingly, we found that IFN-γ mRNA was highly induced in the inflamed colons compared to colons from mice that did not receive T cells (Figure 1A). In contrast to IFN-γ, expression of Th17-associated cytokines is less well characterized; however, elevated IL-17A has been observed in several other mouse models of IBD (Hue et al., 2006; Kullberg et al., 2006). We found that both IL-22 and IL-17A mRNA were highly expressed in the colons of $Rag1^{-/-}$ mice that received naive T cells (Figure 1A). A closer examination of the kinetics of the induction of the mRNA of these cytokines revealed a 10- to 1000-fold increase in the amount of mRNA by 2 weeks after the transfer, and this amount remained elevated throughout the examined disease course (Figure 1B). In addition, when purified via FACS sorting, the population of transferred $CD4^+$ T cells recovered from the inflamed colons expressed mRNA for these cytokines (data not shown). At the protein level, secreted IL-22, as well as IFN-γ, was detected in the supernatant of excised colons cultured in vitro for 3 days (Figure 1C). Thus, IL-22 is expressed during IBD in the colon, which includes a $CD4^+$ T cell source.

IL-22 Is Protective during IBD

Because IL-22R is highly expressed in the gastrointestinal tract and IL-22 expression is highly induced during inflammation of these tissues, we sought to examine the role of IL-22 in IBD. IL-22-deficient mice housed under specific-pathogen-free conditions for up to one year did not develop spontaneous colitis, indicating that IL-22 is not essential for maintenance of normal intestinal homeostasis. We transferred IL-22-deficient or control wild-type $CD45RB^{hi}$ $CD4^+$ T cells into $Il22^{-/-}$ $Rag1^{-/-}$ double-deficient mice. Over the course of several weeks, all mice that received T cells developed colitis. However, mice that received IL-22-deficient T cells had lost significantly more mass than mice that received wild-type cells by 3 weeks after the transfer, and a greater difference was observed at 4 weeks (Figure 2A) (p value week 3 = 0.0367, week 4 = 0.0013). A significant difference was also observed at 5 and 6 weeks after the transfer (week 5 p = 0.0134; week 6 p = 0.0389), and mice that received IL-22-deficient cells had greater mortality (Figure S2). At 3 weeks after the transfer, these mice had greater morphological changes in their ascending colons, the main site of disease, as assessed by histology (Figure 2B). No histopathology was noted in control $Il22^{-/-}$ $Rag1^{-/-}$ double-deficient mice that did not receive transferred cells (Figure 2B). Although in the absence of IL-22 there was greater loss of body mass and destruction of the colonic tissue, we observed little difference in cytokine expression (Figure 2C); with the exception of IL-22, or the total number of infiltrated $CD4^+$ T cells in the inflamed colon (Figure 2D), and these observations agree with our previous data showing that IL-22-deficient T cells do not have a defect in migration or cytokine expression (Zenewicz et al., 2007). Thus, IL-22 is protective to the colonic tissues during IBD.

$Rag1^{-/-}$ Mice Express Protective IL-22 in the Colon during IBD

In addition to $CD45RB^{hi}$ $CD4^+$ T cell transfers into $Il22^{-/-}$ $Rag1^{-/-}$ mice, we also performed experiments with $Rag1^{-/-}$ mice as recipients. Upon transfer of $CD45RB^{hi}$ $Il22^{+/+}$ or $Il22^{-/-}$ $CD4^+$ T cells, these mice also developed colitis, as indicated by their elevated amounts of both IFN-γ and IL-17A mRNA in the colon

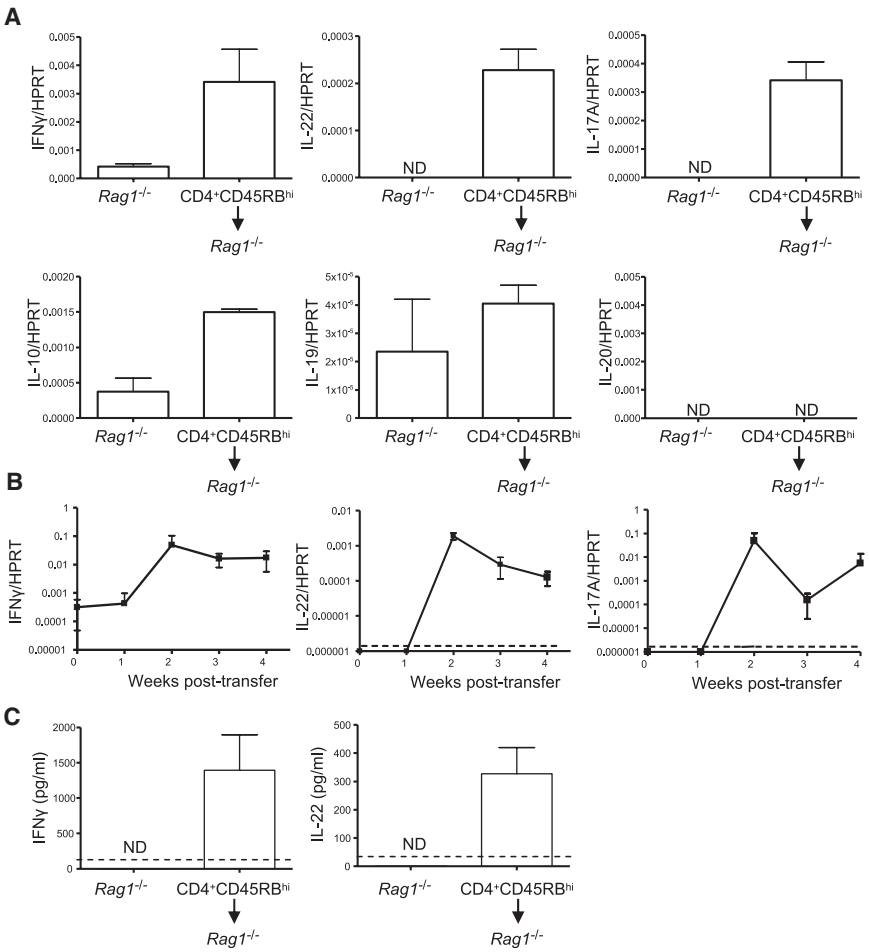

Figure 1. IL-22 Is Expressed in the Inflamed Colon

(A) C57BL/6 $CD4^+$ $CD45RB^{hi}$ $CD25^-$ $NK1.1^-$ T cells (5×10^5) were transferred intraperitoneally to *Rag1*$^{-/-}$ mice, and 5 weeks after the transfer, when clinical signs of IBD were evident, cytokine mRNA in the colon was assessed by real-time RT-PCR. As a control, cytokine in the colons of *Rag1*$^{-/-}$ mice that did not receive T cells was also assessed. Bars represent the mean ± standard deviation (SD) ratio of cytokine gene to HPRT expression as determined by the relative quantification method ($\Delta\Delta C_T$ method). The experiment was performed two times with similar results. There were five to seven mice/group. ND = not detected.

(B) C57BL/6 $CD4^+$ $CD45RB^{hi}$ $CD25^-$ $NK1.1^-$ T cells (5×10^5) were transferred intraperitoneally to *Rag1*$^{-/-}$ mice, and at different weeks after the transfer (0, 1, 2, 3, and 4 weeks), cytokine mRNA expressed in the colon was semi-quantitated by real-time RT-PCR. Mean ± SD; dashed line indicates limit of detection. There were four mice/group.

(C) From the colon samples in (A), a 1 cm section of the ascending colons of the above mice was cultured ex vivo for 3 days. Secreted IFN-γ (left) or IL-22 (right) from the supernatants was quantitated by ELISA; mean ± SD of 5–7 mice/group. ND = not detected; dashed line indicates limit of detection.

(Figure 3A). Surprisingly, *Rag1*$^{-/-}$ mice that received *Il22*$^{-/-}$ $CD4^+$ T cells also had detectable amounts of IL-22 mRNA and protein in their colons (Figures 3A and 3B). The amount of IL-22 expressed in the colon was similar to that expressed by *Il22*$^{+/+}$ $CD4^+$ T cells when they were transferred into *Il22*$^{-/-}$ *Rag1*$^{-/-}$ mice, where the only source of IL-22 is the transferred T cells. This suggests that there is a host-derived source of IL-22 during IBD in *Rag1*$^{-/-}$ mice.

IL-22 expression, whether from transferred T cells or the host, correlated with protection during IBD. Mice that completely lacked IL-22 expression (*Il22*$^{-/-}$ $CD4^+$ T cells transferred into *Il22*$^{-/-}$ *Rag1*$^{-/-}$ mice) had the most severe colitis. There was no difference in disease, inferred from body-mass reduction, of *Rag1*$^{-/-}$ mice that received $CD45RB^{hi}$ *Il22*$^{+/+}$ or *Il22*$^{-/-}$ $CD4^+$ T cells, in stark contrast to the difference observed when *Il22*$^{-/-}$ *Rag1*$^{-/-}$ mice were used as the hosts (Figure 3C). Thus, in *Rag1*$^{-/-}$ mice there is a cell subset(s) that expresses IL-22 in the inflamed colon and is able to provide protection during IBD.

Differential Expression of IL-22 by NK Subsets

IL-22 expression is not limited to Th17 $CD4^+$ T cells. Activated NK cells, NK T cells, $CD8^+$ T cells, and γδ T cells can also express IL-22 (Wolk et al., 2002; Zheng et al., 2007). Because *Rag1*$^{-/-}$ mice lack $CD4^+$ and $CD8^+$ T cells, as well as NK T cells and γδ T cells, and because IL-22 is expressed in *Rag1*$^{-/-}$ mice, we hypothesized that NK cells are responsible for the IL-22 expression in the colons of the mice. We first wanted to better characterize IL-22 expression by NK cells. There are different subsets of NK cells, which in mice can be defined by CD27 expression (Hayakawa et al., 2006). Upon activation, $CD27^{hi}$ NK cells secrete IFN-γ and granzymes, and because of high expression of chemokine receptors, they quickly traffic to sites of inflammation. On the other hand, $CD27^{lo}$ NK cells express high amounts of inhibitory receptors and upon stimulation express low levels of IFN-γ and granzymes; therefore, they are less cytotoxic than $CD27^{hi}$ NK cells. To investigate which subset expresses IL-22 or whether both do, we purified NK cells based on their degree of CD27 expression and then activated the cells with different stimuli. Previous work has shown that activation by IL-12 and IL-18 induces IL-22 in a heterogenous NK cell population (Wolk et al., 2002). However, because IL-23 is a potent stimulator of Th17 cells, has been previously shown to play a role in IL-22 expression of T cells, and is important in IBD pathogenesis, we also examined whether this cytokine is able to induce IL-22.

IL-12 and IL-18 induced IL-22 mRNA and protein in NK cells, but only in the $CD27^{hi}$ population (Figure 4). In contrast, IL-23 was able to induce IL-22 in both subsets, as well as in the absence of IL-18. Unlike IL-12 and IL-18, IL-23, with or without

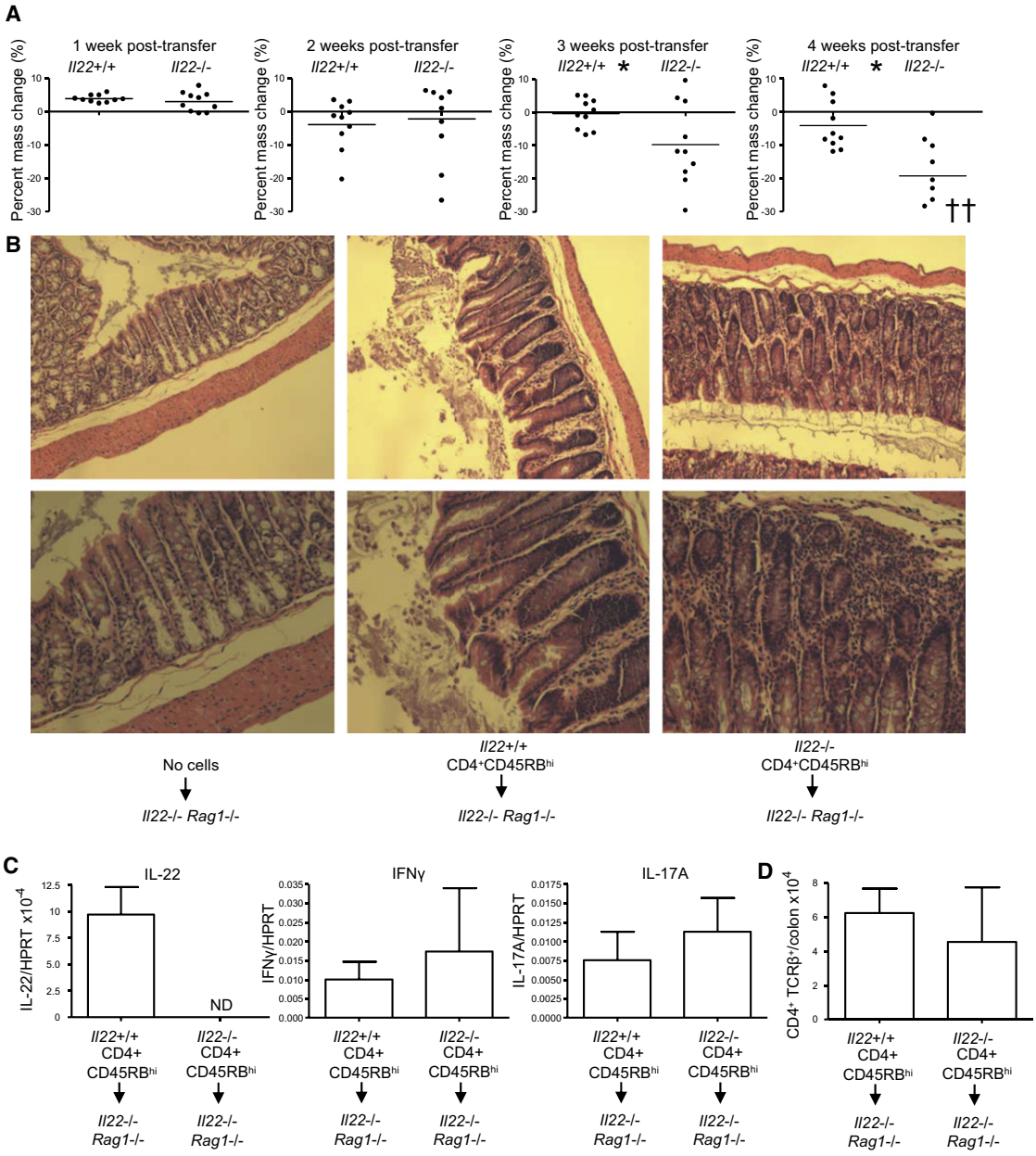

Figure 2. IL-22 Protects the Colon during CD4 T Cell-Mediated Colitis

Il22$^{+/+}$ or *Il22*$^{-/-}$ CD4$^+$ CD45RB^{hi} CD25$^-$ NK1.1- T cells (5 × 10^5) were transferred intraperitoneally into *Il22*$^{-/-}$ *Rag1*$^{-/-}$ double-deficient mice.

(A) Mice were massed weekly, and the percent change from week 0 was calculated. Each dot represents one mouse bar indicates the mean, and crosses represent dead mice or mice that reached 30% mass loss and were euthanized according to protocol. For statistics, dead mice were assigned a mass loss of −30%. An asterisk indicates a p value of < 0.05 determined by an unpaired two-tailed Student's t test.

(B) Histology of H&E-stained sections from the ascending colons of the indicated mice, 3 weeks after the transfer. Shown are representative sections from one mouse out of three to four mice/group. The bottom image is a higher magnification of the top image.

(C) At 4 weeks after the transfer, cytokine mRNA expression was assessed in the colons of the mice by real-time RT-PCR. Bars represent the mean ± SD expression of the cytokine gene to HPRT using the $\Delta\Delta C_T$ method. ND = not detected. The experiment was performed two times with similar results.

(D) Total numbers of CD4$^+$ TCRβ$^+$ cells in the inflamed colons as determined by FACS. Mean ± SD is shown.

IL-18, was not able to induce IFN-γ from either subset. Thus, IL-22 can be differentially expressed; IL-22 can be expressed by highly activated NK cells or from what are termed more inhibitory NK cells, under the proper stimulation conditions.

NK Cells Can Provide Protection during IBD

Unlike the role of T cells, the role of NK cells during IBD has not been extensively studied. Therefore, we examined the colons of mice with CD45RB^{hi}-mediated colitis to see whether

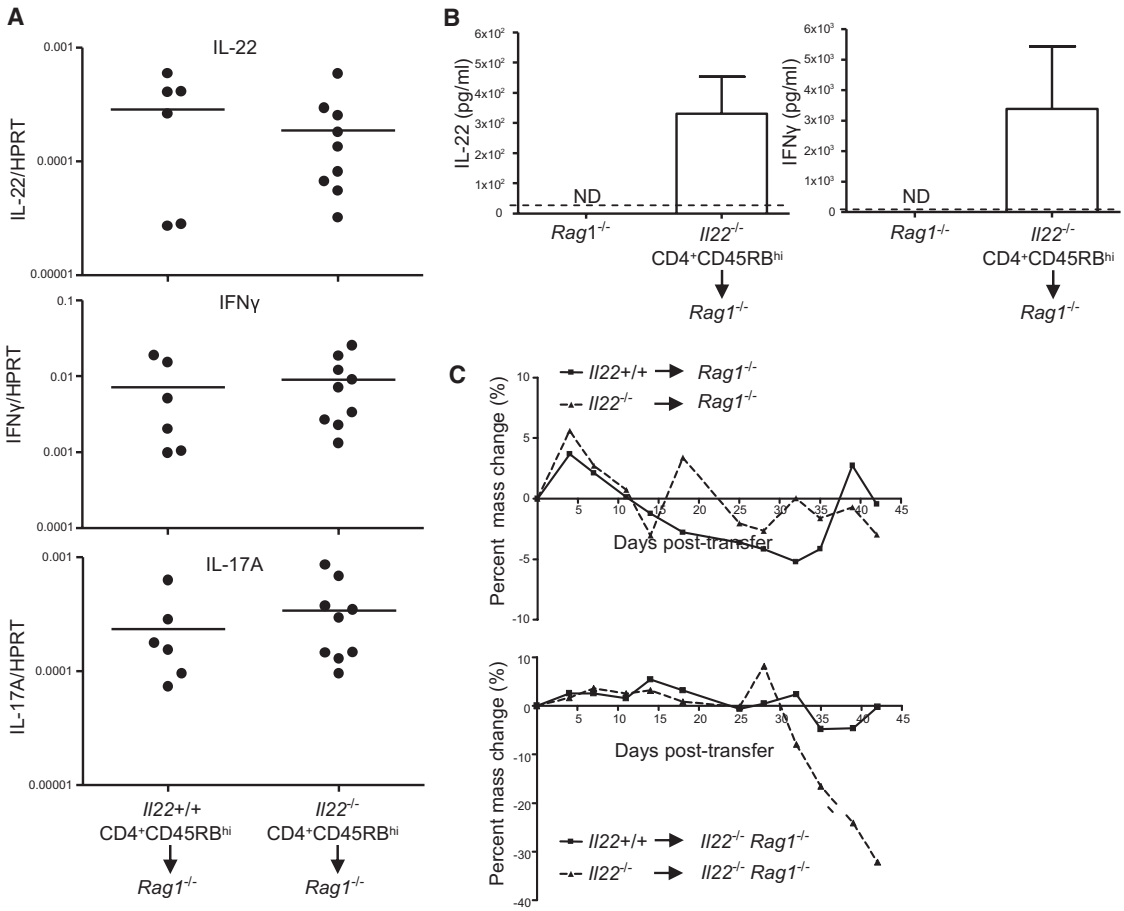

Figure 3. *Rag1*$^{-/-}$ Mice Express Protective IL-22 during IBD

Il22$^{+/+}$ or *Il22*$^{-/-}$ CD4^{+} CD45RB^{hi} CD25^{-} NK1.1^{-} T cells (5 × 10^{5}) were transferred i.p. into *Rag1*$^{-/-}$ mice.

(A) Six-week-post-transfer expression of cytokine mRNA in the ascending colons of the mice was assessed by real-time RT-PCR. The cytokine gene message was compared to that of HPRT via the $\Delta\Delta C_T$ method. Each dot represents one mouse, and the bar indicates the mean. The experiment was performed three times with similar results.

(B) Excised colon sections from either *Rag1*$^{-/-}$ mice that did not receive transferred cells or *Rag1*$^{-/-}$ mice that had received *Il22*$^{-/-}$ T cells 6 weeks previously were cultured for 3 days ex vivo, and then cytokine amounts in the supernatant were quantitated by ELISA. The bar indicates mean ± SD; the dashed line indicates the limit of detection.

(C) Mice from (A) were massed twice weekly, and the percent mass change from day 0 was calculated (top graph). At the same time, *Il22*$^{+/+}$ or *Il22*$^{-/-}$ CD4^{+} CD45RB^{hi} CD25^{-} NK1.1^{-} T cells (5 × 10^{5}) were transferred intraperitoneally into *Il22*$^{-/-}$ *Rag1*$^{-/-}$ mice, and their body mass was also monitored (bottom graph). Points represent the mean percent change in body mass.

NK cell-attracting chemokines were expressed, and if so, whether NK cells were infiltrating the colon. We used reverse transcriptase-polymerase chain reaction (RT-PCR) to examine *Rag1*$^{-/-}$ mice that had received IL-22 wild-type CD45RB^{hi} CD4^{+} T cells 2 weeks earlier and found that the chemokines CXCL9, CXCL10, and CXCL11 were induced approximately 5- to 10-fold more than they were in control mice (Figure 5A). By FACS analysis, we found a population of NK1.1+ CD4- cells in the inflamed colons of these mice (Figure 5B). In contrast, the populations of colonic NK cells in nondiseased C57BL/6 mice or *Rag1*$^{-/-}$ mice were substantially smaller (Figure 5C). Thus, NK cells appear to infiltrate the inflamed colon and in the diseased state constitute approximately 8% of the lymphocyte population.

To examine the relationship between NK cells and IL-22-mediated protection during IBD, we performed CD45RB^{hi} CD4^{+} T cell transfers of IL-22 wild-type or IL-22-deficient cells into *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice. Importantly, in addition to lacking all T cell subsets because they lack both RAG and the signaling of several cytokines (IL-2, IL-4, IL-7, IL-9, and IL-15) through the common gamma chain (γc) receptor, these mice also lack NK cells (Cao et al., 1995; DiSanto et al., 1995). As expected, transfer of *Il22*$^{+/+}$ T cells into *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice over the course of several weeks led to IBD with increased amounts of both IFN-γ and IL-22 in the colons of these mice compared to those that did not receive transferred cells (Figure 5C). The disease course and its severity in the host *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice was similar to that observed in *Rag1*$^{-/-}$ mice. When *Il22*$^{-/-}$ T cells were transferred into *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice, no IL-22 mRNA was detected in their inflamed colons, although there was an approximately 100 fold-increase in IFN-γ amounts in these mice compared to

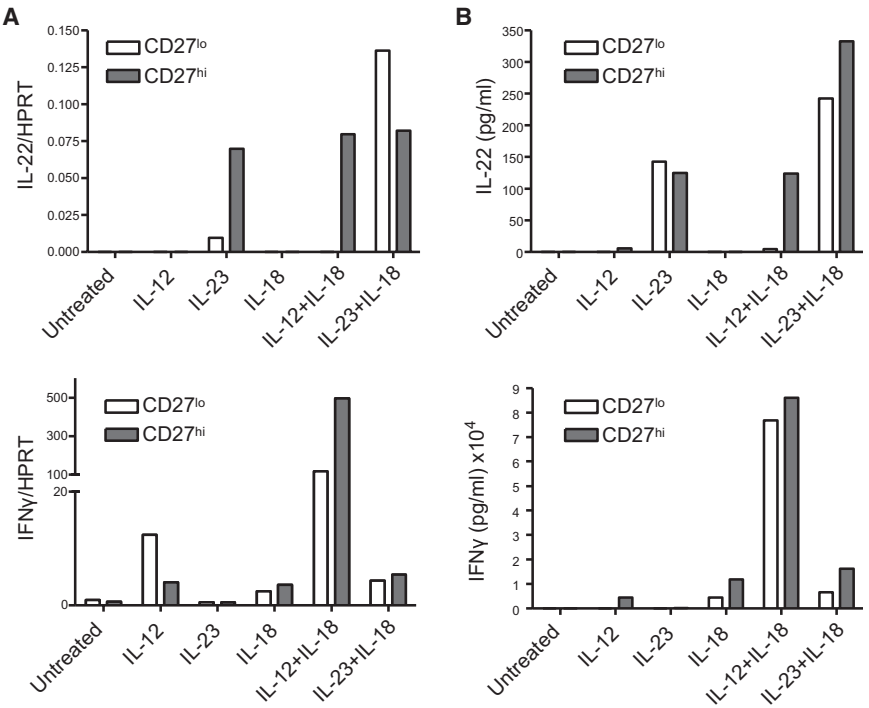

Figure 4. Differential Expression of IL-22 in Activated NK Cells by IL-23 or IL-12

$NK1.1^+$ $TCR\beta^-$ $CD27^{lo}$ ($CD27^{lo}$) or $NK1.1^+$ $TCR\beta^-$ $CD27^{hi}$ ($CD27^{hi}$) NK cells were sorted from the spleen and lymph nodes of C57BL/6 mice. Cells were stimulated in vitro with IL-15 and the indicated cytokines. Eighteen hours after stimulation, IL-22 and IFN-γ mRNA and protein expression were analyzed by (A) real-time RT-PCR or (B) ELISA of the supernatants, respectively. The experiment was performed three times with similar results.

Rag2$^{-/-}$ $\gamma c^{-/-}$ mice that did not receive cells (Figure 5D). IFN-γ amounts were similar to that observed to transfers into *Rag1*$^{-/-}$ mice (Figure 2C). In addition, IL-22-secreted protein from ex vivo-cultured colon tissue sections from these mice was not detected by ELISA. We obtained similar results by performing a similar experiment with the BALB/c strain of mice (data not shown). These data strongly suggest that NK cells are a source of IL-22 in the inflamed colon.

IL-22 Protects in a Model of Colitis Mediated by the Innate Immune System

Colitis in humans is a complex interplay involving both the innate and adaptive immune systems. Because IL-22 expression is not limited to differentiated effector $CD4^+$ T cells and can also be expressed by NK cells, we wished to determine whether IL-22 could also provide protection during colitis driven by the innate immune system, as well as in the adaptive T cell model we presented above. One commonly employed model of innate colitis is dextran sodium sulfate (DSS)-induced colitis (Cooper et al., 1993; Mahler et al., 1998). In this model, DSS is given in the drinking water and is thought to cause disruption of the epithelial integrity of the colon, and this disruption leads to inflammation and colitis within one week. Like $CD45RB^{hi}$-mediated transfer IBD, this is primarily a disease of the colon. IL-22 mRNA is upregulated in the colons of mice after DSS treatment (Brand et al., 2006; te Velde et al., 2007). We confirmed that IL-22 protein secretion is induced by DSS treatment when we detected IL-22 in excised colon cultures of mice that received 3% DSS in their drinking water for 3 days (Figure 6A). Because IL-22 is expressed in the colon during DSS-induced colitis, we compared the disease courses of IL-22-deficient mice and wild-type control mice. IL-22-deficient mice had lost significantly more mass by day 8 after treatment than IL-22 wild-type mice ($p = 0.0030$) (Figure 6B). In addition, IL-22-deficient mice had a higher rate of mortality than the wild-type mice (Figure 6C). Although T cells have previously been shown have little role in DSS-mediated colitis (Axelsson et al., 1996; Dieleman et al., 1994), we nevertheless also compared colitis in *Rag1*$^{-/-}$ mice and *Il22*$^{-/-}$ *Rag1*$^{-/-}$ mice to rule out IL-22 expressed by T cells. As observed for mice with T cells, RAG1-deficient mice also deficient in IL-22 had more severe disease than RAG1-deficient mice (Figure S3). Thus, in addition to being protective during adaptive-immune-mediated colitis, innate-immune-cell-driven IL-22 is also protective during innate-immunity-mediated IBD.

To further provide evidence that NK cells are the primary innate source of IL-22 in the colon during colitis, we used our innate model of DSS-mediated colitis in the absence of T cells. Three days after the initiation of DSS treatment, *Rag1*$^{-/-}$ mice were given either a depleting NK1.1 antibody or a control antibody. Twenty-four hours later, mice were euthanized, and IL-6 and IL-22 secretion from the colon was examined by ex vivo colon culture. Colons from both sets of mice expressed comparable levels of IL-6, indicating similar amounts of inflammation (Figure 7A). However, the colons from the control antibody group secreted significantly more IL-22 than those from mice that received the NK1.1 depleting antibody ($p = 0.0170$) (Figure 7A). Similar results were observed when NK cells were depleted in *Rag1*$^{-/-}$ mice via a different depleting reagent, anti-asialo GM1 antibody (Figure 7B). These data implicate NK cells as an innate source of protective IL-22 in the inflamed colon.

DISCUSSION

IL-22 is a dual-natured cytokine; depending on the context of inflammation, it can have either inflammatory or protective properties. IL-22 has been shown to be an important mediator in dermal inflammation (Ma et al., 2008; Zheng et al., 2007), and alternatively, it can provide protection to hepatocytes during liver inflammation (Radaeva et al., 2004; Zenewicz et al., 2007) and, as we now show here, IBD. Th17 cells have also been termed inflammatory T cells because these cells are hypothesized to participate in, if not instigate, inflammatory responses. IL-17A, the original defining cytokine of this cell subset, is important for neutrophil recruitment and induction of antimicrobial peptides and,

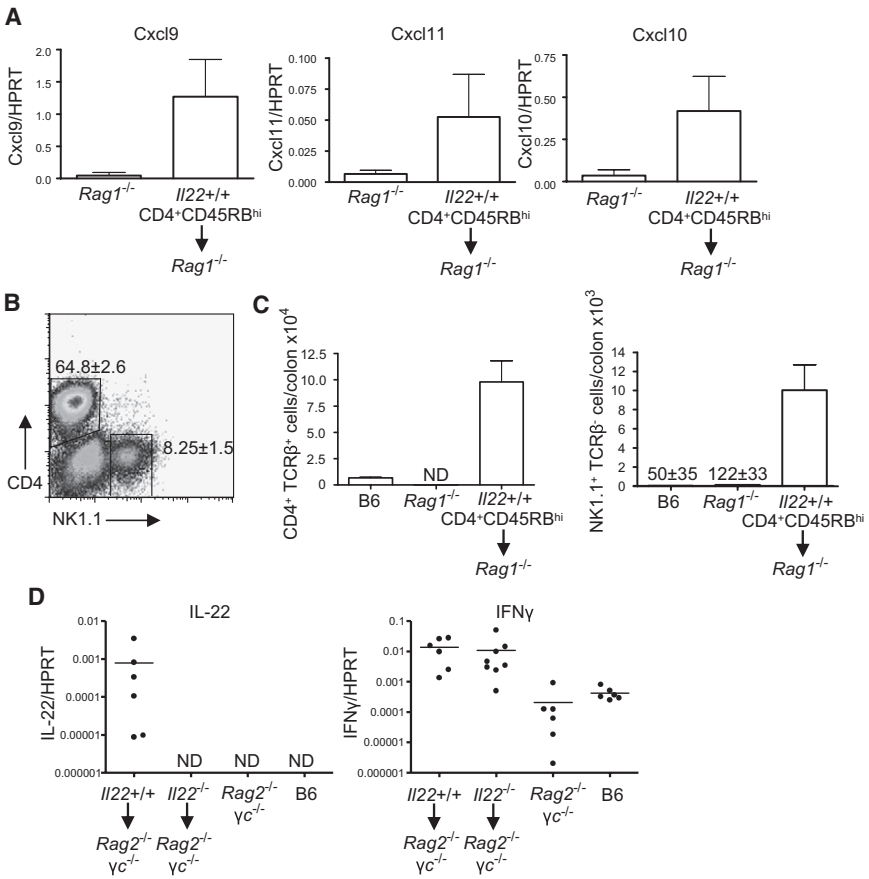

Figure 5. NK Cells Play a Role in IL-22-Mediated Protection during IBD

(A) NK-attracting chemokines are upregulated in the colon during IBD. Two weeks after the transfer of *Il22*$^{+/+}$ CD4$^+$ CD45RB^{hi} CD25$^-$ NK1.1$^-$ T cells (5×10^5) into *Rag1*$^{-/-}$ mice or control *Rag1*$^{-/-}$ mice that did not receive cells, mRNA amounts of the chemokines Cxcl9, Cxcl10, and Cxcl11 were examined in the ascending colon by real-time RT-PCR. Mean ± SD is shown.

(B) *Il22*$^{-/-}$ CD4$^+$ CD45RB^{hi} CD25$^-$ NK1.1$^-$ T cells (5×10^5) cells were transferred into *Rag1*$^{-/-}$ mice, and 45 days after the transfer, when mice exhibited clinical disease, colons were harvested and stained by FACS for CD4 and NK1.1. Numbers indicate the mean ± SD percentage of CD4$^+$ NK1.1$^-$ (top gate) or CD4$^-$ NK1.1$^+$ (right gate) cells out of total lymphocytes isolated from the colon. There were five mice/group.

(C) Total numbers of CD4$^+$ T cells (CD4$^+$ TCRβ$^+$) and NK cells (NK1.1$^+$ TCRβ$^-$) in the colons of mice presented in (B) as determined by FACS. Mean ± SD is shown. B6 = C57BL/6 mouse, ND = not detected.

(D) *Il22*$^{+/+}$ or *Il22*$^{-/-}$ CD4$^+$ CD45RB^{hi} CD25$^-$ NK1.1$^-$ T cells were transferred into *Rag2*$^{-/-}$ γc$^{-/-}$ mice (5×10^5 cells; intraperitoneally). Forty-two days after the transfer, cytokine induction in the ascending colon was semi-quantitated by real-time RT-PCR, as well as in untransferred *Rag2*$^{-/-}$ γc$^{-/-}$ mice or C57BL/6 (B6) mice. Each dot represents one mouse; the bar indicates the mean; ND = not detected.

like IL-22, is upregulated during chronic inflammatory diseases such as IBD, rheumatoid arthritis, and psoriasis (Laan et al., 1999; Liang et al., 2006) (Fujino et al., 2003; Kotake et al., 1999). However, it is increasingly apparent that these cells do not only promote inflammation but also have protective effects. A recent study has put forward the idea, based on data from many groups, that the Th17 subset is not a homogenous population (McGeachy et al., 2007). In that study, based on the cytokine milieu during differentiation, IL-17-expressing cells can be inflammatory or protective depending of the coexpression of IL-10. Additionally, multiple subpopulations defined by the expression of many of the Th17-related cytokines (IL-22, IL-21, IL-17F, IL-6, TNFα) have been observed, and these distinct expression patterns may represent functionally different cell subsets.

CD4$^+$ T cells, as well as NK T cells and γδ T cells, all contribute to IBD pathogenesis (Saubermann et al., 2000; Simpson et al., 1997). The role of another lymphocyte subset, NK cells, has not been as extensively studied in this context. NK cells provide an innate immune defense mechanism that can be rapidly activated to secrete IFN-γ and granzymes to quickly kill pathogen-infected cells or cancerous cells. Because IFN-γ is essential for IBD development, NK cells could be hypothesized to contribute to this pathogenesis. However, although we have not excluded the possibility that they contribute to disease under other circumstances, in the present study we have shown that NK cells serve a protective role during IBD. We observed no difference in colitis in *Rag1*$^{-/-}$ mice that received either IL-22 wild-type or IL-22-deficient T cells. Moreover, *Rag1*$^{-/-}$ mice that received IL-22-deficient T cells surprisingly had high amounts of IL-22 in their inflamed colons, indicating an innate immune source of IL-22. Importantly, this innate IL-22 was protective. When *Il22*$^{-/-}$ *Rag1*$^{-/-}$ double-deficient mice were used as hosts for IBD experiments, mice that received IL-22-deficient T cells had substantially greater disease than mice that received IL-22 wild-type T cells. Thus, the innate IL-22 in *Rag1*$^{-/-}$ mice is able to confer protection. Because NK cell expression of IL-22 had been previously reported, we examined IBD by using *Rag2*$^{-/-}$ γc$^{-/-}$ mice as hosts; such mice differ from *Rag1*$^{-/-}$ mice only in their absence of NK cells. Upon transfer of IL-22 deficient T cells, these mice did not express IL-22 in the inflamed colon, whereas *Rag1*$^{-/-}$ mice did; this provided compelling evidence that NK cells are a source of IL-22. Further experiments in which NK cells were depleted during innate-immune-mediated colitis, in the complete absence of T cells, showed that this depletion reduced amounts of IL-22 in the colon. Thus, NK cells are an important source of IL-22, which protects the colon during IBD.

A previous group showed that NK cells protect during a T cell-mediated mouse model of IBD by performing NK-depletion experiments during colitis; however, their data suggested that this was due to direct effects of the NK cells on perforin production by the activated T cells that mediated disease (Fort et al., 1998). We now provide data showing that NK cells are an innate

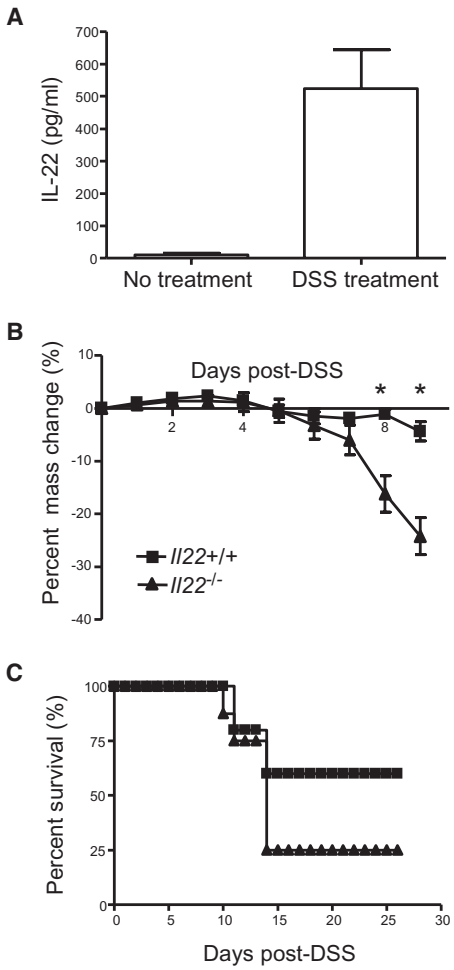

Figure 6. IL-22 Also Provides Protection during Colitis Mediated by the Innate Immune System

(A) IL-22 is secreted from the colon during DSS-mediated colitis. C57BL/6 mice either were given 3% DSS ad libitum in their drinking water or remained untreated, and 3 days later the mice were euthanized and their colons were excised. Colons were cultured for 3 days as described in the Experimental Procedures, and IL-22 was detected in the supernatant by ELISA. Bars represent mean ± SD of seven mice/group.

(B) *Il22*$^{+/+}$ or *Il22*$^{-/-}$ mice were given 3% DSS ad libitum in their drinking water for 7 days. Mice were massed daily, and the percent mass change from day 0 was calculated. Mean ± SD is shown. An asterisk indicates a p value < 0.05. There were eight to ten mice/group.

(C) Survival of *Il22*$^{+/+}$ or *Il22*$^{-/-}$ mice in (B) after they received 3% DSS in their drinking water.

source of IL-22 in the inflamed colon and that these cells contribute to the IL-22-mediated protection of the host tissues during IBD. This effect is independent of effects on T cells, as shown in the innate DSS colitis model. Furthermore, our study is a report of a functional role attributed to IL-22 expression by NK cells, instead of Th17 cells.

NK cell expression of IL-22 is differentially regulated between subsets. Mature NK cells can be defined by surface expression of CD27, a member of the TNFR super family. CD27^{hi} cells have a lower threshold for activation and express high amounts of cytokines and granzymes, making them highly cytotoxic (Hayakawa et al., 2006). On the other hand, CD27^{lo} cells have higher expression of inhibitory receptors, and therefore their activation is more strongly regulated. We show that IL-12 and IL-18 stimulation can only induce IL-22 expression in CD27^{hi} NK cells, but not CD27^{lo} cells. On the other hand, IL-23 is able to induce IL-22 expression in both subsets, independently of IL-18, and is unable to induce IFN-γ. These distinct cytokine expression patterns may allow for optimal immune responses while limiting tissue damage during inflammation.

The cytokines of the IL-10-related cytokine family, with the exception of IL-10 itself, affect tissue responses and not the immune system. IL-22 appears to be the most relevant cytokine for the tissues comprising the gastrointestinal tract. IL-22R expression is greatest in the colon and small intestine when compared to other tissues, such as the skin, where the predominant IL-10 family member receptors are the IL-20Rα and IL-20Rβ chains. We also found that during IBD, IL-19 mRNA was not induced in the colon, and we were unable to detect IL-20 transcripts. In addition, we only observed Stat3 activation in colon epithelial cells upon IL-22 stimulation, but not with IL-10, IL-19, or IL-20. This is in agreement with previous studies showing that IL-10 effects are limited to immune cells and that IL-19 and IL-20 function primarily in the skin (Blumberg et al., 2001; Wolk et al., 2004; Wolk et al., 2005).

Stat3 is the main signaling pathway activated in responsive cells upon stimulation with IL-10-related cytokines. IL-22 activates Stat3 in a wide variety of tissues, including keratinocytes, hepatocytes, and as shown by us and other laboratories, colon epithelial cells (Dumoutier et al., 2000; Wolk et al., 2004; Zenewicz et al., 2007). IL-22 has several different effects on the gastrointestinal epithelium. IL-22 can induce IL-6 and IL-8 secretion from human colonocytes, as well as activate NF-κB and inducible nitric oxide synthase (Andoh et al., 2005; Ziesche et al., 2007). However, these proinflammatory effects on the gastrointestinal tract appear to be of limited effect in vivo. Overexpression of IL-22 in the colon leads to induction of mucus-associated molecules, such as MUC1, MUC3, and MUC13, leading to enhanced mucus production due to the restitution of goblet cells (Sugimoto et al., 2008). This might provide protection to the colonic epithelium during inflammation by reducing the translocation of commensal bacteria across the barrier. In addition, IL-22 induces defensin expression in colonocytes, and these antimicrobial peptides might also provide protection (Brand et al., 2006).

One of the pitfalls with current therapies for chronic inflammation, such as TNFα inhibitors, is that they disrupt and weaken the normal immune response and thus lead to increased disease susceptibility. The benefit of an IL-22-mediated therapy may be that the cytokine only signals to tissues and has no direct effects on the immune response. This specific targeting should allow modulation of tissue responses to alleviate tissue destruction during inflammation while having limited effects on the immune response itself. IL-22 colon-targeted gene expression performed by Sugimoto et al. has already shown that IL-22 holds promise for IBD therapy (Sugimoto et al., 2008). Because IL-22 appears to be both proinflammatory and protective, caution must be used in developing treatments based on this cytokine or blocking its function with antibodies. In addition, IL-22 has proliferative effects on cells (Radaeva et al., 2004), warranting

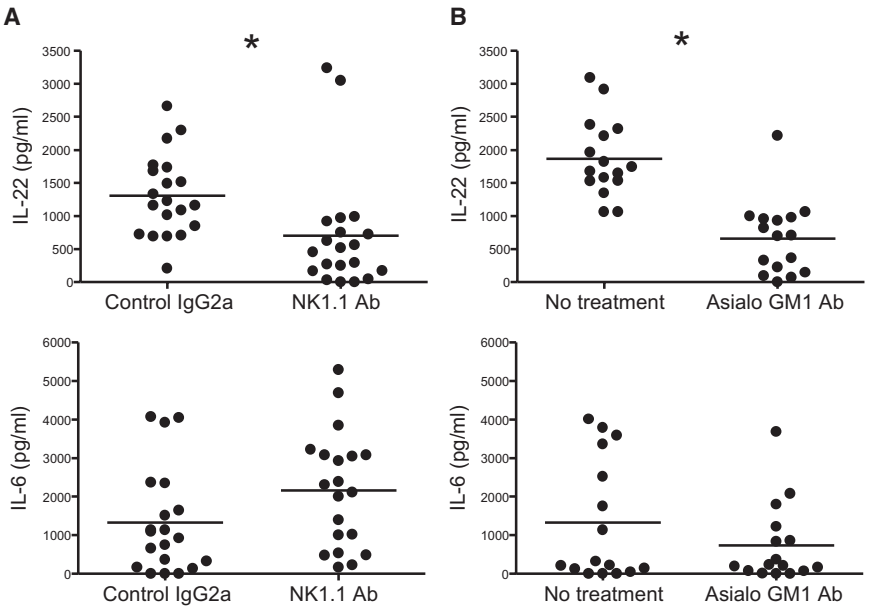

Figure 7. NK Cell Depletion Decreases IL-22 Expression in the Inflamed Colon

Rag1$^{-/-}$ mice were given 3% DSS ad libitum in their drinking water for 4 days. On the third day, mice were either untreated or intraperitoneally injected with (A) 250 μg of NK1.1-depleting antibody or control IgG2a or (B) 500 μg asialo GM1 antibody. Twenty-four hours later, mice were euthanized, and colons were excised. Colons were cultured for three days as described in the Experimental Procedures, and IL-22 or IL-6 was detected in the supernatant by ELISA. Each dot represents one colon segment, bars represent the mean of four to five mice/group; there are four segments per mouse. An asterisk indicates $p < 0.05$. The experiment was performed twice with similar results.

further studies to see whether continuous IL-22 stimulation allows for tumor progression. Gaining a better understanding of both the short-term and long-term effects of IL-22 on different tissues is needed to enable the development of IL-22-related therapeutics for chronic inflammatory diseases such as IBD.

EXPERIMENTAL PROCEDURES

Mice

IL-22-deficient mice were as previously described (Zenewicz et al., 2007). Mice used were at generation 10 of a backcross to the C57BL/6 strain. Mice within experiments were sex and age matched. *Rag1*$^{-/-}$ mice (The Jackson aboratory; Bar Harbor, ME) and *Rag2*$^{-/-}$ γc-/ mice (Taconic Farms; Hudson, NY) were bred in-house. The progeny of a cross of *Il22*$^{-/-}$ mice to *Rag1*$^{-/-}$ mice were intercrossed to generate *Il22*$^{-/-}$ *Rag1*$^{-/-}$ double-knockout mice that, upon genetic screening, were then breed as *Il22*$^{-/-}$ *Rag1*$^{-/-}$ × *Il22*$^{-/-}$ *Rag1*$^{-/-}$. All mice were cared for in accordance with protocols established by the institutional animal care and use committee at the Yale University animal facility.

CD45RB^{hi} Transfers

Splenocytes and inguinal and axillary lymph nodes from *Il22*$^{+/+}$ or *Il22*$^{-/-}$ mice were CD4 MACS (Miltenyi Biotech; Auburn, CA) purified, and naive CD4^{+} T cells were further purified by FACS sorting so that a population of cells that were CD4^{+} CD45RB^{hi} CD25^{-} NK1.1^{-} was collected. A total of 5 × 10^{5} cells were transferred intraperitoneally into the indicated recipient mice (*Rag1*$^{-/-}$, *Il22*$^{-/-}$*Rag1*$^{-/-}$, or *Rag2*$^{-/-}$γc$^{-/-}$). Mice were massed twice a week and euthanized when they had lost 30% of their initial mass.

DSS-Induced Colitis

Mice were given 3% dextran sodium sulfate (DSS) (molecular weight: 36,000-50,000) (MP Biomedicals, Inc.; Solon, OH) ad libitum in their drinking water for 7 days. Body mass was measured every 24 hr, and mice were euthanized when they had lost 30% of their initial mass. Where indicated, mice were given 250 μg of anti-NK1.1 (PK136), 250 μg of control IgG2a, or 500 μg of anti-asialo GM1 (SH-34) (all from BioXCell, West Lebanon, NH) intraperitoneally.

Lymphocyte Preparation and Flow Cytometry

Colons were removed from euthanized mice, placed into Bruff's media, and passed through a wire-mesh screen. In brief, colon homogenate was incubated with 100 U/ml collagenase (Sigma; St. Louis, MO) and 20 μg/ml DNase I (Sigma) for 40 min at 37°C. For removal of colonic debris, homogenates were centrifuged at 300 rpm for 3 min, and then supernatants were centrifuged at 1500 rpm for 10 min. The cells were resuspended in 1 ml complete media and 4 ml of 30% OptiPrep (Axis-Shield; Oslo, Norway) in a sodium phosphate buffer, and 1 ml of media was carefully layered on top. Cells were centrifuged at 2700 rpm for 20 min. The top layer and interface were harvested as the lymphocyte population. Cells were stained with fluorescently conjugated antibodies in 1% BSA in PBS and fixed in 2% PFA. Cells were analyzed with a FACSCalibur (BD Biosciences; San Jose, CA), and data were analyzed by FlowJo v. 6.1 (TreeStar, Inc., Ashland, OR).

Real-Time RT-PCR

RNA from cells or organs was isolated with Trizol reagent (Invitrogen; Carlsbad, CA). RNA was subjected to reverse transcriptase with Superscript II (Invitrogen) and oligo dT primer. cDNA was semi-quantitated with commercially available primer and probe sets (Applied Biosystems; Foster City, CA) and the $\Delta\Delta C_T$ method. Hypoxanthineguanine phosphoribosyltransferase (HPRT) was included as an internal control.

Ex Vivo Colon Culture and ELISAs

Sections of 1 cm of the ascending colon were excised, removed of feces, washed three times with sterile PBS, and then longitudinally halved. The colon sections were then placed into culture in complete Bruff's media (10% FBS, L-glutamine, penicillin, streptomycin, and tetracycline) and cultured at 37°C with 5% CO_2. Supernatants were harvested after 3 days, and the concentration of cytokine was determined by ELISA. IFN-γ and IL-6 ELISA (BD PharMingen; San Diego, CA) or IL-22 ELISA (Antigenix America; Huntington Station, NY) were performed according to the manufacturer's protocols.

NK Cell Stimulation

Spleens and lymph nodes were isolated from C57BL/6 mice and prepared as described previously (Zenewicz et al., 2007), and NK1.1^{+} TCRβ^{-} CD27^{lo} or NK1.1^{+} TCRβ^{-} CD27^{hi} cells were sorted on a FACS Aria. The cells were stimulated in vitro with the indicated combination of the following cytokines: 50 ng/ml IL-15 (Peprotech; Rocky Hill, NJ), 50 ng/ml IL-12 (BD PharMingen), 50 ng/ml IL-23 (eBioscience; San Diego, CA), and 20 ng/ml IL-18 (MPL; Naka-ku Nagoya, Japan) for 18 hr or remained untreated. mRNA was harvested, and cytokine expression was semiquantitated by real-time RT-PCR as described above.

Detection of Activated Stat3

Caco-2 cells (ATCC HTB-37) were grown in DMEM media supplemented with 20% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were stimulated for 20 min with the indicated concentration of recombinant human IL-10, IL-19, IL-20, or IL-22 (all from Peprotech). Cell lysates were

separated under reducing conditions on 4% to 12% gradient gel with the NuPAGE electrophoresis system (Invitrogen). Gels were transferred to Immobolin P membrane (Millipore; Billerica, MA), blocked with 5% dry milk in PBS with 0.01% Tween. Blots were then incubated overnight at 4°C with one of the following primary antibodies: anti-phospho-Stat3 Tyr705 (polyclonal) or anti-Stat3 antibody (polyclonal) (both from Cell Signaling Technology; Danvers, MA). Blots were washed, incubated with appropriate secondary antibodies conjugated to horse radish peroxidase, and then developed with chemiluminescent substrate (Pierce, Rockford, IL) and film.

Histology

Organs were removed and fixed in 4% paraformaldehyde overnight at 4°C, then embedded in paraffin, sectioned, and stained with H&E. Slides were prepared at the Yale University Program for Critical Technologies in Molecular Medicine, Department of Pathology.

Statistics

Prism 4.03 software (Graphpad Software; San Diego, CA) was used for statistical analyses. p values of less than 0.05 were considered statistically significant.

SUPPLEMENTAL DATA

Supplemental Data include three figures and are available with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00507-4.

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