Lyn activity protects mice from DSS colitis and regulates the production of IL-22 from innate lymphoid cells

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Intestinal homeostasis requires a complex balance of interactions between diverse resident microbial communities, the intestinal epithelium, and the underlying immune system. We show that the Lyn tyrosine kinase, a critical regulator of immune cell function and pattern-recognition receptor (PRR) responses, has a key role in controlling gastrointestinal inflammation. *Lyn*$^{-/-}$ mice were highly susceptible to dextran sulfate sodium (DSS)-induced colitis, whereas *Lyn* gain-of-function (*Lyn*^{up}) mice exhibited attenuated colitis during acute and chronic models of disease. *Lyn*^{up} mice were hypersensitive to lipopolysaccharide (LPS), driving enhanced production of cytokines and factors associated with intestinal barrier function, including interleukin (IL)-22. Oral administration of LPS was sufficient to protect antibiotic-treated *Lyn*^{up} but not wild-type mice from DSS, highlighting how Lyn-dependent changes in the nature/magnitude of PRR responses can impact intestinal health. Furthermore, protection from DSS-induced colitis and increased IL-22 production in response to LPS did not depend on the adaptive immune system, with increased innate lymphoid cell-derived IL-22 correlating with Lyn activity in dendritic cells. These data reveal a key role for Lyn in the regulation of innate immune responses and control of intestinal inflammation.

INTRODUCTION

The mammalian intestinal tract has evolved to house vast microbial communities, the microbiota, that exist in a largely symbiotic relationship with their host.[1] Recognition of these microbes by intestinal epithelial and immune cells via pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), has a critical role in intestinal homeostasis, inflammation, and immunity. In the steady state, PRR stimulation supports intestinal epithelial barrier function and maintains homeostasis with the intestinal immune system.[1,2] Indeed, polymorphisms in genes encoding PRRs, such as nucleotide-binding oligomerization domain-containing 2 (NOD2) or TLR4, and downstream signaling molecules, such as MyD88 adaptor-like (MAL), are associated with inflammatory bowel disease (IBD).[3] Furthermore, loss of PRR responses in mice can lead to spontaneous colitis, as in *Tlr5*$^{-/-}$ mice[4], or to increased susceptibility to colitis (*Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, or *Nod2*^{2939iC} mice[5,6]). However, the protective effects of PRRs can be lost if proper regulatory mechanisms are not in place, and as such, uncontrolled PRR-induced inflammatory responses contribute extensively to pathogenesis in IBD and murine models of colitis.[7]

IBD, including Crohn's disease and ulcerative colitis, is associated with production of pro-inflammatory mediators by innate and T cell subsets. Recently, the role of the Type-17 cytokines interleukin (IL)-17, IL-22 and often interferon-γ (IFNγ), and the cells capable of producing them, including T cells and innate lymphoid cells (ILCs), have become a focus of scrutiny in IBD and mouse models of colitis. In IBD patients, levels of IL-22, IL-17 and IFNγ are elevated,[8–10] as are populations of IFNγ- and IL-17-producing ILCs.[11,12] In contrast, IL-22, in particular IL-22 produced by ILCs, is a

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Received 27 July 2012; revised 17 July 2013; accepted 29 July 2013; published online 18 September 2013. doi:10.1038/mi.2013.60

key effector molecule protecting the gut from inflammation in murine models of colitis and enteric infection.[13,14]

IL-22 targets cells in barrier organs, such as the intestinal epithelium, where it induces host defense, pro-survival, and proliferation factors, including β-defensins, RegIII proteins, and mucins.[13] Accordingly, in mouse models of colonic injury,[15] as well as in trinitrobenzene sulfonic acid,[16] T cell transfer,[17] and dextran sulfate sodium (DSS)-induced colitis,[17,18] IL-22 antagonizes inflammation and promotes wound healing. IL-22 is produced by T cell subsets, including Th17 cells. However, multiple subsets of RORγt$^+$ (retinoic acid–related orphan receptor γt-positive) ILCs, including lymphoid tissue-inducer-like cells and NCR$^+$ (natural cytotoxicity receptor-positive) ILC3s, are also major sources of intestinal IL-22.[19,20] Importantly, production of IL-22 is largely influenced by innate immune cell responses to TLR signals. For example, dendritic cells (DCs) are required for IL-22 production by ILCs in response to lipopolysaccharide (LPS) and flagellin.[21,22] These studies support the many reports indicating that appropriate activation of PRRs are required to attenuate inflammation induced by intestinal damage and to enhance barrier function and repair.

Lyn is a Src-family tyrosine kinase (SFK) expressed in all leukocytes except T cells and is activated by ligand binding to adhesion molecules, cytokine receptors, immunoreceptors, and TLRs.[23] Depending on the cell microenvironment, developmental stage, and type of stimulus, Lyn can restrict or amplify signal transduction. The importance of Lyn in regulating TLR signal transduction remains controversial but has been explored using *Lyn*-deficient ($Lyn^{-/-}$) and gain-of-function (Lyn^{up}) mice.[24–27] Lyn^{up} mice contain a tyrosine to phenylalanine mutation in the endogenous *Lyn* gene at the C-terminal negative-regulatory tyrosine phosphorylation site, leading to increased Lyn activity.[28] Our laboratory recently demonstrated that Lyn^{up} DCs exhibit enhanced maturation and distinct cytokine production profiles in response to TLR stimuli, driving increased DC-dependent natural killer (NK) cell activation and IFNγ production, resulting in severely increased susceptibility to LPS.[25]

Perturbations in immune cell function and responses to PRR signals are critical factors in the development of IBD and mouse models of colitis,[3] yet despite the regulation of many immune cell responses by Lyn, there is no known link between Lyn and susceptibility to gastrointestinal inflammation. Herein, we provide evidence that Lyn activity is protective against DSS colitis. Conversely, Lyn deficiency significantly increases susceptibility to colitis. Protection from DSS-induced inflammation in Lyn^{up} mice is associated with elevated levels of IL-22 and IL-22-responsive factors in the colon. We show that LPS hypersensitivity drives enhanced production of IL-22 in Lyn^{up} mice, which requires both DCs and innate CD90$^+$ cells (ILCs) and that increased Lyn activity in DCs is sufficient to enhance IL-22 production by ILCs *in vitro*. Furthermore, augmented responses to LPS by Lyn^{up} mice protect these mice from DSS-induced wasting and morbidity following antibiotic treatment. These results reveal a novel role for Lyn in modulating IL-22 production and ILC function and underscore the importance of this enzyme in the control of intestinal inflammation. Our results also highlight how changes in signaling pathways regulating cellular responses to PRRs can profoundly alter the outcome of intestinal inflammation.

RESULTS

Lyn is protective against experimental colitis

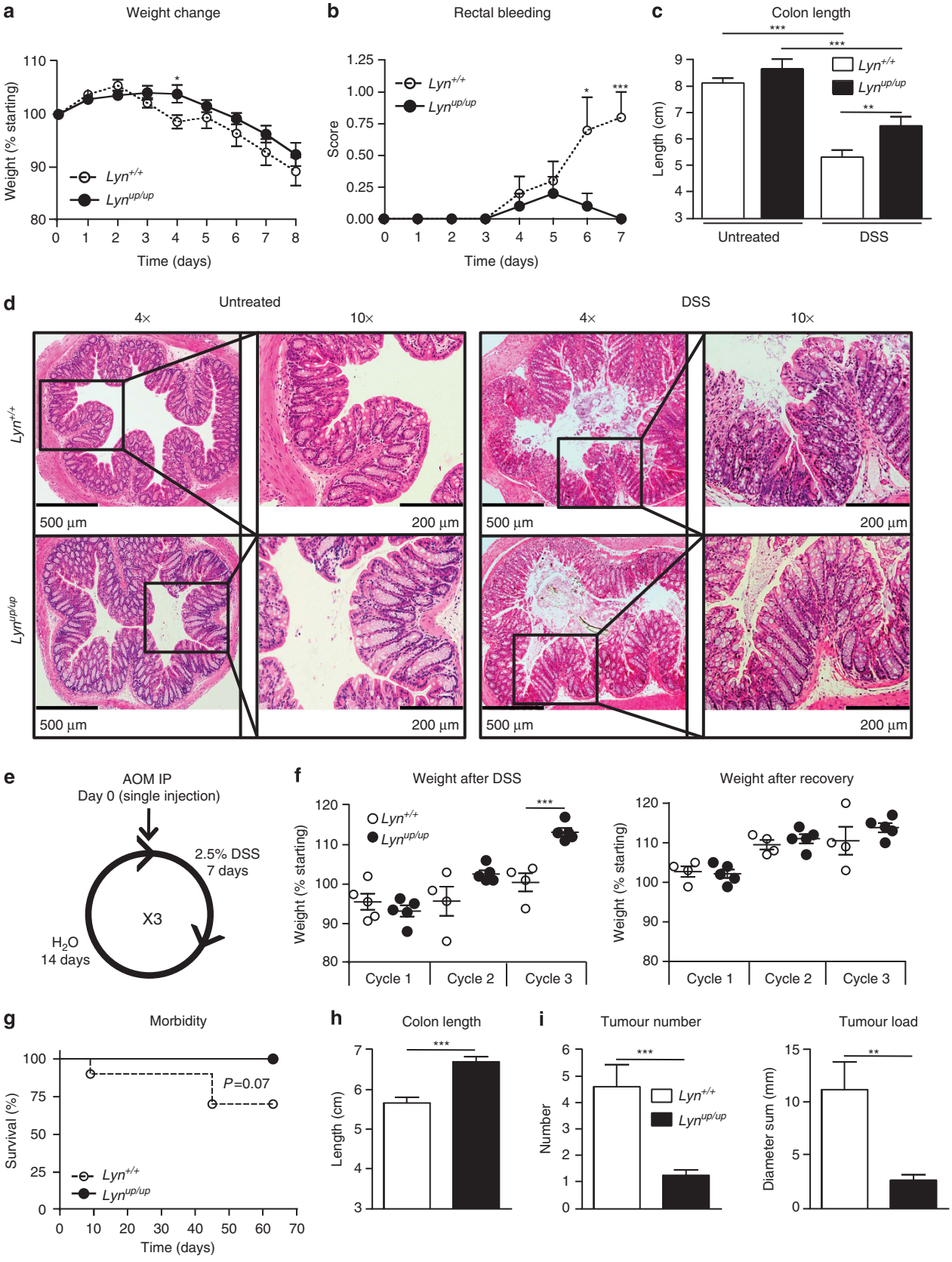
We previously identified Lyn as an important regulator of systemic and DC-intrinsic PRR-induced responses,[25] which are known to dictate the outcome of intestinal inflammation.[2] We therefore questioned how changes in Lyn activity, and associated enhanced innate responses to pathogen-associated molecular patterns, might alter intestinal homeostasis and susceptibility to inflammation. We challenged wild-type (WT), $Lyn^{-/-}$ and Lyn^{up} mice with DSS in their drinking water. $Lyn^{-/-}$ mice were highly susceptible to DSS compared with WT as assessed by body weight, colon length, rectal bleeding, and histopathology (**Supplementary Figures S1A–D** online). By contrast, Lyn^{up} mice lost slightly less weight than WT counterparts, had significantly longer colons, and significantly reduced rectal bleeding, indicating protection from DSS (**Figures 1a–c**). Furthermore, Lyn^{up} colons showed fewer areas of crypt loss and less epithelial sloughing and ulceration compared with WT mice, while changes in crypt length or epithelial cell proliferation were not observed (**Figure 1d**, data not shown). Together, these data demonstrate a protective role of Lyn in acute DSS-induced colitis.

Our investigation of the mechanism(s) underlying susceptibility to gastrointestinal inflammation in $Lyn^{-/-}$ mice revealed a multifactorial role for Lyn involving multiple cell types, with susceptibility to DSS colitis dependent on the adaptive immune system and the development of a distinct microbiota. These findings are the subject of a separate study.

Figure 1 Increased Lyn activity attenuates dextran sulfate sodium (DSS)-induced colitis. (**a–d**) $Lyn^{+/+}$ and $Lyn^{up/up}$ mice were challenged with 2.5% DSS and (**a**) body weight and (**b**) rectal bleeding were monitored for 7 or 8 days. At the experimental endpoint, (**c**) colon length was measured, and (**d**) cross-sections of distal colon were stained with hematoxylin and eosin. Pooled data from two of three independent experiments are shown for **a–c**, $n = 8$–10. (**e–i**) $Lyn^{+/+}$ and $Lyn^{up/up}$ mice were challenged using the azoxymethane (AOM)/DSS model of chronic colitis and colitis-associated cancer. (**e**) Graphical depiction of the experimental protocol. On day 0, $Lyn^{+/+}$ and $Lyn^{up/up}$ mice were injected with 10 mg kg^{-1} AOM and 2.5% DSS was administered for 7 days, followed by 14 days of recovery. DSS and recovery cycles were repeated two more times for a total of 63 days. (**f**) Weight change at the end of each DSS and recovery periods are shown from a representative of two independent experiments, $n = 5$/experiment. (**g**) Moribund mice that lost >20% body weight before day 63 were euthanized. (**h**, **i**) On day 63, (**h**) colon length was measured, and (**i**) macroscopic tumors in colons were counted and diameters were measured. Tumor load indicates the sum of tumor diameters per colon. Data pooled from two independent experiments are shown for colon length, mortality, tumor development, and tumor load, $n = 10$. (**a–i**) Error bars represent s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. No differences in weights of untreated $Lyn^{+/+}$ and $Lyn^{up/up}$ mice were observed (data not shown).

Here we describe how increased Lyn activity protects mice from DSS-induced colitis and the relationship between Lyn activity, PRR responses and ILC cytokine production.

Given Lyn's protective role in acute colitis, we investigated the outcome of chronic inflammation in Lyn^{up} mice. WT and Lyn^{up} mice were treated with the carcinogen azoxymethane



followed by three cycles of DSS (**Figure 1e**). Lyn^{up} mice were resistant to DSS-induced attenuation of weight gain that became apparent following the second cycle and significant by the third (**Figure 1f**). This was associated with a trend towards reduced morbidity in Lyn^{up} mice compared with WT (100% survival of Lyn^{up} vs. 67% survival of WT, $P < 0.07$) (**Figure 1g**). Lyn^{up} mice also had significantly longer colons at the experimental end point (**Figure 1h**), indicating a reduction in chronic inflammation. Consistent with this, Lyn^{up} mice exhibited a dramatic reduction in tumor number and load compared with controls (**Figure 1i**). Notably, on histological examination of distal colon sections containing tumors, no overt differences were observed between the tumors from WT and Lyn^{up} mice, with the tumors confirmed to be adenomas containing low-grade epithelial dysplasia. A minority of the adenomas in each group exhibited characteristics of high-grade dysplasia and intramucosal adenocarcinoma without evidence of muscularis mucosa or submucosal invasion. Together, these data indicate that Lyn activity protects mice from acute and chronic DSS-induced intestinal inflammation and reduces the incidence of colitis-associated cancer.

Increased Lyn activity is associated with an enhanced IL-22 response during experimental colitis

To investigate the contribution of cytokine responses in Lyn-mediated protection from DSS colitis, we screened the colons of DSS-treated WT and Lyn^{up} mice for inflammatory and immunoregulatory mediators. No consistent differences in mRNA or protein expression of tumor necrosis factor-α (TNFα), IL-6, IL-10, or IL-12 were observed (data not shown). By contrast, compared with WT mice, Lyn^{up} colon explants showed a significant fourfold increase in IL-22 production consistent with a trend to an increase in colonic *Il-22* mRNA expression following DSS treatment (**Figures 2a,b**). Increased IL-22 production by Lyn^{up} mice was observed as early as 2 days post-DSS treatment, with a significant increase in the colons (~threefold) and low, but detectable, levels in the serum (**Figure 2c**). IL-23 drives the production of IL-22 by RORγt$^+$ subsets of T cells and ILCs.[29–31] Accordingly, there was a significant 13-fold increase in the production of IL-23 in the colons of Lyn^{up} mice after DSS treatment. This was associated with an increase in *Il-23* mRNA and a significant increase in *Rorc* mRNA (**Figures 2a,b**). IFNγ and IL-17A can also be produced by IL-23-responsive T cell and ILC populations.[30,31] Colons of Lyn^{up} mice produced increased levels of *Ifng* mRNA and protein; however, no consistent differences in *Il-17a* mRNA expression were observed (**Figures 2a,b**). Baseline production of these inflammatory mediators was low to undetectable in the colons of untreated WT and Lyn^{up} mice, with the exception of IFNγ, which was modestly elevated in Lyn^{up} mice compared with controls (data not shown).

IL-22 mediates protection during intestinal inflammation via signal transducer and activator of transcription factor 3 (STAT3) activation in intestinal epithelial cells, promoting cell survival, proliferation, and the production of host-defense molecules.[13,15] Colonic epithelial cells isolated from Lyn^{up} mice 2 days post-DSS exposure showed a small but consistent increase in STAT3 activation, indicated by phosphorylation of tyrosine 705 and serine 727 (**Figure 2d**). This was consistent with a significant increase in DSS-induced colonic IL-22 (**Figure 2c**). Furthermore, increased levels of IL-22 observed 2 and 7 days post-DSS treatment correlated with elevated expression of the antimicrobial lectins *RegIIIg* and *RegIIIb* and mucus protein *Muc1* mRNAs (**Figure 2e**), which are established STAT3-responsive genes induced by IL-22.[15,18,32] No differences in these factors were observed between naive WT and Lyn^{up} mice (data not shown).

Loss of IL-22 responses impairs restitution following DSS treatment in WT mice.[18] As the impact of increased Lyn activity was exaggerated in the chronic/relapsing model of colitis (**Figures 1e–i**), we sought to determine whether recovery of Lyn^{up} mice following DSS treatment was dependent on the increased IL-22 observed in these mice. Anti-IL-22-neutralizing or isotype control antibodies were administered to Lyn^{up} mice starting 4 days before DSS treatment, and body weight was assessed throughout the experiment. No differences in weight change were observed during the acute phase of DSS treatment. However, IL-22 neutralization resulted in a significant delay in recovery of Lyn^{up} mice (**Figure 2f**). Together, these results suggest that increased production of IL-22 in Lyn^{up} mice acts to enhance intestinal repair and may contribute to protection from DSS colitis.

The adaptive immune system is dispensable for protection from acute colitis in Lyn^{up} mice

To investigate whether changes in steady state or DSS-induced inflammatory cell populations were responsible for the diminished inflammation and altered cytokine production profiles in DSS-treated Lyn^{up} mice, we examined the composition of immune cells in the colonic lamina propria (cLP). Untreated, but not DSS-treated, Lyn^{up} colons showed a small but reproducible increase in macrophage (CD11b$^+$F4/80$^+$) and granulocyte (Gr-1$^+$CD11b$^+$) frequencies compared with their WT counterparts (**Supplementary Figure S2A**). B cells were almost completely absent in Lyn^{up} colons, consistent with previous reports of a systemic decrease in B cell populations.[33] Before and after DSS treatment, CD4$^+$ T cell (CD3$^+$CD4$^+$) and DC (CD11c$^+$MHCII^{hi}) frequencies were increased in the Lyn^{up} colons. Within the DC compartment, however, no major differences in DC composition or activation status were observed based on MHCII (major histocompatibility complex II), CD80, CD86, CD11b, and CD103 expression levels (**Supplementary Figure S2A**). Interestingly, no significant changes in total numbers of DCs, macrophages, or neutrophils were found between naive WT and Lyn^{up} mice, although a small but consistent increase in CD4$^+$ and CD8$^+$ T cell numbers, and a 25-fold decrease in B cell numbers, were observed in Lyn^{up} mice (**Supplementary Figure S2B**).

To assess the relative contributions of adaptive vs. innate immune cells in limiting colitis in Lyn^{up} mice, we conducted DSS challenges in $Rag^{-/-}$ and $Rag^{-/-}$ Lyn^{up} mice. Protection from DSS in Lyn^{up} mice was exaggerated in the absence of an

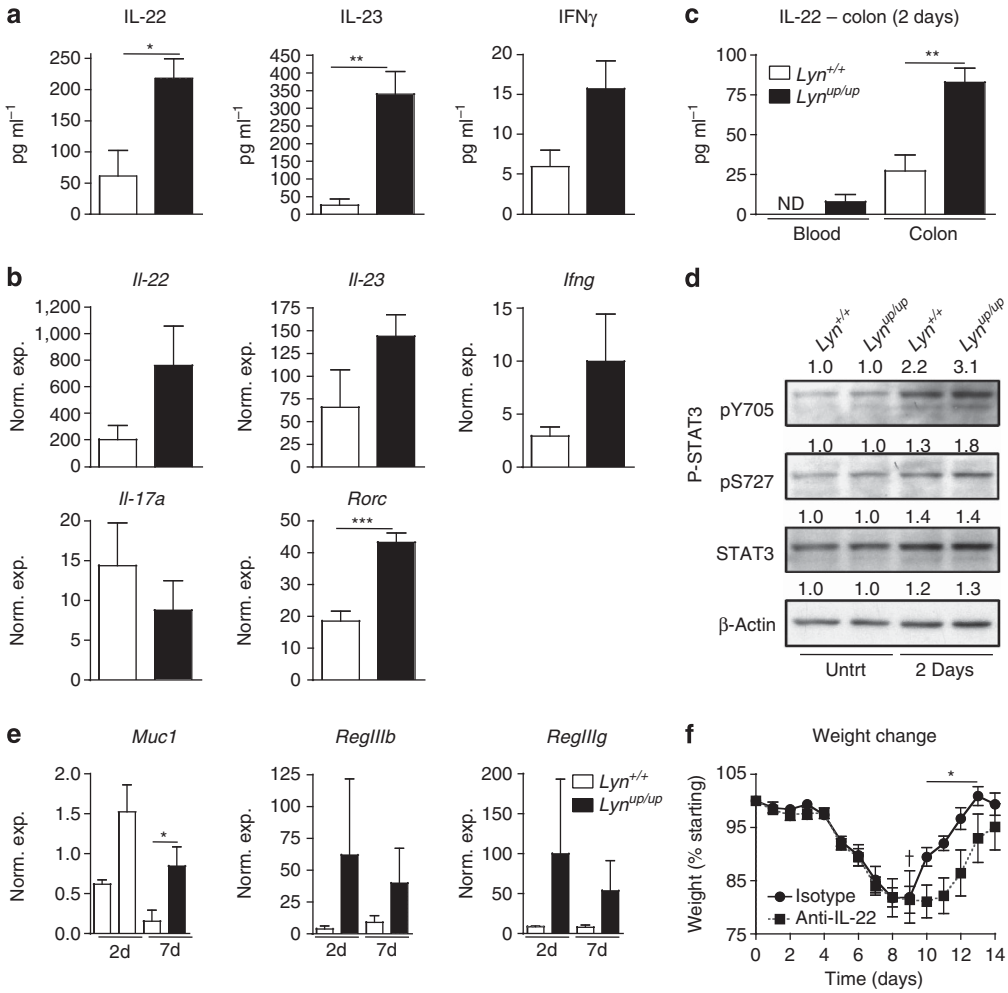


Figure 2 $Lyn^{up/up}$ mice produce increased levels of interleukin (IL)-22 during dextran sulfate sodium (DSS) colitis. $Lyn^{+/+}$ and $Lyn^{up/up}$ mice were treated with 2.5% DSS for 7 days or as indicated. (**a**) Colon tissue was cultured for 24 h, and cytokines were quantified by enzyme-linked immunosorbent assay (ELISA). Representative data are shown from three independent experiments, $n = 4$–5/experiment. (**b**) RNA was extracted from colon sections, and normalized (*Gapdh* (glyceraldehyde 3-phosphate dehydrogenase)) target gene expression (Norm. exp.) was assessed by quantitative PCR. Colon data pooled from two of three independent experiments are shown, $n = 8$. (**c**) IL-22 levels in blood and colon explant cultures were assayed by ELISA. (**d**) Colonic epithelial cells were isolated, and protein expression was analyzed by western blotting for total signal transducer and activator of transcription factor 3 (STAT3) and phospho-STAT3 (pS727 and pY705). β-Actin was used as a loading control. Numbers represent band intensity as quantified by ImageJ software (National Institutes of Health, Bethesda, MD). Representative data are shown, $n = 3$. (**e**) Colonic gene expression was assessed as in panel **b**. Representative data are shown, $n = 2$–4. (**f**) $Lyn^{up/up}$ mice were treated with 2% DSS for 7 days followed by a 7-day recovery period. Neutralizing anti-IL-22 monoclonal antibody (Ab) or isotype control Ab was administered before and throughout the course of DSS treatment, and mice were weighed daily. The symbol † indicates a control mouse that reached humane end point, $n = 5$. (**a**–**f**) Error bars represent s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *Ifn*, interferon; *Muc1*, mucin 1; Rorc, retinoic acid–related orphan receptor c.

adaptive immune system, indicating that the changes observed in B and T cell populations were not responsible for attenuation of DSS colitis. $Rag^{-/-}Lyn^{up}$ mice showed significantly less weight loss and rectal bleeding and had significantly longer and less inflamed colons than $Rag^{-/-}$ mice. In fact, $Rag^{-/-}Lyn^{up}$ mice were almost completely resistant to DSS treatment, with minimal weight loss and rectal bleeding and an absence of significant shortening of the colon (**Figures 3a–d**). In addition, we found that the ceca of $Rag^{-/-}$ mice showed inflammation after DSS treatment, characterized by severe edema and crypt destruction as well as the presence of inflammatory cells in the submucosa and lumen, while $Rag^{-/-}Lyn^{up}$ mice had only slight epithelial sloughing (**Figure 3d**). Similar to Lyn^{up} mice, naive $Rag^{-/-}Lyn^{up}$ mice had a small, but consistent, increase in neutrophil ($CD11b^{+}Gr\text{-}1^{hi}$) frequency (**Supplementary Figure S3**). However, the frequencies of DCs and macrophages were indistinguishable between $Rag^{-/-}$ and $Rag^{-/-}Lyn^{up}$ mice. After DSS challenge, however, the frequency of these populations was moderately reduced in $Rag^{-/-}Lyn^{up}$ compared with $Rag^{-/-}$ animals (**Supplementary Figure S3**). Together, these results suggest that enhanced Lyn activity in innate immune cells is sufficient to protect mice from DSS-induced intestinal inflammation and that protection from colitis is not associated with dramatic changes in cLP innate leukocyte populations.

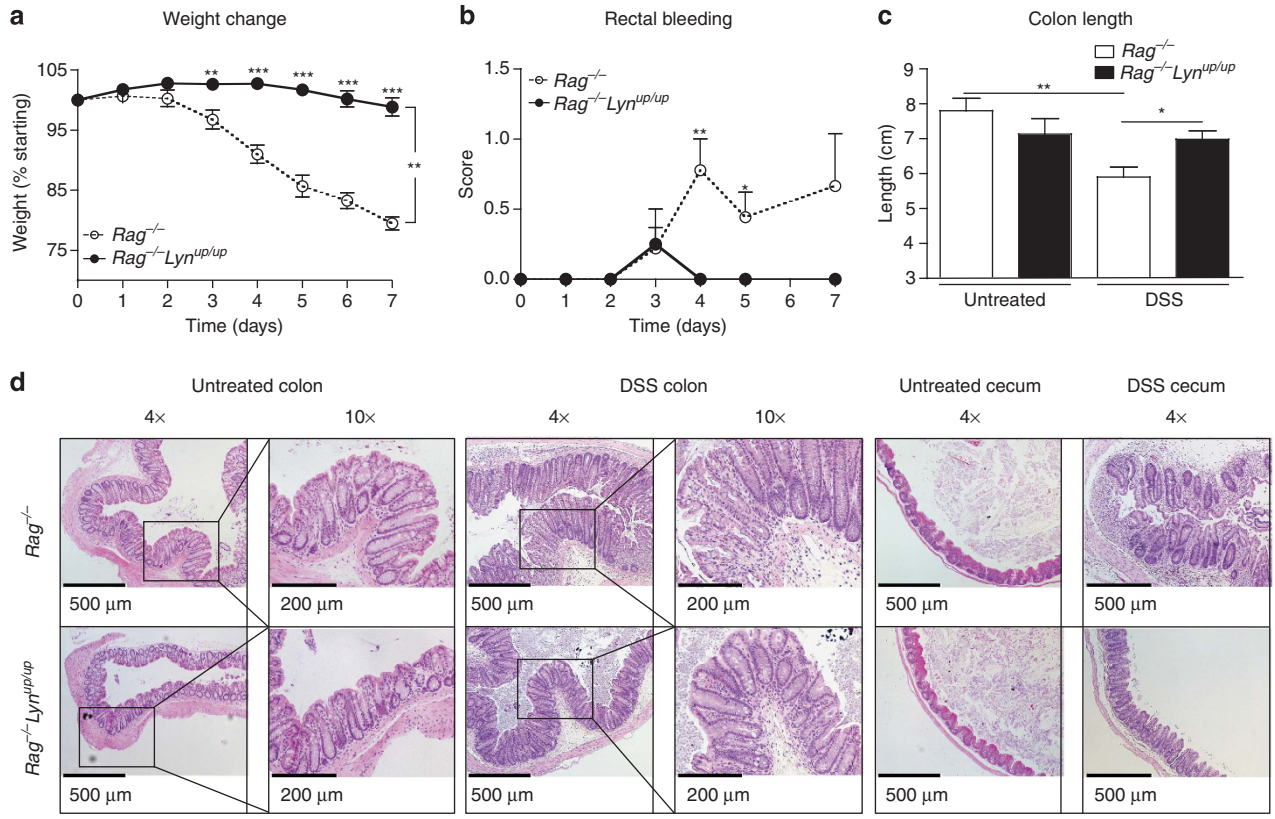


Figure 3 Lyn activity in innate immune cells is sufficient to protect from dextran sulfate sodium (DSS) colitis. $Rag^{-/-}$ and $Rag^{-/-}Lyn^{up/up}$ mice were challenged with 2.5% DSS, and (**a**) body weight and (**b**) rectal bleeding were monitored over 7 days. (**c**) Mice were killed on day 7, and colon length was measured. Pooled data from two of three independent experiments are shown, $n = 7$–9. Error bars represent s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**d**) Cecal cross-sections and longitudinally sectioned colons from untreated and DSS-treated mice (day 7) were stained with hematoxylin and eosin. Representative sections are shown. No differences in weights of untreated $Rag^{-/-}$ and $Rag^{-/-}Lyn^{up/up}$ mice were observed (data not shown).

Enhanced IL-22 production in Lyn^{up} mice occurs in response to LPS and requires DCs and ILCs

To determine the cell types responsible for increased IL-22 production in Lyn^{up} mice, we stimulated total splenocytes or splenocytes depleted of specific cell populations with IL-23 ± LPS. Lyn^{up} splenocytes produced more IL-22 than WT in response to IL-23 with or without LPS. Adaptive immune cells were not required for enhanced IL-22 production by Lyn^{up} splenocytes, but IL-22 production was dependent on the presence of $CD90^{+}$ cells (**Figure 4a**). Flow cytometric analysis revealed that ILCs ($CD90^{+}Lin^{-}$) were the major producers of IL-22 in the spleens of $Rag^{-/-}$ and $Rag^{-/-}Lyn^{up}$ mice (**Supplementary Figure S4**). This is consistent with previous reports that identified ILCs as a major source of IL-22.[21,22,30,34] Interestingly, a twofold increase in frequency and a fivefold increase in ILC numbers were observed in $Rag^{-/-}Lyn^{up}$ compared with $Rag^{-/-}$ spleens (**Figure 4b**). IL-22 production was also slightly enhanced in $Rag^{-/-}Lyn^{up}$ ILCs, based on mean fluorescence intensity of IL-22 staining (**Supplementary Figure S4**). Increased IL-22 production by Lyn^{up} splenocytes also required the presence of DCs, as splenocytes from diphtheria toxin (DT)-treated *Cd11cDTR-*Lyn^{up} mice failed to produce enhanced IL-22 compared with DT-treated *Cd11cDTR* controls (**Figure 4c**). DCs were not, however, a source of IL-22 in splenic cultures, as IL-22 was undetectable in $CD11c^{+}$ cells (data not shown), indicating that these cells were required as accessory cells to drive increased IL-22 by Lyn^{up} ILCs.

Because Lyn^{up} mice are profoundly hypersensitive to LPS[25] and because LPS is known to drive IL-22 production *in vivo*,[21,34] we questioned whether systemic administration of LPS would be sufficient to drive increased IL-22 production in Lyn^{up} mice. Lyn^{up} mice showed significantly increased levels of IL-22 in blood 2 h after LPS intraperitoneal injection and the systemic effects of LPS also resulted in increased IL-22 production in the colon (**Figure 4d**), suggesting that the increased IL-22 response in the colons and blood following DSS treatment may be a result of TLR hyper-responsiveness in Lyn^{up} mice. Enhanced IL-22 production in the blood and colons of Lyn^{up} mice was maintained in the absence of an adaptive immune system as $Rag^{-/-}Lyn^{up}$ mice also produced more IL-22 in response to LPS (**Figure 4e**). As $CD90^{+}$ ILCs were required for enhanced production of IL-22 by Lyn^{up} splenocytes and are known to be a major source of IL-22 during systemic responses to TLR ligands,[21,22,30,34] we questioned whether the increased systemic IL-22 production in Lyn^{up} mice required ILCs. $Rag^{-/-}$ and $Rag^{-/-}Lyn^{up}$ mice were treated with an anti-CD90 monoclonal antibody followed by an

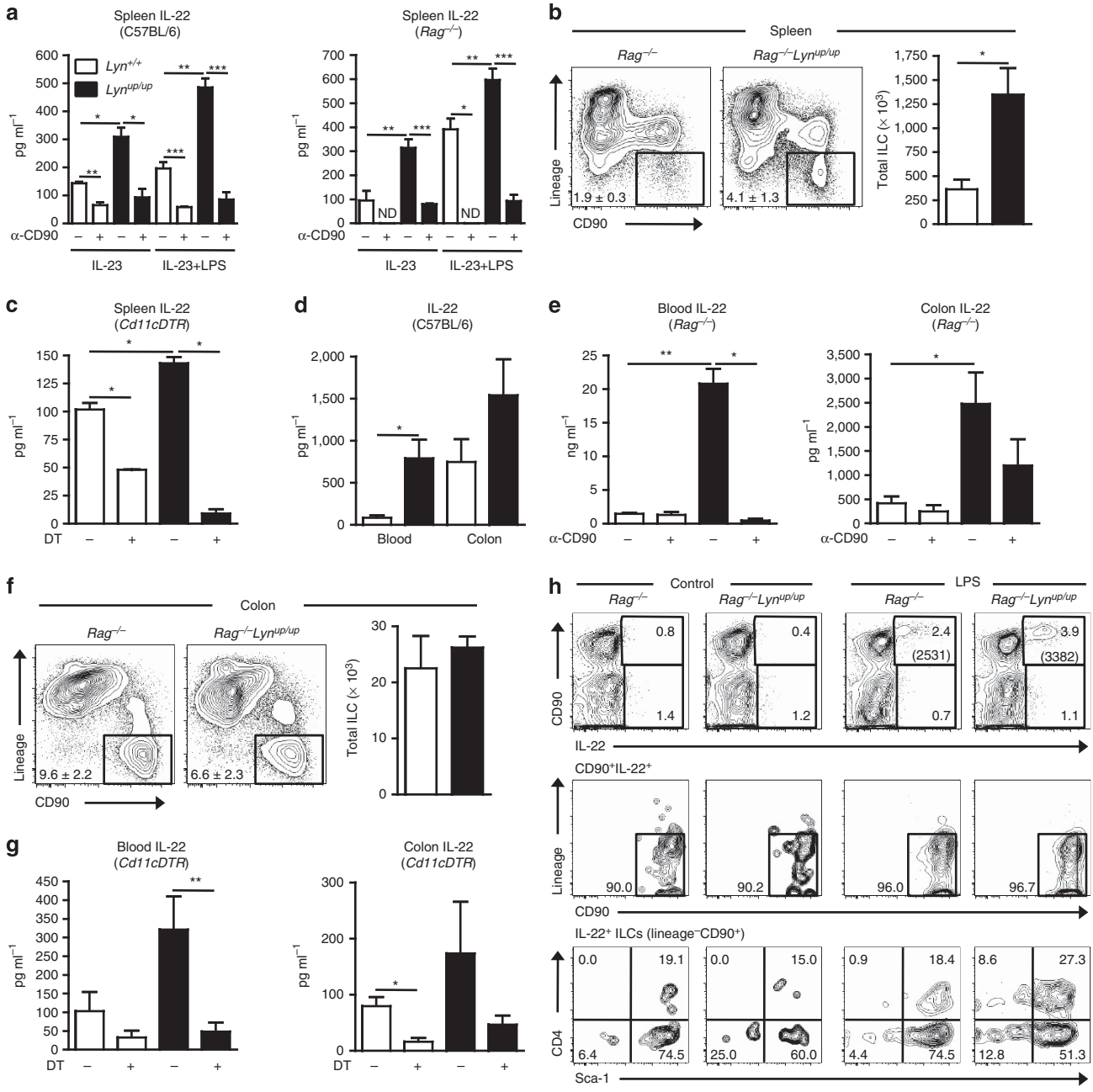


Figure 4 Dendritic cells (DCs) and innate lymphoid cells (ILCs) are required for increased interleukin (IL)-22 production in *Lyn*$^{up/up}$ mice in response to lipopolysaccharide (LPS). (**a**) Total splenocytes (α-CD90 −) or splenocytes depleted of CD90$^+$ cells (α-CD90 +) from naive *Lyn*$^{+/+}$ and *Lyn*$^{up/up}$ (left) or *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Lyn*$^{up/up}$ (right) mice were stimulated with IL-23 ± LPS, and IL-22 production was quantified. Representative data from three independent experiments are shown, $n = 2$/experiment. (**b**) Splenic and (**f**) colonic ILCs (lineage$^-$CD90$^+$) from naive *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Lyn*$^{up/up}$ mice were analyzed by flow cytometry. Graphs and plots represent data pooled from two independent experiments, $n = 6$. Numbers on plots represent mean frequency ± s.e.m. (**c**) *Cd11cDTR* and *Cd11cDTR-Lyn*$^{up/up}$ mice were injected with phosphate-buffered saline (PBS; DT−) or diphtheria toxin (DT; DT+), and 1 day later, splenocytes were isolated and stimulated with IL-23 + LPS and IL-22 production was quantified. Representative data from two independent experiments are shown, $n = 2$–4. (**a**,**c**) Unstimulated cells produced undetectable levels of IL-22 (data not shown). (**d**) *Lyn*$^{+/+}$ and *Lyn*$^{up/up}$ mice were treated with LPS, and IL-22 in blood and colon explant cultures was assessed. Graphs represent data pooled from two independent experiments, $n = 8$. (**e**) *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Lyn*$^{up/up}$ or (**g**) *Cd11cDTR* and *Cd11cDTR-Lyn*$^{up/up}$ mice were injected with PBS (PBS), (**e**) anti-CD90 mAb, or (**g**) DT before LPS injection. IL-22 in blood and colon explants was assessed. (**e**) Representative data from two independent experiments are shown, $n = 3$/experiment. (**g**) Graphs represent data pooled from two independent experiments, $n = 4$–6. (**h**) *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Lyn*$^{up/up}$ mice were left untreated or were injected with LPS followed by brefeldin A, and colonic lamina propria cells were isolated and cultured in the presence of brefeldin A. IL-22 production was analyzed by flow cytometry, $n = 3$ and numbers indicate frequency or mean fluorescence intensity (in brackets) of the highlighted population. (**a**–**g**) Error bars represent s.e.m., $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ND, not detected.

intraperitoneal injection of LPS 2 days later. Depletion of CD90$^+$ ILCs was assessed in the spleen, mesenteric lymph nodes (MLN), and cLP by flow cytometry. Complete depletion of CD90$^+$ cells was observed in the spleen and MLN; however, only partial depletion was achieved in the cLP (**Supplementary Figure S5A**). Consistent with the spleen data, enhanced IL-22

production by $Rag^{-/-}Lyn^{up}$ mice following LPS injection required the presence of $CD90^+$ cells. In $Rag^{-/-}Lyn^{up}$ mice, depletion of $CD90^+$ cells resulted in a significant 10-fold reduction in serum IL-22 levels and a twofold reduction in the colon (**Figure 4e**). Interestingly, unlike in the spleen, increased colonic IL-22 production was not dependent on increased ILC numbers, as no difference in ILC numbers was observed in the colons of $Rag^{-/-}$ and $Rag^{-/-}Lyn^{up}$ mice (**Figure 4f**).

DCs are required for IL-22 production in response to TLR stimulation and during enteric infection,[21,22,35] and we found that DCs were required for enhanced IL-22 production by Lyn^{up} splenocytes (**Figure 4c**). To investigate the contribution of DCs to increased IL-22 production in Lyn^{up} mice *in vivo*, we depleted DCs by DT treatment of *Cd11cDTR* and *Cd11cDTR-*Lyn^{up} mice and measured IL-22 levels in the blood and colon after LPS challenge. DC depletion was verified in the cLP, MLN, and spleen by flow cytometry (**Supplementary Figure S5B-C**). IL-22 levels were reduced up to fivefold in the blood and threefold in the colons of LPS-injected DC-depleted *Cd11cDTR-*Lyn^{up} and *Cd11cDTR* mice (**Figure 4g**). Loss of IL-22 was not due to the depletion of IL-22-producing T cells or ILCs, as no changes were observed in these compartments after DT treatment (**Supplementary Figure S5B** and data not shown).

Finally, we sought to identify the source of IL-22 in the cLP *in vivo*. To do this, $Rag^{-/-}$ and $Rag^{-/-}Lyn^{up}$ mice were left untreated or injected with LPS followed by an intravenous injection of brefeldin A. Mice were killed 4 h later, and cLP cells were isolated, incubated for 6 h with brefeldin A, and then analyzed by flow cytometry to assess cellular production of IL-22. As in the spleen, IL-22 was produced by ILCs but not DCs (**Figure 4h**, data not shown). Furthermore, $Rag^{-/-}Lyn^{up}$ mice had an increased frequency of IL-22-producing cells and produced more IL-22 on a per cell basis. As in the spleen, IL-22 was produced by a heterogeneous population of colonic ILCs, including both $CD4^+$ and $CD4^-$ populations. Taken together, our results demonstrate that Lyn activity enhances IL-22 production by ILCs in both the gastrointestinal tract and systemically in response to TLR stimulation and that this response is dependent on DCs.

Increased Lyn activity in DCs is sufficient to drive enhanced IL-22 production by ILCs

Although well characterized in the majority of the hematopoietic system, the expression of Lyn has, to the best of our knowledge, not been assessed in ILCs or in cLP leukocytes. We therefore isolated cLP cells from naive WT and $Lyn^{-/-}$ mice and assessed Lyn expression in various cell types by flow cytometry. Lyn was expressed in macrophages, neutrophils, and NK cells but not in T cells, consistent with previous reports.[23] Interestingly, very low levels of Lyn were also observed in $CD45^-$ cells, which may include intestinal epithelial cells (**Supplementary Figure S6**). Lyn expression was found to differ in ILC subsets. $CD4^-Sca\text{-}1^+$ ILCs expressed Lyn, whereas $CD4^+Sca\text{-}1^+$ ILCs did not, which, given the expression of IL-22 by both populations in the spleen and colon, suggests that changes in Lyn activity in other cell types drives enhanced IL-22 production by ILCs (**Figure 5a**). We also confirmed the expression of Lyn in cLP DCs by flow cytometry and found that although all DCs expressed Lyn, expression varied between DC subsets, with $CD11b^+CD103^-$ DCs exhibiting the highest Lyn expression (**Figure 5b**).

We have previously shown that Lyn activity in DCs regulates DC maturation, cytokine production, and NK cell IFNγ production in response to LPS.[25] Here we identified DCs as necessary for increased IL-22 production in Lyn^{up} mice. We therefore questioned whether similar interactions were driving increased IL-22 production by group 3 ILCs. Innate $CD90^+$ cells (ILCs) were isolated from $Rag^{-/-}$ spleens and cultured with WT or Lyn^{up} bone marrow–derived DCs with or without LPS. In response to LPS, ILCs cultured with Lyn^{up} DCs produced more IL-22 than those cultured with WT DCs, indicating that enhanced Lyn activity in DCs is sufficient to drive increased IL-22 production by ILCs (**Figure 5c**). Neither ILCs nor DCs cultured alone produced IL-22 in response to LPS, confirming the necessity of both cell types for IL-22 production. Consistent with their ability to enhance ILC production of IL-22, Lyn^{up} DCs produced more IL-23 in response to LPS (**Figure 5d**). This increase in IL-23 production correlated with increased expression of *Il-12p40*, as Lyn^{up} DCs expressed more *Il-12p40* mRNA compared to WT with no differences in *Il-23p19* expression (**Figure 5e**). Collectively, these results highlight the importance of DC function in regulating ILC activity and suggest that increased Lyn activity in DCs regulates systemic responses to microbial products like LPS, leading to enhanced IL-22 production by ILCs.

LPS hypersensitivity is sufficient to protect microbiota-depleted Lyn^{up} mice from DSS

Intestinal responses to microbial products are required to limit destructive inflammation resulting from DSS-induced injury[5,6] and radiation-induced gastrointestinal damage.[36,37] Given the systemic LPS hypersensitivity and increased IL-22 production by ILCs in response to LPS in Lyn^{up} mice, we hypothesized that intestinal hypersensitivity to LPS might have a protective role following DSS treatment. WT and Lyn^{up} mice were treated with antibiotics to reduce their microbiota and received a low dose ($10\ \mu g\ ml^{-1}$) of LPS in their drinking water, which provides marginal protection to WT mice from DSS-induced pathology,[5] before and during DSS treatment. Mice were then left to recover for 7 days (**Figure 6a**). The antibiotic regime was effective at significantly reducing bacterial load in the gut as assessed by fecal DNA and bacterial 16S ribosomal RNA gene content (**Figure 6b**). Corresponding with the induction of IL-22 by LPS (**Figure 4**), the reduction in intestinal bacteria resulted in a significant reduction in DSS-induced IL-22 production in WT and Lyn^{up} mice (**Figure 6c**). In the absence of LPS, WT and Lyn^{up} mice exhibited similar DSS-induced weight loss; however, WT mice exhibited profound sensitivity to DSS, as 80% of mice became moribund by day 43, reaching 100% by day 44 (**Figures 6d,e**). Strikingly, microbiota-reduced Lyn^{up} mice were protected from DSS, with 75% of mice surviving the experiment. The presence of a low dose of LPS in the drinking

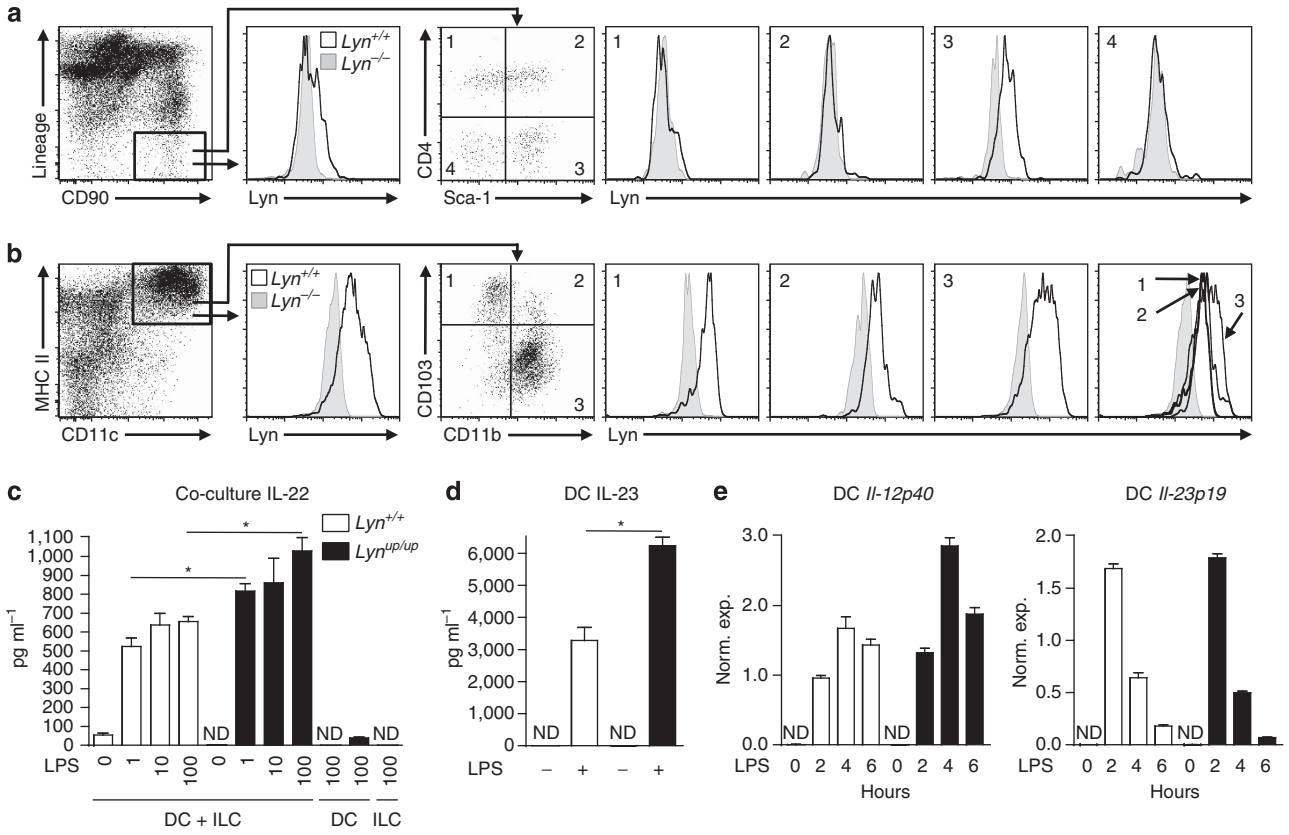


Figure 5 Increased Lyn activity in dendritic cells (DCs) drives enhanced interleukin (IL)-22 production by innate lymphoid cells (ILCs). (**a**–**b**) Colonic lamina propria cells were isolated from naive $Lyn^{+/+}$ (black open histograms) and $Lyn^{-/-}$ (gray filled histograms) mice and Lyn expression in (**a**) ILCs (lineage$^-$CD90$^+$) and (**b**) DCs (CD11c$^+$MHCII$^+$) was assessed by flow cytometry. All cells were first gated on live CD45$^+$ populations. Representative histograms are shown. (**c**, **d**) $Lyn^{+/+}$ or $Lyn^{up/up}$ bone marrow–derived DCs (BM-DCs) were cultured alone or with CD90$^+$ cells isolated from $Rag^{-/-}$ mice for 24 h with (**c**) the indicated doses (ng ml^{-1}) or (**d**) 0 ng ml^{-1} (−) or 100 ng ml^{-1} (+) of lipopolysaccharide (LPS). (**c**) IL-22 and (**d**) IL-23 in culture supernatants were assessed by enzyme-linked immunosorbent assay. Representative data from three independent experiments are shown. (**e**) $Lyn^{+/+}$ or $Lyn^{up/up}$ BM-DCs were stimulated with LPS for the indicated time points, and *Il-12p40* and *Il-23p19* expression levels were assessed by quantitative PCR (normalized to *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase)). Error bars represent s.e.m., $^*P<0.05$. MHC, major histocompatibility complex; ND, not detected.

water, mimicking TLR stimulation by intestinal bacteria, did not rescue WT mice from DSS-induced weight loss or morbidity. By contrast, LPS had a profound effect in Lyn^{up} mice, with 100% survival of the LPS-treated Lyn^{up} mice and significantly reduced weight loss observed (**Figures 6d,e**). Overall these data suggest that Lyn activity modulates intestinal responses to microbial products, which significantly impact the outcome of disease following intestinal damage.

DISCUSSION

Perturbations in the immune system and its responses to PRR signals are critical factors in the development of IBD and experimental colitis;[3] however, despite Lyn's role in immune cell responses, the function of Lyn has never been explored in the context of gastrointestinal inflammation. There are, however, various human malignancies associated with dysregulation of Lyn expression or activity.[23] Drugs targeting SFKs, such as Dasatinib used to inhibit SFKs in tumors may also affect Lyn and other SFKs systemically. Importantly, colitis can develop as a side effect of Dasatinib treatment,[38,39] implicating a role for SFKs such as Lyn, or other targets of this tyrosine kinase inhibitor, in modulating gastrointestinal immune responses. Here we present evidence that Lyn has a role in regulating intestinal inflammation and disease in acute and chronic models of DSS-induced colitis. Moreover, we suggest a potential mechanism of protection from disease in Lyn^{up} mice, showing that hypersensitivity to TLR stimuli leads to increased protective responses during inflammatory insult.

Chronic intestinal inflammation is linked with the development of colon cancer both in mice and in patients with IBD.[40–42] Accordingly, increased Lyn activity in Lyn^{up} mice resulted not only in decreased chronic colitis but also the development of fewer tumors following azoxymethane/DSS treatment. Whether protection from colitis-associated cancer is afforded by enhanced Lyn activity within the hematopoietic system alone or other Lyn-expressing compartments such as the epithelium still remains to be determined and would provide important mechanistic insights into the role of Lyn in colon cancer. Nonetheless, the data presented here may have implications for IBD patients, as a recent study identified a protective association between increased levels of *Lyn* mRNA in the rectal mucosa of ulcerative colitis patients and diminished

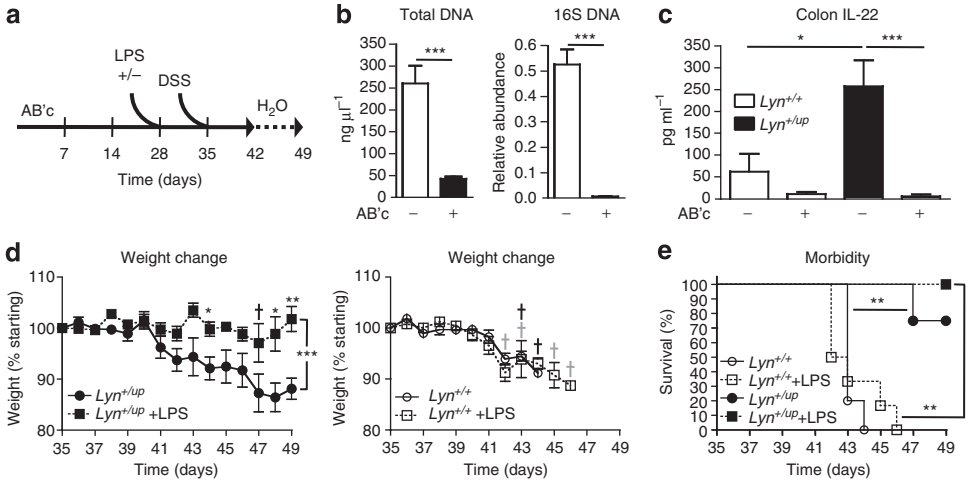


Figure 6 Hypersensitivity to lipopolysaccharide (LPS) protects antibiotic-treated $Lyn^{+/up}$ mice from dextran sulfate sodium (DSS)-induced weight loss and death. (**a**) Diagram depicting the experimental protocol. $Lyn^{+/+}$ and $Lyn^{+/up}$ mice were given a cocktail of antibiotics (AB'c) in their drinking water for 5 weeks. LPS was added during the fourth week to half the group of mice and 2.5% DSS was added to all the cages for the fifth week. All mice were then given sterile drinking water for an additional week. (**b**, **c**) Colon sections and fresh fecal pellets were harvested from $Lyn^{+/+}$ and $Lyn^{+/up}$ mice 4 days post-DSS treatment. (**b**) Total concentration of DNA and relative abundance of 16S bacterial DNA is shown. (**c**) Interleukin (IL)-22 in colon explant cultures was quantified by enzyme-linked immunosorbent assay, $n=4$. (**d**, **e**) $Lyn^{+/up}$ and $Lyn^{+/+}$ mice were treated as in panel **a**, and (**d**) body weight and (**e**) animal health were assessed daily from days 35–49 and mice that became moribund were euthanized. The symbol † indicates the death of a mouse treated with (gray) or without (black) LPS. Representative data from two independent experiments are shown, $n=4$–6/experiment. Error bars represent s.e.m., $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

development of colitis-associated cancer.[43] SFK inhibitors are being used and developed for the treatment of various cancers, including colon cancer;[44] however, our results suggest that the use of these inhibitors be carefully monitored for gastrointestinal complications.

Uncontrolled inflammatory responses downstream of PRR signaling contribute extensively to pathogenesis in IBD and murine models of colitis.[7] However, these same signaling pathways are required to maintain intestinal homeostasis and limit inflammation.[1–6] Given the DC-dependent hypersensitivity of Lyn^{up} mice to LPS,[25] we questioned whether enhanced PRR responses by innate immune cells were responsible for the protection from DSS in Lyn^{up} mice. We found that the adaptive immune system was dispensable for this phenotype and that LPS hypersensitivity in Lyn^{up} mice was sufficient to rescue antibiotic-treated mice from DSS-induced disease and death. A previous study demonstrated that introduction of a high dose (50 mg ml^{-1}) of LPS into drinking water of mice following antibiotic treatment was sufficient to protect mice from DSS-induced colitis and mortality, whereas a lower dose of LPS (10 µg ml^{-1}) was less effective and rescued only ~30% of WT mice.[5] By contrast, we found that a low dose of LPS (10 µg ml^{-1}) had little effect on DSS-induced weight loss and mortality in WT mice, possibly due to differences in animal facilities, sources of LPS, microbiota, and/or the antibiotic treatment regime used in our study. Nonetheless, heterozygosity for the *Lyn* gain-of-function allele ($Lyn^{+/up}$) was sufficient to confer sensitivity to the low dose of LPS, rescuing all Lyn^{up} mice from DSS-induced weight loss and morbidity. Together, these studies suggest that the hypersensitivity to PRR ligands, such as LPS, by innate immune cells is sufficient to provide protection from DSS-induced disease. However, further studies using immunodeficient mice are currently ongoing to address this question directly, as it remains a possibility that increased innate responses to microbes in Lyn^{up} mice enhance protective adaptive immunity.

Notably, in the absence of LPS treatment, Lyn^{up} mice exhibited increased survival compared with WT. The antibiotic treatment regime used in our studies has been shown to significantly reduce the microbial burden within the intestine; however, some bacteria persist following the treatment.[45] We therefore speculate that hypersensitivity to these remaining microbes in Lyn^{up} mice provides some protection from DSS insult. Interestingly, recent studies have highlighted the importance of IL-22 and antimicrobial responses in maintaining appropriate anatomical localization of intestinal microbes that have the potential to induce systemic inflammation.[46,47] Whether *Lyn* gain-of-function mice possess a distinct microbiota or a superior ability to control pathobionts that may emerge during the course of antibiotic treatment remains to be determined and is currently being investigated.

IL-22 production is induced *in vivo* in response to TLR ligands, including LPS or flagellin.[21,22,34] We therefore hypothesized that hypersensitivity to microbial products is involved in modulating IL-22 production in Lyn^{up} mice. Systemic administration of LPS alone was sufficient to induce increased amounts of IL-22 in Lyn^{up} mice compared with WT. Furthermore, antibiotic treatment profoundly decreased DSS-induced IL-22 production in both WT and Lyn^{up} mice. These data suggest that increased Lyn activity serves to enhance TLR responses that modulate IL-22 production and does not drive IL-22 production through PRR-independent mechanisms. This is in agreement with previous studies indicating that increased Lyn activity in Lyn^{up} mice does not qualitatively affect immune

cell signaling pathways but instead serves to modulate signaling thresholds in Lyn-regulated pathways.[28] To our knowledge, this is the first report demonstrating regulation of IL-22 production by Lyn or any other SFK. Although IL-22 levels increase early in Lyn^{up} mice during DSS treatment, neutralizing experiments suggested that IL-22 has a more important role in restitution and repair. This is consistent with the exaggerated protection observed in Lyn^{up} mice during the course of the chronic DSS model compared with acute DSS. However, we hypothesize that subtle increases in intestinal IL-22 throughout the lifetime of a Lyn^{up} mouse may result in predisposition towards resistance to the initial DSS-induced epithelial damage, resulting in protection from acute intestinal damage. We are currently investigating this possibility.

ILCs have emerged as major sources of IL-22 in response to systemic administration of TLR ligands.[21,22,30,34] However, whether Lyn is expressed in ILCs or has a role in ILC activation and function has not been studied. Interestingly, in the colon Lyn was only expressed by $CD4^-$ $Sca\text{-}1^+$ ILCs, but IL-22 was produced by numerous subsets of ILCs, including $CD4^+$ subsets. This suggests that ILC-intrinsic changes in Lyn activity are not solely responsible for the increased IL-22 production by ILCs in Lyn^{up} mice. It is now evident that DCs are required to respond to microbial products and convey signals to ILCs in order to drive IL-22 production.[21,22] Furthermore, the importance of DC interactions with members of the ILC family has been extensively studied in the context of NK cells.[48] However, the pathways that mediate DC interactions with IL-22-producing group 3 ILCs, including lymphoid tissue-inducer-like cells and NCR^+ ILC3s, are just beginning to be elucidated.[20] Our previous work highlighted the importance of DC-intrinsic Lyn signaling in DC regulation of NK cell (ILC1) activation and effector responses. LPS challenge of Lyn^{up} mice led to DC and NK cell–dependent morbidity in Lyn^{up} mice.[25] The data presented here extend the importance of Lyn in modulating DC interactions with other members of the ILC family. Although DCs did not produce IL-22, both DCs and innate $CD90^+$ cells (ILCs) were required to drive increased IL-22 production *in vivo* and in *in vitro* culture systems. Furthermore, increased Lyn activity in DCs drove increased IL-22 production by ILCs *in vitro*. Collectively, we show that modulation of Lyn signaling in DCs may be a common mechanism that regulates the activity of distinct ILC family members. Given the importance of various ILC subsets in regulating inflammation and immunity at mucosal sites[13,20] and in maintaining anatomical localization of commensal organisms,[46] we are currently investigating the molecular mechanisms that dictate Lyn-mediated changes in DC–ILC interactions.

This study reveals a novel role for Lyn in regulating intestinal inflammation and IL-22 production by ILCs and demonstrates how changes in signaling pathways that regulate PRR-responses can have a profound impact on intestinal inflammation. This work should pave the way for future studies investigating the importance of Lyn and other SFKs in regulating host–microbiota interactions during homeostasis and disease, the molecular interactions that dictate the outcome of innate immune cell interactions, and the outcome of clinical studies utilizing Lyn and other SFK inhibitors that may lead to colitis.

METHODS

Reagents and detailed methods are provided in the **Supplementary Material**.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGEMENTS

We thank Alex Sio and Danielle Krebs for technical support and advice; Yiannis Himaris and Joshua Jiang for technical assistance; David Artis and David Hill for advice on antibiotic cocktail formulations; and John Priatel and Amit Bhavsar for thoughtful discussions. This work was supported by a grant from the Canadian Institutes of Health Research (CIHR) (CIHMOP-86694 to K.W.H.), a fellowship from the Canadian Association of Gastroenterology, Crohn's and Colitis Foundation Canada and CIHR (to J.L.B) and scholarships from the Michael Smith Foundation, CIHR and CIHR Training Program in Transplantation (to M.E.R and M.K.C.). K.W.H. holds a Tier 2 Canada Research Chair.

DISCLOSURE

The authors declared no conflict of interest.

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