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IL-36 receptor promotes resolution of intestinal damage

Oscar Medina-Contreras[*,1,2], **Akihito Harusato**[*,1], **Hikaru Nishio**[†,3], **Kyle L. Flannigan**[*], **Vu Ngo**[*], **Giovanna Leoni**[†,4], **Philipp-Alexander Neumann**[†,5], **Duke Geem**[*,†], **Loukia N. Lili**[†], **Ravisankar A. Ramadas**[‡,6], **Benoit Chassaing**[*], **Andrew T. Gewirtz**[*], **Jacob E. Kohlmeier**[§], **Charles A. Parkos**[†,¶], **Jennifer E. Towne**[‖,7], **Asma Nusrat**[†,¶], and **Timothy L. Denning**[*]

[*]Institute for Biomedical Sciences, Georgia State University, Atlanta, GA 30303

[†]Department of Pathology & Laboratory Medicine, Emory University, Atlanta, GA 30322

[‡]Department of Pediatrics, University of Florida, Gainesville FL 32607

[§]Department of Microbiology and Immunology, Emory University, Atlanta, GA 30322

[¶]Department of Pathology, University of Michigan, Ann Arbor, MI 48109

[‖]Department of Inflammation Research, Amgen, Seattle, WA 98119

Abstract

Interleukin-1 family members are central mediators of host defense. Here we show that the novel IL-1 family member, IL-36γ, was expressed during experimental colitis and human inflammatory bowel disease (IBD). In response to dextran sodium sulfate (DSS)-induced damage, germ-free (GF) mice failed to induce IL-36γ, suggesting that gut microbiota are involved in its induction. Surprisingly, IL-36R-deficient (*Il1rl2*$^{-/-}$) mice exhibited defective recovery following DSS-induced damage and impaired closure of colonic mucosal biopsy wounds, which coincided with impaired neutrophil accumulation in the wound bed. Failure of *Il1rl2*$^{-/-}$ mice to recover from DSS-induced damage was associated with a profound reduction in IL-22 expression, particularly by colonic neutrophils. Defective recovery of *Il1rl2*$^{-/-}$ mice could be rescued an aryl hydrocarbon receptor (AhR) agonist, which was sufficient to restore IL-22 expression and promote full recovery from DSS-induced damage. These findings implicate the IL-36/IL-36R axis in the resolution of intestinal mucosal wounds.

Address correspondence to Dr. Timothy L. Denning, Institute for Biomedical Sciences, Georgia State University, Atlanta, GA 30303. Phone: 404-413-3609; Fax: 404-413-3580; tdenning@gsu.edu.

[1]O.M.-C. and A.H. contributed equally to this work.

[2]Oscar Medina-Contreras current address: Department of Research in Immunology, National Institute for Respiratory Diseases, and National Council for Science and Technology, Mexico City, Mexico.

[3]Hikaru Nishio current address: Project Creation Group, Innovation Promotion Dept., Research Institute, Ajinomoto Pharmaceuticals Co., Ltd, Kanagawa, Japan.

[4]Giovanna Leoni current address: Institute for Cardiovascular Prevention, Ludwig-Maximilians-University, Munich, Germany.

[5]Philipp-Alexander Neumann current address: Department of General and Visceral Surgery, University of Muenster, Muenster, Germany.

[6]Ravisankar A. Ramadas current address: Department of Immunology, Oncology and IMRs, Merck Research laboratories, Boston, MA, USA.

[7]Jennifer E. Towne current address: Immunology Discovery, Janssen Research and Development, San Diego, CA, USA.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC), the two major clinical phenotypes of IBD, are associated with dysregulated innate and adaptive immune responses towards gut microbiota (1). Members of the IL-1 family of cytokines are up-regulated in the inflamed mucosa during experimental colitis and human IBD and contribute to intestinal inflammation (2). Interestingly however, mice deficient in IL-1β or IL-18, or components of their processing, are more susceptible to DSS-induced colitis (3–6) and polymorphisms leading to decreased Nlrp3 expression in humans are associated with increased risk for developing CD (7). Thus, IL-1 family members may contribute not only to pro-inflammatory responses, but also resolution of inflammation (2, 8, 9).

Recently, information on the role of IL-36 family members, which are members of the IL-1 superfamily, has begun to emerge (10, 11). However, little data exists about the function of the IL-36/IL-36R axis in the intestine. Here we demonstrate that IL-36γ was induced during intestinal injury/inflammation and that IL-36R influenced neutrophil accumulation, IL-22 production, and repair of intestinal damage following injury. Treatment with an AhR agonist was sufficient to induce intestinal IL-22 expression and promote recovery in *Il1rl2*$^{-/-}$ mice. Collectively, these findings support a novel contribution of the IL-36/IL-36R axis in repair of damaged intestinal mucosa.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 and *Rag1*$^{-/-}$ mice were from The Jackson Laboratory. *Il1rl2*$^{-/-}$ mice were from Amgen. GF mice were maintained as previously described (12). Protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Reagents

Antibodies were from eBioscience except for CD45, CD103, CD4 (Becton Dickinson). Dead cells were identified using the Aqua dead cell staining kit (Invitrogen). Murine IL-36γ was from R&D. ELISAs for IL-36γ (antibodies-online.com) and IL-22, CXCL1, and CXCL2 (eBioscience) were performed following the manufacturer's instructions.

Isolation of colonic lamina propria (cLP) cells and flow cytometry

Isolation of cLP cells, staining, and analyses were performed as described (13). cLP cells were defined as: CD45+IAb+CD11b+F4/80+CD103-macrophages (Mφ), CD45+IAb +CD11c+F4/80-CD103+ dendritic cells (DC); Lin-CD90+RORgt +NKp46+CD117intCD127+ innate lymphoid cells (ILC), CD45+CD11b+Ly6C+Ly6G+ cells neutrophils (Nφ).

Microarray analysis

Microarray processing was done by Vanderbilt Microarray Shared Resource using Murine Genome 430 2.0 microarray chips (Affymetrix). GEO accession number is GSE68269

(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=olgvmwkanfgzlcd&acc=GSE68269).

Real-time PCR

RNA isolation and first-strand cDNA synthesis were performed as previously described (13). Primers were as previously described (14) except for: m*Il1f9* (F, TTGACTTGGACCAGCAGGTGTG; R, GGGTACTTGCATGGGAGGATAG), h*Il1f9* (F, GTCTATCAATCAATGTGTAAACC; R, ATCTTCTGCTCTTTTAGCTGCAAT). cDNA from human IBD tissues were obtained in plate arrays (OriGene).

In vitro stimulation

Bone marrow derived macrophages (1×10^6 cells/ml) were stimulated with TLR agonists for 12 h.

DSS-induced colitis

Mice were provided 3% (wt/vol) DSS (MP Biomedicals) in the drinking water for 5 d and then switched to normal drinking water for recovery. Daily clinical assessment of DSS-treated animals was performed as described previously (13).

$CD4^+CD45RB^{hi}$-induced colitis

Purified WT $CD4^+CD45RB^{hi}$ T cells (4×10^5) were injected i.p. into *Rag1*$^{-/-}$ recipients. Colons were harvested when clinical signs of chronic colitis were evident (4 wk).

Helicobacter hepaticus-induced colitis

Helicobater hepaticus (1 X 10^8 CFU strain 51449; ATCC) was gavaged to mice on 0, 2, 4 d. Mice also received 1mg of anti-IL-10R (BioXCell; 1B1.2) antibody via i.p. injection on 0, 7, 14, 21 d after *H. hepaticus* infection (15).

Wound healing assays

Mucosal wound healing assays were performed as previously described (16).

In vivo AhR treatment

FICZ (Enzo Life Sciences; 1μg/mouse) was injected i.p. at 3 d of DSS treatment for 5 d.

Histology

Colons were fixed in 10% formalin. Paraffin embedding, sectioning, Hematoxylin/Eosin staining, and slide scanning was performed at the Emory's WCI-Pathology Core.

Statistical analyses

Statistical analyses were performed using unpaired Student's *t* test or one-way ANOVA.

Results and Discussion

IL-36γ is expressed during experimental colitis and human IBD

Resident macrophages ($Cx3cr1^{hi}Ly6C^{-}$) are abundant in the healthy cLP, while a distinct subset of $Cx3cr1^{low/int}Ly6C^{+}$ cells infiltrate the colon after DSS treatment ((17) and Supplemental Fig. 1A), which are predominantly inflammatory macrophages (Mφ). To identify genes that are preferentially expressed by these inflammatory Mφ, we conducted microarray analysis between FACS-sorted $CD45^{+}IAb^{+}CD11b^{+}F4/80^{+}$ cLP Mφ isolated from healthy mice (>80% $Cx3cr1^{hi}Ly6C^{-}$ "resident") or 5 d DSS-treated mice (>50% $Cx3cr1^{lo/int}Ly6C^{+}$ "infiltrating"). Using hierarchical clustering, 820 genes were differentially expressed > 2-fold (log2 scale) between cLP Mφ isolated from healthy control (CTL) or inflamed (DSS) colon (Supplemental Fig. 1B middle panel). Among these genes, the gene ontology (GO) processes enriched by DAVID showed Immune response as top ranked process. Of the 62 genes in this category, the top 10 (fold change) are shown. Interestingly, the novel IL-1 family member Il1f9 (renamed IL-36γ was the top-ranked gene that was most preferentially expressed by cLP Mφ isolated from DSS-treated mice (5.4-fold increase log2 scale) while other members of the IL-1 family only showed modest increases or remained unchanged (data not shown). Validation of these observations by qPCR confirmed that IL-36γ mRNA, but not IL-36α or IL-36β, was highly expressed in the inflamed colonic of DSS-treated mice (Fig. 1A), and correspondingly, protein levels were also increased as detected by western blot (WB) and ELISA (Fig. 1B). This increased IL-36γ expression (~9-fold) in the inflamed colon following DSS treatment was recapitulated in additional models of colitis including the $CD45RB^{hi}$ transfer model (~13-fold), and the *Helicobacter hepaticus* model (15) (~5-fold) (Fig. 1C). Importantly, IL-36γ mRNA expression was also increased in the human colonic mucosa from individuals with IBD when compared to CTL, and no significant differences were noted between UC and CD samples (Fig. 1D).

To investigate cellular sources of IL-36γ in the inflamed intestine, IL-36γ mRNA expression was analyzed among cLP DCs, Mφ, intestinal epithelial lymphocytes (IEL), and intestinal epithelial cell (IEC). cLP Mφ and IECs expressed ~600-fold and ~85-fold higher levels of IL-36γ, respectively, as compared to DCs or IEL (Fig. 1E). As DSS treatment disrupts the epithelial barrier and exposes immune cells to microbes, the role of the microbiota in IL-36γ induction was explored. Conventionally-housed (CNV) or GF mice were treated with DSS for 5 d and IL-36γ mRNA expression was assessed. As shown in Fig. 1F, colonic tissue from GF mice expressed ~25-fold lower levels of IL-36γ mRNA than CNV mice. Consistent with this observation, stimulation of bone marrow-derived macrophages with LPS or CpG significantly induced IL-36γ mRNA expression (Supplemental Fig. 1C).

IL-36R contributes to colonic wound healing

To evaluate the biological functions of IL-36γ *in vivo*, $Il1rl2^{-/-}$ mice were treated with DSS for 5 d and the severity of colonic inflammation was assessed. Consistent with a pro-inflammatory role for the IL-36 pathway (10), we observed modestly decreased DAI in $Il1rl2^{-/-}$ mice when compared to WT ($Il1rl2^{+/+}$) mice as early as 1 d of DSS treatment that remained until 5 d (Fig. 2A). To evaluate recovery and repair from acute mucosal injury,

DSS was replaced with normal water at 5 d and a progressive increase in the DAI for *Il1rl2*$^{-/-}$ mice was observed, which eventually matched that of *Il1rl2*$^{+/+}$ mice on 7 d (Fig. 2A). While *Il1rl2*$^{+/+}$ mice eventually recovered from colitis, the DAI of *Il1rl2*$^{-/-}$ mice continued to increase until they had to be euthanized due to being moribund and having met humane endpoint criteria. Examination of *Il1rl2*$^{-/-}$ colons at sacrifice revealed significant shortening compared to *Il1rl2*$^{+/+}$ colons (Fig. 2B). Additionally, histological analyses of *Il1rl2*$^{-/-}$ colons showed significantly enhanced mucosal damage/inflammation as compared to *Il1rl2*$^{+/+}$ colons (Fig. 2C, 2D). Since *Il1rl2*$^{-/-}$ mice failed to recover from DSS-induced colitis, the role of IL-36R in colonic wound recovery was further examined by mechanically generating mucosal wounds using an endoscope equipped with biopsy forceps. Images captured from the wound sites were used to quantify colonic mucosal wound healing at 2 d and 4 d post-injury (Fig. 2E). Analogous to the defective repair observed with acute DSS treatment, *Il1rl2*$^{-/-}$ mice showed a significant delay in wound healing (35.1% wound closure compared with 51.7% in *Il1rl2*$^{+/+}$ mice; Fig. 2F). Interestingly, impaired wound healing in *Il1rl2*$^{-/-}$ mice correlated with a significant reduction in neutrophil accumulation in the wound bed (Supplemental Fig. 2A, 2B). Since neutrophils can aid in wound repair (18), these cells were examined in the wound beds of *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ mice following mucosal biopsy. As shown in Supplementary Fig. 2A, 2B, wound beds of from *Il1rl2*$^{-/-}$ mice exhibited a significant reduction in neutrophil accumulation as compared to *Il1rl2*$^{-/-}$ mice. Consistent with these observations, treatment of colonic explants or intestinal epithelial cell cultures with IL-36γ significantly induced expression of the neutrophil chemokines CXCL1 and CXCL2 (Supplemental Fig. 2C, 2D). These data were also supported by evidence showing that intestinal epithelial cells constitutively expressed Il1rl2 mRNA during the steady-state and following treatment of mice with DSS (data not shown). Collectively, these results suggest an important role for IL-36R in promoting wound healing of damaged intestinal mucosa.

IL-36R contributes to DSS-induced IL-22 production

IL-22 is a barrier-protective cytokine that stimulates epithelial proliferation and restitution, the secretion of antimicrobial peptides, and protection from intestinal inflammation (19). Since *Il1rl2*$^{-/-}$ mice displayed impaired recovery from DSS-induced damage, the role of IL-36R in IL-22-mediated intestinal barrier protection was investigated. Interestingly, upon DSS treatment IL-36γ expression preceded that of IL-22 with IL-36γ peaking on 3 d followed by IL-22 beginning on 4 d and peaking on 5 d (Fig. 3A). These data suggested that IL-36γ may regulate IL-22 production following DSS-induced damage. Therefore, IL-22 expression in the colon of *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ mice following DSS treatment was quantitated. After 5 d of DSS treatment, *Il1rl2*$^{-/-}$ mice had significantly reduced IL-22 protein expression when compared to *Il1rl2*$^{+/+}$ mice (Fig. 3B). IL-22 protein expression in *Il1rl2*$^{+/+}$ colons returned to baseline by 8 d as these mice repaired intestinal damage, while *Il1rl2*$^{-/-}$ mice failed to induce IL-22 at 8 d as intestinal damaged progressed. Interestingly, robust IL-22 expression in *Il1rl2*$^{+/+}$ colons following DSS was abolished in GF mice, similar to IL-36γ expression (data not shown). These data demonstrate that the IL-36 pathway is involved in DSS-induced IL-22 expression.

In the DSS model, IL-22-producing neutrophils provide a major contribution to the resolution of colonic injury (14). Thus, we explored whether there may be defects in IL-22-producing neutrophils following DSS treatment in the absence of IL-36R. Indeed, a significant decrease in IL-22 production by neutrophils from *Il1rl2*$^{-/-}$ mice was observed when compared to *Il1rl2*$^{+/+}$ mice for both frequency (Fig. 3C) and absolute cell number (Fig. 3D), while T cells and ILC did not exhibit significant differences. These data are consistent with the observations of Zindl et al (14), showing that IL-22-producing neutrophils are important contributors to resolution of DSS-induced colonic damage and suggest that IL-36R is involved in the differentiation of this important cell type. Of note, very low levels of IL-22 were detected in *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ wound beds following mucosal biopsy (data not shown), suggesting that the extent and type of injury dictates whether the IL-36 pathway may predominantly control neutrophil recruitment and/or differentiation into IL-22 producers.

FICZ induces IL-22 and resolution of colonic damage in *Il1rl2*$^{-/-}$ mice

Since IL-22-producing neutrophils were significantly reduced in colons of DSS-treated *Il1rl2*$^{-/-}$ mice (Fig. 3C/D), we next investigated whether boosting IL-22 production from other cellular sources in vivo could complement this defect. The AhR pathway has been shown to induce IL-22 production from CD4+ T cells and ILC3s in the intestine (20, 21), therefore, we treated *Il1rl2*$^{-/-}$ mice with the AhR agonist 6-formylindolo(3, 2-b) carbazole (FICZ) during the course of DSS treatment beginning at 3 d. While *Il1rl2*$^{-/-}$ mice failed to recover from DSS-induced intestinal mucosal damage and had to be euthanized consistent with Fig. 2A and 2B, FICZ treated *Il1rl2*$^{-/-}$ mice showed a significant reduction in DAI (Fig. 4A) and histological damage (Fig. 4B, 4C), and were able to fully recover from intestinal damage similar to *Il1rl2*$^{+/+}$ mice or *Il1rl2*$^{+/+}$ mice treated with FICZ. Importantly, FICZ treatment enhanced IL-22 mRNA expression in *Il1rl2*$^{-/-}$ mice during DSS-induced damage (Fig. 4D), however, we do not exclude the possibility that FICZ mediated protective effects independent of IL-22 induction.

Collectively, these data implicate the IL-36/IL-36R axis in the repair of intestinal mucosal wounds. While the IL-36 pathway plays a pathogenic role in chronic psoriatic disorders (10), it is beneficial during acute intestinal damage. Neutrophils may play a central role in IL-36-mediated resolution of intestinal damage by phagocytosing and killing bacteria and/or producing IL-22, and other pro-resolving factors (22), that contribute to epithelial barrier repair and control of inflammation. Future insight into the functions of the IL-36 pathway during acute and chronic mucosal inflammation may contribute to the development of novel therapeutic strategies aimed at manipulating this cytokine axis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CD	Crohn's disease
cLP	colonic lamina propria
CTL	healthy control
CNV	conventionally-housed
DAI	disease activity index
DC	dendritic cell
DSS	dextran sodium sulfate
FICZ	6-formylindolo (3, 2-b) carbazole
GF	germ-free
GO	gene ontology
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
ILC	innate lymphoid cells
LI	large intestine
Mφ	macrophages
Nφ	neutrophils
UC	ulcerative colitis
WB	western blot
WT	wild-type

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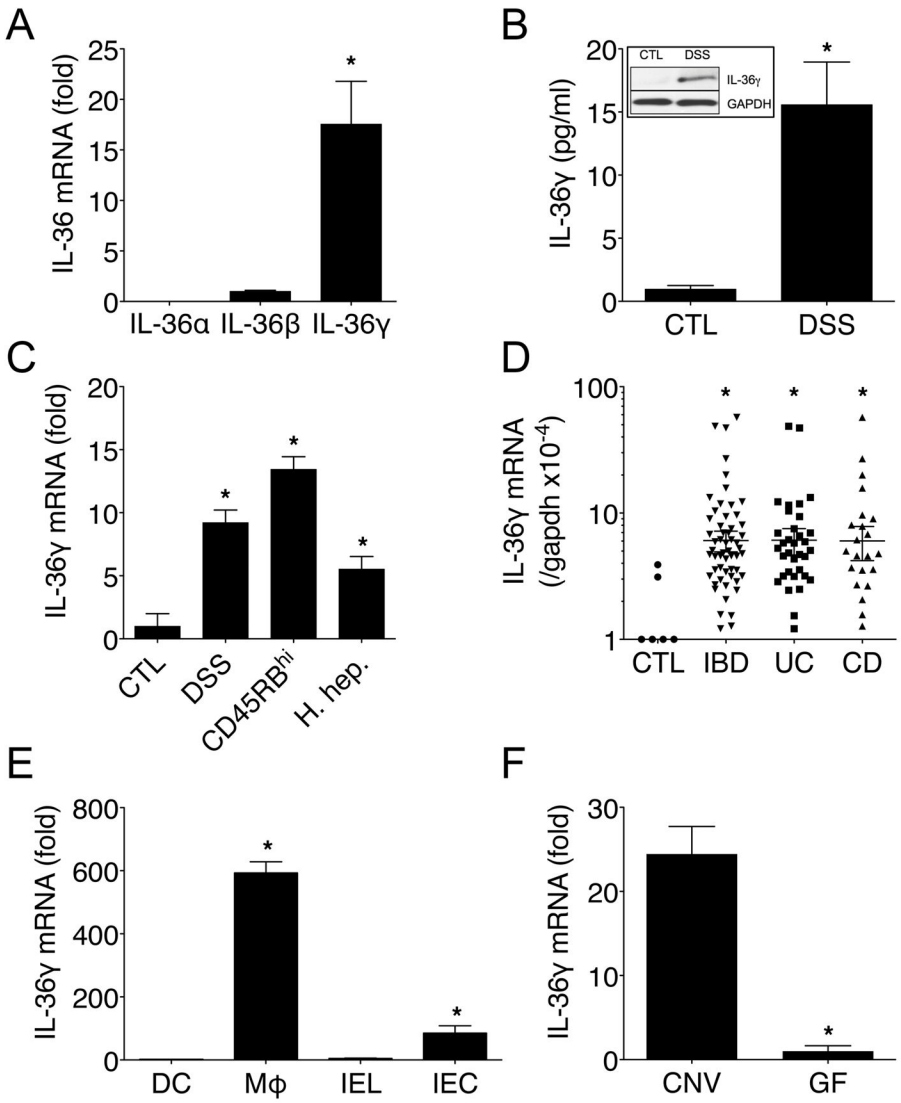

FIGURE 1.

IL-36γ is expressed during experimental colitis and human IBD. (**A**) IL-36γ, IL-36γ, and IL-36γ mRNA expression in colons of WT mice treated for 5 d with DSS. (**B**) ELISA and WB analyses of IL-36γ in colons of WT mice treated as in (A). (**C**) IL-36γ mRNA expression in colonic tissue from CTL, DSS (5 d), CD45RB^{hi}, and *H. hepaticus* models of colitis. (**D**) IL-36γ mRNA expression in CTL, IBD, UC, or CD human mucosa samples. (**E**) IL-36γ mRNA expression from cLP DC, Mφ, IEL, and IEC from WT mice treated as in (A). (**F**) IL-36γ mRNA expression in colons of WT mice housed under CNV or GF conditions and treated as in (A). Data are representative of two or three independent experiments with four or five mice per group. *$P < 0.05$.

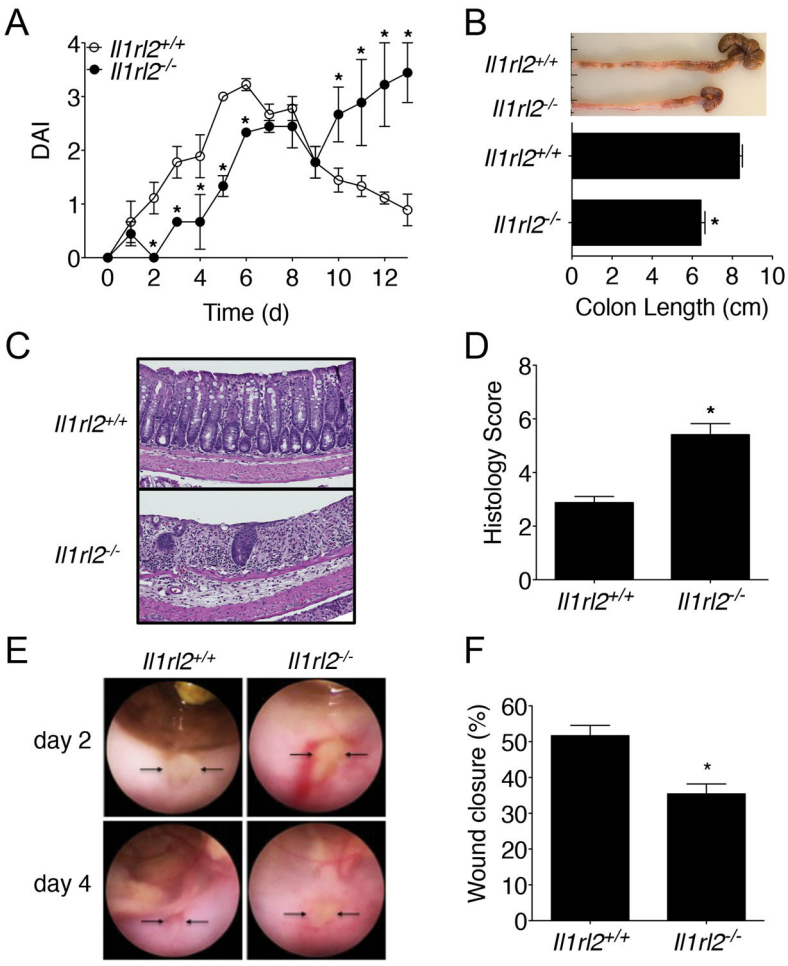

FIGURE 2.

IL-36R contributes to colonic wound healing. (**A**) DAI of *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ mice treated for 5 d with DSS followed by normal water for 8 d. (**B**) Image and length of *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ colons from mice treated as in (A) at 13 d. (**C**) H&E staining and (**D**) histology scoring of colon sections from *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ mice treated as in (A) at 13 d. (**E**) Endoscopic images at 2 d and 4 d and (F) Quantification at 4 d of wound repair in *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ mice after biopsy-induced injury. Arrows indicate the wound bed. Data are representative of three independent experiments with five mice per group. *$P < 0.05$.

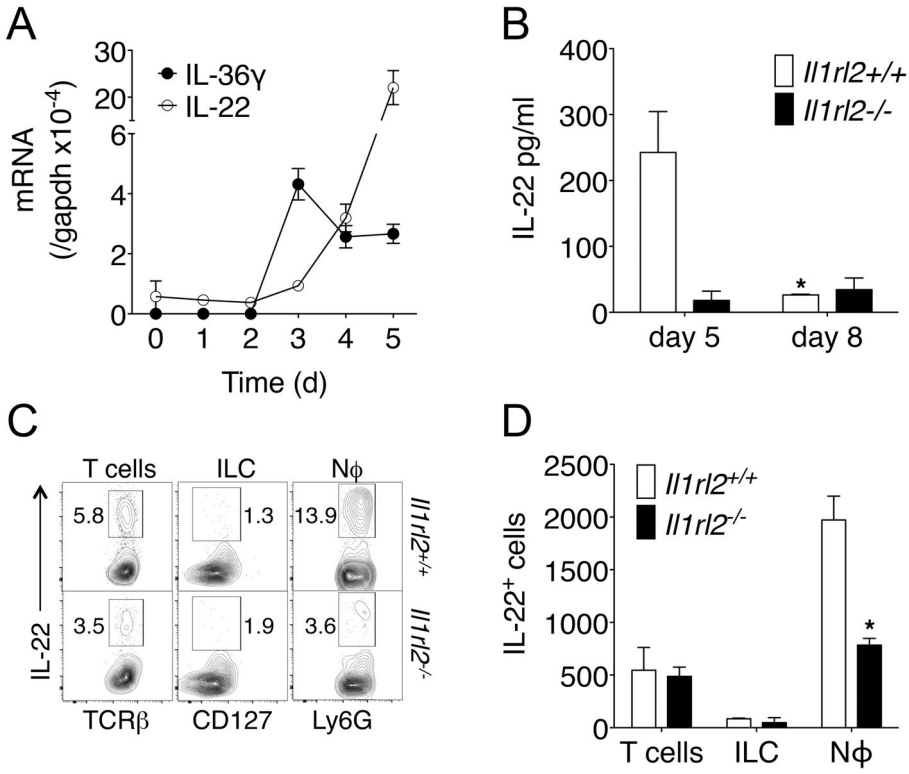

FIGURE 3.

IL-36R contributes to DSS-induced IL-22 production. (**A**) Time course of IL-36γ and IL-22 mRNA expression in colons of WT mice treated for 5 d with DSS. (**B**) ELISA of IL-22 in colons of *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ mice treated for 5 d or 8 d with DSS. (**C**) FACS plots and (**D**) absolute cell numbers of IL-22-producing colonic T cells, ILCs and Nφ in the colon of *Il1rl2*$^{+/+}$ or *Il1rl2*$^{-/-}$ mice treated as in (A). Data are representative of two or three independent experiments with four or five mice per group. **P* < 0.05.

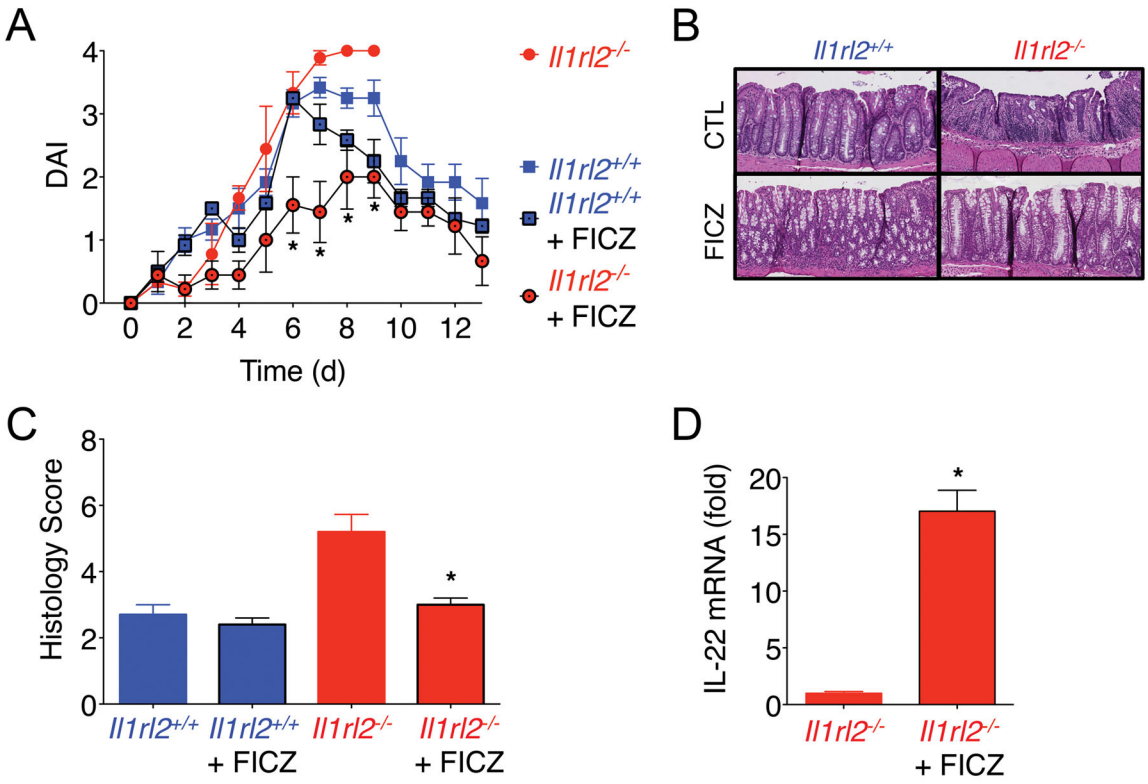

FIGURE 4.

FICZ induces IL-22 and resolution of colonic damage in *Il1rl2*$^{-/-}$ mice. (**A**) DAI of *Il1rl2*$^{+/+}$ (blue) and *Il1rl2*$^{-/-}$ (red) mice treated for 5 d with DSS followed by normal water for 8 days, in the presence (black line) or absence (no line) of FICZ. (**B**) H&E staining and (**C**) histology scoring of colon sections of *Il1rl2*$^{+/+}$ and *Il1rl2*$^{-/-}$ mice treated as in (A). (**D**) IL-22 mRNA expression in colons from DSS-treated *Il1rl2*$^{-/-}$ mice in the presence or absence of FICZ (5 d). Data are representative of three independent experiments with five mice per group. *$P < 0.05$.