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KSR1 protects from interleukin-10 deficiency-induced colitis in mice by suppressing T lymphocyte interferon-γ production

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Abstract

BACKGROUND AND AIMS—Immunological disorders of the gastrointestinal tract such as inflammatory bowel disease (IBD) often result in recurrent and persistently elevated levels of pro-inflammatory cytokines. Kinase suppressor of Ras 1 (KSR1) is involved in TNF-mediated colon epithelial cell survival, yet its role in chronic inflammation has not been defined. In this study, we tested the hypothesis that KSR1 is protective against spontaneous experimental colitis.

METHODS—*KSR1*$^{-/-}$*Interleukin-10 (Il10)*$^{-/-}$ mice were generated and histolopathologic parameters of intestinal inflammation were scored. Bone marrow transplants performed on wild-type (WT) and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice determined the contribution of KSR1 in hematopoietic lineages. Mucosal T helper (Th)1 and Th17 cytokine were also examined. *In vitro* Th1 and Th17

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J.A.G. wrote the paper, J.A.G., H.M.S.A., D.O.V. designed experiments and acquired the data, M.K.W., R.C., K.T.W., and L.V.K. assisted with data interpretation, and D.B.P supervised the study.

polarization assays were conducted and interleukin-17A (IL-17A) and interferon-γ (IFN-γ) production analyzed by flow cytometry. Neutralizing antibodies against IgG, IL-17A, or IFN-γ were administered to 3-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice for 3 weeks and scored for colitis.

RESULTS—*KSR1*$^{-/-}$*Il10*$^{-/-}$ mice developed accelerated and severe spontaneous colitis by 4 weeks of age. KSR1 expression in hematopoietic lineages was protective against colitis. Both IFN-γ and IL-17A transcripts were elevated in colons of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. IFN-γ production was increased in lamina propria T cells isolated from *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Additionally, *in vitro* Th1 polarization was increased while Th17 polarization was impaired in KSR1 deficient naïve T cells. Finally, administration of IFN-γ neutralizing antibodies attenuated colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice.

CONCLUSIONS—Mice lacking both KSR1 and IL-10 develop exacerbated colitis due to dysregulated IFN-γ production in T lymphocytes.

Keywords

KSR1; IL-10; IFN-γ; IL-17A

Introduction

Pathogen defense mechanisms, such as innate and adaptive immunological responses, have greatly contributed to the success of vertebrate evolution. While acute inflammatory responses are beneficial to the host, chronic inflammation can be deleterious. Thus, identification of the molecules involved in regulating these responses is critical for understanding the mechanisms that can contribute to immunological disorders. For patients suffering from inflammatory bowel disease (IBD), dysregulated immune responses result in sustained immune cell activation and elevated cytokine production, which can lead to an increased risk for developing colon cancer over their lifetime.[1, 2] Therefore, it is imperative to develop strategies and identify novel targets that modulate immune responses to complement current therapeutic options for IBD patients.

Much of our current understanding of the molecular mechanisms involved in IBD has come from knockout, transgenic, and chemically-induced mouse models. Although mouse models of IBD do not fully recapitulate the human disease, interleukin-10 deficient (*Il10*$^{-/-}$) mice display similar characteristics to that of human Crohn's disease.[3, 4] Since the anti-inflammatory effects of IL-10 are required to regulate Th1 cytokine production and promote immune homeostasis,[5, 6] loss of IL-10 in mice results in spontaneous enterocolitis driven by an aberrant immunological response to enteric antigens.[7, 8]

Kinase suppressor of Ras-1 (KSR1) functions as a kinase or molecular scaffold of the Raf/MEK/ERK signaling module to regulate proliferation, apoptosis or function in a cell/tissue context-dependent manor.[9–11] For example, KSR1 is required for colon epithelial cell survival downstream of TNF signaling both *in vitro* and *in vivo*.[12, 13] Given that pro-inflammatory cytokines, including TNF, are elevated in *Il10*$^{-/-}$ mice, we hypothesized that loss of KSR1 in *Il10*$^{-/-}$ mice would exacerbate colitis. Therefore, we crossed *KSR1*$^{-/-}$ mice with *Il10*$^{-/-}$ mice to generate *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice to examine the role of KSR1 as a protective mediator during chronic inflammation.

In the present study, we found that *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice developed an early onset form of severe colitis resulting from loss of KSR1 expression in hematopoietic lineages. Specifically, KSR1 deficient T cells produced more IFN-γ, had a greater propensity to polarize along the Th1 axis *in vitro*, and neutralizing IFN-γ attenuated disease in

KSR1$^{-/-}$*Il10*$^{-/-}$ mice. The data presented here, along with recent reports, reveal an emerging role for KSR1 in immune function.

Materials and Methods

Animals

BALB/c (WT), *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were bred and maintained on a BALB/c background. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

Colonoscopy

10-week old mice were sedated with an intraperitoneal injection of ketamine (5 mg/ml)/ xylazine (0.5 mg/ml) solution (100 μl/10g body weight). Mouse colons were visualized using a KARL STORZ Veterinary Endoscope (KARL STORZ Imaging, Inc., Goleta, CA) according to procedures previously described.[14]

Histopathology

Colon sections were stained with Hematoxylin and Eosin (H&E) and analyzed by light microscopy. A pathologist (M.K.W.) blinded to the genotypes and conditions used a scoring method previously described to evaluate colitis.[15]

Reagents

Fluorochrome coupled anti-CD4, -TCRβ, -IFN-γ, and anti-IL-17 as well as unconjugated anti-CD3, -CD28, -IFN-γ, -IL-4 and rmIL-6 were all purchased from BD Pharmingen (San Jose, CA). rhTGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Lymphocyte isolation

Splenic were isolated using conventional procedures. Lamina propria T cells were obtained as previously described.[16]

Intracellular cytokine staining

Stimulation, surface and intracellular staining was performed as described.[16] Cells were acquired in a FACSCalibur instrument and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

In vivo cytokine neutralization

3-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were given intraperitoneal injections of 100 μg/mouse anti-IgG (eBioscience, cat#16-4301, San Diego, CA), anti-IFN-γ (eBioscience, clone XMG 1.2), or anti-IL-17A (eBioscience, clone: eBioTC11-18H10.1) neutralizing antibodies twice per week for 3 weeks. Mouse colons were harvested and scored as before.

Statistics

Histopathology, immunohistochemistry, and qPCR statistical analysis were compared using one-way ANOVA followed by Dunnett's post-test. Bone marrow transplant statistical analysis was conducted using one-way ANOVA followed by Bonferroni's multiple comparison test. Th1/Th17 polarization, intracellular cytokine staining, and cytokine neutralization statistical analysis were performed using an unpaired Student's t test. P-values of < 0.05 were considered significant. All data was analyzed using Prism 5 (GraphPad Software, Inc.).

Bone marrow transplantation

Please see detailed Supplementary Materials and Methods.

Generation of Th17 and Th1 cells

Please see detailed Supplementary Materials and Methods.

Results

KSR1$^{-/-}$Il10$^{-/-}$ mice develop accelerated spontaneous colitis

Although KSR1 inhibits epithelial cell apoptosis downstream of TNF signaling,[13] the role of KSR1 during chronic inflammation has not been examined. Since *Il10*$^{-/-}$ mice on the BALB/c (WT) strain under SPF conditions develop spontaneous colitis between 12–16 weeks of age, we examined the effect of KSR1 deficiency on colitis in *Il10*$^{-/-}$ mice. The clinical onset of disease was assessed for each group of mice by examination of fecal occult blood and monitoring whole body weight from 3 to 10 weeks. *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice frequently suffered from chronic diarrhea and failed to thrive beginning at 5 weeks of age (Figure 1*A*). By colonoscopic examination, the colons of 10-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice appeared thickened with a loss of translucency compared to the colons of WT, *KSR1*$^{-/-}$, and *Il10*$^{-/-}$ mice (Figure 1*B*). Since colon weight per unit length is often indicative of inflammation, the colon length-to-weight (l/w) ratio was quantified for each group. *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had an average l/w ratio of 15.03 ± 1.5 whereas WT, *KSR1*$^{-/-}$, and *Il10*$^{-/-}$ mice had ratios of 44.36 ± 2.96, 41.64 ± 3.24, and 38.19 ± 2.34 respectively (Figure 1*C*). Paraffin-embedded colon sections from 10-week old mice were stained with Hematoxylin and Eosin (H&E) and a blinded pathologist scored each section for inflammation and injury. While 10-week old WT, *KSR1*$^{-/-}$, and *Il10*$^{-/-}$ were relatively free of inflammation and injury, *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice suffer from severe colitis (score of 9.83 ± 0.6) (Figures 1*D*–1*E*). Barrier permeability was assed by administering FITC-dextran enemas to 10-week old mice, peripheral blood serum collected one hour later, and quantified for the presence of translocated FITC. *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had increased barrier permeability, possibly due to the increased epithelial cell turnover as determined by apoptotic and proliferative markers (Supplemental Figure 1). We then evaluated the developmental time course of colitis and found that by 4 weeks of age, *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice already have histological signs of disease (Figure 1*F*). These data indicate that loss of KSR1 expression in *Il10*$^{-/-}$ mice results in accelerated and severe spontaneous colitis with retarded growth similar to findings in children with Crohn's disease.[17]

Expression of KSR1 in hematopoietic lineages is protective against colitis

The disease that develops in *Il10*$^{-/-}$ mice is attributed to immune hypersensitivity to enteric microflora.[7, 8] Therefore, we investigated whether KSR1 expression in hematopoietic lineages mediated protection from disease in *Il10*$^{-/-}$ mice. Since the *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$ *Il10*$^{-/-}$ mice used in this study were engineered as global deletions, we performed bone marrow transplantation on irradiated 4-week old WT or *KSR1*$^{-/-}$*Il10*$^{-/-}$ recipient mice. Following bone marrow transplantation, recipient mice were sacrificed at 10 weeks of age and colitis was assessed as before. Irradiated *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice reconstituted with WT bone marrow (BM) developed mild colitis scores (2.8 ± 1.12) while reconstitution with *KSR1*$^{-/-}$*Il10*$^{-/-}$ bone marrow resulted in severe colitis as expected (11 ± 1) (Figures 2*A* and 2*B* panels 1 & 4). Interestingly, restoring IL-10 to the immune system in *KSR1*$^{-/-}$*Il10*$^{-/-}$ recipient mice did not ameliorate colitis (8.1 ± 1.72) (Figures 2*A* and 2*B* panel 3). However, restoring KSR1 to the immune system in *KSR1*$^{-/-}$*Il10*$^{-/-}$ recipient mice (5.5 ± 1.25) attenuated the disease (Figures 2*A* and 2*B* panel 2). Interestingly, reconstitution of irradiated WT mice with *Il10*$^{-/-}$ BM was insufficient to cause disease, while reconstitution with

KSR1$^{-/-}$*Il10*$^{-/-}$ BM was sufficient to drive colitis (5.25 ± 0.84) (Figure 2). Though suppression of colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice required KSR1 expression in cells of the immune system, KSR1 was not required for leukocyte differentiation or stimulated T cell proliferation *in vitro* (Supplemental Figures 2*A* & 2*B*). Taken together, our data suggest that KSR1 expression in hematopoietic lineages plays a significant role in suppressing colitis in *Il10*$^{-/-}$ mice.

Lamina propria T cells isolated from KSR1$^{-/-}$ and KSR1$^{-/-}$Il10$^{-/-}$ mice have increased IFN-γ production

Pro-inflammatory cytokines including TNF, IFN-γ, and IL-17 are increased in the intestinal mucosa of Crohn's disease patients and in mouse models of IBD.[18–22] Therefore, we determined relative transcript levels of Th1 and Th17 cytokines in the colonic mucosa of WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice by quantitative real-time PCR (qRT-PCR). Both IFN-γ and IL-17A transcripts were increased KSR1 deficient mice (Figure 3*A*). In addition, colon mucosal lysates were screened and cytokine protein quantified using a Milliplex cytokine bead array. Consistent with the qRT-PCR data, IFN-γ was also increased in colons of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (8.79 ± 0.6 pg/mg and 93.7 ± 52.6 pg/mg) compared to WT (0.02 ± 0.0 pg/mg) and *Il10*$^{-/-}$ mice (Figure 3*B*). Neither message nor protein levels of IL-1β, IL-21, IL-22, or IL-23 were altered by KSR1 expression status (data not shown). We then examined IFN-γ and IL-17A production in T cells isolated from the lamina propria of WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice and stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin. The percentage of TCRβ^{+} lamina propria T cells producing IFN-γ was significantly increased in *KSR1*$^{-/-}$ mice (10.58% ± 1.21%) and elevated in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (5.24% ± 1.22%) compared to *Il10*$^{-/-}$ mice (1.4% ± 0.12%) and WT mice (2.95% ± 0.52%) (Figure 4). Interestingly, IL-17A production was decreased in KSR1 deficient lamina propria T cells. Consistent with these data, isolated CD4^{+}TCRβ^{+} splenocytes from *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice also exhibited elevated IFN-γ production (Supplemental Figure 3). Thus, KSR1 expression modulates IFN-γ production in TCRβ^{+} T lymphocytes.

In vitro Th1 polarization is enhanced while Th17 polarization is impaired in KSR1 deficient T cells

IL-17A production by Th17 effector cells has been implicated in the pathogenesis of diseases including rheumatoid arthritis, multiple sclerosis, and Crohn's disease.[23–25] Our previous observations that IL-17A transcripts and protein levels were altered in mice lacking KSR1 suggested that KSR1 may be involved in T cell development along the Th1/Th17 axis. To test this, we isolated splenocytes from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice and cultured them under Th1 or Th17 polarizing conditions. We then stimulated with PMA/ionomycin for 5 hours in the presence of GolgiPlug and measured intracellular IL-17A and IFN-γ production by flow cytometry. We found that under Th17 polarizing conditions, the number of IL-17A expressing cells was reduced in both *KSR1*$^{-/-}$ (3.20% ± 1.5%) and *KSR1*$^{-/-}$*Il10*$^{-/-}$ (5.9% ± 0.48%) CD4^{+}TCRβ^{+} T cells compared to WT (13.48% ± 1.34%) and *Il10*$^{-/-}$ (37.43% ± 8.3%) CD4^{+}TCRβ^{+} T cells (Figures 5*A*–*B*). In fact, the number of CD4^{+}TCRβ^{+} T cells producing IFN-γ was increased for *KSR1*$^{-/-}$ (10.32% ± 3.0%) and *KSR1*$^{-/-}$*Il10*$^{-/-}$ (6.8% ± 1.7%) T cells compared to WT (0.89% ± 0.4%) and *Il10*$^{-/-}$ (2.84% ± 0.7%) T cells even when cultured under Th17 polarizing conditions (Figures 5*A*–*B*). We then determined if loss of KSR1 enhanced T cell development along the Th1 axis *in vitro*. Splenocytes were isolated and cultured under Th1 polarizing conditions and analyzed as before. We found that *KSR1*$^{-/-}$ T cells expressing IFN-γ under Th1 polarization was increased (56.1% ± 1.9%) compared to WT (44.8% ± 3.4%) (Figures 5*C*–*D*). Interestingly, culturing splenocytes from *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice under Th1 polarizing conditions resulted in

reduced T cell viability. These findings suggest that *KSR1*$^{-/-}$ T cells have a greater propensity to develop along the Th1 axis *in vitro*.

Neutralization of IFN-γ attenuates severity of disease in KSR1$^{-/-}$Il10$^{-/-}$ mice

Our data showing that colons of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had increased in IFN-γ transcript and protein levels, together with the data indicating that *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ CD4^{+}TCRβ^{+} T cells have enhanced IFN-γ production, suggest a potential mechanism driving the pathogenesis of colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Since colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice was detected by 4 weeks of age, we administered intraperitoneal injections of anti-IgG (αIgG), anti-IFN-γ (αIFN-γ), or anti-IL-17A (αIL-17A) neutralizing antibodies in 3-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice twice per week for 3 weeks to determine the role of IFN-γ in disease *KSR1*$^{-/-}$*Il10*$^{-/-}$ pathogenesis. Three days following the final antibody injection mice were sacrificed and colons were flushed, measured, and weighed and then paraffin embedded. The colon length to weight ratio for mice receiving αIFN-γ was increased (26.92 ± 1.86) compared to mice receiving αIgG or αIL-17A (18.70 ± 0.90 and 21.60 ± 1.29 respectively), indicative of reduced inflammation (Figure 6*A*). H&E stained mouse colon sections were obtained and scored for inflammation and injury as before. While neutralization of IL-17A caused no statistical decrease in colitis severity over isotype control (8.75 ± 1.5 vs. 9.75 ± 1.0), treatment with αIFN-γ significantly reduced the severity of colitis (6.3 ± 1.1) in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (Figures 6*B* & 6*C*). In addition, cytokine transcript levels were measured for TNF, IFN-γ, IL-1β, IL-17A, IL-22, and KC for all three groups of mice. While neutralizing IFN-γ resulted in diminished TNF and IFN-γ mRNA levels, neutralizing IL-17A significantly reduced KC transcription, indicating IL-17A inhibition (Supplemental Figure 4). Collectively, these data indicate that the severity of colitis in *KSR1*$^{-/-}$ *Il10*$^{-/-}$ mice is mediated, at least in part, by the increase in IFN-γ that is associated with loss of KSR1.

Discussion

While distinct roles for KSR1 have been reported for intestinal epithelial cells,[12, 13] T cell proliferation,[26] T cell differentiation,[27, 28] and recently NK cell-mediated cytolysis,[29] the role of KSR1 in inflammatory diseases has not been defined. In this study, we utilized the IL-10 deficiency-induced mouse model of spontaneous experimental colitis to investigate the role of KSR1 during chronic inflammation. We found that *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice developed accelerated severe spontaneous colitis with 100% penetrance by 4 weeks of age. The pathogenesis of the disease was predominantly attributed to loss of KSR1 in hematopoietic lineages. In addition, IFN-γ transcript and protein levels were elevated in colons of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. The percent of IFN-γ producing TCRβ^{+} T cells isolated from the lamina propria of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were increased compared to WT and *Il10*$^{-/-}$ mice. KSR1 deficient naïve T cells also exhibited a greater propensity to develop along the Th1 axis *in vitro* while *in vitro* Th17 development was impaired. Finally, administration of αIFN-γ neutralizing antibody attenuated colitis severity in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Collectively, the data presented here implicate KSR1 as a regulatory molecule that functions to suppress IFN-γ production in T cells and promotes Th1/Th17 developmental homeostasis.

Early reports characterizing the spontaneous colitis that develops in *Il10*$^{-/-}$ mice established the requirement for intestinal bacterial flora in mediating disease.[7, 8] Since *KSR1*$^{-/-}$ *Il10*$^{-/-}$ mice develop colitis by 4 weeks of age and enteric microbial populations are established just post-weaning,[30] we initially suspected that loss of KSR1 might contribute to epithelial barrier defects. Though epithelial barrier permeability was increased in diseased 10-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice, barrier permeability in 10-week old *KSR1*$^{-/-}$ and *Il10*$^{-/-}$ mice were similar to WT mice (Supplemental Figure 1*A*). Although KSR1 does not appear to be

required for epithelial barrier function in the absence of challenging conditions, we cannot rule out the effect of KSR1 deficiency in the presence of inflammatory conditions since KSR1 protects colon epithelial cells from TNF-induced apoptosis.[12, 13]

We were surprised, however, to find that the protective role of KSR1 was attributed to expression in cells of the immune system (Figure 2). Interestingly, *Il10*$^{-/-}$ BM was insufficient to cause disease in irradiated WT mice, and is consistent with a previous report.[31] The finding that *KSR1*$^{-/-}$*Il10*$^{-/-}$ BM was sufficient to cause disease in irradiated recipient WT mice established a role for KSR1 in disease pathogenesis and immune cell function. Since IFN-γ and IL-17A transcripts were elevated in the colons of disease-free *KSR1*$^{-/-}$ mice and diseased *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (Figure 3*A*), it seemed plausible that KSR1 was involved in T cell development or function. Though current data on the pathogenesis of IL-17A in inflammatory diseases remains unclear,[32–35] the colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice appears to be exacerbated by IFN-γ and not IL-17A (Figure 6). The observation that IFN-γ production and *in vitro* Th1 polarization was elevated in KSR1 deficient T cells suggests a potential pathway by which KSR1 deficiency might exacerbate colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (Figures 4*B* & 5*D*). KSR1 expression modifies signal transduction pathways through association with multiple protein kinases and protein phosphatases including protein phosphatase 2A (PP2A) and 2B (PP2B).[36,37, 38] Suppression of PP2A activity increases IFN-γ production in NK cells.39 It is attractive to speculate that antigenic stimulus in the absence of KSR1 perturbs signaling pathways normally regulated by protein phosphatases. It will be interesting to see if KSR1-associated phosphatases in T lymphocytes are involved in this process.

The crosstalk between innate immune defenses and acquired immunity is actively being investigated for autoimmune diseases and IBD.[40–42] While our data support a role for KSR1 in lymphocyte function in IL-10 deficiency-induced colitis, we cannot exclude a role for KSR1 in innate immune cell function. Consistent with a functional role for KSR1 in innate immune system, we observed decreased nitric oxide production from stimulated bone marrow derived *KSR1*$^{-/-}$ macrophages (unpublished observations). In a secondary mouse model of colitis using dextran sulfate sodium (DSS), which is not dependent on lymphocyte function, [43] *KSR1*$^{-/-}$ mice also exhibited greater sensitivity to DSS-induced injury compared to WT mice (Supplemental Figure 5). While emerging data implicate KSR1 in innate immune cell function,[29] further investigations are necessary to elucidate if KSR1 is involved in innate immune responses and susceptibility to other models of inflammation-associated diseases.

Finally, there may be a connection between vitamin D and KSR1 in immune regulation. Vitamin D receptor (VDR) signaling regulates T cell development and helps maintain immunological tolerance with implications for multiple sclerosis, type-1 diabetes mellitus, and IBD.[44, 45] Recently, polymorphisms at the *VDR* locus were linked to IBD and other autoimmune disorders.[46] Vitamin D supplementation holds promise as a therapeutic agent in the treatment of Crohn's disease by increasing NOD2 expression that then couples to the expression the antimicrobial peptide defensin β2.[47] Interestingly, the *KSR1* promoter region contains a vitamin D responsive element and moreover, KSR1 protein is upregulated by 1, α25-dihydroxyvitamin D_3.[28] In fact, *VDR*$^{-/-}$*Il10*$^{-/-}$ mice develop severe accelerated spontaneous colitis harboring many phenotypic similarities to those observed in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice including increased levels of IFN-γ.[48, 49] It is attractive to speculate that vitamin D-mediated suppression of pathogenic immune responses is, in part, regulated by KSR1 expression and suppression of IFN-γ production in Th1 effector cells.

We conclude that KSR1 expression suppresses IFN-γ production in T lymphocytes and promotes T cell developmental homeostasis along the Th1/Th17 axis. Therefore, induction

of KSR1 expression may be an ideal strategy for modulating IFN-γ in Th1-mediated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CD	cluster of differentiation
DSS	dextran sulfate sodium
FITC	fluorescein isothiocyanate
H&E	Hematoxylin and Eosin
IBD	inflammatory bowel disease
IFN-γ	interferon-gamma
IgG	Immunoglobulin G
IL-10	interleukin-10
IL-17A	interleukin-17A
KSR1	kinase suppressor of Ras 1
NK	natural killer
qRT-PCR	quantitative real-time polymerase chain reaction
SPF	specific pathogen free
TCR	T cell receptor
Th	T helper
TNF	tumor necrosis factor
VDR	Vitamin D receptor
WT	wild-type

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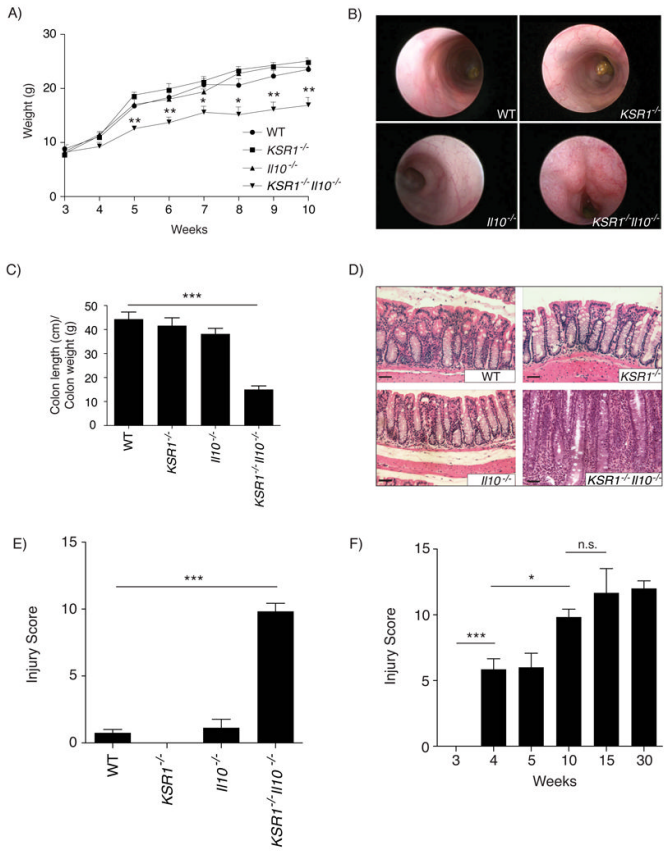

Figure 1. *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice develop accelerated spontaneous colitis

A) WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were weighed weekly from 3 weeks of age to 10 weeks. Plotted data are the mean weight for each group (n ≥ 5). Error bars represent the SEM. B) Mouse colonoscopic images taken on 10-week old mice. C) Colons were removed from 10-week old mice and flushed, weighed, and measured from the cecum to anus. Solid bars represent the mean (n ≥ 3) of the length/weight ratios. Error bars are the SEM. D) Paraffin embedded colon sections from 10-week old mice stained with Hematoxylin and Eosin (H&E). Images were taken at 20X magnification (*scale bars, 50 μm*). E) H&E stained 10-week old mouse colon sections were scored by a pathologist blinded to the genotype. Solid bars represent the mean injury and inflammation score for each group (n ≥ 6) and error bars are the SEM. F) *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were sacrificed at each time point indicated and scored for inflammation and injury as before. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

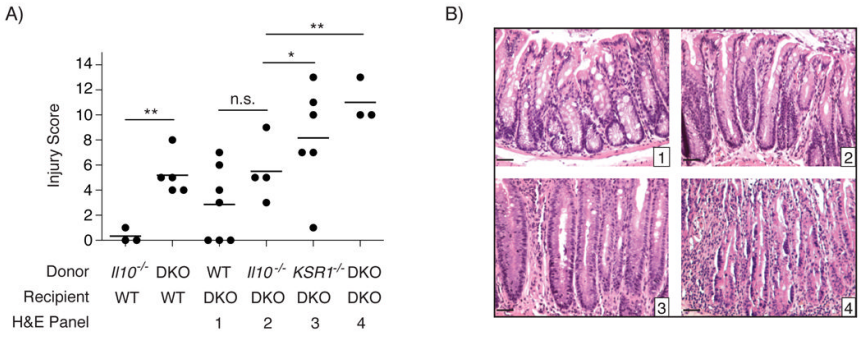

Figure 2. KSR1 in hematopoietic lineages suppresses colitis in *Il10*$^{-/-}$ mice

A) 4-week old WT and *KSR1*$^{-/-}$*Il10*$^{-/-}$ (DKO) recipient mice were irradiated with 9 Gy ^{137}Cesium. Bone marrow transplants using the indicated donor mice were performed and mice were sacrificed at 10-weeks of age. Each individual colonic injury and inflammation score is plotted with a solid line indicating the mean score for each group from three independent experiments and error bars are the SEM. B) Representative H&E stained paraffin embedded colon sections from recipient DKO mice transplanted with WT (1), *Il10*$^{-/-}$ (2), *KSR*$^{-/-}$ (3), or *KSR1*$^{-/-}$*Il10*$^{-/-}$ (4) bone marrow as indicated. * $P < 0.05$, ** $P < 0.01$

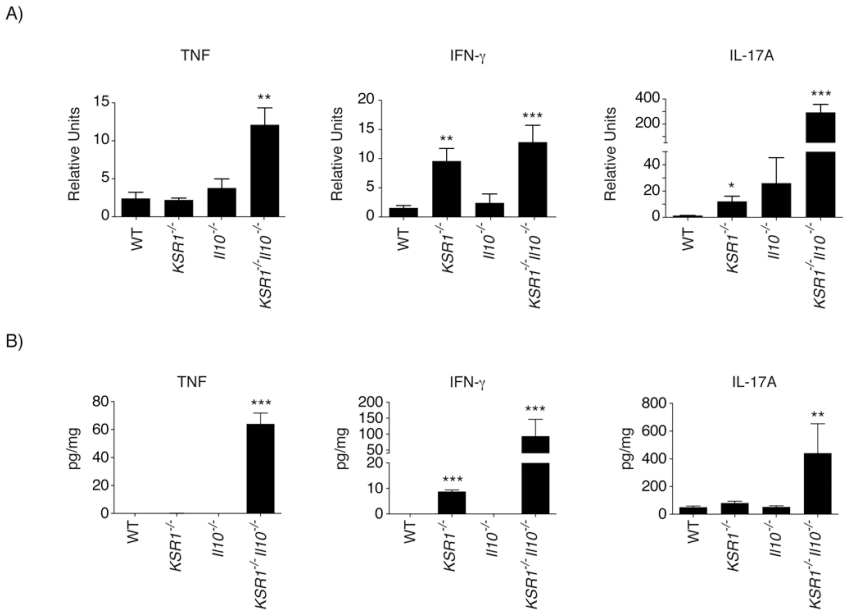

Figure 3. Colons of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice have elevated IFN-γ

A) Total RNA was isolated from homogenized whole colon tissue from 10 week-old WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. TNF, IFN-γ, and IL-17A cytokine transcript levels were analyzed by quantitative real-time PCR. Solid bars represent the mean for each genotype and error bars are the SEM. B) Total protein from homogenized mouse colons was analyzed for the detection and quantitation of TNF, IFN-γ, and IL-17A. Solid bars represent the mean quantity of each cytokine per mg of protein. Error bars are the SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

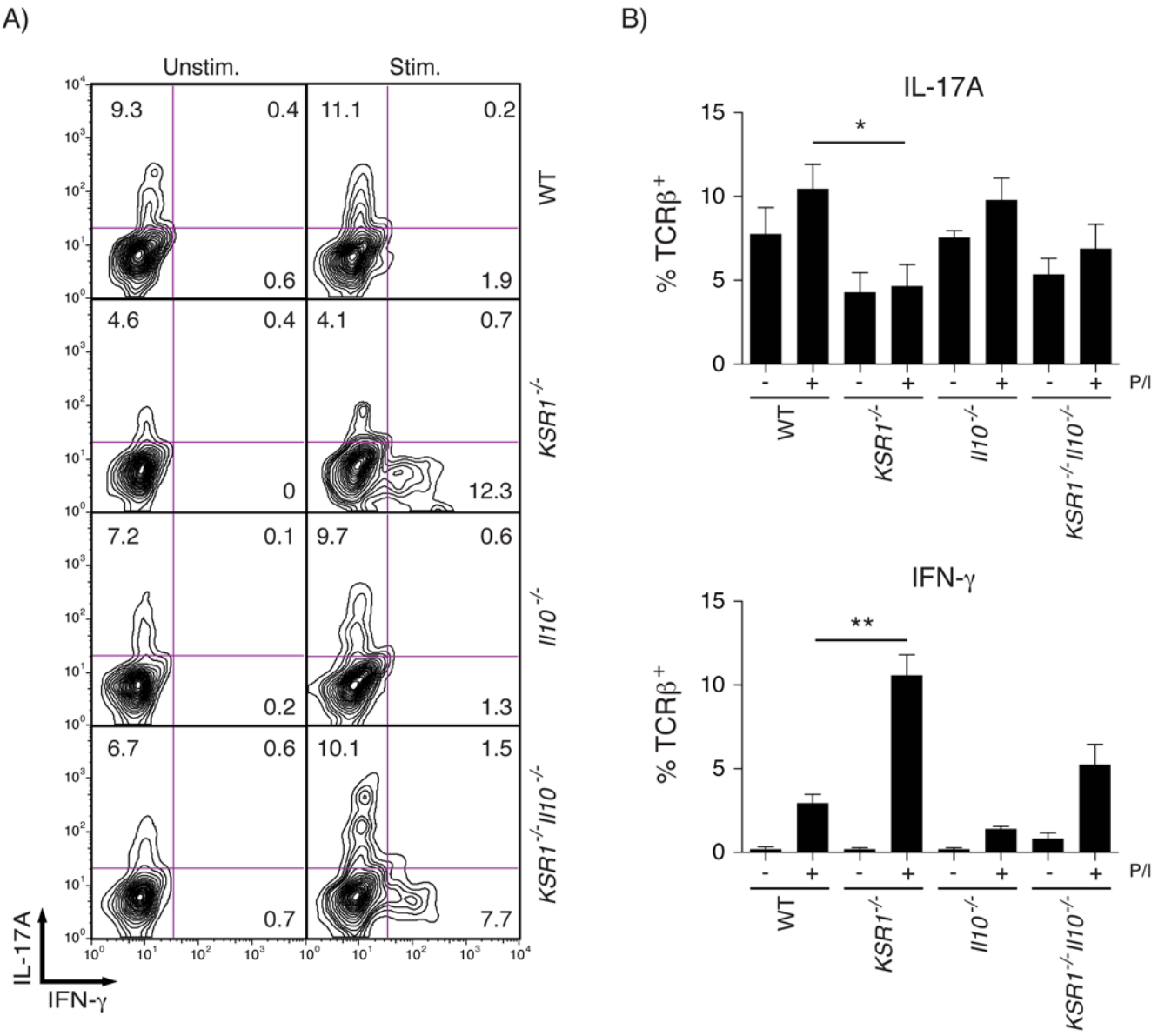

Figure 4. Lamina propria T cells isolated from *KSR1*$^{-/-}$ mice have increased IFN-γ production

Lamina propria T cells were isolated from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice and cultured for 5 hours in the presence of the protein transport inhibitor GolgiPlug and treated with or without PMA/Ionomycin. Cells were stained for cell surface TCRβ and intracellular IFN-γ and IL-17A. Samples were analyzed by flow cytometry gated on lymphocyte geometry and TCRβ^{+} staining. A) Representative flow cytometry contour plots of unstimulated and stimulated WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ lamina propria TCRβ^{+} T cells stained for intracellular IFN-γ and IL-17A. B) The percent of TCRβ^{+} T cells positive for intracellular IFN-γ or IL-17A was determined and solid bars represent the mean while error bars are the SEM. * $P < 0.05$, ** $P < 0.01$

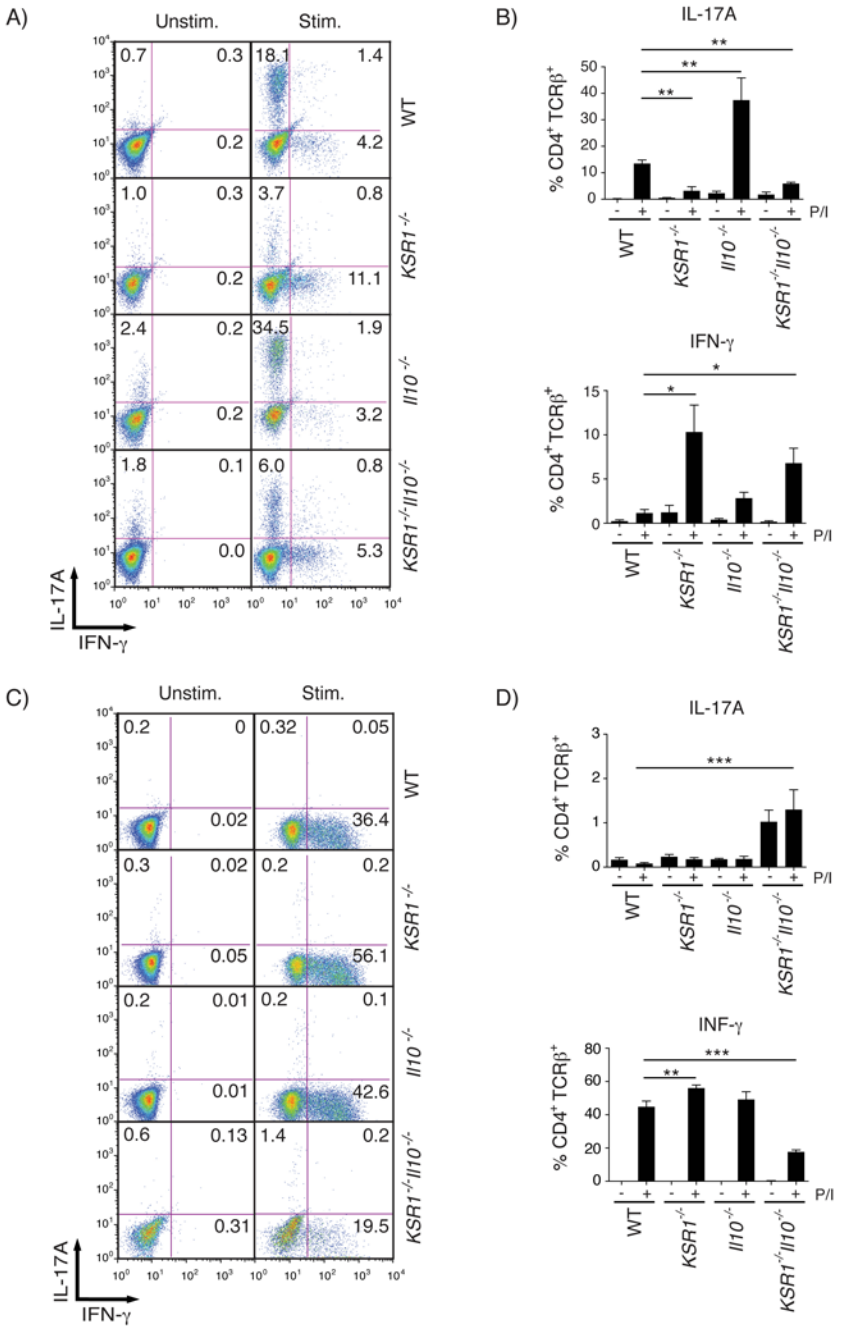

Figure 5. *In vitro* Th17 polarization is impaired while *in vitro* Th1 polarization is increased in *KSR1*$^{-/-}$ T cells

Splenocytes isolated from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were cultured under Th17 or Th1 polarizing conditions. Lymphocytes were stained for cell surface CD4 and TCRβ and intracellular IFN-γ and IL-17A. Samples were analyzed by flow cytometry gated on lymphocyte geometry and CD4^{+}TCRβ^{+} cell surface staining. A) Representative flow cytometry dot plots of unstimulated and stimulated T cells cultured under Th17 polarizing conditions. B) Intracellular cytokine staining on Th17 polarized cells was quantified and reported as the percent CD4^{+}TCRβ^{+} cells staining positive for IL-17A or IFN-γ. Bar graphs represent the mean for each cultured T cell population from two

independent experiments. Error bars are the SEM. C) Representative flow cytometry dot plots of unstimulated and stimulated T cells cultured under Th1 polarizing conditions. D) Intracellular cytokine staining on Th1 polarized cells was quantified and reported as the percent $CD4^{+}TCR\beta^{+}$ cells staining positive for IL-17A or IFN-γ. Bar graphs represent the mean for each cultured T cell population. Error bars are the SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

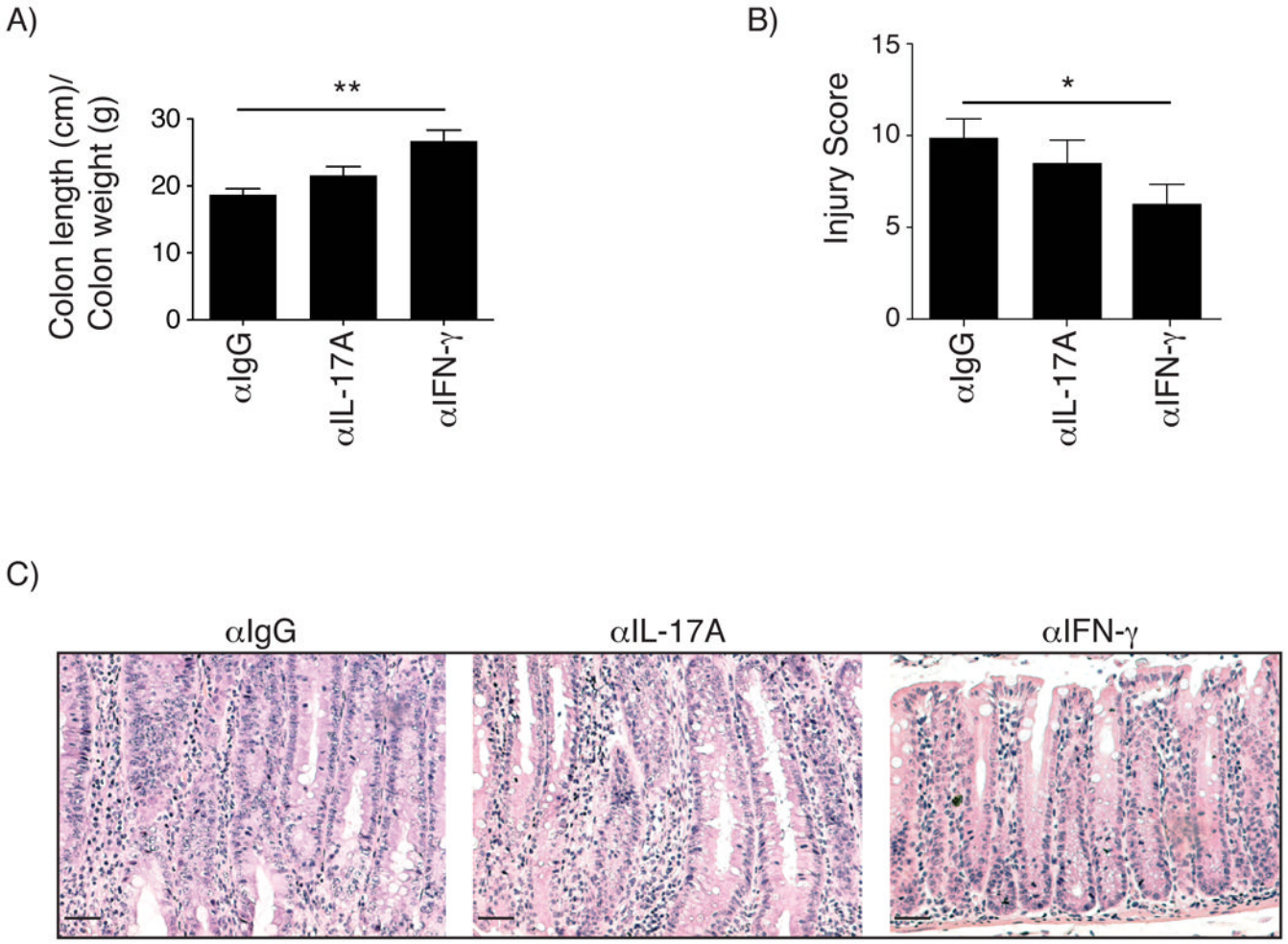

Figure 6. Neutralizing IFN-γ attenuates colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice

3-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were administered 100 μg/mouse neutralizing antibodies against αIgG, αIFN-γ, or αIL-17A intraperitoneally twice per week for a period of 3 weeks and assessed for colitis. A) Mouse colons were removed, flushed, weighed, and measured from the cecum to anus. Solid bars represent the mean length/weight ratio. Error bars are the SEM. B) Colon sections were scored as before for inflammation and injury. Solid bars represent the mean and error bars represent the SEM. C) Representative H&E stained colon sections from *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice administered αIgG, αIFN-γ, or αIL-17A neutralizing antibody. * $P < 0.05$, ** $P < 0.01$