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Interleukin-19 contributes as a protective factor in experimental Th2-mediated colitis

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Abstract

Inflammatory bowel disease results from chronic dysregulation of the mucosal immune system and aberrant activation of both the innate and adaptive immune responses. IL-19 is a member of the IL-10 family, and IL-10 plays important role in inflammatory bowel disease. We have previously shown that IL-19 knockout mice are more susceptible to innate-mediated colitis. Next, we ask whether IL-19 contributes to T cells-mediated colitis. Here, we investigated the role of IL-19 in a mouse model of Th2 cell-mediated colitis. Inflammatory responses in IL-19-deficient mice were assessed using a Th2-mediated colitis induced by oxazolone. The colitis was evaluated by analyzing body weight loss and histology of the colon. Lymph node cells were cultured *in vitro* to determine cytokine production. IL-19 knockout mice exacerbated oxazolone-induced colitis by stimulating the transport of inflammatory cells into the colon, and by increasing IgE production and the number of circulating eosinophil. The exacerbation of oxazolone-induced colonic inflammation following IL-19 knockout mice was accompanied by increased production of IL-4 and IL-9, but no changes in the expression of IL-5 and IL-13 in lymph node cells. IL-19 plays an anti-inflammatory role in the Th2-mediated colitis model, suggesting that IL-19 may represent potential

therapeutic target for reducing colonic inflammation.

Key Words

IL-19, anti-inflammatory cytokine, inflammatory bowel disease

Abbreviations

CD Crohn's disease

DSS dextran sulfate sodium

HE hematoxylin and eosin

IBDs inflammatory bowel diseases

IFN interferon

KO IL-19-knockout

MPO myeloperoxidase

UC ulcerative colitis

Introduction

IL-19 was originally found by sequence homology to IL-10 (Gallagher et al., 2000), and is a member of the IL-10 family, which also includes IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29 (Sabat et al., 2007). Our previous studies showed that IL-19-knockout (KO) mice are more susceptible to experimental acute colitis induced by dextran sulfate sodium (DSS), and this increased susceptibility is correlated with the accumulation of macrophages and the increased production of interferon (IFN)-γ, IL-1β, IL-6, IL-12, TNF-α, and CXCL1 (Azuma et al., 2010a).

DSS-induced colitis is a well-characterized model of colonic inflammation. The adaptive immune system obviously does not play a major part in DSS-induced colitis model, because T- and B-cell-deficient mice also develop severe colonic inflammation in this model (Dieleman et al., 1994). Clinically, Ulcerative colitis (UC) and Crohn's disease (CD) are two of the most common types of inflammatory bowel diseases (IBDs) and are characterized by dysregulated intestinal inflammation and mucosal tissue damage in parts of the gastrointestinal tract (Tong et al., 2013). CD is characterized by transmural and discontinuous inflammation, which is associated with a type-1 response mainly driven by IL-12 and IFN-γ

(Monteleone et al., 2005). In contrast, UC involves the superficial mucosal and submucosal layers of the colon and is driven by type-2 cytokines, such as IL-4, IL-5, and IL-13 (Tanaka et al., 2010). However, little is known about the exact immunological role of IL-19 in the regulation of IBD such as T-cell mediated colonic inflammation.

To determine the potential role of IL-19 in IBD, we previously examined the role of IL-19 in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model which shows a clear type-1 phenotype (Kitani et al., 2000). We demonstrated that IL-19-KO mice aggravated TNBS-induced colitis (Matsuo et al., 2015). Since TNBS-induced colitis resembles the symptom seen in CD, the exact immunological role of IL-19 in the regulation of UC remains still unclear. In this study, we investigated the role of IL-19 in oxazolone-induced colitis which is useful for the study of the type-2 inflammatory response and resembles the symptom seen in UC (Heller et al., 2002; Boirivant et al., 1998).

Materials and Methods

Mice

We constructed targeting vectors for the IL-19 gene and used them to generate IL-19-KO mice. C57BL/6-IL-19 mice were backcrossed onto the Balb/c

genetic background for at least 10 generations. Balb/c-IL-19 heterozygous mice were intercrossed to generate mutant and control mice. Age-matched mice (10–15 weeks old) were used in all experiments. All procedures used in this study complied with institutional policies of the Osaka Prefecture University Animal Care and Use Committee.

Oxazolone-induced colitis

To presensitize mice, a 2 × 2 cm field of the abdominal skin was shaved, and 150 μL of a 4% (w/v) solution of oxazolone in 100% ethanol was applied. Seven days after presensitization, colitis was induced by intrarectal administration with 100 μL of 3% of oxazolone in 50% ethanol using a plastic catheter. Control mice were administered a 50% ethanol solution without oxazolone using the same technique. Body weights were monitored daily.

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction

Total RNA was isolated from the distal colon as previously described (Azuma et al., 2010b), with minor modifications. RNA was used to synthesize complementary cDNA using SuperScript Reverse Transcriptase (Roche, Madison, WI, USA). The primers used for amplification of IL-19 were as

follows: 5′-ATGAAGACACAGTGCGCGTC-3′ and 5′-GTGTCAGGCTGCAGGAG-3′. mRNA expression levels were quantified using real-time PCR analysis based on the intercalation of SYBR Green (Toyobo, Osaka, Japan). The amplification of *HPRT* mRNA was used as an endogenous control to account for differences in the amount and quality of RNA added to each reaction.

Histology

The distal colon was fixed with 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). The severity of intestinal inflammation was graded using a previously described system for colitis from 0 to 4 as follows: 0, no leukocyte infiltration; 1, low level of leukocytic infiltration; 2, moderate level of leukocytic infiltration; 3, high level of leukocytic infiltration, high vascular density, thickening of the colon wall; and 4, transmural infiltration, loss of goblet cells, high vascular density, thickening of the colon wall (Fuss et al., 1999). All sections were scored in a blinded fashion by a pathologist.

Myeloperoxidase (MPO) activity in the colon

Neutrophil infiltration into the colon was quantified by measurement of MPO activity. The distal colon was homogenized in PBS containing Complete Lysis-M (Roche). Homogenized samples were placed on a rotary shaker at 300 rpm on ice for 30 min and centrifuged at 12,000 × *g* for 10 min. Supernatants were then collected, and protein concentrations were quantified and adjusted to 0.9 mg/mL. MPO activity was determined using an EnzChek MPO Activity Assay Kit (Life Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions.

Immunofluorescence staining

The distal colon was fixed with 4% paraformaldehyde. Frozen sections (5 μm thick) were prepared for immunofluorescence staining as previously described (Azuma et al., 2008), with minor modifications. Macrophages were detected using rat anti-mouse F4/80 monoclonal antibodies conjugated to Alexa Fluor 647 (CI:A3-1; AbD Serotec). Neutrophils were detected using rat anti-mouse Gr-1 monoclonal antibodies conjugated to Alexa Fluor 488 (RB6-8C5; AbD Serotec). Confocal images were obtained under a laser-scanning microscope (C1si; Nikon Corporation, Tokyo, Japan).

IgE ELISA

Serum IgE levels were measured by ELISA (Koma Biotech, Seoul, Korea) according to the manufacturer's instructions.

Differential white blood cell counts

Blood smears slides were stained with Giemsa (Wako, Osaka, Japan). Differential cell counts were determined for 2000 cells per slide under oil at 60× magnification. During cell counting, the researcher was blinded to the experimental treatment.

Eosinophil staining

Eosinophil infiltration into the distal colon was stained by Eosinophil Stain Kit (ScyTek Laboratories, Inc., UT, USA). Simultaneously, acidic polysaccharide was stained by Astra Blue (ScyTek Laboratories, Inc., UT, USA).

Cell isolation and cytokine production

Mesenteric lymph nodes were isolated. Lymph node cells were isolated by grinding the tissue and filtering the cell suspension through a 70-μm filter. Then, $CD4^+$ T-cells were enriched from lymph node cells by positive selection with CD4 MicroBeads, according to the manufacturer's instructions (Miltenyi

Biotec, Bergisch Gladbach, Germany). Simultaneously, we collected and used lymph node cells by negative selection with CD4 microbeads. $CD4^+$ T-cells from lymph node were stimulated with BioCoat anti-mouse CD3 T-cell activation plates (BD, Franklin Lakes, NJ, USA), whereas lymph node cells by negative selection with CD4 MicroBeads were cultured with normal plates. After 48 h of culture, cytokine concentrations were determined in the culture supernatants using enzyme-linked immunosorbent assays (ELISAs; eBioscience, San Diego, CA, USA) and multiplex assays (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Histological scores were statistically analyzed using the Mann-Whitney U test. Differences in parametric data were evaluated using Student's t tests. Differences with *P* values of less than 0.05 were considered significant.

Results

Increased susceptibility of IL-19-KO mice to oxazolone-induced colitis

We initially investigated the susceptibility of IL-19-KO mice to the development of oxazolone-induced colitis by analyzing body weight loss and

histology of the distal colon. IL-19-KO mice showed severe weight loss compared with WT control mice from day 1 to day 2 (Fig. 1A). As shown in Figure 1B, the administration of oxazolone to WT mice induced the expression of *IL-19* mRNA in the distal colon 2 days after oxazolone administration. Simultaneously, the administration of oxazolone to IL-19-KO mice induced no expression of IL-19 mRNA on day 2 (data now shown). Histological analysis revealed that the distal colon in IL-19-KO mice exhibited increased numbers of infiltrating cells and a general loss of tissue architecture 2 days after oxazolone administration (Fig. 1C). The severity of colitis, as represented by the histological score, was significantly increased in IL-19-KO mice compared with WT mice (Fig. 1C).

In IL-19-KO mice, oxazolone treatment increased colonic MPO activity (Fig. 2A), consistent with a marked increase in neutrophil infiltration as evaluated using anti-Gr-1 antibodies (Fig. 2B). Interestingly, macrophage infiltration (evaluated using anti-F4/80 antibodies) was also highly increased in IL-19-KO mice with oxazolone-induced colitis (Fig. 2B).

IL-4 induces type-2 inflammatory responses, thereby stimulating production of IL-13, B-cell-dependent production of serum IgE and recruitment of eosinophils in mice with oxazolone-induced colitis (Hoving et al., 2012). Therefore, we assessed serum IgE levels and blood eosinophil

levels in mice with oxazolone-induced colitis. Interestingly, circulating IgE levels were significantly elevated in IL-19-KO mice in comparison with WT mice (Fig. 2C). The number of circulating eosinophils was also significantly elevated in IL-19-KO mice as compared with WT mice (Fig. 2D upper panel). To estimate the eosinophil infiltration of IL-19-KO tissue preparations in the distal colon, we used Eosinophil Stain Kit. Administration of oxazolone to IL-19-KO mice showed marked eosinophil infiltrations as indicated red color (Fig. 2D lower panels). However, WT showed little eosinophil infiltrations. Collectively, these results showed that IL-19 deficiency exacerbated oxazolone-induced colitis by facilitating the transport of inflammatory cells into the colon.

Expression of inflammatory cytokines in the colons of mice with oxazolone-induced colitis

To investigate the potential inflammatory mediators that may be altered by IL-19 during oxazolone-induced colitis, we analyzed the expression of various inflammatory mediators (i.e., *IL-1β*, *IL-4*, *IL-6*, *TNF-α*, and *IL-10*) by real-time PCR. Interestingly, administration of oxazolone to IL-19-KO mice increased the expression of *IL-1β* and *IL-4* mRNA in the distal colon (Fig. 3). In contrast, IL-19-KO mice decreased the expression of *IL-10* mRNA.

However, the levels of *IL-6* and *TNF-α* were similar in WT and IL-19-KO mice. These results suggested that IL-19 played an important role in the IL-4-mediated inflammatory responses in mice with oxazolone-induced colitis.

Altered cytokine production in lymph node cells from IL-19-KO mice with oxazolone-induced colitis

Because the oxazolone-induced colitis model has been associated with increases in Th2 responses (Heller et al., 2002; Boirivant et al., 1998), we examined the levels of several types of cytokines in lymph node cells. As shown in Figure 4A, $CD4^+$ T-cells isolated from lymph node of IL-19-KO mice with oxazolone-induced colitis produced elevated amounts of IL-2, IL-4, IL-9 and CCL5 upon stimulation with anti-CD3 and anti-CD28 antibodies *in vitro*. However, there are no marked changes in the amounts of IL-5, IL-13 (Fig. 4A) and IL-17 (data not shown). These results suggested that IL-19 controlled Th2 cytokine IL-4. At the same time, we measured the production of several cytokines in lymph node cells by negative selection with $CD4^+$. As shown in Figure 4B, IL-19-KO mice produced high levels of IL-12, G-CSF and CXCL1, but not TNF-α, IL-1β and IL-6, suggesting that the production of these cytokines is controlled by IL-19.

Discussion

In this study, our experiments highlighted several novel aspects of the anti-inflammatory effects of IL-19 in Th2-mediated colitis *in vivo*. We found that the exacerbation of oxazolone-induced colitis by IL-19 deficiency was accompanied by increased production of IL-4 and IL-9 without increased production of IL-5 and IL-13. The oxazolone-induced colitis and UC include characteristic ulcers of the colon, which form through an IL-4-driven, Th2-mediated response to oxazolone (Heller et al., 2002; Boirivant et al., 1998). In addition, IL-4, IL-5, and IL-13 are upregulated in keratin 8-KO mice, which develop chronic spontaneous Th2-mediated colitis (Habtezion et al., 2005). IL-4 and IL-5 are correlated with the clinical and histological severity of colitis in UC patients (Inoue et al., 1999). IL-13 was found to be upregulated in UC patients and linked to an impaired epithelial barrier function in the gut (Heller et al., 2005). Indeed, IL-5 and IL-13 as well as IL-4 have important roles in colonic inflammation. Why did IL-19-KO mice show increased production of IL-4, but not IL-5 and IL-13? Although IL-4 and IL-13 are structurally analogous and share functional receptors, their function is quite different. IL-4 acts mainly as a regulatory cytokine and IL-13 acts as

an effector cytokine. IL-5 plays a key role in eosinophil proliferation, differentiation, maturation, migration to tissue sites and survival (Sitkauskiene et al., 2004). IL-4 was mainly produced by Th2, and Th2 produced IL-5 and IL-13. IL-5 and IL-13 were also produced by innate lymphoid cells and invariant natural killer T cells. IL-4 and IL-5 promote the clonal expansion and maturation of B cells and induces the IgM/IgE switch. Released IgE activate mast cells to secrete IL-4, IL-5 and IL-13. In contrast, a recent study reported that IL-9 plays an important role in driving UC by regulating intestinal epithelial cells and that IL-9 represents a promising target for the treatment of chronic colitis (Gerlach et al., 2014). Thus, it is possible that IL-19 regulates IL-4 and IL-9 production, but not IL-5 and IL-13 production, because all of these cytokines are significant pathological factors in oxazolone-induced colitis (Heller et al., 2002; Boirivant et al., 1998). In line with these previous findings, our data suggest that IL-19 plays important role in the gut by regulating Th2 cells to produce IL-4, but not innate lymphoid cells and invariant natural killer T cells to produce IL-5 and IL-13.

It is not surprising to find that IL-2 was increased in IL-19-KO compared to WT. IL-2 plays a critical role in the polarization of naive CD4 T cells to the Th2 phenotype as well as Th1 clearly needs IL-2 (Cote-Sierra et al., 2004). CCL5 is a chemotactic agent for T cells, eosinophils, and basophils

and plays an active role in recruiting leukocytes to sites of inflammation. CCL5 does not play the survival for eosinophils although IL-3, IL-5 and GM-CSF play the survival for eosinophils. We found that IL-19-KO showed increased production of CCL5 though no alteration on the production of IL-3, IL-5 and GM-CSF (Fig. 4A and data not shown), suggesting that IL-19 control migration and infiltration to the tissue of eosinophils, but not proliferation and survival for eosinophils.

IL-10 is a well-known anti-inflammatory and immunosuppressive cytokine. Anti-inflammatory effect of IL-19 is reminiscent of the widely accepted regulation of IL-10. There is the possibility that altered production of cytokines in IL-19-KO was due to downregulation of IL-10.

Th17 is responsible for the IL-17 production, a key pro-inflammatory cytokine that is increased in IBD (Zhu et al., 2015). There is the possibility that altered production of cytokines in IL-19-KO was due to upregulation of IL-17. However, both WT and IL-19-KO secreted equivalent amounts of IL-17 (data not shown), suggesting that IL-17 is not likely to mediate the suppressive effect of IL-19.

Interestingly, we observed increased levels of IL-12, G-CSF and CXCL1 in IL-19-KO mice with oxazolone-induced colitis. G-CSF stimulates the survival, proliferation, differentiation, and functions of

neutrophil precursors and mature neutrophils and can induce the production of granulocytes in the bone marrow. CXCL1 has neutrophil chemoattractant activity and has been shown to function by signaling through the chemokine receptor CXCR2, which is expressed on the surface of neutrophils. In a previous study, researchers found that the colons of mice with oxazolone-induced are flooded with inflammatory cells, including Gr-1$^+$ neutrophils, γδT cells, eosinophils, NKT cells, and CD4$^+$ T-cells (Camelo et al., 2012). Consistent with previous result, we found here that IL-19-KO mice exhibited increased Gr-1$^+$ neutrophils and eosinophils, supporting the role of IL-19 in mediating intestinal inflammation. IL-12 is a well-known Th1 cytokine. In addition, IL-12 mediates enhancement of the cytotoxic activity of NK cells and CD8$^+$ cytotoxic T-cells. Thus, it is possible that IL-19 may regulate NK cells and CD8$^+$ cytotoxic T-cells in oxazolone-induced colitis.

It is well known that pro-inflammatory cytokines play an important role in inflammation of the intestinal mucosa. For instance, IL-6, IL-1β, and TNF-α have been shown to play pivotal roles in the pathogenesis of IBD (Fiocchi, 1998; Bouma and Strober, 2003). Both IL-6-deficient mice and caspase-1 (IL-1β converting enzyme)-deficient mice have been shown to have less severe colitis induced by DSS, suggesting that IL-6 and IL-1β both play a role in promoting T-cell-independent acute colitis (Siegmund et al., 2001;

Naito et al., 2004). In contrast, TNF-α-deficient mice have been shown to be more susceptible to DSS-induced colitis (Naito et al., 2003), whereas a low dose TNF-α monoclonal antibody treatment protected against chronic colitis (Kojouharoff et al., 1997). In our study, lymph node cells showed a similar production of IL-6, IL-1β and TNF-α between WT and IL-19-KO. Interestingly, we also found that real-time RT-PCR analysis of the distal colons of mice showed increased expression of IL-1β, but not IL-6 and TNF-α, in IL-19-KO compared to WT. It is likely that non-immune cells in the gut tissue produced IL-1β in IL-19-KO with oxazolone-induced colitis. These results indicate that IL-19 may plays as anti-inflammatory effector by acting both immune cell and non-immune cell, regardless of the mechanism.

In a previous study, we showed that DSS-induced colitis is exacerbated in IL-19-KO mice (Azuma et al., 2010a). The increased severity of colonic inflammation following genetic ablation of IL-19 was accompanied by increased production of many inflammatory cytokines, including IFN-γ, IL-1β, IL-6, IL-12, TNF-α, and CXCL1, several of which have been implicated in the pathogenesis of DSS-induced colitis. In contrast, $CD4^+$ T-cells have been shown to play a central role in IBD. We showed that IL-19 deficiency aggravated TNBS-induced colitis. Additionally, the exacerbation of TNBS-induced colonic inflammation following genetic ablation of IL-19 was

accompanied by increased production of IFN-γ, IL-12, IL-17, IL-22 and IL-33 (Matsuo et al., 2015). IL-19 can have anti-inflammatory roles in the gut, regardless the type of colonic inflammation. In type-1 responses, such as in DSS and TNBS-induced colitis, we revealed that IL-19 clearly plays anti-inflammatory role. In type-2 responses, our present study provides solid evidence for the immunopathological relevance of IL-19 as an anti-inflammatory cytokine in colonic inflammation. Consistent with our data, recent studies have reported that IL-19 is impaired in patients with active UC (Fonseca-Camarillo et al., 2014). Our data add a new key player as colonic inflammation, showing that IL-19 act as potent anti-inflammatory cytokine.

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Conflict of interest The authors declare that they have no conflicts of interest to declare.

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Fig. 1. Increased susceptibility of IL-19-KO mice to oxazolone-induced colitis. (A) The percent weight loss of WT (n = 15) and IL-19-KO (n = 17) mice was monitored daily. *P < 0.05 compared with oxazolone-WT. (B) Expression of *IL-19* mRNA in the distal colon of WT mice (n = 5) on day 2 post-oxazolone administration. *P < 0.05 compared with 0 day. (C) Histological evaluation in WT (n = 4) and IL-19-KO (n = 7) mice on day 2 post-oxazolone administration. Representative sections are shown. Scale bar, 100 μm. Histological scores were determined. *P < 0.05 compared with WT.

Fig. 2. The transport of inflammatory cells into the colon, and IgE and eosinophil level. (A) MPO activity in WT (n = 3) and IL-19-KO (n = 8) mice on day 2 post-oxazolone administration. (B) Neutrophil and macrophage infiltrations in WT (n = 4) and IL-19-KO (n = 4) mice on day 2 post-oxazolone administration. Gr-1 and F4/80 immunofluorescence staining of representative sections are shown. Scale bar, 10 μm. Each positive cell was enumerated. (C) serum IgE levels, (D) serum eosinophils levels and eosinophils infiltrations in WT (n = 4) and IL-19-KO (n = 7) mice on day 2 post-oxazolone administration. Representative sections are shown. Scale bar, 20 μm. *P < 0.05 compared with WT.

Fig. 3. Altered levels of cytokines. Cytokine expressions in WT (n = 7) and IL-19-KO (n = 8) mice on day 2 post-oxazolone administration. The expression levels of the indicated mRNAs were examined by quantitative real-time PCR. *$P < 0.05$ compared with WT.

Fig. 4. Cytokine productions in (A) $CD4^+$ T-cells and (B) cells by negative selection with $CD4^+$ from lymph node. Cytokine production in WT (n = 4) and IL-19-KO (n = 7) mice on day 2 post-oxazolone administration. *$P < 0.05$, **$P < 0.01$ compared with WT.

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Figure 1

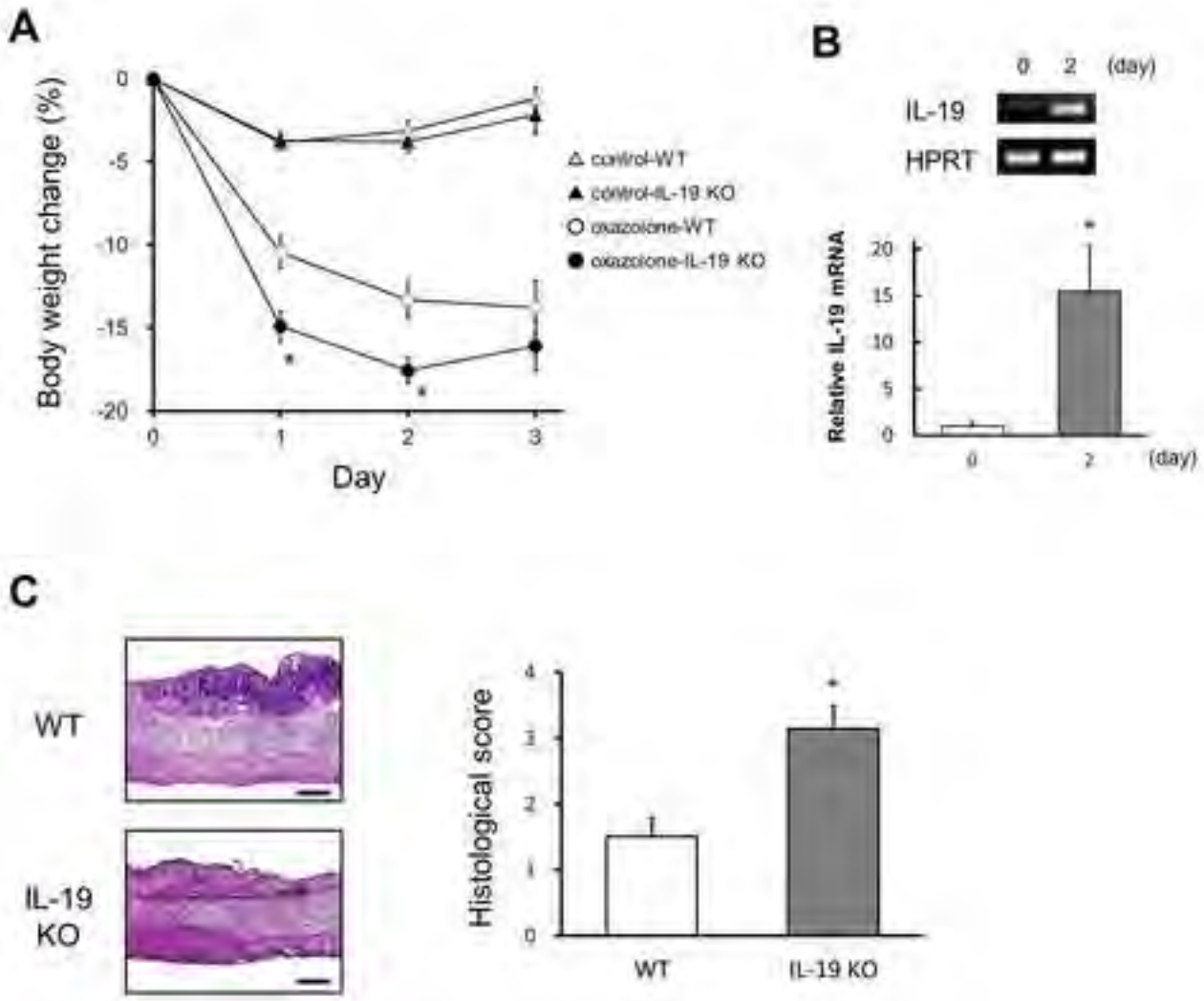

Figure 2

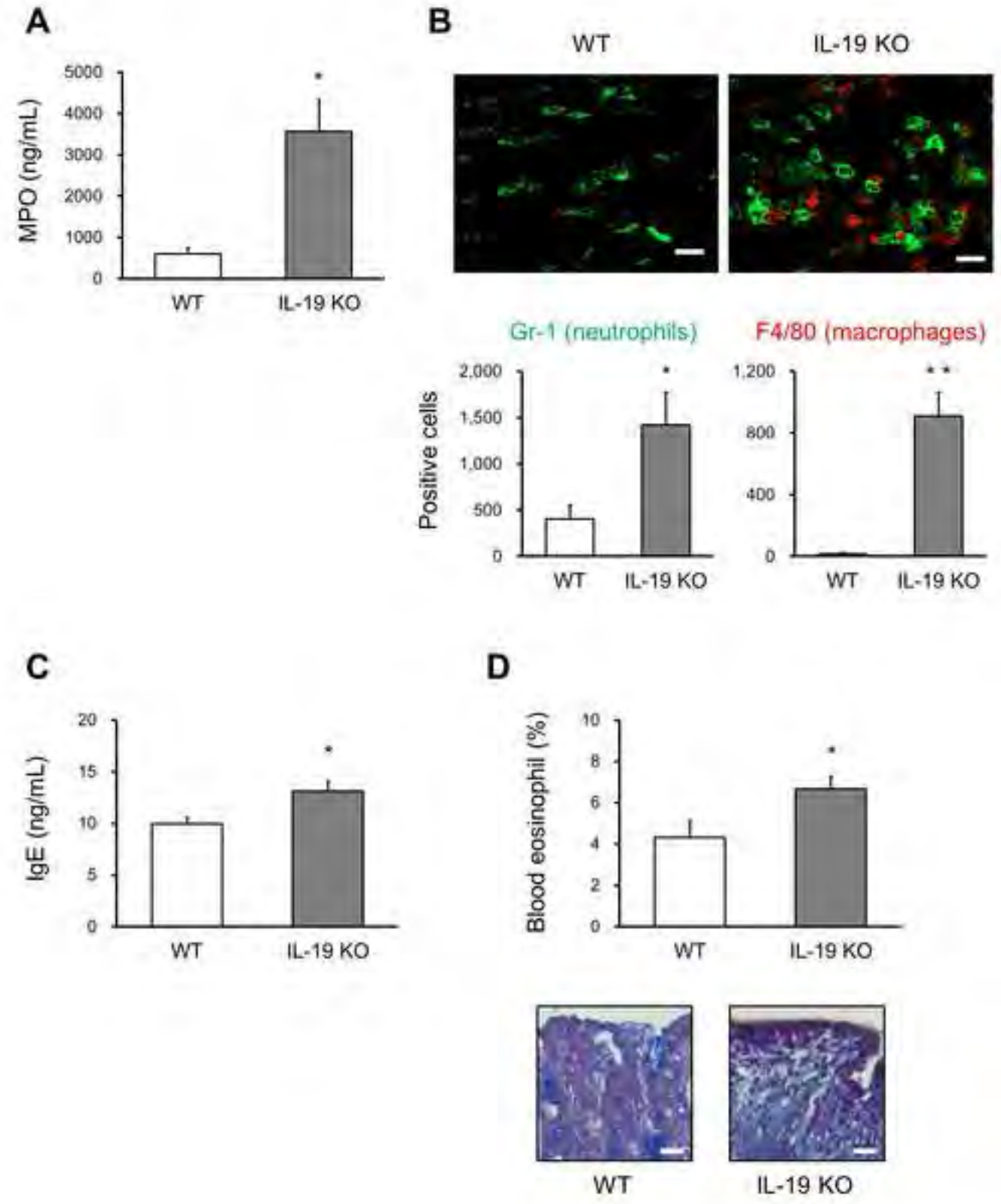

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Figure 3

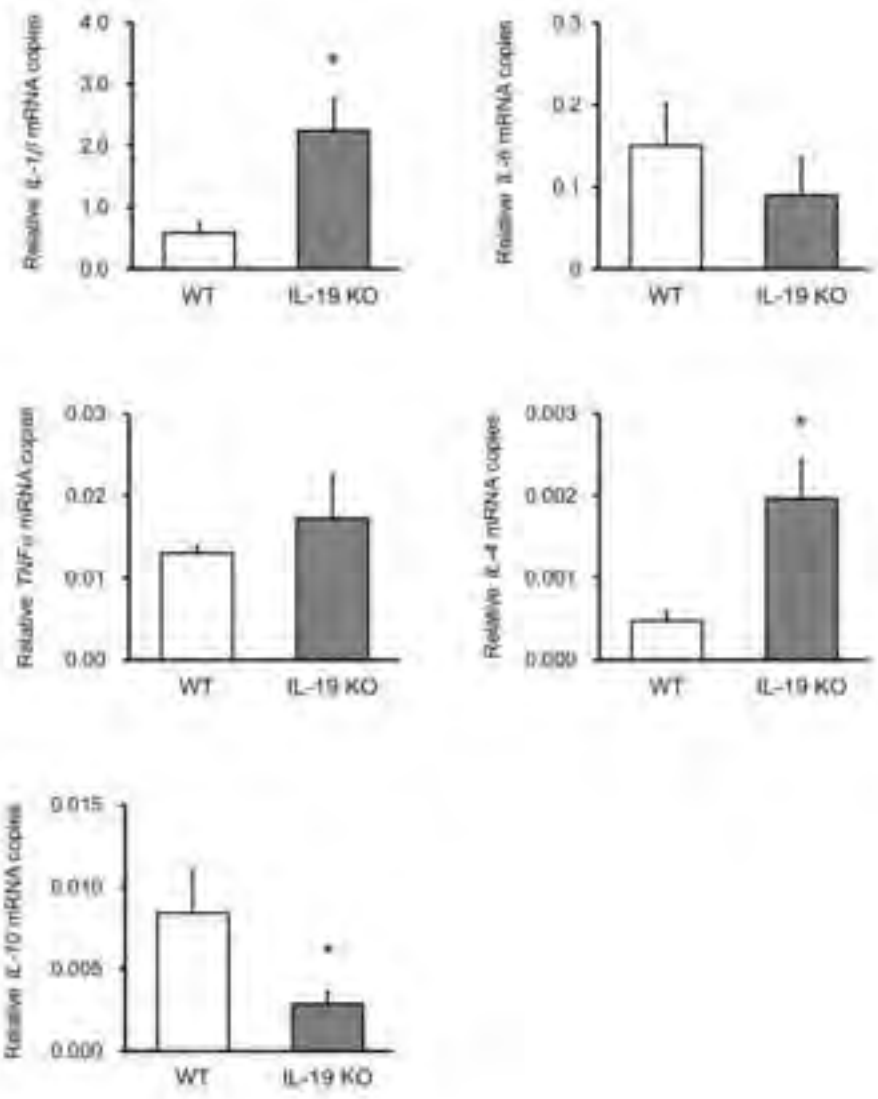

Figure 4