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Dysregulated balance of retinoid-related orphan receptor γt-dependent innate lymphoid cells is involved in the pathogenesis of chronic DSS-induced colitis

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ABSTRACT

Retinoid-related orphan receptor (ROR) γt-expressing and IL-22-producing NKp46^{+} innate lymphoid (ILC22) cells reside in the lamina propria of the intestine in mice, suggesting that ILC22 cells contribute to host defense during intestinal damage in models of colitis in mice. Nevertheless, another set of pathological interferon (IFN)-γ and/or IL-17A-producing innate lymphoid cells (ILC1 and ICL17) may participate in the pathogenesis in different models of colitis. We here showed that RORγt^{+}IL-22^{+} ILC22 cells were localized in Thy-1^{high}SCA-1^{high} and/or Thy-1^{high}SCA-1^{low} subpopulations of the intestine in normal and dextran sodium sulfate (DSS)-induced colitic RORγt-sufficient Rag-2$^{-/-}$ mice. RORγt-deficient Rag-2$^{-/-}$ mice developed more severe DSS-induced colitis accompanied with lower expression of REG3β and REG3γ in the colon, but with a lower ratio and absolute number of IFN-γ-producing ILC1 cells as compared to control RORγt-sufficient Rag-2$^{-/-}$ mice. Collectively, not only the presence of ILC22 cells but also the balance of protective and pathogenic ILCs may be involved in the prevention of colitis.

1. Introduction

Retinoid-related orphan receptor (ROR) γt is a transcription factor that regulates a variety of physiological processes [1,2]. Besides RORγt-dependent Th17 T cells [3], evidence is emerging of a new RORγt-dependent IL-17A-producing cell population, innate lymphoid cells (ILCs), that plays crucial roles in formation and maintenance of GALT, and pathogenesis of colitis in animals and humans [4]. There are various types of ILCs categorized by location, cytokine production, and expression of specific surface markers. Among them, the first-described ILCs, lymphoid tissue inducer (LTi) cells, are involved in GALT formation during the fetal period [5]. Similar LTi cells are present in the gut and tonsils after birth, and are called adult LTi-like cells [6]. Although LTi cells produce both IL-17A and IL-22, several groups have identified NKp46^{+} in mice [7] and NKp44^{+} in humans [8,9], ILCs that express RORγt and produce IL-22 but not IL-17A in the intestines, designated as NK22 [8,9], NKR-LTi [10], NCR-22 [11], NKR^{+}RORγt^{+} ILCs [12], and ILC22 cells [13]. Importantly, IL-22-specific receptor seems to be expressed only on non-hematopoietic cells including epithelial cells [7]. IL-22$^{-/-}$ mice develop more severe colitis with marked epithelial damage in the dextran sodium sulfate (DSS)-induced colitis model and *Citrobacter rodentium* colitis than do control wild-type mice [11,14].

The next critical issue is whether ILCs are pathologically involved in the development of inflammatory bowel disease (IBD) [2]. Powrie's group described colitogenic ILCs (ILC1, ILC17 or ILC17/1 cells) that produce IFN-γ and/or IL-17 in two models of innate colitis [15].

Given that ILCs play both protective and pathological roles in the pathogenesis at the different models of colitits, we here attempted to clarify the role of ILCs in the development of colitis by assessing the small intestine (SI) and colon separately. We used RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice to exclude the impact of Th17 cells, and adopted chronic DSS colitis model.

2. Materials and methods

See Supplemental materials and methods for more details.

2.1. Mice

We used 8–10-wk-old mice with a green fluorescent protein (GFP) RORγt reporter cDNA knocked-in at the site for initiation

Abbreviations: H.h., *Helicobacter hepaticus*; IBD, inflammatory bowel disease; IFN, interferon; ILC, innate lymphoid cell; LP, lamina propria; LTi, lymphoid tissue inducer; REG, regenerating gene; RORγt, retinoid-related orphan receptor; SCA, stem cell antigen; SI, small intestine.

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of RORγt translation on the C57BL/6 (Ly5.2) background [16,17]. RORγt$^{gfp/gfp}$ mice were intercrossed into RAG-2$^{-/-}$ mice to generate RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice. Mice were maintained under specific pathogen-free conditions. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and regulations.

2.2. DSS-induced colitis

Colitis was induced by giving 2.0% DSS (50 kDa; Ensuiko Co. Ltd., Kanagawa, Japan) dissolved in sterile distilled water (DW) *ad libitum* for 7 d followed by regular drinking water for 7 d. This cycle was repeated twice and then, mice were administrated 2.0% DSS for 6 d to establish a chronic DSS colitis model (Fig. 2A). Body weight and stool score were measured during the experiments. The disease activity index (DAI) was assessed by trained individuals blinded to the mouse groups [18].

2.3. Cell isolation

Small intestine and colon were removed and placed in Ca^{2+}, Mg^{2+}-free HBSS (Nacalai Tesque, Japan). Peyer's patches were carefully excised, and the intestine was opened longitudinally. The intestines were thoroughly washed in HBSS and cut into small pieces. The dissected mucosa was incubated with HBSS containing 1 mM DTT (Sigma–Aldrich, St. Louis, MO) and 5 mM EDTA (Gibco, Carlsbad, CA) for 30 min at 37 °C. The pieces of intestine were washed and placed in digestion solution containing 1.5% FBS, 1.0 mg/mL collagenase A (Roche Diagnostics GmbH, Germany) and 0.1 mg/mL DNase (Sigma–Aldrich) for 1 h at 37 °C. Intestine supernatants were resuspended in the 40% fraction of a 40:75 Percoll gradient, and overlaid on the 75% fraction. Percoll gradient separation was performed by centrifugation at 840*g* for 20 min at room temperature. Mononuclear cells were collected at the interphase of the Percoll gradient, washed, and resuspended in FACS buffer or RPMI-1640 (Sigma–Aldrich) containing 10% FBS and penicillin/streptomycin (Gibco).

2.4. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Groups of data were compared by Student's *t* test. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Distinct types of Lin$^-$ Thy-1$^+$SCA-1$^+$ cells reside in normal intestine

To investigate the role of RORγt in the pathogenesis of colitis without any effect of lymphocytes, including RORγt-expressing Th17 and T-cell receptor γδ$^+$ T cells, we used lymphocyte-lacking and RORγt-lacking RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ and lymphocyte-lacking and RORγt-expressing RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice. Both mice appeared healthy up to 40 wk old (data not shown). Histological examination revealed no abnormal structure and inflammation of the SI or colon (Fig. 1A). An increasing number of studies [4] have revealed that ILCs, including adult LTi-like cells, NK-22 cells (ILC22), and colitogenic IFN-γ/IL-17A-producing Thy-1^{high}SCA-1$^+$ ILCs (ILC1, ILC17, and/or ILC1/17) reside in the mouse intestine under steady state and colitic conditions. Therefore, we stained Thy-1 and SCA-1 on lineage maker (CD11b, B220 and Gr1)-negative (Lin$^-$) population in the SI and colon of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice (Fig. 1B). In this regard, we could classify three Lin$^-$ subpopulations in the SI: Thy-1^{high}SCA-1^{high}, Thy-1^{int}SCA-1^{high}, and Thy-1^{high}SCA-1^{low} (Fig. 1B). All three subpopulations resided in the SI of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice. However, the Thy-1^{high}SCA-1^{low} subpopulation almost disappeared in the SI of RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice, and the ratio of those cells was significantly reduced as compared to RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 1C). Conversely, the ratios of Thy-1^{high}SCA-1^{high} and Thy-1^{int}SCA-1^{low} subpopulations in the SI of RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice were significantly increased as compared to those of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 1C). Intriguingly, only the Thy-1^{high}SCA-1^{high} subpopulation was found in the colon of both RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice, and the ratio of this subpopulation in the colon of RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice was significantly increased as compared to that of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 1C), suggesting that the residency of Thy-1^{high}SCA-1^{high} cell subpopulation is not entirely dependent on RORγt expression.

3.2. Thy-1^{high}SCA-1^{high} and Thy-1^{high}SCA-1^{low} RORγt$^+$ ILCs produce IL-22

To determine the localization of RORγt expression in these three subpopulations in the SI and colon of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice, we evaluated GFP and IL-22 expression in reporter (RAG-2$^{-/-}$ × RORγt$^{gfp/+}$) and knockout (RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$) mice. In the SI of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ reporter mice, IL-22-expressing Lin$^-$ cells preferentially resided in Thy-1^{high}SCA-1^{high} and Thy-1^{high}SCA-1^{low} subpopulations in accordance with GFP (RORγt) expression (Fig. 1D and Supplemental Fig. 1). In the colon of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ reporter mice, unlike Thy-1^{high}SCA-1^{high} cells in the SI, a few but substantial proportion of Thy-1^{high}SCA-1^{high} cells expressed IL-22 and GFP (Fig. 1D and Supplemental Fig. 1). In contrast, irrespective of in the SI and colon, IL-22 expression was completely dependent on RORγt, as almost all IL-22-expressing Lin$^-$ cells disappeared in RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice, in accordance with no expression of GFP (Fig. 1D and Supplemental Fig. 1).

3.3. RORγt-expressing ILCs may be protective in chronic DSS-induced colitis

Given the evidence that IL-22 expression in the intestine of RAG-2$^{-/-}$ mice is dependent on the presence of RORγt-expressing ILCs, and is preferentially localized in the SI rather than colon, of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice, we next evaluated the role of RORγt expression in innate immune cells during colonic damage and inflammation induced by sequential DSS administration (Fig. 2A). As shown in Fig. 2B, DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice constantly developed more severe wasting disease after the first cycle of DSS administration as compared to the paired DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice. Consistently, DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice showed a more severe reduction in colonic length than did DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 2C). This was confirmed by statistical analysis (Fig. 2D). Expectedly, the total DAI score (Fig. 2E) and the absolute cell number of colonic LPMCs (Fig. 2F) in DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice were significantly increased as compared to those of DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice.

Consistently, DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice showed more severe histopathology accompanying crypt destruction, mucosal ulcers, and erosions in the mucosal tissue than DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ littermates (Fig. 2G). This was statistically confirmed by the histological scores (Fig. 2H). Nevertheless, curiously, the production of IFN-γ and IL-17A in the colon of DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice was significantly reduced as compared to that of DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 2I). As expected, however, the expression of IL-22 mRNA in

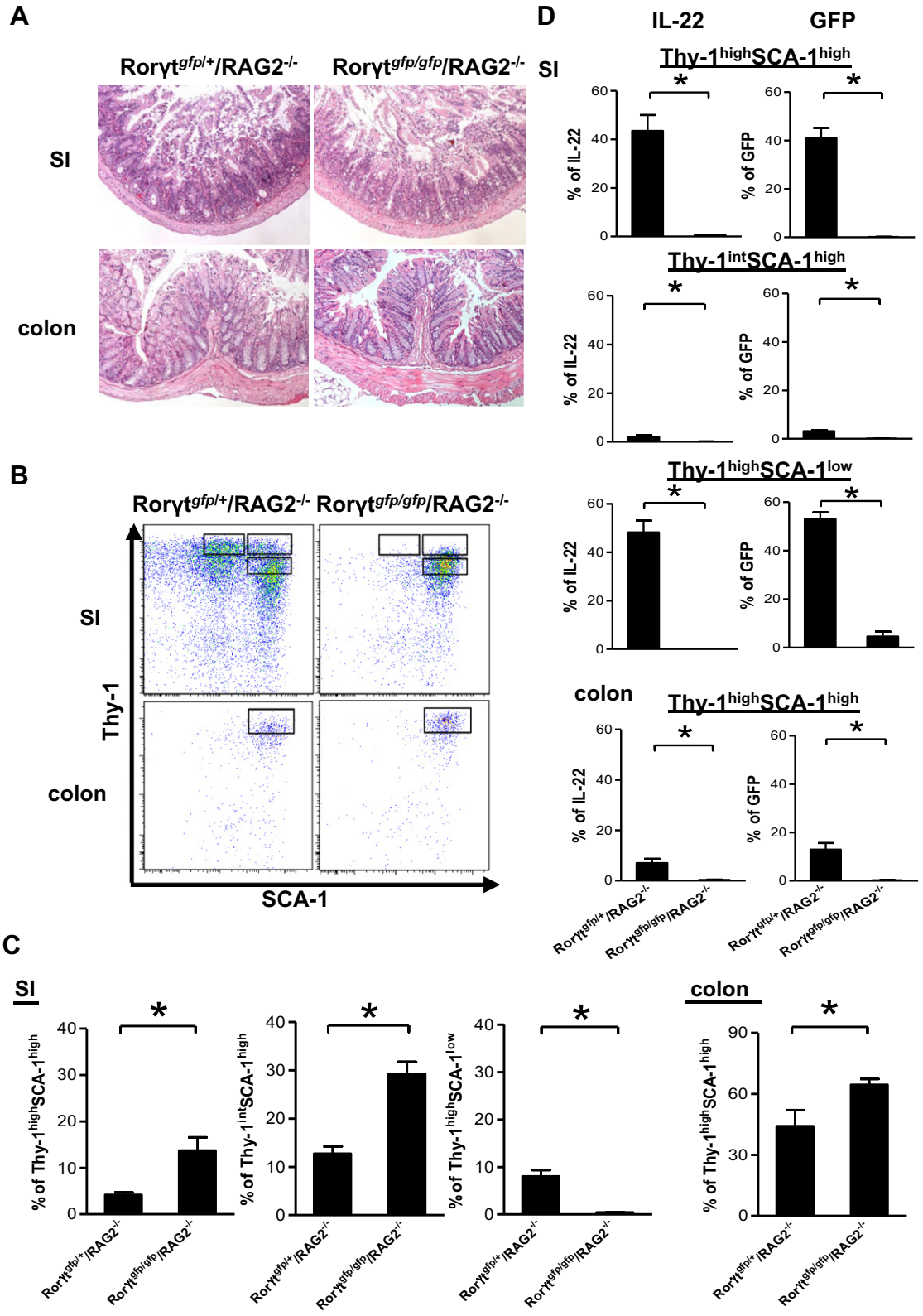

Fig. 1. Distinct types of Lin⁻Thy-1⁺SCA-1⁺ cells reside in SI and the colon of normal mice. (A) Histology of SI and colon in untreated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice. Original magnification, ×100. (B) Thy-1^{high}SCA-1^{high}, Thy-1^{int}SCA-1^{high}, and Thy-1^{high}SCA-1^{low} cells in CD45.2^{+} Lin^{-} (CD11b, B220 and Gr1) population in the SI and colon from untreated RAG-2$^{-/-}$ × RORγt $^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice. Gated CD45.2^{+} and Lin^{-} cells were stained with anti-Thy-1 and anti-SCA-1 mAbs, and analyzed by flow cytometry. (C) The percentages of Thy-1^{high}SCA-1^{high}, Thy-1^{int}SCA-1^{high}, and Thy-1^{high}SCA-1^{low} cells in the SI and Thy-1^{high}SCA-1^{high} cells in the colon. Data show the mean ± SEM (n = 4/group). *P < 0.05. (D) Percentages of IL-22^{+} and GFP^{+} cells in Lin^{-} Thy-1^{high}SCA-1^{high}, Thy-1^{int}SCA-1^{high}, and Thy-1^{high}SCA-1^{low} cells in the SI and Thy-1^{high}SCA-1^{high} cells in the colon. Data show the mean ± SEM (n = 4/group). *P < 0.05.

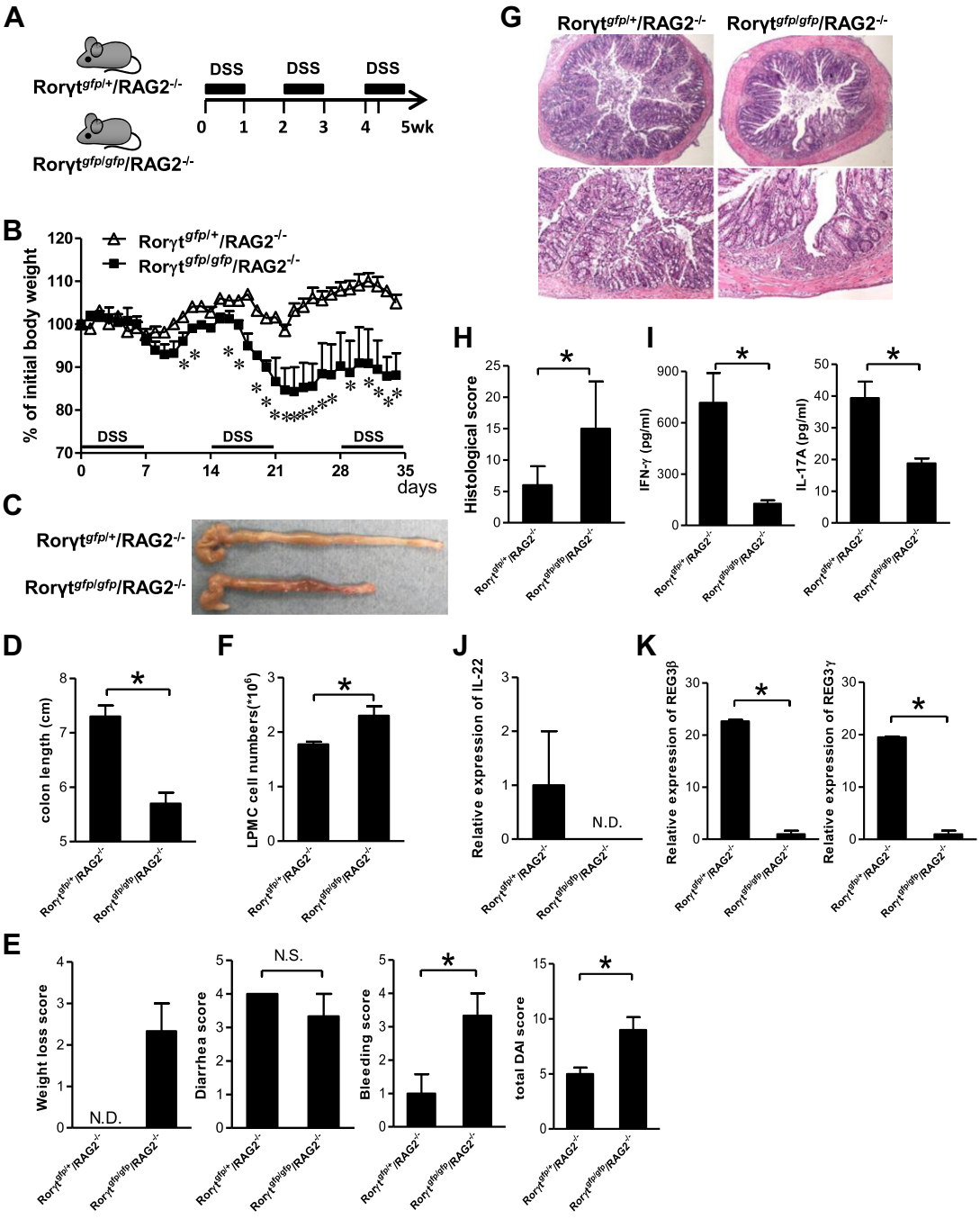

Fig. 2. RORγt-expressing innate cells are protective in chronic DSS-induced colitis. (A) Experimental protocol. RAG-$2^{-/-}$ × RORγt$^{gfp/+}$ and RAG-$2^{-/-}$ × RORγt$^{gfp/gfp}$ mice were treated with two cycles of 2.0% DSS administration for 7 d followed by water for 7 d, and then these mice were added 2.0% DSS administration for 6 d. (B) Change in body weight over time is expressed as a percentage of original weight. Data are expressed as mean ± SEM. (C) Gross appearance of the colon. (D) Colon length in each group. (E) DAI score. (F) Absolute number of colonic LP cells. Data are expressed as mean ± SEM of each group mice (n = 3 or 4). Data are representative one of three individual experiments. *P < 0.05. (G) Representative hematoxylin and eosin staining for DSS-treated RAG-$2^{-/-}$ × RORγt$^{gfp/+}$ and RAG-$2^{-/-}$ × RORγt$^{gfp/gfp}$ mice. Original magnification, ×40 and ×100. (H) Histological scores of distal colon. Data are indicated as the mean ± SEM of each group of mice (n = 4). *P < 0.05. (I) Production of IFN-γ and IL-17A proteins in supernatants of colonic LP stimulated with PMA and ionomycin for 8 h. (J) Relative expression of IL-22 mRNA in Lin$^-$ Thy-1^{high}SCA-1^{high} cells of the colonic LP from RAG-$2^{-/-}$ × RORγt$^{gfp/+}$ and RAG-$2^{-/-}$ × RORγt$^{gfp/gfp}$ mice treated with DSS. (K) Relative expression of REG3β and REG3γ mRNAs in the colonic epithelial cells from RAG-$2^{-/-}$ × RORγt$^{gfp/+}$ and RAG-$2^{-/-}$ × RORγt$^{gfp/gfp}$ mice treated with DSS.

colon tissue of DSS-treated RAG-$2^{-/-}$ × RORγt$^{gfp/gfp}$ mice was significantly lower than that of DSS-treated RAG-$2^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 2J). Consistent with previous findings that key proteins that maintain epithelial barrier function in the intestine are tightly controlled by IL-22 [19], the expression of REG3β and REG3γ mRNAs in the colonic epithelial cells from DSS-treated RAG-$2^{-/-}$ × RORγt$^{gfp/gfp}$ mice was significantly reduced as compared to that of DSS-treated RAG-$2^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 2K). Furthermore, the expression of mucin protein MUC-2 (Supplemental Fig. 2A) and tight junction composition protein occludin (Supplemental Fig. 2B) in the DSS-treated RAG-$2^{-/-}$ × RORγt$^{gfp/gfp}$ mice was markedly reduced as compared to that in the DSS-treated RAG-$2^{-/-}$ × RORγt$^{gfp/+}$ mice. These results suggested that the defect of RORγt expression in mice affected the epithelial repair system

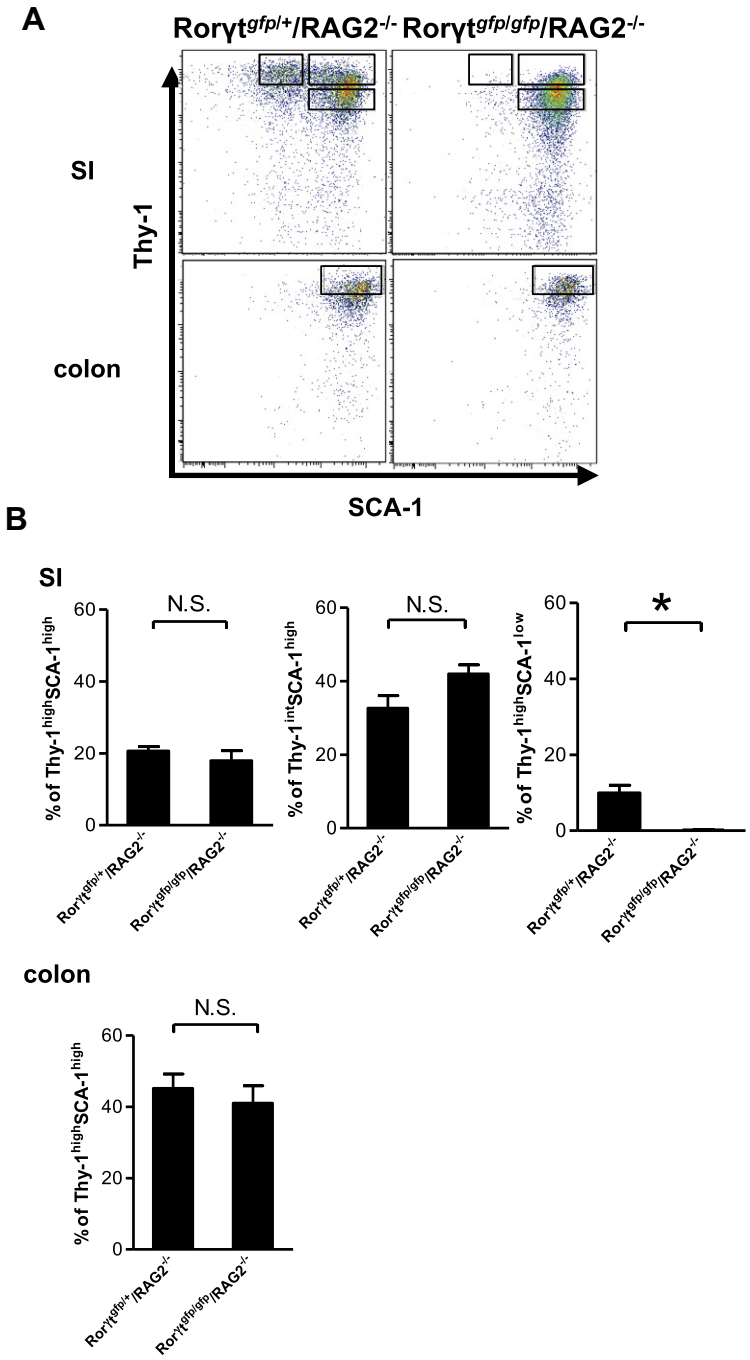

Fig. 3. Colonic Lin⁻ Thy-1^{high}SCA-1^{high} RORγt^{+} ILC22 cells reduce in the intestinal LP of repeated DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice. (A) Thy-1^{high}SCA-1^{high}, Thy-1^{int}SCA-1^{high}, and Thy-1^{high}SCA-1^{low} cells in CD45.2^{+} Lin⁻ (CD11b, B220 and Gr1) population in the SI and colon from DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice. Gated CD45.2^{+} and Lin⁻ cells were stained with anti-Thy-1 and anti-SCA-1 mAbs, and analyzed by flow cytometry. (B) Percentages of Thy-1^{high}SCA-1^{high}, Thy-1^{int}SCA-1^{high}, and Thy-1^{high}SCA-1^{low} cells in the SI and Thy-1^{high}SCA-1^{high} cells in the colon.

rather than the activation of immune compartments, although expression of IL-22 in the colon irrespective of steady state or colitic conditions seemed to be low.

3.4. Colonic Lin⁻ Thy-1^{high}SCA-1^{high} RORγt^{+} ILC22 cells reduce in the intestinal LP of DSS-treated Rag-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice

We suggest that ILC22 cells that are involved in the prevention of colitis, in terms of epithelial repair via IL-22 production, are located in the Thy-1^{high}SCA-1^{high} or Thy-1^{high}SCA-1^{low} subpopulations in the SI and Thy-1^{high}SCA-1^{high} subpopulation in the colon (Fig. 3). Powrie's group have previously demonstrated that pathological ILCs (ILC17 or ILC17/1) located in the Thy-1^{high} SCA-1^{+} subpopulation produce IL-17A and IFN-γ and are markedly increased in the colonic LP of *Helicobacter hepaticus* (*H.h.*)-induced innate colitis [15]. However, we found that the ratio of Lin⁻ Thy-1^{high}SCA-1^{high} cells in the colon was comparable among the two groups, although a subpopulation of Thy-1^{high}SCA-1^{low} cells, but not Thy-1^{high}SCA-1^{high} and Thy-1^{int}SCA-1^{high} cells, in the SI LP of DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice was significantly reduced as compared to that of the paired RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 3A and B).

Consistent with the result under physiological conditions, IL-22-expressing Lin⁻ cells preferentially resided in the

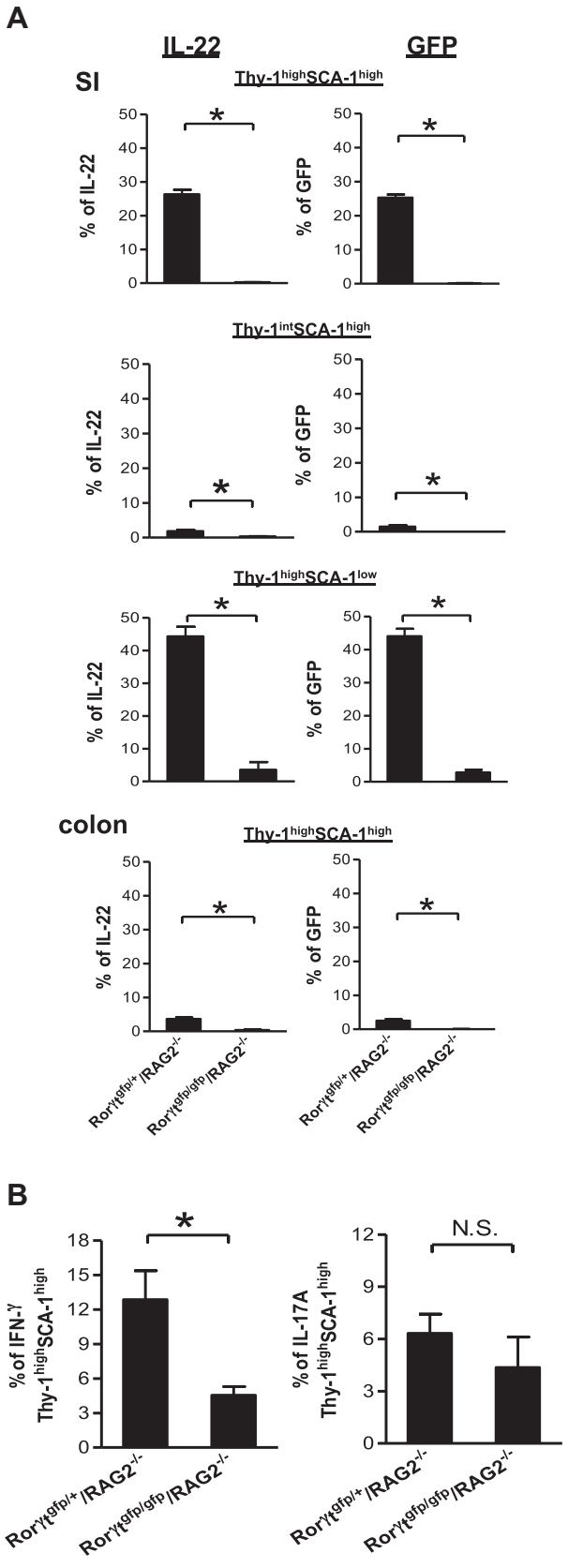

Fig. 4. IL-22 production is increased in Lin$^-$Thy-1^{high}SCA-1^{high} RORγt$^+$ cells from DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice. (A) Percentages of IL-22$^+$ and GFP$^+$ cells in Lin$^-$ Thy-1^{high}SCA-1^{high}, Thy-1^{int}SCA-1^{high}, and Thy-1^{high}SCA-1^{low} cells in the SI and Thy-1^{high}SCA-1^{high} cells in the colon. Data show the mean ± SEM (n = 4/group). *P < 0.05. (B) Percentages of IFN-γ$^+$ and IL-17A$^+$ cells in Lin$^-$ Thy-1^{high}SCA-1^{high} cells in the colonic LP from DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ and RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice. Data show the mean ± SEM (n = 4/group). *P < 0.05.

Thy-1^{high}SCA-1^{high} and Thy-1^{high}SCA-1^{low} subpopulations of the SI of colitic DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice, in the accordance with GFP expression (Fig. 4A). In the colon of colitic DSS-treated RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice, only a small proportion of Thy-1^{high}SCA-1^{high} cells expressed IL-22 and GFP (Supplemental Fig. 3A). In contrast, the proportion of Thy-1^{high}SCA-1^{high} cells in the SI and the colon, IL-22 expression was completely dependent on RORγt (Supplemental Fig. 3A). These results were also confirmed statistically (Fig. 4A).

In light of pathological ILCs, the ratio of IFN-γ expression in Lin$^-$ Thy-1^{high}SCA-1^{high} cells of colitic RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice was significantly decreased as compared to that in Lin$^-$ Thy-1^{high} SCA-1^{high} cells of colitic RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice (Fig. 4B and Supplemental Fig. 3C), suggesting that pathological ILCs that produce IFN-γ are not positively involved in this model of colitis, but rather IL-22 is crucial for the protection and repair of epithelia. Although the ratio of IL-17A expression in Lin$^-$ Thy-1^{high}SCA-1^{high} cells of colitic RAG-2$^{-/-}$ × RORγt$^{gfp/gfp}$ mice tended to decrease (Fig. 4B and Supplemental Fig. 3C), that was not significant.

4. Discussion

In this study, we demonstrated that innate RORγt-expressing cells comprehensively function as a negative regulator to suppress the development of colitis. Consistently, RORγt-deficient RAG-2$^{-/-}$ mice developed more severe DSS-induced colitis compared to the paired RORγt-sufficient RAG-2$^{-/-}$ mice with lower expression of REG3β and REG3γ, but with a lower ratio and absolute number of IFN-γ-producing ILC1 cells in the colon as compared to control RORγt-sufficient RAG-2$^{-/-}$ mice. The subpopulation of protective RORγt$^+$IL-22$^+$ ILC22 cells preferentially resided in the Lin$^-$ Thy-1^{high}SCA-1^{high} cell subpopulation in the colon of RORγt-sufficient RAG-2$^{-/-}$ mice. Importantly, however, the subpopulation of possibly pathological RORγt$^+$ IFN-γ$^+$ ILC1 cells in the colon of colitic DSS-treated RORγt-deficient RAG-2$^{-/-}$ mice was also significantly reduced as compared to that in the paired colitic RORγt-sufficient RAG-2$^{-/-}$ mice. Therefore, we concluded that RORγt-expressing ILC22 cells may be protectively involved in the development of DSS-induced colitis, but the pathogenesis of colitis in terms of ILC family members seems to be not as simple. Namely, we need to know about the balance between protective and pathological RORγt-dependent ILCs and the distinctive mechanisms due to different types of colitis to establish the exact mechanisms of the pathogenesis of innate colitis in mice.

Previous studies have suggested the protective role of IL-22 in the pathogenesis of colitis in mice [7]. For instance, Ouyang's group has demonstrated innate immune function of IL-22 that induces the production of REG3β and REG3γ from colonic tissues in a model of *Citrobacter rodentium* infection, although this group suggested that IL-22 is derived from Th17 cells and dendritic cells in that setting [19]. Furthermore, IL-22R seems to be expressed only on non-hematopoietic cells, such as epithelial cells [20]. These findings suggest that IL-22 is crucially involved in intestinal epithelial cell repair through IL-22R on epithelial cells and the following production of mucin and REG3 family proteins. Furthermore, Eberl's group previously demonstrated that not only IL-22 expression was markedly increased in ILCs of the intestine of RAG-2$^{-/-}$ mice after DSS administration, and RAG-2$^{-/-}$ × RORγt$^{-/-}$ mice rapidly showed more severe wasting disease as compared to the control RAG-2$^{-/-}$ × RORγt$^{-/-}$ mice [21]. However, this group always used the samples of SI, and did not assess the severity of colitis in detail [21]. However, we found in this study that RORγt$^+$IL-22$^+$ ILC22 cells were abundant in the SI, but scarce in the colon of RAG-2$^{-/-}$ × RORγt$^{gfp/+}$ mice under both physiological and DSS-induced inflammatory conditions. Therefore, ILC22 cells in the colon may

not be positively involved in protection against chronic DSS-induced colitis.

Emerging evidence suggests that another subpopulation, IFN-γ- and/or IL-17A-producing ILCs (ILC1, ILC17 and ILC1/17), is involved in the pathogenesis of animal models of IBD. In this regard, Powrie's group has described pathological ILCs in models of innate colitis [15]. In the model of *H.h.*-infected $RAG^{-/-}$ mice, the proportion and absolute number of unique $Thy1^{high}SCA\text{-}1^{+}$ ILCs that produce IL-17A and IL-22 and IFN-γ (ILC17 or ILC1/17 cells) were markedly increased in the colonic LP. Like LTi/LTi-like cells under physiological conditions, ILCs in colitic *H.h.*-infected $RAG^{-/-}$ mice are Lin^{-} $ROR\gamma t^{+}NKp46^{-}IL\text{-}7R\alpha^{+}$. This group attempted using another innate colitis model, in which $RAG^{-/-}$ mice were administered anti-CD40 mAb to activate antigen-presenting cells (APCs) [15]. In this model, increased $Thy\text{-}1^{high}SCA\text{-}1^{+}$ ILCs also emerged in inflamed colon mucosa, and anti-Thy-1 mAb treatment blocked development of colitis. Intriguingly, these ILCs produced IFN-γ and IL-22, but not IL-17A. In sharp contrast to $ROR\gamma t^{-/-} \times RAG\text{-}2^{-/-}$ mice in our DSS-induced colitis model, $ROR\gamma t^{-/-} \times RAG^{-/-}$ mice were resistant to this anti-CD40 mAb administration model, suggesting that RORγt is essential for the generation of the second type of pathological ILC1 cells. It remains unknown whether ILC22 cells are also involved in this anti-CD40 mAb administration colitis model, and if so, whether treatment with anti-Thy-1 mAb depletes these possibly protective ILC22 cells. In our study, we showed that both protective IL-22-producing ILC22 and pathogenic IFN-γ-producing ILC1 cells in colitic $RAG\text{-}2^{-/-} \times ROR\gamma t^{gfp/gfp}$ mice were impaired in the model of chronic DSS colitis. Therefore, the balance of both types of ILCs may determine whether RORγt-deficient mice develop colitis. In this regard, it is notable that the DSS colitis model seems to be due to epithelial damage, whereas *H.h.*-induced or anti-CD40-treated RAG-deficient colitis models are due to direct activation of APCs. Further studies should address the developmental pathway and plasticity of colitogenic ILCs, their relationship to ILC22 cells, and the reason these two pathological ILCs produce IL-22.

Collectively, the presence of RORγt-dependent protective ILC22 cells and pathogenic ILC1/ILC17 cells, and the strict balance of protective and pathogenic ILCs may be involved in the prevention of chronic DSS-induced colitis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.091.

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