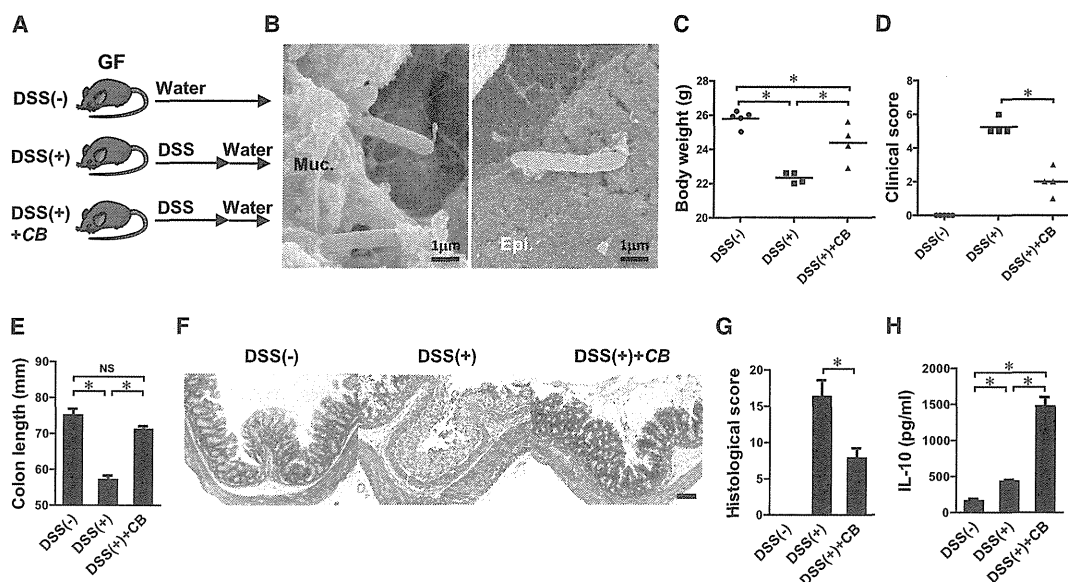

Figure 1. *CB* Monoassociation Suppresses the Development of Acute DSS-Induced Colitis

(A) Experimental design. GF mice were either untreated or orally inoculated with *CB*, and 1.0% DSS treatment followed.

(B) Scanning electron microscopy showing the distal colon of *CB*-monoassociated mice treated with DSS. Epi., epithelial cells; Muc., mucus.

(C and D) Body weight (C) and clinical score (D) on day 10. Each symbol represents an individual mouse; horizontal lines indicate the mean values.

(E) Colon length of each group.

(F) Histopathology of distal colon.

(G) Histological scores of the distal colon.

(H) LPMCs obtained from the colon were restimulated with heat-killed *CB* for 24 hr, and the culture supernatants were analyzed for IL-10 by CBA assay.

Error bars represent the mean ± SEM (n = 4–5 mice per group). $^*p < 0.05$. NS, not significant. Original magnification, ×100. The scale bar represents 100 μm. See also Figure S1.

induced IL-10 production by $F4/80^{+}CD11b^{+}CD11c^{int}$ intestinal macrophages, but not $CD4^{+}$ T cells, in inflamed mucosa, thereby suppressing experimental colitis by an IL-10-dependent mechanism.

RESULTS

CB Suppresses Development of Acute DSS-Induced Colitis

Mounting evidence suggests that commensal *Clostridium* strains have major effects on the development of the gut immune system, thereby regulating gut homeostasis (Atarashi et al., 2011; Ivanov et al., 2009). The *Clostridium* strain *CB* is a noncommensal bacterium in the intestine yet has been used for various human gastrointestinal diseases in clinical settings (Seki et al., 2003). Although *CB* has a major impact on the treatment of gastrointestinal diseases, the precise mechanism by which it prevents disease remains to be elucidated. To determine whether colonization by *CB* has an immunomodulatory role in intestinal inflammation, we used DSS to induce acute colitis in germ-free (GF) or *CB*-monoassociated mice (Figure 1A). We confirmed that colonization of *CB* was retained in the distal colon (the most inflamed area) even after DSS administration (Figure 1B). Body weight (BW) of GF mice was significantly reduced by DSS administration and was significantly attenuated in *CB*-monoassociated mice (Figure 1C). As shown in Figure 1D, monoassociation with *CB* significantly ameliorated the clinical score compared with GF DSS mice. Macroscopic findings showed bloody stools and shortened colon length in GF DSS mice, which was reduced in *CB*-monoassociated mice (Figure 1E). Histological scoring confirmed the clinical improvement due to *CB* monoassociation (Figures 1F and 1G). Anti-inflammatory effects of *CB* were also observed in a different colitis model. Similar to the acute DSS colitis model, *CB* treatment prevented colitis development in an oxazolone-induced colitis model (Figures S1A–S1F available online). Although *CB* exhibited a colitis-preventing effect in two different experimental colitis models, the mechanism by which *CB* suppressed colitis development remains unclear. To address this, we isolated lamina propria mononuclear cells (LPMCs) from inflamed colons, and assessed cytokine profiles. Intriguingly, production of IL-10, an anti-inflammatory cytokine, by LPMCs was greatly enhanced in DSS-treated *CB* mice compared with other groups (Figure 1H), suggesting that IL-10 production in the LP might play a role in the therapeutic effect of *CB*.

Protective Effect of *CB* on DSS-Induced Colitis Is Dependent on IL-10

To further examine the involvement of IL-10 in the anticolitic effect of *CB*, we continuously administered neutralizing anti-IL-10 monoclonal antibody (mAb) to DSS-treated SPF mice with or without *CB* feeding (Figure 2A). Analysis of bacterial DNA in feces showed that feeding with *CB* did not significantly affect the representative species of microbiota resident in the

colon of SPF mice (Figure 2B). Interestingly, the anti-IL-10 mAb treatment of DSS-administered mice, irrespective of *CB* administration, significantly decreased the amount of *Clostridium coccoides* group and *Clostridium leptum* subgroup when compared with paired mice without anti-IL-10 mAb treatment (Figure 2B). In accordance with a previous report (Heimesaat et al., 2007), the amount of *Enterobacteriaceae*, a pathological bacterial candidate, was significantly increased after DSS administration, but was not altered among DSS-administered groups of mice (Figure 2B). DSS-administered mice developed colitis as assessed by the body weight curve (Figure 2C), disease activity index (DAI) (Figure 2D), colon length (Figure 2E), histological findings (Figure 2F), and histological score (Figure 2G). Of note, all of these manifestations of DSS-induced colitis were significantly attenuated by *CB* feeding (Figures 2C–2G). Notably, the protective effect of *CB* was completely abrogated by coadministration of anti-IL-10 mAb, assessed with the above parameters (Figures 2C–2G), and in mice with similar levels of colitis symptoms to those observed in anti-IL-10 mAb-treated and DSS-administered mice. This confirmed the IL-10-dependent probiotic effect of *CB* on colitis. These results, as well as the monoassociation of *CB* shown in Figure 1, indicated that the probiotic effect of *CB* is not mediated through modification of the commensal microbial community (e.g., increased anti-inflammatory commensals or suppressed colitogenic pathobionts), but rather through the direct induction of IL-10-mediated anti-inflammatory immunity by *CB* itself.

A Treg-Cell-Mediated Anti-inflammatory Role Might Be Dispensable for the Suppression of Colitis by *CB*

Since a major protective effect of *Clostridium* strains against colitis is mediated by the induction of IL-10-producing transcription factor forehead box P3 (Foxp3)$^+$ iTreg cells (Atarashi et al., 2011), whether the anti-inflammatory effect of *CB* was mediated through Treg cells was assessed. Unexpectedly, the frequency and number of CD4$^+$Foxp3$^+$ Treg cells were not increased in *CB*-treated mice compared with non-*CB* treatment group in colitis conditions (Figures 3A and 3B). Instead, the frequency of intestinal IL-10-expressing CD11b$^+$F4/80$^+$ macrophages in *CB*-administered DSS mice was significantly increased compared with that in DSS-administered mice or in mice that had not been treated with DSS (Figure 3C). These results suggested that *CB* could induce IL-10-producing macrophages but not Treg cells in the inflamed colon and that it might regulate intestinal inflammation through these regulatory cells. Although *CB* treatment did not increase the number Foxp3$^+$Treg cells in the inflamed intestine, the functional involvement of Treg cells in the suppression of colitis cannot be ruled out by only quantification of cell numbers in the intestine. To address whether the anti-inflammatory effect of *CB* was conserved in Treg-null mice, we depleted CD4$^+$CD25$^+$ Treg cells by injection of anti-CD25 mAb (PC61) and administered mice DSS with or without *CB* treatment (Figure 3D). We confirmed that the PC61 treatment protocol induced CD4$^+$CD25$^+$ cell depletion in the spleen irrespective of *CB* treatment (Figure 3E). Depletion of Treg cells by PC61 significantly worsened DSS colitis in terms of colon length and histology/histological scores (Figures 3F–3I), suggesting that Treg cells are partly involved in the prevention of acute DSS colitis. Consistent with our earlier observation, *CB* administration almost completely prevented DSS colitis in Treg-intact mice (Figures 3F–3I). Notably, *CB* administration significantly suppressed colitis even in Treg-cell-depleted mice (Figures 3F–3I), suggesting that *CB* might suppress intestinal inflammation by a Treg-cell-independent anti-inflammatory mechanism. However, *CB* administration did not fully prevent the development of colitis in the absence of Treg cells (Figures 3F–3I), indicating that Treg cells also have a partial role in the *CB*-mediated anti-inflammatory effect.

Protective Effect of *CB* in DSS-Induced Colitis in *Rag2*$^{-/-}$ Mice Is IL-10 Dependent

To confirm the T cell independent anti-inflammatory mechanism of *CB* on colitis, we determined whether *CB* induced a suppressive effect on colitis in T-cell-deficient conditions. It was previously reported that recombination activating gene 2 (Rag-2)-deficient (*Rag2*$^{-/-}$) mice developed colitis after DSS administration (Katakura et al., 2005). As shown in Figure 4, *CB* treatment significantly prevented DSS-induced colitis in *Rag2*$^{-/-}$ mice. Notably, this anti-inflammatory effect of *CB* was prevented by the administration of IL-10 neutralizing antibody (Figures 4A–4F), suggesting that the anti-inflammatory effect was mediated through the promotion of T-cell-independent IL-10 production. Consistent with the in vivo results, *CB* induced robust IL-10 production in isolated colonic LPMCs from colitic *Rag2*$^{-/-}$ mice (Figure S2A). In addition, another commensal bacterial strain *Enterococcus faecalis* (*EF*), induced a minimal amount of IL-10 isolated colonic LPMCs (Figure S2A). The *CB*-induced IL-10 production was observed in CD11b$^+$ cells but not in CD11b$^-$ cells from the LPMCs (Figure S2B). Moreover, flow cytometric analysis indicated that a CD11b$^+$CD11c^{int} macrophage subset was the major producer of IL-10 after *CB* stimulation (Figures S2C and S2D).

CB Induces IL-10 Production by Macrophages in Inflammatory Conditions

Next, we attempted to confirm IL-10 production by intestinal macrophages in vivo using *Il10*^{Venus} reporter mice. DSS colitis was triggered in *Il10* ^{Venus} reporter mice with or without *CB* treatment. In the inflamed colonic LP, IL-10 (Venus) expression was significantly enhanced in intestinal CD11b$^+$CD11c^{int} macrophages by *CB* administration, but not in CD4$^+$ T cells (Figures 5A and 5B). Consistent with flow cytometric analysis, immunohistochemical analysis demonstrated the majority of IL-10-Venus$^+$ cells in the LP of *CB*-treated mice were CD11b$^+$, but not CD4$^+$, cells (Figure 5C). We next confirmed IL-10 production by CD11b$^+$ macrophages in *CB*- or *EF*-stimulated LPMCs from DSS-treated colitic *Il10*^{Venus} mice. IL-10 production was markedly induced in response to *CB*, but not *EF*, in colitis *Il10*^{Venus} CD11b$^+$ CD11c^{int} macrophages but not in CD4$^+$ T cells and Foxp3$^+$CD4$^+$ Treg cells (Figures 5D and 5E).

IL-10 Induction in *CB*-Stimulated Macrophages Is TLR2-MyD88 Dependent

Taken together, the results suggested that *CB* treatment promoted IL-10 production by intestinal macrophages in inflamed mucosa. To determine whether *CB*-induced IL-10 secretion by intestinal macrophages was directly induced by bacterial components of *CB* or mediated through secreted metabolites of

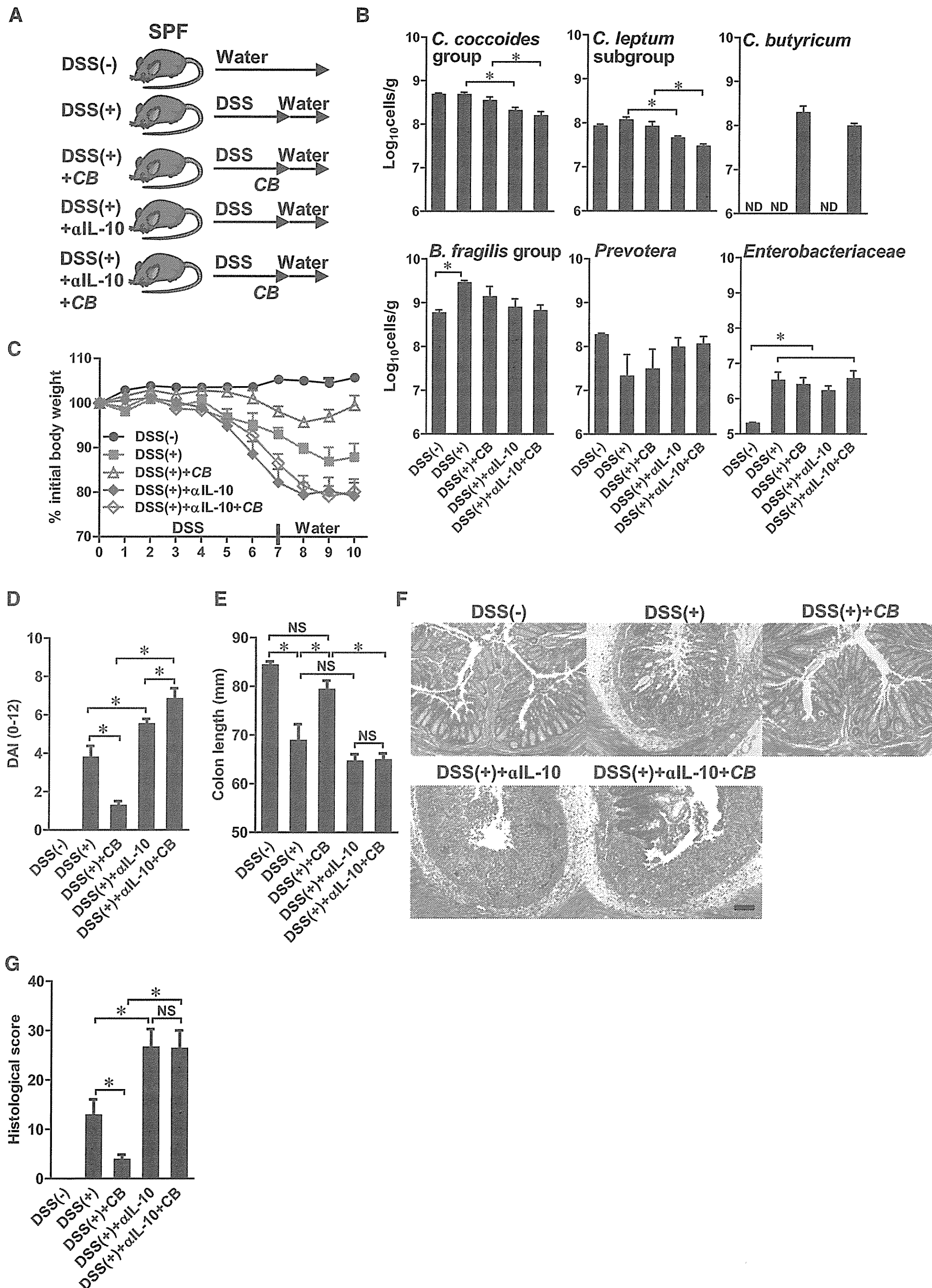

Figure 2. Protective Effect of *CB* Treatment in DSS-Induced Colitis Is IL-10 Dependent

(A) Experimental design. SPF mice were treated with *CB*. After 1 week, neutralizing anti-IL-10 mAb or control IgG (0.4 mg/200 μl) were administered 2 hr before 1.0% DSS treatment and then every 48 hr.

(B) Bacterial DNA from the feces in each group of mice was extracted and analyzed by quantitative PCR (qPCR) for the 16S ribosomal RNA coding gene.

(C) Change in body weight.

(D and E) DAI score (D) and colon length (E) on day 10.

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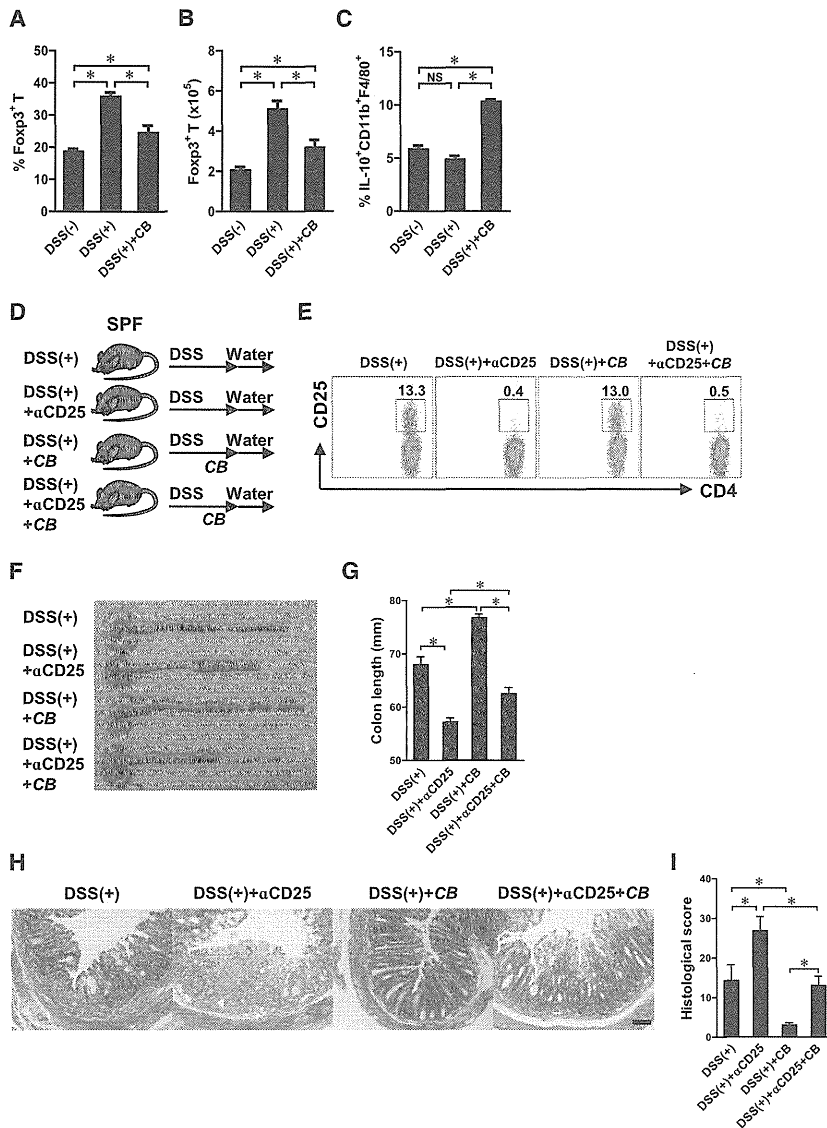

Figure 3. A Treg-Cell-Mediated Anti-inflammatory Role Might Be Dispensable for the Suppression of Colitis by *CB*

SPF mice were treated with *CB*, followed by 1.0% DSS treatment.

(A and B) The percentage (A) and absolute number (B) of Foxp3$^+$ cells in colonic LP CD3$^+$CD4$^+$ T cells.

(C) Statistical analysis of the percentages of IL-10$^+$ CD11b$^+$F4/80$^+$ macrophages.

(D) Experimental design. SPF WT mice were treated with *CB*. After 1 week, depleting anti-CD25 mAbs (PC61) or control IgGs (1 mg/500 μl) was administered 1 day before and 4 days after 1.0% DSS treatment.

(E) The percentage of CD25$^+$ cells in splenic CD3$^+$CD4$^+$ T cells.

(F and G) Representative gross appearance of colon (F) and colon length (G) on day 10.

(H) Histopathology of distal colon.

(I) Histological score.

Error bars represent the mean ± SEM (n = 4–5 mice per group). *p < 0.05. Original magnification, ×100. The scale bar represents 100 μm.

CB, we stimulated intestinal macrophages with heat-killed *CB* or *CB* culture supernatant in vitro. IL-10 production by inflamed colonic LPMCs was significantly induced by *CB* compared with *EF* (Figure 6A). Although the culture supernatant from *CB* slightly induced IL-10 by LPMCs, heat-killed bacteria was a more potent inducer of IL-10 (Figure 6A). Since neither heat-killed bacteria nor culture supernatant of *EF* induced measurable production of IL-10 by LPMCs compared with *CB*, bacterial components specific to *CB*, but not *EF*, might be capable of inducing IL-10. We further explored the mechanism by which *CB* induced IL-10 from intestinal macrophages in inflammatory conditions. Toll-like receptor 2 (TLR2) and myeloid differentiation factor 88 (MyD88) have been implicated in the recognition of, and signaling in response to, a variety of pathogen-associated molecular patterns (PAMPs) from gram-positive *CB* by innate immune cells including macrophages. Therefore, we isolated LPMCs from DSS-treated wild-type (WT), *Tlr2*$^{-/-}$, or *Myd88*$^{-/-}$ mice and stimulated them with heat-killed *CB* or *EF*. Cytokine profiling showed that *CB* induced significantly higher amounts of IL-10 and lower amounts of interferon γ in WT LPMCs compared with *EF* (Figure 6B). Of note, the induction of these cytokines was almost completely abolished in the absence of TLR2 or MyD88. These data suggest that PAMPs from *CB* are required to induce IL-10 production from intestinal macrophages through the TLR2-MyD88 signaling pathway (Figures 6C and 6D).

IL-10 Production from Macrophages Is Responsible for Protection against Colitis by *CB*

The results obtained suggested that *CB*-treatment induced IL-10 secretion by intestinal macrophages, but not T cells, in the inflamed colon. Although we demonstrated that IL-10 is the key factor for the colitis-preventing effect of *CB* and that *CB* induced IL-10 production by intestinal macrophages rather than T cells, there is still no direct evidence that macrophage-produced IL-10 prevents colitis after *CB* treatment. To address this, we

(F) Histopathology of distal colon.

(G) Histological score.

Error bars represent the mean ± SEM (n = 4–5 mice per group). *p < 0.05. NS, not significant; ND, not detectable. Original magnification, ×100. The scale bar represents 100 μm.

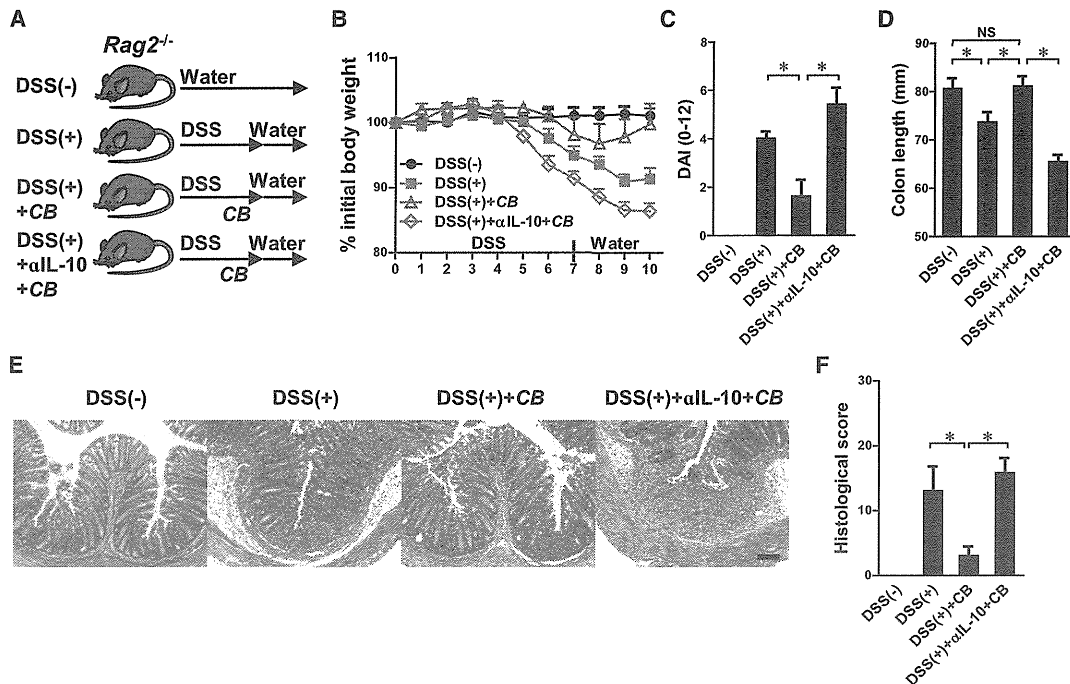

Figure 4. Protective Effect of *CB* Treatment on DSS-Induced Colitis in *Rag2*$^{-/-}$ Mice Is IL-10 Dependent

(A) Experimental design. SPF *Rag2*$^{-/-}$ mice were treated with *CB*. After 1 week, neutralizing anti-IL-10 mAb or control IgG (0.4 mg/200 μl) was administered 2 hr before 1.5% DSS treatment and then every 48 hr.

(B) Change in body weight.

(C and D) DAI score (C) and colon length (D) on day 10.

(E) Histopathology of distal colon.

(F) Histological score.

Error bars represent the mean ± SEM of 4–5 mice in each group. *$p < 0.05$. NS, not significant. Original magnification, ×100. The scale bar represents 100 μm. See also Figure S2.

used macrophage-specific *IL-10*-deficient (*Il10*$^{FL/FL}$ *lysM-Cre*$^+$) mice and administered DSS to *CB*-fed *Il10*$^{FL/FL}$ *Cre*$^-$ and *Il10*$^{FL/FL}$ *lysM-Cre*$^+$ mice (Figure 7A). *CB*-fed *Il10*$^{FL/FL}$ *Cre*$^-$ mice showed significantly improved DSS colitis in terms of weight loss (Figure 7B), colon length (Figure 7C), and histological findings/score (Figures 7D and 7E). In contrast, the protective effects of *CB* were canceled in *Il10*$^{FL/FL}$ *lysM-Cre*$^+$ mice (Figures 7B–7E), suggesting that IL-10 production by macrophages was crucial for the prevention of colitis by *CB*. Consistent with this in vivo phenotype, *CB*-stimulated LPMCs from *Il10*$^{FL/FL}$ *Cre*$^-$ mice produced robust amounts of IL-10, while limited amounts of IL-10 were observed in *CB*-stimulated LPMCs from *Il10*$^{FL/FL}$ *lysM-Cre*$^+$ mice (Figure 7F). Next, the adoptive transfer of LP CD11b$^+$ cells was assessed to confirm the role of IL-10 production by macrophages to control colitis. We obtained colitic LP CD11b$^+$ cells from WT and *Il10*$^{-/-}$ mice stimulated with or without *CB* in vitro and transferred them into DSS-treated WT mice (Figure 7G). Adoptive transfer of *CB*-stimulated WT macrophages but not of unstimulated WT macrophages significantly improved colitis (Figures 7H–7L). In contrast, when LP macrophages from *Il10*$^{-/-}$ mice were transferred, in vitro *CB* treatment failed to ameliorate the severity of DSS colitis (Figures 7H–7L). Taken together, these data indicated that IL-10 production by macrophages is indispensable for *CB*'s probiotic effects against colitis.

DISCUSSION

Although emerging evidence shows that specific commensal bacteria can affect the differentiation of mucosal Th17 and iTreg cells under normal conditions (Atarashi et al., 2011; Geuking et al., 2011; Ivanov et al., 2009; Littman and Pamer, 2011; O'Mahony et al., 2008; Round et al., 2011), it has remained largely unknown which specific bacteria influence mucosal-specific antigen-presenting cell (APC) phenotypes. Here we demonstrated that *CB* induced the production of IL-10 by colonic F4/80$^+$CD11b$^+$CD11c^{int} macrophages rather than Treg cells in inflammatory conditions and suppressed acute experimental colitis in an IL-10-dependent manner.

IL-10 has important roles in the regulation of gut homeostasis during host defense (Maloy and Powrie, 2011; Maynard et al., 2007). The association between IL-10 and inflammatory bowel disease (IBD) has been demonstrated by several findings in both humans and in animal models. For example, *Il10*$^{-/-}$ mice spontaneously develop colitis in the presence of commensal bacteria (Kühn et al., 1993; Spencer et al., 1998), while a genome-wide association study in humans has shown that a single-nucleotide polymorphism in the *Il10* gene is closely associated with IBD (Franke et al., 2008). In this regard, various immune cell types produce IL-10 in the gut. Treg cells are a major

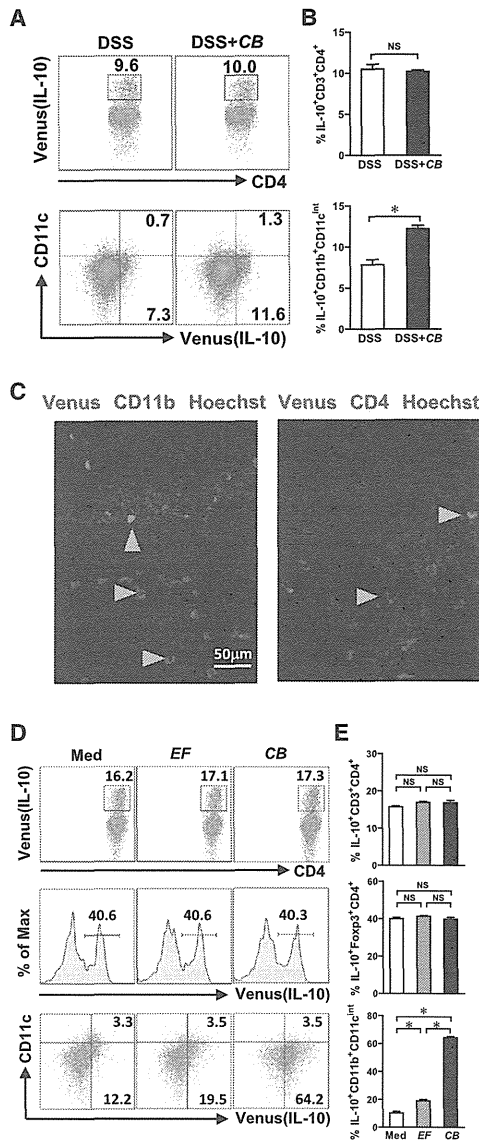

Figure 5. *CB* Induces IL-10 Production by Macrophages in Inflammatory Conditions

(A) LPMCs from DSS-administered *Il10*^{Venus} mice with or without *CB* treatment were isolated, and IL-10 (Venus) expression was determined in CD3$^+$CD4$^+$ T cells (top) and CD11b$^+$ macrophages (bottom).

(B) Statistical analysis of the percentages of IL-10 (Venus)$^+$ in CD3$^+$CD4$^+$ T cells (top) and IL-10 (Venus)$^+$ in CD11b$^+$CD11c^{int} cells (bottom).

(C) Immunohistochemistry of CD11b (red)/IL-10 (Venus) (left) and CD4 (red)/IL-10 (Venus) (right) in the colon of DSS-administered *Il10*^{Venus} mice with *CB* treatment.

(D) LPMCs from colitic DSS (+) SPF *Il10*^{Venus} mice were cultured without (Med) or with heat-killed *CB* or *EF* for 24 hr. Expression of Venus (IL-10) in CD4$^+$ T cells, Foxp3$^+$CD4$^+$ T cells, and CD11b$^+$ macrophages from colitic *Il10*^{Venus} mice is shown.

(E) Statistical analysis of the percentages of IL-10$^+$ cells in CD3$^+$CD4$^+$ T cells (top), IL-10$^+$ cells in Foxp3$^+$CD3$^+$CD4$^+$ T cells (middle) and IL-10$^+$ cells in CD11b$^+$CD11c^{int} cells (bottom).

Data are representative of three independent experiments. Error bars represent the mean ± SEM of triplicates. *$p < 0.05$. int, intermediate; NS, not significant.

source of IL-10 in the gut (Honda and Littman, 2012; Maynard et al., 2007), and Treg-specific *Il10*$^{-/-}$ mice spontaneously develop colitis, indicating the importance of Treg-derived IL-10 in the suppression of colitis (Roers et al., 2004; Rubtsov et al., 2008).

A recent report demonstrated that colonization of GF mice with a mixture of 46 *Clostridium* cluster IV and XIVa strains promoted the accumulation of IL-10-producing iTreg cells in the colon and induced resistance to acute DSS-induced colitis. Thus, a diverse set of metabolites produced by these 46 strains of *Clostridium* might be required for the optimal induction of iTreg cells (Atarashi et al., 2011).

Unlike these commensal *Clostridium* strains, the probiotic *Clostridium* strain *CB* primarily induced IL-10 production from colonic F4/80$^+$CD11b$^+$CD11c^{int} intestinal macrophages rather than Foxp3$^+$ Treg cells and prevented experimental colitis. In addition to Treg cells, intestinal mononuclear phagocytes, such as intestinal macrophages, are major producers of IL-10 and play pivotal roles in the regulation of intestinal homeostasis and inflammation (Liu et al., 2011). Yet the responsible commensal bacterial strains remain unidentified, although it was reported that IL-10 production by intestinal macrophages was enhanced by colonization with commensal microbiota (Rivollier et al., 2012; Ueda et al., 2010). However, unlike these unidentified commensal bacteria, monoassociation of *CB* in GF mice failed to increase the production of IL-10 by intestinal macrophages in the steady state (data not shown). However, in inflamed mucosa, *CB* directly stimulated intestinal macrophages through the TLR2/MyD88 signaling pathway and induced IL-10 production by macrophages, suggesting that *CB* preferentially induces IL-10 by newly recruited and/or proliferating macrophages during intestinal inflammation but does not activate resident macrophages in the intestine. This possibility is also supported by evidence that resident intestinal macrophages are hyporesponsive to microbial stimulation, including TLR2 ligands (Mowat and Bain, 2011), while newly recruited inflammatory monocytes and macrophages during inflammation can respond to these bacterial ligands (Murray and Wynn, 2011; Smith et al., 2011). Thus, this difference in responsiveness to microbial ligands between resident and inflammatory macrophages might explain the failed induction of macrophage-mediated IL-10 production by *CB* under physiological conditions.

The role of bacterial pattern recognition receptor signaling in commensal bacteria-mediated development of anti-inflammatory immunity is controversial for each bacterium. MyD88 signaling is dispensable for Foxp3$^+$ Treg induction by cocktail of 46 *Clostridium* species, whereas Treg induction by colonization with a cocktail of eight defined commensals, called altered Schaedler flora, is MyD88 dependent (Atarashi et al., 2011; Geuking et al., 2011). A human commensal bacterium, *Bacteroides fragilis*, promoted IL-10-producing Tregs and/or macrophages through the TLR2 dependent pathway that was activated by its outer membrane vesicle component polysaccharide A (PSA) (Cohen-Poradosu et al., 2011; Mazmanian et al., 2008; Round et al., 2011; Shen et al., 2012). In the present study, we demonstrated that *CB* could induce IL-10 from intestinal macrophages through the TLR2/MyD88-mediated pathway. Although the precise active component of *CB* is

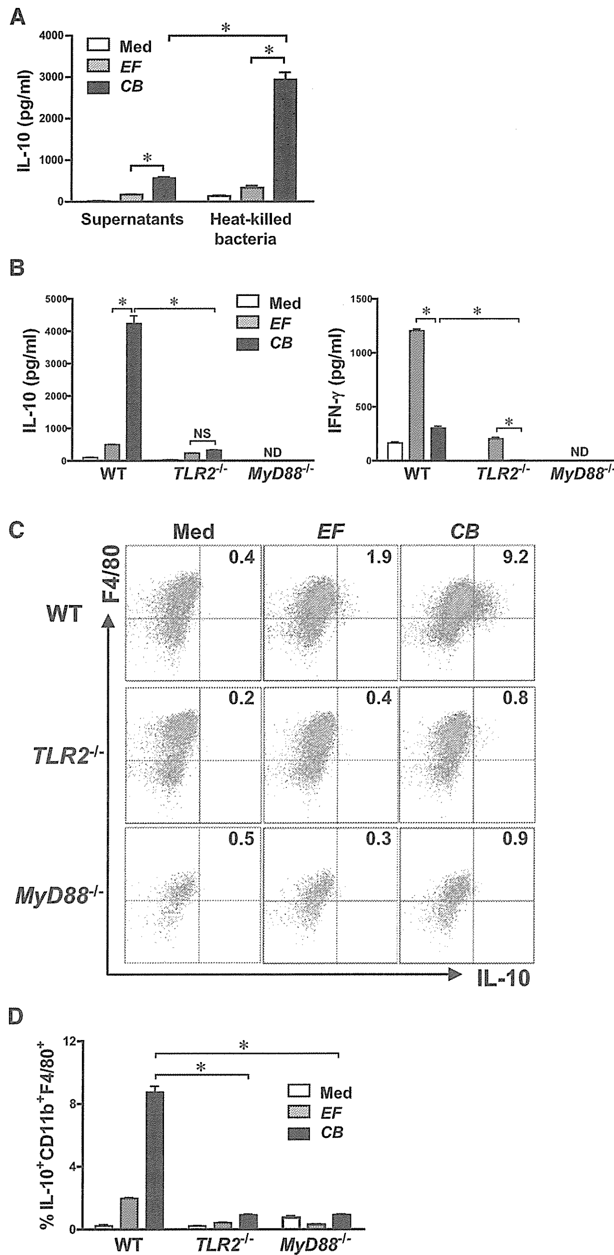

Figure 6. IL-10 Induced in *CB*-Stimulated Macrophages Is TLR2-MyD88 Dependent

(A) LPMCs from colitic DSS-administered mice were cultured with heat-killed *CB* or *EF*, or supernatant of cultured *CB* or *EF* for 24 hr. IL-10 concentrations were determined by CBA assay.

(B) LPMCs were isolated from DSS-treated WT, $Tlr2^{-/-}$, or $Myd88^{-/-}$ mice and stimulated without (Med) or with heat-killed *CB* or *EF* for 24 hr. IL-10 in the culture supernatants was measured by CBA assay.

(C) Intracellular IL-10 in $CD11b^+F4/80^+$ macrophages was analyzed by flow cytometry.

(D) Statistical analysis of the percentages of $IL-10^+$ cells in $CD11b^+F4/80^+$ macrophages.

Data are representative of three independent experiments. Error bars represent the mean ± SEM. $^*p < 0.05$. NS, not significant; ND, not detectable.

unclear, heat-killed *CB*, but not culture supernatant that may include secreted components and/or metabolites, induced IL-10 production by macrophages by the TLR2/MyD88 pathway. Unlike *CB*, an anti-inflammatory subset of human commensal *Clostridium leptum* group strains, *Faecalibacterium prausnitzii*, secretes its active component into culture supernatant, such that culture supernatant of *F. prausnitzii* prevents colitis through the induction of IL-10 (Sokol et al., 2008). Thus, certain kinds of anti-inflammatory commensal microbiota and probiotic bacteria might harbor a specific structure to promote IL-10 production by T cells and/or non-T cells, thus regulating gut homeostasis and intestinal inflammation.

It is noteworthy that in addition to individual immune regulatory functions of IL-10-producing T cells or non-T cells, interplay between IL-10-producing T cells and IL-10-producing non-T cells is also important for the regulation of immune homeostasis and disease suppression. It was reported that intestinal $F4/80^+CD11b^+$ macrophages can promote IL-10-producing $Foxp3^+$ Treg development by an IL-10-mediated mechanism (Denning et al., 2007). In addition to the homeostatic immune responses, IL-10 secretion by dendritic cells (DCs) is required for the induction of IL-10-producing T cell development and prevention of colitis by *B. fragilis* PSA (Shen et al., 2012). Likewise, the anticolitic effect of phosphoglycerol transferase gene-deleted *Lactobacillus acidophilus* was negated by depletion of Treg cells with anti-CD25 antibody (PC61) treatment, despite this bacterial strain inducing IL-10 from either DCs or Treg cells (Mohamadzadeh et al., 2011). In the current study, macrophage-specific IL-10 depletion and IL-10-deficient macrophage adoptive transfer experiments strongly suggested that IL-10 production by intestinal macrophages was the key factor for *CB*-mediated anti-inflammatory effects. However, although *CB* treatment induced an anti-inflammatory effect in Treg-cell-depleted mice, *CB* failed to prevent colitis completely in these mice. This indicated a potential pathway where IL-10 produced by *CB*-treated intestinal macrophages might contribute to further anti-inflammatory responses by inducing IL-10-producing Treg cells.

Probiotic treatment is an effective treatment option for gastrointestinal disorders, including IBD (Sartor, 2004). A major therapeutic effect of probiotics is mediated by their metabolites, such as oligosaccharides and short-chain fatty acids (Macia et al., 2012). Metabolites produced by probiotic bacterial strains improve the ecosystem of the intestinal tract via promoting the growth of healthy symbionts and/or enhancing the barrier function of epithelial cells (Round and Mazmanian, 2009). However, in this study, *CB* administration did not alter the composition of gut microbiota in DSS-treated mice, suggesting that the suppressive effect of *CB* on DSS-induced colitis was not mediated by altered commensal bacteria composition. Furthermore, the ability of *CB* to increase IL-10 production from macrophages was not due to metabolites produced by live *CB*. Commensal bacterial strains are thought to be innocuous in the healthy intestinal tract; however, it is evident that inflammatory macrophages, which accumulate in the intestine of IBD patients, robustly respond to commensal bacteria and produce proinflammatory cytokines, such as IL-23 and tumor necrosis factor α (Kamada et al., 2008). This evidence suggests commensal bacteria might penetrate the LP, and thus enhance pathogenic responses, in

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inflammatory conditions such as those seen in IBD patients and that this might worsen intestinal inflammation. Because *CB* induced an IL-10-mediated immune-suppressive effect even under inflammatory conditions, *CB* might be a "safer" therapeutic option as a probiotic bacterium for the treatment of IBD patients experiencing an active inflammatory phase.

Collectively, these findings clarify the role of a probiotic strain of *Clostridium* species, *CB*, which regulates IL-10 production by intestinal macrophages in inflamed mucosa and prevents the development of colitis.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice were purchased from Japan Clea, and *Rag2*$^{-/-}$ mice were obtained from Taconic Laboratory and Central Laboratories for Experimental Animals. *Il10*$^{-/-}$ C57BL/6 mice were purchased from Jackson Laboratories. *Il10*^{Venus} mice were previously described (Atarashi et al., 2011). *Il10*$^{FL/FL}$*lysM-Cre*$^{+}$ mice were established previously (Roers et al., 2004; Siewe et al., 2006). *Tlr2*$^{-/-}$ mice were purchased from Oriental Bio Service. *Myd88*$^{-/-}$ mice were a kind gift from Dr. Shizuo Akira (Osaka University). GF C57BL/6 mice were purchased from Sankyo Laboratories (Supplemental Experimental Procedures). All experiments were approved by the regional animal study committees and were performed according to institutional guidelines.

Heat-Killed Bacteria and Culture Supernatants

A gram-positive commensal strain of *EF* (catalog number 29212; American Type Culture Collection) was cultured in brain-heart infusion (BHI) medium. *Clostridium butyricum* MIYAIRI 588 used in this study was obtained from Miyarisan Pharmaceutical (Supplemental Experimental Procedures). *EF* or *CB* was cultured in BHI medium. After 24 hr culture at 37°C, the culture broth was centrifuged at 6,000 × *g* for 10 min and filtrated through a 0.2 μm filter. The supernatants were collected and stored at −80°C.

Tissue Preparations

Intestinal LPMCs were prepared as previously described (Totsuka et al., 2008), with slight modifications (Supplemental Experimental Procedures). LP CD11b^{+} and CD11b^{-} cells were obtained by positive selection with anti-CD11b MACS beads. The resultant cells analyzed by a FACSCanto II (Becton Dickinson) contained >95% CD11b^{+} cells.

Stimulation of LPMC

LPMCs were seeded on 96-well tissue culture plates (1×10^6 cells/ml) and stimulated with each strain of heat-killed bacteria (1×10^7 cells/ml) for 24 hr at 37°C in a humidified incubator with 5% CO_2. For measurement of secreted cytokines, culture supernatants were collected and stored at −80°C.

DSS-Induced Colitis

For induction of acute experimental colitis, SPF or GF C57BL/6 background WT, *Myd88*$^{-/-}$, *Tlr2*$^{-/-}$, *Il10*$^{-/-}$, *Il10*^{venus}, or *Rag2*$^{-/-}$ mice received 1.0%–2.0% DSS (molecular weight 50 kDa; Ensuiko) dissolved in sterile distilled water ad libitum. GF WT mice were inoculated with a single *CB* (1×10^8 cells/200 μl). Three weeks after inoculation, GF and *CB*-monoassociated mice were administered 1.0% DSS for 7 days followed by regular drinking water for 3 days. SPF mice were fed a normal diet or a diet containing 5×10^8 colony-forming units/g *CB*. After 1 week, mice were administered 1.0% DSS for 7 days followed by regular drinking water for 3 days (Supplemental Experimental Procedures). Mice were weighed daily and visually inspected for diarrhea and rectal bleeding. The DAI was assessed blinded to the mouse groups (Siegmund et al., 2001) (Supplemental Experimental Procedures).

Oxazolone-Induced Colitis

Acute Oxazolone colitis was induced as previously described (Wirtz et al., 2007). In brief, C57BL/6 mice were presensitized with 150 μl 3% oxazolone (Sigma-Aldrich) in 100% ethanol. Seven days after presensitization, mice were rechallenged intrarectally with 100 μl 1% oxazolone in 50% ethanol.

Clinical and Histological Scorings of Colitis

All mice were observed for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. The clinical score of mice was assessed at autopsy (Mikami et al., 2010). Histological activity score (Mennigen et al., 2009) (maximum total score 40) was assessed as the sum of three parameters, extent, inflammation, and crypt damage as described in the Supplemental Experimental Procedures.

Electron Microscopy

Dissected 0.5 cm pieces of the colon were cut open and fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. The samples were fixed with 2% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 2 hr and were then dehydrated through a series of graded ethanol wash steps for scanning electron microscopy. The samples were transferred into tert-butyl alcohol three times for 30 min each and frozen at 4°C. After drying, the samples were coated with a thin layer (35 nm) of osmium with an osmium plasma coater (NL-OPC80NS, Nippon Laser & Electronics Laboratory). The samples were observed with a scanning electron microscope (S-800, Hitachi) at an acceleration voltage of 10 kV, and images were captured with a digital camera.

Immunohistochemistry

Consecutive cryostat colon sections were used for immunohistochemistry with purified rat anti-CD4 (RM4-5; BD PharMingen), rat anti-CD11b (M1/70; Abcam) and rabbit anti-GFP (green fluorescent protein; MBL) mAb. In brief, tissue samples were fixed in PBS containing 4% paraformaldehyde for 2 hr and were then placed in PBS containing 15% sucrose overnight, replaced in PBS containing 30% sucrose overnight, mounted in optimal cutting temperature (OCT) embedding compound, and frozen at −80°C. OCT-embedded tissue samples were cut into 10-μm-thick serial sections, placed on coated slides, and fixed with 2% paraformaldehyde phosphate buffer solution for 5 min. Slides were then incubated with the primary antibody at room temperature for 2 hr and stained with Alexa Fluor 568 goat anti-rat IgG (Invitrogen) for CD4 and CD11b detection and with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) for GFP detection at room temperature for 2 hr. All slides were counterstained with Hoechst (Invitrogen) and observed by confocal microscopy.

Flow Cytometry

For surface and intracellular staining, we used the same protocol as previously described (Atarashi et al., 2011) (Supplemental Experimental Procedures). FITC-, PE-, PerCP-Cy5.5-, APC-, PE-Cy7-, APC-Cy7- or Alexa Fluor 647-conjugated mAbs against CD11c (HL3), CD3 (145-2C11), and CD4 (RM4-5) were from BD Biosciences, CD11b (M1/70), F4/80 (BM8), Foxp3 (FJK-16S), and IL-10 (JES5-16E3) were from eBioscience, and CD25 (PC61) was from BioLegend (Supplemental Experimental Procedures).

Cytokine Assay

A mouse inflammatory cytometric bead array (CBA) kit (BD Biosciences) was used for cytokine measurements, according to the manufacturer's instructions. Samples were analyzed with a FACSCanto II flow cytometer (BD Biosciences).

Fecal DNA Extraction for qPCR

Bacterial genomic DNA was extracted from fecal pellets with phenol-chloroform extraction as previously reported (Matsuki et al., 2004). qPCR analysis was performed with a Thermal Cycler Dice Real Time System TP800 (Takara). The primer sets used in this study are described in the Supplemental Experimental Procedures.

Statistical Analysis

The results are expressed as mean ± SEM. Groups of data were compared with the Mann-Whitney U test, Tukey-Kramer test, and Student's t test. Differences were considered to be statistically significant when $p < 0.05$.

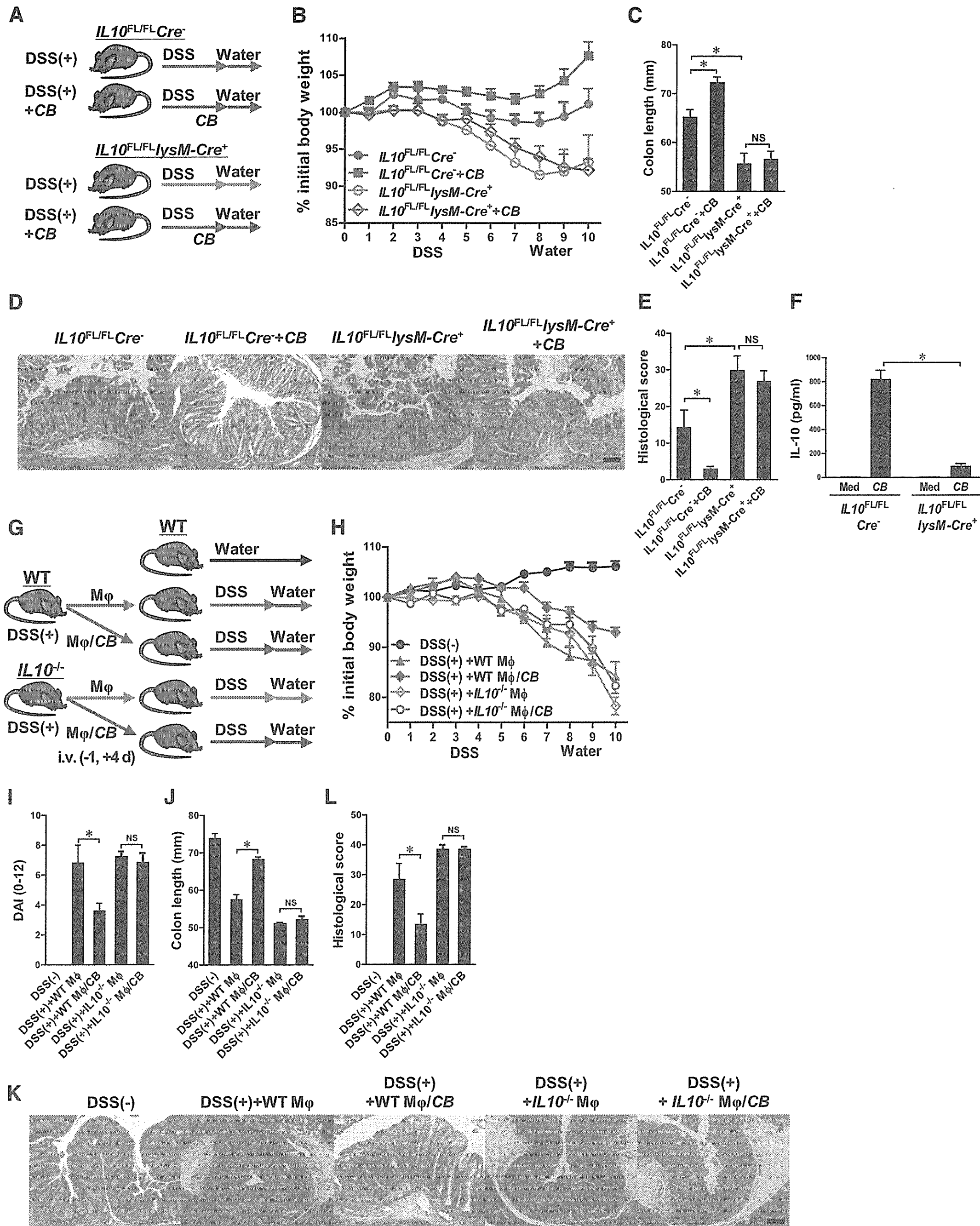

Figure 7. Macrophage-Specific IL-10 Deficiency Negated the Probiotic Effect of *CB*

(A) Experimental design 1. $Il10^{FL/FL}$ Cre^{-} and $Il10^{FL/FL}$ $lysM\text{-}Cre^{+}$ mice were treated with *CB*, followed by 1.0% DSS treatment.

(B) Change in body weight.

(C) Colon length on day 10.

(D) Histopathology of distal colon.

(E) Histological score.

(F) Colitic LPMCs obtained from $Il10^{FL/FL}$ Cre^{-} and $Il10^{FL/FL}$ $lysM\text{-}Cre^{+}$ mice were stimulated with or without heat-killed *CB* for 24 hr, and the culture supernatants were analyzed for IL-10 by CBA assay.

(G) Experimental design 2. LP $CD11b^{+}$ macrophages isolated from WT and $Il10^{-/-}$ mice treated with DSS were stimulated with or without *CB* for 24 hr in vitro. The stimulated macrophages (1×10^{6} cells/100 μl) were intravenously injected into WT mice on 1 day before and 4 days after 1.0% DSS treatment.

(H) Change in body weight.

(I and J) DAI score (I) and colon length (J) on day 10.

(legend continued on next page)

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.05.013.

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(L) Histological score.
Error bars represent the mean ± SEM of five mice in each group. *p < 0.05. NS, not significant; Mφ, macrophage. Original magnification, ×100. The scale bar represents 100 μm.

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OPEN ACCESS

ORIGINAL ARTICLE

Bone marrow-mesenchymal stem cells are a major source of interleukin-7 and sustain colitis by forming the niche for colitogenic CD4 memory T cells

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ABSTRACT

Objective Interleukin (IL)-7 is mainly produced in bone marrow (BM) that forms the niche for B cells. We previously demonstrated that BM also retains pathogenic memory CD4 T cells in murine models of inflammatory bowel disease (IBD). However, it remains unknown whether BM-derived IL-7 is sufficient for the development of IBD and which cells form the niche for colitogenic memory CD4 T cells in BM.

Design To address these questions, we developed mice in which IL-7 expression was specific for BM, and identified colitis-associated IL-7-expressing mesenchymal stem cells (MSC) in the BM.

Results IL-7$^{-/-}$×RAG-1$^{-/-}$ mice injected with BM cells from IL-7$^{+/+}$×RAG-1$^{-/-}$ mice, but not from IL-7$^{-/-}$×RAG-1$^{-/-}$ mice, expressed IL-7 in BM, but not in their colon, and developed colitis when injected with CD4^{+}CD45RB^{high} T cells. Cultured BM MSC stably expressed a higher level of IL-7 than that of primary BM cells. IL-7-sufficient, but not IL-7-deficient, BM MSC supported upregulation of Bcl-2 in, and homeostatic proliferation of, colitogenic memory CD4 T cells in vitro. Notably, IL-7$^{-/-}$×RAG-1$^{-/-}$ mice transplanted with IL-7-sufficient, but not IL-7-deficient, BM MSC expressed IL-7 in BM, but not in their colon, and developed colitis when transplanted with CD4^{+}CD45RB^{high} T cells.

Conclusions We demonstrate for the first time that BM MSC are a major source of IL-7 and play a pathological role in IBD by forming the niche for colitogenic CD4 memory T cells in BM.

Significance of this study

What is already known on this subject?

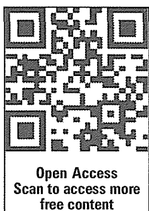
- IL-7 is mainly produced in BM.
- BM stromal cells forms the niche for B cells.
- We have previously reported that colitogenic memory CD4 T cells are retained in BM of IBD model mice in a IL-7-dependent manner.
- MSC have the ability to downmodulate inflammation and improve tissue repair, so their use to treat inflammatory diseases is being explored.

What are the new findings?

- IL-7$^{-/-}$×RAG-1$^{-/-}$ mice injected with BM cells from IL-7$^{+/+}$×RAG-1$^{-/-}$ mice, but not from IL-7$^{-/-}$×RAG-1$^{-/-}$ mice, expressed IL-7 in BM, but not in the colon, and developed colitis when injected with CD4^{+}CD45RB^{high} T cells.
- BM MSC produced larger amount of IL-7 than that of primary BM cells.
- IL-7-sufficient, but not IL-7-deficient, BM MSC supported upregulation of Bcl-2 in, and homeostatic proliferation of, colitogenic memory CD4 T cells in vitro.
- IL-7$^{-/-}$×RAG-1$^{-/-}$ mice transplanted with IL-7-sufficient, but not IL-7-deficient, BM MSC expressed IL-7 in BM, but not in the colon, and developed colitis when transplanted with CD4^{+}CD45RB^{high} T cells.

How might it impact on clinical practice in the foreseeable future?

- The present study may be a new example for changing concepts of IBD from intestinal to systemic disease, and a therapeutic approach targeting BM MSC-derived IL-7 may be feasible in the treatment of chronic immune diseases.



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INTRODUCTION

Inflammatory bowel disease (IBD) is characterised by chronic inflammation of the gastrointestinal tract. Accumulating evidence suggests that IBD is caused by an inappropriate response of the innate and acquired immune systems to commensal microbiota.[1] Even if IBD enters remission as a result of treatment, it often relapses, leading to its lifelong duration. Therefore, we hypothesised that even in remission, colitogenic memory T cells survive for a long period as 'pathogenic memory stem cells'[2 3] in IBD patients.

Interleukin (IL)-7 is an important cytokine involved in supporting the survival of naive and memory, but not effector, CD4 T cells.[4–6] IL-7 is secreted by stromal cells in the bone marrow (BM) and thymus, and by epithelial cells.[7] In a series of studies, we have consistently focused on IL-7 as an essential factor for the persistence of chronic T-cell-mediated colitis. We have shown that: (1) IL-7 is constitutively produced by intestinal goblet cells;[8] (2) IL-7 transgenic mice, in which strong promoters drive systemic overexpression, develop colitis that mimics the histopathological characteristics of human IBD;[9] (3) colitogenic CD4 effector memory T (T_{EM}) cells, which express high levels of IL-7Rα, reside in the inflamed lamina propria (LP)

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of RAG-2$^{-/-}$ mice injected with CD4$^+$CD45RB^{high} T cells;[10] and (4) IL-7$^{-/-}$×RAG-1$^{-/-}$ mice injected with CD4$^+$CD45RB^{high} T cells or colitogenic LP CD4$^+$ T$_{EM}$ cells do not develop colitis.[11]

However, we have found that the IL-7 level of colitic intestine is less than that of normal intestine as a result of the disappearance of goblet cells.[12] Therefore, we hypothesise that colitogenic memory CD4 T cells are maintained outside the intestine as memory stem cells. Because the spleen and lymph nodes are dispensable for the development of chronic colitis,[13] we found that BM is the main source of IL-7.[14 15] We previously demonstrated that, in addition to a major subpopulation of T$_{EM}$ cells, a substantial proportion of colitogenic CD4 central memory cells preferentially reside in the BM of colitic mice.[16 17] Importantly, these resident BM CD4 memory T cells are closely associated with IL-7-producing stromal cells and retain a significant potential to induce colitis after adoptive retransfer into new SCID/RAG-2$^{-/-}$ mice. BM CD4 memory T cells cannot induce colitis when they are transferred into IL-7$^{-/-}$×RAG-1$^{-/-}$ mice, suggesting that IL-7 plays an essential role in the maintenance of CD4 memory T cells in BM.[16] Using intrarectal administration of CD4 T cells, we also demonstrated that colitogenic memory CD4 T cells constantly recirculate from the LP to BM.[18]

However, two important questions remain: whether BM-derived IL-7 is sufficient for the maintenance of colitogenic CD4 memory T cells in the absence of IL-7 produced at other sites, and which cells in BM mainly produce IL-7 and form the niche for colitogenic memory CD4 T cells. To address these questions, we established mice in which IL-7 expression is specific to BM, and attempted to identify the IL-7-expressing cells in BM. Although it is generally accepted that mesenchymal stem cells (MSC) have the ability to downregulate inflammation, and their use to treat inflammatory diseases is being explored, we propose a new hypothesis in which BM MSC,[19–21] a candidate for the IL-7-producing stromal cells or their progenitors in BM, play a pathological role in the maintenance of colitogenic CD4 memory T cells.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from Japan CLEA (Tokyo, Japan). RAG-2-deficient mice (RAG-2$^{-/-}$) were obtained from Taconic Laboratory (Hudson, New York, USA) and Central Laboratories for Experimental Animals (Kawasaki, Japan). RAG-1$^{+/-}$ and IL-7$^{+/-}$ mice on the C57BL/6 background were kindly provided by Dr Rose Zamoyska (National Institute for Medical Research, London, UK). These mice were intercrossed to generate RAG-1$^{-/-}$ and IL-7$^{-/-}$×RAG-1$^{-/-}$ littermate mice. All mice are originally derived from C57BL/6 mice. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and performed according to institutional guidelines and Home Office regulations.

See 'more information' in supplementary materials and methods (available online only) for details.

RESULTS

IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transplanted with BM cells from RAG-1$^{-/-}$ mice develop colitis after adoptive transfer of CD4$^+$CD45RB^{high} T cells

We first assessed whether BM-derived IL-7 is sufficient for the development of colitis in the absence of IL-7 produced at other sites. To this end, we used a model of chronic colitis induced by the adoptive transfer of CD4$^+$CD45RB^{high} T cells into RAG-1$^{-/-}$ mice in combination with bone marrow transplantation (BMT) of donor BM cells from RAG-1$^{-/-}$ or IL-7$^{-/-}$×RAG-1$^{-/-}$ littermate mice. First, RAG-1$^{-/-}$ and IL-7$^{-/-}$×RAG-1$^{-/-}$ mice were treated with intraperitoneal busulfan and underwent total body irradiation to ablate their BM. The next day, mice were reconstituted with donor BM cells from RAG-1$^{-/-}$ or IL-7$^{-/-}$×RAG-1$^{-/-}$ mice. Mice were divided into four groups as follows (figure 1A): IL-7$^{-/-}$×RAG-1$^{-/-}$ mice transplanted with RAG-1$^{-/-}$ BM cells (IL-7$^{+/+}$→IL-7$^{-/-}$); IL-7$^{-/-}$×RAG-1$^{-/-}$ mice transplanted with IL-7$^{-/-}$×RAG-1$^{-/-}$ BM cells (IL-7$^{-/-}$→IL-7$^{-/-}$); RAG-1$^{-/-}$ mice transplanted with RAG-1$^{-/-}$ BM cells (IL-7$^{+/+}$→IL-7$^{+/+}$) and RAG-1$^{-/-}$ mice transplanted with IL-7$^{-/-}$×RAG-1$^{-/-}$ BM cells (IL-7$^{-/-}$→IL-7$^{+/+}$). Four weeks after recovery from BMT, all groups of mice were injected intraperitoneally with CD4$^+$CD45RB^{high} T cells (figure 1A). As expected, IL-7$^{+/+}$→IL-7$^{+/+}$ mice developed severe colitis. Interestingly, IL-7$^{-/-}$→IL-7$^{+/+}$ mice also developed colitis at a level similar to that of IL-7$^{+/+}$→IL-7$^{+/+}$ mice (figure 1B–E), indicating that non-hematopoietic cells, including IL-7-producing BM stromal cells and/or BM stem cells that differentiated into IL-7-producing stromal cells, could not be completely ablated by the current busulfan/irradiation protocol. In contrast, IL-7$^{-/-}$→IL-7$^{-/-}$ mice did not develop colitis because of the lack of IL-7 (figure 1B–E). However, to our surprise, the clinical findings revealed that IL-7$^{+/+}$→IL-7$^{-/-}$ mice injected with CD4$^+$CD45RB^{high} T cells developed a wasting disease and severe colitis to a similar extent to that of control IL-7$^{+/+}$→IL-7$^{+/+}$ mice (figure 1B–E), indicating that the BMT protocol led to successful transplantation of IL-7-producing BM cells in IL-7$^{+/+}$→IL-7$^{-/-}$ mice.

A quantitative evaluation of T-cell expansion was performed by counting LP, spleen and BM CD4 T cells. While only a few CD4 T cells were recovered from all sites examined in IL-7$^{-/-}$→IL-7$^{-/-}$ mice, approximately 100-fold higher numbers of LP, spleen and BM CD4 T cells were recovered from IL-7$^{+/+}$→IL-7$^{-/-}$, IL-7$^{+/+}$→IL-7$^{+/+}$ and IL-7$^{-/-}$→IL-7$^{+/+}$ mice (figure 1F). In addition, on in-vitro stimulation, LP CD4 T cells from IL-7$^{+/+}$→IL-7$^{-/-}$, IL-7$^{+/+}$→IL-7$^{+/+}$ and IL-7$^{-/-}$→IL-7$^{+/+}$ mice produced equal and significantly higher amounts of interferon (IFN)-γ, tumour necrosis factor (TNF)α and IL-17 than those from IL-7$^{-/-}$→IL-7$^{-/-}$ mice (figure 1G). Flow cytometric analysis revealed that the CD4 T cells isolated from the LP, mesenteric lymph nodes, spleen and BM of all recipients at 8 weeks after transfer of CD4$^+$CD45RB^{high} T cells had a characteristic CD69$^+$IL-7Rα^{high} phenotype (see supplementary figure S1, available online only), indicating that the transferred CD4$^+$CD45RB^{high} T cells differentiated into activated T$_{EM}$ cells irrespective of the presence of IL-7. These results suggest that BM-derived IL-7 promotes the development and persistence of colitis primarily by supporting the expansion of colitogenic CD4 T$_{EM}$ cells in the colon.

We performed highly sensitive reverse transcription PCR for the detection of IL-7 messenger RNA using samples obtained from the BM and colon. As shown in figure 1H, IL-7 mRNA was detected in the BM of IL-7$^{+/+}$→IL-7$^{-/-}$, IL-7$^{+/+}$→IL-7$^{+/+}$ and IL-7$^{-/-}$→IL-7$^{+/+}$ mice with colitis, but not in that of IL-7$^{-/-}$→IL-7$^{-/-}$ mice without colitis. Of note, IL-7 mRNA was detected in the LP of IL-7$^{+/+}$→IL-7$^{+/+}$ and IL-7$^{-/-}$→IL-7$^{+/+}$ mice, but not in IL-7$^{+/+}$→IL-7$^{-/-}$ or IL-7$^{-/-}$→IL-7$^{-/-}$ mice regardless of the development of colitis, indicating that after BMT, IL-7$^{+/+}$ BM cells led to the establishment of IL-7-producing stromal cells in BM. Consistent with this result, immunohistochemistry revealed that IL-7 was in the BM of IL-7$^{+/+}$→IL-7$^{-/-}$, IL-7$^{+/+}$→IL-7$^{+/+}$ and IL-7$^{-/-}$→IL-7$^{+/+}$ mice with colitis, but not in IL-7$^{-/-}$→IL-7$^{-/-}$ mice without colitis (figure 1I). However, IL-7 protein

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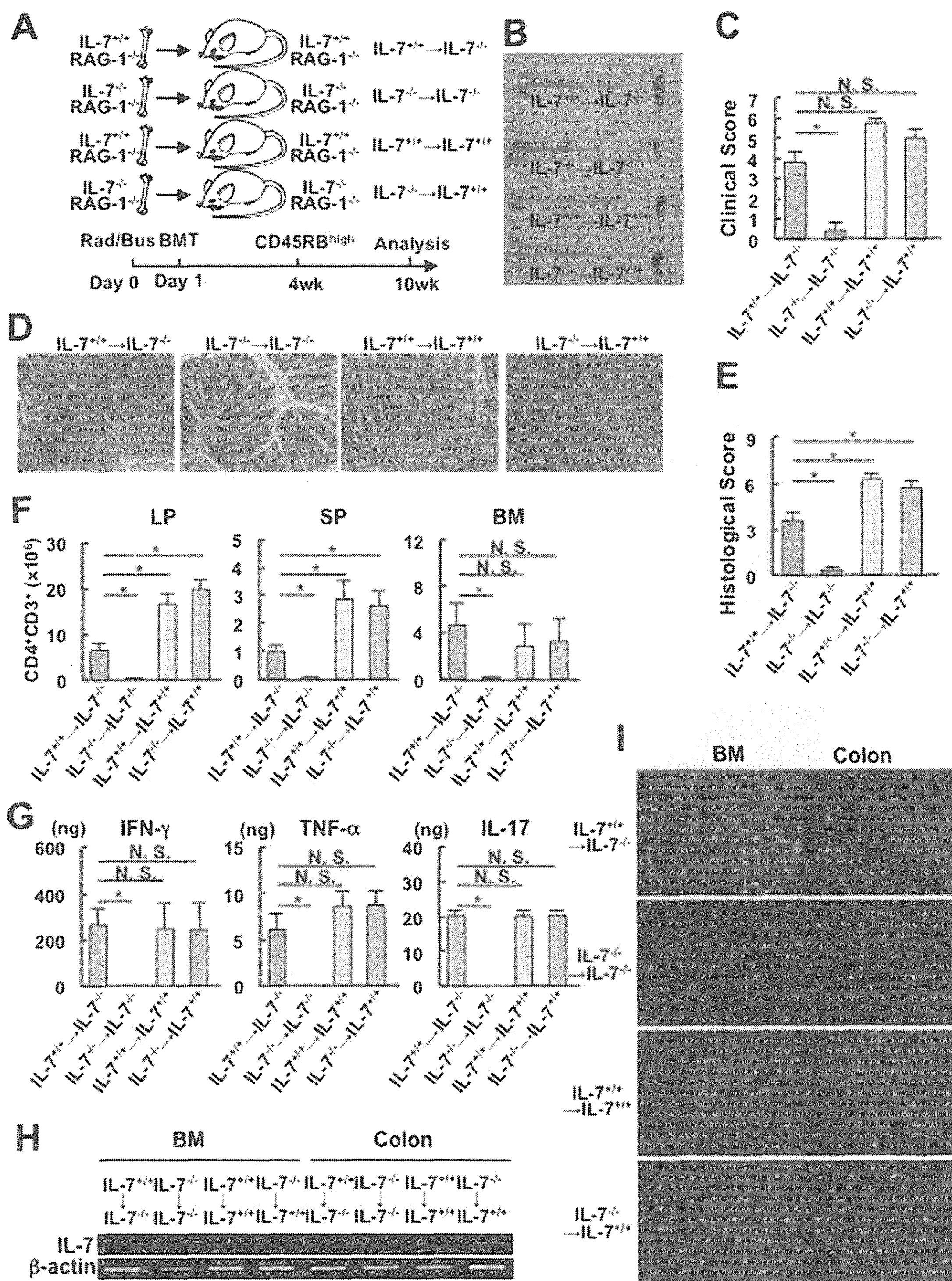

Figure 1 $CD4^+CD45RB^{high}$ T-cell-injected IL-$7^{-/-}$×RAG-$1^{-/-}$ recipients pretransplanted with bone marrow (BM) cells from IL-$7^{+/+}$×RAG-$1^{-/-}$ mice develop colitis. (A) Experimental design. Mice were divided into four groups (n=8). Each group was injected intraperitoneally with $CD4^+CD45RB^{high}$ T cells at 4 weeks after bone marrow transplantation. (B) Gross appearance of the colon, mesenteric lymph nodes and spleen (SP) from mice of each group at 10 weeks after cell administration. (C) Clinical scores determined at 10 weeks after administration as described in Materials and methods section. Data are shown as the mean±SEM for eight mice in each group, *p<0.01. (D) Histopathology of the distal colon of the indicated mice. Original magnification, ×200. (E) Histological scores. Data are shown as the mean±SEM for eight mice in each group, *p<0.05. (F) Absolute number of lamina propria (LP) $CD3^+CD4^+$ T cells from the colon. Data are shown as the mean±SEM. N.S., not significant, *p<0.01. (G) Cytokine production by LP CD4 T cells. LP CD4 T cells were isolated and stimulated in vitro. IFN-γ, TNFα and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown as the mean±SEM for eight mice in each group, *p<0.05. (H) Expression of IL-7 mRNA in colonic BM and the colon from the indicated mice as detected by reverse transcription PCR. (I) Frozen sections of BM and the colon from each mouse were stained with a polyclonal anti-IL-7 antibody. Original magnification ×400.

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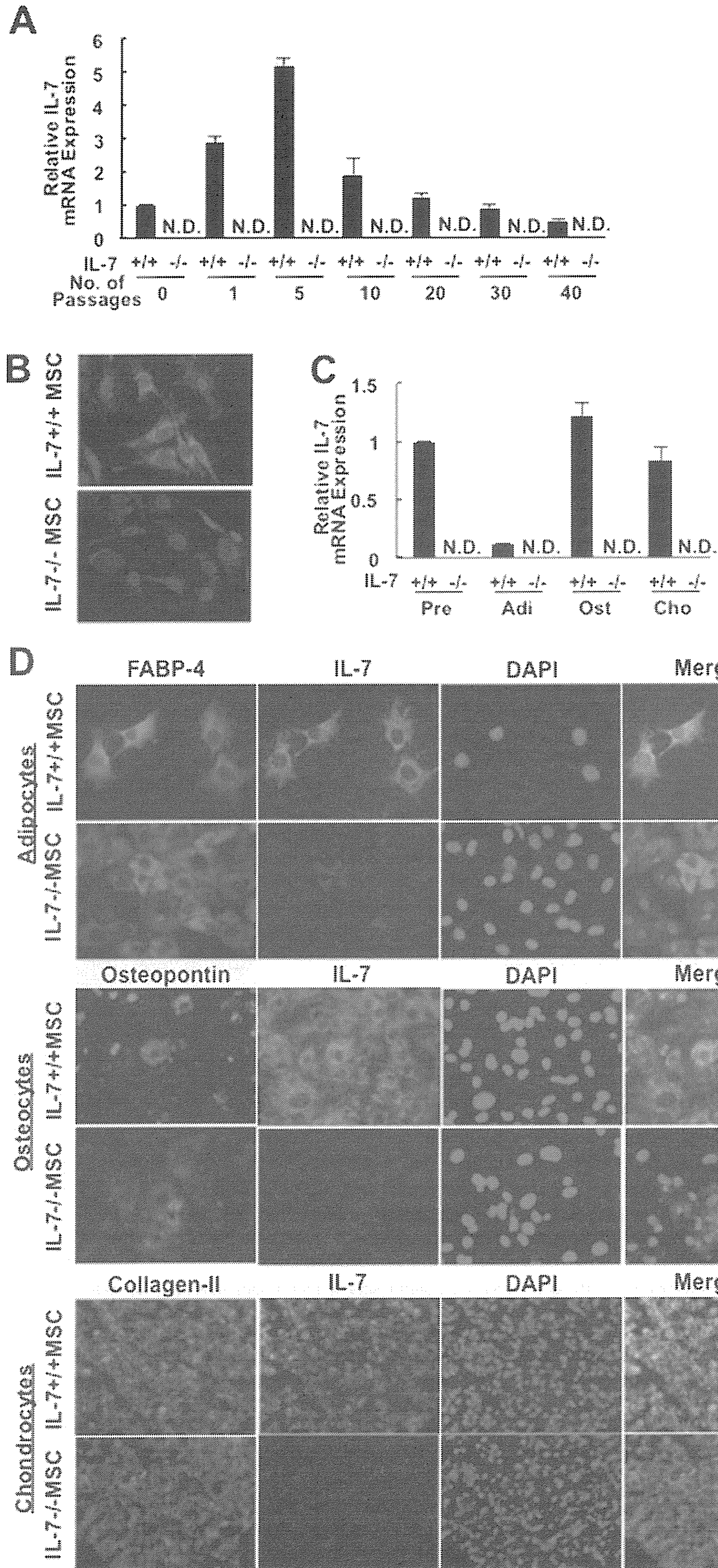

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was not detected in any group regardless of the presence of colitis by immunohistochemistry, indicating that IL-7-producing goblet cells were depleted in the inflamed mucosa of colitic mice, a phenomenon known as 'goblet depletion' in the pathology of human IBD.[12] Collectively, these data suggest that BM-derived stem cells become established in the BM after BMT and thereafter produce IL-7.

BM MSC from RAG-1$^{-/-}$ mice express IL-7 mRNA and protein

As shown in figure 1I, IL-7-producing cells were colonised in the BM, but not in the colon of IL-7$^{+/+}$ → IL-7$^{-/-}$ mice even at 10 weeks after BMT, suggesting that the BM cells of RAG-1$^{-/-}$ mice include some progenitors of IL-7-producing cells, which preferentially migrate to the BM. BM cells include not only haematopoietic stem cells, which can differentiate into granulocytes, macrophages, natural killer cells, B cells, T cells and other haematopoietic cells, but also MSC, which can differentiate into adipose tissue, cartilage, bone and other mesenchymal tissues, and both stem cells preferentially migrate to the BM after BMT.[20] Because it is well known that IL-7 is not secreted from haematopoietic cells, but from stromal cells in BM, we hypothesised that MSC are the progenitors of IL-7-producing cells in BM. To test this hypothesis, we cultured MSC derived from the BM of RAG-1$^{-/-}$ and IL-7$^{-/-}$ ×RAG-1$^{-/-}$ mice using a current standard protocol.[21] First, IL-7 mRNA expression in cultured MSC at passages 0–40 was assessed by real-time PCR. Surprisingly, MSC derived from the BM of RAG-1$^{-/-}$ mice (IL-7$^{+/+}$ MSC), but not from IL-7$^{-/-}$×RAG-1$^{-/-}$ mice (IL-7$^{-/-}$ MSC), strongly expressed IL-7 mRNA at passages 1–40, which peaked at passage 5 (figure 2A). Furthermore, a substantial proportion of cultured IL-7$^{+/+}$ MSC, but not IL-7$^{-/-}$ MSC, expressed IL-7 protein that was detected by immunohistochemistry (figure 2B). MSC derived from both RAG-1$^{-/-}$ and IL-7$^{-/-}$ ×RAG-1$^{-/-}$ mice expressed MSC markers Sca-1, CD140a, CD73 and CD105, but not haematopoietic markers CD11b, Gr-1, TER119, CD3, CD45 and CD34 or endothelial markers CD146, FLK1 and VEGFR3 (see supplementary figure S2, available online only). In contrast, whole BM cells from RAG-1$^{-/-}$ and IL-7$^{-/-}$ ×RAG-1$^{-/-}$ mice included various types of haematopoietic cells (see supplementary figure S2, available online only). We confirmed that these cultured BM MSC had the ability to differentiate into three mesenchymal lineages: adipocytes, osteocytes and chondrocytes (figure 2D). Importantly, IL-7$^{+/+}$ MSC and IL-7$^{+/+}$ MSC-derived adipocytes, osteocytes and chondrocytes expressed IL-7 mRNA in sharp contrast to that of cells derived from IL-7$^{-/-}$ mice (figure 2C). This result was also confirmed by immunohistochemistry analysis (figure 2D). We thus identified MSC not only as the progenitors of IL-7-producing cells, but also the highly IL-7-producing cells in BM.

MSC suppress activation of CD4 T cells, but support maintenance of memory CD4 T cells in vitro

Given the evidence of IL-7$^{+/+}$ BM MSC that express high levels of IL-7, we next assessed the role of BM MSC in the maintenance of colitogenic CD4 memory T cells in vitro. First, we attempted to confirm the hallmark character of the immunosuppressive ability of MSC.[19] To this end, CD4^{+}CD25^{-} T cells isolated from the spleen of wild-type mice were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and then co-cultured with IL-7$^{+/+}$ or IL-7$^{-/-}$ MSC in the presence of an anti-CD3 antibody and mitomycin-C-treated CD4 negative cells, which are used as antigen presenting cells. At 4 days after co-culture, CD4^{+}CD25^{-} T cells were collected and analysed (see supplementary figure S3A, available online only). Both IL-7$^{+/+}$ and IL-7$^{-/-}$ MSC suppressed the proliferation of responder T cells, while those cultured without MSC proliferated extensively (see supplementary figure S3B–D, available online only).

We next conducted long-term co-culture with colitogenic CD4 T_{EM} cells and MSC to assess the ability of MSC to support memory CD4 T cells (figure 3). CFSE-labelled CD4 T cells isolated from colonic LP of colitic RAG-2$^{-/-}$ mice, which had been pre-injected with CD4^{+}CD45RB^{high} T cells, were incubated with IL-7$^{+/+}$ or IL-7$^{-/-}$ MSC, or in conditioned medium alone (figure 3A), and CFSE was evaluated after 4 weeks of culture. The absolute number of surviving CD3^{+}CD4^{+} T cells that were co-cultured with IL-7$^{+/+}$ MSC was significantly higher than that of the other groups (figure 3B). Consistently, CD4 T cells co-cultured with IL-7$^{+/+}$ MSC slowly divided up to five times in 4 weeks, suggesting that these cells were quiescent and divided intermittently, much like homeostatic proliferation in vivo.[22] In sharp contrast, CD4 T cells co-cultured with IL-7$^{-/-}$ MSC or cultured in medium alone did not proliferate (figure 3C). Furthermore, the expression of Bcl-2 in CD4 T cells co-cultured with IL-7$^{+/+}$ MSC was significantly higher than that in other groups (figure 3D, E). These results indicate that IL-7$^{+/+}$ MSC support not only homeostatic proliferation but also the survival of colitogenic CD4 T_{EM} cells in a manner dependent on IL-7-producing MSC. To exclude the possibility that these activities of IL-7$^{+/+}$ MSC are mediated by secondary effects, rather than their production of IL-7, we performed an IL-7-blocking experiment. As shown in figure 3F–H, the ability of IL-7$^{+/+}$ MSC to support colitogenic CD4 T_{EM} cells was almost completely abrogated by an IL-7-blocking antibody (figure 3F–H).

IL-7$^{-/-}$×RAG-1$^{-/-}$ mice pretransplanted with IL-7-sufficient BM MSC develop colitis after adoptive transfer of CD4^{+}CD45RB^{high} T cells

We next conducted an adoptive transfer experiment in conjunction with the transplantation of BM MSC. First, we checked whether cultured MSC could migrate to the BM, spleen and colon, because it is unknown whether these cultured MSC traffic to the same organs compared with those of freshly isolated MSC.[20] IL-7$^{+/+}$ MSC were transferred to IL-7$^{-/-}$ ×RAG-1$^{-/-}$ mice without the busulfan/irradiation protocol that was used in the previous BMT experiment. At 1, 2 and 4 weeks after the transfer, the expression of IL-7 mRNA and protein in

Figure 2 Bone marrow (BM) mesenchymal stem cells (MSC) from IL-7$^{+/+}$×RAG-1$^{-/-}$ mice express IL-7 mRNA and protein. (A) Expression of IL-7 mRNA in IL-7$^{+/+}$ and IL-7$^{-/-}$ MSC at various passages and in freshly isolated BM cells from RAG-1$^{-/-}$ (IL-7$^{+/+}$ BM cells) and IL-7$^{-/-}$×RAG-1$^{-/-}$ (IL-7$^{-/-}$ BM cells) mice were determined by real-time PCR. Data are relative mIL-7 expression levels in MSC at each passage compared with that in IL-7$^{+/+}$ BM cells. (B) Adherent monolayer of cultured MSC stained with a polyclonal anti-IL-7 antibody. Original magnification ×400. (C) Real-time PCR was performed to check the expression of IL-7 mRNA in differentiated tissues derived from IL-7$^{+/+}$ and IL-7$^{-/-}$ MSC. Data are relative mIL-7 expression levels in each differentiated cell type compared with that in IL-7$^{+/+}$ pre-MSC. (D) Adherent monolayer of differentiated tissues derived from IL-7$^{+/+}$ and IL-7$^{-/-}$ MSC were stained with anti-FABP-4, anti-osteopontin, anti-collagen-II (all red) and anti-IL-7 (green) antibodies and DAPI (blue). Original magnification ×400.

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the BM, spleen and colon of recipient mice was determined (figure 4A). As shown in figure 4B and C, at each time point, IL-7 mRNA and protein was detected in the BM, but not in the spleen or colon.

Given the evidence of IL-7-producing MSC that migrate to the BM, 1×10^6 IL-$7^{+/+}$ or IL-$7^{-/-}$ MSC were transplanted into IL-$7^{-/-}\times$RAG-$1^{-/-}$ mice. At 4 weeks after transplantation, $CD4^+CD45RB^{high}$ T cells were injected into the pretransplanted mice (figure 5A). At 6 weeks after transfer, the colon from IL-$7^{+/+}$ MSC-transplanted mice, but not that from IL-$7^{-/-}$ MSC-transplanted mice, was enlarged and had a greatly thickened wall (data not shown). Overall, the assessment of colitis by clinical scoring showed a clear difference between mice that received IL-$7^{+/+}$ or IL-$7^{-/-}$ MSC (figure 5B). This result was confirmed by histological examination of multiple colon sections (figure 5C, D). The absolute number of $CD3^+CD4^+$ T cells recovered from the BM, spleen and LP of IL-$7^{+/+}$ MSC-transplanted mice was significantly higher than that from IL-$7^{-/-}$ MSC-transplanted mice (figure 5E). Flow cytometric analysis revealed that CD4 T cells isolated from the BM, spleen and LP of IL-$7^{+/+}$ MSC-transplanted mice at 6 weeks after transfer of $CD4^+CD45RB^{high}$ T cells had a characteristic $CD44^+$ $CD62L^-CD69^+$IL-$7R\alpha^{high}$ effector memory phenotype (see supplementary figure. S4, available online only). Furthermore, on in-vitro stimulation, LP CD4 T cells from IL-$7^{+/+}$ MSC-transplanted mice produced significantly higher amounts of IFN-γ, TNFα and IL-17 than those from IL-$7^{-/-}$ MSC-transplanted mice (figure 5F). More importantly, IL-7 mRNA and protein were detected in the BM, but not the colon, of IL-$7^{+/+}$ MSC-transplanted mice, and not in either the BM or colon of IL-$7^{-/-}$ MSC-transplanted mice (figure 5G, H). We further compared the number of MSC transplanted into this colitis model, and found that transplantation of 1×10^6 MSC, but not 1×10^5 or 1×10^4 MSC, induced colitis in terms of the clinical score, histology, absolute number of recovered LP CD4 T cells, and cytokine expression (see supplementary figure S5A–H, available online only). This result suggested that insufficient numbers of MSC appropriately migrated to induce colitis because of the loss of their homing receptors during culture.

In addition to the above results, BM MSC have been identified as progenitors of mesenchymal tissues because they can migrate to injured tissues to repair them.[21] Therefore, transplantation of BM MSC for tissue repair has been proposed based on their stem cell qualities observed in animal models of IBD. In fact, Duijvestein *et al*[23] recently reported the feasibility of autologous BM MSC for the treatment of patients with refractory Crohn's disease. Moreover, recent studies suggest that MSC play a second role in inducing peripheral tolerance by

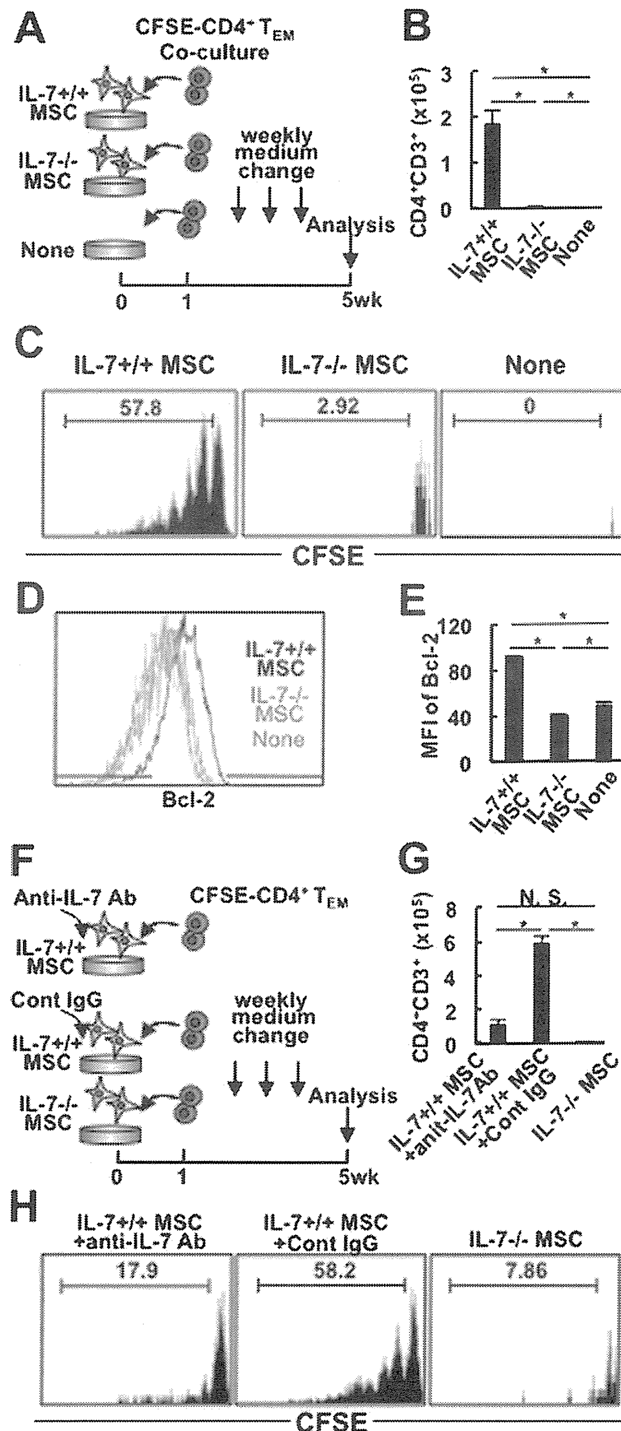

Figure 3 IL-$7^{+/+}$, but not IL-$7^{-/-}$, mesenchymal stem cells (MSC) induce extensive proliferation of colitogenic CD4 T cells in vitro. (A) Experimental design. Colitogenic memory CD4 T cells were isolated from colonic lamina propria (LP) of colitic RAG-$2^{-/-}$ mice re-injected with $CD4^+CD45RB^{high}$ T cells, and labelled with CFSE. CFSE-labelled colitogenic memory CD4 T cells (1×10^6) were then co-cultured with IL-$7^{+/+}$ or IL-$7^{-/-}$ MSC. CFSE-labelled memory CD4 T cells were incubated in conditioned medium as a negative control. (B) Cell counts of recovered $CD3^+CD4^+$ cells were performed by flow cytometry. Data are shown as the mean±SEM of six samples in each group, *p<0.05. (C) After 4 weeks of co-culture, CFSE in memory CD4 T cells was evaluated by flow cytometry. Representative data of six samples are shown. (D) Intracellular staining of Bcl-2 in colitogenic CD4 T_{EM} cells in each group. Colitogenic CD4 T_{EM} cells were co-cultured with IL-$7^{+/+}$ or IL-$7^{-/-}$ MSC, or incubated in conditioned medium alone. (E) Mean fluorescence intensity of Bcl-2 in $CD3^+CD4^+$ cells in each group. Data are shown as the mean±SEM. (F) Experimental design. Colitogenic memory CD4 T cells were isolated from colonic LP of colitic RAG-$2^{-/-}$ mice pre-injected with $CD4^+CD45RB^{high}$ T cells, and labelled with CFSE. CFSE-labelled colitogenic memory CD4 T cells (1×10^6) were co-cultured with IL-$7^{+/+}$ MSC in medium containing a polyclonal anti-IL-7 antibody or isotype control IgG. CFSE-labelled memory CD4 T cells were co-cultured with IL-$7^{-/-}$ MSC as a negative control. (G) Cell counts of recovered $CD3^+CD4^+$ cells were performed by flow cytometry. Data are shown as the mean±SEM of three samples in each group, *p<0.05. (H) After 4 weeks of co-culture, CFSE in memory CD4 T cells was detected by flow cytometry. Representative data for six samples are shown.

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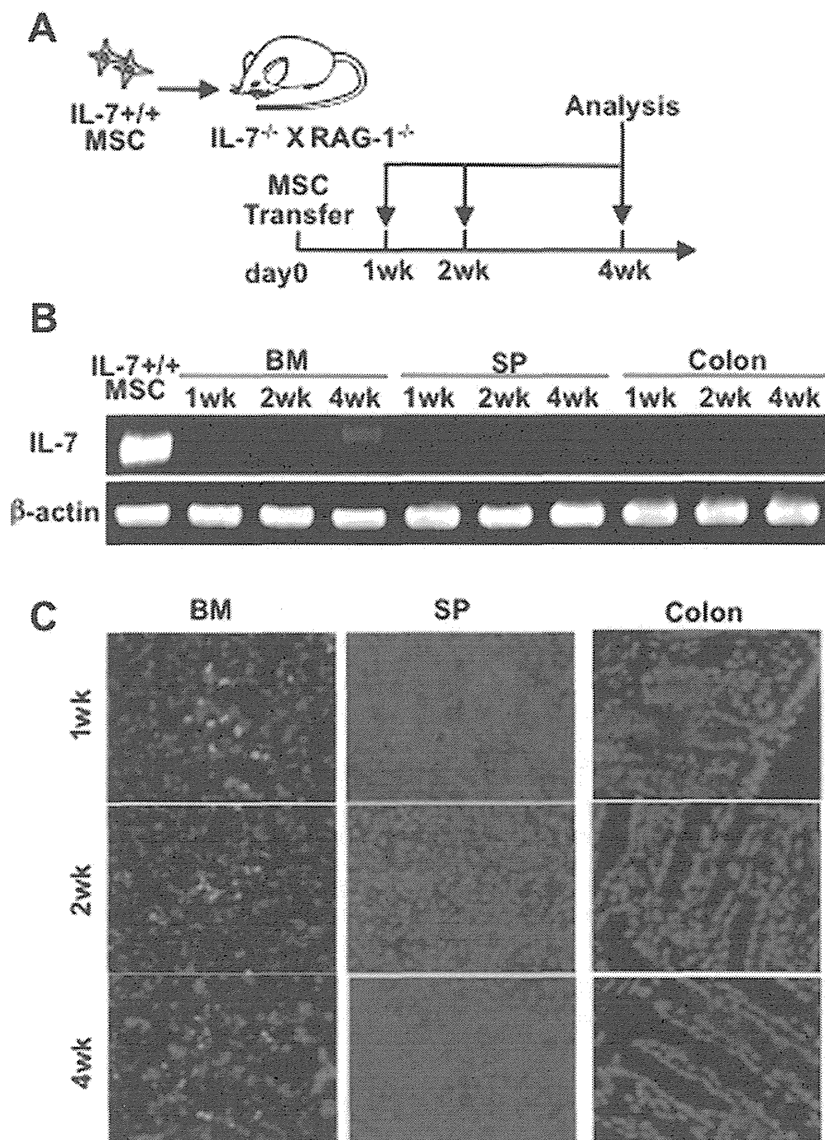

Figure 4 Time course analysis of IL-7 expression in the bone marrow (BM), spleen and colon of IL-$7^{-/-}$×RAG-$1^{-/-}$ mice pre-injected with IL-$7^{+/+}$ mesenchymal stem cells (MSC). (A) Experimental design. IL-$7^{-/-}$×RAG-$1^{-/-}$ mice were injected intravenously with IL-$7^{+/+}$ MSC. At 1, 2 and 4 weeks after the transfer, the BM, spleen (SP) and colon of the mice were collected and checked for the expression of IL-7. (B) IL-7 mRNA expression in the BM, spleen and colon at each time point as assessed by quantitative reverse transcription PCR. (C) IL-7 protein expression in the BM, spleen and colon at each time point as assessed by immunohistochemistry. IL-7 (green) and DAPI (blue).

inhibiting the release of proinflammatory cytokines and interacting with various kinds of immune cells.[19] Therefore, we checked whether our cultured MSC could suppress colitis when they were transferred together with $CD4^+CD45RB^{high}$ T cells to RAG-$2^{-/-}$ mice as a preventive protocol. Mice were divided into four groups as follows: RAG-$1^{-/-}$ mice injected with $CD4^+CD45RB^{high}$ T cells and IL-$7^{+/+}$ MSC (IL-$7^{+/+}$ MSC); RAG-$1^{-/-}$ mice injected with $CD4^+CD45RB^{high}$ T cells and IL-$7^{-/-}$ MSC (IL-$7^{-/-}$ MSC); RAG-$1^{-/-}$ mice injected with $CD4^+CD45RB^{high}$ T cells (RB^{high}) as a positive control; and RAG-$1^{-/-}$ mice injected with $CD4^+CD45RB^{high}$ T cells and $CD4^+CD25^+$ T cells ($RB^{high}+T_{reg}$) as a negative control (figure 6A). Both IL-$7^{+/+}$ MSC and IL-$7^{-/-}$ MSC groups developed a wasting disease and colitis with a thickened colon and splenomegaly to the same extent as that in the RB^{high} group, while the $RB^{high}+T_{reg}$-negative control group gained weight and did not develop colitis (figure 6B–F). A large number of LP CD4 T cells was recovered from IL-$7^{+/+}$ MSC, IL-$7^{-/-}$ MSC, and RB^{high} groups, while only a small number of LP CD4 T cells was recovered from the $RB^{high}+T_{reg}$ group (figure 6G). As shown in figure 6H, on in-vitro stimulation, LP CD4 T cells from IL-$7^{+/+}$ MSC, IL-$7^{-/-}$ MSC and RB^{high} groups produced equal and significantly higher amounts of IFN-γ, TNFα and IL-17 than those by the $RB^{high}+T_{reg}$ group. These data indicated that, at least in our present in-vivo model, neither IL-$7^{+/+}$ MSC nor IL-$7^{-/-}$ MSC could suppress the development of colitis, even when they were transferred in combination with $CD4^+CD45RB^{high}$ T cells. Although we performed multiple injections of IL-$7^{+/+}$ and IL-$7^{-/-}$ MSC to suppress colitis and evaluate a therapeutic effect, colitis could not be suppressed in terms of clinical and histological scores, the number of infiltrated LP CD4 T cells and cytokine production (see supplementary figure S6A–G, available online only).

DISCUSSION

BM MSC have previously been identified as progenitors of mesenchymal tissues by migrating to injured tissues to repair them,[19–21] and transplantation of BM MSC for tissue repair has been proposed based on their stem cell qualities. Moreover, recent studies suggest that cultured MSC play a second role in the induction of peripheral tolerance by inhibiting the release of proinflammatory cytokines and interacting with various kinds of

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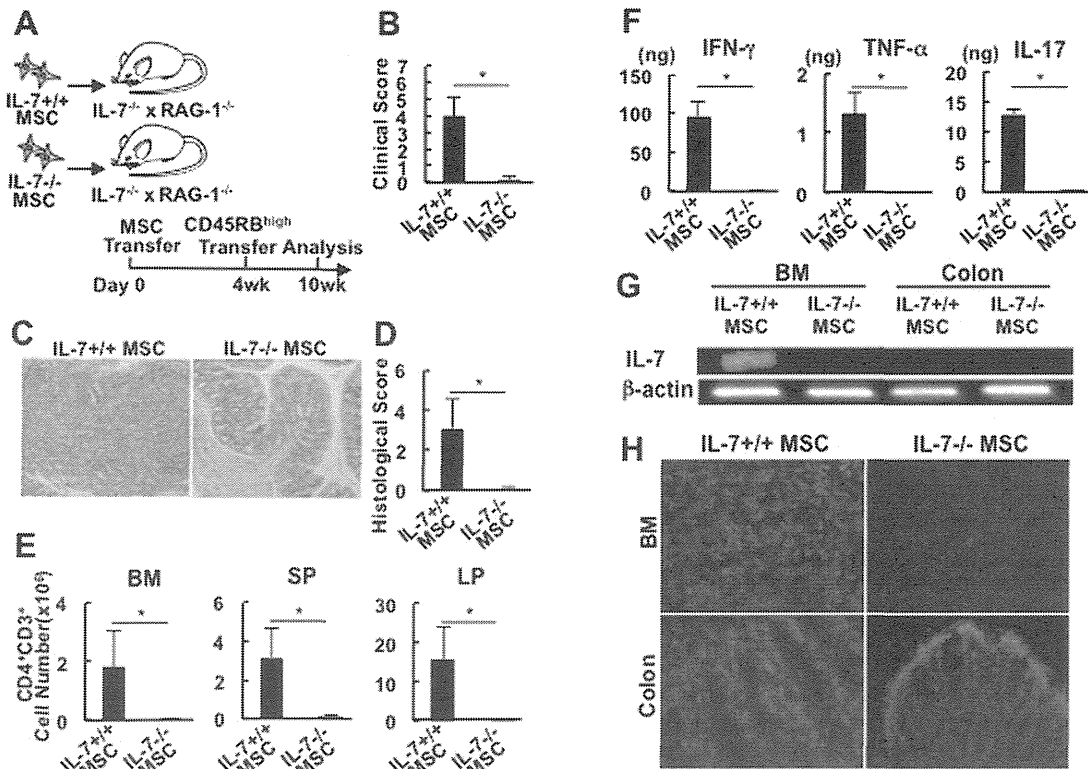

Figure 5 CD4$^+$CD45RB^{high} T-cell-injected IL-7$^{-/-}$×RAG-1$^{-/-}$ mice pretransplanted with IL-7$^{+/+}$, but not IL-7$^{-/-}$, mesenchymal stem cells (MSC) develop colitis. (A) Experimental design. Mice were divided into two groups (n=5). Each group was injected intraperitoneally with CD4$^+$CD45RB^{high} T cells at 4 weeks after MSC transfer. (B) Clinical scores. Data are shown as the mean±SEM for five mice in each group, *p<0.05. (C) Histological results for the colons of each group. Original magnification ×200. (D) Histological scores. Data are shown as the mean±SEM for five mice in each group, *p<0.05. (E) Absolute number of lamina propria (LP) CD3$^+$CD4$^+$ T cells from the colon at 10 weeks after transfer. Data are shown as the mean±SEM. N.S. not significant, *p<0.01. (F) Cytokine production by LP CD4 T cells stimulated in vitro. IFN-γ, TNFα and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown as the mean±SEM for five mice in each group, *p<0.05. (G) Expression of IL-7 mRNA in the bone marrow (BM) and LP of IL-7$^{+/+}$ MSC- or IL-7$^{-/-}$ MSC-transplanted mice as measured by reverse transcription PCR. (H) Expression of IL-7 protein in the BM and LP of each group of mice as detected by immunohistochemistry.

immune cells. However, the present study clearly shows that: (1) BM MSC produce IL-7; (2) MSC have the potential to support the proliferation and survival of colitogenic CD4 T_{EM} cells; and notably (3) transplantation of BM MSC into IL-7$^{-/-}$×RAG-1$^{-/-}$ mice induces colitis when the mice are later injected with CD4$^+$CD45RB^{high} T cells; and (4) IL-7 expression is maintained in the BM of IL-7$^{-/-}$×RAG-1$^{-/-}$ mice transplanted with BM MSC. The present study thus suggests the possible participation of IL-7-producing BM MSC as niche cells to maintain colitogenic CD4 memory T cells. Although it is possible that IL-7 produced in BM leads to levels of circulating IL-7 that support local (intracolonic) expansion of T-cell populations, rather than facilitating the formation of a niche in BM for these cells, we previously demonstrated that intrarectally administered colitogenic CD4 T cells surprisingly egress to the colon, migrate to BM.[18] In addition, we have reported that IL-7$^{-/-}$×RAG-1$^{-/-}$ host mice combined with colitic RAG-2$^{-/-}$ donor mice as a parabiosis develop colitis without IL-7 expression in any organ.[12] Therefore, we concluded that colitogenic memory CD4 T cells as 'memory stem cells' may be supported in some specific niche, such as BM, in which IL-7 is abundant even when in the acute phase of colitis.

Because we used IL-7 as a marker of MSC in this setting, it is possible that transferred MSC can spread to many tissues including inflamed colonic mucosa to become differentiated cells but lose expression of IL-7. However, it remains unknown why IL-7 was not detected in the inflamed colon of IL-7$^{-/-}$×RAG-1$^{-/-}$ mice after transfer of IL-7$^{+/+}$ MSC with CD4$^+$CD45RB^{high} T cells, although MSC-derived adipocytes that expressed IL-7 could not be detected (figure 2C,D). Nevertheless, it is noteworthy that IL-7 production by the transferred MSC was maintained only in the BM regardless of their differentiation status. Therefore, we propose that, in addition to the two major roles previously reported, namely tissue repair[19–21] and immune suppression,[19 20] BM MSC-derived IL-7 is positively involved in the perpetuation of chronic inflammatory diseases by forming the niche for pathogenic CD4 memory T cells in BM (figure 7). Although many differences exist between our colitis model induced by a lymphopenic driver, other murine models, such as dextran sodium sulfate-induced acute colitis model, and human IBD, we propose a pathological role of IL-7-producing MSC at least in our model. Furthermore, the present study supports a conceptual change of IBD from an intestinal to a systemic disease, and suggests therapeutic approaches that target BM MSC-derived IL-7 for the treatment of IBD.

From a clinical viewpoint, we have previously demonstrated that IL-7 protein in the serum of patients with ulcerative colitis (UC) is higher than that in healthy controls.[24] Furthermore, *IL7R* has previously been identified as one of the disease susceptibility genes of UC.[25] Therefore, it may be interesting to compare the IL-7 levels in BM, especially in BM MSC, between IBD patients and healthy controls to determine whether BM

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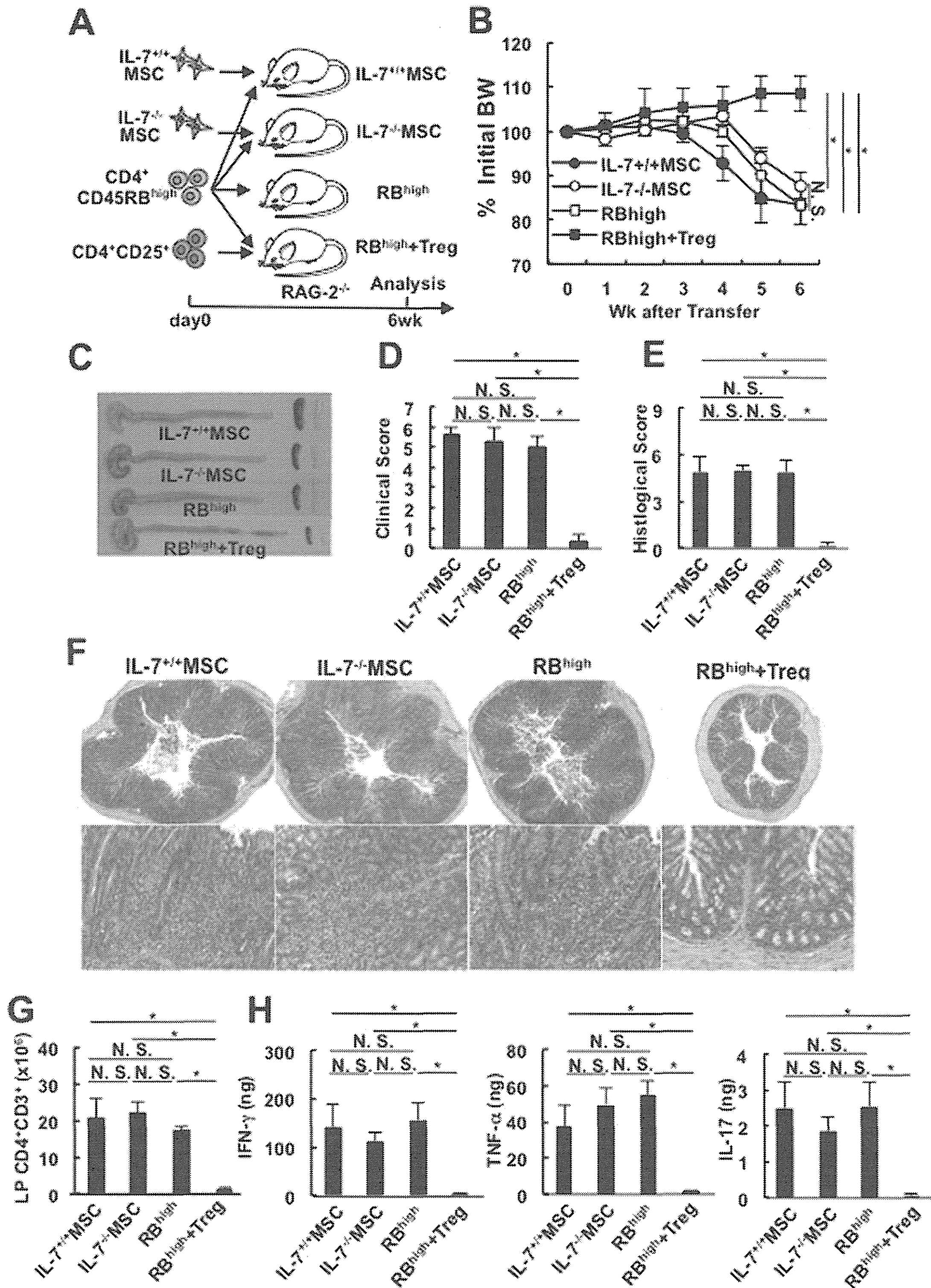

Figure 6 $CD4^+CD45RB^{high}$ T-cell-injected RAG-$1^{-/-}$ recipients pre-injected with either IL-$7^{+/+}$ or IL-$7^{-/-}$ mesenchymal stem cells (MSC) develop colitis. (A) Experimental design. Mice were divided into four groups as follows: RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells and IL-$7^{+/+}$ MSC (n=3, IL-$7^{+/+}$ MSC); RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells and IL-$7^{-/-}$ MSC (n=3, IL-$7^{-/-}$ MSC); RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells (n=3, RB^{high} cells); and RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells and $CD4^+CD25^+$ T cells (n=3, RB^{high} + T_{reg} cells). (B) Percentage of the initial body weight (BW) of each group. Data are shown as the mean±SEM for three mice in each group, *p<0.05. (C) Representative gross appearance of the spleen, mesenteric lymph nodes and colon of each group. (D) Clinical scores. Data are shown as the mean±SEM for three mice in each group, *p<0.05. (E) Histological scores. Data are shown as the mean±SEM for three mice in each group, *p<0.05. (F) Histopathology of the distal colon of the indicated mice at 6 weeks after transfer. Original magnification, ×40 upper panel and ×200 lower panel. (G) Absolute number of lamina propria (LP) $CD3^+CD4^+$ T cells from the colon at 6 weeks after transfer. Data are shown as the mean±SEM. N.S. not significant, *p<0.01. (H) Cytokine production by LP CD4 T cells. LP CD4 T cells were isolated at 6 weeks after transfer and stimulated with anti-CD3 and anti-CD28 antibodies for 48 h. IFN-γ, TNFα and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown as the mean±SEM for three mice in each group, *p<0.05.

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