

Regulatory role of B-1 B cells in chronic colitis

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Abstract

According to the 'hygiene hypothesis', enhanced microbial exposure due to early childhood infections leads to a reduction of T_H2-mediated allergic diseases and inflammatory bowel disease. To begin to elucidate the mechanisms underlying this hypothesis, we studied development of T_H2-mediated colitis of the TCR α knockout (KO) mouse in both a specific pathogen-free (SPF) facility and a conventional (CV) facility. After more than five generations in each facility, TCR α KO mice kept in the CV facility developed dramatically less colitis than mice that were kept in the SPF facility. Surprisingly, the suppression of colitis in the CV facility correlated with a significant increase in natural IgM production by B-1 B cells. In contrast, B cell-deficient TCR α double-knockout ($\alpha\mu$ DKO) mice maintained in the CV facility continued to develop severe colitis, strongly suggesting that B-1 B cells contributed to the suppression of colitis. Indeed, the adoptive transfer of B-1 B cells isolated from the peritoneal cavity of TCR α KO mice (SPF) into $\alpha\mu$ DKO mice (CV) suppressed the development of colitis in the recipient mice. We conclude that B-1 cells play a regulatory role in T_H2-mediated colitis under non-hygienic conditions, possibly by generating natural antibodies in response to microbial flora.

Introduction

The 'hygiene hypothesis' is the concept that the frequent use of antibiotics and improved sanitary conditions in the industrialized countries has led to decreased childhood infections and increased susceptibility to T_H2-mediated allergic diseases such as asthma and atopic dermatitis (1–3). The hygiene hypothesis may also explain the association of inflammatory bowel disease (IBD) and autoimmunity with modern living conditions (4, 5). However, the immunological mechanisms involved in the hygiene hypothesis have not been fully defined.

Since the mucosal immune system is constantly exposed to a variety of microbial antigens in the intestinal lumen, it is essential that there is development of tolerance to enteric commensal flora without compromising the ability to effectively respond to infectious microbes. Dysregulation of immune response to normal enteric flora leads to the development of colitis (6–9). Recent studies indicate that bacterial products contribute to the suppression of colitis by activating innate immune responses that are required for the preservation of intestinal homeostasis (10, 11). Beneficial effects of certain bacteria (probiotics) on IBD support the significance of relative balance between protective and ag-

gressive micro-organisms in the pathogenesis of this disease (12, 13). The role of micro-organisms in the colitis pathogenesis may vary depending on the phase and the type of colitis as well as the genetic background (14, 15).

Chronic colitis in TCR α knockout (KO) is mediated by an IL-4-dependent T_H2 pathway and shares some features with human ulcerative colitis (UC) (6, 16, 17). In contrast, IL-23/IL-17 pathway has recently been demonstrated to cause chronic colitis resembling human Crohn's disease (CD) (18–20). Both T_H2-mediated colitis in TCR α KO and T_H17-mediated colitis in IL-10 KO mice (19, 20) spontaneously develop in specific pathogen-free (SPF) facilities but not when maintained under germ-free conditions (6, 21). Although IL-10 KO mice maintained in non-hygienic conventional (CV) facilities develop a colitis of greater severity than those maintained in SPF conditions (21), TCR α KO mice maintained in CV facilities do not develop colitis (22). The mechanism by which non-hygienic conditions contribute to the suppression of this colitis was not determined.

In the present study, colitis development was suppressed when TCR α KO mice were maintained for five generations

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under CV facilities that are less hygienic as compared with SPF conditions. The suppression of colitis required the presence of B cells, which was associated with increased levels of IgM natural antibodies. Since the unique B cell population of B-1 cells that reside primarily in the peritoneal cavity (PerC) are a major source of IgM natural antibodies and participate in the early immune responses against many bacteria and viruses (23), we hypothesized that this subset of B cells may be involved in the suppression of colitis in CV facilities. The transfer of PerC B-1 cells into B cell-deficient TCR α double-knockout ($\alpha\mu$ DKO) mice under CV conditions resulted in the suppression of colitis and the production of IgM antibodies in the spleen with presence in the sera. These results indicate that PerC B-1 cells play a regulatory role in T_H2-mediated colitis under non-hygienic conditions.

Methods

Mice

TCR α KO and Ig μ (Igh6) KO mice of C57BL/6 (H-2b) background were crossed to generate the $\alpha\mu$ DKO mice (24). The litters from TCR α KO and $\alpha\mu$ DKO breeding pairs of SPF were either maintained under the SPF or moved to CV facility. After continuous generations of a family lines from the CV first TCR α KO and $\alpha\mu$ DKO mice at the CV facility, the fifth generation of mice were used as CV mice. The CV mice were maintained in cages without filtered lid in a room without negative pressure and drinking water and food without autoclaving was supplied to the mice. Pathogenic bacteria, worm or parasites were not detectable in the CV mice and serological test against pathogenic viruses such as murine hepatitis virus was negative in these mice.

Histological examination

Specimens of colon were fixed in 10% buffered formalin and stained with hematoxylin and eosin. The severity of colitis was determined using a combination of gross and histological criteria as previously described (25, 26). The gross score was rated as 0, presence of normal beaded appearance; 1, absence of beaded appearance of colon; 2, focal thickened colon and 3, marked thickness of the entire colon. The histological score was based upon the extent of intestinal wall thickening (0–3) and lamina propria cell infiltration (0–3). The disease score (0–9) was estimated by the addition of both gross and histological findings.

Cell preparation

Peyer's patches (PP), cecal patches/appendix lymphoid follicles (ALF) and mesenteric lymph node (MLN) cells were extracted as described previously (27). For purification of B cells, the cells were incubated with PE-conjugated anti-Ig μ (Caltag/Invitrogen, Carlsbad, CA, USA) at 4°C for 30 min, and then incubated with the anti-PE microbeads at 4°C for 30 min. After washing, the labeled cells were subjected to MACS system (Miltenyi Biotec Inc., Auburn, CA, USA).

Cell transfer

CD11b⁺ B-1 B cells were purified from the PerC TCR α KO mice. The peritoneal cells were collected using a modification

of a previously described method (28). Briefly, a PerC lavage was carried out by flushing the PerC with 8 ml of sterile PBS. The PerC lavage cells were plated in tissue culture dish (Falcon 3802) for 2 h at 37°C, 5% CO₂ to remove macrophages; the non-adherence cells were collected and erythrocytes were removed. CD11b⁺ B-1 B cells were purified using FITC-anti-CD11b (M1/70) (BioLegend, San Diego, CA USA) and MACS system (Miltenyi Biotec Inc.); 2–5 × 10⁵ purified cells or PBS (as a control) were injected intra-peritoneally into 1-month-old $\alpha\mu$ DKO mice maintained in the CV facilities. The $\alpha\mu$ DKO mice transferred with B-1 B cells or injected with PBS were sacrificed at 6 months of age for the presence of colitis.

Flow cytometry

Cells from the spleen and PerC were stained with labeled anti-mouse FITC-CD11b (M1/70) (BioLegend), PE-conjugated anti-Ig μ (Caltag/Invitrogen) and PerCP-B220 (RA3-6B2) (BD Bioscience, San Jose, CA, USA). Data analyzed with CellQuest.

Fecal pellet collection

Fresh fecal pellets were collected as described previously (29) and weighed. PBS containing protease inhibitor was added in the ratio of 1 ml per 100 mg fecal pellets and the mixture incubated for 15 min at room temperature. Samples were vortexed, left to settle for 15 min and re-vortexed until all material was suspended. The tubes were centrifuged at 3000 × *g* for 15 min at 4°C and the supernatant stored at –70°C till assayed.

ELISPOT assay

Enzyme-linked immune spot (ELISPOT) assay was performed as described previously (27). A 96-well plate with a nitrocellulose base (Millititer HA; Millipore Corporation, Bedford, MA, USA) was coated overnight at 4°C with 100 μ l of goat anti-mouse Ig (5 mg ml^{–1}; Dako, Glostrup, Denmark) in D-PBS. After wash, the wells were blocked with 3% BSA/PBS at 37°C for 1 h. Cells (1 × 10⁵ to 5 × 10² cells per well) in 100 μ l of Iscove's modified Dulbecco's medium complete medium containing 10% FCS were added to each well and incubated at 37°C for 16 h. Following subsequent washes with PBS, water, 0.25% Tween/PBS and 0.05% Tween/PBS, the wells were re-blocked with 3% BSA/2% goat serum/PBS for 1 h. The wells were washed again and incubated with HRP antibodies specific to Ig isotypes (IgM, IgA and IgG) (Serotec, Raleigh, NC, USA; Southern Biotechnology, Birmingham, AL, USA) in 0.5% BSA/0.05% Tween/PBS overnight at 4°C. The wells were washed and developed with 3-amino-9-ethyl carbazole. The reaction was stopped by a wash with tap water.

ELISA for detection of antigen-specific-binding Ig

A 96-well plate (Polysorb, Nunc; Thermo Fisher Scientific Inc., Waltham, MA, USA) was coated 100 μ l per well with 10 μ g ml^{–1} phosphoryl choline (PC)-BSA (Biosearch Technologies Inc., Novato, CA, USA) in coating buffer (Na₂CO₃/NaHCO₃, pH 9.4) overnight at 4°C, then incubated with blocking buffer (0.05% Tween 20 and 0–25% gelatin in PBS) for 30 min at room temperature and then washed twice with distilled water. Sera or fecal pellet supernatants were diluted

1/10 in blocking buffer, 50 μ l added to the plates and then incubated for 1 h at room temperature. Plate was then washed in PBS containing 0.05% Tween 20 and 50 μ l per well of HRP-conjugated goat anti-mouse IgA or anti-mouse IgM (Serotec) was diluted 1/2000 in blocking buffer and 50 μ l was added to corresponding wells for 1 h. After a further wash, 100 μ l of substrate solution [prepared by mixing equal volumes of substrate A and B (BD Bioscience)] was added to the plate, incubated for 30 min at room temperature, followed by addition of 50 μ l of 1 M *ortho*-phosphoric acid stop

solution to each well. The plate was read at 405 nm using a BioRad 680 ELISA plate reader and results were statistically analyzed using Mann–Whitney *U*-test.

Results

Non-hygienic environment suppresses development of colitis in TCR α KO mice

Young TCR α KO mice that had been housed in SPF facility were transferred to CV facility to determine if the less

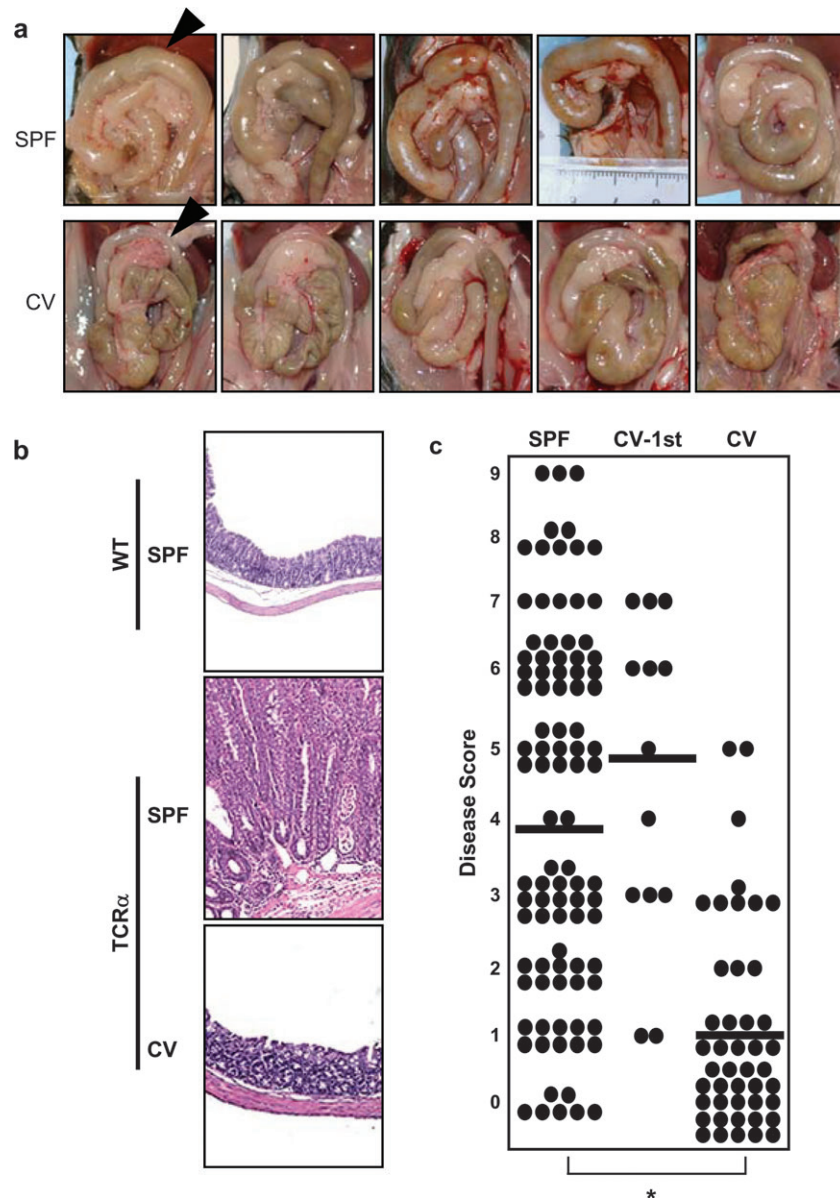


Fig. 1. Suppression of colitis under CV conditions. The litters from TCR α KO breeding pairs from SPF facility were either maintained under the SPF conditions or moved to CV facility (CV-1st). The CV first TCR α KO mice were maintained for more than five generations at the CV facility. The SPF, CV first and fifth generation CV TCR α KO mice (CV) were sacrificed at 6 months of age and the disease severity evaluated by a combination of gross and histological examinations. (a) The gross findings of the colon of SPF versus CV TCR α KO mice are shown. (b) The representative histology of the colon from SPF wild-type mice (WT), SPF TCR α KO mice (SPF) and CV TCR α KO mice (CV) are shown. The colons are markedly thickened in SPF TCR α KO mice, whereas the normal beaded appearance of colon is present in the CV TCR α KO mice. (c) The disease scores of SPF, CV first and CV (fifth generation) TCR α KO mice are summarized. The average of disease scores is indicated by black bars (SPF, 3.9; CV first, 4.8 and CV, 1.0). **P* < 0.0001 compared SPF and CV fifth TCR α KO mice.

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hygienic conditions present in CV facility would have an effect on the development of colitis. The majority of CV first TCR α KO mice, which were directly transferred from SPF to CV facility, continued to develop colitis (Fig. 1c). However, the incidence of colitis development decreased gradually during the subsequent generations of mice in the CV facility. The fifth generation of TCR α KO mice bred in the CV facility exhibited a significant decrease in the incidence and severity of colitis as compared with SPF TCR α KO mice (Fig. 1a–c). These findings suggest that continuous exposure to incompletely sanitized environment of CV facility over several generations is needed to fully suppress colitis development in TCR α KO mice, maintained previously in SPF conditions.

We next performed terminal restriction fragment-length polymorphism (T-RFLP) analysis using the luminal contents and intestinal tissues from the distal part of the small intestine, cecum and distal part of the large intestine to determine the effect of CV facility on the bacterial diversity. Although T-RFLP is a powerful approach for the assessment of diversity of bacterial flora (30), we did not find any major differences in the bacterial colonization in the colon of CV TCR α KO mice as compared with SPF TCR α KO mice (data not shown).

B cells are required for the suppression of the colitis development in TCR α KO mice under CV conditions

Since our previous studies clearly indicate that B cells regulate the colitis of TCR α KO mice (24, 31), we investigated the possibility that B cell-related immune mechanisms play a role in the suppression of colitis observed in CV environment. We next evaluated the development of colitis in $\alpha\mu$ DKO mice maintained in CV facility for more than five generations. As previously described (24), the severity of colitis in SPF $\alpha\mu$ DKO mice was markedly more than in SPF TCR α KO mice (Figs 1c and 2b). Interestingly, like SPF $\alpha\mu$ DKO mice, the CV $\alpha\mu$ DKO mice continued to develop severe colitis as judged by thickening of colonic wall, elongation of crypts and diffuse inflammatory cell infiltration. There was no statistically significant difference in the incidence and severity of colitis between CV $\alpha\mu$ DKO mice and SPF $\alpha\mu$ DKO mice (Fig. 2). These results indicate that suppression of colitis in TCR α KO mice under CV environment does not occur in the absence of B cells.

B cells are activated under CV conditions

To determine whether activation of B cells occurs under CV environment, phenotypic and functional characteristics of B cells from different tissues including spleen, colon and

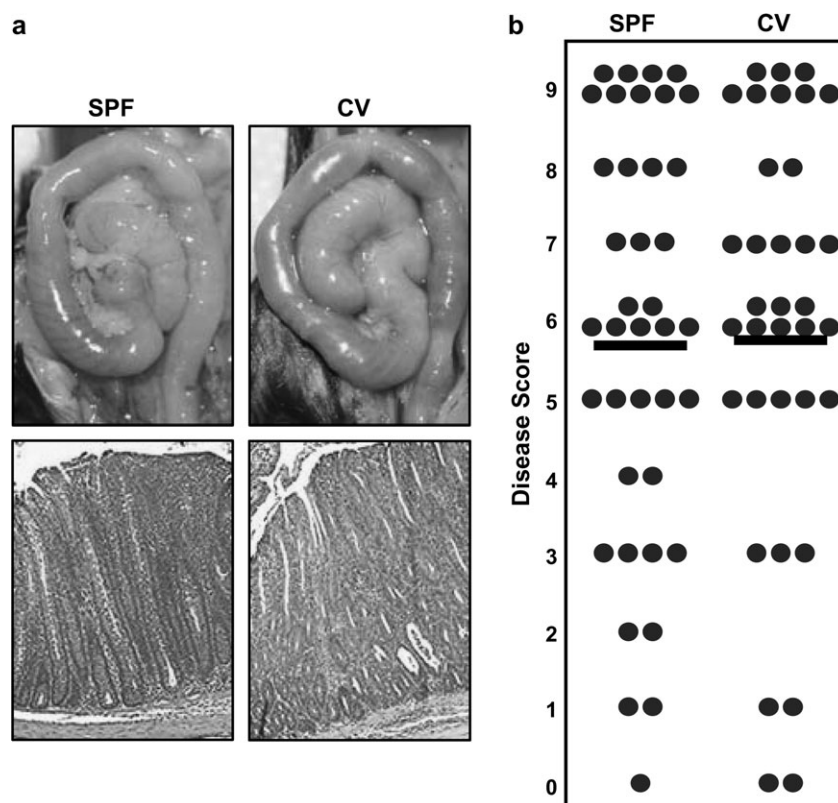


Fig. 2. Development of colitis in CV $\alpha\mu$ DKO mice. The litters from $\alpha\mu$ DKO breeding pairs from SPF facility were either maintained under the SPF conditions or moved to CV facility and subsequently maintained for more than five generations in CV facilities. The SPF and fifth generation CV $\alpha\mu$ DKO mice were sacrificed at 6 months of age. (a) The gross findings (top panels) and the representative histology (bottom panels) of the colon from SPF and CV $\alpha\mu$ DKO mice are shown. (b) The disease scores of SPF versus CV $\alpha\mu$ DKO mice are summarized. The average of disease score is indicated by black bar (SPF, 5.8 and CV, 5.9).

gut-associated lymphoid tissues (GALT), such as ALF, PP and MLNs, of CV TCR α KO mice were compared with B cells from SPF TCR α KO mice. ELISPOT assay for the detection of spontaneous Igs production showed that IgA and IgM productions by splenic B cells was significantly up-regulated in the CV TCR α KO mice compared with SPF TCR α KO mice (Fig. 3a and b and data not shown). These findings suggest that B cells are significantly and constitutively activated under CV conditions.

Since our previous experiments have identified an IL-10-producing B cell subset that is involved in the attenuation of ongoing colitis in TCR α KO mice maintained in SPF facilities (24, 31, 32), B cells from the GALT and colonic lamina propria were evaluated for IL-10 production. There was no difference in the level of IL-10 production by between B cells from CV TCR α KO mice and SPF TCR α KO mice (data not shown).

CV conditions activate B-1 B cells

B-1 cells are well known to participate in the early phases of immune response against bacteria (23) and characteristically produce natural IgM antibodies (23, 33, 34). Since IgM production by the splenic B cells was significantly enhanced under the CV condition, we next assessed the effect of CV conditions on B1 cells. Interestingly, the proportion of B-1 B cells in the PerC was increased in the CV TCR α KO mice compared with the SPF TCR α KO mice (Fig. 4a and b). However, there were no differences between the number of PerC CD5 $^{+}$ B-1a cells in the SPF and CV TCR α KO mice,

suggesting an increase in the CD5 $^{-}$ B-1b but not CD5 $^{+}$ B-1a cells under CV (data not shown).

Natural antibodies produced by B1 cells react with ubiquitous bacterial antigens such as PC (34). Therefore, the production of the IgA and IgM antibodies specific for PC was evaluated. Anti-PC IgM antibodies were significantly increased in the sera of CV TCR α KO mice as compared with SPF TCR α KO mice, whereas there was no significant difference in the production of anti-PC IgA antibodies among these mice groups (Fig. 4c). Since the amount of anti-PC antibody in the fecal pellets corresponds with the number of host B-1 B cells (28), the amount of IgA and IgM antibodies specific for PC in the fecal pellets was measured. Anti-PC IgM but not IgA antibodies were significantly increased in the fecal pellets from CV TCR α KO mice as compared with SPF TCR α KO mice (Fig. 4d).

B-1 B cells contribute to the inhibition of colitis under CV conditions

The presence of significantly activated B-1 B cells in the CV TCR α KO mice raised the possibility that B-1 B cells may be involved in the suppression of colitis in CV TCR α KO mice. Since B1 B cells are preferentially located in the PerC and are the major source of natural antibodies, PerC B-1 B cells from SPF TCR α KO mice were adoptively transferred into B cell-deficient CV $\alpha\mu$ DKO mice at 1 month of age and the recipient mice were then sacrificed at 6 months of age. Interestingly, the reconstitution of B-1 B cells in CV $\alpha\mu$ DKO mice resulted in significant suppression of colitis in the recipient $\alpha\mu$ DKO mice (Fig. 5a and b). Flow cytometric analysis confirmed reconstitution of donor-derived IgM $^{+}$ CD11b $^{+}$ B-1 B cells in the PerC of the recipient $\alpha\mu$ DKO mice. These reconstituted B-1 B cells did not express CD5 (Fig. 5c). Of note, IgM-producing cells were detectable in the spleen of recipient $\alpha\mu$ DKO mice following B-1 B cell reconstitution (Fig. 5d). The reconstitution of B-1 B cells in the recipient $\alpha\mu$ DKO mice was also associated with an increase in the serum IgM specific for PC (Fig. 5e). These findings indicate that B-1 B cells regulate colitis and produce natural IgM antibodies under CV conditions.

Discussion

The increased incidence of IBD in the last several decades has been associated with an improvement in living conditions (4, 5). Although in the Western countries, the hygiene hypothesis has been considered to play a role in the pathogenesis of human CD but not UC (4, 5), the incidence of UC but not CD has continued to increase in Asian countries (Japan and Korea) as the sanitary conditions have improved during the last two decades (35). It is likely that the effect of hygienic conditions on CD pathogenesis in the Western population is influenced by genetic susceptibility as an association of IBD development with the variants in major IBD-susceptible genes (NOD2, autophagy-related 16-like 1 and IL-23) has been observed in the Western population, but not in the Japanese population (36). The differences in the criteria used for the evaluation of sanitized conditions (4) may have also influenced results of these epidemiological studies.

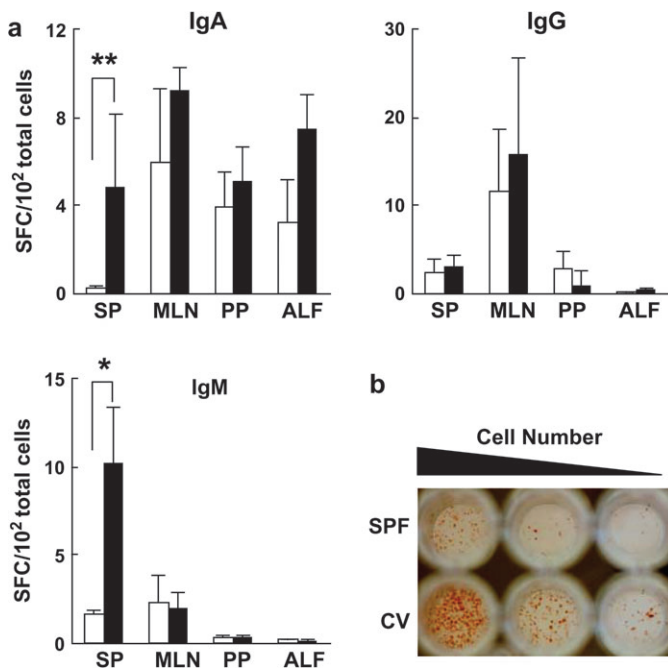


Fig. 3. Enhanced activation of B cells under CV facility. (a and b) Productions of IgA, IgG and IgM by the cells isolated from the spleen (SP), MLN, PP and ALF of TCR α KO mice in SPF (open bars) and CV (solid bars) were assessed by ELISPOT assay (* P < 0.01 and ** P < 0.001). (a) Spot forming cells of IgM from the splenic B cells of SPF (upper wells) versus CV (lower wells) are shown in (b).

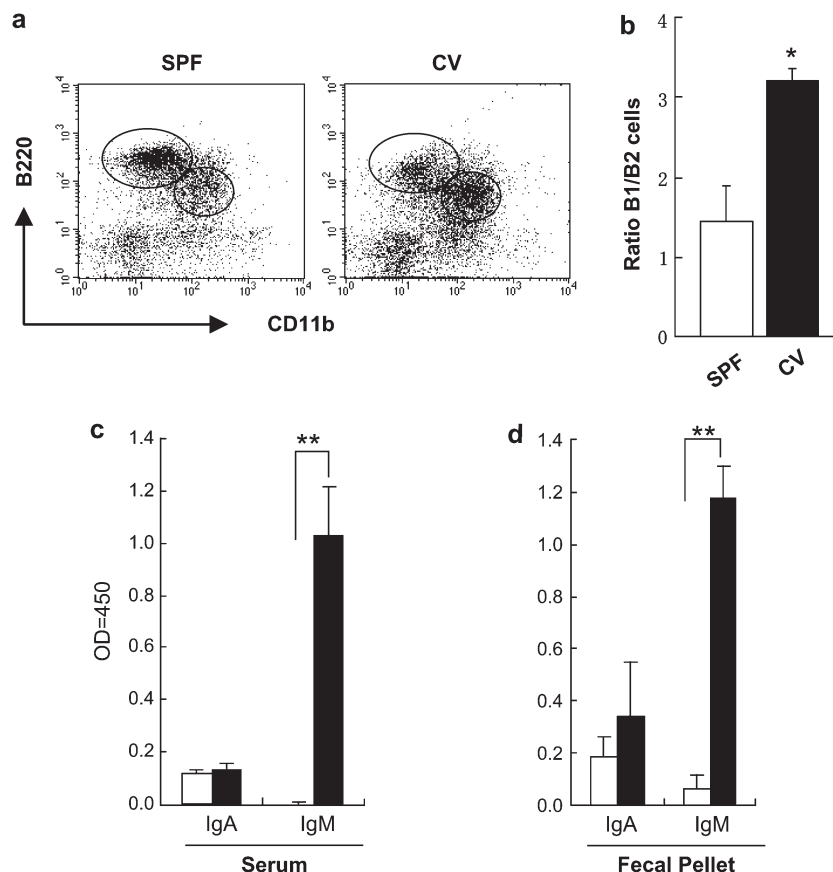


Fig. 4. Activation of B-1 B cells in CV TCR α KO mice. (a) PerC cells from SPF and CV TCR α KO mice were analyzed for the expression of CD11b and B220. The upper circles include B-2 B cells (B220⁺ CD11b⁻) and the lower circles include B-1 B cells (B220^{low} CD11b⁺). (b) The average ratio of B-1 B cells (B220^{low} CD11b⁺)/B-2 B cells (B220⁺ CD11b⁻) of SPF (open bars) ($n = 9$) and CV (solid bars) ($n = 9$) is shown. *Indicates a statistically significant difference ($P < 0.05$). (c and d) Sera and fecal pellets from SPF (open bars) and CV (solid bar) TCR α KO mice were subjected to ELISA for detection of IgM and IgA specific for PC. **Indicates a statistically significant difference ($P < 0.001$).

The experimental models of IBD have helped dissect the different components of chronic intestinal inflammation (6, 37, 38). Many of these experimental models are T_H1 mediated and are considered to resemble CD. The T_H2-mediated colitis model in TCR α KO mice shares some features with human UC including restriction of inflammation to the colon, presence of circulating autoantibodies and the protective role of appendectomy in the development of colitis (6, 16, 17). Interestingly, the T_H1-mediated colitis in IL-10 KO mice (21) is exacerbated under CV conditions. In contrast, we demonstrate here that the CV environment suppresses the T_H2-mediated colonic inflammation in the TCR α KO mice.

In our study, the TCR α KO from SPF facilities had to be maintained for five generation in CV facilities to fully observe the suppressive effect of the CV environment on colitis development (Fig. 1). This may reflect the time taken for the mice to be colonized completely by the microbial flora of CV environment as in the previous report that used embryo transfer technique to re-derive mice for breeding under CV (22) and the suppression of colitis occurred in the first generation of mice (19). The T-RFLP analysis, which is a powerful approach for the assessment of diversity of bacterial flora

(30), showed no major differences in the bacterial colonization in the intestinal luminal contents and intestinal tissues between TCR α KO mice maintained in SPF versus CV facilities. A more detailed quantification of microbial burden as well as differences in the colonization of all microbes including fungi and viruses may be required to demonstrate the effect of hygiene on the microbial diversity in the intestine.

Our previous experiments have established that B cells play an important regulatory role in the suppression of colitis in TCR α KO mice maintained in SPF facilities (24, 31, 32). A regulatory B cell subset termed 'Breg' (31) that produces IL-10 has been implicated in the attenuation of inflammation not only in TCR α KO mice but also in several other inflammatory conditions including experimental allergic encephalomyelitis and experimental arthritis (31, 39). However, the IL-10-producing B cell subset in the GALT was not increased in the CV TCR α KO mice as compared with the SPF TCR α KO mice (our unpublished data). Furthermore, the IL-10-producing B cell subset is involved in the amelioration of ongoing colitis but not in the suppression of colitis development (27). Therefore, we considered the possibility that a different B cell population is involved in the suppression of colitis development under CV environment.

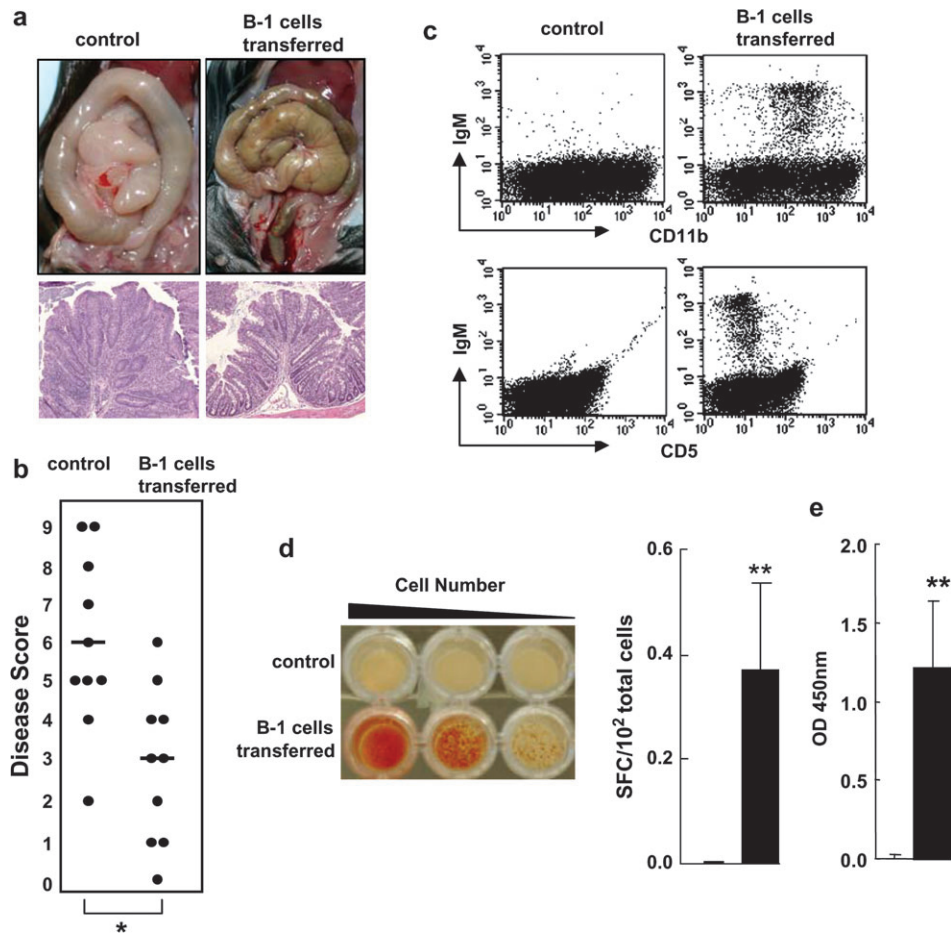


Fig. 5. Suppression of colitis in $\alpha\mu$ DKO mice by reconstitution of CD11b⁺ B-1 B cells. CD11b⁺ B-1 B cells from SPF TCR α KO mice or PBS (as a control) were injected into 1-month-old B cell-deficient $\alpha\mu$ DKO mice maintained in CV condition and the recipient mice sacrificed at 6 months of age. (a) The gross findings (top panels) and the representative histology (bottom panels) of the colon from CV $\alpha\mu$ DKO mice that received PBS (left panels) or CD11b⁺ B-1 B cells (B-1 cells transferred) are shown. (b) Disease scores of CD11b⁺ B-1 B cells (B-1 cells transferred) versus PBS (control) injected CV $\alpha\mu$ DKO mice are summarized. The average of disease score is indicated by black bars (control, 6.0 and B-1 B cells transferred, 2.9; $*P < 0.005$). (c) PerC lavage cells from control CV $\alpha\mu$ DKO mice or B-1 B cells transferred CV $\alpha\mu$ DKO mice and subjected to flow cytometric analysis for detection of IgM versus CD11b (top panels) or IgM versus CD5 (bottom panels). The data shown are representative of three independent experiments. (d) Production of IgM by the splenocytes from CV $\alpha\mu$ DKO mice without or with B-1 cell reconstitution was assessed by ELISPOT assay. The average numbers of IgM-secreting B cells from control (open bars) versus B-1 B cells transferred (solid bars) CV $\alpha\mu$ DKO mice are shown in right panel. **Indicates a statistically significant difference ($P < 0.0001$). (e) Sera from control (open bars) or B-1 B cells transferred (solid bars) CV $\alpha\mu$ DKO mice were subjected to ELISA for detection of IgM specific for PC. **Indicates a statistically significant difference ($P < 0.0001$).

The enhanced production of natural IgM capable of binding to PC (Fig. 4a and b) under CV conditions indicated that activation of B-1 B cells, particularly B-1b subset, is involved in the production of natural antibodies and are known to participate in the initial phase of T-independent microbial immune responses (23, 33). The IgM-producing B cells were detected preferentially in the spleen (Fig. 3a). This finding is consistent with a recent report that the activation of B-1 B cells induces their mobilization from PerC to the spleen (33). Since proportionately the number of B-1 B cells in the spleen is very low and it is difficult to obtain a pure population of B1 cells without significant contamination by other splenic cells, in particular B2 cells, we decided to use peritoneal B1 cells for the transfer studies. The transfer of B-1 B cells to CV B cell-deficient $\alpha\mu$ DKO led to the inhibition of colitis (Fig. 5a and b), indicat-

ing a regulatory function of this B cell subset. Interestingly, B-1 B cells show a stronger regulatory gene expression signature than CD4⁺CD25⁺ regulatory T cells (40). Since B-1 B cells have also been reported to be involved in the development of several autoimmune diseases including systemic lupus erythematosus (41) and autoimmune hemolytic anemia (42), the functional (pathogenic versus regulatory) role of B-1 B cell population may depend on the disease pathogenesis. It is likely that activated B-1 B cells mediate suppression through production of natural IgM antibodies. However, it is technically difficult to purify adequate quantities of natural IgM antibodies for transfer studies to demonstrate suppression of colitis. Since we did not perform B-2 cell transfer studies, we cannot exclude a role of B-2 B cells in the suppression of colitis observed in CV facilities.

This study provides a novel insight into the regulatory role of B-1 B cells in environmentally induced suppression of colitis. In contrast to the previously described regulatory B cell subsets that produce IL-10 or IL-12 (24, 31, 32) and regulate ongoing chronic colitis, B-1 B cells prevent the development of colitis. Furthermore, IL-10- and IL-12-producing regulatory B cell subsets are generated during chronic intestinal inflammation, whereas regulatory B-1 B cells are induced without intestinal inflammation under non-hygienic conditions.

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Abbreviations

ALF	cecal patches/appendix lymphoid follicles
CD	Crohn's disease
CV	conventional
$\alpha\mu$ DKO	B cell-deficient TCR α double knockout
ELISPOT	enzyme-linked immune spot
GALT	gut-associated lymphoid tissues
IBD	inflammatory bowel disease
KO	knockout
MLN	mesenteric lymph node
PC	phosphoryl choline
PerC	peritoneal cavity
PP	Peyer's patches
SPF	specific pathogen free
T-RFLP	terminal restriction fragment-length polymorphism
UC	ulcerative colitis

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