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Th1/Th17-Mediated Interstitial Pneumonia in Chronic Colitis Mice Independent of Intestinal Microbiota

Yasuhiro Nemoto,* Takanori Kanai,† Masahiro Takahara,*‡ Shigeru Oshima,* Ryuichi Okamoto,* Kiichiro Tsuchiya,* Satoshi Matsumoto,§ and Mamoru Watanabe*

Although intestinal microbiota are essential for the development of T cell–mediated colitis, it remains undetermined whether they enhance or suppress the chronic extraintestinal inflammation that often complicates inflammatory bowel diseases. In this study, we investigate the role of intestinal microbiota in the development of colitis and extraintestinal manifestations in a mouse model in which colitis was induced in SCID mice by adoptive transfer of CD4+CD45RBhigh T cells. Under specific pathogen-free conditions, these mice developed both colitis and extraintestinal interstitial pneumonia, whereas mice given a mixture of antibiotics did not develop colitis, but, surprisingly, developed Th1/Th17-mediated IP. Irrespective of antibiotic treatment, cotransfer of CD4+CD25+ regulatory T cells suppressed the development of pneumonitis and colitis, with all local CD4+CD45RBhigh T cell–derived cells converted to CD44highCD62L−IL-7Rαhigh effector–memory T cells. Retransfer of CD4+ effector–memory T cells from the lungs of antibiotic-treated mice with IP not only induced IP in both antibiotic-treated and -untreated recipients but also induced colitis in the untreated recipients. In summary, we have established a unique model of Th1/Th17-mediated IP in microbiota-free and antibiotic-treated mice. This model may be valuable in investigating the immunological mechanisms underlying extraintestinal disorders in patients with inflammatory bowel disease. \textit{The Journal of Immunology}, 2013, 190: 6616–6625.

Inflammatory bowel diseases (IBD) are caused by an excessive, tissue-damaging chronic inflammatory response, due largely to inappropriate activation of the immune system, with most patients having chronic disease (1, 2). The presence of commensal microbiota in the gut appears to be of crucial importance in the pathogenesis of IBD (1, 2). For example, germ-free (GF) rodent models of IBD are characterized by the absence of intestinal inflammation (3), indicating that intestinal microbiota are indispensable contributors to the pathogenesis of chronic T cell–mediated inflammation. A hallmark of T cell–mediated immune reactions to intestinal microbiota in IBD would be the continual generation of microbiota-specific colitogenic memory CD4+ T cells (4). Surviving memory T cells become functionally impaired in the absence of MHC class II signals (5), providing further evidence that persistent Ags help maintain CD4+ memory T cells. In addition, using a mouse model of colitis, generated by transfer of CD4+CD45RBhigh T cells, with a reduction in regulatory T (Treg) cells, we previously reported that large numbers of CD4+CD44highCD62L−IL-7Rαhigh effector–memory T (T_{EM}) cells proliferated in the colonic lamina propria (LP) (6). These cells reacted with intestinal bacterial Ags in vitro (7) and induced colitis when transferred to new SCID mice (8). Taken together, these findings suggest that memory CD4+ T cells reactive against intestinal bacteria are essential in the pathogenesis of chronic colitis.

IBD is often associated with extraintestinal manifestations, including conjunctivitis, uveitis, iritis, erythema nodosum, pyoderma gangrenosum, primary sclerosing cholangitis, and arthritis (9). These manifestations affect not only sites at which intestinal bacteria may be present, such as the skin and bile duct, but sites far from intestinal bacterial Ags, such as the eyes and joints. To date, it remains undetermined whether intestinal bacteria are essential for the development of extraintestinal manifestations of IBD.

Several recent studies have demonstrated that intestinal microbiota also affects the development of chronic systemic diseases, including diabetes mellitus, multiple sclerosis, and rheumatoid arthritis (10). For example, commensal gut flora are essential in triggering the activation of autoantibody-producing B cells in mice with experimental autoimmune encephalomyelitis, a model for multiple sclerosis in humans (11).

We therefore hypothesized that the intestinal microbiota modulate the development of extraintestinal manifestations in IBD. To investigate the role of these bacteria, we assessed the development of colitis and extraintestinal manifestations in a mouse model in which colitis was induced in SCID mice by adoptive transfer of CD4+CD45RBhigh T cells.

Materials and Methods

\textbf{Mice}

C57BL/6, BALB/c, C3HeJ, and CB17-isc SCID mice (6–8 wk old) were purchased from Japan CLEA (Tokyo, Japan). C57BL/6 RAG-2−/− mice...
were obtained from Taconic Farms (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). IL-2/- mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Specific pathogen-free (SPF) and GF breeding colonies of C3Hc-SCID mice were established and maintained at the Animal Facilities (SPF) and the Gnotobiatic Facilities (GF) of Yakult Central Institute for Microbiological Research (Kunitachi, Tokyo). Sterility in the Gnotobiatic Facilities was tested monthly by culturing of feces and bedding as well as gram staining. RORγt-GFP reporter mice were provided by Dr. D. R. Littman (Departments of Microbiology and Pathology, New York University School of Medicine, New York, NY). The experimental protocols were approved by the Institutional Committees on Animal Research at the Tokyo Medical and Dental University and the Yakult Central Institute, and the experiments were approved by the regional animal study committees and were performed according to institutional guidelines and home office regulations.

Antibodies

The following mAbs were obtained from BD Pharmingen (San Diego, CA) and used to purify cell populations and for flow cytometric analysis: FITC-, PE-, PerCP-, and allophycocyanin-conjugated anti-mouse CD4 (RM4-5); FITC- and PerCP-conjugated anti-mouse CD3 (145-2C11); PE-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); PE-conjugated anti-mouse IL-17A; FITC-conjugated anti-mouse IFN-γ; PE-conjugated anti-mouse integrin-β2 (DXTK32); Alexa Fluor 647–conjugated anti-mouse CD49a (H4/31); FITC-conjugated anti-mouse CD29 (H1.2F3); FITC-conjugated anti-mouse CD45RB (16A); PE-conjugated anti-mouse CD25 (PC61); and PE-conjugated streptavidin. Biotin-conjugated anti-mouse CCR6; biotin-conjugated anti-rat CD29 (Ha2/5), and rat anti-mouse IL-7R (DATK32); Alexa Fluor 647–conjugated anti–IL-17A mAb (TC11-18H10; BD Pharmingen) or FITC-conjugated anti-mouse CD4 (RM4-5); and PE-conjugated anti-mouse CCR7 (A7R34) and PerCP-Cy5.5–conjugated anti-mouse CCR7 (4B12) were obtained from ebioscience (San Diego, CA).

Isolation of mononuclear cells from LP

The entire colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosae were incubated in Ca2+,Mg2+-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 30 min to remove mucus and incubated with 3 mg/ml collagenase A (Roche) for 2–3 h. The HBSS containing 1 mM DTT (Sigma-Aldrich) for 30 min to remove mucus and incubated with 3 mg/ml collagenase A (Roche) for 2–3 h. The cells were subjected to Percoll (GE) density gradient centrifugation (40% 75%).

Isolation of mononuclear cells from lung tissue

After reflux with PBS, the lungs were resected, washed with PBS, cut into small pieces, incubated with 3 mg/ml collagenase A (Roche) for 2–3 h, and subjected to Percoll (GE) density gradient centrifugation (40% 75%).

Histological examination

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. Two tissue samples from the proximal and distal parts of each colon were prepared and analyzed without prior knowledge of the type of T cell reconstitution or treatment. The area most affected in each colon was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated as the sum of three parameters: crypt elongation, 0–3; mononuclear cell infiltration, 0–3; and frequency of crypt abscesses, 0–3 (6). The mean degree of inflammation in the colon was calculated as the sum of three parameters: crypt elongation, 0–3; mononuclear cell infiltration, 0–3; and frequency of crypt abscesses, 0–3 (6). The mean degree of inflammation in the lung, with scores of 0, 1, 2, 3, 4, and 5 indicating 0, 10–30, 30–50, 50–80, and 80–100%, respectively (12).

Flow cytometry

Isolated spleen (SP), mesenteric lymph nodes (MLN), colonic LP, and lung cells were incubated with each Ab for 20 min on ice; cells stained with PerCP-Cy5.5–conjugated anti-mouse CCR7 were incubated with Ab for 30 min at room temperature and protected from light, as recommended by the manufacturer’s protocol. The cells were subsequently analyzed by four-color FACScalibur flow cytometry and CellQuest software (BD Biosciences, San Jose, CA).

Intracellular staining of cytokines

Isolated CD4+ T cells were cultured for 12 h with ionomycin (500 ng/ml), PMA (50 ng/ml) and BD GolgiPlug (1 μl/ml; BD Pharmingen) and collected, and their surface molecules were stained. After cell fixation using a Cytofix/Cytoperm Kit (BD Pharmingen), the cells were stained with PE-conjugated anti–IL-17A mAb (TC11-18H10; BD Pharmingen) or FITC-conjugated anti–IFN-γ mAb (XMG1.2; BD Pharmingen) for 20 min.

Cytokine ELISA

To measure cytokine production, 3 × 106 CD4+ T cells from LP were cultured in 200 ml culture medium at 37°C in a humidified atmosphere containing 5% CO2 in 96-well plates (Costar, Cambridge, MA) precoated with 5 μg/ml hamster anti-mouse CD3ε mAb (145-2C11; BD Pharmingen) and 2 μg/ml hamster anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C. After 48 h, the culture supernatants were removed, and cytokine concentrations were assayed using specific ELISA kits, as recommended by the manufacturer (R&D Systems, Minneapolis, MN).

Immunohistochemistry

Consecutive colon cryostat sections (5 μm) were fixed and stained with rat anti-mouse CD4 mAb (BD Pharmingen). After washing, the cells were incubated with 488 goat anti-hamster IgG (Molecular Probes). The cells were examined by fluorescence microscopy, using a BioZERO BZ8000 (Keyence, Tokyo, Japan). Staining of lung tissue with Masson–Trichrome stain was performed by GenoStaff (Tokyo, Japan).

Induction of transfer colitis with CD4+ CD45RBhigh T cells

CD4+ T cells were isolated from SPs of BALB/c or C3HeJ mice using the anti–CD3 (L3T4)-MACS system (Miltenyi Biotec, Auburn, CA), as recommended by the manufacturer. Enriched CD4+ T cells were labeled with PE-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD45RB, and CD4+CD45RBhigh T cells were isolated using a FACS Aria II. These cells were >98% pure on reanalysis. Each SP CB17-isc SCID or GF C3HeJ-SCID mouse was injected i.p. with 3 × 106 cells of these cells. The mice were weighed initially and then three times per week. They were also evaluated for clinical symptoms such as hunched posture, piloerection, diarrhea, and blood in the stool. The mice were sacrificed 8 wk after transfer, and the clinical score of each was assessed as the sum of four parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, none; 1, mild; 2, moderate; and 3, extensive); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; and 3, bloody stool) (6).

In some experiments, SP CD4+ T cells were labeled with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD25. CD4+CD25+ T cells were isolated using a FACS Aria II, and 3 × 104 CD4+CD25+ T cells were cotransferred with CD4+CD45RBhigh T cells into SPF CB17-isc SCID mice as a negative control.

Antibiotics treatment

Some CB17-isc SCID mice were administered drinking water containing ampicillin (1 g/l), vancomycin (500 mg/l), neomycin sulfate (1 g/l), and metronidazole (1 g/l) 2 wk prior to beginning the adoptive transfer and during the course of the experiment. Control mice received drinking water without antibiotics.

Coculture of LP or lung CD4+ T cells with APCs previously pulsed with cecal bacterial Ags

Cecal bacterial Ags (CBA) were prepared from colitic CB17-isc SCID mice previously transferred with CD4+CD45RBhigh T cells. The cecum of each was opened, immersed in 1 ml PBS, and vortexed. DNAse (10 μg/ml) was added, and 1 ml of each suspension was added to 1 ml glass beads. The cells were disrupted at 5000 rpm in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) for 3 min and placed on ice. The glass beads and unlysed cells were removed by centrifuging at 5000 × g for 5 min. The lysates were filter-processed in a similar manner.

APCs were cocultured with CD4+ T cells. SP cells from normal BALB/c mice were prepared, and 2 × 105 cells were incubated with 5 ml lysate solution in a 15-ml tube overnight at 37°C. These APCs were washed twice, treated with mitomycin c, and added to T cell cultures. LP and lung CD4+ T cells obtained from normal mice and from colitic CD4+CD45RBhigh T cell–transferred SCID mice were cultured in the presence of APCs pretreated with CBA in complete media. The culture supernatants were collected on day 5, and IFN-γ and IL-17 concentrations were assayed by ELISA.

Retransfer of LP CD4+ T cells from colitic mice previously transfused with RORYigFPp CD4+CD45RBhigh T cells

Splenic CD4+CD45RBhigh T cells from RORYigFPp mice were transferred into Rag-2−/− mice. Ten weeks later, the mice were sacrificed. LP CD4+ T cells were isolated from colitic mice and CD3+CD4+GFP+ and CD3+CD4+GFP−, and CD3+CD4+ populations were sorted by FACSAria II, and 3 × 105 cells were injected into each Rag-2−/− mouse.
Retransfer of lung \( T_{EM} \) cells into SCID mice

In the first experiment, CB17-icr SCID mice were pretreated with antibiotics. After 2 wk, they received \( 3 \times 10^4 \) CD4+CD45RB\(^{high}\) T cells. Ten weeks later, these mice were sacrificed, and their lung CD4+ T cells were isolated. CD3+CD4+CD45RB\(^{high}\) CD62L+ \( T_{EM} \) cells were sorted by FACSAria II, and \( 3 \times 10^4 \) of these cells were injected into each new CB17-icr SCID mouse, with or without pretreatment with antibiotics.

In the second experiment, GF C3HeJ SCID mice were injected with \( 3 \times 10^4 \) CD4+CD45RB\(^{high}\) T cells. Ten weeks later, these mice were sacrificed, and lung CD4+ T cells were isolated. CD3+CD4+CD45RB\(^{high}\) CD62L+ cells were sorted with FACSAria II, and \( 3 \times 10^4 \) of these cells were injected into each new GF or SPF C3HeJ SCID mouse.

Statistical analysis

Variables not normally distributed in either group were compared using the Mann–Whitney U test. If a variable was normally distributed in both groups, we assessed the variance within each group using the F test. If both groups were homoscedastic, between group differences were evaluated using Student t tests; if not, they were compared using Welch’s t test. All statistical analyses were performed using Statcell software, with \( p < 0.05 \) defined as statistically significant.

Results

Antibiotics treatment prevents the development of colitis, but not pneumonitis, in SCID mice injected with CD4+CD45RB\(^{high}\) T cells

SCID mice injected with CD4+CD45RB\(^{high}\) T cells, alone or with \( T_R \) cells, were treated with a mixture of antibiotics (ABX) (vancomycin, neomycin, metronidazole, and ampicillin) or left untreated (Fig. 1A). Consistent with a previous report (7), SCID mice injected with CD4+CD45RB\(^{high}\) T cells without ABX treatment (hereafter called DW RB\(^{high}\) mice) rapidly developed severe wasting disease (Fig. 1Ba), with marked thickening of the colon wall (Fig. 1Bb). In contrast, mice injected with both CD4+ CD45RB\(^{high}\) T cells and \( T_R \) cells without ABX treatment (DW RB\(^{high}/T_R \) mice) showed no wasting disease (Fig. 1Ba), and their colons appeared normal (Fig. 1Bb). Body weight loss differed significantly between these two groups (Fig. 1Ba). As expected, mice injected with CD4+CD45RB\(^{high}\) T cells, either alone or with CD4+CD25\(^{high}\) \( T_R \) cells, and treated with ABX (ABX RB\(^{high}\) and ABX RB\(^{high}/T_R \), respectively) showed no evidence of wasting disease (Fig. 1Ba) with normal colon walls (Fig. 1Bb). Interestingly, ABX RB\(^{high}\) mice maintained body weight, whereas ABX RB\(^{high}/T_R \) mice gradually gained body weight, with their weights differing significantly 14 wk after transfer (Fig. 1Bb). Histological examination revealed that DW RB\(^{high}\) mice alone showed marked infiltration of mononuclear cells into the colonic LP (Fig. 1Ca) and a higher colitis score than the other three groups (Fig. 1Cb). The colitis scores of the ABX RB\(^{high}\) and ABX RB\(^{high}/T_R \) mice were comparable (Fig. 1Cb). Moreover, DW RB\(^{high}\) and ABX RB\(^{high}\) mice showed massive infiltration of mononuclear cells into the lungs, regardless of ABX treatment, whereas DW RB\(^{high}/T_R \) mice did not (Fig. 1Da). The interstitial pneumonia (IP) scores of mice that were and were not injected with \( T_R \) cells differed significantly (Fig. 1Db). Extraintestinal lesions in mice treated with ABX in the absence of \( T_R \) cells were specific to the lungs, as we confirmed that other sites, including the thyroid, salivary glands, heart, stomach, small intestine, liver, kidneys, adrenal glands, ovaries, and joints, were almost normal in all groups of mice, even in the absence of \( T_R \) cells (data not shown).

Infiltration of CD3+CD4+ T cells was quantitatively evaluated by flow cytometry (Fig. 1E). The numbers of CD3+CD4+ T cells recovered from the colonic LP and MLNs of DW RB\(^{high}\) mice far exceeded the number originally injected, indicating extensive T cell proliferation and survival in the inflamed colons and MLNs of DW RB\(^{high}\) mice but not in the other three groups (Fig. 1E). In contrast, the absolute numbers of CD3+CD4+ T cells isolated from the lungs of DW RB\(^{high}\) and ABX RB\(^{high}\) mice were significantly higher than those from the lungs of DW RB\(^{high}/T_R \) and ABX RB\(^{high}/T_R \) mice (Fig. 1E). Immunochemistry analysis revealed marked infiltration of CD4+ T cells into the lungs of DW RB\(^{high}\) and ABX RB\(^{high}\) mice but not of DW RB\(^{high}/T_R \) and ABX RB\(^{high}/T_R \) mice (Fig. 1F).

The concentrations of IFN-\( \gamma \) and IL-17A secreted into the media by colonic LP CD4+ T cells of DW RB\(^{high}\) mice was significantly higher than those of the other three groups (Fig. 1G). Consistent with the pathology of pneumonitis in the lungs of DW RB\(^{high}\) and ABX RB\(^{high}\) mice, the concentrations of IFN-\( \gamma \) and IL-17A secreted by their lung CD4+ T cells were significantly higher than those of that of DW RB\(^{high}/T_R \) and ABX RB\(^{high}/T_R \) mice (Fig. 1G). Intriguingly, the production of IFN-\( \gamma \) by lung CD4+ T cells of ABX RB\(^{high}\) mice was significantly lower than that of DW RB\(^{high}\) mice, whereas their production of IL-17A was comparable. Regardless of the presence or absence of \( T_R \) cells, and with or without ABX treatment, the cells recovered from the colon and lungs of these mice had the CD44\(^{high}\)CD62L\(^{low}\) IL-7Ro\(^{high}\) T cell phenotype (Supplemental Fig. 1A). Masson–Trichrome staining of the lung showed fibrosis, indicating chronic inflammation, in the lungs of ABX RB\(^{high}\) and DW RB\(^{high}\) mice, but no fibrosis in the lungs of ABX RB\(^{high}/T_R \) and DW RB\(^{high}/T_R \) mice (Supplemental Fig. 1B). Collectively, these results indicate that ABX modulation of intestinal microbiota suppresses the development of colitis but not extraintestinal pneumonitis, whereas \( T_R \) cells suppress the development of both conditions.

IL-2/ABX mice developed both colitis and IP

To exclude the possibility that IP was specific to the SCID transfer model, we evaluated IL-2/ABX mice, which spontaneously develop both autoimmune diseases and colitis because of \( T_R \) reduction. After they developed colitis, IL-2/ABX mice were treated with ABX for 4 wk, resulting in the disappearance of colitis symptoms in all ABX-treated IL-2/ABX mice. A comparison of age matched ABX-treated IL-2/ABX mice (ABX IL-2/ABX), colitic IL-2/ABX mice (DW IL-2/ABX), and C57BL/6J mice (wild type [WT]) showed that DW IL-2/ABX mice developed severe colitis, ABX IL-2/ABX mice developed very slight colitis, and control WT mice did not develop colitis (Supplemental Fig. 2A, 2B). Consistent with this finding, the absolute numbers of colonic LP CD3+CD4+ T cells were significantly higher in DW IL-2/ABX than in ABX IL-2/ABX and WT mice (Supplemental Fig. 2C). We also found that large numbers of mononuclear cells had infiltrated the lung interstitial lesions of both DW IL-2/ABX and ABX IL-2/ABX mice but not of WT mice (Supplemental Fig. 2D). IP scores and the absolute number of lung CD3+CD4+ cells were significantly higher in DW IL-2/ABX and ABX IL-2/ABX mice than in WT mice (Supplemental Fig. 2E, 2F), confirming that IL-2/ABX mice also developed T cell–mediated IP regardless of ABX treatment. Intracellular staining of cytokines revealed that colonic LP and lung CD4+ T cells of IL-2/ABX mice were abundant in Th1, Th17/Th1, and Th17 subpopulations (Supplemental Fig. 2G).

Infiltrating lung CD4+ T cells in colitic mice produce IFN-\( \gamma \) and IL-17A in response to cecal bacterial Ags

Because DW RB\(^{high}\) and ABX RB\(^{high}\) mice developed IP, with marked infiltration of Th17 and Th1 TEM cells in the lungs, we assessed whether these colitic lung CD4+ T cells could respond to Ags derived from commensal bacteria (CBA) by synthesizing and secreting IFN-\( \gamma \) and IL-17A in vitro. Significantly higher levels of IFN-\( \gamma \) and IL-17A were produced by colitic LP and lung CD4+ T cells in response to CBA than by normal colonic LP and lung...
CD4+ T cells, respectively (Fig. 2A), with responses to CBA being dose dependent. Although colitic lung CD4+ T cells produced significantly less IFN-γ than colitic LP CD4+ T cells upon stimulation by the same concentration of CBA, the two cell types produced comparable levels of IL-17A in response to CBA (Fig. 2A). These results indicated that colitic lung CD4+ T cells can respond to intestinal bacterial Ags and may therefore be colitogenic, like colitogenic LP CD4+ T cells. To determine whether lung CD4+ TEM cells generated in ABX-treated mice can respond to intestinal bacterial Ags, lung and LP CD4+ T cells of ABX-treated and DW-treated SCID mice injected with CD4+CD45RBhigh T cells were cocultured with CBA-pulsed APC. Surprisingly, lung CD4+ T cells from ABX-treated mice produced very small amounts of IL-17 and IFN-γ in response to CBA (Fig. 2B) while producing larger amounts both in response to anti-CD3/CD28 Abs (Fig. 1G). This result suggested that most lung CD4+ T cells in ABX-treated mice were not “intestinal microbiota–reactive TEM cells.”

**IP occurs independent of Th17**

Consistent with our finding that colitic lung CD4+ TEM cells preferentially produce IL-17 rather than IFN-γ in response to CBA (Fig. 2A), a recent study suggested that lung IL-17-producing CD4+ T cells, including Th17 cells, are critically involved in inducible BALTs during acute influenza virus infection of mice (13). We therefore hypothesized that colitogenic RORγt-dependent Th17 cells, rather than colitogenic Th1 cells, are involved in the development of IP in this transfer model. To assess this, we isolated colitogenic CD3+CD4+RORγt+ (GFP+) and RORγt– (GFP–) cells from the colons of colitic RAG-2−/− mice previously injected...
with CD4+CD45RBhigh T cells from RORγt-GFP reporter mice and then retransferred colitogenic GFP+ or GFP− cells to new RAG-2−/− mice (RORγt+ and RORγt− mice, respectively). The RORγt+ T cells were a mixture of IL-17+IFNγ+, IL-17+IFNγ−, and IL-17+IFNγ− cells, whereas most RORγt− T cells are IL-17−IFNγ+, because RORγt−IFNγ−IL-17+ Th17 cells differentiate to RORγt−IFNγ+IL-17− “alternative Th1” cells (14). As positive and negative controls, RAG-2−/− mice were transferred with whole colitic LP CD3+CD4+ cells with or without splenic CD4+CD25+ Treg cells (colitic CD4+ and CD4+ + Treg mice, respectively). We also assessed the dominance of RORγt+ and RORγt− cells in the lungs when these cells were transferred with whole colitogenic CD3+ CD4+ cells by evaluating GFP expression (Fig. 3A). RORγt+, RORγt−, and colitic CD4+ mice developed severe colitis, whereas colitic CD4+ + Treg mice did not (Fig. 3A). The histological scores of RORγt+, RORγt−, and colitic CD4+ mice were comparable but were significantly higher the scores of colitic CD4+ + Treg mice (Fig. 3C). Large numbers of mononuclear cells infiltrated the lung interstitial areas of RORγt+, RORγt−, and colitic CD4+ mice, with small numbers of cells found in the same area in colitic CD4+ + Treg mice (Fig. 3D). In addition, IP scores in RORγt+, RORγt−, and colitic CD4+ mice were comparable and significantly higher than in colitic CD4+ + Treg mice (Fig. 3E). The numbers of CD3+CD4+ cells in the lungs and colonic LP were comparable in RORγt+, RORγt−, and colitic CD4+ mice, with all three being significantly higher the numbers in colitic CD4+ + Treg mice (Fig. 3F). We also assessed the proportion of GFP+ cells in the lungs, colonic LP, MLN, and SP of the four groups. There were almost no GFP+ cells in any organ of RORγt− mice, whereas one-half to one-third of GFP+ cells were lost in the organs of the other groups, and no difference in the number of IFN-γ–producing cells in the RORγt+, RORγt−, and CD4+ groups (Supplemental Fig.

**FIGURE 2.** CD4+ T cells in the lungs of colitic mice respond to cecal Ags. (A) Cytokine production by lung, LP and SP CD4+ T cells from colitic and normal mice. Lung, LP and SP CD4+ T cells were isolated and stimulated with various concentrations of CBA-pulsed APC in vitro. IFN-γ and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown as the mean ± SEM for five wells per group. (B) SCID mice were pretreated with ABX or DW, followed by transfer of CD4+CD45RBhigh T cells. Six weeks later, the lung and LP CD4+ T cells in both groups were isolated and stimulated in vitro with various concentrations of CBA-pulsed APC. IFN-γ and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown as the mean ± SEM for three wells per group. *p < 0.05 compared with negative control, 0 µg/ml CBA.
3B). Immunohistochemistry showed that most of the mononuclear cells infiltrating the lung interstitial lesions of these three groups were CD4+ T cells (Fig. 3G).

Colitogenic CD4+ TEM cells in the colon and lungs share similar expression patterns of gut- and lung-homing receptors

To evaluate the possible developmental correlation between colitis and pneumonitis in this transfer model, we assessed the expression of homing receptors on lung and LP CD3+CD4+ T cells in colitic SCID mice previously injected with CD4+CD45RBhigh T cells from colitic RAG-2−/− mice (CD4+). As a negative control, RAG-2−/− mice were injected with whole colitogenic CD3+CD4+ cells from colitic RAG-2−/− mice (CD4+). As a positive control, RAG-2−/− mice were injected with whole colitogenic CD3+CD4+ cells from colitic RAG-2−/− mice and splenic CD4+CD25+ T cells from normal mice (CD4+ + Treg). (B) Histopathology of the distal colon of the indicated mice 10 wk after retransfer. Original magnification ×100. (C) Histological scores. Data are shown as the mean ± SEM for four mice per group. (D) Histopathology of the lungs of the indicated mice 10 wk after transfer. Original magnification ×100. (E) IP scores. Data are shown as the mean ± SEM for four mice per group. (F) Absolute number of CD3+CD4+ cells in the lung, colonic LP, SP, and MLN 10 wk after retransfer. Data are shown as the mean ± SEM. (G) Expression of CD4 by frozen sections of lung from each group. Tissue samples obtained 10 wk after the retransfer were incubated with anti-CD4 Abs. Red indicates CD4; blue indicates DAPI. Original magnification ×200. *p < 0.01.

Lung CD4+ T cells of SCID mice injected with CD4+CD45RBhigh T cells preferably migrate to the lungs

We also assessed whether colitic lung and LP CD4+ T cells, which express high levels receptors for homing to the lungs and intestine, can migrate to the lung and LP after transfer into new SCID mice. Both lung CD4+ T cells and LP CD4+ T cells from colitic SCID mice were transferred into new SCID mice (Supplemental Fig. 4A), sacrificed 3 wk later, and analyzed. Interestingly, larger numbers of lung than of LP CD4+ T cells migrated to the lungs, whereas almost the same number of both migrated to LPs, MLNs and SPs (Supplemental Fig. 4B).

Lung CD4+ T cells of SCID mice injected with CD4+CD45RBhigh T cells and treated with ABX have the potential to induce colitis

We next examined whether colitic lung CD4+ T cells have the potential to induce pneumonitis and/or colitis if transferred to new SCID mice with or without ABX treatment (Fig. 5A). As expected, colitis developed only in SCID mice injected with CD4+ T cells from the lungs of ABX RBhigh mice without (DW Lung Re-Tr mice) not with (ABX Lung Re-Tr mice) subsequent ABX treatment (Fig. 5B, 5C). Furthermore, significantly fewer LP cells...
were recovered from ABX Lung Re-Tr than DW Lung Re-Tr mice (Fig. 5D), whereas both groups developed pneumonitis ∼6 wk after retransfer (Fig. 5E) with comparable IP scores (Fig. 5F) and numbers of recovered lung CD3+CD4+ T cells (Fig. 5G), suggesting that pneumonitis is induced independently of the presence of living intestinal microbiota.

Intestinal microbiota are not essential for the development of pneumonitis in SCID mice injected with CD4+CD45RBhigh T cells

To exclude the possibility that lung harbor bacteria may cause IP, even after ABX treatment, we used a GF system to completely exclude the possible contribution of living bacteria in the intestine or in the lung from inhalation. Again, we found that SCID mice injected with CD4+CD45RBhigh T cells under SPF conditions (SPF RBhigh mice) developed severe colitis and IP 6 wk later, whereas SCID mice injected with CD4+CD45RBhigh T cells under GF conditions (GF RBhigh mice) developed IP but not colitis (Fig. 6A, 6C). The colitis and IP scores of these two groups provided statistical confirmation (Fig. 6B, 6D). When lung CD3+CD4+ T cells isolated from GF RBhigh mice were retransferred to new SPF (SPF Lung Re-Tr mice) or GF SCID (GF Lung Re-Tr mice) mice (Fig. 6E), the former group developed pneumonitis and colitis, whereas the latter developed pneumonitis but not colitis (Fig. 6F, 6H), similar to the ABX treatment system. These observations were also confirmed by statistical analysis (Fig. 6G, 6I).

CD4+CD45RBhigh T cells of GF donor mice also induce IP

We thought that the TCR repertoires of naive T cells may differ in GF donors, in which all intestinal bacteria-reactive T cells remain naive, and SPF donors, in which some intestinal bacteria-reactive T cells, have already become memory T cells. We also assessed whether CD4+CD45RBhigh T cells from GF donor mice can induce colitis upon transfer to new SCID mice, with or without ABX treatment (Fig. 7A). We found that these GF CD4+CD45RBhigh T cells could induce Th1/Th17-mediated colitis and pneumonitis (Fig. 7B–I). The expression of CD49a and integrin α4β7 by lung CD3+CD4+ T cells was slightly lower in ABX-treated than in DW-treated mice (Fig. 7J). Lung and LP CD4+ T cells generated from GF donor mice in DW-treated SCID mice produced large amount of IL-17 and IFN-γ in response to CBA in vitro, whereas cells in ABX-treated SCID mice produced very small amounts of IL-17 and IFN-γ (data not shown).
FIGURE 6. Commensal bacteria are not essential for the development of IP. (A–D) GF or SPF C3HeJ SCID mice were injected with CD4+CD45RB<sup>high</sup>T cells. (A) Histopathology of the distal colon of the indicated mice 10 wk after transfer. (B) Histopathology of the lungs of the indicated mice 10 wk after transfer. (C) Histopathology of the lungs of the indicated mice 10 wk after transfer. (D) IP scores. Data are shown as the mean ± SEM for eight GF and four SPF mice. (E) GF C3HeJ SCID mice were injected with CD4+CD45RB<sup>high</sup>T cells and sacrificed 10 wk later. Lung CD4<sup>+</sup>T cells were isolated, and CD3<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup> T cells were sorted with FACS Aria II and injected into new GF (GF Lung Re-Tr) or SPF (SPF Lung Re-Tr) C3HeJ SCID mice. (F) Histopathology of the distal colon of the indicated mice 10 wk after retransfer. (G) Histopathological scores. Data are shown as the mean ± SEM for three mice per group. (H) Histopathology of the lungs of the indicated mice 10 wk after retransfer. Original magnification 100×. (I) IP scores. Data are shown as the mean ± SEM for three mice per group. Original magnification 100×. *p < 0.01.

Discussion

We have shown in this study that living intestinal microbiota are required for the development of intestinal, but not lung, inflammation in an adoptive transfer model of colitis. Furthermore, we showed that infiltrating lung CD4<sup>+</sup>TEM cells generated under ABX-treated or GF conditions have the potential to induce colitis if transferred under SPF conditions. These surprising findings clearly indicate that intestinal microbiota are essential for the development of intestinal, but not lung, inflammation, at least during lymphopenia-driven cell expansion.

In assessing the mechanisms that mediate the development of lung inflammation in ABX-treated mice under GF conditions, it was surprising to find that chronic inflammation in GF SCID mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup>T cells occurs only in lung but not in any other tissue examined. Over 90% of SPF SCID mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup>T cells develop colitis, with 84, 44, 26, and <10% also developing low levels of inflammation of the stomach, liver, lungs, and other tissues, respectively (17). Differences in level of inflammation may be due to the use of immune-deficient recipient mice obtained from different animal facilities or to environmental factors that drive the inflammatory response, because the nature of the endogenous microbiota in immune-deficient recipients may be important and may vary among facilities. Although GF SCID mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup>T cells do not develop colitis, the absence of inflammation in other tissues under GF conditions has not been experimentally established. Because we found that SPF SCID mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup>T cells developed both colitis and IP, we initially hypothesized that colitogenic CD4<sup>+</sup>TEM cells were originally generated in response to intestinal bacterial Ags presented by professional APCs at intestinal sites, subsequently migrating to the lungs to establish lung inflammation. It was unclear, however, how aberrantly migrating CD4<sup>+</sup>TEM cells could be maintained in the lungs. Surprisingly, however, we found that GF- and ABX-treated SCID injected with CD4<sup>+</sup>CD45RB<sup>high</sup>T cells developed IP with infiltration of CD4<sup>+</sup>TEM cells but did not develop colitis. These TEM cells may not just be retained in the lungs but may be pathogenic because lung CD4<sup>+</sup>T cells from ABX-treated SCID mice produced large amount of INF-γ and IL-17 in response to anti-CD3/CD28 stimulation but produced very small amounts of IL-17 and INF-γ in response to CBA in vitro. These results suggested that most lung CD4<sup>+</sup>T cells in ABX-treated mice were not “intestinal microbiota-reactive TEM cells,” but became intestinal microbiota-reactive TEM cells upon injection into SPF SCID mice. This result suggests that, in GF- and ABX-treated mice, CD4<sup>+</sup>T cells expand in the lungs in response to self-Ags. Because these cells produced very small amounts of INF-γ and IL-17 in response to intestinal bacterial Ags, some may have cross-reactivity to intestinal Ags. Upon transfer into new SCID mice under SPF conditions, the cell population reactive to intestinal bacteria expands and induces colitis. Because these “colitogenic” CD4<sup>+</sup>T cells were generated in the lungs, they have homing receptors to the lungs. Finally, these cells migrate to the lungs, where they also induce intestinal pneumonitis. Another hypothesis is that lung CD4<sup>+</sup>TEM-like cells were generated in ABX-treated mice were not “effector” or “memory” cells but had a “memory-like” phenotype generated through homoeostatic expansion in lymphopenic hosts (18). Upon injection into ABX-treated GF SCID mice, these naïve CD4<sup>+</sup>T cells expand in the lungs in the absence of cognate Ag activation, making them memory–phenotype (MP) cells. Injection of these MP cells into new SCID mice under SPF conditions results in the expansion of MP cells specific to intestinal bacterial Ags, thus inducing colitis.

Although athymic BALB/c nude mice reconstituted with CD4<sup>+</sup>CD25<sup>+</sup>T<sub>R</sub> cells spontaneously develop histologically and serologically evident autoimmune diseases, such as thyroiditis, gastritis, insulinitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, and polyarthritis, but not colitis or pneumonitis, under SPF conditions (19, 20), it is not known whether they develop these diseases...
under GF conditions. Even if GF nude mice injected with CD4+CD25− T cells develop IP or other autoimmune diseases, a different mechanism appears to underlie the development of IP in GF SCID mice injected with CD4+CD45RBhigh T cells. First, the nude transfer autoimmune model requires a large enough number of donor CD4+CD25− T cells (2 × 10^6–1 × 10^7 cells/mouse) to block lymphopenia-driven T cell expansion, compared with the lower number of donor CD4+CD45RBhigh T cells (∼3–4 × 10^5 cells/mouse) in the SCID/RAG-1/2 transfer colitis model. Consistent with this, RAG-1/2 mice with over 1 × 10^7 CD4+CD45RBhigh T cells did not develop colitis, even under SPF conditions, owing to the lack of lymphopenia-driven T cell expansion (21). Moreover, autoantigen-reactive CD4+CD25− T_pom cells in the CD4+CD45RBhigh subpopulation may expand slowly (22). Consistent with this finding, the period required to establish disease is longer for the nude transfer (over 15 wk) than for the SCID/RAG-1/2 transfer (∼6–8 wk) colitis model. These findings indicate that our model of microbiota-reactive T cell–mediated pneumonitis in the absence of microbiota (GF) or in the presence of small numbers (ABX treatment) is a unique and valuable tool to investigate the immunological mechanisms of extraintestinal disorders in patients with IBD.

From a clinical perspective, although pulmonary diseases are thought to be rare in IBD patients, prospective studies have suggested that a significant proportion of these patients have complicating pulmonary diseases, including chronic bronchitis, bronchiectasis, bronchiolitis obliterans with organizing pneumonia, and parenchymal nodules (23). Our model seems to be similar to bronchiolitis obliterans with organizing pneumonia, a typical interstitial lung disorder. Although the manifestations of pulmonary disease usually parallel intestinal disease activity, as seen in our model under SPF conditions, our model suggests that pulmonary lesions may be maintained, at least at an immunological level, even after intestinal...
remission and act as a reservoir of microbiota-reactive T_{EM} cells that can cause recurrence. Consistent with this, we showed that adoptive transfer of T_{EM} cells from ABX-treated or GF mice into SPF recipient mice reproduces colitis comparable with the original colitis in this model. Further studies are required on a range of clinical issues, such as recent overuse of ABX and the increased incidence of autoimmune diseases due to excessive hygiene.

In summary, we have established a unique microbiota-reactive T cell–mediated pneumonitis model in microbiota-free or decreasing conditions. This model may be a valuable tool for investigating the immunological mechanisms of extraintestinal disorders in patients with IBD.

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Disclosures
The authors have no financial conflicts of interest.

References

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