Classical Th1 Cells Obtain Colitogenicity by Co-existence of RORγt-expressing T Cells in Experimental Colitis

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Background: Both Th1 and Th17 cell types are involved in the pathogenesis of chronic intestinal inflammation. We recently demonstrated that retinoid-related orphan receptor gamma t (RORγt)-expressing Th17 cells are progenitor cells for alternative Th1 cells, which have the potential to induce colitis. However, the involvement of classical Th1 (cTh1) cells generated directly from naive T cells without RORγt expression in the pathogenesis of colitis remains poorly understood.

Methods: We performed a series of in vivo experiments using a murine chronic colitis model induced by adoptive transfer of splenic $CD4^+CD45RB^{high}$ T cells obtained from wild-type, $ROR\gamma t^{gfp/gfp}$, or $ROR\gamma t^{gfp/+}$ mice into RAG-$2^{-/-}$ mice.

Results: RAG-$2^{-/-}$ mice receiving transfer of in vitro-manipulated $ROR\gamma t^{gfp/gfp}$ Th1 cells developed colitis. RAG-$2^{-/-}$ mice co-transferred with splenic $CD4^+CD45RB^{high}$ T cells obtained from wild-type mice and $ROR\gamma t^{gfp/gfp}$ mice developed colitis with a significant increase in $ROR\gamma t^{gfp/gfp}$ cTh1 cell numbers when compared with noncolitic mice transferred with splenic $CD4^+CD45RB^{high}$ T cells obtained from $ROR\gamma t^{gfp/gfp}$ mice. Furthermore, RAG-$2^{-/-}$ mice transferred with in vivo-manipulated $ROR\gamma t^{gfp/gfp}$ cTh1 cells developed colitis with a significant increase in $ROR\gamma t^{gfp/gfp}$ cTh1 cell numbers.

Conclusions: These findings indicate that both alternative Th1 cells and cTh1 cells have the potential to be colitogenic in an adaptive transfer model. The development of cTh1 cells was dependent on the co-existence of RORγt-expressing T cells, suggesting a critical role for the interactions of these cell types in the development of chronic intestinal inflammation.

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Key Words: colitis, alternative Th1, classical Th1, RORγt, $CD4^+CD45RB^{high}$ T cell

Inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, are chronic, relapsing, and remitting inflammatory conditions of the gastrointestinal tract.[1,2] The activation and expansion of colitogenic $CD4^+$ T cells are required for colitis induction.[3–7]

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T helper (Th) 1 cells are characterized by the production of Th1 cytokines, such as interferon-γ (IFN-γ), and the expression of Th1-polarizing transcription factor, T-bet,[8] whereas Th17 cells are characterized by the production of Th17 cytokines such as interleukin (IL)-17A and the expression of the Th17-polarizing transcription factor, retinoid-related orphan receptor gamma t (RORγt).[9,10] Previous studies have shown that either $CD4^+CD45RB^{high}$ T cells obtained from T-$bet^{-/-}$ mice,[11] which are defective in Th1 cell generation but have Th17 cell differentiation, or from $ROR\gamma t^{-/-}$ mice, which are defective in Th17 cell generation but retain Th1 cell differentiation, do not develop colitis.[12,13] These studies indicated an indispensable role for both cell types in colitis. Both Th1 and Th17 cells are known as colitogenic $CD4^+$ T cells that play important roles in chronic intestinal inflammation of human inflammatory bowel disease and murine colitis models.[14–16] Th17/Th1 double-positive cells characterized by the production of both IL-17A and IFN-γ, were involved in the pathogenesis of human inflammatory bowel disease and murine colitis models.[17–20] Moreover, several recent studies demonstrated the plasticity of T cells[21–27]; for example, Th17 cells can be converted to IFN-γ–producing cells.[18,28–31] Although a critical role for each cell type has been established, the impact of each cell type in the development of intestinal inflammation is not fully understood.

Using an adoptive T-cell transfer model, we recently demonstrated that Th17 ($ROR\gamma t^+$ T-bet^-) cells further

differentiated into Th1 (RORγt$^-$ T-bet$^+$) cells via Th17/Th1 double-positive cells (RORγt$^+$ T-bet$^+$), independent of classical Th1 (cTh1) cells, which directly differentiated from naive T cells.[32] Th1 cells differentiated from Th17 cells were termed alternative (aTh1) cells. These results indicate that Th17 cells are progenitors of colitogenic aTh1 cells, which are key cells in the development of colitis.

Since Th1 cells were identified, it was proposed that they play critical roles in adaptive immune responses,[33] although the phenotypic classification of Th1 cells has not been fully investigated. In previous studies, we identified 2 types of Th1 cells, aTh1 cells and cTh1 cells, and demonstrated the contribution of aTh1 cells in intestinal inflammation.[32] However, the role of cTh1 cells in the development and promotion of intestinal inflammation remains unclear. Therefore, in this study, we aimed to clarify the role of cTh1 cells in the development of intestinal inflammation.

MATERIALS AND METHODS

Mice

C57BL/6 (Ly5.1) and C57BL/6-background RAG-2$^{-/-}$ (Ly5.2) mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan), respectively. Mice with a green fluorescent protein (GFP) reporter complementary DNA knocked-in at the site for initiation of RORγt translation on the C57BL/6 (Ly5.2) background were previously described.[34] Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Keio University School of Medicine. All experiments were approved by the regional animal study committees.

Cell Isolation

Single-cell suspensions of spleen were aseptically prepared by mechanical mashing. Single-cell suspensions of intestinal lamina propria mononuclear cells were prepared as previously described[27] with slight modifications. Briefly, colons were removed and placed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (Nacalai Tesque, Kyoto, Japan). After removal of residual mesenteric fat tissue, the colons were opened longitudinally, washed in Hanks' balanced salt solution, and cut into small pieces. The dissected mucosa was incubated with Hanks' balanced salt solution containing 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) and 5 mM EDTA (Gibco, Carlsbad, CA) for 30 minutes at 37°C to remove the epithelial layer. The pieces of colons were washed and placed in digestion solution containing DNase (Sigma-Aldrich) for 1 hour at 37°C. Colon supernatants were washed, resuspended in 40% Percoll, and overlaid on 75% Percoll fraction. Percoll gradient separation was performed by centrifugation at 840*g* for 20 minutes at room temperature. Mononuclear cells were collected at the interphase of the Percoll gradient, washed, and resuspended in FACS buffer or RPMI-1640 (Sigma-Aldrich) containing 10% fetal bovine serum and penicillin/streptomycin (Gibco). Lamina propria (LP) CD4$^+$ cells were isolated from colon lamina propria mononuclear cells using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA).

In Vitro Induction of Th1 Cells

CD4$^+$ T cells were isolated from spleens of C57BL/6 (Ly5.1) mice, RORγt$^{gfp/+}$ mice, or RORγt$^{gfp/gfp}$ mice using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec). Enriched CD4$^+$ T cells were stained with CD4 and CD45RB monoclonal antibodies and then were sorted to yield a CD4$^+$CD45RB^{high} T-cell fraction by FACSAria (Becton, Dickinson, NJ). Naive splenic CD4$^+$CD45RB^{high} T cells were cultured in 96-well plates containing 5 ng/mL of plate-bound anti-CD3 (BD Pharmingen, San Diego, CA), 1 ng/mL of soluble anti-CD28 (BD Pharmingen), 10 μg/mL of anti-IL-4 (BD Pharmingen), and 0.5 ng/mL of rmIL-12 (R&D systems, Minneapolis, MN) for 3 days.

In Vitro Induction of Th17 Cells

Naive splenic CD4$^+$CD45RB^{high} T cells were cultured in 96-well plates containing 5 ng/mL of plate-bound anti-CD3 (BD Pharmingen), 1 ng/mL of soluble anti-CD28 (BD Pharmingen), 5 ng/mL of rhTGF-β1 (R&D systems), 30 ng/mL of rmIL-6 (PeproTech, Rocky Hill, NJ), 10 μg/mL of anti–IFN-γ (BD Pharmingen), and 10 μg/mL of anti-IL-4 (BD Pharmingen) for 3 days.

Adoptive Transfer

Purified CD4$^+$CD45RB^{high} T cells (3×10^5 cells per mouse) or LP CD4$^+$ T cells (3×10^5 cells per mouse) were intraperitoneally injected into RAG-2$^{-/-}$ mice. RAG-2$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells or LP CD4$^+$ T cells developed chronic colitis 6 to 8 weeks after cell transfer.[7,35,36] Mice were killed at the indicated time point after transfer.

Histological Scoring of Colitis

Tissue samples were fixed in phosphate-buffered saline containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm thick) were stained with hematoxylin and eosin. The most affected area of pathological specimens was assessed for histological score as the sum of 3 criteria: cell infiltration, crypt elongation, and the number of crypt abscesses. Each was scored on a scale of 0 to 3 in a blind fashion.[27,37]

Flow Cytometry and Antibodies

For intracellular cytokine staining, cells were incubated for 5 or 12 hours with 50 ng/mL of phorbol-12-myristate-13-acetate (Sigma, St. Louis, MO), 1000 ng/mL of ionomycin (Sigma), and 1 μL/mL of GolgiPlug (eBioscience, San Diego, CA) in an incubator at 37°C. Surface staining was performed with the corresponding cocktail of FITC-, PE-, PerCP-Cy5.5-, APC-, PE-Cy7-, APC-Cy7- or Alexa Fluor 647-conjugated monoclonal antibodies for 20 minutes at 4°C. After staining surface molecules, the cells were resuspended in fixation/permeabilization solution (BD Pharmingen), and intracellular staining was performed. Standard six-color flow cytometry analyses were performed using the FACSCanto II (Becton Dickinson) and analyzed by FlowJo software (Tree Star,

Inc., Ashland, OR). The following monoclonal antibodies were obtained for purification of cells population and flow cytometry analysis: CD3 (145-2C11) (BD Pharmingen), CD4 (RM4-5) (BD Pharmingen), CD45RB (C363.16A) (eBioscience), CD45.1 (Ly5.1; A20) (BD Pharmingen), CD45.2 (Ly5.2; 104) (BD Pharmingen), IFN-γ (XMG1.2) (BD Pharmingen), and IL-17A (eBio17B7) (eBioscience).

Statistical Analysis

Results are expressed as the mean ± standard error of mean. Groups of data were compared using the Student's *t* test. A *P*-value of ≤0.05 was considered statistically significant.

RESULTS

RORγt-deficient Naive T Cells Differentiate into Th1 Cells

We first investigated whether in vitro differentiated cTh1 cells, differentiated directly from RORγt-deficient ($ROR\gamma t^{gfp/gfp}$) naive T cells, could induce colitis. Splenic $CD4^+CD45RB^{high}$ T cells obtained from control mice ($ROR\gamma t^{gfp/+}$) or $ROR\gamma t^{gfp/gfp}$ mice, were cultured in vitro for 3 days under Th1 or Th17 polarizing conditions. We confirmed that $CD4^+CD45RB^{high}$ T cells obtained from $ROR\gamma t^{gfp/+}$ displayed a normal induction of Th17 cells, although $CD4^+CD45RB^{high}$ T cells obtained from $ROR\gamma t^{gfp/gfp}$ showed a marked reduction in Th17 cell numbers after in vitro polarization as reported previously.[9] In Th1 polarizing conditions, the same number of Th1 cells was acquired from each mouse strain (Fig. 1). These data demonstrate that RORγt-deficient naive T cells retain the ability to differentiate into Th1 cells under in vitro Th1 polarizing conditions. The in vitro differentiated RORγt-deficient Th1 cells represent cTh1 cells because of the lack of Th17/Th1 cell generation.

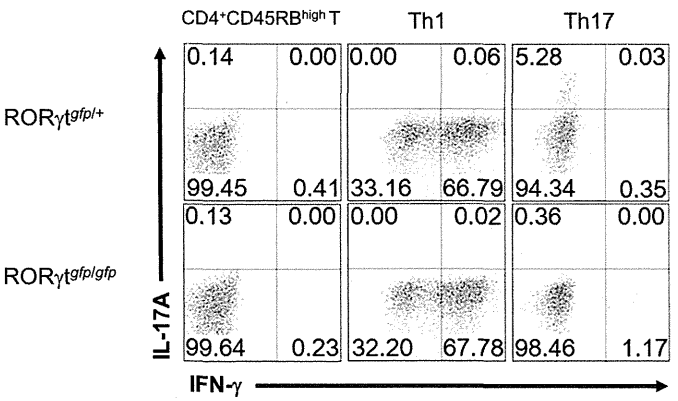

FIGURE 1. RORγt-deficient naive T cells differentiate into Th1 cells. Expression of IL-17A and IFN-γ in splenic $CD4^+CD45RB^{high}$ T cells obtained from $ROR\gamma t^{gfp/gfp}$ or $ROR\gamma t^{gfp/+}$ mice cultured for 3 days under Th1 or Th17 polarizing conditional medium.

RORγt-independent cTh1 Cells are colitogenic Upon In Vitro Manipulation

We next transferred splenic naive $ROR\gamma t^{gfp/+}$ $CD4^+$ T cells, (Group [Gr]. 1^A), splenic naive $ROR\gamma t^{gfp/gfp}$ $CD4^+$ T cells (Gr. 2^A), in vitro-manipulated $ROR\gamma t^{gfp/+}$ Th1 cells (Gr. 3^A), and in vitro-manipulated $ROR\gamma t^{gfp/gfp}$ Th1 cells (Gr. 4^A) into RAG-$2^{-/-}$ mice (Fig. 2A).

As expected, Gr. 1^A and Gr. 3^A mice developed colitis, as assessed by weight loss (Fig. 2B), stool scores (Fig. 2C), gross colon appearance (Fig. 2D), microscopic mucosal appearance, histological scores (Fig. 2E, F), and the absolute number of LP $CD4^+$ cells (Fig. 2G). Gr. 2^A mice developed a wasting disease but did not develop colitis. Unexpectedly, Gr. 4^A mice developed severe colitis. Additionally, the percentage of LP Th1 cells in Gr. 4^A mice was similar to that in Gr. 1^A and Gr. 3^A mice but it was significantly higher than that in Gr. 2^A mice (Fig. 2H, I). Although naive RORγt-deficient $CD4^+$ T cells had an impaired ability to differentiate into Th1 cells in vivo and failed to induce colitis, because of the lack of aTh1 cell generation as previously shown,[13,32] in vitro-differentiated RORγt-deficient Th1 cells (cTh1) expanded and induced colitis. This suggested that cTh1 cells become colitogenic following differentiation.

Differentiation of RORγt-independent cTh1 Cells is Accelerated in the Presence of RORγt-expressing T Cells In Vivo

We hypothesized that impaired Th1 cell differentiation of splenic naive RORγt-deficient $CD4^+$ T-cell transferred mice (Gr. 2^A) was because of a lack of co-existence with $ROR\gamma t^+CD4^+$ T cells. To examine this, we used splenic $CD4^+CD45RB^{high}$ T cells obtained from wild-type mice (Ly5.1) and $CD4^+CD45RB^{high}$ T cells obtained from $ROR\gamma t^{gfp/gfp}$ mice (Ly5.2). Ly5.2 cells (Gr. 1^B), Ly5.1 cells (Gr. 3^B), or both Ly5.1 and Ly5.2 (Gr. 2^B) cells were transferred to RAG-$2^{-/-}$ mice (Fig. 3A).

Gr. 3^B mice and Gr. 2^B mice developed colitis, whereas Gr. 1^B mice developed wasting disease but did not develop colitis, as assessed by weight loss (Fig. 3B), stool scores (Fig. 3C), gross colon appearance (Fig. 3D), macroscopic mucosal appearance and histological scores (Fig. 3E, F), and the absolute number of LP $CD4^+$ cells (Fig. 3G). The percentage of Th1 cells was significantly higher in Gr. 3^B mice than in Gr. 1^B mice. Surprisingly, the percentage of $Ly5.2^+$ Th1 cells in Gr. 2^B mice increased significantly compared with the percentage of Th1 cells derived from the $Ly5.2^+$ T-cell population in Gr. 1^B mice (Fig. 3H, I). These results suggested that RORγt-independent cTh1 cells obtained colitogenicity when present with RORγt-expressing T cells in vivo and that RORγt-expressing T cells might strengthen the cTh1 differentiation pathway, allowing RORγt-independent cTh1 cells to participate in the development of colitis.

RORγt-independent cTh1 Cells are Colitogenic upon In Vivo Manipulation

We further investigated whether RORγt-independent cTh1 cells could induce colitis upon in vivo manipulation.

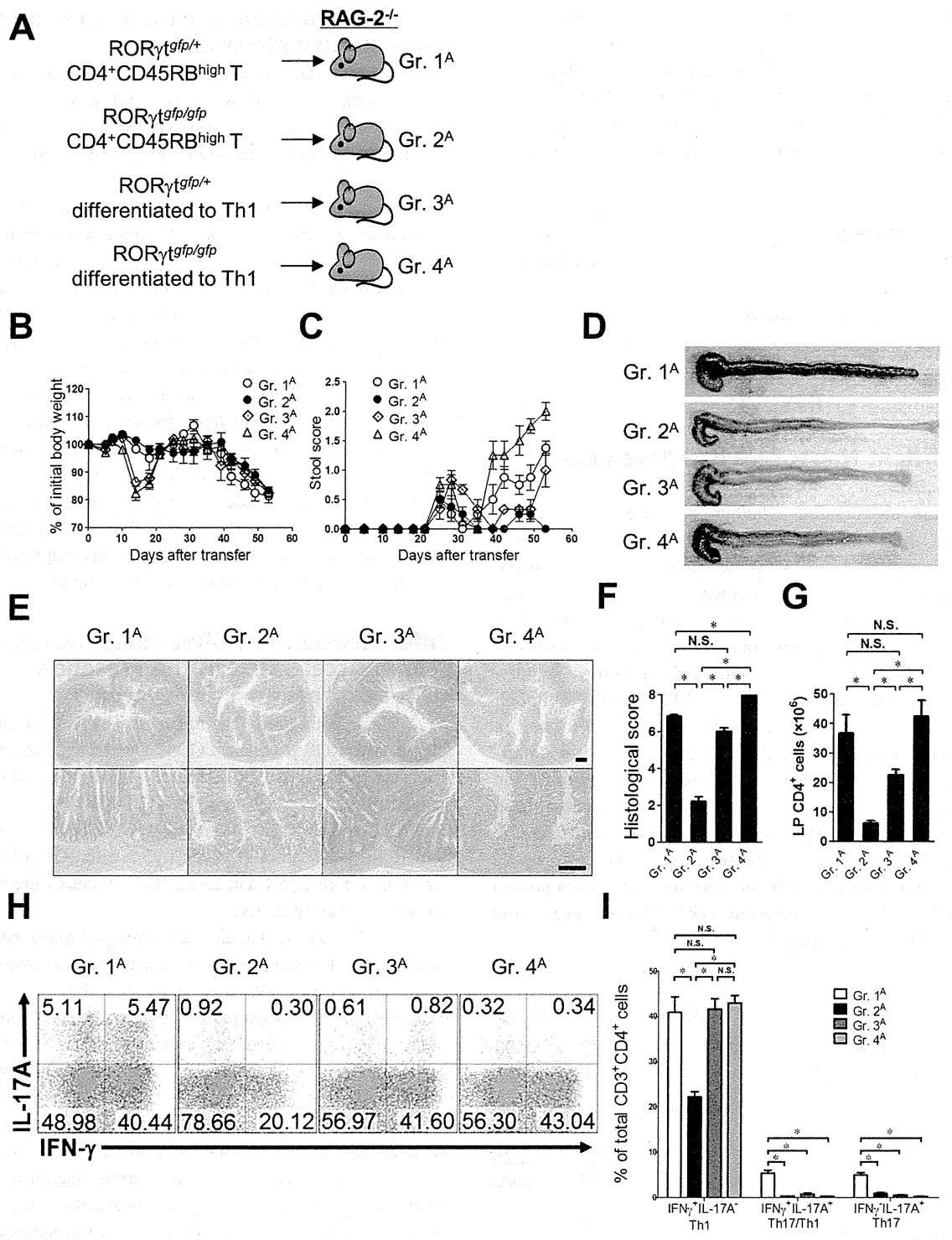

FIGURE 2. RORγt-deficient Th1 cells are colitogenic upon in vitro manipulation. A, Transfer protocol. The following cells types were transferred into RAG-2$^{-/-}$ mice: splenic CD4$^+$CD45RB^{high} T cells obtained from RORγt$^{gfp/+}$ (Gr. 1^A, n = 5), RORγt$^{gfp/gfp}$ (Gr. 2^A, n = 5), or in vitro-manipulated Th1 cells under Th1 polarizing conditions from RORγt$^{gfp/+}$ (Gr. 3^A, n = 5) or RORγt$^{gfp/gfp}$ (Gr. 4^A, n = 5) mice. Mice were killed 6 to 8 weeks after transfer. B, Change in body weight. C, Stool score. D, Representative gross appearance of colon. E, Histopathology of distal colon at 6 to 8 weeks after transfer (original magnification ×40 and ×100). The scale bar represents 200 μm. F, Histological score. G, Absolute cell number of recovered LP CD4$^+$ T cells at 6 to 8 weeks after transfer. H, Expression of IL-17A and IFN-γ in LP CD4$^+$ T cells. Data are representative of 5 mice in each group. I, Mean percentage of Th17, Th17/Th1, and Th1 cells in LP CD4$^+$ T cells. Data (F, G, I) show mean ± standard error of mean (n = 5 per group). *$P <$ 0.05. NS, not significant.

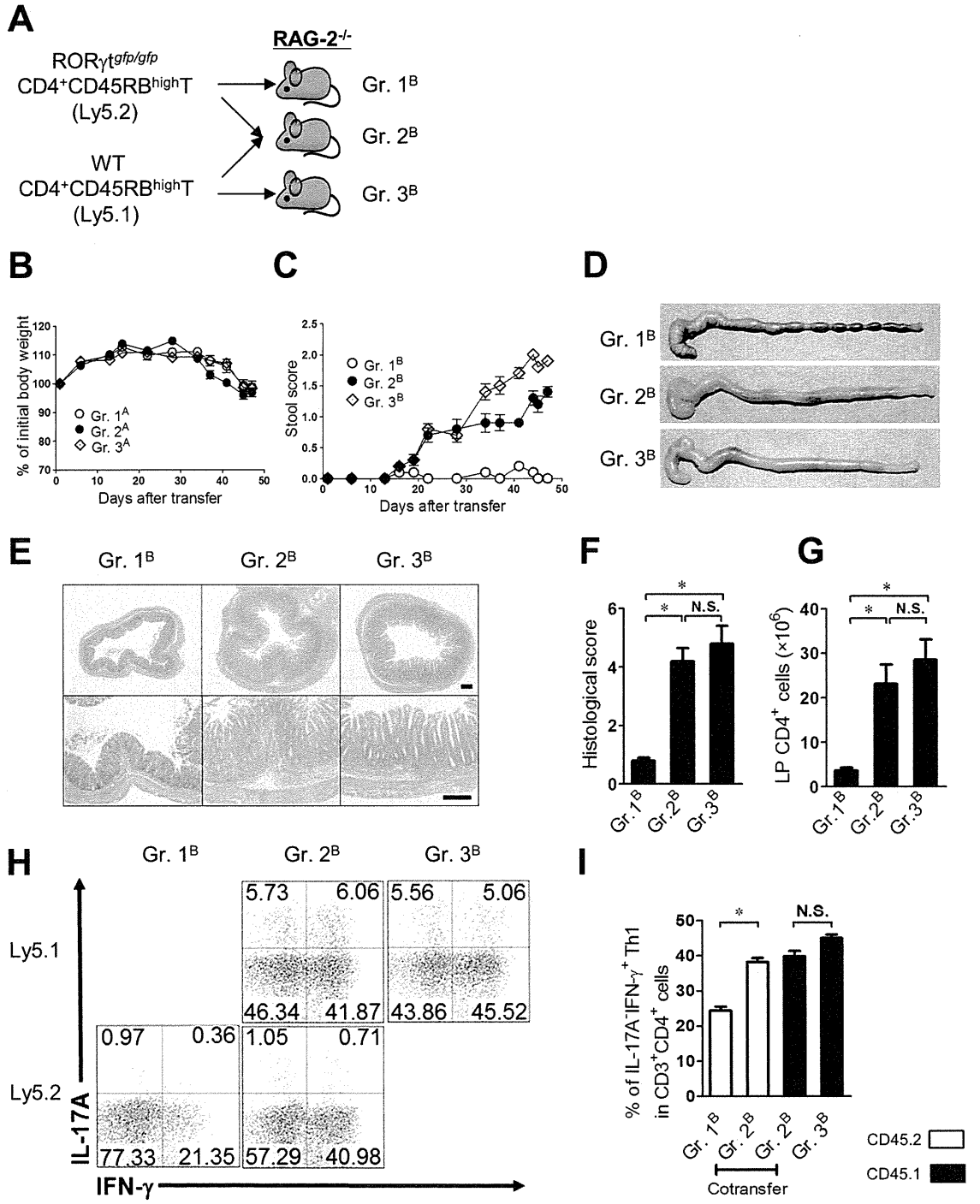

FIGURE 3. Differentiation of RORγt-independent cTh1 cells is accelerated in the presence of RORγt-expressing T cells in vivo. A, Transfer protocol. The following cell types were transferred to RAG-$2^{-/-}$ mice: splenic $CD4^+CD45RB^{high}$ T cells obtained from $ROR\gamma t^{gfp/gfp}$ (Ly5.2) (Gr. 1^B, n = 5), C57BL/6 (Ly5.1) (Gr. 3^B, n = 5), or both groups (Gr. 2^B, n = 5). B, Change in body weight. C, Stool score. D, Representative gross appearance of the colon. E, Histopathology of distal colon at 6 to 8 weeks after transfer (original magnification ×40 and ×100). The scale bar represents 200 μm. F, Histological score. G, Absolute cell number of recovered LP $CD4^+$ T cells at 6 to 8 weeks after transfer. H, Expression of IL-17A and IFN-γ in LP $CD4^+$ T cells. Data are representative of 5 mice in each group. I, Mean percentage of Th1 cells in LP $CD4^+$ T cells. Data (F, G, I) show mean ± standard error of mean (n = 5 per group). *$P < 0.05$. NS, not significant.

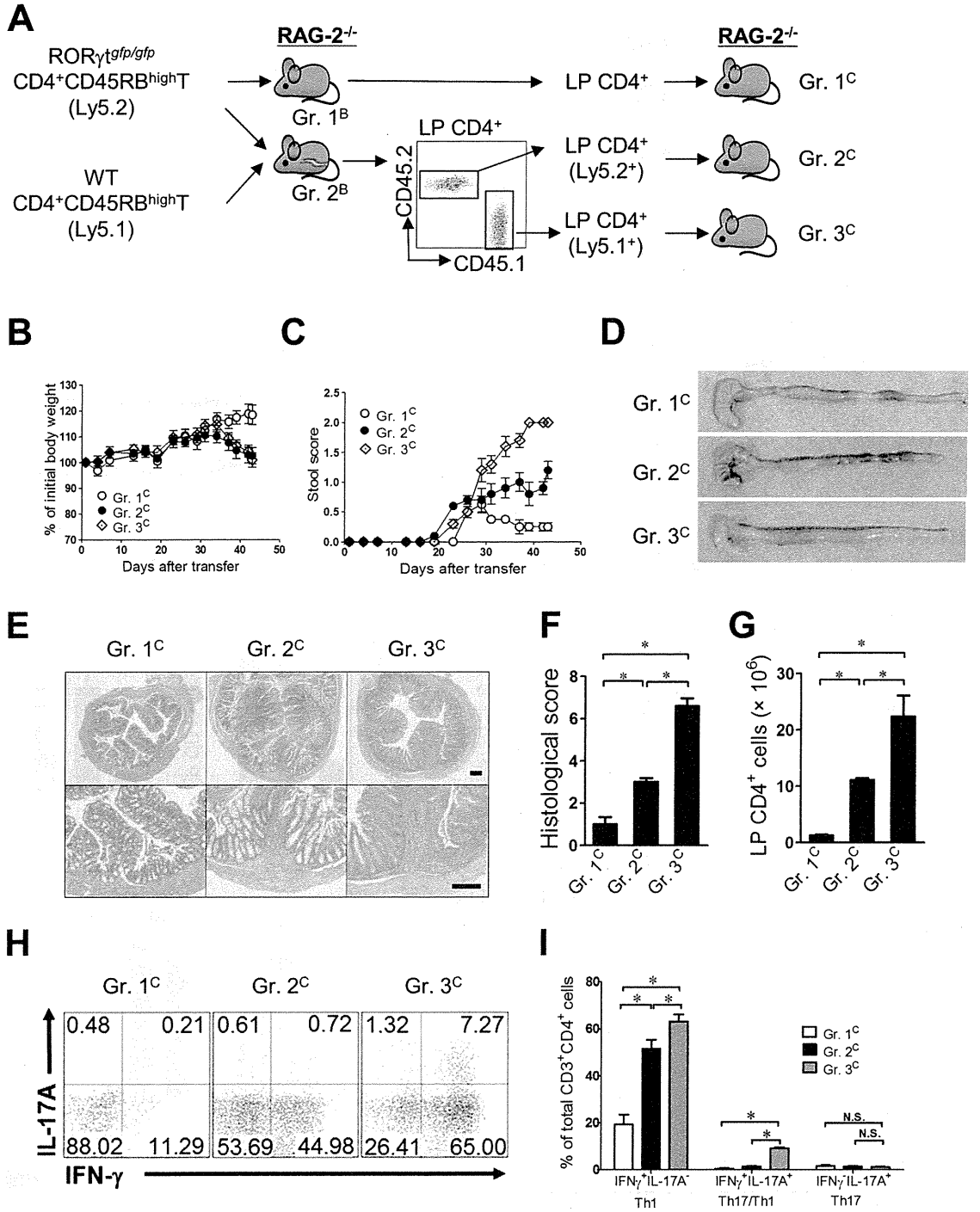

FIGURE 4. RORγt-deficient cTh1 cells are colitogenic upon in vivo manipulation. A, Transfer protocol. The following cells types were transferred to RAG-2$^{-/-}$ mice: Ly5.2$^+$ or Ly5.1$^+$ LP CD4$^+$ cells obtained from colitic Gr. 2^B mice (Gr. 2^C, n = 5) (Gr. 3^C, n = 5) or LP CD4$^+$ cells obtained from noncolitic Gr. 1^B mice (Gr. 1^C, n = 5). B, Change in body weight. C, Stool score. D, Representative gross appearance of colon. E, Histopathology of distal colon at 6 to 8 weeks after transfer (original magnification ×40 and ×100). The scale bar represents 200 μm. F, Histological score. G, Absolute cell number of recovered LP CD4$^+$ T cells at 6 to 8 weeks after transfer. H, Expression of IL-17A and IFN-γ in LP CD4$^+$ T cells. Data are representative of 5 mice in each group. I, Mean percentages of Th17, Th17/Th1, and Th1 cells in LP CD4$^+$ T cells. Data (F, G, I) show mean ± standard error of mean (n = 5 per group). *$P < 0.05$. NS, not significant.

LP Ly5.1$^+$ CD4$^+$ cells or LP Ly5.2$^+$ CD4$^+$ cells obtained from colitic Gr. 2^B mice were transferred to naive RAG-2$^{-/-}$ mice (Gr. 2^C and Gr. 3^C). LP Ly5.2$^+$ CD4$^+$ cells obtained from Gr. 1^B mice were transferred to new RAG-2$^{-/-}$ mice (Gr. 1^C) as a control.

We confirmed that Ly5.1$^+$ cells and Ly5.2$^+$ cells were present at the same proportion in LP CD4$^+$ cells obtained from colitic Gr. 2^B mice (Fig. 4A). We also confirmed that the degree of colitis did not depend on the number of transferred CD4$^+$CD45RB^{high} T cells (data not shown).

Gr. 3^C mice developed colitis as assessed by weight loss (Fig. 4B), stool score (Fig. 4C), colon appearance (Fig. 4D), microscopic mucosal appearance and histological scores (Fig. 4E, F), and the absolute number of LP CD4$^+$ cells (Fig. 4G). As expected, Gr. 1^C mice did not develop colitis but unexpectedly did not develop a wasting disease either. Interestingly, Gr. 2^C mice developed colitis with a significantly increased percentage of Th1 cells when compared with the percentage of Th1 cells in Gr. 1^C (Fig. 4H, I). These results suggest that RORγt-independent cTh1 cells obtain colitogenicity in the presence of RORγt-expressing T cells in vivo. Thus, RORγt-expressing T cells strengthen the cTh1 differentiation pathway, resulting in the cTh1 cells becoming colitogenic.

DISCUSSION

We recently classified Th1 cells into 2 subsets, aTh1 cells and cTh1 cells, based on their dependence of RORγt expression. However, the role of each cell type has not been extensively studied. In the current study, we demonstrated that RORγt-independent cTh1 cells, generated directly from naive T cells without RORγt expression, also obtain colitogenicity in the presence of RORγt-expressing T cells in the pathogenesis of colitis using an adoptive transfer model of colitis and Ly5.1/5.2 congenic system. These studies also suggested RORγt-expressing T cells have a role as both progenitor cells for aTh1 cells, but also as T cells that strengthen the cTh1 differentiation pathway, resulting in the development of experimental T-cell–dependent colitis.

To investigate a role for cTh1 cells, we first transferred in vitro manipulated RORγt-deficient Th1 cells to RAG-2$^{-/-}$ mice. A previous study reported that RORγt-deficient T cells failed to induce colitis.[13] Unexpectedly, our results clearly demonstrated that in vitro-differentiated Th1 cells derived from RORγt$^{gfp/gfp}$ mice strongly induced colitis in an adoptive transfer model. This result indicated that RORγt-independent cTh1 cells, at least those differentiated in vitro, are colitogenic and that they might also be colitogenic in vivo.

Thus, we next examined whether cTh1 cells were colitogenic in vivo. Based on a recent report by Huh et al[38] reporting antagonizing RORγt activity suppressed colitis in experimental colitis, we assumed that cTh1 cells were colitogenic and contribute to development of colitis in co-existence with RORγt-expressing T cells in vivo. We co-transferred naive T cells obtained from wild-type mice and RORγt$^{gfp/gfp}$ mice into RAG-2$^{-/-}$ mice.

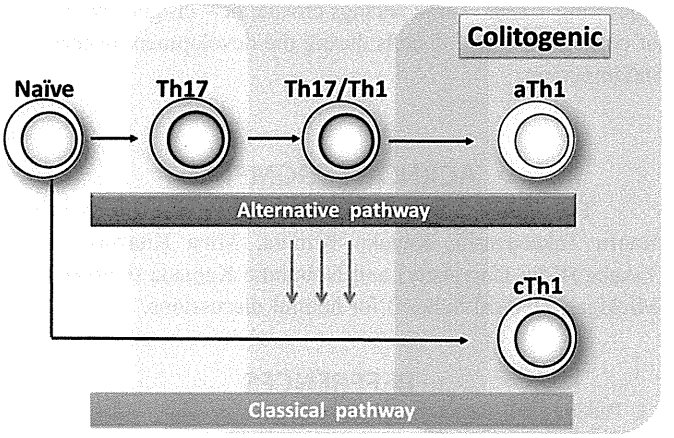

FIGURE 5. Model of the development of colitogenic cTh1 cells from naive T cells in the presence of RORγt-expressing T cells. Th17 cells differentiate into Th17/Th1 cells and then become colitogenic aTh1 cells. Stimulation will switch differentiation toward colitogenic Th1 cells. Similar to Th17 cells, Th1 cells are colitogenic in the presence of RORγt-expressing T cells.

Using a surface staining method that precisely discriminates RORγt-expressing T cells from RORγt-deficient T cells by using the Ly5.1/5.2 congenic system identified an unexpected mechanism. The co-transfer of RORγt-expressing T cells resulted in an increased ratio of cTh1 cells that were polarized from RORγt-deficient T cells. These results indicated that differentiation of RORγt-independent cTh1 cells was accelerated in the presence of RORγt-expressing T cells during the development of colitis.

However, it was still unclear how cTh1 cells acquired colitogenicity in vivo. To investigate this, we transferred RORγt-independent cTh1 cells to naive RAG-2$^{-/-}$ mice a second time, by in vivo manipulation, and these mice developed colitis. These results clearly indicated that cTh1 cells develop colitogenicity in vivo and are consistent with previous reports that CD4$^+$CD45RB^{high} T cells obtained from T-bet$^{-/-}$ mice[11] or from RORγt$^{-/-}$ mice do not induce colitis.[12,13] Our current immunologic scenario is depicted in Figure 5.

Although, we showed that cTh1 also obtained colitogenicity in the presence of RORγt-expressing T cells in vivo, the mechanism of how RORγt-expressing T cells affect cTh1 cells in developing colitis has not been extensively studied. Our previous study showed that the differentiation status of macrophage/dendritic cells determined the level of IL-12/IL-23 production.[39] Thus, one possibility is that RORγt-expressing T cells may induce macrophage/dendritic cells to produce proinflammatory cytokines such as IL-12 or IL-23. These proinflammatory macrophage/dendritic cells may contribute to the cTh1 differentiation pathway. Another possibility is that direct cell-to-cell contact between RORγt-expressing T cells and RORγt-deficient T cells may exist. Further studies will be warranted to address these possibilities.

In conclusion, our data show for the first time that cTh1 cells developed colitogenicity in the presence of RORγt-

expressing T cells. These findings provide new insights into the role of colitogenic $CD4^+$ T cells during the development of intestinal inflammation.

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Cross-talk Between RORγt$^+$ Innate Lymphoid Cells and Intestinal Macrophages Induces Mucosal IL-22 Production in Crohn's Disease

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Background: Interleukin (IL)-22–producing RORγt$^+$ innate lymphoid cells (ILCs) play a pivotal role in intestinal immunity. Recent reports demonstrated that ILCs contribute to mucosal protection and intestinal inflammation in mice. In humans, numbers of RORγt$^+$ ILCs are significantly increased in the intestine of patients with Crohn's disease (CD), suggesting that ILCs may be associated with intestinal inflammation in CD. However, the mechanism by which ILCs are regulated in the intestine of patients with CD is poorly understood. This study aimed to determine the activation mechanism of intestinal ILCs in patients with CD.

Methods: CD45$^+$ lineage marker$^-$ ILCs were isolated from intestinal lamina propria of patients with CD. ILCs were then subdivided into 4 distinct populations based on the expression of CD56 and CD127. Purified ILC subsets were cocultured with intestinal CD14$^+$ macrophages, and IL-22 production was evaluated.

Results: CD127$^+$CD56$^-$ and CD127$^+$CD56$^+$ ILC, but not CD127$^-$CD56$^+$ or CD127$^-$CD56$^-$ ILC, subsets expressed RORγt and produced IL-22. IL-22 production by these ILC subsets was enhanced when ILCs were cocultured with intestinal macrophages. IL-23 or cell-to-cell contact was required for macrophage-mediated activation of ILCs. IL-22 production by ILCs was perturbed in inflamed mucosa compared with noninflamed mucosa. IL-22 induced the expression of Reg1α and Claudin-1 in human intestinal epithelial organoids.

Conclusions: RORγt$^+$ ILCs might enhance mucosal barrier function through the upregulation of Reg1α through production of IL-22. Although CD14$^+$ macrophages augment intestinal inflammation in patients with CD, macrophages also promote a negative feedback pathway through the activation of IL-22 production by RORγt$^+$ ILCs.

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Key Words: RORγt$^+$ ILC, IL-22, macrophages, Crohn's disease

Crohn's disease (CD) is one of the major forms of human inflammatory bowel disease (IBD), and sustains inflammation throughout the entire gastrointestinal tract. It is well known that genetic background, food, intestinal flora, and immunological factors can affect the course of its pathological condition.[1] Although several studies have revealed various different immunological pathways involved in the pathogenesis of IBD, the precise mechanism(s) remain poorly understood.

Innate lymphoid cells (ILCs) are newly identified subsets of immune cells. ILCs comprise several functionally distinct subsets.

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Recently, a uniform nomenclature of ILCs was proposed, and ILCs are currently categorized into 3 subsets: group 1, 2, and 3 ILCs. All 3 ILC groups arise from common progenitor cells, but differentiation is driven by different transcriptional factors. Group 1 ILCs (ILC1s) are induced by the expression of T-bet and are known as interferon-producing cells and include natural killer (NK) cells. GATA-3 expression drives the differentiation of group 2 ILCs (ILC2s), which express chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)[2] and produce interleukin (IL)-5, IL-9, and IL-13. Group 3 ILCs (ILC3s) including ILC3s and lymphoid tissue inducer (LTi) cells express retinoic acid receptor-related orphan receptor (ROR)γt and produce the effector cytokines IL-17A and IL-22.

Among these ILC subsets, recent evidence suggests that RORγt+ ILC3s play important roles mucosal tissues, including the intestine.[3] IL-22 produced by RORγt+ ILC3s is considered a key molecule that has an important role in mucosal protective function. Mice deficient in IL-22 exhibit significantly increased susceptibility to enteric pathogen infection and experimental colitis.[4–6] Thus, IL-22–producing RORγt+ ILC3s protect the host from mucosal damage caused by intestinal inflammation.

However, several reports have indicated that RORγt+ ILC3s are involved in the induction of inflammation and the pathogenesis of certain autoimmune diseases, including IBD.[7] It was reported that intestinal RORγt+ ILC3s promote intestinal inflammation through the production of IL-17, IL-22, and interferon in a mouse innate colitis model.[8] Consistent with the finding in mice, homologous ILC populations have been identified in the human intestine. IL-17 and IL-22 seemed to be produced by $CD3^-CD127^+CD56^-$ or $CD56^+$ ILC subsets. Notably, $CD127^+$ ILCs accumulate in inflamed mucosa in patients with CD compared with control subjects or patients with ulcerative colitis, suggesting RORγt+ ILCs may be involved in intestinal inflammation in IBD, particularly CD.[9]

Furthermore, ILCs can be activated in response to various stimuli, such as Toll-like receptor 2 stimulation and cytokines, such as IL-2 and IL-23.[10] IL-23 is an important activator of RORγt+ ILCs in the intestine.[9] In a mouse model of innate colitis, IL-23 activates RORγt+ ILCs, thereby, inducing production of IL-17, IL-22, and interferon that promotes intestinal inflammation.[11] Consistent with the mouse colitis model, the IL-23–ILC–IL-22 axis might be involved in the pathogenesis of human IBD. Accumulating evidence suggests that IL-22 or IL-23 is upregulated in the inflamed mucosa of patients with IBD, particularly in CD.[9] Thus, evidence strongly implies a link between IL-22 and IL-23 in the pathogenesis of CD. However, the mechanism(s) of how the IL-23–IL-22 axis is involved in intestinal inflammation in patients with CD remains to be elucidated. Moreover, the precise mechanism by which IL-22–producing ILCs are activated within the intestine is unclear. We previously reported that $CD33^+CD14^+$ intestinal macrophages produced high levels of IL-23 compared with other myeloid cells within the inflamed mucosa,[12] and another group further characterized the $CD14^+$ intestinal macrophages, and reported that $CD14^+CD163^{low}$ subset of intestinal myeloid cells is a major source of IL-23.[13] Because IL-23 is known to be a key regulator of ILCs, the $CD14^+$ intestinal macrophages may participate in the induction of mucosal IL-22 through activation of ILCs in patients with CD. However, to date, there are no studies clearly reporting cross-talk between intestinal macrophages and ILCs in the regulation of mucosal IL-22 production.

In this study, we hypothesized that cross-talk between intestinal macrophages and ILCs is important for IL-23–mediated induction of mucosal IL-22 production by RORγt+ ILCs in patients with CD. Moreover, we have tried to clarify the precise role of ILC-produced IL-22 in intestinal inflammation in patients with CD.

MATERIALS AND METHODS

Tissue Samples

Inflamed intestinal mucosa was obtained from surgically resected specimens from patients with CD and diagnosed on the basis of clinical, radiographic, endoscopic, and histological findings according to established criteria. In all inflamed samples from patients with CD, the degree of inflammation was histologically moderate to severe. Noninflamed intestinal mucosa was obtained from macroscopically unaffected areas or inactive lesions of patients with CD. See tissue samples text, Supplemental Materials and Methods, Supplemental Digital Content 1, http://links.lww.com/IBD/A505.

Preparation of Lamina Propria Mononuclear Cells

Lamina propria mononuclear cells (LPMCs) were isolated from intestinal specimens using modifications of previously described techniques.[14] Briefly, dissected mucosa was incubated in calcium and magnesium-free Hanks balanced salt solution (Sigma-Aldrich, St. Louis, MO) containing 1.5% heat-inactivated fetal bovine serum (GE Healthcare Life Sciences, Little Chalfont, England) and 1 mM dithiothreitol (Sigma-Aldrich) to remove mucus. The mucosa was then incubated twice in Hanks balanced salt solution containing 1 mM EDTA (Sigma-Aldrich) for 30 minutes at 37°C. Tissues were collected and incubated in Hanks balanced salt solution containing 1 mg/mL collagenase type 3 (Worthington Biochemical, Lakewood, NJ) and 0.1 mg/mL DNase I (Sigma-Aldrich) for 60 minutes at 37°C. The fraction was pelleted and resuspended in a 40% Percoll solution (GE Healthcare Life Sciences), then layered on 60% Percoll before centrifugation at 700*g* for 20 minutes at room temperature. Viable LPMCs were recovered from the 40% to 60% layer interface.

Flow Cytometric Analysis

Cell-surface fluorescence intensity was assessed using a FACSCanto II flow cytometer (BD Bioscience, Franklin Lakes, NJ) and analyzed using FlowJo software (TreeStar, Ashland, OR). Dead cells were excluded by 7AAD staining. To detect the surface and intracellular expression of various molecules, isolated LPMCs were preincubated with an FcγR-blocking antibody (human FcR Blocking Reagent; Miltenyi Biotech, Bergisch Gladbach, Germany) for 20 minutes followed by incubation with specific FITC-, PE-,

PE-Cy7-, APC-, or APC-Cy7-conjugated antibodies for 30 minutes on ice. Monoclonal antibodies for CD3, CD14, CD16, CD19, CD20, CD127, CD56, CD117, NKp46, and NKp44 were purchased from BD Biosciences, NKG2D and CD161 were from eBioscience, and CD45 was from BioLegend (San Diego, CA).

For intracellular staining of cytokines, after staining of the surface molecules was complete, the cells were resuspended in fixation/permeabilization solution (BD Biosciences), and intracellular staining was performed. Background fluorescence was assessed by staining with control-irrelevant isotype-matched monoclonal antibodies. Anti-IL-22 monoclonal antibody was purchased from R&D Systems (Minneapolis, MN) and anti-RORγ(t) monoclonal antibody was from eBioscience.

Isolation of ILCs

ILCs were sorted from LPMCs using FACSAria II cell sorter (BD Biosciences with Diva software). According to the above protocol, ILCs were stained with the following antibodies: CD3 FITC (UCHT1), CD14 FITC (M5E2), CD16 FITC (3G8), CD19 FITC (4G7), CD20 FITC (L27), CD127 PE (HIL-7R-M21), CD56 PE-Cy7 (B159) (all BD Biosciences), and CD45 APC-Cy7 (H130) (BioLegend). After sorting, isolated ILCs were measured on the FACSAria II, and the purity was routinely more than 95%.

Isolation of LP CD14$^+$CD33$^+$ Macrophages

LP CD14$^+$CD33$^+$ macrophages were isolated from LPMCs using EasySep Human CD14$^+$ (StemCell Technologies Inc., Vancouver, Canada). The percentage of each subset of cells isolated using this method was evaluated by flow cytometry and was routinely more than 95%.

Stimulation of ILCs and Macrophages

Isolated ILCs or macrophages were seeded on 96-well round-bottom culture plates (ILCs, 1×10^4 cells per milliliter; macrophages, 1×10^5 cells per milliliter) and stimulated with 1 μg/mL lipopolysaccharide (L2630; Sigma-Aldrich) for 24 hours.

Recombinant human IL-2 and IL-23 (PeproTech, Rocky Hill, NJ) were used for stimulation according as previously described.[10] Briefly, ILCs were plated at 1×10^4 cells per milliliter with recombinant human IL-2 (10 U/mL) and IL-23 (50 ng/mL) in 96-well round-bottom plates for 6 days. Neutralizing antihuman IL-23p19 antibody (R&D Systems) was added at 10 μg/mL in culture medium from the start of the incubation.

Transwell Assay

CD14$^+$ macrophages were seeded in medium in the upper or lower chamber of an 8-μm pore size 96-well transwell plate (Corning Inc., Corning, NY). ILCs were seeded in the medium in the lower chamber. Cells were incubated in the same lower chamber or separate chambers for 24 hours.

Reverse Transcription-polymerase Chain Reaction

Quantitative reverse transcription-polymerase chain reaction was performed as described previously.[15] Total RNA from ILCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from 100 ng total RNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). According to the manufacturer's protocol, complementary DNA was analyzed by quantitative reverse transcription-polymerase chain reaction using TaqMan Universal PCR Master Mix (Applied Biosystems) in Applied Biosystems StepOne/StepOnePlus. Cycling conditions for polymerase chain reaction amplification were 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute. Relative quantification was determined by normalizing to human 18S RNA (Applied Biosystems). The following probes were purchased from Applied Biosystems: IL-23R (Hs00332759_m1) and RORC (Hs01076122_m1).

Measurement of Cytokines by Enzyme-linked Immunosorbent Assay

The concentration of IL-22 in cell culture supernatants of patients was assayed using a human IL-22 Quantikine enzyme-linked immunosorbent assay kit (R&D Systems) with a detection limit of 15.6 pg/mL. IL-23 was assayed using a human IL-23 enzyme-linked immunosorbent assay Ready-SET-Go! Set (BioLegend) with limit of 15 pg/mL. See immunohistochemical staining text, Supplemental Materials and Methods, Supplemental Digital Content 1, http://links.lww.com/IBD/A505.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Groups of data were compared by Student's *t* test. For multiple comparisons, statistical analysis was performed using Kruskal–Wallis one-way analysis of variance and the Tukey–Kramer test. Differences were considered statistically significant when the *P*-value was less than 0.05.

ETHICAL CONSIDERATIONS

All experiments were approved by the Institutional Review Board of Keio University School of Medicine, Yokohama Municipal Hospital and Social Health Insurance Medical Center, Japan. Written informed consent was obtained from all patients.

RESULTS

Majority of CD127$^+$ ILCs Express CD161 and CD117

Previous studies defined ILCs as lineage marker (Lin) negative and CD45 positive cells.[7] Lineage marker was defined as CD3, CD14, CD16, CD19, and CD20 to exclude T cells, B cells, and macrophages. We divided Lin negative cells into 4 populations along with the expression CD56 and CD127 (also known as IL-7Rα) as follows: CD127$^+$CD56$^-$ (CD127 single positive [SP]) cells, CD127$^+$CD56$^+$ (double positive [DP]) cells, CD127$^-$CD56$^-$ (double negative) cells, and CD127$^-$CD56$^+$ (CD56 SP) cells (Fig. 1A). The transcriptional factor RORγt is required for the development of ILC3s

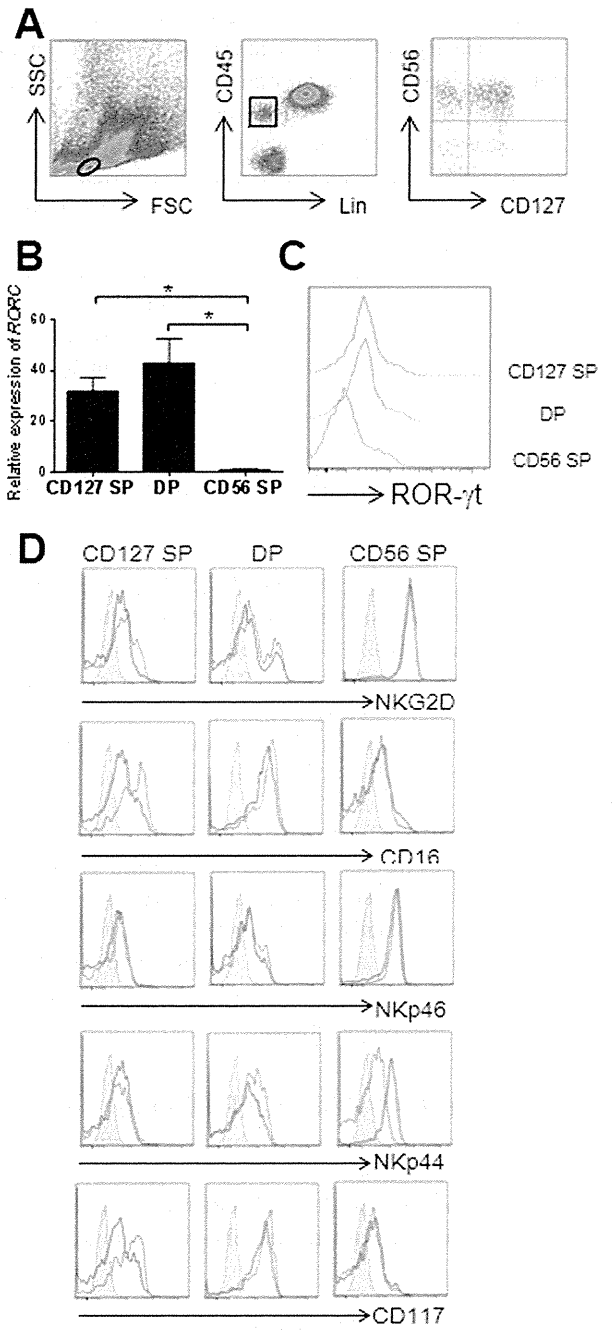

FIGURE 1. A, Cell surface staining. LPMCs were gated on the lymphocyte gate forward scatter (FSC)/side scatter (SSC) and Lin$^-$CD45$^+$, and classified according to the staining pattern with antibodies to CD56 and CD127. B, *RORC* messenger RNA was quantified and normalized to 18S RNA expression (n = 4). C, Intracellular staining. Whole LPMCs from a patient with CD were stimulated with LPS. Two hours later, GolgiStop was added and incubated for 4 hours. The green line shows CD127 SP cells. The red line shows DP cells, and the blue line shows CD56$^+$ SP cells. D, Cell surface molecule of Lin$^-$CD45$^+$ cells from inflamed lesions and noninflamed lesions. The filled histograms show the isotype control. The open histograms with black lines show noninflamed lesions and the open histograms with gray lines show inflamed lesions. *$P < 0.05$.

but not ILC1 and ILC2.[4,16,17] To confirm that Lin(−) CD45(+) CD127 SP and DP cells contained the ILC3 population, we analyzed *RORC* gene, which encodes RORγt, expression, in each subset of Lin (−) CD45(+) cells. *RORC* gene was expressed in CD127 SP cells and DP cells but not in CD56 SP cells (Fig. 1B). This was confirmed by flow cytometry. Consistent with gene expression, RORγt was expressed in CD127 SP cells and DP cells (Fig. 1C).

Next, to characterize further the surface phenotypes of human intestinal ILC3s, we investigated surface markers of each population by flow cytometry. All CD56 SP cells expressed NKG2D, NKp46 (also known as natural cytotoxicity triggering receptor [NCR]1) and NKp44. The ratio of NKp44$^+$ cells in the CD56 SP population of noninflamed lesions was higher than that of inflamed lesions as previously reported.[18,19] Most DP cells expressed CD161 (also known as NKRP1) and CD117 (also known as c-kit). Some CD127 SP cells expressed NKG2D and CD117 (Fig. 1E). These findings are consistent with a previous report that CD127 SP cells comprise part of NCR$^-$ ILC3s, and DP cells comprise a large part of NCR$^+$ ILC3s. Thus, CD56 SP cells can be thought of as mostly conventional NK cells comprised ILC1s.

Macrophages Enhance IL-22 Production from CD127 SP and DP Cells

Next, we determined the role of ILCs in mucosal inflammation and analyzed their capability to produce IL-22. We confirmed that both RORγt$^+$ ILC3 subsets (CD127$^+$CD56$^-$ and CD127$^+$CD56$^+$) produced IL-22 (Fig. 2A). Previous reports showed that IL-23 was essential for IL-22 production by ILC3s.[9,10] Consistently, IL-23 induced IL-22 production from CD127 SP cells and DP cells (Fig. 2B). However, the source of IL-23 within the intestine is still unclear. Because intestinal CD14$^+$ macrophages are increased at the site of inflammation and produce robust levels of IL-23,[12,20] we postulated that intestinal CD14$^+$ macrophages play a critical role in stimulating IL-22 production by ILCs in inflamed mucosa. To address this, we isolated CD14$^+$ macrophages from the intestine and then cocultured them with purified CD127 SP or DP ILCs. CD14$^+$ intestinal macrophages were stimulated with lipopolysaccharide (LPS), whereas they were cultured with ILCs. Recombinant IL-23 plus IL-2 stimulation was used as control stimulation of ILCs. Consistent with previous reports, exogenous stimulation with IL-23 plus IL-2 significantly upregulated the production of IL-22 by CD127 SP and DP ILCs (Fig. 2B). Coculture with CD14$^+$ intestinal macrophages triggered a robust induction of IL-22 secretion by ILCs compared with addition of IL-2 plus IL-23 (Fig. 2B). Notably, ILCs cocultured with macrophages did not produce IL-22 in the absence of LPS stimulation (Fig. 2C). Because LPS stimulation did not affect IL-22 production by ILCs, this suggests that LPS-activated CD14$^+$ macrophages induce IL-22 production by ILCs (Fig. 2C). CD56 SP and CD127 CD56 DN ILCs did not secrete IL-22 even after coculture with LPS-primed intestinal macrophages (Fig. 2C). Thus, CD127$^+$CD56$^-$ and CD127$^+$CD56$^+$ ILCs, which express RORγt, might be a major source of IL-22

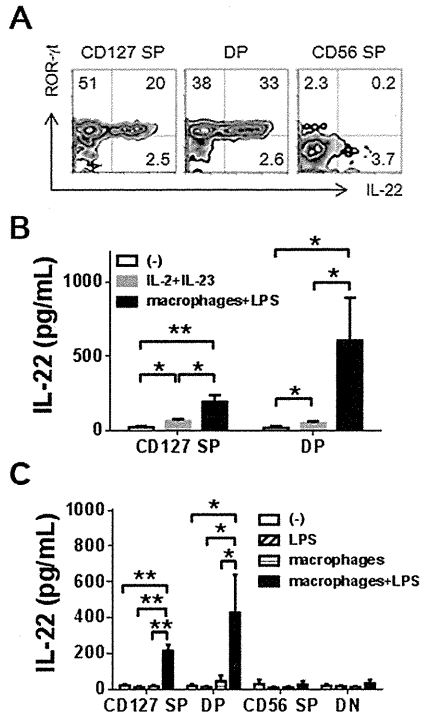

FIGURE 2. A, Intracellular staining for IL-22 and RORγt after LPS stimulation in LPMCs isolated from a patient with CD. The numbers are the ratio of each population. B, CD127 SP and DP cells were sorted by flow cytometry, and stimulated with IL-2 (50 U/mL) and IL-23 (50 ng/mL) for 6 days. CD127 SP and DP cells cocultured with purified macrophages under LPS (1 μg/mL) stimulation for 24 hours. C, Sorted each ILCs population was cultured as indicated for 24 hours. $*P < 0.05$. $**P < 0.01$.

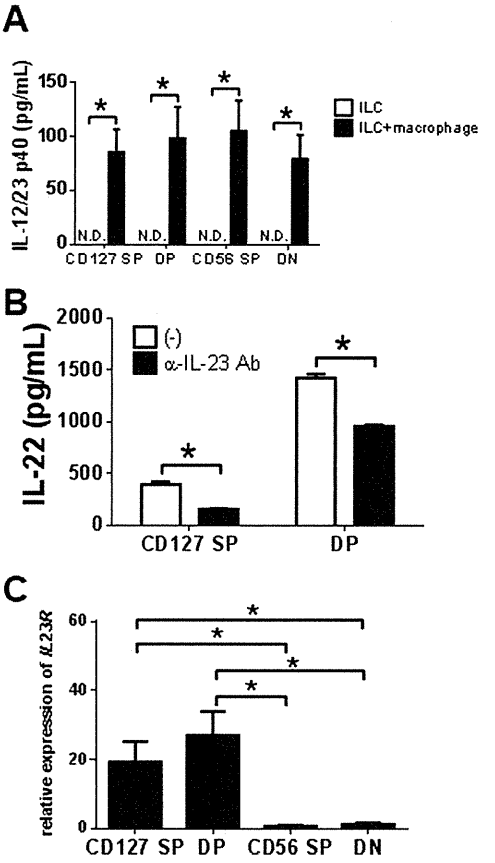

FIGURE 3. A, IL-23 secretion of sorted ILCs populations cocultured with or without macrophages, as determined by enzyme-linked immunosorbent assay (ELISA), n = 3. B, The open bars show IL-22 secretion of sorted cells cocultured with macrophages under LPS stimulation measured by ELISA. The filled bars show sorted cells cocultured with macrophages and stimulated with LPS and anti-IL-23 antibody (10 μg/mL), n = 3. C, IL-23 receptor messenger RNA was quantified and normalized to 18S RNA expression, n = 12. $*P < 0.05$.

among intestinal ILC subsets, and are activated through interactions with intestinal CD14$^+$ macrophages.

Macrophages Stimulate CD127 SP and DP Cells Through IL-23 Dependent and Independent Pathways

Because intestinal CD14$^+$ macrophages produce abundant amounts of IL-23, we hypothesized that macrophages promote IL-22 secretion from ILCs through IL-23. To address this, IL-23 was measured in ILC-macrophage coculture supernatant. As expected, IL-12/23p40 was detected in the ILC-macrophage coculture supernatant but not in ILC single culture supernatant (Fig. 3A). Next, we inhibited the IL-23-mediated IL-22 induction pathway in the ILC-macrophage coculture system. Blockade of IL-23 significantly suppressed the induction of IL-22 in the ILC-macrophage coculture, suggesting IL-23 was involved in the induction of IL-22 by ILCs after coculture with intestinal macrophages (Fig. 3B). Consistent with the involvement of IL-23, IL-23 receptor was expressed by CD127 SP and CD127 CD56 DP ILCs (Fig. 3C). It is noteworthy that IL-22 production by ILCs induced by macrophages was partially, but not completely, suppressed by blocking IL-23 signaling (Fig. 3B). This indicates additional factor(s) may contribute to macrophage-induced activation of intestinal RORγt$^+$ ILCs.

IL-22 Production Is Mediated by Interactions Between CD127$^+$ ILCs and Macrophages

To examine the detailed mechanism of the IL-23-independent IL-22–producing pathway, we focused on direct interactions between macrophages and ILCs. Isolated CD14$^+$ macrophages and ILC3s were cocultured or cultured separately using a Transwell system, and IL-22 production by ILCs was measured. IL-22–producing levels in the separation culture assay were significantly lower than in the coculture assay (Fig. 4A). This indicated that direct interactions between ILCs and macrophages may have a role in the IL-22–producing pathway. In addition, blocking IL-23 completely suppressed the remaining IL-22 production in the separation culture, suggesting that cell-to-cell contact and secreted IL-23 cooperatively activate ILC3s (Fig. 4B).

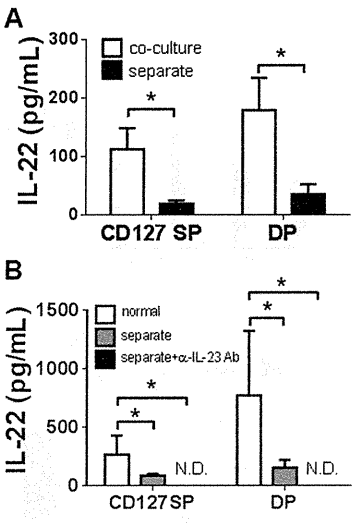

FIGURE 4. A, The open bars show IL-22 secretion levels of ILCs cocultured with macrophages with LPS stimulation in the same chambers of a 96-well transwell plate. The filled bars show that ILCs cocultured with macrophages in different chambers of the same well. The secretion levels were measured by enzyme-linked immunosorbent assay, n = 3. B, The open bars show IL-22 secretion levels of ILCs cocultured with macrophages with LPS stimulation in 96-well round-bottom plates. The gray filled bars show ILCs cocultured with macrophages in different chambers of the same well in a transwell plate. IL-22 secretion levels of anti-IL-23 antibody added to the transwell system were under the detection limit, n = 3. *$P < 0.05$. ND, not detected.

CD127+ ILCs in Noninflamed Mucosa Display a Higher Capacity for IL-22 Production Those in Inflamed Mucosa

A recent report indicated that IL-22 is important for mucosal protection within the intestine, and therefore impaired IL-22 signaling increases susceptibility to colitis-inducing stimuli.[21] These observations suggested that IL-22 negatively regulates intestinal inflammation in IBD by facilitating mucosal barrier functions. Thus, we determined whether IL-22 contributes to mucosal protection in the intestine of patients with CD.

The percentages of RORγt+ CD127 SP and CD127 CD56 DP ILC3s were not significantly different between noninflamed mucosa and inflamed mucosa of patients with CD (Fig. 5A). In contrast, IL-22 production by CD127 SP and CD127 CD56 DP ILC3s isolated from noninflamed mucosa was significantly higher than from those isolated from inflamed mucosa when they cocultured with intestinal macrophages isolated from same lesion (Fig. 5B and see Table, Supplemental Digital Content 2, http://links.lww.com/IBD/A506). This result suggested that IL-22 production is somehow perturbed in inflamed mucosa compared with noninflamed mucosa. Because the ILCs and macrophages used in the coculture experiment were isolated from same lesion of mucosa, it is hard to know whether ILCs or macrophages contribute more to this impaired IL-22 production in inflamed mucosa. To address this issue, we cultured ILCs cross-coupled with macrophages: inflamed ILCs were cocultured with noninflamed macrophages and noninflamed ILCs were cocultured with inflamed macrophages (see Fig., Supplemental Digital Content 3, http://links.lww.com/IBD/A507). As a result, inflamed ILCs produce less

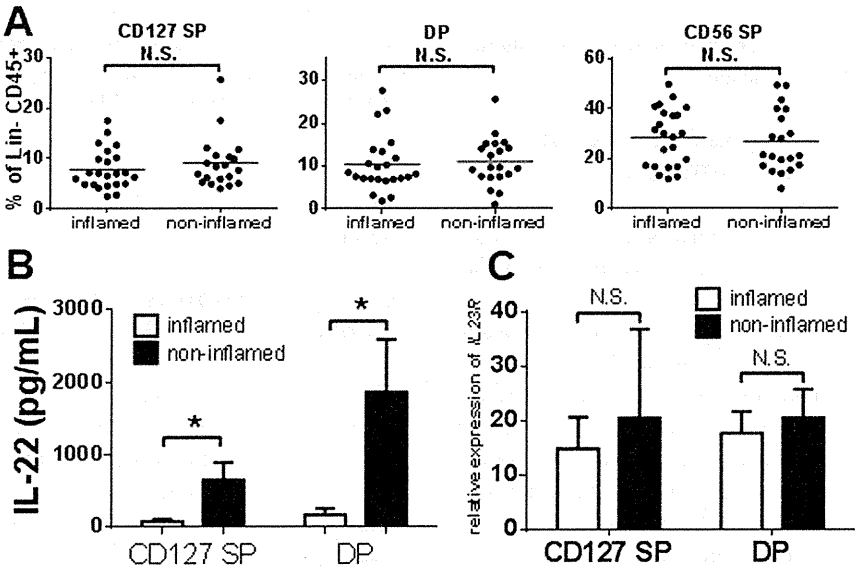

FIGURE 5. A, The percentage of each population from inflamed lesions (n = 23) and noninflamed lesions (n = 20). B, The open bars show IL-22 secretion levels of ILCs cocultured with macrophages from inflamed lesions. The filled bars show ILCs cocultured from noninflamed lesions in the same individuals. The secretion levels were measured by enzyme-linked immunosorbent assay, n = 4. C, The open bars show IL-23 receptor expression of ILCs from inflamed lesions. The filled bars show IL-23 receptor expression of ILCs from noninflamed lesions. IL-23 receptor messenger RNA was quantified and normalized to 18S RNA expression, n = 5. *$P < 0.05$. NS, not significant.

IL-22 even when they are cocultured with noninflamed macrophages, suggesting that ILCs, rather than macrophages, in inflamed mucosa may have intrinsic defect in production of IL-22. Because the IL-23 receptor expression levels were not significantly different between ILCs isolated from noninflamed or inflamed mucosa, the impaired production of IL-22 by ILCs in inflamed mucosa is not likely to be a hyporesponsiveness to the IL-23 (Fig. 5C).

IL-22 May Play a Protective Role in Intestinal Mucosa in Patients with CD

It is known that the IL-22 receptor (IL-22R) is expressed in nonhematopoietic cells within the intestine, such as epithelial cells.[22] Consistent with previous reports, we showed that IL-22R was expressed by epithelial cells but not ILCs (Fig. 6A). IL-22 acts on intestinal epithelial cells to promote the production of antimicrobial peptides or enhance barrier function through the activation of epithelial STAT-3 signaling.[23] To address how ILC3s-produced IL-22 acts within the intestine in humans, we generated intestinal epithelial organoids from purified crypts of the intestinal mucosa from a healthy control. Because the organoids expressed IL-22R (data not shown), we stimulated them with IL-22, which significantly upregulated mRNA levels of REG1α, which encodes an antimicrobial peptides (Fig. 6B). We also investigated the expression of claudin-1, important components of tight junction. The induction of claudin-1 after IL-22 treatment was not obvious by immunohistochemistry (Fig. 6C); however, quantitative polymerase chain reaction result showed that significant upregulation of claudin-1, which encodes one of tight junction proteins, on stimulation with IL-22 in human colonic organoids (Fig. 6D). These results suggest that IL-22, produced by RORγt$^+$ ILCs, plays a protective role by improving the barrier function of the intestine through upregulation of both antimicrobial peptide and tight junction protein expression.

DISCUSSION

This study demonstrated important cross-talk between RORγt$^+$ ILCs and intestinal macrophages that induced the activation of ILCs, triggering IL-22 production. Intestinal macrophages producing IL-23 partially contributed to the activation of RORγt$^+$ ILCs. In addition to the IL-23–mediated IL-22 induction pathway, direct interactions with intestinal macrophages seemed to be essential for maximal IL-22 production by ILCs. In addition, we observed that IL-22 produced by intestinal RORγt$^+$ ILCs was

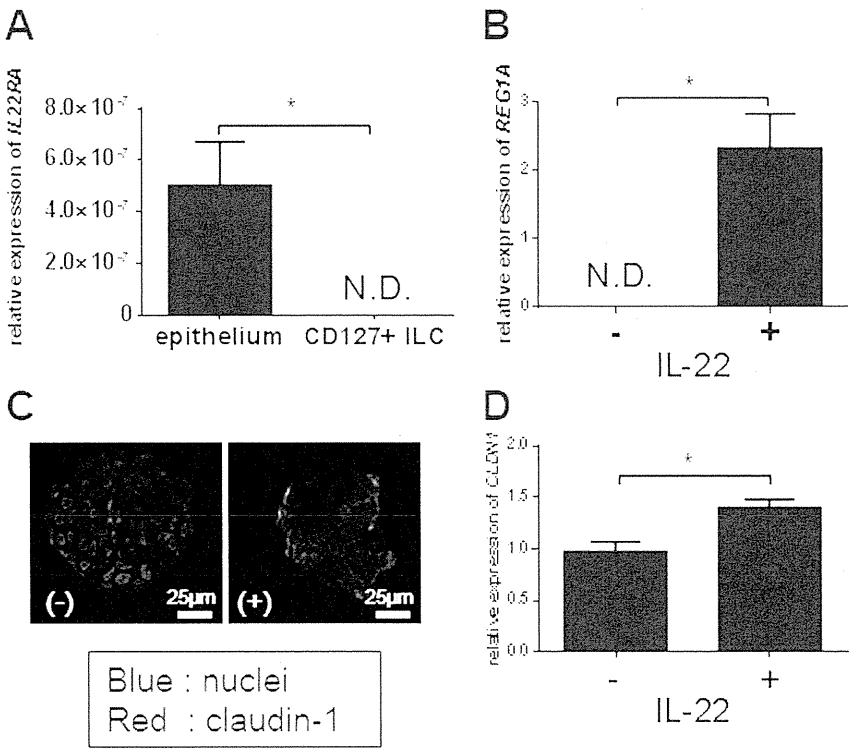

FIGURE 6. A, IL-22 receptor messenger RNA (mRNA) was quantified and normalized to 18S RNA expression. Epithelial cells and CD127$^+$ ILCs were obtained from the intestine of the same patient with CD, n = 3. B, *REG1α* mRNA was quantified and normalized to Hprt1 expression. The bars show the expression level of REG1α from organoids. The organoids were incubated with no stimulation or IL-22 stimulation (100 ng/mL) for 24 hours. C, Representative immunofluorescence images of organoids with claudin-1 (red) and nuclei (blue). The organoids were incubated for 24 hours with no stimulation (left panel) or IL-22 stimulation (right panel; 100 ng/mL). D, Claudin-1 mRNA was quantified and normalized to Hprt1 expression. The bars show the expression level of claudin-1 from organoids. The organoids were incubated with no stimulation or IL-22 stimulation (100 ng/mL) for 24 hours. These assays were performed in triplicate (B, D). *$P < 0.05$. ND, not detected.

important for the tuning of mucosal barrier function, including antimicrobial peptide REG1α production. Notably, IL-22–mediated mucosal protection was impaired in the inflamed mucosa of patients with CD.

Previous studies of human ILCs suggest that they produce IL-22 through various stimuli, such as IL-2, IL-15, IL-23, and toll-like receptor ligands.[8–10] However, most reports focused on ILCs from human tonsils or expanded cell lines and few reports have directly assessed ILCs from human intestines and their IL-22–producing capacity. However, murine studies revealed the mechanisms that control ILCs. In vitro stimulation with IL-23 induced the production of IL-22 by NKp46+ cells in Peyer's patches.[24] *Citrobacter rodentium* infection induced IL-22 production mainly from CD4+ LTi cells by an IL-23–dependent mechanism.[25] These observations raise questions about the correlation of ILCs between humans and mice, and the importance of IL-23 in human intestinal ILCs. Recently, IL-22–producing ILCs were found in the human intestine, where IL-23 seems to be an activator of ILCs.[10] In patients with IBD, some ILC subsets are dramatically increased compared with healthy individuals.[18,19] Thus, IL-22–producing ILCs may play an important role in the pathogenesis of human IBD. However, the precise mechanism of how ILCs are activated during intestinal inflammation and what the role of IL-22 is in intestinal inflammation is still unclear. Here, we characterized ILC subsets within human intestines. We confirmed that CD127+ ILCs express RORγt and that CD127+CD56+ ILCs express CD117, NKG2D, and CD161 (homologous to human NK1.1) similar to murine NCR+-ILCs.[26] The expression of NKp44 in CD56+ ILCs is higher in noninflamed lesions compared with inflamed lesions. This is consistent with our previous report that the NKp44/NKp46 ratio was significantly higher in noninflamed sites.[18,19]

Previous studies revealed that IL-23 could activate both ILC1 and ILC3 in humans and mice.[10,27,28] Consistent with these reports, the combination of IL-2 and IL-23 induced IL-22 production by RORγt+ ILC3s in patients with CD. This indicated that IL-23 is a key inducer of IL-22 by ILC3s in CD.[9] We previously reported that CD33+ CD14+ macrophages are the major source of IL-23 within the intestine.[12] Thus, we hypothesized that CD14+ intestinal macrophages participate in the activation of ILC3s from patients with CD through production of IL-23. Unexpectedly, ILC3s did not produce IL-22 after coculture with intestinal macrophages in the absence of LPS stimulation. It is noteworthy that intestinal macrophages could activate ILC3s when they were stimulated with LPS. LPS stimulation does not induce IL-23 production by CD14+ intestinal macrophages from patients with CD,[12] indicating that LPS might induce other factors that are involved in the activation of ILC3s. Consistently, direct cross-talk between ILC3s and intestinal macrophages is required for the maximal activation of ILC3s in addition to the role of soluble IL-23 secreted by intestinal macrophages. This implies that LPS induces molecules that are involved in cell-to-cell contact between ILCs and intestinal macrophages. ILC3 is activated by either noninflamed (Fig. 5B) or inflamed (see Fig., Supplemental Digital Content 3, http://links.lww.com/IBD/A507) macrophages, indicating that ILC3s, rather than macrophages, play a pivotal role in IL-22 production.

What are the molecules involved in direct interactions between RORγt+ ILCs and intestinal macrophages? It is known that tumor necrosis factor receptor family, NCRs, and receptor tyrosine kinases are expressed on CD127 SP cells or DP cells. We blocked these surface molecules using blocking antibodies (anti-CD58 antibody, anti-HLA-DR antibody, anti-NKG2D antibody, anti-OX40 ligand antibody, and anti-CXCL2 antibody). However, none of the tested antibodies could suppress IL-22 production from ILCs (data not shown). Although this study did not identify the molecules that play an important role in interactions between ILCs and intestinal macrophages, blocking or stimulation of interacting molecules may shut down or prevent the activation of ILCs, and may be used as a novel therapeutic strategy for targeting ILC3s.

Mounting evidence suggests that IL-22–producing RORγt+ ILCs protect the host from tissue damage.[5,29,30] Thus, the enhanced production of IL-22 might contribute to feedback regulation of intestinal damage caused by inflammation. However, it was also reported that IL-22 has a pathological role in murine colitis models. Whether IL-22 has a capacity for mucosal healing remains controversial.[31] Therefore, we adopted a "mini-gut" system to assess the direct interaction of IL-22 with human intestinal epithelial cells and addressed the role of IL-22 on human intestinal epithelium. The mini-gut was developed from a single intestinal stem cell, and 3-dimensional epithelial structures were built. Mini-gut retains the hallmarks of in vivo epithelium.[32,33] Using this system, we demonstrated that IL-22 could enhance the expression of the antimicrobial peptide REG1α in intestinal epithelium, suggesting IL-22 produced by RORγt+ ILCs may enhance mucosal protective functions including antimicrobial peptide production. Collectively, although CD14+ macrophages augment intestinal inflammation in patients with CD, macrophages also promote a negative feedback pathway through the activation of IL-22 production by RORγt+ ILCs.

However, intestinal inflammation may interfere with the feedback regulation by ILC3s. We demonstrated that the IL-22–producing capacity of RORγt+ ILCs was impaired in inflamed mucosa of patients with CD compared with noninflamed mucosa, whereas absolute numbers of RORγt+ ILCs were comparable in both inflamed and noninflamed mucosa. Notably, IL-23 receptor expression in ILC3s from noninflamed and inflamed mucosa was comparable, and there was no significant difference in IL-23–induced IL-22 production between noninflamed and inflamed ILC3s (Fig. 5C and data not shown). This indicated that mucosal inflammation in patients with CD perturbs IL-23–independent ILC activation mechanisms, most likely by direct interactions with intestinal macrophages. Thus, the impairment of mucosal protection by IL-22 may be involved in chronic intestinal inflammation in patients with CD. To investigate whether this cross-talk between ILCs and macrophages is specific for CD, we examined patients with ulcerative colitis as an inflammatory control. Although the number of RORγt+ ILCs was comparable

(see Fig. A and B, Supplemental Digital Content 4, http://links.lww.com/IBD/A508), the IL-22 production ability of the ILCs seemed lower than those in patients with CD (see Fig. C, Supplemental Digital Content 4, http://links.lww.com/IBD/A508).

After the emergence of anti-tumor necrosis factor therapy, sustaining mucosal healing has been an important therapeutic goal in CD treatments. Our results suggest that the modification of CD127$^+$ ILC functions contribute to mucosal healing in patients with CD. Further experiments are required to investigate target molecules involved in the interactions between CD127$^+$ ILCs and macrophages. This will hopefully lead to a new treatment strategy in patients with CD.

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REVIEW

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KJIM

Diet, microbiota, and inflammatory bowel disease: lessons from Japanese foods

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The incidence and prevalence of inflammatory bowel diseases (IBDs) including ulcerative colitis and Crohn disease are rapidly increasing in Western countries and in developed Asian countries. Although biologic agents targeting the immune system have been effective in patients with IBD, cessation of treatment leads to relapse in the majority of patients, suggesting that intrinsic immune dysregulation is an effect, not a cause, of IBD. Dramatic changes in the environment, resulting in the dysregulated composition of intestinal microbiota or dysbiosis, may be associated with the fundamental causes of IBD. Japan now has upgraded water supply and sewerage systems, as well as dietary habits and antibiotic overuse that are similar to such features found in developed Western countries. The purpose of this review article was to describe the association of diet, particularly Japanese food and microbiota, with IBD.

Keywords: Diet; Microbiota; Inflammatory bowel diseases; Probiotics; Japanese food

INTRODUCTION

In Japan, approximately 140,000 patients with ulcerative colitis (UC) and 40,000 with Crohn disease (CD) are currently registered by the Japanese Health, Labor and Welfare Ministry [1]. Because health-care costs for inflammatory bowel diseases (IBDs), including UC and CD, of registered patients are generally covered by the government, most patients voluntarily join the registry. Registration, however, is not mandatory, and some patients with mild to moderate IBD may refuse to register because of privacy concerns. Therefore, the actual numbers of IBD patients in Japan may be 20% to 40% higher than the numbers in the registry. The incidence of IBD in Japan ranks it as a low- to moderate-frequency country [2-4], although the incidence and prevalence is rapidly increasing [1].

Advances in next-generation gene sequencing technology have resulted in the identification of over 160 IBD-associated susceptibility genes within the past 10 years [5]. However, these susceptibility genes are unlikely to be the primary cause of IBD in Asia, because in the past 30 years the numbers of IBD patients in Japan have increased 100-fold. It is more likely that dramatic changes in the Japanese social environment, especially dietary habits that lead to an unhealthy composition of microbiota, known as dysbiosis, are fundamental causes of IBD [6-15]. Japan now has an upgraded water supply and sewerage systems along with dietary habits and overuse of antibiotics [16] that are similar to those found in developed Western countries (Fig. 1). Indeed, residents of Tokyo can live in an environment with exactly the same food and hygienic conditions as in New York.

RAPID DIETARY HABIT CHANGES IN JAPAN

Until about 150 years ago, Japan was officially sealed off from the outside world. Most Japanese individuals

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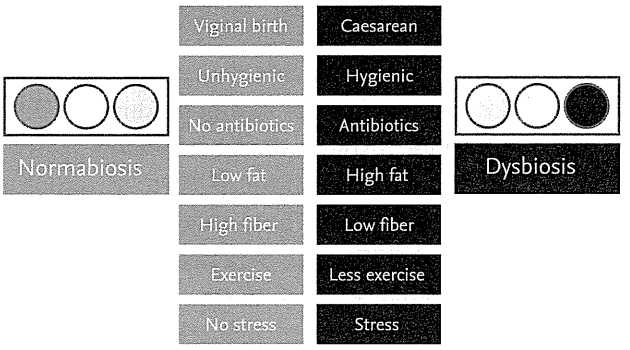

Figure 1. Dramatic lifestyle changes in Japan. Modern lifestyles, including delivery mode, hygiene environment, antibiotic use, food, exercise, and stress, could induce dysbiosis, which seems to be prediseased composition of microbiota.

had no contact with Western people or Western dietary habits, and ate traditional Japanese foods. After the end of the Edo era in 1868, the new Japanese government opened the country to Westerners and began diplomatic and cultural contract with many Western countries. Concurrently, the Japanese government promoted a Western lifestyle, including Western diets, housing, clothes, and culture. However, only a small proportion of Japanese people, known as the favored classes, could afford Western foods, while the vast majority continued to eat frugal Japanese foods for an additional 100 years. A typical Japanese diet at that time was a simple vegetarian meal composed of unthreshed rice mixed with barley, miso soup with root vegetables and/or tofu, small grilled fermented fish, and fermented pickled vegetables. Fermentation was essential to preserve foods in the absence of cooling systems. After the end of World War II in 1945, democracy emerged in Japan, with many people choosing Westernization. Annual reports by the Japanese Health, Labor and Welfare Ministry have shown rapid increased intake of sugar-rich carbonated beverages, fat- and carbohydrate-rich Western snacks (e.g., potato chips), and animal protein and fat, and a concurrent rapid decrease in the intake of dietary fiber.

CORRELATION BETWEEN DIET AND MICROBIOTA

The microbiota/microbiome field has been described in many recent review articles [17-23]. The availability of next-generation sequencing has had the greatest impact on this field, as it can be used to sequence numerous bacterial DNA sequences simultaneously using a shot-gun method [24-29]. Each human being contains 100 trillion bacteria, composed of over 200 species with 50-fold more genes that in the human genome [28,29]. Dysbiosis and loss of microbiome diversity is thought to result in many kinds of diseases and predisease conditions. These include not only intestinal immune diseases (e.g., IBD) [9,10,30] and functional diseases (e.g., irritable bowel syndrome) [31,32] but also extraintestinal diseases such as obesity, arteriosclerosis, allergy, and autism disorders [33-37]. The incidence of all these diseases is increasing in developed Western and Asian countries, irrespective of whether they are T helper (Th) 1- or Th2-mediated diseases.

The organisms constituting a healthy composition of microbiota, or normabiosis, remain unclear for humans or animals. Moreover, it is unclear whether normabiosis is similar in healthy individuals and between Western and Asian people. However, striking differences in the composition of microbiota have been observed, not only between diseased (e.g., IBD) and healthy individuals but also between different diseased individuals. For example, the composition of microbiota in healthy African children from Burkina Faso, a country with a low incidence of IBD, included greater amounts of *Prevotella*, greater microbial diversity, and higher levels of short chain fatty acids (SCFA) than the microbiota of healthy European children from Italy, a country with a high incidence rate of IBD [38]. Similar results were observed when the microbiota of healthy individuals from South America and South Asia were compared with the microbiota of healthy individuals from an industrialized country such as the United States [39].

POOR EVIDENCE OF SPECIFIC DIETARY COMPONENTS IN THE ETIOLOGY OF IBD

Dramatic changes in dietary components, including increased sugar/refined carbohydrates and animal fat/protein and reductions in dietary fibers (prebiotics, fermentable oligosaccharides), fruits/vegetables, and fermented products containing probiotics, have been

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proposed as major etiologic factors in the development of both UC and CD [40,41]. Additionally, the hygienic environment in industrial countries may be closely associated with a lower likelihood of coming in contact with fermented bacteria, which may be identical to probiotics [42]. Surprisingly, however, there is little evidence showing that specific dietary components are risk factors for the development of UC and CD [43-45]. However, it may be difficult or impossible to determine the real causes of IBD, because some individuals may consume both Western snacks and Japanese foods. Nevertheless, many researchers and clinicians strongly believe that current dietary habits, of high fat/low fiber and less fiber/probiotics, may be improved by returning to diets consumed during the era before modernization.

SOLID EVIDENCE OF SPECIFIC DIETARY COMPONENTS IN THE ETIOLOGY OF IBD IN ANIMAL MODELS

Results from animal disease models provide clearer evidence of the involvement of specific dietary components in the etiology of IBD [46,47]. However, a direct translation of animal results to human diseases is problematic. For example, mice without colitis co-housed with colitic mice developed similar colitis with a shift to dysbiosis [48], while germ-free mice transplanted with feces of obese mice became obese and those transplanted with feces of lean mice became lean [49].

Several hypotheses have been proposed to explain the critical roles of probiotic microbiota in the prevention of IBD in mouse models. This may be shown using a gnotobiotic system, in which germ-free mice are inoculated with one or several specific strains of bacteria, allowing the specific roles of these bacteria to be evaluated *in vivo* (Fig. 2). Germ-free mice inoculated with a mixture of 46 mouse-derived *Clostridium coccoides* and *Clostridium leptum* strains had a normal proportion of mucosal interleukin (IL)-10-producing regulatory T-cells, equivalent to those in specific pathogen-free normal mice [50]. These T-cells stimulated the production of transforming growth factor-b from colonic epithelial cells, resulting in resistance to experimental colitis [50]. Furthermore, *C. coccoides* and *C. leptum* from healthy human volunteers were similarly able to induce regulatory T-cells in mice [51]. Additionally, fermented Clostridia probiotics locally produced SCFAs, including butyrate, through the fermentation process, and these SCFA directly induced IL-10-producing regulatory T-cells [52-54]. These results indicate that probiotic-induced SCFAs are beneficial in maintaining colonic epithelial cells and in providing energy for hepatocytes. In contrast to the induction of regulatory T-cells by probiotics, we recently proposed a distinctive mechanism of probiotic actions, based on findings that a probiotic strain, *Clostridium butyricum*, which preferentially produces butyrate, suppressed the development of acute experimental colitis in mice by inducing IL-10-producing mucosal macrophages in a toll-like receptor-2/MyD88-dependent manner [55].

The mechanism by which a high-fat diet is associated with IBD onset remains unknown. The incidence of colitis was markedly increased in milk fat-fed IL-10-deficient mice, but not in normal mice or polyunsaturated fat-fed IL-10-deficient mice, with *Bilophila wadsworthi* observed in the feces of milk fat-fed mice, indicating dysbiosis [56]. This finding was clearly linked with tau-

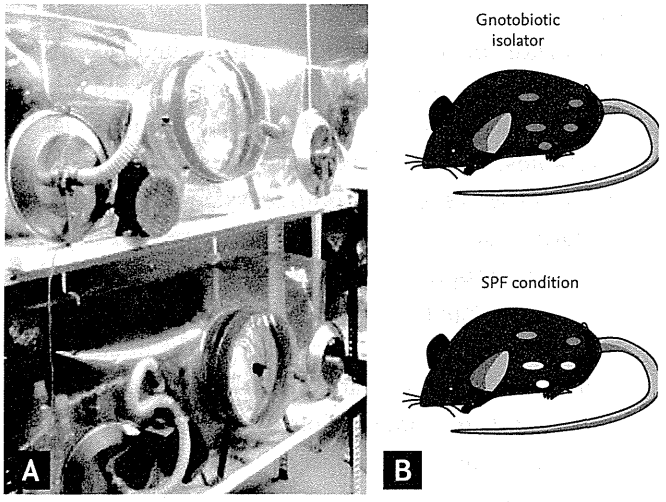

Figure 2. A gnotobiotic system. (A) Gnotobiotic isolator. One germ free isolator can be installed in four to six small cages, each containing four to five mice. Each unit may contain eight to 12 isolators, occupying a space of approximately 30 m^2, requiring one specific technician. (B) Because over 200 microbiota reside in the colon of each healthy mouse, the exact role of specific bacteria can be assessed using a gnotobiotic system. SPF, specific pathogen-free.