form the organized lymphoid structures, while lamina propria lymphocytes (LPLs) and IELs represent diffusely distributed immunocompetent cells. Numerous IELs reside above the basement membrane, together with columnar epithelial cells (IECs) and, due to their anatomical location, IELs are believed to encounter intestinal antigens early, thus allowing these cells to play an important role in activating and regulating intestinal mucosal immunity. However, the details of the physiological function of IELs have remained unclear. The mechanisms and site of IEL production have also remained unknown for a long period of time.

In 1996, small clusters of lymphocytes were first identified in the lamina propria (LP) near crypts throughout the mouse small intestine, and these were named cryptopatches (CPs). There are about 1000–1500 CPs in the mouse small intestine,[3] and immunohistochemical analyses have shown that the majority of these CP lymphocytes (70%–80%) are undifferentiated lymphocytes expressing c-kit, interleukin-7 receptor (IL-7R), Thy-1, and leukocyte function-associated antigen-1 (LFA-1), but not CD3, TCRαβ, TCRγδ, sIgM, or B220. In addition to lymphocytes, dendritic cells that express CD11c/CD18 integrin account for 20%–30% of CP cells, and these dendritic cells are widely distributed in the margin of CPs surrounding lymphocytes.[3] These findings indicate that CPs are more than simple lymphocyte clusters; rather, they consist of legitimate small lymphoid tissues.

In subsequent studies, lymphocytes isolated from CPs, MLNs, or PPs from 4-week-old nude mice were transplanted into severe combined immunodeficiency (SCID) mice. The results showed that IELs had developed only in SCID mice receiving CP lymphocytes, confirming the compartmentalization of undifferentiated IEL progenitor cells in gut CPs.[4] Furthermore, when athymic common cytokine receptor γ-chain mutant mice that lacked thymus, PPs, CPs, and IELs[5] were transplanted with wild-type bone marrow (BM) cells, CPs filled with BM-derived c-kit$^+$ cells were first reconstituted, then IELs appeared to develop and differentiate from the BM-derived CP lymphocytes.[6] Taken together, these results support the notion that CPs are an extrathymic IEL-producing plant in the mouse small intestine.

While CPs[3–6] were described in mice, ascertaining whether similar lymphoid tissues are present in the GIT of other animals, including humans, is very important. Several groups have actively sought for evidence of CPs in the human GIT, and, although lymphocyte-filled villi (LFVs), comprising villi containing mature lymphocytes,[7] have been found, clusters of undifferentiated lymphocytes, similar to mouse CPs, have not.[7,8] After humans and mice, various monoclonal antibodies (mAbs) to lymphocyte cell surface antigens have become available for rats, enabling detailed immunohistochemical analysis of rat lymphoid tissues. In fact, LFVs not resembling PPs have been described in the rat intestinal mucosa.[9–11] According to these studies, LFVs can be detectable from around 10-day-old suckling rats. Although recent evidence supports the extrathymic origin of intestinal γδ T cells in normal rats[12,13] and lymphoid progenitor cells are believed to develop and differentiate in the rat LFVs, details of the underlying mechanisms remain elusive.

To investigate whether rats possess lymphoid tissues similar to mouse CPs, we carried out an immunohistchemical study on rat small-intestinal tissue. The result showed that rat intestinal mucosa did not possess lymphocyte clusters in crypt lamina propria (LP) that were structurally comparable to the mouse CPs. However, as reported by Mayrhofer and colleagues,[9] about 1600 lymphocyte clusters were found in crypts and villi, thus representing LFVs. Next, we addressed whether the possible progenitor IELs equivalent to those present in the mouse CPs were present in the rat LFVs. As a result, we have identified for the first time that rat LFVs contain abundant c-kit$^+$ and IL-7R$^+$ cells. These findings suggest that these structures may be similar to the mouse CPs, in that they harbor immature lymphocytes expressing c-kit and/or IL7-R molecules and in that they are found to develop after birth. In the present article, all of these newly elucidated aspects of rat LFVs are described, with respect to their transfiguration with age.

Materials and methods

Rats

We purchased Jcl:Wistar (Wistar) rats from Clea Japan (Tokyo, Japan).

Antibodies and peanut agglutinin

The following monoclonal antibodies (mAbs) and polyclonal Abs (pAbs) were used: murine anti-rat TCR-αβ mAb (R73, 0.625 μg/ml; BD Pharmingen, Franklin Lakes, NJ, USA); murine anti-rat IgM mAb (G53-238, 5 μg/ml; BD Pharmingen), murine anti-rat IgA mAb (A93-3, 2.5 μg/ml; BD Pharmingen), murine anti-rat pan B cell mAb (RLN-9D3, 20 μg/ml; Serotec, Raleigh, NC, USA), goat anti-c-kit pAb (5 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-IL-7R pAb (5 μg/ml; Santa Cruz Biotechnology). Biotinylated peanut agglutinin (PNA, 7.5 μg/ml; Vector Laboratories, Burlingame, CA, USA) was also used in this study. For flow cytometric analysis, 1–3 × 10^5 cells were stained in 50 μl of staining medium containing the above mAbs.

Immunohistochemical procedures

The small intestine was longitudinally opened along the mesenteric wall, and a 10-mm length of intestine that had been either kept flat for horizontal sections or rolled up for vertical sections was embedded in OCT compound (Tissue-Tek; Miles, Elkhalt, IN, USA) at −80°C. Tissue segments were sectioned using a cryostat at 5 μm, and sections were preincubated with Blockace (Dainippon Pharmaceutical, Osaka, Japan) to block nonspecific binding of Abs. Sections were then incubated with mouse, goat, or rabbit Ab for 30 min at 37°C and rinsed three times with phosphate-buffered saline (PBS), followed by incubation with biotin-conjugated goat anti-mouse Ig Ab (1.25 μg/ml; BD Pharmingen), biotin-conjugated donkey anti-goat IgG (10 μg/ml; Jackson ImmunoResearch, West Grove, PA, USA), or biotin-conjugated donkey anti-rabbit IgG (10 μg/ml; Jackson ImmunoResearch). In staining with biotinylated PNA, the second biotin-conjugated anti-IgG Ab was not used. Sections were subsequently washed three times with PBS and then incubated with avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories). Histochemical color development was achieved using a Vectastain DAB (3,3'-diaminobenzidine) substrate kit (Vector Laboratories) according to the manufacturer's instructions. Finally, sections were counterstained with hematoxylin for microscopy. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 and 0.1% NaN_3 in distilled water for 10 min at room temperature. Tissue sections incubated either with isotype-matched normal mouse IgG, normal goat IgG, or normal rabbit IgG showed only minimal background staining.

In vivo labeling and in situ immunohistochemical visualization of proliferating lymphocytes

Rats were administered drinking water containing 1 mg/ml bromodeoxyuridine (BrdU) for 20 h. Small intestines were opened along the mesenteric wall. Next, 10-mm segments of intestine that had been rolled up were embedded in OCT compound at −80°C. Cryostat tissue sections 9-μm-thick were fixed in 4% paraformaldehyde for 15 min at 4°C, washed three times with PBS, and treated with 2M HCl for 20 min at 37°C, followed by neutralization with 0.1M sodium tetraborate. Subsequent immunohistochemical color development using the first anti-BrdU mAb (B44; BD Biosciences, San Jose, CA, USA) and the second biotinylated goat anti-mouse Ig Ab (20 μg/ml; Cappel, Aurora, OH, USA) was performed according to the methods described above.

Flow cytometry

A single lymphoid cell suspension was prepared, and nucleated cells were counted using a hemocytometer. LFV cells were isolated using essentially the same technique as that used for the isolation of murine CP and ILF cells.[4–6] In brief, the small intestine was opened longitudinally along the mesenteric wall, and mucus and feces were removed using filter paper. Subsequently, a 10-mm-long segment of intestine was pasted on a plastic culture dish. An 18-gauge needle (inner diameter, 940 μm) was cut off at the proximal end of the tapering tip. The needle was then bent in the middle, and the cross-section was sharpened using a small UA12A electric dental grinder (Urawa Kogyo, Saitama, Japan). This needle was finally fitted onto a 1-ml syringe. LFVs were identified under transillumination stereomicroscopy and a tiny fragment of the small intestine containing one LFV was isolated using the needle described above. Lymphoid cells were incubated first with biotinylated mAb, then with streptavidin phycoerythrin (PE; BD Biosciences)—conjugated second mAb. Stained cells were suspended in staining medium (Hanks' solution without phenol red, 0.02%, NaN_3, and 2% heat-inactivated fetal bovine serum [FBS], containing 0.5 μg/ml propidium iodide [PI]) and analyzed using FACScan with CellQuest software (BD Biosciences). Dead cells were excluded by PI gating.

Results

We have recently described CPs and ILFs in the mouse small intestine and have characterized the histogenetic, cellular, and functional aspects of these newly identified GALTs.[3–6,14,15] Mice display about 1500 CPs throughout the small intestinal mucosa,[3] and 140–180 ILFs mostly on the antimesenteric wall of the small intestine.[15] Several research groups, including our own, have been actively searching for clusters of undifferentiated lymphocytes in the human GIT, but no such clusters have been identified.[7,8] Rat GIT displays LFVs, and, because these structures predominantly comprise $CD3^-$, $\alpha\beta TCR^-$, $CD25^+$, and $CD44^+$ cells, LFVs in rats appear comparable to CPs in mice.[10] We therefore investigated whether clusters of undifferentiated lymphocytes expressing c-kit and IL-7R existed in the rat LFVs. We first prepared and examined hematoxylin-and-eosin (H&E)-stained sections of small-intestinal mucosa from 13-day-old Wistar rats under microscopy. The results showed clusters of lymphoid cells in the small-intestinal mucosa (Fig. 1a, b). While most CPs in mice were localized near crypt LP,[3] lymphocyte clusters on the rat small intestinal mucosa were densely distributed inside villi, and were morphologically classified as LFVs.[9–11] Fur-

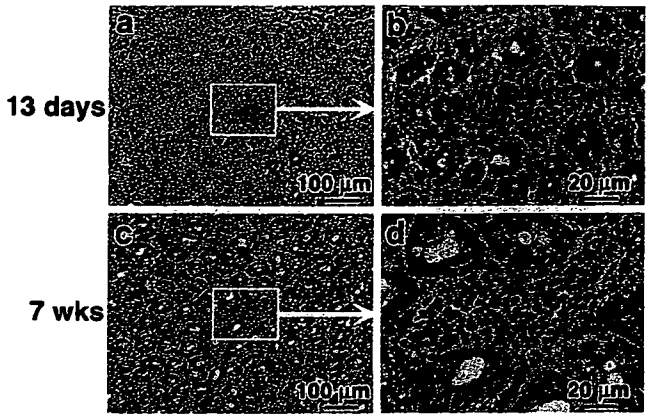

Fig. 1a–d. Localization of lymphoid cell aggregations (lymphocyte-filled villi, LFVs) in the hematoxylin-and-eosin-stained tissue sections of small intestine from 13-day-old (**a** and **b**) and 7-week-old (**c** and **d**) Wistar rats

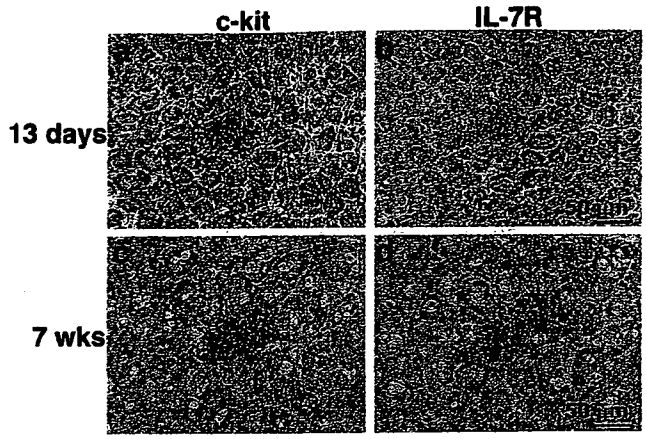

Fig. 2a–d. Immunohistochemical visualization of **a** and **c** c-kit$^+$ and **b** and **d** interleukin 7 receptor (*IL-7R*)$^+$ lymphocytes in small-intestinal LFVs from 13-day-old (**a** and **b**) and 7-week-old (**c** and **d**) Wistar rats

ther, small-intestinal villi containing LFVs were thicker than other villi (see below). We next examined small-intestinal mucosa from 7-week-old young adult rats, and found that lymphoid cell clusters were localized throughout the small-intestinal mucosa (Fig. 1c, d). It was also verified that about 1600 of such lymphoid cell clusters were present in 7-week-old young adult rats, and that these clusters were also morphologically classified as LFVs by H&E staining (data not shown). We analyzed extensively the small intestine from three 10-week-old rats and noted that LFVs were not evenly distributed in the small-intestinal mucosa. The average number of LFVs in the duodenum and jejunum was 1.6 ± 0.2 and 1.1 ± 0.1/100 villi, compared to 0.2 ± 0.1/100 villi ($P < 0.005$) in the distal half of the ileum.

Neither CPs nor ILFs were detected in the small intestine of fetal mice. In mice, CPs can be first detected at 14–17 days after birth,[3] and ILFs can be first detected at 7 (BALB/c mice) and 25 (C57BL/6 mice) days after birth.[15] We therefore investigated the development of LFVs in the Wistar rats, and found that LFVs could not be detected in fetal and neonatal rats, but became detectable without exception by 13 days after birth (Fig. 1a, b). These findings clearly indicate that rat LFVs develop after birth, like mouse CPs and ILFs.

Mayrhofer and Brooks[10] stated that, because LFVs contain few mature T and B cells, they represent organized GALTs populated mainly by undifferentiated lymphocytes. With this possibility in mind, we aimed to investigate whether rat LFVs consisted of clusters of undifferentiated lymphocytes, like mouse CPs. Rat LFVs were immunohistochemically stained for c-kit, a marker for undifferentiated lymphocytes, and examined under microscopy. Rat LFVs were found to contain numerous c-kit$^+$ cells (Fig. 2a, c). Furthermore, the rat LFVs were immunohistochemically stained for IL-7R, a cell-surface antigen found on mouse CP cells, revealing that rat LFVs also contained numerous IL-7R-expressing lymphocytes (Fig. 2b, d). These findings suggest that, as claimed by Mayrhofer and colleagues,[9] LFVs represent CP-like lymphoid tissues consisting of clusters of undifferentiated cells expressing c-kit and IL-7R. Next, to analyze the lymphoid cells forming rat LFVs in greater detail, we explored the expression of various cell-surface antigens.

The distribution of B cells in LFVs was immunohistochemically analyzed using antibody to rat IgM, and it was revealed that LFVs could be divided into those with and without IgM$^+$ cells (IgM$^+$ and IgM$^-$ LFVs, respectively; Fig. 3b–d). Most IgM$^+$ LFVs were arranged linearly on the antimesenteric wall (Fig. 3b, c), and numbered about 400 in small intestine from 7-week-old rats. IgM$^-$ LFVs were found throughout the intestinal mucosa (Fig. 3b, d), and numbered about 1200 in small intestine from 7-week-old rats, although fewer IgM$^-$ LFVs were present at the end of the ileum (data not shown). A small number of $\alpha\beta^+$ T cells was interspersed among both IgM$^+$ and IgM$^-$ LFVs (Fig. 3f–h). On vertical sections of LFVs, IgM$^+$ B cells were found to occupy the core and the base of villi (Fig. 4a), whereas $\alpha\beta^-$ T cells were distributed mainly over the upper half of both IgM$^+$ and IgM$^-$ LFVs (Fig. 4b, f). On the basis of these vertical profiles, it was also evident that LFVs were thicker than surrounding villi that were devoid of lymphocyte congregation (Fig. 4a–h) and that both IgM$^+$ and IgM$^-$ LFVs were filled with closely packed c-kit$^+$ (Fig. 4c, g) and IL-7R$^+$ (Fig. 4d, h) lymphocytes.

To confirm that LFVs found on the antimesenteric wall of the small intestine contained B cells, we located an LFV under a transillumination stereomicroscope

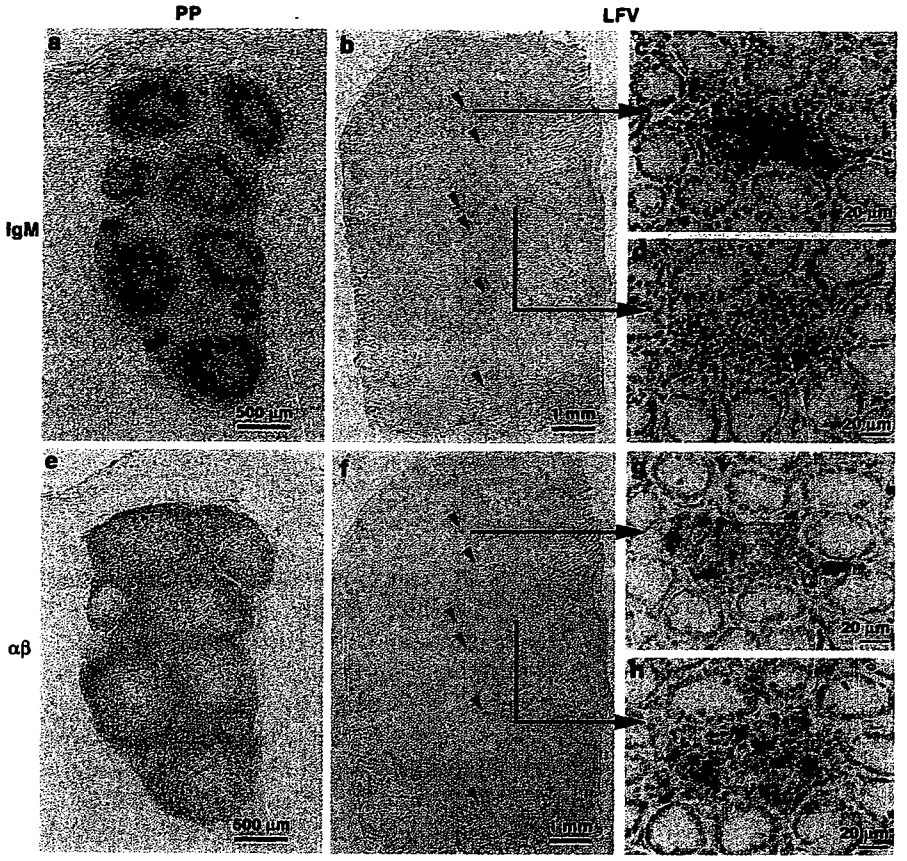

Fig. 3a–h. Immunohistochemical visualization of **a**, **b**, **c**, **d** IgM$^+$ **e**, **f**, **g**, **h** T cell receptor (TCR)-αβ$^+$ lymphocytes in Peyer's patches (*PPs*) and LFVs. Frozen sections of small intestine from 7-week-old Wistar rats were prepared, and PP (**a** and **e**) and LFV (**b** and **f**) were immunohistochemically analyzed. Consecutive tissue sections were stained with anti-IgM (**a** and **b**) and anti-TCR-αβ (**e** and **f**) monoclonal antibody (mAb), respectively. Multiple LFVs are aligned along the antimesenteric wall of the mucosa (*arrowheads*) and LFVs are also interspersed throughout the mucosa. Magnified views of the representative LFVs are also presented (**c**, **d**, **g**, **f**)

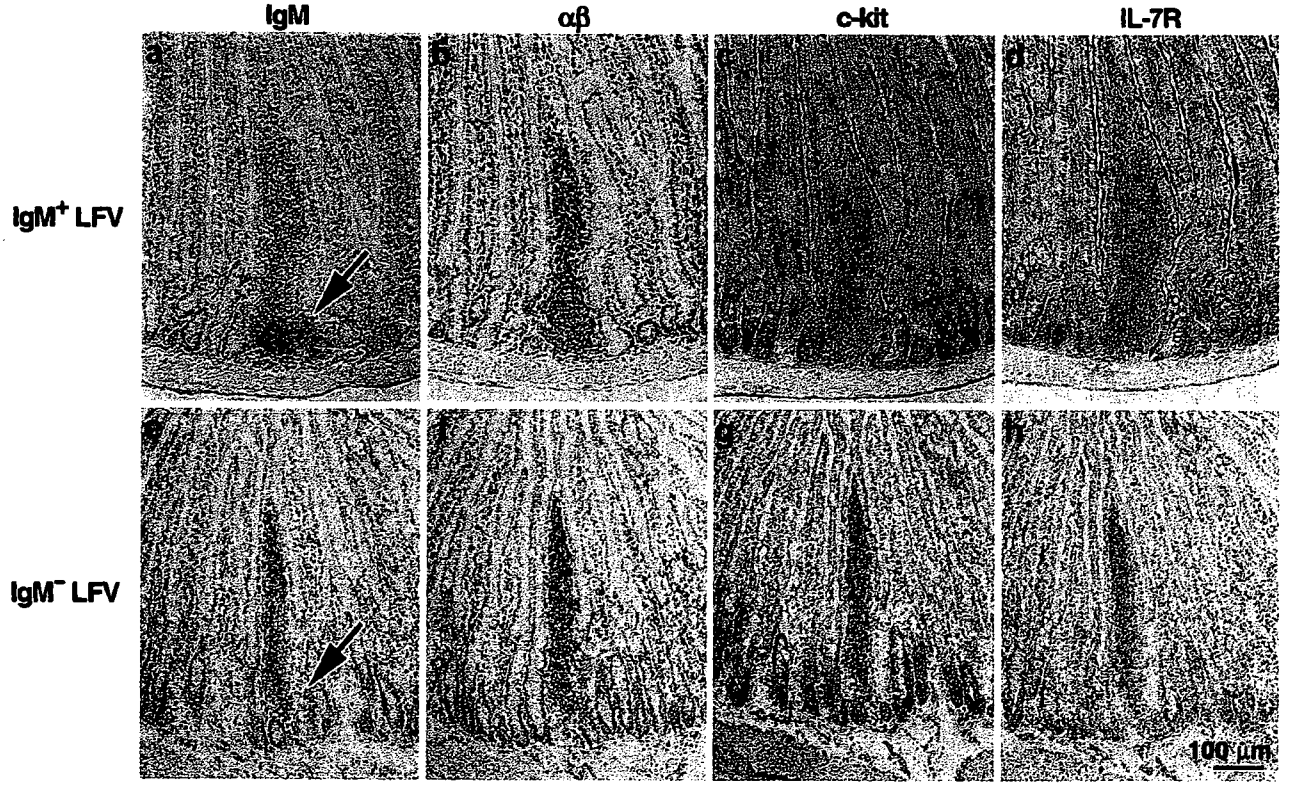

Fig. 4a–h. Immunohistochemical characterizations of **a**, **b**, **c**, **d** IgM$^+$ and **e**, **f**, **g**, **h** IgM$^-$ LFVs in consecutive longitudinal tissue sections of small intestine from 7-week-old Wistar rats. IgM$^+$ cells are found on the bottom of IgM$^+$ LFV (**a**), but IgM$^-$ LFV lacks such IgM$^+$ cells (**e**) (*arrows*). αβ T cells tend to be localized in the subepithelial regions of both IgM$^+$ (**b**) and IgM$^-$ (**f**) LFVs. Numerous c-kit$^+$ cells are present in both IgM$^+$ (**c**) and IgM$^-$ (**g**) LFVs, and numerous IL-7R$^+$ cells are also present in both IgM$^+$ (**d**) and IgM$^-$ (**h**) LFVs

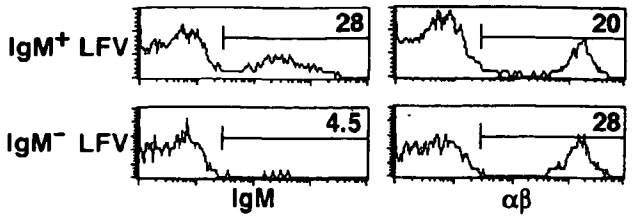

Fig. 5. Flow cytometric analysis of lymphocytes isolated from IgM$^+$ and IgM$^-$ LFVs. Lymphocytes were isolated from LFVs of 7-week-old Wistar rats and stained using anti-IgM and anti-TCR-αβ mAbs. A large fraction of lymphocytes in LFVs from the antimesenteric side express IgM molecules (IgM$^+$ LFVs), whereas only a small fraction of lymphocytes in LFVs from the non-antimesenteric side express IgM molecules (IgM$^-$ LFVs). The ratios of αβ T cells are comparable in the two types of LFVs

and isolated a tiny fragment of the small intestine containing one LFV by a technique described elsewhere.[4,15] Lymphocytes isolated from LFVs that were located in tandem on the antimesenteric wall (Fig. 3b, c); i.e., IgM$^+$ LFV, and those from LFVs localized throughout the mucosa (Fig. 3b, d); i.e., IgM$^-$ LFV, were stained using anti-IgM and anti-αβ-TCR mAbs, and subjected to flow cytometry. About 30% of the lymphocytes isolated from LFVs on the antimesenteric wall were IgM$^+$, compared to less than 5% of those isolated from LFVs localized in other areas (Fig. 5). It also became evident that the composition of αβ$^+$ T cells was about 20%–30% for both IgM$^+$ and IgM$^-$ LFVs (Fig. 5). Thus, LFVs that are localized throughout the length of the antimesenteric wall of the small intestine from young adult rat contain a large number of IgM$^+$ B cells. In the mouse small-intestinal mucosa, ILFs are found on the antimesenteric wall.[15] Like mouse ILFs,[15] these rat LFVs are located on the antimesenteric wall and contain B cells, suggesting that rat IgM$^+$ LFVs are comparable to mouse ILFs. We also confirmed that, on day 13 of the postnatal period, IgM$^+$ B cells were very few in number in both antimesenteric (Fig. 6a) and scattered (Fig. 6c) LFVs and that only a marginal number of αβ$^+$ T cells were detected in both types of LFVs (Fig. 6b, d). Overall, these results indicate that IgM$^+$ LFVs in the rat develop between the ages of 13 days and 7 weeks.

In view of the finding that the cellular composition of LFVs changes with age, we investigated LFVs in 22-week-old rats. Clusters of IgM$^+$ B cells (Fig. 7a) composed of anti-rat pan B-cell mAb-positive cells (Fig. 7b) were found not only in LFVs that were arranged linearly on the antimesenteric side but also in LFVs that were distributed randomly throughout the intestinal mucosa. Furthermore, IgM$^+$ LFVs contained an area where the slightly larger cells were congregated, suggesting the development of a germinal center in these LFVs from aged rats. We therefore examined whether such areas truly represented germinal centers, using PNA staining.[16] This B-cell area turned out to be occupied by PNA$^+$ cells, indicating the presence of a secondary follicle with a germinal center (Fig. 7c). Furthermore, B cells concentrated in the germinal center were found to contain a substantial number of IgA$^+$ B cells (Fig. 7d).

The proliferation of rat LFVs was investigated by a technique of BrdU uptake, and BrdU$^+$ cells were found in both IgM$^+$ (Fig. 8a, b) and IgM$^-$ (Fig. 8c, d) LFVs in 7-week-old rats, accounting for about 10% of cells (Fig. 8b, d). The ratio of BrdU$^+$ cells was higher in LFVs than in rat thymic medulla (5%–10%), but lower than that in the thymic cortex (<30%). As might have been expected, numerous BrdU$^+$ proliferating lymphocytes were observed in PPs from 7-week-old rats (Fig. 8e).

Discussion

In addition to PPs and MLNs, LFVs were reported to represent one of the organized GALTs compartmentalized in the rat small intestine.[9,10] Although Mayrhofer and coworkers[9] envisaged rat LFVs as a diffuse "gut thymus" and interpreted that T cells were generated in LFVs from precursors similar to triple-negative thymocytes, the details of these issues have remained to be explored to date. In this study, we aimed to investigate whether rat LFVs were comparable to mouse CPs, and whether any other rat GALTs comparable to mouse CPs were detectable.

In mice, the organogenesis of 6 to 12 PPs involves at least three distinct steps in the late embryonic stage,[17] while the development of CPs[3] and ILFs[15] commences after birth. In rat small-intestinal mucosa, no lymphoid tissues that were morphologically identical to mouse CPs (lymphocytes distributed mainly in the crypt LP) were noted,[9,10] but lymphocyte clusters that were clearly different from PPs were observed. As described above, Mayrhofer and co-workers[9] have reported the existence of rat small-intestinal villi that are somewhat thicker in a longitudinal section than the surrounding classical villi. The villi were, in fact, distinguished by containing a LP that was replaced by closely packed lymphocytes; thus, these structures were named "LFVs". In the present study, we confirmed the presence of LFVs in the GIT of Wistar rats and were able to show, for the first time, that, like mouse CPs, rat LFVs contained numerous c-kit$^+$ and IL-7R$^+$ undifferentiated lymphocytes.

The present results also clarified the existence of two types of LFVs in the rat small intestine; that is, those consisting of c-kit$^+$ and IL-7R$^+$ lymphocytes with and without IgM$^+$ B cells. It was also noteworthy that the distribution and ratios of IgM$^+$ and IgM$^-$ LFVs changed

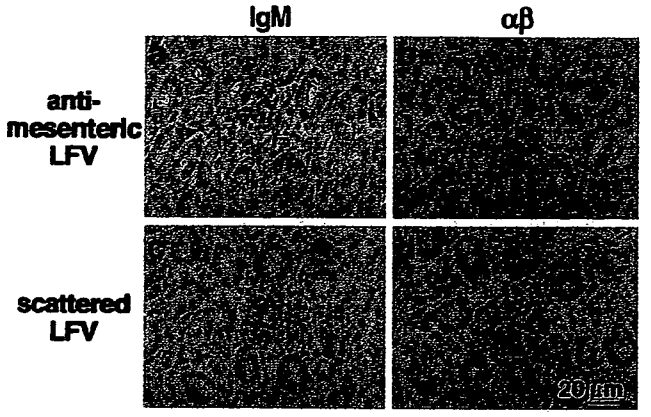

Fig. 6a–d. Immunohistochemical analysis of **a**, **b** antimesenteric and **c**, **d** scattered LFVs in young suckling Wistar rats. Expression of IgM (**a** and **c**) and TCR-αβ (**b**, **d**) was assessed in LFVs from 13-day-old Wistar rats. Unlike LFVs in 7-week-old Wistar rats, LFVs on the antimesenteric side display only a few IgM^+ cells on day 13 after birth (**a**)

with age. In rats at 7 to 8 weeks of age, most IgM^+ LFVs were found on the antimesenteric wall, while IgM^- LFVs were found throughout the intestinal mucosa. At age 22 weeks, however, IgM^+ LFVs were found throughout the intestinal mucosa and retained a germinal center. Mouse ILFs, which are composed of a large B-cell area, including a germinal center, are distributed predominantly on the antimesenteric wall.[15] In ILFs from normal mice, the collection of B cells was surrounded by a layer of cells expressing c-kit and IL-7R molecules,[15] and $c\text{-}kit^+$ cells replaced $B220^+$ B cells as the predominant population in ILFs from TCR-$\beta^{-/-}$, recombination-activating gene-2 $(RAG\text{-}2)^{-/-}$, and $\mu m^{-/-}$ mutant mice.[15]

According to Mayrhofer and colleagues,[9] the phenotype of the major population of lymphocytes in rat LFVs remains $CD3^-$, TCR-$\alpha\beta^-$, $CD4^-$, $CD8^-$, $CD25^+$, $CD43^+$, and $CD161^+$. Thus, we have confirmed and extended their observations,[9] by showing that the majority

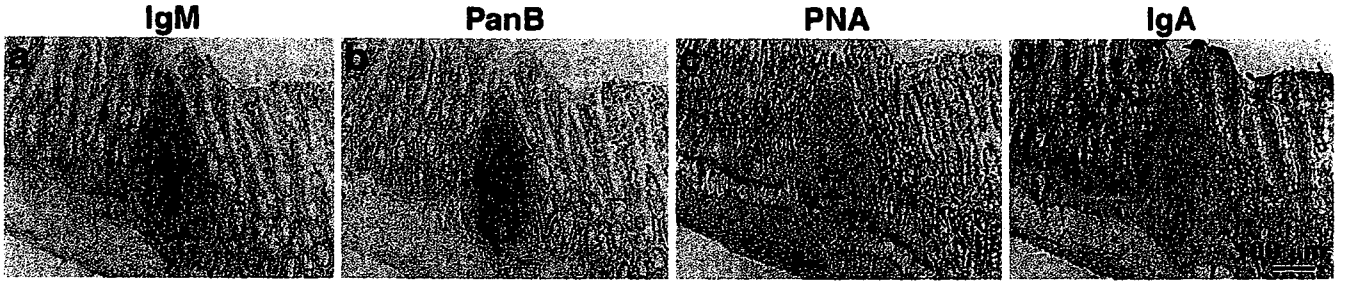

Fig. 7a–d. Immunohistochemical characterization of LFVs from 22-week-old Wistar rats. In 22-week-old Wistar rats, lymphocytes in both antimesenteric (**a**, **b**) and scattered (not shown) LFVs are abundant in B cells. Some of these B cells were peanut agglutinin $(PNA)^+$ (**c**) and IgA^+ (**d**). These findings suggest that LFVs from aged Wistar rars are isolated lymphoid follicles (ILFs) with a germinal center

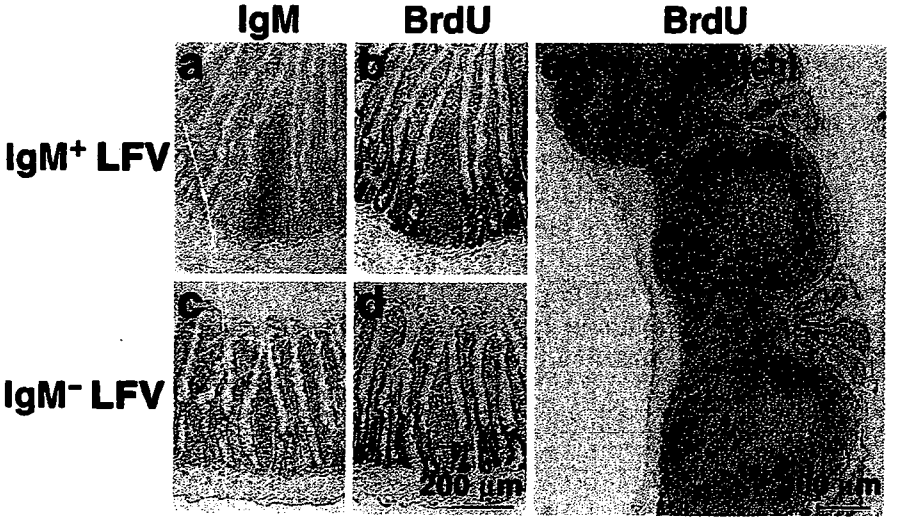

Fig. 8a–e. Bromodeoxyuridine (*BrdU*)-incorporated lymphocytes in IgM^+ LFV, IgM^- LFV, and PP. After allowing 7-week-old Wistar rats to drink water containing BrdU for 20 h, we prepared frozen sections of small intestine and subjected them to immunohistochemical analysis using mAbs. Distributions of IgM^+ cells (**a**, **c**) and $BrdU^+$ cells (**b**, **d**) were assessed in LFVs found on the antimesenteric side (**a**, **b**) and in other areas (**c**, **d**). PP displays many $BrdU^+$ cells in the germinal center (**e**)

of lymphocytes congregating in rat LFVs are also c-kit$^+$ and IL-7R$^+$. Further, in agreement with what was observed by Mayrhofer and coworkers,[9] $\alpha\beta^+$ T cells appeared to be localized in the subepithelial regions of LFVs (Fig. 6b, f). However, we were unable to verify in the present study whether these rat LFVs containing undifferentiated lymphocytes represented clusters of extrathymic progenitor T cells, and this issue needs clarification in the future.

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Is there a role for apheresis in gastrointestinal disorders?

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APHERESIS is now a common therapeutic modality, with proven clinical benefits for several gastrointestinal disorders including inflammatory bowel disease (IBD),[1] pouchitis, intestinal Bechét's disease, alcoholic hepatitis and hepatitis C. Gastroenterologists have limited experience with apheresis, however, owing to the complexity of the process.

The etiology and pathogenesis of IBD remain obscure, but it is known that immunologic factors are involved and there is consensus that the inflamed mucosa of IBD patients are infiltrated by large numbers of inflammatory cells. The treatment of IBD with leukocyte apheresis is based on the hypothesis that removal of the circulating cells involved in intestinal inflammation will re-establish the balance between uncontrolled and controlled mucosal inflammation. The efficacy of leukocyte apheresis might not be restricted to cell removal, however, as contact activation of cells with the treatment apparatus or a change in proportions of regulatory T cells might also have immunomodulatory effects.

A single session of leukocyte apheresis removes approximately 3×10^9–10×10^9 granulocytes, which may be only ~10% of those circulating in the peripheral blood. A temporal decrease in the numbers of peripheral blood leukocytes and/or granulocytes is observed during the procedure, followed by a rapid return to the pretreatment level or higher. This rise in leukocyte numbers could be explained by the mobilization of leukocytes from the marginal pool. This results in a decrease in activated leukocytes and an increase in immature leukocytes in the peripheral blood. Under inflammatory conditions, the number and survival time of granulocytes are increased, and their serial removal could contribute to the anti-inflammatory effect of apheresis. Apheresis results in downregulation of several adhesion molecules and chemokine receptors on leukocytes, which might inhibit their migration to inflamed tissues. Furthermore, the capacity of peripheral leukocytes to produce proinflammatory cytokines is suppressed. The concomitant depletion of platelets could also contribute to their immunomodulatory effects. Thus, the overall effects of apheresis might be due to reduced levels of activated leukocytes followed by an increase in the numbers of immature leukocytes; diminished leukocyte migration to the inflamed tissue; and a reduction in the levels of proinflammatory cytokines. Studies are needed to identify the exact mode of action of apheresis.

Several devices have been developed to improve the efficiency and simplify the process of leukocyte removal. Granulocyte and monocyte adsorption apheresis (GCAP), an adsorptive type of extracorporeal apheresis, is performed with the Adacolumn® apheresis system (Japan Immunoresearch Laboratory, Japan). The device contains cellulose-coated acetate beads, and adsorbs ~65% of granulocytes, 55% of monocytes, 2% of lymphocytes and a small number of platelets from the blood. A single procedure removes a total of $\sim4.0\times10^9$ granulocytes and monocytes from the blood circulation. Leukocytapheresis (LCAP) performed with the Cellsorba® system (Asahi Medical, Japan), uses a filter consisting of nonwoven hollow fibers to trap leukocytes. LCAP nonselectively removes $\sim13.0\times10^9$ leukocytes and 5.2×10^{11} platelets from the circulating blood during one session of treatment. The removal efficacy of leukocytes passing through the filter is close to 100%. In both procedures, blood inlet and outlet are via suitable veins in the bilateral forearms of the patient and small amounts of anticoagulants are added to the circulation. Sessions are performed at a flow rate of 30–50 ml/min for 1 h and weekly for 5–10 sessions. GCAP and LCAP have both been approved by the Japanese Government for the treatment of ulcerative colitis. LCAP is also approved for the treatment of rheumatoid arthritis.

Several uncontrolled trials of plasmapheresis[2] and lymphocyte apheresis[3] have shown efficacy in active Crohn's disease; however, it has also been reported that the latter was unable to prevent relapse in those patients with corticosteroid-induced remission.[4] The results of previous studies in Crohn's disease using the centrifugal

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method to remove leukocytes are controversial. Studies of GCAP and LCAP in Crohn's disease are limited and only small pilot studies have been reported. Weekly treatment with GCAP or LCAP was, however, reported to be effective in >50% of treated patients.[5]

Nationwide multicenter trials have been carried out in Japan to assess the efficacy and safety of GCAP or LCAP compared with corticosteroid treatment in patients with active ulcerative colitis refractory to conventional medication.[6,7] GCAP and LCAP showed clinical improvement in ~66% of patients, which was significantly higher than in patients treated with corticosteroids. In addition, significantly fewer adverse effects were seen in the GCAP and LCAP treatment groups; only mild adverse effects such as dizziness and nausea were observed in ~20% of the patients.

A double-blind controlled trial of LCAP showed that weekly treatment was more efficacious and safer than sham apheresis treatment; clinical improvement was shown in 80% of LCAP-treated patients compared with 33% of the sham-treated patients.[8] A significant advantage was demonstrated, but the study was rather small (10 patients per treatment group), performed for active ulcerative colitis refractory to conventional drug therapy, and most patients were also receiving simultaneous corticosteroids.

The efficacy of GCAP in patients with active ulcerative colitis who were not on concomitant corticosteroids has also been reported.[9] More than 80% of steroid-naive patients showed a response to GCAP.

Weekly GCAP or LCAP induced remission in 3–4 weeks in ~66% of patients with moderate to severe active ulcerative colitis. Moreover, only minor side effects such as nausea, fever and rashes were observed in ~20% of the patients.

We have found that triweekly treatment with GCAP is more effective than weekly treatment, and therefore shortens the time needed to achieve remission without increasing the incidence of side effects (T Hibi and A Sakuraba, unpublished data). Furthermore, in a retrospective analysis, we found that weekly GCAP therapy was effective in patients who were steroid-refractory as well as in patients with steroid-dependent ulcerative colitis, thus allowing the dosage of corticosteroids to be reduced in these patients.[10] In another preliminary study we confirmed that biweekly treatment of GCAP was as effective as 6-mercaptopurine in maintaining remission of ulcerative colitis. Several reports have also been published on the efficacy of GCAP in pediatric patients with ulcerative colitis, for whom treatment with corticosteroids should ideally be avoided.

The standard therapeutic strategies used to treat IBD are reliant on drugs and carry with them associated adverse side effects. A treatment that is highly effective and safe has been long awaited and leukocyte apheresis might be the right candidate. This treatment should be considered as a nonpharmacologic adjunct to conventional therapy, however, as a single round of GCAP or LCAP will cost nearly US$1,000. At present, its use should be limited to those patients who are steroid-refractory, or for whom corticosteroids are contraindicated.

Preliminary studies indicate that GCAP or LCAP is efficacious and safe in patients with IBD. To obtain maximum efficacy, we recommend that it should be performed triweekly for a total of 6–10 sessions. To prove the efficacy and assess the most efficient frequency of apheresis, further larger controlled studies should be conducted.

Supplementary information, in the form of a figure is available online on the *Nature Clinical Practice Gastroenterology & Hepatology* website.

GLOSSARY

APHERESIS
The removal of blood from the body and withdrawal of a specific component. Blood is retransfused to the body once the process is complete

Competing interests
The authors declared they have no competing interests.

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Abnormally Differentiated Subsets of Intestinal Macrophage Play a Key Role in Th1-Dominant Chronic Colitis through Excess Production of IL-12 and IL-23 in Response to Bacteria[1]

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Disorders in enteric bacteria recognition by intestinal macrophages (Mϕ) are strongly correlated with the pathogenesis of chronic colitis; however the precise mechanisms remain unclear. The aim of the current study was to elucidate the roles of Mϕ in intestinal inflammation by using an IL-10-deficient (IL-10$^{-/-}$) mouse colitis model. GM-CSF-induced bone marrow-derived Mϕ (GM-Mϕ) and M-CSF-induced bone marrow-derived Mϕ (M-Mϕ) were generated from bone marrow CD11b$^+$ cells. M-Mϕ from IL-10$^{-/-}$ mice produced abnormally large amounts of IL-12 and IL-23 upon stimulation with heat-killed whole bacteria Ags, whereas M-Mϕ from wild-type (WT) mice produced large amounts of IL-10 but not IL-12 or IL-23. In contrast, IL-12 production by GM-Mϕ was not significantly different between WT and IL-10$^{-/-}$ mice. In ex vivo experiments, cytokine production ability of colonic lamina propria Mϕ (CLPMϕ) but not splenic Mϕ from WT mice was similar to that of M-Mϕ, and CLPMϕ but not splenic Mϕ from IL-10$^{-/-}$ mice also showed abnormal IL-12p70 hyperproduction upon stimulation with bacteria. Surprisingly, the abnormal IL-12p70 hyperproduction from M-Mϕ from IL-10$^{-/-}$ mice was improved by IL-10 supplementation during the differentiation process. These results suggest that CLPMϕ and M-Mϕ act as anti-inflammatory Mϕ and suppress excess inflammation induced by bacteria in WT mice. In IL-10$^{-/-}$ mice, however, such Mϕ subsets differentiated into an abnormal phenotype under an IL-10-deficient environment, and bacteria recognition by abnormally differentiated subsets of intestinal Mϕ may lead to Th1-dominant colitis via IL-12 and IL-23 hyperproduction. Our data provide new insights into the intestinal Mϕ to gut flora relationship in the development of colitis in IL-10$^{-/-}$ mice. *The Journal of Immunology,* **2005, 175: 6900–6908.**

Macrophages (Mϕ),[3] the major population of tissue-resident mononuclear phagocytes, play key roles in bacterial recognition and elimination as well as in polarization of innate and adaptive immunities. Mϕ are activated by microbial pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors, such as TLRs (1, 2), and produce proinflammatory cytokines such as IL-12 and IL-23, thereby leading to development of Th1 immunity (3). Besides these classical antibacterial immune roles, it has recently become evident that Mϕ also play important roles in homeostasis maintenance, such as inflammation dampening, via production of anti-inflammatory cytokines such as IL-10 and TGF-β, debris scavenging, angiogenesis, and wound repair (4–6). IL-10 and IL-12 are two key players in these processes, usually acting in opposition, with IL-10 inhibiting IL-12 production. Therefore, loss of balance between IL-12 and IL-10 can lead to disproportionate pathology or immunosuppression.

Although precise etiologies of inflammatory bowel diseases (IBDs) including Crohn's disease and ulcerative colitis remain unclear, pathogenic roles of the gut flora in initiation and perpetuation of intestinal inflammation have been proposed (7). Recently, it has become evident that abnormal innate immune responses to bacteria are responsible for the pathogenesis of IBD. For instance, mutations in nucleotide-binding oligomerization domain (NOD)2 highly correlated with disease incidence in a substantial subgroup of patients with Crohn's disease (8, 9). NOD2 mutant Mϕ were reported to produce large amounts of IL-12 in response to stimulation with microbial components, compared with wild-type (WT) cells, and to promote Th1 immunity (10). Thus, disorders in bacterial recognition by Mϕ strongly correlate with pathogenesis of IBDs (11–13).

IL-10-deficient (IL-10$^{-/-}$) mice develop spontaneous chronic colitis and are widely used as a colitis animal model for human IBDs (14). IL-10$^{-/-}$ mice show Th1 polarized immunity upon exposure to bacteria, whereas germfree conditions prevent development of intestinal inflammation (15), and treatment with antibiotics attenuates intestinal inflammation (16, 17). These facts suggest that enteric bacteria play essential roles in onset and development of colitis in IL-10$^{-/-}$ mice, similar to human IBDs. Recently, the following pathogenic aspects of Mϕ in IL-10$^{-/-}$ mice have been reported: APC such as Mϕ and dendritic cells (DC), from IL-10$^{-/-}$ mice were potent activators of Th1 responses

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[3] Abbreviations used in this paper: Mϕ, macrophage; WT, wild type; DC, dendritic cell; IBD, inflammatory bowel disease; NOD, nucleotide-binding oligomerization domain; BM, bone marrow; M-Mϕ, M-CSF-induced BM-derived Mϕ; GM-Mϕ, GM-CSF-induced BM-derived Mϕ; PGN, peptidoglycan; MDP, muramyl-dipeptide; PAMP, pathogen-associated molecular pattern; MOI, multiplicity of infection; CBA, cytometric beads array; CLPMϕ, colonic lamina propria macrophage.

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from naive or immune T cells (18, 19); Mϕ from IL-$10^{-/-}$ mice were hyperreactive to microbial components (20); and Mϕ depletion prevented chronic colitis in IL-$10^{-/-}$ mice (21). Based on these reports, Mϕ and DC are considered to play key roles in the pathogenesis of colitis in IL-$10^{-/-}$ mice, although mechanisms for bacterial recognition by APC that induces a Th1 bias and development of intestinal inflammation remain unclear. Previous studies suggested that IL-12 was crucial for development of colitis in IL-$10^{-/-}$ mice because mice deficient for both IL-10 and IL-12p40 showed no intestinal inflammation, and treatment with anti-IL-12p40 Abs markedly reduced intestinal inflammation (22, 23). Until now, however, how IL-10 deficiency affects IL-12 production from Mϕ in mice has not been thoroughly analyzed.

In the present study, we examined whether IL-10-deficient conditions affected differentiation and functions of bone marrow (BM)-derived Mϕ subsets and intestinal Mϕ, and investigated how bacteria recognition by Mϕ induced a Th1 polarization and intestinal inflammation in IL-$10^{-/-}$ mice. We found that M-CSF-induced BM-derived Mϕ (M-Mϕ) and intestinal Mϕ, but not GM-CSF-induced BM-derived Mϕ (GM-Mϕ) or splenic Mϕ from IL-$10^{-/-}$ mice showed abnormal hyperproduction of IL-12 and IL-23 upon stimulation with bacteria. More importantly, our results suggested that endogenous IL-10 played an essential role in differentiation of the anti-inflammatory Mϕ subset induced by M-CSF.

Materials and Methods

Reagents

Recombinant mouse GM-CSF, M-CSF, and IL-10 were purchased from R&D Systems. Gel filtration grade LPS (*Escherichia coli* O111:B4), muramyl-dipeptide (MDP), and *Staphylococcus aureus* peptidoglycan (PGN) were obtained from Sigma-Aldrich. Pam_3CSK_4 and *E. coli* ssDNA were obtained from InvivoGen.

Bacteria heat-killed Ags

A Gram-negative nonpathogenic strain of *E. coli* (25922; American Type Culture Collection (ATCC)) was cultured in Luria-Bertani medium, and a Gram-positive strain of *Enterococcus faecalis* (29212; ATCC) was cultured in brain-heart infusion medium. Bacteria were harvested and washed twice with ice-cold PBS. Then, bacterial suspensions were heated at 80°C for 30 min, washed, resuspended in PBS, and stored at −80°C. Complete killing was confirmed by 72 h incubation at 37°C on plate medium.

Mice

Specific pathogen-free WT C57BL/6J mice were purchased from Charles River Breeding Laboratories. WT and IL-$10^{-/-}$ (C57BL/6J background) mice were housed under specific pathogen-free conditions at the animal center of Kitasato Institute Hospital and Keio University (Tokyo, Japan). All experiments using mice were approved by and performed according to the guidelines of the animal committee of Keio University and Kitasato Institute Hospital.

Preparation of BM-derived Mϕ

BM cells were isolated from femora of 7- to 12-wk-old mice. After separation of BM mononuclear cells by gradient centrifugation, $CD11b^+$ cells were purified using a magnetic cell separation system (MACS; Miltenyi Biotec) with anti-mouse CD11b microbeads. To generate BM-derived GM-Mϕ and M-Mϕ, $CD11b^+$ cells (5×10^5 cells/ml) were cultured for 7 days with GM-CSF (20 ng/ml) and M-CSF (20 ng/ml), respectively. In some experiments, to determine effects of IL-10 during differentiation of M-Mϕ, BM $CD11b^+$ cells from IL-$10^{-/-}$ mice were cultured with M-CSF and various concentrations of exogenous IL-10. After differentiation, cells were washed three times with HBSS to remove residual IL-10.

Flow cytometry analysis

Day 7 BM-derived GM-Mϕ and M-Mϕ were harvested with EDTA and washed with ice-cold PBS. Then, cells were preincubated with 1 μg/ml mAb CD16/CD32 to block FcγR, and stained with mAbs CD11b, Gr-1, TLR4/MD2, or TLR2 (all from eBiosciences), mAbs CD80 or CD86 (both from BD Pharmingen) or their isotype control Abs for 20 min at 4°C. After staining, cells were washed with PBS, stained with propidium iodide, and analyzed using a FACSCalibur (BD Pharmingen). The CellQuest software was used for data analysis.

Activation of BM-derived Mϕ by PAMPs

Day 7 BM-derived GM-Mϕ and M-Mϕ were harvested, plated on 96-well tissue culture plates (1×10^5 cells/well) in RPMI 1640 medium supplemented with 10% FBS, antibiotics, and 20 ng/ml GM-CSF or M-CSF, and incubated for 12–16 h. Before each experiment, Mϕ were washed three times with HBSS (Sigma-Aldrich) to completely remove secreted or supplemented cytokines from the supernatant, and were stimulated with either LPS (100 ng/ml), PGN (2 μg/ml), Pam_3CSK_4 (500 ng/ml), *E. coli* ssDNA (10 μg/ml), MDP (10 μg/ml), or heat-killed bacteria (multiplicity of infection (MOI) = 100) for 24 h. Culture supernatants were collected, passed through 0.22-μm pore size filters, and then stored at −80°C until the cytokine assay.

Isolation of colonic lamina propria Mϕ (CLPMϕ) and splenic Mϕ

Lamina propria mononuclear cells were isolated using a modified protocol as previously described (25). Briefly, mice were sacrificed, and colonic tissues were removed. Isolated colons were washed with HBSS, dissected into small pieces, and incubated in HBSS containing 2.5% FBS and 1 mM DTT (Sigma-Aldrich) to remove any mucus. Then, the pieces were incubated in HBSS containing 1 mM EDTA (Sigma-Aldrich) twice for 20 min each at 37°C, washed three times with HBSS, and incubated in HBSS containing 1 mM collagenase type IV (Sigma-Aldrich) for 2 h at 37°C. Digested tissues were filtered and washed twice with HBSS. Isolated cells were resuspended in 40% Percoll (Pharmacia Biotech), layered onto 75% Percoll, and centrifuged at 2000 rpm for 20 min. Cells were recovered from the interphase and washed with PBS. CLPMϕ and splenic Mϕ were purified by positive selection from lamina propria mononuclear cells or unfractionated splenocytes using MACS CD11b microbeads (Miltenyi Biotec) as previously described (24, 26).

Activation of Mϕ by whole bacteria Ags

BM-derived Mϕ, and isolated CLPMϕ and splenic Mϕ were plated on 96-well tissue culture plates (1×10^5 cells/well) in RPMI 1640 medium supplemented with 10% FBS and antibiotics, and stimulated by heat-killed bacterial Ags (MOI = 100) for 24 h at 37°C. Culture supernatants were collected, passed through a 0.22-μm pore size filter, and stored at −80°C until the cytokine assay.

Cytokine assay

A mouse inflammatory cytometric beads array (CBA) kit (BD Pharmingen) was used for cytokine measurements, according to the manufacturer's instructions. Samples were analyzed using a FACSCalibur (BD Pharmingen).

Quantitative RT-PCR

After 8 h of stimulation by bacterial Ags, total RNA was isolated from Mϕ using an RNeasy Mini kit (Qiagen). In some experiments, RNA was isolated from colonic tissues and spleen. cDNA was synthesized with OmniScript reverse transcriptase (Qiagen). For quantitative RT-PCR, TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays for murine IL-12p35, IL-12p40, IL-23p19, M-CSF, GM-CSF, and β-actin (Applied Biosystems) were used. PCR amplifications were conducted in a thermocycler DNA Engine (OPTICON2; MJ Research). Cycling conditions for PCR amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Statistical analysis

Statistical significance of differences between two groups was tested using a Student's *t* test. For comparison of more than two groups, ANOVA was used. If the ANOVA was significant, Dunnett's multiple comparison test or Scheffe's test were used as a post hoc test.

Results

GM-Mϕ and M-Mϕ derived from BM $CD11b^+$ cells from IL-$10^{-/-}$ mice do not differ significantly from those derived from WT mice in morphology and cell surface Ag expressions

When BM-derived $CD11b^+$ cells from WT mice were cultured in M-CSF or GM-CSF for 7 days, they showed morphological

changes characteristic of Mϕ such as increases in size and adherence, and were stained with nonspecific esterase (data not shown). As shown in Fig. 1*A*, GM-CSF and M-CSF induced differentiation of BM CD11b$^+$ cells into two distinct subsets of adherent Mϕ, which corresponded to human GM-Mϕ and M-Mϕ (27). GM-Mϕ derived from BM CD11b$^+$ cells had a rounded morphology and possessed dendrites, similar to DCs. In contrast, M-Mϕ derived from BM CD11b$^+$ cells had an elongated spindle-like morphology. FACS analysis revealed that GM-Mϕ expressed higher levels of MHC class II molecules and costimulatory molecules CD80 compared with M-Mϕ (Fig. 1*B* and Table I). Expression of Gr-1 was also different between them; i.e., GM-Mϕ but not M-Mϕ expressed Gr-1 (Fig. 1*B* and Table I). However, CD11c, a DC marker, was not expressed on both Mϕ (data not shown).

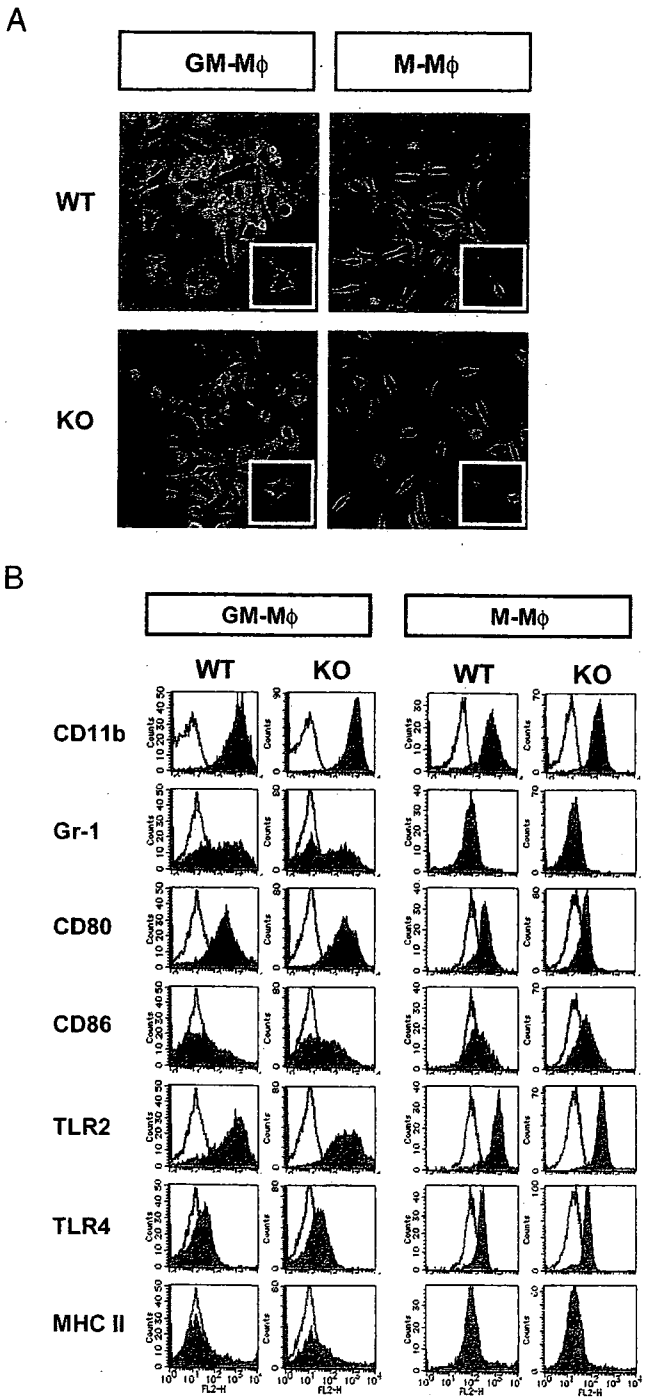

FIGURE 1. In vitro differentiated Mϕ from WT and IL-10$^{-/-}$ mice do not differ in morphology and surface marker expressions. *A*, BM CD11b$^+$ cells from WT and IL-10$^{-/-}$ mice were polarized into Mϕ with GM-CSF or M-CSF for 7 days. *B*, Polarized Mϕ from WT and IL-10$^{-/-}$ mice (KO) were stained with the indicated mAbs and analyzed by flow cytometry. Profiles of specific Ab staining (shaded histograms) and staining with isotype controls (open histograms) are shown. Data shown are representative of five independent experiments.

Next, we compared the two Mϕ subsets derived from IL-10$^{-/-}$ BM CD11b$^+$ cells with those from WT BM CD11b$^+$ cells. As shown in Fig. 1*A*, both GM-Mϕ and M-Mϕ from IL-10$^{-/-}$ BM CD11b$^+$ cells showed normal morphological characteristics. Flow cytometric analysis further revealed that these Mϕ subsets from IL-10$^{-/-}$ mice did not differ from those in WT mice in their cell surface Ag expressions (Fig. 1*B* and Table I). These results suggest that GM-Mϕ and M-Mϕ from IL-10$^{-/-}$ mice are similar to those in WT mice, at least in terms of morphology and cell surface Ag expression.

BM-derived M-Mϕ from WT mice show an anti-inflammatory phenotype in response to PAMPs and whole bacterial Ags

To determine the immunological responses of GM-Mϕ and M-Mϕ from WT mice to PAMPs stimulation, Mϕ were stimulated with various kinds of PAMPs for 24 h, and production levels of IL-12p70 and IL-10 in culture supernatant were measured. As shown in Fig. 2*A*, none of the stimuli tested induced IL-12p70 production from both Mϕ in WT mice. In contrast, the TLR4 ligand LPS, TLR2 ligands PGN and Pam_3CSK_4, and TLR9 ligand *E. coli* ssDNA induced IL-10 production by these Mϕ, although the amounts produced were higher in M-Mϕ compared with GM-Mϕ. The NOD2 ligand MDP did not induce either IL-12p70 or IL-10 in either subset from WT mice.

Next, we examined the effects of whole bacterial Ags on these Mϕ. In contrast to stimulation with PAMPs, stimulation of GM-Mϕ with heat-killed *E. coli* and *E. faecalis* induced IL-12p70 production (Fig. 2*B*). However, M-Mϕ from WT mice did not produce IL-12p70, but did produce large amounts of IL-10 in response to the whole bacterial Ags (Fig. 2*B*). These results suggested that M-Mϕ, but not GM-Mϕ, in WT mice act as anti-inflammatory Mϕ in the recognition of bacteria.

BM-derived M-Mϕ but not GM-Mϕ from IL-10$^{-/-}$ mice reveal abnormal hyperproduction of IL-12 and IL-23 in response to whole bacterial Ags

We next examined the effects of PAMPs and whole bacteria Ags on GM-Mϕ and M-Mϕ from IL-10$^{-/-}$ mice. In contrast to the results obtained from Mϕ in WT mice, IL-10$^{-/-}$ Mϕ produced IL-12p70 by stimulation with LPS or Pam_3CSK4, although the amounts were very low, and no significant differences were observed between GM-Mϕ and M-Mϕ (Fig. 3*A*). The use of 10-fold higher concentrations of these PAMPs did not induce higher levels of IL-12p70 either (data not shown).

Upon whole bacteria stimulation, such as with heat-killed *E. coli* and *E. faecalis*, GM-Mϕ from IL-10$^{-/-}$ mice produced similar levels of IL-12p70 to WT GM-Mϕ, although they lacked IL-10 production ability (Fig. 3*B*). Surprisingly, in contrast to WT M-Mϕ, M-Mϕ from IL-10$^{-/-}$ mice produced significantly large amounts of IL-12p70 upon stimulation with whole bacterial Ags (Fig. 3*B*). In addition, a lower dose of the whole bacteria Ag (MOI = 10) also induced abnormally large IL-12p70 production (data not shown).

To further confirm this abnormal IL-12p70 hyperproduction by IL-10$^{-/-}$ M-Mϕ, gene transcriptions of IL-12p35, p40, and IL-23p19 were analyzed using real-time quantitative PCR. Results revealed that basal expressions of these genes before stimulation

Table I. *Expression of surface Ags on BM-derived different subsets of macrophage*[a]

	ΔMFI			
	WT		KO	
	GM-Mϕ	M-Mϕ	GM-Mϕ	M-Mϕ
CD11b	1174.2 ± 155.5*	509.5 ± 58.7	1229.7 ± 15.5*	388.2 ± 58.5
Gr-1	93.1 ± 3.4*	0.0 ± 0.0	63.7 ± 7.6*	0.0 ± 0.0
CD80	290.4 ± 34.2*	73.4 ± 27.5	383.0 ± 4.1*	38.6 ± 2.5
CD86	14.5 ± 1.6	36.2 ± 8.1	26.7 ± 0.9	35.9 ± 3.4
TLR2	348.6 ± 18.6	342.4 ± 118.8	386.0 ± 48.8	220.0 ± 4.4
TLR4/MD2	7.6 ± 2.1	18.4 ± 0.8	8.7 ± 4.3	18.8 ± 4.8
MHC-II	20.6 ± 3.7*	1.7 ± 0.4	24.4 ± 5.9*	1.5 ± 0.3

[a] Data indicated as ΔMFI (geometric mean fluorescence intensity (MFI) of each Ab staining minus MFI of control IgG staining), and are expressed as mean ± SEM of five independent experiments. *, $p < 0.01$ compared with M-Mϕ (Sheffe's test)

were almost undetectable in GM-Mϕ and M-Mϕ from both WT and IL-$10^{-/-}$ mice (data not shown). Upon stimulation with *E. coli*, their expressions were detected in GM-Mϕ from both WT and IL-$10^{-/-}$ mice, and expression levels in GM-Mϕ from IL-$10^{-/-}$ mice were ~3- to 4-fold higher than levels in WT mice (Fig. 3*C*). In contrast, expression levels of those genes were quite different in M-Mϕ from WT and IL-$10^{-/-}$ mice. These transcripts remained at very low levels in WT M-Mϕ even after stimulation; however, IL-$10^{-/-}$ M-Mϕ showed markedly high expressions of IL-12 and IL-23 genes (Fig. 3*C*).

These results suggest that GM-Mϕ can produce IL-12 and IL-23 in response to bacterial stimuli, whereas M-Mϕ cannot produce these cytokines but rather produce anti-inflammatory cytokine IL-10. The results also suggested that endogenous IL-10 strongly contributes to inhibition of IL-12p70 production in M-Mϕ but not GM-Mϕ. Thus, IL-$10^{-/-}$ M-Mϕ may contribute to Th1 polarization by producing IL-12p70 upon stimulation with bacteria.

Exogenous IL-10 supplementation at the time of stimulation with whole bacteria Ag inhibits abnormal IL-12p70 hyperproduction by IL-$10^{-/-}$ M-Mϕ

Because M-Mϕ, but not GM-Mϕ, from IL-$10^{-/-}$ mice showed abnormal IL-12 and IL-23 hyperproduction in response to stimulation with *E. coli*, we further examined how absence of IL-10 led to IL-12 and IL-23 hyperproduction from M-Mϕ. M-Mϕ from IL-$10^{-/-}$ mice were stimulated with heat-killed bacterial Ags concomitant with a supplementation of exogenous IL-10 (Fig. 4*A*). Abnormal IL-12p70 hyperproduction by M-Mϕ from IL-$10^{-/-}$ mice was completely suppressed by exogenous IL-10 in a dose-dependent manner (Fig. 4*B*). In addition, IL-10 had similar inhibitory effects on productions of other proinflammatory cytokines (TNF-α and IL-6) from IL-$10^{-/-}$ Mϕ. These findings were consistent with a previous report showing that IL-10 inhibited production of several proinflammatory cytokines by Mϕ, including IL-12 (28). These results suggest that IL-10 inhibits the production of proinflammatory cytokines by M-Mϕ in response to stimulation with whole bacteria Ags.

Exogenous IL-10 supplementation during the differentiation process attenuates abnormal IL-12p70 hyperproduction by IL-$10^{-/-}$ M-Mϕ

As described, IL-10 production by M-Mϕ in response to bacteria is important for suppression of IL-12p70 production, as well as for other cytokine productions; however, how IL-10 acts on the differentiation process of BM CD11b$^+$ cells still remains unclear. Therefore, we examined the role of IL-10 in differentiation of M-Mϕ from BM CD11b$^+$ cells. BM CD11b$^+$ cells from IL-$10^{-/-}$ mice were differentiated into M-Mϕ with M-CSF in the presence of exogenous IL-10. Polarized M-Mϕ were thoroughly washed to remove any residual IL-10, and then stimulated by heat-killed *E. coli* without exogenous IL-10 (Fig. 5*A*). Interestingly, M-Mϕ differentiated from IL-$10^{-/-}$ mice in the presence of M-CSF and exogenous IL-10 was unable to induce large amounts of IL-12p70 production in response to stimulation with *E. coli*, although exogenous IL-10 was removed from the culture supernatant before bacterial Ags were added (Fig. 5*B*).

Contrary to the production of IL-12p70, the other proinflammatory cytokines (TNF-α and IL-6) were only partially or not significantly suppressed by IL-10 supplementation during the differentiation process. Moreover, consistent with IL-12p70 results, levels of IL-12p35, IL-12p40, and IL-23p19 mRNA transcripts were significantly reduced in IL-$10^{-/-}$ M-Mϕ differentiated in the presence of exogenous IL-10 (Fig. 5*C*). These results suggest that endogenous IL-10 is an essential cytokine for functional differentiation of M-Mϕ, especially for maturation of the phenotype as anti-inflammatory Mϕ, which cannot produce IL-12p70 while producing large amounts of IL-10.

CLPMϕ but not splenic Mϕ show functional similarity to BM-derived M-Mϕ in the production of IL-10 and IL-12

It became evident that in vitro differentiated M-Mϕ, but not GM-Mϕ, from IL-$10^{-/-}$ mice showed abnormal responses to whole bacteria Ags. Hence, we further analyzed CLPMϕ from WT and IL-$10^{-/-}$ mice to investigate how intestinal Mϕ act in vivo and contribute to trigger and develop Th1-dominant inflammation in IL-$10^{-/-}$ mice. CLPMϕ from WT mice did not produce IL-12p70 upon stimulation with heat-killed *E. coli*, but instead produced large amounts of IL-10, and the levels were similar to those of BM-derived M-Mϕ (Fig. 6*A*). In contrast, CLPMϕ from IL-$10^{-/-}$ mice produced significantly larger amounts of IL-12p70 in response to stimulation with the bacteria, and levels were similar to those of BM-derived M-Mϕ from IL-$10^{-/-}$ mice (Fig. 6*A*). In contrast to CLPMϕ, abnormal IL-12p70 hyperproductions by bacteria were not observed in splenic Mϕ from IL-$10^{-/-}$ mice, although TNF-α induction levels were similar to those of CLPMϕ (Fig. 6*A*). Similar results were obtained when CLPMϕ from WT and IL-$10^{-/-}$ mice were stimulated with heat-killed *E. faecalis* (data not shown).

These results suggest that CLPMϕ revealed a functional similarity to that of M-Mϕ, that CLPMϕ from WT mice act as anti-inflammatory Mϕ via production of large amounts of IL-10, and that CLPMϕ from IL-$10^{-/-}$ mice contribute to the development of Th1-dominant colitis via the abnormal hyperproduction of IL-12p70.

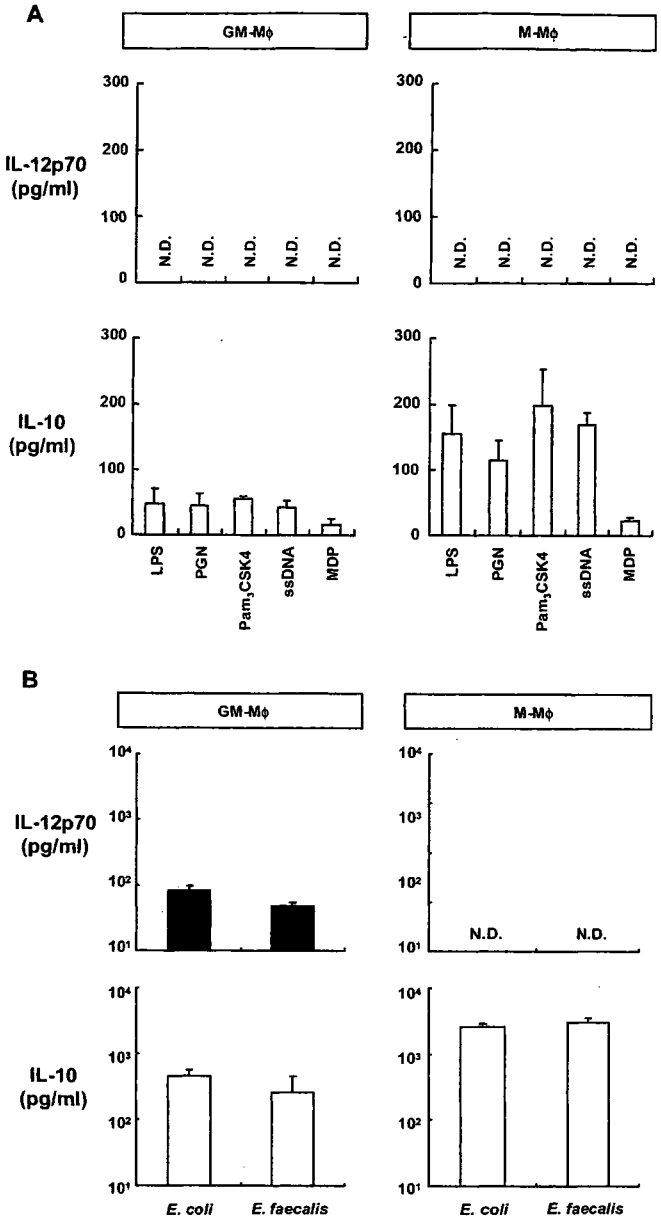

FIGURE 2. BM-derived M-Mϕ from WT mice reveal an anti-inflammatory phenotype in response to PAMPs and whole bacterial Ags. *A*, Polarized GM-Mϕ and M-Mϕ (1×10^5 cells) from WT mice were stimulated with LPS (100 ng/ml), PGN (2 μg/ml), Pam_3CSK_4 (500 ng/ml), *E. coli* ssDNA (10 μg/ml), or MDP (10 μg/ml) for 24 h. The amounts of IL-12p70 and IL-10 in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from five independent experiments for LPS, PGN, Pam_3CSK_4, and ssDNA and three independent experiments for MDP. *B*, GM-Mϕ and M-Mϕ (1×10^5 cells) from WT mice were stimulated with a heat-killed Gram-negative strain of *E. coli* or a heat-killed Gram-positive strain of *E. faecalis* (MOI = 100). Data are expressed as the mean ± SEM from seven independent experiments. N.D., Not detected.

Therefore, we next assessed the expression of M-CSF and GM-CSF in colonic tissues and spleen because M-CSF and GM-CSF are different in their activity to induce anti-inflammatory Mϕ as described. M-CSF to GM-CSF expression level ratios in murine colonic tissues were dramatically higher than in spleen (Fig. 6*B*).

These results demonstrate that M-CSF rich environment in colonic tissues may contribute the differentiation of intestinal Mϕ into anti-inflammatory M-Mϕ phenotype.

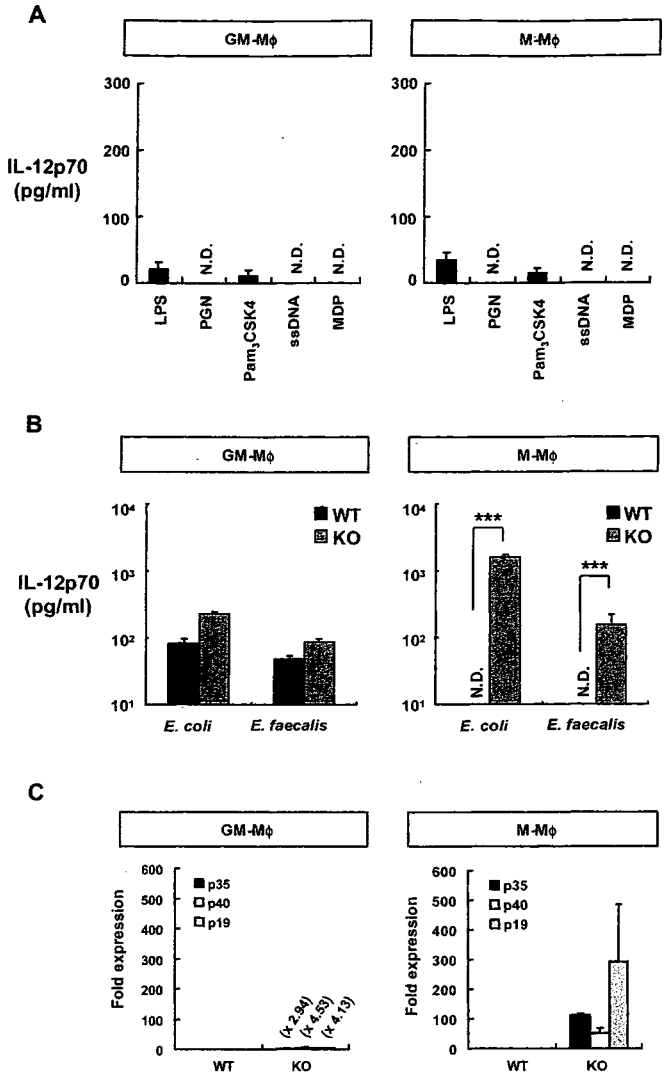

FIGURE 3. BM-derived M-Mϕ from IL-$10^{-/-}$ mice produced large amounts of IL-12p70 in response to whole bacteria Ags. *A*, GM-CSF- or M-CSF-induced Mϕ (1×10^5 cells) from IL-$10^{-/-}$ mice (KO) were stimulated with LPS (100 ng/ml), PGN (2 μg/ml), Pam_3CSK_4 (500 ng/ml), *E. coli* ssDNA (10 μg/ml), or MDP (10 μg/ml) for 24 h. Data are expressed as the mean ± SEM from five independent experiments for LPS, PGN, Pam_3CSK_4, ssDNA and three independent experiments for MDP. *B*, GM-CSF- or M-CSF-induced Mϕ (1×10^5 cells) from WT and mice were stimulated with heat-killed *E. coli* or *E. faecalis* (MOI = 100) for 24 h. Data are expressed as the mean ± SEM from seven independent experiments. N.D., Not detected. ***, $p < 0.001$ compared with WT Mϕ (Student's *t* test). *C*, GM-Mϕ and M-Mϕ (1×10^5 cells) were stimulated by heat-killed *E. coli* (MOI = 100) for 8 h. The gene expressions of IL-12p35, IL-12p40, and IL-23p19 were analyzed by real-time quantitative PCR and normalized by the amount of β-actin transcripts. Data indicate the fold expression compared with WT mice and are expressed as the mean ± SEM from three independent experiments.

Discussion

The results we present revealed that CLPMϕ and M-CSF-induced M-Mϕ in WT mice produce large amounts of IL-10, but not IL-12 and IL-23 upon stimulation with whole bacteria Ags. In contrast, GM-CSF-induced GM-Mϕ in WT mice produce IL-12 and IL-23 despite of IL-10 production. In contrast, we first demonstrated that CLPMϕ and BM-derived M-Mϕ in IL-$10^{-/-}$ mice produced abnormal large amounts of IL-12 and IL-23 upon stimulation with bacteria, but splenic Mϕ and BM-derived GM-Mϕ in IL-$10^{-/-}$ mice were not significantly different from those in WT mice. These

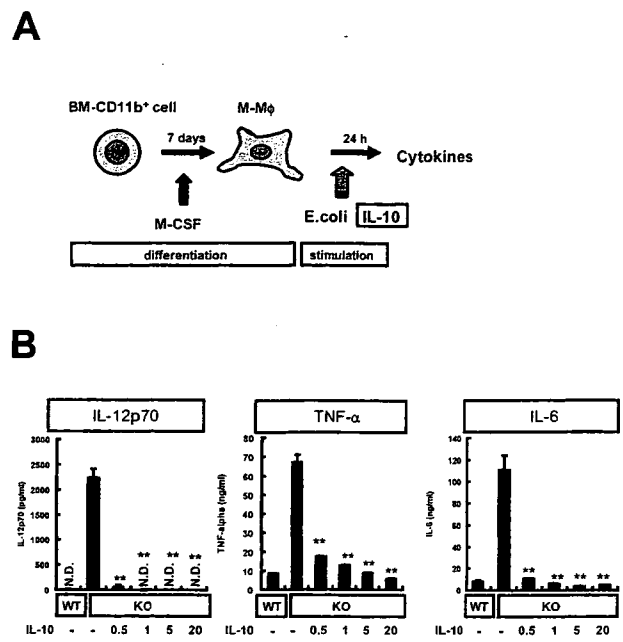

FIGURE 4. Exogenous IL-10 prevents the production of proinflammatory cytokines by M-Mϕ from IL-10$^{-/-}$ mice. *A*, Schema of the experiment. *B*, M-Mϕ (1×10^5 cells) from WT and IL-10$^{-/-}$ mice (KO) were stimulated with heat-killed *E. coli* (MOI = 100) for 24 h with or without various concentrations of exogenous IL-10. The amounts of IL-12p70, TNF-α, and IL-6 in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from three independent experiments. N.D., Not detected. **, $p < 0.01$ compared with IL-10$^{-/-}$ Mϕ without IL-10 supplementation (Dunnett's test).

results indicate that CLPMϕ usually acts as anti-inflammatory Mϕ, however, CLPMϕ in IL-10$^{-/-}$ mice play key roles in Th1-dominant chronic colitis through excess production of IL-12 and IL-23.

In the present study, we demonstrated that GM-Mϕ and M-Mϕ were different not only in morphology or cell surface Ag expression but also in the production of proinflammatory cytokines IL-12, IL-23, and anti-inflammatory cytokine IL-10 in response to heat-killed bacteria, such as *E. coli* and *E. faecalis*. Such differences in cytokine production by Mϕ generated under the influence of M-CSF and GM-CSF were also reported in human monocyte-derived Mϕ. Human monocyte-derived GM-Mϕ show potent Ag-presenting functions, produce IL-12p40 and IL-23p19, but none to low levels of IL-10 in response to mycobacteria and their components, and promote development of Th1 immunity (27, 29, 30). In contrast, human monocyte-derived M-Mϕ show low Ag-presenting activity and produce large amounts of IL-10 but no IL-12 or IL-23 (27, 29, 30). Thus, it is considered that these two subsets of Mϕ play opposite roles both in mice and humans; GM-Mϕ act as proinflammatory and M-Mϕ act as anti-inflammatory Mϕ in response to bacteria. In contrast to mice BM-derived GM-Mϕ, human monocyte-derived GM-Mϕ can produce IL-23 but not IL-12 (29). Reasons for differences between our mice study and previously reported human studies might be attributed to differences in the type of cells used (mouse BM-derived Mϕ and human monocyte-derived Mϕ) or in the stimulus used (*E. coli* and *E. faecalis* vs mycobacteria and their components).

Because the intestinal mucosa of the gut is always exposed to numerous enteric bacteria including both pathogenic and nonpathogenic bacteria, it is considered that the gut may possess regulatory mechanisms preventing excessive inflammatory responses. In fact, it was previously reported that human intestinal Mϕ does

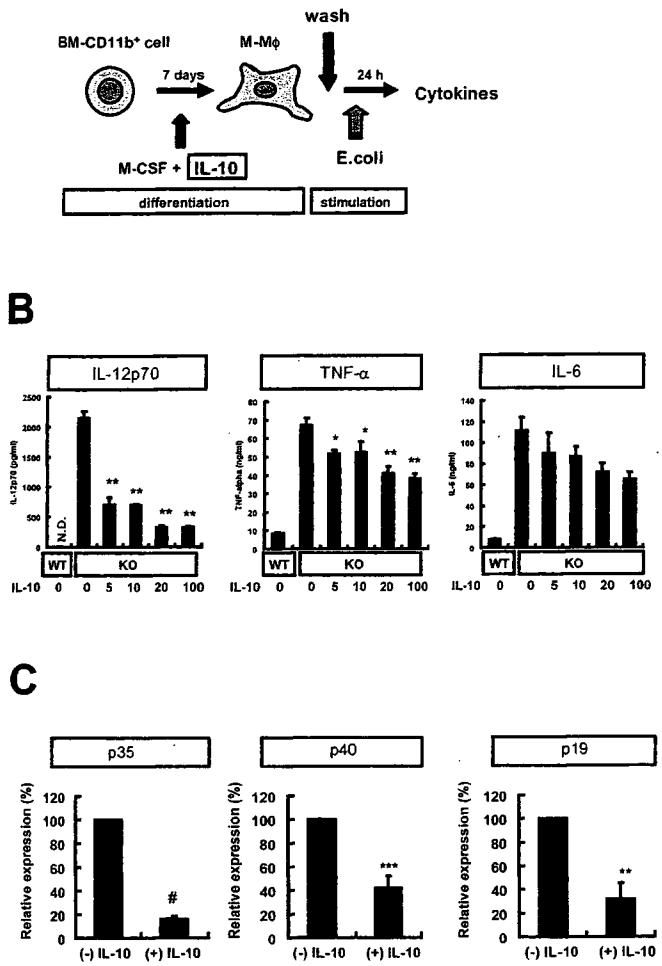

FIGURE 5. IL-10 supplementation during the differentiation process of M-Mϕ improves the abnormal IL-12 production upon stimulation with whole bacterial Ags. *A*, Schema of the experiment. *B*, BM CD11b$^+$ cells from IL-10$^{-/-}$ mice (KO) were differentiated with 20 ng/ml M-CSF and various concentrations of exogenous IL-10 (0–100 ng/ml) for 7 days. The M-Mϕ were harvested, seeded at 1×10^5 cells/well, and incubated for 15–16 h. The cells were washed three times to remove any residual cytokines and then stimulated by heat-killed *E. coli* (MOI = 100) for 24 h. The amounts of IL-12p70, TNF-α, and IL-6 in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from 10 independent experiments. N.D., Not detected. *, $p < 0.05$; **, $p < 0.01$ compared with IL-10$^{-/-}$ Mϕ differentiated without IL-10 supplementation (Dunnett's test). *C*, BM CD11b$^+$ cells from IL-10$^{-/-}$ (KO) were polarized with M-CSF (20 ng/ml) alone or M-CSF plus IL-10 (20 ng/ml). The polarized Mϕ were stimulated by heat-killed *E. coli* (MOI = 100) for 8 h. The mRNA expressions are shown as relative percentages of the levels in knockout mice without IL-10 supplementation. Data are expressed as the mean ± SEM from five independent experiments. **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.0001$ compared with levels in KO mice without IL-10 supplementation (Student's *t* test).

not express innate response receptors (31, 32). Although these cells retained their phagocytic and bacteriocidal functions, they did not produce proinflammatory cytokines in response to several inflammatory stimuli such as microbial components (31, 32). Thus, recent studies have suggested that Mϕ located in the intestinal mucosa play important roles in the maintenance of intestinal homeostasis by protecting the host from foreign pathogens (33) and regulating excess immune responses to enteric bacteria (32).

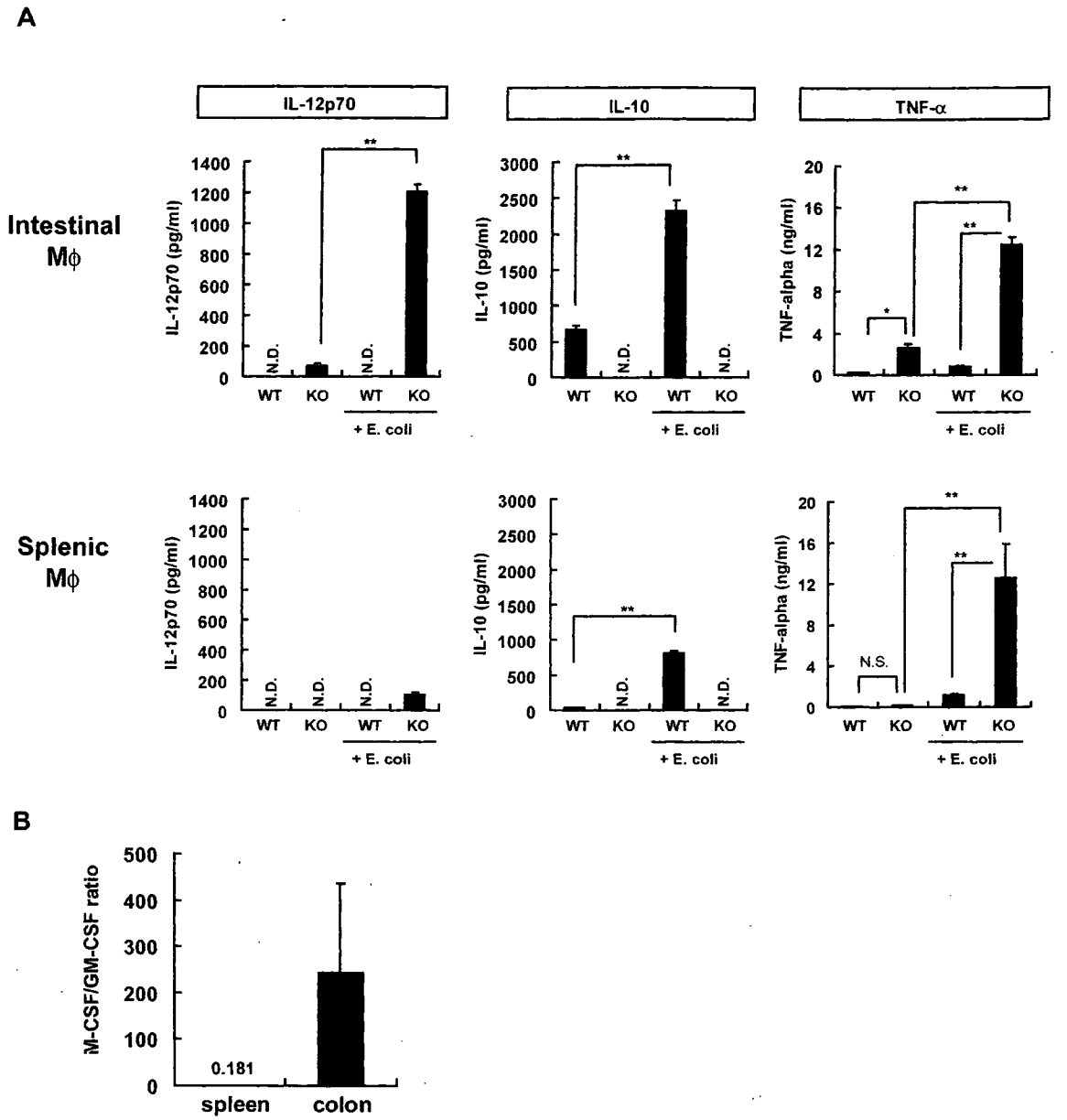

FIGURE 6. CLPMϕ, but not splenic Mϕ, from IL-$10^{-/-}$ mice produce a large amount of IL-12 upon stimulation with whole bacterial Ags. *A*, CLPMϕ and splenic Mϕ were isolated from WT and IL-$10^{-/-}$ mice (KO). Mϕ were seeded at 1×10^5 cells/well and incubated with or without heat-killed *E. coli* (MOI = 100) for 24 h. The amounts of IL-12p70, IL-10, and TNF-α in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from six independent experiments. N.D., Not detected; N.S., not significant. **, $p < 0.01$ (Sheffe's test). *B*, Expression of M-CSF and GM-CSF in murine tissues from WT mice. Data are expressed as the ratio of M-CSF to GM-CSF expression.

Consistent with these human studies, our present study shows that CLPMϕ in WT mice does not produce proinflammatory cytokines IL-12 and IL-23, and produced just few amounts of TNF-α and IL-6 (data not shown) but large amounts of IL-10 upon stimulation with heat-killed *E. coli* and *E. faecalis* Ags. Thus, CLPMϕ may act as anti-inflammatory Mϕ in vivo, when encountering bacteria. These behaviors of CLPMϕ were very similar to those of in vitro differentiated M-Mϕ. In agreement with this observation, our present study showed that M-CSF to GM-CSF expression level ratios in murine colonic tissues were higher than in other organs, such as spleen. These results suggest that M-CSF-rich conditions in colonic tissues might play an important role in differentiation of CLPMϕ as anti-inflammatory Mϕ. In accordance with this idea, recent studies suggested that M-CSF is an essential growth factor for development of intestinal Mϕ. The number of intestinal Mϕ in M-CSF-deficient *op/op* mice was significantly decreased (33, 34), and M-CSF was expressed in the lamina propria in the human intestine (35).

IL-$10^{-/-}$ mice develop Th1 polarized spontaneous chronic colitis and are widely used as a colitis animal model for human IBDs (14). It has been reported that enteric bacteria play essential roles in onset and development of colitis in IL-$10^{-/-}$ mice, similar to human IBDs (15). However, functional roles of enteric bacteria in development of colitis in IL-$10^{-/-}$ mice have not been identified. We demonstrated in this study that bacteria induce abnormal production of proinflammatory cytokines IL-12 and IL-23 from intestinal Mϕ, but not splenic Mϕ in IL-$10^{-/-}$ mice. Because IL-12 and IL-23 are key cytokines, which induce Th1 immune responses, and IL-12 plays a critical role for the development of colitis in IL-$10^{-/-}$ mice (22, 23), these abnormal responses of intestinal Mϕ in IL-$10^{-/-}$ mice to bacteria may cause Th1 polarization and development of colitis.

In the present study, only stimuli from whole bacteria, but not from PAMPs could induce the production of IL-12p70, IL-12 bioactive form consisted of p35-p40 heterodimer, in differentiated Mϕ from BM CD11b$^+$ cells. In general, TLR ligands such as LPS only induce IL-12p40 subunits, but fail to induce IL-12p70 production from Mϕ without IFN-γ costimulation (36, 37). Consistent with this, we also demonstrated that GM-Mϕ and M-Mϕ produced none or just low levels of IL-12p70 in response to stimulation with various TLR ligands. In contrast, whole bacterial Ags induced IL-12p70 production by GM-Mϕ in WT and IL-10$^{-/-}$ mice, and by M-Mϕ in IL-10$^{-/-}$ mice without IFN-γ. Moreover, intestinal Mϕ from IL-10$^{-/-}$ mice also produced high levels of IL-12p70 in response to stimulation with whole bacteria Ags without IFN-γ, but did not induce IL-12p70 in response to LPS alone (data not shown). These findings imply that TLR ligands and whole bacteria may activate IL-12p70 production through distinct mechanisms. Because whole bacteria are internalized into Mϕ by phagocytosis, it is possible that cell surface receptors involved in phagocytosis are different from TLRs, and can stimulate signaling for IL-12p70 production, and/or internalized bacteria stimulate IL-12p70 production via an intracellular recognition pathway. These are important observations that will help in understanding the pathogenesis of enteric bacteria in development of colitis in IL-10$^{-/-}$ mice, and clarification of these points are underway.

Mϕ and DCs can produce both IL-12 and IL-10, but IL-10 is known to inhibit the production of not only IL-12 but also other proinflammatory cytokines through several transcriptional regulations (38–40). In fact, in the present study, we demonstrated that abnormal IL-12p70 hyperproduction by M-Mϕ in IL-10$^{-/-}$ mice were completely suppressed by exogenous IL-10 supplementation concomitant with bacterial stimulation. These results indicated that the lack of IL-10 production by bacterial stimulation may account for the abnormal IL-12p70 hyperproduction by IL-10$^{-/-}$ M-Mϕ. In the present study, however, we found that IL-10 also plays a novel role for the differentiation of Mϕ with anti-inflammatory phenotype. We demonstrated that abnormal IL-12p70 production by IL-10$^{-/-}$ M-Mϕ in response to stimulation with bacteria was significantly reduced by supplementation with IL-10 during the differentiation process from BM cells to M-Mϕ. Interestingly, the effect of IL-10 on the differentiation of Mϕ differs from that on the concomitant stimulation with bacteria. In the former case, only IL-12p70 production was significantly reduced, and TNF-α and IL-6 productions were suppressed just a little or not at all. In contrast, in the latter case, not only IL-12p70 but also other proinflammatory cytokines (TNF-α and IL-6) were completely suppressed. These results indicated that, in anti-inflammatory Mϕ subsets, IL-12 was regulated during the differentiation process by endogenous IL-10, but TNF-α and IL-6 were not regulated in this process. Thus, endogenous IL-10, which is induced during differentiation of M-Mϕ, functionally regulates Mϕ to acquire an anti-inflammatory phenotype such as the hypoproduction of IL-12. Moreover, abnormally differentiated BM-derived M-Mϕ and CLPMϕ in IL-10$^{-/-}$ mice may show an abnormal response to bacteria, produce extraordinary amounts of IL-12 and IL-23, and contribute to the pathogenesis of colitis in IL-10$^{-/-}$ mice. Similarly to these results, we previously demonstrated that endogenous IL-10 plays a key role in phenotype determination of M-Mϕ in humans (27, 41), and IL-10 is produced during the differentiation of monocyte-derived M-Mϕ, but not of GM-Mϕ (30). In human IBDs, monocytes obtained from some patients who have Crohn's disease did not differentiate normally with M-CSF stimulation (T. Hisamatsu, unpublished observation). This observation suggests the possibility that M-Mϕ from patients with Crohn's disease show an abnormal phenotype and contribute to the pathogenesis of intestinal inflammation.

In conclusion, results of the present study demonstrate that BM-derived M-Mϕ and intestinal Mϕ show an anti-inflammatory phenotype, which involves the production of large amounts of IL-10, but a failure to produce IL-12 and IL-23 upon stimulation with bacteria, and intestinal Mϕ may play important roles in gut homeostasis. However, IL-10 deficiency during differentiation of these Mϕ altered their characteristics into a proinflammatory phenotype, which was characterized by the production of huge amounts of IL-12 and IL-23 after bacteria recognition. Thus, these abnormal responses of intestinal Mϕ upon the bacteria may contribute to Th1 polarization, and cause chronic colitis via IL-12 and IL-23 hyperproductions. Our data provide new insights into the intestinal Mϕ to gut flora relationship in the development of colitis in IL-10$^{-/-}$ mice.

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Disclosures

The authors have no financial conflict of interest.

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ORIGINAL ARTICLE

Therapeutic efficacy of infliximab on active Crohn's disease under nutritional therapy

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Abstract

Objective. The aim of this investigation was to elucidate retrospectively the therapeutic effect of infliximab in patients with active Crohn's disease (CD) under nutritional therapy. ***Material and methods***. Using a review of the clinical records in 24 nationwide institutions specializing in inflammatory bowel disease, the short-term effect of infliximab in 97 patients with active CD was retrospectively investigated. The Crohn's disease activity index (CDAI) at baseline and after 2 weeks of a single infliximab administration (5 mg/kg) was compared among patients under total parenteral nutrition (TPN group, $n=36$), those following an elemental or polymeric diet (EN group, $n=49$) and those without TPN and EN (NN group, $n=12$). A decrease in CDAI ≥ 70 or a CDAI value <150 at 2 weeks was regarded as effective. ***Results***. There was no difference in CDAI at baseline among the three groups. In each group, CDAI decreased significantly (from 250 (195–290) [median (interquartiles)] to 152 (123–233) in the TPN group, $p<0.0001$; from 259 (200–325) to 180 (130–238) in the EN group, $p<0.0001$; from 278 (222–291) to 164 (132–196) in the NN group, $p=0.003$). Infliximab was effective in 63.9% of patients in the TPN group, in 55.1% of those in the EN group and in 75% of the NN group. There was no statistical difference in efficacy among the three groups ($p=0.4$). Multivariate logistic regression analysis revealed younger age to be a significant factor related to the efficacy of infliximab. ***Conclusions***. Infliximab is effective in patients with CD under TPN or EN. Age at infliximab administration may be predictive of response to infliximab.

Key Words: *Crohn's disease, infliximab, nutritional therapy*

Introduction

Infliximab, a chimeric antibody to tumor necrosis factor (TNF)-α, has become widely accepted as an effective agent for the treatment of active and refractory Crohn's disease (CD) [1]. It has also been shown that infliximab prolongs remission in CD [2,3]. Based on an accumulation of a large number of clinical trials, guidelines for the clinical use of infliximab in patients with CD have been defined [4,5]. Recently, various clinical parameters and medications for the prediction of the efficacy have been identified for the use of infliximab [6–10].

In Japan, however, total parenteral nutrition (TPN) and enteral nutrition (EN) have been approved as primary therapies for the treatment of active CD [11]. These nutritional therapies have been shown to have a therapeutic effect equal to or less than that of steroids [12,13]. In addition, recent investigations confirmed that EN is effective in CD, regardless of nitrogen sources [14–16]. However, the effect of infliximab under nutritional therapy has not been examined previously.

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On the basis of the results of a preliminary multicenter trial of infliximab in Japanese patients with CD [17], the agent has become available in Japan. In this study, we retrospectively investigated the effect of infliximab on Japanese patients with CD from a multicenter database in order to elucidate the short-term effect of infliximab under TPN or EN, and to identify the clinical characteristics predictive of response to infliximab in Japanese patients with CD.

Material and methods

Subjects

This study was performed retrospectively by reviewing the clinical records in 24 institutions specializing in the treatment of chronic inflammatory bowel diseases in Japan (see Appendix). Since the Japanese Ministry of Labor and Welfare approved infliximab as a therapeutic agent in June 2002, the practical use and the side effects of the drug have been monitored by Tanabe Seiyaku Corporation (Tokyo, Japan) during a period from November 2002 to March 2003. The purpose of monitoring was to specify the incidence of adverse events and infusion reaction of infliximab in Japanese patients with CD. The clinical data of 138 subjects, in whom infliximab was administered at a dose of 5 mg/kg, were obtained from the attending physicians (Figure 1). In 25 patients, the Crohn's disease activity index (CDAI) values prior to treatment were less than 150. In 16 patients, TPN or EN was changed to the other nutritional treatment or was discontinued after infliximab administration. These 41 patients were excluded from the analysis. The remaining 97 patients were the subjects of the present investigation; 85 patients were continuously treated with TPN or EN during a period from at least 1 week prior to and 2 weeks after infliximab. The other 12 patients were not treated with either TPN or EN. We directly contacted each attending physician and the following clinical items were reviewed.

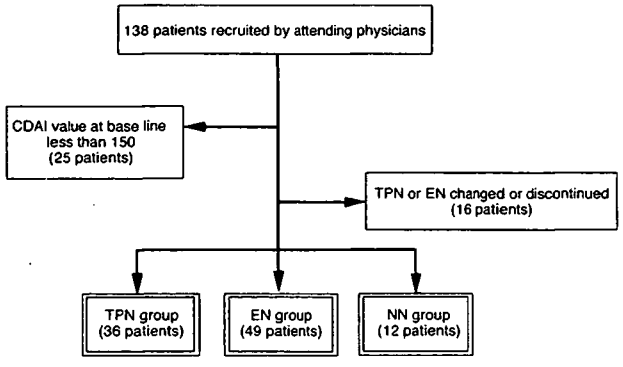

Figure 1. Scheme for recruitment of study subjects. Clinical data of 138 patients were collected; 41 patients were excluded from the analysis because they had inactive disease or they were treated with different nutritional therapies during observation. Thirty-six patients were continuously treated with total parenteral nutrition (TPN group) and 49 patients with enteral nutrition (EN group). Twelve patients were not given any nutritional treatment (NN group).

Parenteral and enteral nutrition

The TPN group comprised 36 subjects who were treated with intravenous hyperalimentation through catheterization in the superior vena cava at a dose of over 1500 kcal/day. Similarly, the EN group comprised 49 subjects in whom either an elemental or polymeric diet of over 1500 kcal/day was applied. The formula of EN included an elemental diet (Elental; Ajinomoto Farma Co., Tokyo, Japan) and polymeric diet (Ensure Liquid; Abot Japan Co., Racall; Otsuka Seiyaku Co., Tokyo, Japan). Twelve patients without TPN or EN treatment were classified as the NN group.

Data collection

Information collected included age, gender, duration and site of CD, prior surgery, intestinal complications (stricture and fistula), concomitant medications and smoking habits.

The location of the disease was divided into three types: small intestinal disease only, colonic disease only and both small and large intestinal disease. Intestinal stricture was defined as an obvious intestinal stricture demonstrated by radiography or endoscopy, regardless of abdominal symptoms. Fistulous disease was regarded to be positive when there was a fistulous tract between the intestine or between the intestine and the skin, or when there was a perianal fistula. Patients without any fistulous disease were categorized as having inflammatory disease. As for the prior surgery, intestinal resection and strictureplasty were counted as positive findings.

Treatments with corticosteroids and immunosuppressive drugs were analyzed separately. When any dose of prednisolone was administered during 2 weeks after infliximab treatment, the subject was regarded to be positive for steroid treatment. Similarly, the use of thiopurines (azathioprine or 6-mercaptopurine) was judged to be positive for immunosuppressive drugs. None of the subjects was treated with methotrexate.

Disease activity and treatment efficacy

Determination of response was based on the CDAI [18], which was calculated using a review of the clinical records of each patient. CDAI was determined prior to and 2 weeks after infliximab