Naive T cells preferentially recirculate between blood and secondary lymphoid tissues, entering lymph nodes from the blood by crossing high endothelial venules. After encountering the activated dendritic cells undergoing antigen presentation in the mesenteric lymph nodes, the naive T cells become activated, proliferate and differentiate into activated effector T cells. These effector T cells then acquire the gut-homing receptors, integrin $\alpha_4\beta_7$. Thus, colitogenic effector T cells, unlike naive T cells, can migrate efficiently to sites of inflammation [104], subsequently entering afferent lymphatic vessels and travelling to local lymph nodes [104-108]. In parabiotic mouse models, endogenous memory T cells in most peripheral tissues react in equilibrium with migrating blood-borne donor T cells within a week [109], suggesting that there is rapid recirculation of T cells in peripheral tissues. These recent understandings suggest that selective removal of these colitogenic activated effector T cells by leukocytapheresis should reduce the cellular components of IBD.

Factors believed to contribute to granulocyte activation and its increased survival time in IBD include inflammatory cytokines [110] and, paradoxically, corticosteroids [53], which are given to most patients with active IBD. Indeed, corticosteroids are known to reactivate quiescent UC and may precipitate the first UC attack [1]. These again in part explain why IBD shows poor response to drug therapy, and strengthen the assumption that activated leukocytes are involved in the initiation, exacerbation and perpetuation of IBD. Activated leukocytes and their cytokines, together with corticosteroids, might constitute a vicious cycle whereby leukocytes produce cytokines that then support the former in addition to promoting inflammation, and both are enhanced by corticosteroids. Hence, peripheral blood leukocytes should be the most appropriate target of therapy in IBD. Based on this thinking, leukocytapheresis should be equivalent to removing inflammatory cytokines at a point upstream of inflammatory drive.

To continue the above arguments, it could be said that the effectiveness of certain cytokine antagonists, such as infliximab, might be viewed as a solid evidence for the involvement of TNF-α (in this case) in the immunopathogenesis of IBD. Hence, given that major sources of TNF-α (and other inflammatory cytokines) include activated leukocytes, depleting these cells from patients' body should represent biological therapy, a natural medication that is safe and, as it removes the effector cells from the body rather than adding them, is not likely to cause refractoriness. Alternatively, leukocytapheresis as an adjunct to conventional medication should spare most patients with active IBD from additional drug therapy and reduce the number of patients who require colectomy.

The word apheresis means to take away, to purify. In fact, today's selective removal of activated peripheral blood leukocytes to achieve a therapeutic effect by apheresis is reminiscent of the rather crude practice of bloodletting (phlebotomy) and its therapeutic application at the time of Hippocrates (460 – 377 BC) in Ancient Greece. The perception then was that disease reflected the presence of disease-causing agents in the blood, and bloodletting was to expel the disease. Bloodletting was routinely and extensively performed for diseases such as inflammation, infection and fever. This practice was subsequently popularised by another Greek physician, Claudius Galen (129 – 203 AD), in Rome, who became a very well-respected authority on medicine for > 1500 years. In the age of modern medicine, bloodletting may be viewed as just a folly of the past, but it is difficult to imagine that this procedure would have been so widely practiced for such a long time if it had not been associated with efficacy.

5. Expert opinion

UC and CD together represent the chronic idiopathic IBDs, and produce symptoms that impair ability to function and quality of life. The aetiology of IBD is inadequately understood and, therefore, drug therapy has been empirical instead of based on sound understanding of the disease mechanisms. This has been a major factor for poor drug efficacy and treatment-related side effects that often add to disease complications. The development of biologicals, notably infliximab, to block TNF-α reflects some progress towards an ideal goal of having a medication with adequate efficacy margin, yet there is concern about their side effects and lack of long-term safety and efficacy profiles. However, IBD by its very nature is exacerbated and perpetuated by inflammatory cytokines, including TNF-α, IL-6, IL-12, for which activated peripheral blood granulocytes and monocytes/macrophages are major sources. Furthermore, in patients with active IBD, peripheral leukocytes are elevated with activation behaviour, increased survival time and are believed to be major factors in the immunopathogenesis of IBD. Hence, depleting activated leukocytes should be considered as a safe and effective natural biological therapy, equivalent to reducing inflammatory cytokine release at an upstream point. Published data show leukocytapheresis producing impressive efficacy, strong drug-sparing effects, with potential to reduce the number of patients with severe disease who must undergo colectomy or be exposed to potent immunosuppressors such as CyA. Based on data available at present, the safety of leukocytapheresis is no longer a concern; however, there are many other aspects of the treatment that require further investigation, not least the importance of large controlled studies to fully evaluate its therapeutic efficacy and its precise place in the treatment of IBD.

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Blockade of NKG2D signaling prevents the development of murine CD4 T cell-mediated colitis

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Ito Y, Kanai T, Totsuka T, Okamoto R, Tsuchiya K, Nemoto Y, Yoshioka A, Tomita T, Nagaishi T, Sakamoto N, Sakanishi T, Okumura K, Yagita H, Watanabe M. Blockade of NKG2D signaling prevents the development of murine CD4 T cell-mediated colitis. Am J Physiol Gastrointest Liver Physiol 294: G199–G207, 2008. First published October 25, 2007; doi:10.1152/ajpgi.00286.2007.—It has been recently demonstrated that NKG2D is an activating costimulatory receptor on natural killer (NK) cells, natural killer T (NKT) cells, activated CD8 T cells, and T cells, which respond to cellular stress, such as inflammation, transformation, and infection. Here we show that intestinal inflammation in colitic SCID mice induced by adoptive transfer of CD4 CD45RB^{high} T cells is characterized by significant increase of CD4 NKG2D T cells and constitutive expression of NKG2D ligands, such as H60, Mult-1, and Rae-1, by lamina propria CD11c dendritic cells. Furthermore, treatment with nondepleting and neutralizing anti-NKG2D MAb after transfer of CD4 CD45RB^{high} T cells into SCID mice significantly suppressed wasting disease with colitis, abrogated leukocyte infiltration, and reduced production of IFN- by lamina propria CD4 T cells. These findings demonstrate that NKG2D signaling pathway is critically involved in CD4 T cell-mediated disease progression and suggest a new therapeutic target for inflammatory bowel diseases.

NKG2D; CD4 T cells; chronic colitis; inflammatory bowel disease

INFLAMMATORY BOWEL DISEASES (IBDs), such as Crohn's disease and ulcerative colitis, are chronic inflammatory diseases characterized by massive infiltration of activated effector-memory CD4 T cells, macrophages, and dendritic cells in the inflamed mucosa (3, 24). Although their etiology remains unclear, it has been shown that production of proinflammatory cytokines by infiltrating activated CD4 T cells and macrophages plays a critical role in the pathogenesis of IBD (2, 4, 10, 13).

It is well known that the activation of CD4 T cells requires two distinct signals: signal 1 derived from the interaction between the T cell receptor (TCR) and peptide-major histocompatibility complex (MHC), and signal 2, the costimulatory signal, derived from the interaction between costimulatory molecules of CD28 family on CD4 T cells and their ligands of the B7 family on antigen-presenting cells (APC) (7, 8, 16). In addition to the CD28/B7 family pathway (27, 28), accumulating evidence shows that many other costimulatory systems, such as the TNF- /TNF- receptor family pathway, are also involved in the pathogenesis of IBDs and animal models of chronic colitis (18, 21, 26, 29).

NKG2D was first shown as a novel costimulatory molecule expressed on NK cells, which was also demonstrated to be expressed on CD8 T cells, T cells, and NKT cells that have cytotoxic activity (23, 25). In CD8 T cells, NKG2D is expressed on activated effector-memory, but not resting naive, CD8 T cells. The ligands of NKG2D are poorly expressed on normal cells but are upregulated on stressed, transformed, or infected cells (23, 25). To date, it has been reported that mouse NKG2D ligands include the retinoic acid early inducible (Rae)-1 family of proteins (6), the minor histocompatibility antigen H60 (20), and murine UL-16-binding protein-like transcript (Mult)-1 glycoprotein (5). It has been demonstrated that the activation via NKG2D receptor can enhance negative signals by MHC class I-specific NK cell inhibitory receptors (9, 15).

Although it had been believed that both human and mouse CD4 T cells do not express NKG2D, recent studies have suggested that NKG2D expression could be observed in a fraction of CD4 T cells residing in the peripheral blood and synovial tissue from patients with rheumatoid arthritis (12). To characterize the role of NKG2D molecule expressed on colitogenic CD4 effector-memory T cells in the development of colitis, we utilized a Th1-type CD4 T-cell-mediated Crohn's disease-like colitis model induced in SCID mice by adoptive transfer of CD4 CD45RB^{high} T cells (27). We here demonstrated that the lamina propria (LP) CD4 CD44^{high} memory T cells in inflamed mucosa express NKG2D. Furthermore, we show that neutralizing anti-mouse NKG2D MAb suppressed the development of colitis.

MATERIALS AND METHODS

Animals. Six- to 8-wk-old female C.B-17 SCID mice and BALB/c mice were purchased from Japan Clea (Tokyo, Japan). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility at Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 wk of age. All experiments were approved by the regional animal study committees.

Antibodies. The anti-murine NKG2D MAb (HMG2D, hamster IgG) was generated by immunizing an American hamster with mouse NKG2D-Fc fusion protein (R&D Systems, Minneapolis, MN). This MAb inhibited the binding of NKG2D-Fc to Rae-1 transfectants and blocked the killing of Rae-1 transfectants by NK cells but did not activate NK cells in vitro or deplete NK cells in vivo. We confirmed that this activity of HMG2D was comparable to another clone of anti-NKG2D MAb, CX5 (17) (data not shown). The following MAbs were obtained from BD Pharmingen (San Diego, CA) and used for purification of cell populations and flow cytometric analysis: Fc R

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(CD16/CD32)-blocking MAb (2.4G2); FITC-, phycoerythrin (PE)-, and phycoerythrin-cyanin5 (PECy5)-conjugated anti-mouse CD4 (RM4-5); FITC-conjugated anti-mouse CD3 (145-2C11); FITC-conjugated anti-mouse CD45RB (16A); FITC-conjugated anti-mouse CD11b (M1/70); FITC-conjugated anti-mouse CD11c (HL3); FITC-conjugated anti-mouse DX5 (DX5); APC-conjugated anti-mouse CD28 (37.51); FITC-conjugated anti-mouse inducible T-cell costimulator (ICOS) (C398.4); FITC-conjugated anti-mouse PD-1 (RMP1-30); PE-conjugated streptavidin; biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG.

In vivo experimental design. Colitis was induced in SCID mice by adoptive transfer of CD4 $CD45RB^{high}$ T cells as previously described (27). CD4 T cells were isolated from splenic mononuclear cells from BALB/c mice by using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. Enriched CD4 T cells were then labeled with PE-conjugated anti-mouse CD4 MAb (RM4-5) and FITC-conjugated anti-CD45RB MAb (16A) and sorted into $CD45RB^{high}$ (highest staining 30%) and $CD45RB^{low}$ (lowest staining 30%) fractions on a FACS Vantage (Becton Dickinson, Sunnyvale, CA). Each SCID mouse was injected with 200 l ip of PBS containing 3 10^5 cells of CD4 $CD45RB^{high}$ T cells. These mice were then administered with 250 g ip of anti-NKG2D MAb in 250 l PBS three times per week, starting at the day of T cell transfer, over a period of 7 wk in the preventive protocol. An equivalent amount of control hamster IgG (Sigma-Aldrich, St. Louis, MO) was administered in control mice. Negative control SCID mice were also transferred with a mixture of 3 10^5 CD4 $CD45RB^{high}$ T cells and 3 10^5 CD4 $CD45RB^{low}$ T cells.

Disease monitoring and clinical scoring. Mice were weighed and monitored for appearance and signs of soft stool and diarrhea once a week. Clinical score (27) was assessed at 7–8 wk after T cell transfer as the sum of three parameters as follows: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, gross bloody stool).

Histological examination. Tissue samples were fixed in 6% phosphate-buffered formalin. Paraffin-embedded sections (5 m) were stained with hematoxylin and eosin. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of the type of treatment. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated via a modification of a previously described scoring system (19).

Immunohistochemistry. For immunohistochemistry, colonic samples were snap-frozen in liquid nitrogen and stored at 80°C. Cryostat sections (5 m) were fixed in 4% paraformaldehyde and detection of mouse CD4 and NKG2D was performed by the avidin-biotin complex method. Briefly, after blocking, the sections were incubated with primary rat anti-mouse NKG2D MAb (clone 191004, R&D Systems) and goat anti-mouse CD4 MAb (AF554, R&D Systems), followed by biotin-conjugated goat anti-rat IgG (1:200, Vector Laboratories, Burlingame, CA) and biotin-conjugated rabbit anti-goat IgG (1:200, Vector Laboratories). The deposition of the biotin on tissue sections was detected by streptavidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector) and diaminobenzidine. Then the sections were counterstained with hematoxylin.

To assess the colocalization of CD4 and NKG2D on colitic LP CD4 T cells, we further performed an immunofluorescent staining experiment. Sections were incubated with rat anti-mouse NKG2D MAb (clone 191004) and goat anti-mouse CD4 MAb (AF554), followed by biotin-conjugated goat anti-rat IgG and amino acid polymer conjugated with rabbit anti-goat IgG and peroxidase (NICHIREI Bioscience, Tokyo, Japan). The samples were then incubated with Alexa 488-conjugated tyramide at a 1:100 dilution (Molecular Probes, Eugene, OR) for detection of CD4 and streptavidin-Alexa Fluor 594 conjugate (Molecular Probes) for detection of NKG2D. Cell nuclei were counterstained with DAPI (Molecular Probes). Stained sections were examined by a BioZERO BZ8000 (KEYENCE, Osaka, Japan).

Preparation of LP lymphocytes and splenocytes. For the isolation of LP lymphocytes from the colon, the entire length of intestine was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated two times with Ca^{2} - and Mg^{2} -free Hanks' balanced salt solution containing 1 mM dithiothreitol (Sigma-Aldrich) for 30 min each to remove mucus. The supernatants containing intraepithelial cells and epithelial cells were removed. Collected tissues were treated with 3 mg/ml collagenase A (Worthington Biomedical, Freehold, NJ) and 0.01% DNase (Worthington) in RPMI 1640 medium for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution and then further purified by Ficoll-Hypaque density gradient centrifugation (40%/75%). LP CD4 T cells were obtained by positive selection via the anti-CD4 (L3T4) MACS magnetic separation system. The cells were 95% CD4 when analyzed by flow cytometry. Splenic mononuclear cells were obtained from the same animals by mechanical dissociation of the spleen followed by Ficoll-Hypaque density gradient centrifugation.

Flow cytometry. Isolated LP mononuclear cells or splenocytes were preincubated with Fc R-blocking MAb (2.4G2) for 20 min, followed by incubation with FITC-, PE-, or biotin-labeled MAb for 30 min on ice. Biotinylated antibodies were detected with PE- or CyChrome-streptavidin. Standard two- or three-color flow cytometric analyses were obtained by use of the FACS Calibur using CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched MAbs.

Quantitative RT-PCR. Whole colon from colitic SCID mice and age-matched normal BALB/c mice were opened longitudinally, washed, and cut into pieces, then homogenized three times by homogenizer. Each cell population of LP cells from colitic mice was isolated by the anti-CD4, CD11b, or CD11c MACS magnetic separation (Miltenyi Biotec). Colonic intestinal epithelial cells (IECs) were also isolated as previously described (11). Total RNA was extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan). Aliquots of 3 g of total RNA were used for complementary DNA synthesis in 14 l of reaction volume by using random primers. One l of complementary DNA was amplified with 12.5 l of SYBR Green PCR master mix (Qiagen, Hilden, Germany) in a 25- l reaction volume. Sense and antisense primers for the amplification of each gene were as follows: sense H60, 5 -GTGTGATGACGATT-TGTTGAG-3 and antisense H60, 5 -ATTGATGGATTCTGGGC-CATA-3 ; sense Mult-1, 5 -CTCATAGGAACAGCATGA-3 and an-

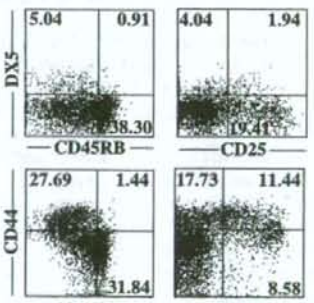

Fig. 1. DX5 NKT cells do not reside in splenic CD4 $CD45RB^{high}$ cell population. Phenotypic characterization of splenic CD4 $CD45RB^{high}$ cells and CD4 CD25 cells in terms of expression of DX5 and CD44. Data represent FACS profiles from 3 independent experiments.

tisense Mult-1 5 *-TCCTGTGAAATGTTTGTC-3 ; sense isoforms of retinoic acid early transcript* 1 (**R***ae-1*) *molecules* (**R***ae-1*), 5 *-ATAATGGATCCATGGCCAAGGCAGCAGTGACCAA-3 and antisense* **R***ae-1* , 5 *-ATATTATAGCGGCCGCTCACATCG-CAAATGCAAATGCAAATAAT-3 ; and sense glyceraldehyde-3-phosphate dehydrogenase (G3PDH),* 5 *-TGAAGGTCGGTGTGAA-CGGATTTGGC-3 and antisense G3PDH,* 5 *-CATGTAGGC-CATGAGGTCCACCAC-3 . Analysis was performed on Applied Biosystems* 7500 *real-time PCR system* (**A***pplied Biosystems, Foster City, CA*). *mRNA level of each ligand was normalized by the corresponding level of G3PDH mRNA.*

Cytokine production assay. To measure cytokine production, LP CD4 T cells (1 10^5) *were cultured in* 200 *l of culture medium at* 37°*C in a humidified atmosphere containing* 5% CO_2 *in* 96*-well plates (Costar, Cambridge, MA) precoated with* 5 *g/ml of hamster anti-mouse CD3 MAb* (145-2C11, *BD Pharmingen*) *and* 2 *g/ml of*

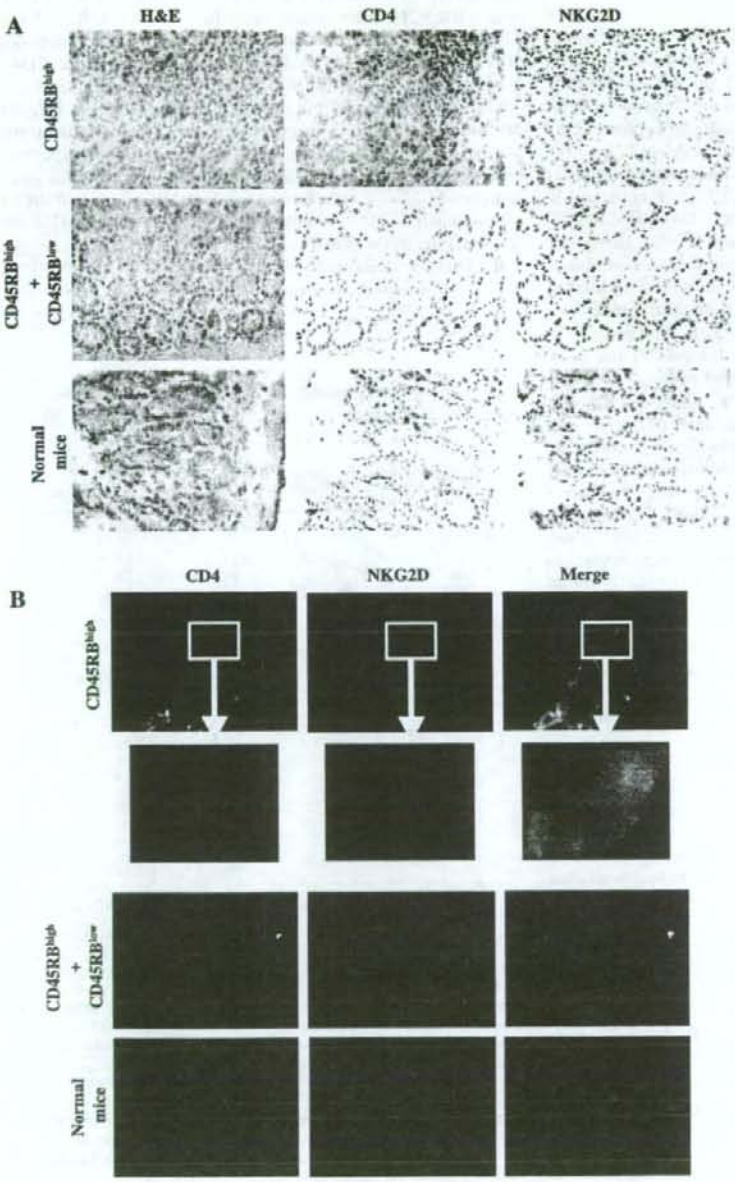

Fig. 2. Expression of NKG2D by colitic *LP* CD4 T cells. A: frozen serial sections of the colons from colitic SCID mice transferred with CD4 $CD45RB^{high}$ T cells, noncolitic SCID mice transferred with a mixture of CD4 $CD45RB^{high}$ T cells and CD4 $CD45RB^{low}$ T cells, and age-matched normal BALB/c mice were stained with anti-CD4 or anti-NKG2D MAb, or hematoxylin and eosin (H&E). Representative of 5 separate samples in each group. Original magnification 100. B: fluorescence image of CD4 and NKG2D. Frozen sections were doubly stained with anti-CD4 in green and anti-NKG2D MAbs in red. Representative of 5 separate samples in each group. Original magnification 60.

hamster anti-mouse CD28 MAb (37.51, BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were collected after 48 h of culture and assayed for cytokine production. Cytokine concentrations were measured by mouse Cytokine CBA kit (BD Biosciences, San Jose, CA) per the manufacturer's recommendation.

Statistical analysis. The results were expressed as means SE. Groups of data were compared by Mann-Whitney U-test. Differences were considered to be statistically significant when P 0.05.

RESULTS

Expression of NKG2D in CD4 CD45RB^{high} T cell-transferred SCID mice. To investigate whether interactions between NKG2D and its ligands are involved in the development of chronic colitis, we used a murine model of chronic colitis induced in C.B-17 SCID mice by adoptive transfer of CD4 CD45RB^{high} T cells of normal BALB/c mice (27), where high refers to the brightest staining 30% fluorescence intensity of CD4 T cells. In this colitis model, CD8 T cells and B cells are absent as we transferred only CD4 CD45RB^{high} T cells into SCID mice lacking both T and B cells. However, it should be noted that CD4 donor population contains a small number of CD4 NKT cells that can express inducible NKG2D in addition to a majority of conventional CD4 TCR T cells. To precisely evaluate this issue, we performed three-color flow cytometry analysis of the cells that were prepared for transfer. As shown in Fig. 1, a small number of DX5 cells were surely observed in the CD4 CD45RB$^{low\text{-}moderate}$ population, but not in the CD4 CD45RB^{high} population. Furthermore, we found that CD4 CD25 cells, which are another population of donor cells commonly used to induce colitis in SCID mice (17), contained substantial number of DX5 cells (Fig. 1, top). Furthermore, almost all CD4 CD45RB^{high} T cells had CD44^{low} (low refers to the dullest 30% CD4 T cells, and moderate refers to the intermediate staining population between the previous two populations) naive phenotype, whereas CD4 CD25 T cells contained substantial numbers of CD44^{high} memory T cells along with CD44^{low} naive T cells (Fig. 1, bottom). Thus we decided to use CD4 CD45RB^{high} T cells as donor cells to assess the possible role of NKG2D expression by conventional CD4 T cells in the development of colitis, so as to exclude the involvement of NKT cells.

After adoptive transfer of CD4 CD45RB^{high} cells, the recipient mice manifested weight loss from 3 wk after transfer and clinical symptoms of colitis such as diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 6 wk (data not shown). The colons from these mice were enlarged and had a greatly thickened wall due to severe colonic inflammation (Fig. 2A, left). In contrast, when transferred with a mixture of CD4 CD45RB^{high} and CD4 CD45RB^{low} cells, the recipient mice did not develop colitis at all as well as control BALB/c mice (Fig. 2A, left). We then examined the expression of CD4 and NKG2D in this model by immunohistochemistry. Colonic samples were obtained from colitic SCID mice transferred with CD4 CD45RB^{high} cells, noncolitic SCID mice transferred with CD4 CD45RB^{high} and CD4 CD45RB^{low} cells at 6 wk after the transfer, and age-matched BALB/c mice as a control. Figure 2A shows that CD4 T cells were markedly increased in the inflamed mucosa of colitic SCID mice transferred with CD4 CD45RB^{high} T cells (Fig. 2A, middle), but not in noncolitic SCID mice transferred with CD4 CD45RB^{high} and CD4 CD45RB^{low} T cells or normal mice. The majority of CD4 T cells in colitic SCID mice located in the LP and submucosa, but some of the cells appeared to locate also in the tunica muscularis and subserosa. Of note, in colitic mice, the distribution of NKG2D cells were scattered within the location of CD4 T cells (Fig. 2A, right), suggesting that NKG2D was expressed by a part of colitic CD4 LP T cells. In contrast, a small number of CD4 T cell were indeed found in the LP of noncolitic SCID mice transferred with CD4 CD45RB^{high} and CD4 CD45RB^{low} T cells and in normal mice, but expression of NKG2D could never be detected on these normal LP CD4 T cells (Fig. 2A, middle and right). To further confirm that NKG2D is expressed on CD4 T cells in colitic LP, we performed a double-staining experiment by fluorescent immunostaining. As shown in Fig. 2B, NKG2D was surely expressed by a part of CD4 T cells in colitic mice. In contrast, a small number of CD4 T cell showed scattered distribution in the LP of noncolitic SCID or normal BALB/c mice, but NKG2D was not detected in these mice.

To further confirm the expression of NKG2D on CD4 T cells in colitic mice, we next performed two-color flow cytom-

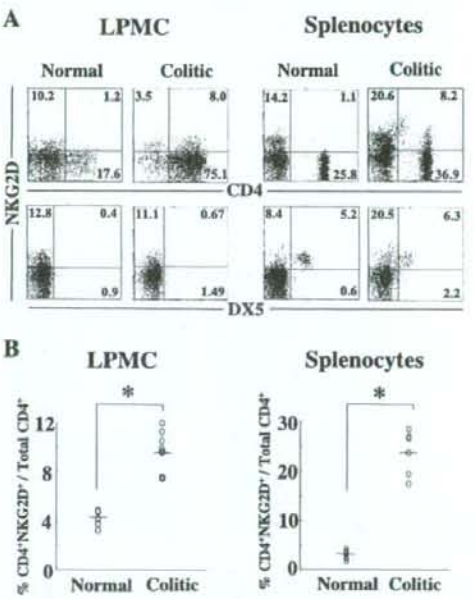

Fig. 3. NKG2D is expressed on lamina propria (*LP*) and splenic CD4 T cells in colitic mice. Expression of NKG2D on splenic and *LP* CD4 and DX5 cells in colitic SCID mice (6 wk after transfer) and age-matched normal BALB/c mice. A: freshly isolated cells from colitic mice and normal BALB/c mice were stained with anti-NKG2D MAb, anti-CD4, or DX5 MAb. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side scatter profiles. Data are displayed as dotted plot (4-decade log scale), and quadrant markers were positioned to include 98% of control Ig-stained cells in the bottom left. *P*ercentages in each quadrant are indicated. Data are representative of 6 mice in each group. B: expression of NKG2D on *LP* and splenic CD4 T cells from colitic mice is significantly increased compared with that on the paired samples from normal mice. Data are shown as means SE of 6 mice in each group. **P* 0.05. *LP*MC, lamina propria mononuclear cells.

etry analysis. In the colitic samples, NKG2D expression on CD4 T cells was significantly increased compared with that in normal mice (Fig. 3, A and B). Although it has been reported that murine NKG2D molecule is usually expressed on DX5 NK or NKT cells (23, 25), DX5 cells could not be detected on cells isolated from colonic tissues through conventional DTT/collagenase treatment (Fig. 3A). In the spleen of colitic mice, a significantly increased proportion of CD4 T cells coexpressed NKG2D, which was not observed in normal mice (Fig. 3, A and B). Interestingly, we detected DX5 cells in the spleen of both colitic and normal mice, where we found that almost all DX5 cells expressed NKG2D with high intensity (Fig. 3, A and B).

Correlation between CD4 NKG2D T cells and other costimulatory molecules. It has been recently reported that both CD4 T cells and CD8 T cells in the peripheral blood or the synovial tissue of patients with rheumatoid arthritis do not express CD28 but express NKG2D (12), indicating that NKG2D can function as a complementary molecule of other CD28 family molecules, such as CD28, ICOS, and PD-1. To address this, we assessed phenotypic correlation between these CD28 family molecules on CD4 T cells using three-color flow cytometry. Since normal splenic CD4 T cells do not express NKG2D (Fig. 3, A and B), we assessed whether three splenic populations, normal CD4 NKG2D , colitic CD4 NKG2D , colitic and CD4 NKG2D cells, express CD28, ICOS, or PD-1 on the cell surface. Unlike peripheral CD4 CD28 NKG2D cells in patients with rheumatoid arthritis (12), CD28 was expressed on colitic CD4 NKG2D to a similar extent with that on normal and colitic CD4 NKG2D cells (Fig. 4A). This was statistically confirmed by assessing the mean fluorescence intensity (MFI) of NKG2D expression (Fig. 4B, left). In contrast, ICOS and PD-1 molecules were markedly upregulated on colitic CD4 NKG2D and CD4 NKG2D cells but were not on normal CD4 NKG2D cells (Fig. 4, A and B). The results indicated that NKG2D expression is not associated with other representative T cell costimulatory molecules.

*E*xpression of NKG2D ligands in the colon of CD4 CD45RB^{high} T cell-transferred SCID mice. To clarify the expression of NKG2D ligands in the colon, we next conducted quantitative real-time PCR analysis using whole colonic tissues from colitic mice or age-matched BALB/c mice. This quantitative PCR analysis revealed that mRNA expression of H60 was significantly increased in colitic colon samples compared with that in normal colon samples (Fig. 5A). In contrast, mRNA expression of Mult-1 and Rae-1 was not significant in colitic colon samples compared with that in normal colon samples (Fig. 5A). Because of the limitation of recovered cell number of immune cells from normal colon samples, we isolated I*E*Cs and LP CD4 , CD11b , and CD11c cells only from the colon of colitic mice to evaluate which NKG2D ligand is expressed in each cell population of colitic mice. As shown in Fig. 5B, H60 mRNA was expressed in every population, but was significantly increased in CD11c cells compared with that in other populations (Fig. 5B, top). Mult-1 mRNA was expressed exclusively in CD11c cells (Fig. 5B, middle). Rae-1 mRNA in CD11c cells was significantly increased compared with that in I*E*Cs and CD4 T cell populations, indicating that the major population expressing NKG2D ligands is CD11c dendritic cells rather than I*E*Cs in colitic mice.

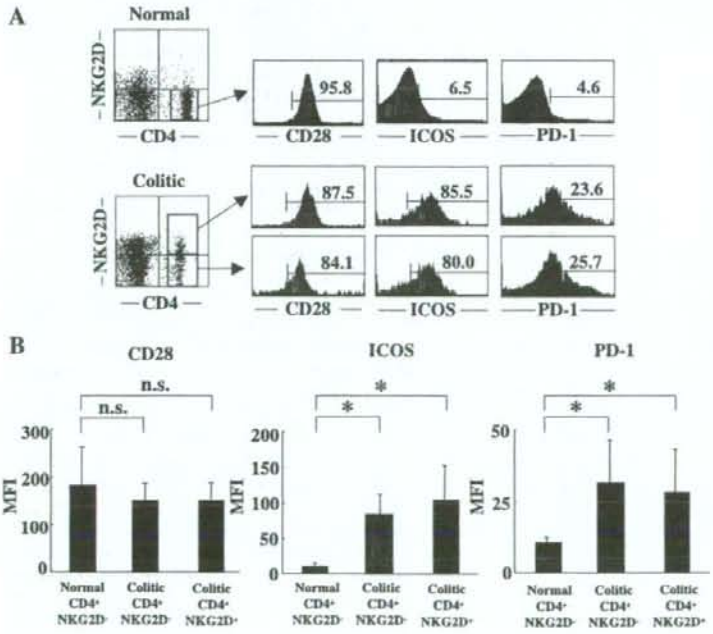

Fig. 4. Correlation between NKG2D molecule and other costimulatory molecules. A: expression of CD28, inducible T-cell costimulator (ICOS), and *P*D-1 on CD4 NKG2D and CD4 NKG2D subpopulations of splenic T cells obtained from control mice or colitic mice. Thick histograms represent staining with MAbs against the indicated markers. B: mean fluorescence intensity (MFI) of each costimulatory molecule on each CD4 NKG2D and CD4 NKG2D subpopulation are compared by flow cytometry. Data are shown as mean SE of 6 mice in each group. *P 0.05; n.s., not significant.

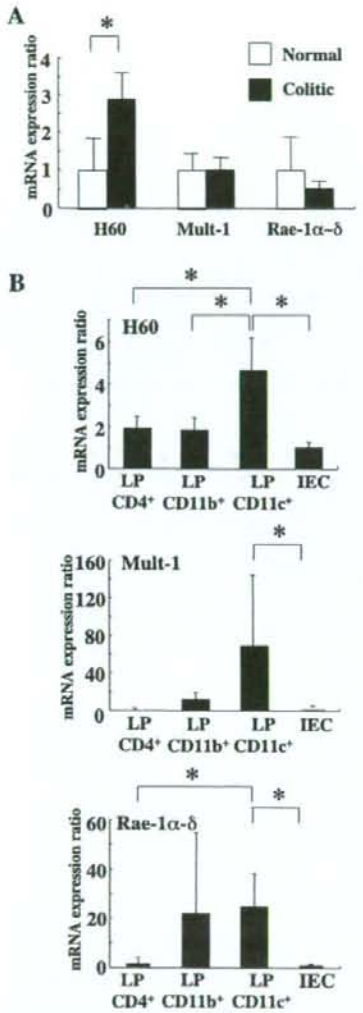

Fig. 5. Expression of H60, Mult-1, and Rae-1 mRNA in colonic samples. A: expression of H60, Mult-1, Rae-1, and G3*P*DH mRNA was determined by quantitative real-time RT-*P*CR using 3 whole colon samples each from normal and colitic mice. Relative mRNA expression of each ligand in colitic colon is compared with that in normal colon. Expression of H60 mRNA in colitic colon was significantly increased compared with that in normal colon. Data represent means SE of 3 independent experiments. B: expression of H60, Mult-1, and Rae-1 mRNA is determined by quantitative real-time RT-*P*CR using 3 samples each from colitic L*P* CD4 , CD11b , CD11c cells, and intestinal epithelial cells (IECs). Expression of H60 mRNA in colitic L*P* CD11c cells was significantly increased compared with other populations. Expression of Mult-1 mRNA and Rae-1 mRNA in colitic L*P* CD11c cells was also increased compared with that in colitic L*P* CD4 , CD11b cells or colitic IECs. H60 mRNA was mainly expressed in populations of CD11c cells. Data represent mean SE of 3 independent experiments.

Administration of neutralizing anti-NKG2D MAb prevents the development of colitis. The expression of NKG2D on the infiltrating LP CD4 T cells and expression of NKG2D ligands in the colitic LP suggested a possible involvement of NKG2D signaling pathway in the pathogenesis of chronic colitis. Thus, to explore the contribution of NKG2D signaling pathway in chronic colitis, nondepleting and neutralizing anti-NKG2D MAb (HMG2D) were administered to the recipient SCID mice transferred with CD4 CD45RB^{high} T cells started from the day of transfer and then three times a week for 7 wk. As shown in Fig. 6A, control IgG-treated mice manifested progressive weight loss (wasting disease) from 3 wk after transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 5–6 wk after transfer. In contrast, anti-NKG2D MAb-treated mice appeared healthy with gradual increase of body weight, and no diarrhea was observed throughout the whole period of observation (Fig. 6A). At 7 wk after transfer, the colon of control IgG-treated mice was enlarged and had a greatly thickened wall, which was not observed in the anti-NKG2D MAb-treated mice or mice transferred with CD4 CD45RB^{high} and CD4 CD45RB^{low} T cells (Fig. 6B). In addition, enlargement of the spleen and mesenteric lymph nodes were also evident in control IgG-treated mice, but not in anti-NKG2D MAb-treated mice (Fig. 6B). A comprehensive assessment of colitis by clinical scores showed a clear difference between control IgG-treated mice and anti-NKG2D MAb-treated mice (Fig. 6C).

Histological examination showed prominent epithelial hyperplasia with glandular elongation with a massive infiltration of mononuclear cells in the LP in the colon of control IgG-treated mice (Fig. 6D). In contrast, inflammation was mostly abrogated and only few mononuclear cells were observed in the LP of the colon from anti-NKG2D MAb-treated mice and in mice transferred with CD4 CD45RB^{high} and CD4 CD45RB^{low} T cells (Fig. 6D). This difference was also confirmed by histological scoring of multiple colon sections, which was 5.2 1.1 in the control rat IgG-treated mice, 2.2 0.8 in anti-NKG2D MAb-treated mice, and 1.6 0.6 in mice transferred with CD4 CD45RB^{high} and CD4 CD45RB^{low} T cells (P 0.01) (Fig. 6*E*). A further quantitative evaluation of CD4 T cell infiltration was done by isolating LP mononuclear cells from resected colons. Only a few CD4 T cells were recovered from the colonic tissue of anti-NKG2D MAb-treated mice and mice transferred with CD4 CD45RB^{high} and CD4 CD45RB^{low} T cells compared with control IgG-treated mice (Fig. 6F). Furthermore, the number of CD4 splenocytes from control IgG-treated mice was significantly increased compared with that from age-matched normal BALB/c mice (data not shown). In contrast, the number of CD4 splenocytes from anti-NKG2D MAb-treated mice was significantly less than that from control IgG-treated mice (data not shown).

We also examined the cytokine production by LP CD4 cells of control IgG- or anti-NKG2D MAb-treated mice. As shown in Fig. 6G, LP CD4 cells from anti-NKG2D MAb-treated mice produced significantly less IFN- compared with those from control IgG-treated mice upon in vitro stimulation. These results suggested that anti-NKG2D MAb prevented the development of colitis primarily by inhibiting the expansion and/or infiltration of pathogenic T cells in the colon and secondarily by inhibiting the development of pathogenic Th1 cells.

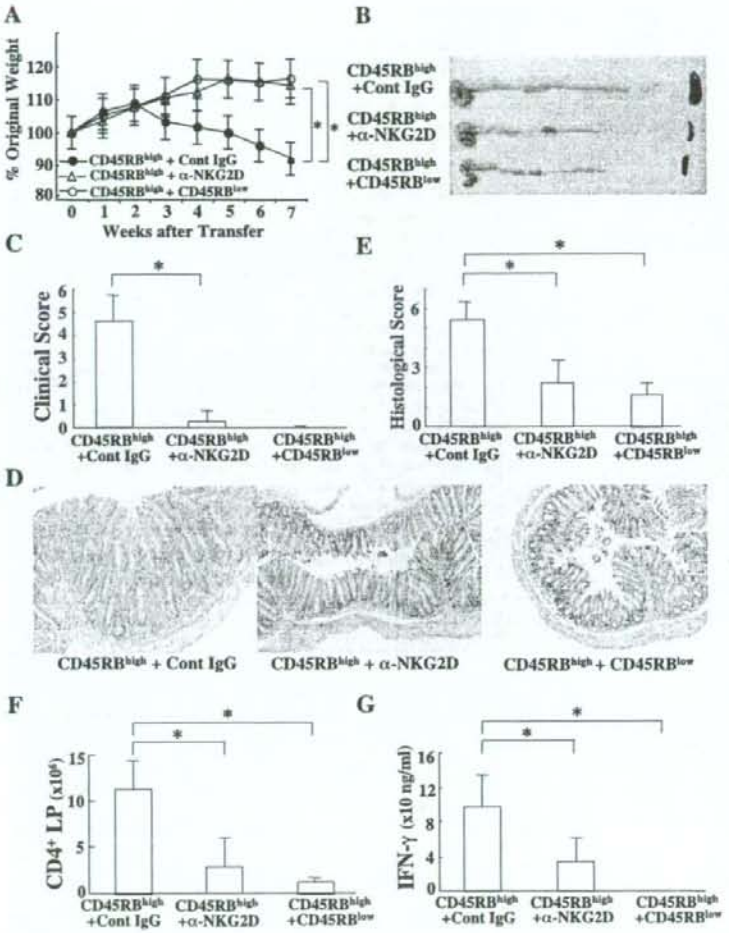

Fig. 6. Preventive effect of anti-NKG2D MAb on the development of colitis. The recipient mice were administered with anti-NKG2D MAb or control hamster IgG for 7 wk starting from the time of CD4 CD45RB^{high} T cell transfer. Other mice were transferred with CD4 CD45RB^{high} T cells and CD4 CD45RB^{low} T cells. A: change in body weight over time is expressed as percent of the original weight. Data represent means SE of 7 mice in each group. *P 0.05 compared with control IgG. B: gross appearance of the colon, mesenteric lymph nodes, and spleen 7 wk after transfer in CD4 CD45RB^{high} T cell-transferred SCID mice treated with control IgG (top) or anti-NKG2D MAb (middle), and mice transferred with CD4 CD45RB^{high} T cells and CD4 CD45RB^{low} T cells (bottom). C: clinical scores were determined at 7 wk after transfer as described in MATERIALS AND METHODS. Data indicate means SE of 7 mice in each group. *P 0.05. D: histological examination of the colons at 7 wk after T cell transfer. Original magnification 100. E: histological scoring of colitis at 7 wk after T cell transfer. Data indicate means SE of 7 mice in each group. *P 0.05. F: lamina propria lymphocytes (LPL) were isolated from the colon at 7 wk after transfer, and the number of CD4 cells were determined by flow cytometry. Data indicate means SE of 7 mice in each group. *P 0.01. G: IFN- production by LP CD4 T cells. Isolated LP CD4 T cells were stimulated with anti-CD3 and anti-CD28 MAbs for 48 h. IFN- concentration in culture supernatants were measured by mouse Th1/Th2 CBA kit. Data indicate means SE of 7 mice in each group. *P 0.05.

DISCUSSION

In the present study, we demonstrated that NKG2D signaling pathway is critically involved in the development of CD4 T cell-mediated chronic colitis by showing that LP CD4 T cells obtained from colitic SCID mice induced by adoptive transfer of CD4 CD45RB^{high} T cells express NKG2D and neutralizing anti-NKG2D MAb treatment ameliorates the development of the colitis model.

Very recently, Kjellev and colleagues (17) demonstrated that NKG2D is expressed on CD4 T cells obtained from colitic SCID mice induced by adoptive transfer of normal splenic CD4 CD25 T cells, and treatment by a neutralizing anti-NKG2D MAb (CX5) prevents the development of colitis. Their results are quite similar to ours, but it is noteworthy that normal splenic CD4 CD25 T cells that are a distinct cell population from CD4 CD25 regulatory T cells, include DX5 CD44^{high}CD45RB^{low} CD4 NKT cells along with CD44^{high} memory and CD44^{low} naive CD4 T cells (Fig. 1). Although they demonstrated that CD4 CD25 donor cells did not express NKG2D before transfer, it remained possible that NKG2D expression could be inducible on activated NKT cells rather than on activated conventional CD4 T cells especially in case of mouse models (30). In contrast, we used CD4 CD45RB^{high} T cells as donor cells, which were characterized to have a cell-surface phenotype of DX5 CD44^{low}, whereas NKT cells were DX5 CD44^{high} (Fig. 1). Thus we believe that NKG2D was solely induced on donor conventional CD4 cells after transfer in our colitis model. Interestingly, their group showed that anti-NKG2D MAb (CX5) treatment significantly decreased histological score in the colon but did not prevent the wasting disease, compared with control IgG-treated mice. In contrast, however, we here showed that our anti-NKG2D MAb (HMG2D) treatment ameliorated both the histological score and the wasting disease, although the protocol was quite similar except the phenotype of transferred donor cells (CD4 CD25 vs. CD4 CD45RB^{high}).

Notably, although the numbers of the donor cells used in these two studies were exactly the same, the kinetic of the development of colitis in control-IgG-treated SCID mice transferred with CD4 CD25 T cells in Kjellev's preventive protocol (17) was obviously more rapid (3 wk of experimental period) compared with those transferred with CD4 CD45RB^{high} T cells in our protocol (7 wk of experimental period). Although there are several explanations for the discrepancy, including differences in the status of Helicobacter hepaticus infection, the type of animal model, the type of blocking agents, and dosing regimens used, further studies will be needed to address this issue.

In this study, we also demonstrated using whole colonic samples by quantitative real-time RT-PCR analysis that NKG2D ligands were also expressed in colonic tissues in both colitic mice and normal mice. *E*xpectedly, mRNA expression of H60 was significantly increased in colitic colon samples compared with that in normal colon samples, although no significant change was observed in mRNA expression of Mult-1 and Rae-1 between colitic and normal samples. This indicates a possibility that NKG2D signaling pathway modulates the development of chronic colitis and also tunes the degree of colitis.

Recent human studies have demonstrated that not only MICA, which is one of NKG2D ligands in humans, is markedly upregulated on cell surface of inflammatory *I*ECs but also CD8 NKG2D intestinal epithelial lymphocytes (IELs) are significantly increased in patients with active celiac disease (14, 22), suggesting a possible contribution to the pathogenesis of celiac disease due to IEL-mediated damage of *I*ECs by NKG2D-MICA interaction. Furthermore, Allez and colleagues (1) have very recently reported that MICA and NKG2D expression is significantly increased on *I*ECs and LP CD4 T cells, respectively, in inflamed mucosa of active Crohn's disease. Although all these studies in humans focused on MICA expression in inflamed I*E*Cs, interestingly, we found that NKG2D ligands were mainly expressed in CD11c dendritic cells rather than I*E*Cs in inflamed mucosa of colitic SCID mice. Collectively, in our system, it is likely that NKG2D signaling pathway is critically involved in the interactions between T cells and APCs (especially CD11c dendritic cells) rather than I*E*Cs in intestinal mucosal immune system of chronic colitis. However, it remains unclear which ligand of NKG2D plays the dominant role in the pathogenesis of our colitic model. Further studies will be needed to address this issue.

In summary, our present findings suggest that the regulation of NKG2D signaling pathway may be of a key importance in successful treatment of chronic colitis, and targeting of NKG2D-expressing pathogenic CD4 T cells may be a useful strategy for the treatment of Th1-mediated chronic intestinal inflammations such as Crohn's disease.

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Lamina Propria c-kit$^+$ Immune Precursors Reside in Human Adult Intestine and Differentiate Into Natural Killer Cells

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Background & Aims: **Recent studies have revealed that murine intestinal mucosa contains several kinds of lineage markers (lin)$^-$ c-kit$^+$ immune precursor cells. However, immune precursors in the human adult intestine have not been studied extensively.** ***Methods:*** **Lamina propria mononuclear cells and intraepithelial lymphocytes from surgically resected human adult intestine were examined for the surface antigen expression and cytokine profile by immunohistochemistry and flow cytometry. The transcriptional profile of these cells was analyzed by reverse-transcription polymerase chain reaction. The phenotypic and functional characterization of the in vitro differentiating cells from the precursors was examined by flow cytometry.** ***Results:*** **We identified lin$^-$ c-kit$^+$ cells scattered throughout lamina propria of the human adult intestine. These intestinal immune precursors expressed CD34, CD38, CD33, interleukin-2Rα, and interleukin-7Rα, and they had much more abundant expression of Id2, PU.1, SpiB1, and lymphotoxin than thymocytes. The lin$^-$ c-kit$^+$ immune precursors mainly differentiated into CD56$^+$ c-kit^{dim} cells during in vitro culture. These in vitro differentiating cells corresponded to intestinal natural killer (NK) cells, which had distinct characteristics from their peripheral counterparts, such as CD83 and integrin α_E expression, less cytotoxic activity, and higher interferon-γ production. Furthermore, both c-kit^{dim} cells and NK cells were increased in lamina propria of Crohn's disease, although there was no change for peripheral blood NK cells.** ***Conclusions:*** **The human intestine may have the unique NK cell differentiation system, which may contribute to maintenance of immune homeostasis in the intestine.**

The cellular components of the immune system, such as T cells, B cells, monocytes, granulocytes, macrophages, dendritic cells, and natural killer (NK) cells, are derived from common hematopoietic stem cells (HSCs) in the bone marrow. As a first step, HSCs differentiate into 2 distinct subsets: common myeloid progenitors and common lymphoid progenitors. Although common myeloid progenitors ultimately differentiate into myeloid cells such as monocytes, granulocytes, macrophages, and dendritic cells,[1] common lymphoid progenitors differentiate into B-cell precursors and common T- and NK-cell precursors (T/NKPs).[2] T/NKPs subsequently differentiate into NKPs and T-cell precursors.[3–5] These steps are assumed to proceed mainly in the bone marrow, which is regarded as the most important site for primary immune cell differentiation.

A unique immune system has developed in the intestine. The intestinal immune system includes Peyer's patches, isolated lymphoid follicles, mesenteric lymph nodes (MLN), lamina propria mononuclear cells (LPMCs), and intraepithelial lymphocytes (IELs). This intestinal immune system maintains immunologic homeostasis against gut luminal antigens. In addition to these components, the intestine has become recognized as a site for differentiation of immune cells. Recent studies have revealed that murine intestinal mucosa contains immune precursor cells, which are lymphoid tissue inducer cells (LTi)[6,7] in the fetus and cryptopatch (CP) cells[8] in the adult. Both LTi and CP cells express c-kit, IL-7 receptor α subunit (IL-7Rα), IL-2Rα, CD44, and CD4^{dim}. These surface phenotypes are similar to those of common lymphoid progenitors in bone marrow, and LTi and CP cells have been reported to develop in situ into Peyer's patches[6,7,9] and extrathymic T cells of IELs,[10,11] respectively. In addition, a recent study suggested that CP cells can function as adult LTi by developing into isolated lymphoid folli-

Abbreviations used in this paper: CP, cryptopatch; HSC, common hematopoietic stem cell; IENK, intraepithelial natural killer cell; IFN, interferon; IL, interleukin; IL-7Rα, IL-7 receptor α subunit; Lin, lineage markers; LPMCs, lamina propria mononuclear cells; LPNKs, lamina propria natural killer cells; LTi, lymphoid tissue inducer cells; MLN, mesenteric lymph nodes; NK, natural killer; PBL, peripheral blood lymphocytes; PBNKs, peripheral blood natural killer cells; PCR, polymerase chain reaction; pTα, pre-T cell receptor chain α; RAG, recombination activating gene; SEM, standard error of the mean; T/NKPs, common T and natural killer cell precursors; TNF, tumor necrosis factor.

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cles rather than IELs in normal adult mice.[12] The immune precursor cells in the murine intestine have been investigated extensively; however, only a few reports have referred to immune precursor cells in the human intestine. A recent study showed that $CD3^-$ $CD7^+$ cells in the human fetal intestine express messenger RNA (mRNA) for pre–T-cell receptor chain α (pTα), which is essential for early T-cell differentiation.[13] It also shows that these cells can give rise to $CD3^+$ T cells in vitro and in vivo, using severe combined immunodeficient (SCID) mice engrafted with human fetal intestine.[14] It also has been reported that recombination activating gene (RAG)-1 and RAG-2 mRNA can be detected in the intestinal mucosa of human infants.[15,16] Moreover, $CD3^-$ $CD2^+$ $CD7^+$ cells in the human adult jejunum have been shown to express RAG mRNA as well as pTα mRNA.[16] All these reports have examined intestinal immune precursors in light of extrathymic T-cell differentiation. However, considering the reports on the murine intestine, we assume that more immature immune precursor cells, such as LTi, also may reside in the human adult intestine.

To verify this hypothesis, we first analyzed human adult intestine immunohistochemically, focusing on expression of c-kit, which is a receptor for stem cell factor and is known to be expressed on immune precursor cells such as HSCs.[17] Although intensive analysis did not reveal any $c\text{-}kit^+$ cell clusters such as CP, we found a considerable number of $c\text{-}kit^+$ cells scattered in the lamina propria. We next characterized with flow cytometry these $c\text{-}kit^+$ cells in LPMCs isolated from human adult intestine, which revealed that the $c\text{-}kit^+$ cells in the intestine have phenotypes identical to T/NKPs in the fetal liver[18] and thymus.[19] The $c\text{-}kit^+$ cells mainly were committed to the NK cell lineage in vitro. We also found unique characteristics of mature NK cells residing in the human adult intestine. These results suggest that $c\text{-}kit^+$ cells should differentiate into intestinal NK cells. Furthermore, NK cell differentiation is accelerated in Crohn's disease (CD), indicating that this intestinal NK cell differentiation system may play a role in the pathogenesis of chronic intestinal inflammation. Thus, we were able to show differentiation of intestinal NK cells from $c\text{-}kit^+$ cells in the human adult intestine, which may contribute to maintenance of intestinal immune homeostasis.

Materials and Methods

Tissue Samples

Normal intestinal mucosa and MLN were obtained from macroscopically and microscopically unaffected areas of patients with colon cancer. Intestinal mucosa also was obtained from surgically resected specimens from patients with CD or ulcerative colitis (UC), diagnosed on the basis of clinical, radiographic, endoscopic, and histologic findings, according to established criteria.[20,21] In all samples from patients with CD or UC, the degree of inflammation was histologically moderate to severe. All experiments were approved by the institutional review board and written informed consent was obtained from all the patients.

Histologic Analysis

Tissue sections were treated according to well-established methods. Intestinal specimens were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin. Sections from paraffin-embedded blocks were deparaffinized and stained with H&E (Sakura Finetech Japan, Tokyo, Japan). For immunohistochemical staining, deparaffinized sections were heated at 100°C for 20 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) in a microwave oven. For the enzyme-labeled antibody method, each section was treated with 3% H_2O_2 (Wako) in 100% methanol and then incubated with normal rabbit serum (Nichirei Biosciences, Tokyo, Japan) for 15 minutes at room temperature to block nonspecific reactions. Thereafter, sections were treated with rabbit anti-human c-kit Ab (Dako Cytommation, Glostrup, Denmark) and incubated at 4°C overnight. Primary antibodies were washed out and sections were incubated with Histofine anti-rabbit Simplestain Max-PO (Nichirei), and visualized with 3-3′-diaaminobenzidine (Nichirei) for peroxidase and counterstained with hematoxylin. Sections incubated with the IgG fraction of normal rabbit serum (Dako) served as negative controls. For identification of mast cells, deparaffinized sections were stained with .05% toluidine blue solution, pH 4.1 (Wako). Mast cells were stained red-purple and other cells were stained blue.

Preparation of LPMCs, IELs, Peripheral Blood Lymphocytes (PBLs), and MLN Cells

LPMCs and IELs were isolated from intestinal specimens using modifications of previously described techniques.[22,23] Briefly, dissected mucosa was incubated in calcium and magnesium-free Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO) containing 2.5% heat-inactivated fetal bovine serum (BioSource, Camarillo, CA) and 1 mmol/L dithiothreitol (Sigma-Aldrich) to remove mucus. The mucosa then was incubated in Hanks' balanced salt solution containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) for 60 minutes at 37°C. During this treatment, IELs and epithelial cells were removed from the tissue. Tissues were collected and incubated in Hanks' balanced salt solution containing .02% collagenase type 3 (Worthington Biochemical, Freehold, NJ) for 60 minutes at 37°C. The fraction was pelleted and resuspended in 40% Percoll solution (Amersham Biosciences, Piscataway, NJ), then layered on 60% Percoll before centrifugation at 2000 rpm for 20 minutes at room temperature. Viable LPMCs were recovered from the 40%–60% layer interface. For isolation of IELs, after EDTA treatment the supernatants were

collected and filtered through a glass-wool column to deplete cell debris and sticky cells. Cells were centrifuged over a 40%–60% Percoll solution density gradient. IELs were recovered from the layer interface. PBLs were isolated from heparinized peripheral blood samples by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). For isolation of MLN cells, MLN were squeezed and passed through sterile nylon mesh to create single-lymphocyte suspensions.[24]

Giemsa Stain

The lin$^-$ c-kit$^+$ cells and the mast cells in LPMCs were sorted by Epics Altra with the HyPerSort cell sorting system (Beckman-Coulter, Fullerton, CA). The purity of the sorted cells was greater than 98% by postsorting analysis. After spreading the sorted cells on glass slides they were air dried, then the cells were fixed with methanol and stained with pH 6.4 Giemsa solution (Merck, Whitehouse Station, NJ), and they were observed by light microscope.

Flow Cytometric Analysis of c-kit$^+$ LPMCs Differentiation Markers

Cell surface fluorescence intensity was assessed using a FACSCalibur analyzer and analyzed with Cell Quest software (BD Biosciences, San Jose, CA). Dead cells were excluded with propidium iodide staining. The lineage marker monoclonal antibodies that were used were the available Lineage Cocktail 1 (BD Biosciences). Lineage Cocktail 1 included CD3 (SK7), CD14 (MϕP9), CD16 (3G8), CD19 (SJ25C1), CD20 (L27), and CD56 (NCAM16.2). All the antibodies were purchased from BD Biosciences except for CD2, CD20, CD56 (MEM188), and NKG2D, which were purchased from eBiosciences (San Diego, CA); CCR7, CXCR5, and IL-18Rα were purchased from R&D systems (Minneapolis, MN); CD133 was purchased from Miltenyi Biotec (Bergisch, Gladbach, Germany); and CX3CR1 was purchased from Medical & Biological Laboratories (Nagoya, Japan).

Quantitative Real-Time, Reverse-Transcription Polymerase Chain Reaction Analysis

Cells were sorted by Epics Altra with the HyPerSort cell sorting system (Beckman-Coulter). The purity of the sorted cells was always greater than 98% by postsorting analysis. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany), and total thymocyte RNA was purchased from BD Biosciences. Total RNA was treated with Qiagen DNase I to remove any contaminating genomic DNA. Absence of amplification of contaminating genomic DNA was ascertained by polymerase chain reaction (PCR) in which RNA was used as a template. Complementary DNA was synthesized using the Superscript first-strand synthesis system for reverse-transcription PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Semiquantitative real-time, reverse-transcription PCR was performed using TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and on-demand gene-specific primers. The fluorogenic probes were as follows: RAG-1, RAG-2, preTCRα, Id2, PU.1, SpiB1, lymphotoxin α, lymphotoxin β, and β-actin, which all were purchased from Applied Biosystems. Cycling conditions for PCR amplification were 95°C for 10 minutes, followed by 45 cycles of 94°C for 15 seconds, and 60°C for 1 minute. Transcription of mRNA was assessed on a DNA Engine Opticon 2 System and analyzed with Opticon monitor software (MJ Research, Waltham, MA). All samples were analyzed in triplicate.

Lin$^-$ c-kit$^+$ LPMCs In Vitro Culture

CD3$^+$ and CD56$^+$ cells were removed from LPMCs using a magnetic cell-sorting system (MACS; Miltenyi Biotec) according to the manufacturer's instructions. The CD3$^-$ CD56$^-$ LPMCs were cultured at a concentration of 1×10^6/mL in complete medium consisting of 1640 RPMI (Sigma-Aldrich) supplemented with GlutaMAX (Invitrogen), 10% heat-inactivated fetal bovine serum (BioSource), 10 mmol/L HEPES (Invitrogen), 50 μmol/L 2-mercaptoethanol (Wako), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). The cultures were maintained in a humidified atmosphere at 37°C in 5% CO_2 for 72 hours. All samples were cultured in duplicate.

Isolation of Peripheral Blood NK Cells and Lamina Propria NK Cells

CD56^{dim} (CD3$^-$ CD14$^-$ CD16$^+$ CD56$^+$) peripheral blood NK (PBNK) cells and CD56^{bright} (CD3$^-$ CD14$^-$ CD16$^-$ CD56$^+$) PBNKs were isolated from PBLs by using MACS (Miltenyi Biotec) according to the manufacturer's instructions. Lamina propria NK (LPNK) cells (CD3$^-$ CD14$^-$ CD56$^+$) also were isolated with MACS. The percentage of each isolated NK cell was evaluated by flow cytometry and routinely was greater than 95%.

Cytotoxicity Assay

The cytotoxicity of NK cell subsets against the NK-sensitive K562, a human erythroleukemic cell line (American Type Culture Collection, Rockville, MD), was measured by using a previously described protocol[25] with minor modifications.

NK Cell Cytokine Production

A total of 1×10^6 cells in 1 mL complete RPMI 1640 medium (Sigma-Aldrich) were stimulated with 10 ng/mL IL-12 (Medical & Biological Laboratories) and 100 ng/mL IL-15 (R&D) or 10 ng/mL IL-12 (Medical & Biological Laboratories) and 100 ng/mL IL-18 (Medical & Biological Laboratories) for 8 hours at 37°C. After stimulation, interferon-γ (IFN-γ) or tumor necrosis factor-α

(TNF-α) production were detected using the Cytokine Secretion Assay Cell Detection Kit (Miltenyi Biotec) according to the manufacturer's instructions, defining cells with antibodies against CD3, CD56, or CD117 (BD Biosciences) and propidium iodide. IFN-γ and TNF-α in cell culture supernatant also were measured using human Th1/Th2 cytokine beads array kit (BD Biosciences) according to the manufacturer's protocol.

Transplantation of the Human lin⁻ c-kit⁺ LPMCs Into RAG-2⁻/⁻ Mice

C57BL/6J background RAG-2$^{-/-}$ mice (Central Institute for Experimental Animals, Kanagawa, Japan) were housed under specific pathogen-free conditions at the animal center of Keio University (Tokyo, Japan). All experiments using mice were approved by and performed according to the guidelines of the animal committee of Keio University. The human lin⁻ c-kit⁺ LPMCs cells ($1.3–1.5 \times 10^5$) were injected intraperitoneally into 8-week-old RAG-2$^{-/-}$ mice. LPMCs, IELs, and splenic lymphocytes were isolated and examined for human lineage markers and human CD117 monoclonal antibody (BD Biosciences) by flow cytometry. For in vitro differentiation, isolated LPMCs were cultured with complete medium in the presence of 100 U/mL human recombinant IL-2 (eBiosciences) or 100 ng/mL IL-15 (R&D) for 7 days. Cultured cells were stained by human CD3 and CD56 monoclonal antibody (BD Biosciences) and assessed by flow cytometry. All samples were cultured in duplicate.

Statistical Analysis

Statistical analysis was performed by using STATVIEW software version J-5.0 (Abacus Concepts, Berkeley, CA), StatMate III software version 3.05 (ATMS, Tokyo, Japan), and GraphPad Prism software version 4.0 (GraphPad Software Inc., San Diego, CA). Differences at a P value of less than .05 were considered significant. All data are expressed as means ± standard error of the mean (SEM).

Results

Presence of Non–Cluster-Forming Lineage Markers (lin)⁻ c-kit⁺ Lymphocytes in the Human Adult Intestine

We first tried to identify c-kit⁺ cells in the human adult intestine by immunohistochemistry. We could not find any c-kit⁺ cell clusters such as murine CP cells; however, we found a considerable number of c-kit⁺ cells scattered in the intraepithelial space, lamina propria, and submucosal layers of both the ileum and colon (Figure 1*A* and *B*). The c-kit expression on IELs was dimmer than that on LPMCs (Figure 1*B* and *I*). Furthermore, the c-kit⁺ IELs were found only in the intraepithelial space of crypts, but not in that of villi (data not shown). Because c-kit also is expressed on mast cells,[26] sequential sections were stained metachromatically with toluidine blue, which is useful for identifying mast cells (Figure 1*C*). Although most of the c-kit⁺ cells in the submucosal layer and a part of the c-kit⁺ cells in the lamina propria presented metachromasia (Figure 1*G* and *H*), a considerable proportion of the c-kit⁺ LPMCs and IELs did not (Figure 1*E*, *F*, *I*, and *J*). We next analyzed with flow cytometry LPMCs isolated from human adult intestine. Mast cells were excluded by taking advantage of their complex granular morphology and higher c-kit expression[27] (Figure 2*A*). The other c-kit⁺ population, distinct from mast cells, was consistent with a typical lymphoid cell gate in forward- and side-scatter diagrams and was used for further analysis. We then examined these c-kit⁺ cells for lineage markers as follows: CD3, CD14, CD16, CD19, CD20, and CD56. The blot diagram clearly identified 2 distinct c-kit⁺ populations: lin⁻ c-kit⁺ and lin⁺ c-kit^{dim} cells (Figure 2*B*). The lin⁻ c-kit⁺ subset occupied 1.97% ± .15%, whereas the lin⁺ c-kit^{dim} subset occupied 1.29% ± .22% of the total LPMCs (n = 10). The lin⁻ c-kit⁺ cells were different from mast cells morphologically, and they seemed to be small immature lymphoid cells about 7 μm in diameter (Figure 2*C*). A similar number of lin⁻ c-kit⁺ cells were detected in LPMCs from both the ileum and colon; however, they barely were recognized in IELs, MLN cells, or PBLs (Figure 2*B*). In contrast, the lin⁺ c-kit^{dim} subset also was present in IELs, which was consistent with the immunohistochemical finding that c-kit expression on IELs was dimmer than that on LPMCs (Figure 1*B* and *I*). We assumed that these lin⁻ c-kit⁺ cells were immune precursor cells in the intestine.

Characterization of the lin⁻ c-kit⁺ and lin⁺ c-kit^{dim} Subsets

To characterize further the 2 c-kit⁺ populations in LPMCs, we examined the expression of various surface stem cell markers. The lin⁻ c-kit⁺ cells also expressed CD34 (Figure 3*A*), which is another marker for HSCs.[28] However, CD34 expression on lin⁻ c-kit⁺ cells was lower than that on HSCs (Figure 3*A*). It is reported that the expression of CD34 decreases during differentiation, therefore, lower CD34 expression may reflect a later stage in the hematopoietic lineage.[29] The lin⁻ c-kit⁺ cells in LPMCs expressed CD38^{dim}, Thy-1, and CD45RA (Figure 3*A*), which corresponds to the phenotype of T/NKPs in fetal liver[18] and thymus.[19] Furthermore, they expressed IL-7Rα, IL-2Rα, CD44, CCR7, and CXCR5 (Figure 3*B*), which are expressed on immune precursor cells such as mouse LTi and CP cells. Importantly, a small number of the lin⁻ c-kit⁺ cells in LPMCs expressed IL-2Rβ and CD161, which are known as NK cell markers (Figure 3*B*). The lin⁻ c-kit⁺ cells were negative for CD56 when examined with clone NCAM 16.2 and MEM 188; however, a small fraction of the cells were weakly positive for clone B159. It is interesting to note that this subset expressed CD33, a known marker of myeloid lineage cells (Figure 3*A* and *B*).[30]

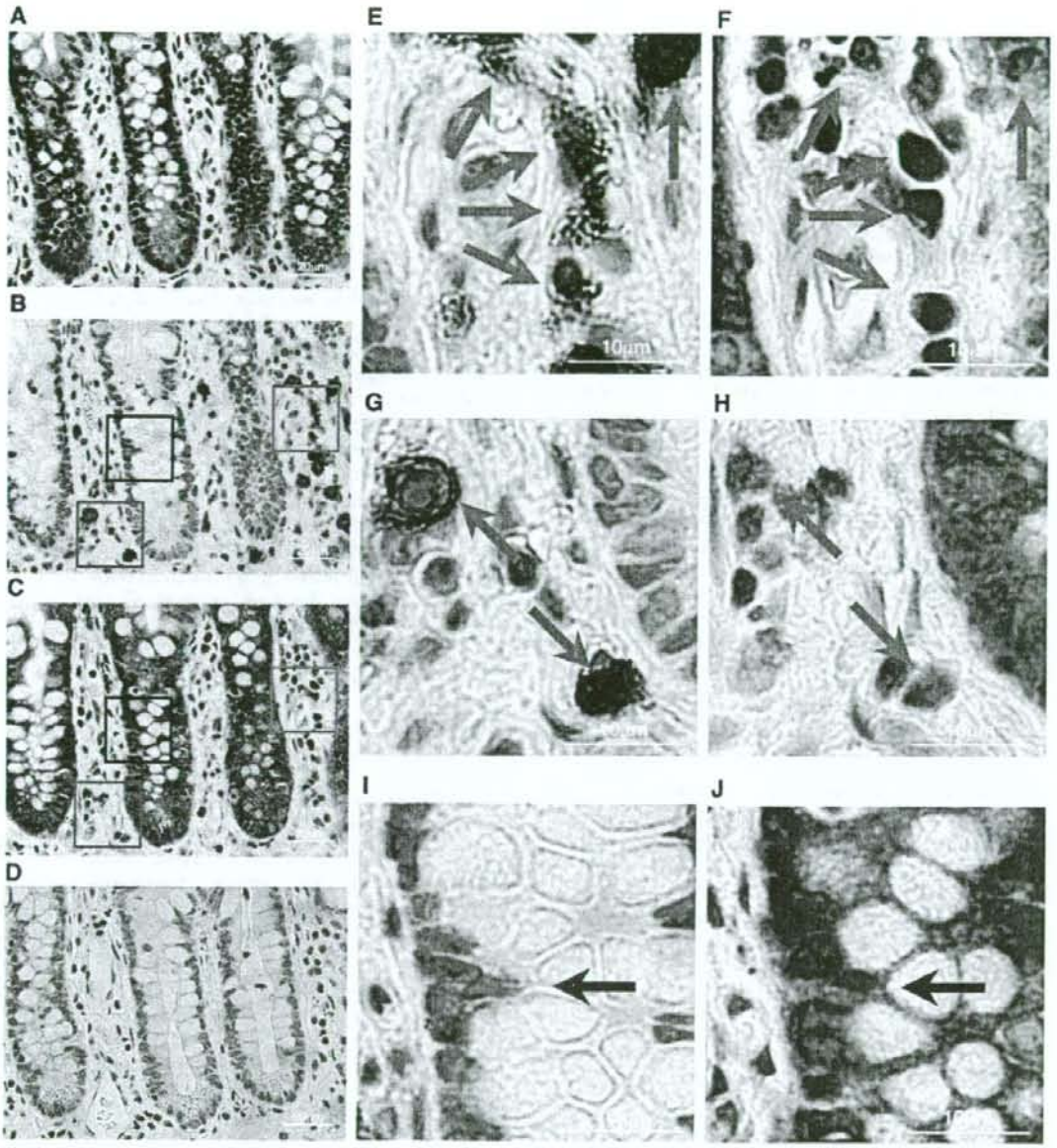

Figure 1. Identification of c-kit–expressing cells in the human adult intestine. Sequential sections of ileal mucosa were stained with (*A*) H&E, (*B*) c-kit antibody by 3-3'-diaaminobenzidine–enhanced immunoperoxidase and counterstained with hematoxylin, (*C*) toluidine *blue* to identify mast cells, and (*D*) negative control. The high-power image of representative c-kit$^+$ cells (*G*, *H*) with metachromatic staining (*red frame*) or (*E*, *F*) without metachromatic staining (*blue frame*) metachromatic staining. The high-power image of a (*I*) representative c-kit$^+$ IEL without metachromatic staining is shown in the (*J*) *black frame*.

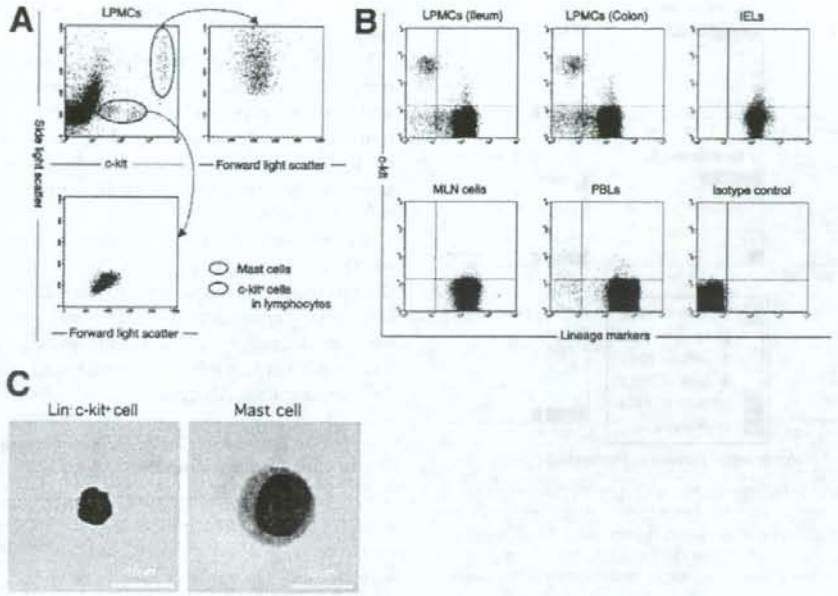

Figure 2. Flow cytometry of lin⁻ c-kit⁺ subset of LPMCs isolated from human adult intestine. (*A*) LPMCs were highlighted with c-kit expression and side-light scatter blotting (*upper left*). Side-light scatter high and c-kit high cells (red oval), which represented mast cells, were expanded further with side- and forward-side–scatter blotting (*upper right*). The cells with lower c-kit expression and lower side light scatter (*blue oval*) were expanded with the side- and forward-side–scatter blotting (*bottom left*). These cells were within a typical lymphoid cell gate. (*B*) LPMCs from ileum or colon, IELs, PBLs, and MLN cells in the lymphoid gate were analyzed for expression of c-kit and lineage markers (CD3, CD14, CD16, CD19, CD20, and CD56). The data shown are representative of 6 independent experiments. (*C*) The lin⁻ c-kit⁺ cells and the mast cells from LPMCs were stained with Giemsa solution.

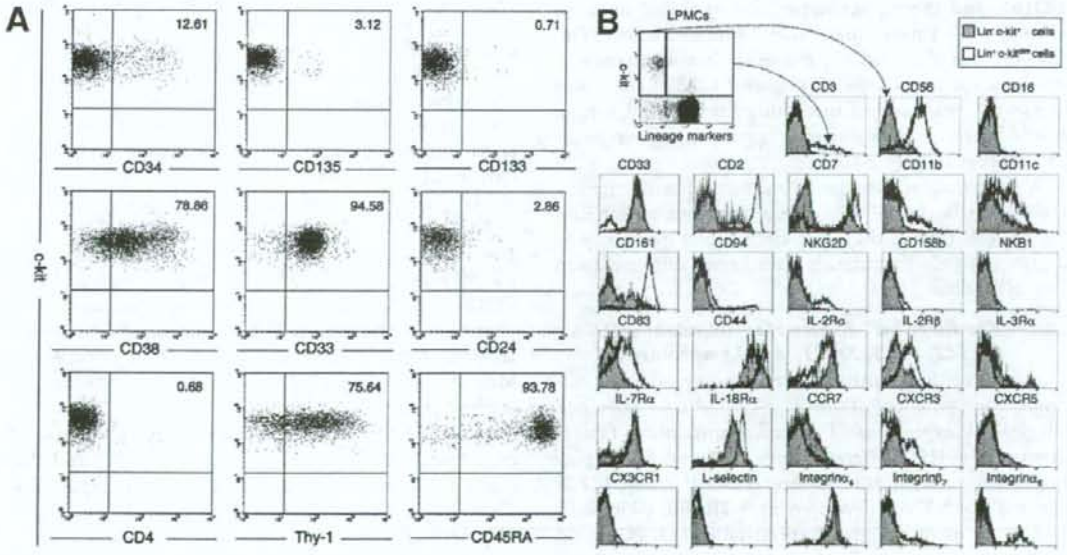

Figure 3. Characterization of the lin⁻ c-kit⁺ and lin⁻ c-kit^{dim} subsets of LPMCs. (*A*) Stem cell marker expression was analyzed in the lin⁻ c-kit⁺ subset of LPMCs. (*B*) The lin⁻ c-kit⁻ subset (*gray histograms*) and the lin⁻ c-kit^{dim} subset (*black lines*) of LPMCs were analyzed for the expression of several cell surface antigens. The data shown are representative of at least 5 experiments for each surface marker.