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]

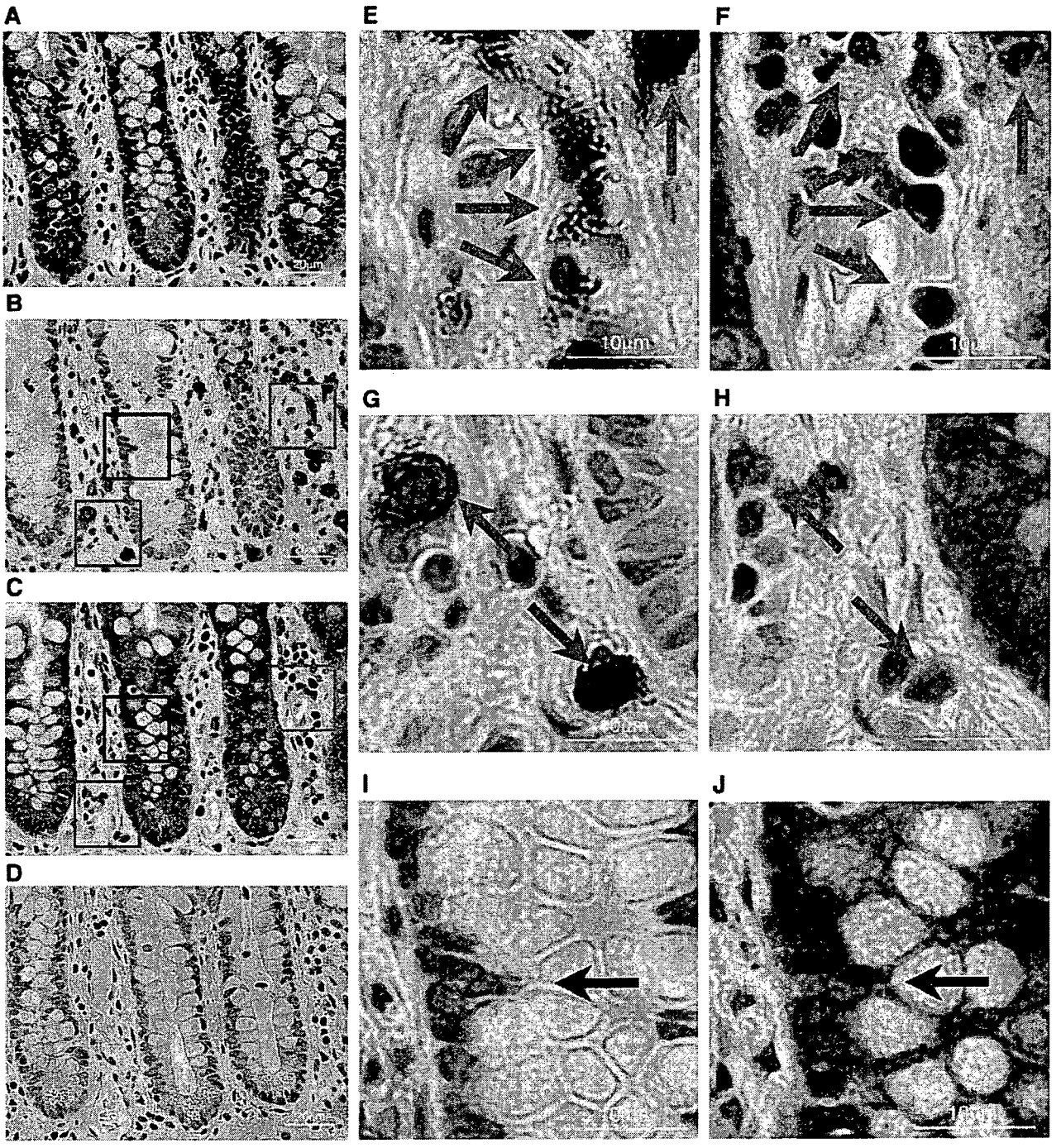


Figure 1. Identification of c-kit–expressing cells in the human adult intestine. Sequential sections of ileal mucosa were stained with (*A*) H&E, (*B*) c-kit antibody by 3-3′-diaaminobenzidine–enhanced immunoperoxidase and counterstained with hematoxylin, (*C*) toluidine *blue* to identify mast cells, 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*.

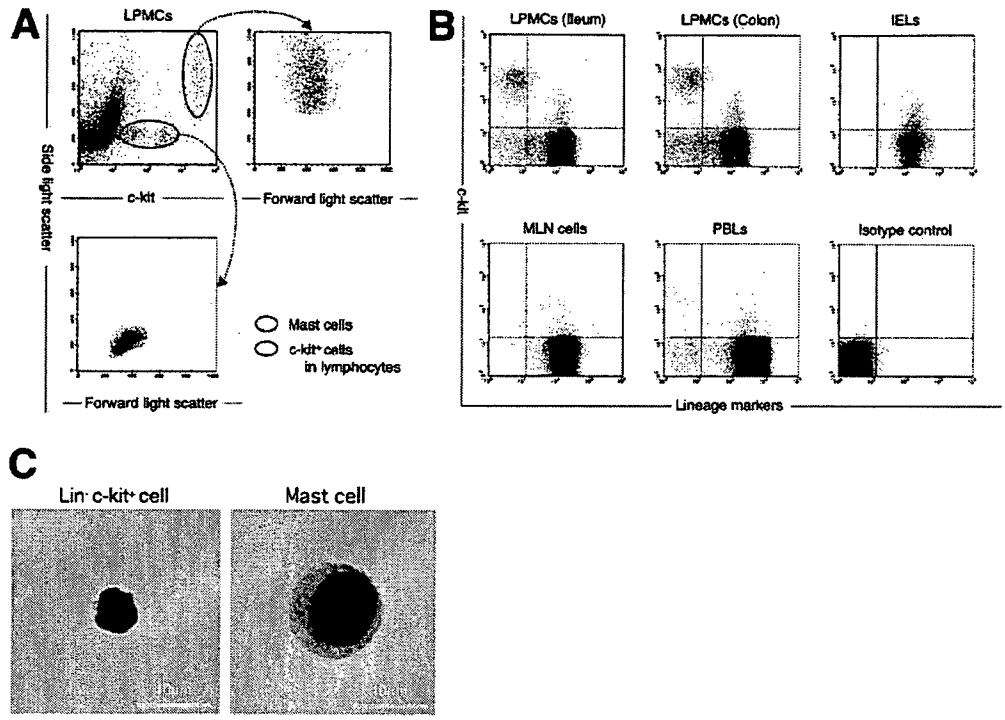


Figure 2. Flow cytometry of lin$^-$ c-kit$^+$ subset of LPMCs isolated from human adult intestine. (*A*) LPMCs were highlighted with c-kit expression and side-light scatter blotting (*upper left*). Side-light scatter high and c-kit high cells (red oval), which represented mast cells, were expanded further 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.

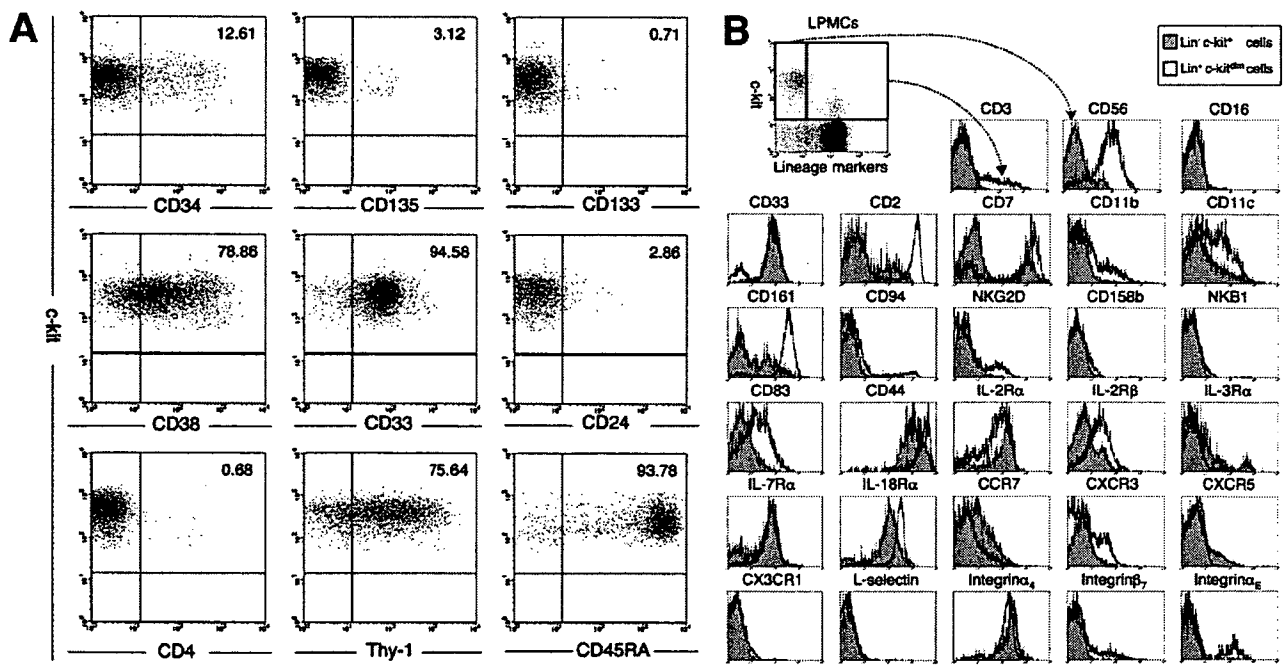


Figure 3. Characterization of the lin$^-$ c-kit$^+$ and lin$^+$ c-kit^{dim} subsets of LPMCs. (*A*) Stem cell marker expression was analyzed in the lin$^-$ c-kit$^+$ subset of LPMCs. (*B*) The lin$^-$ c-kit$^+$ subset (*gray histograms*) and the lin$^+$ c-kit^{dim} subset (*black lines*) of LPMCs were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

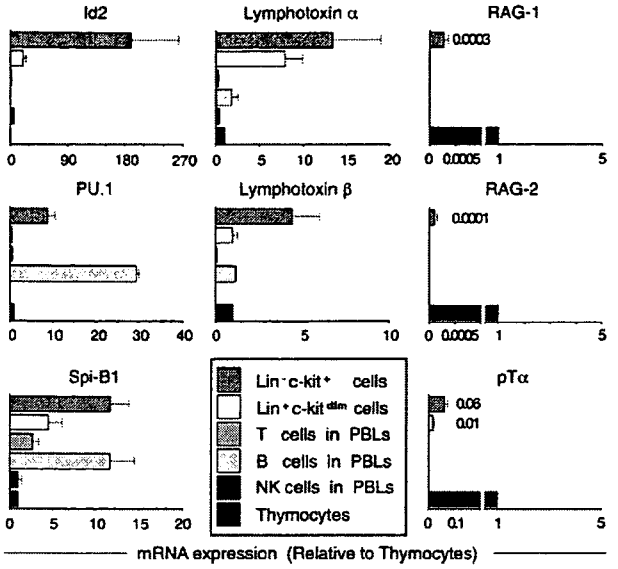


Figure 4. The lin⁻ c-kit⁺ subset has abundant mRNA for Id2, PU.1, SpiB1, and lymphotoxin α and β mRNA transcripts for each gene were quantified using real-time reverse-transcription PCR. In each sample, mRNA transcripts were normalized to those of β-actin. The expression value of each mRNA is shown as a ratio against that of thymocytes. Peripheral T, B, and NK cells were used as controls. Results are expressed as means ± SEM. The data are representative of 4 samples.

We next analyzed the lin⁺ c-kit^{dim} subset in LPMCs (Figure 3*B*). The lin⁺ c-kit^{dim} cells expressed only CD3 and CD56 among the lineage markers, and CD56⁺ cells predominated over CD3⁺ cells. The lin⁺ c-kit^{dim} subset also expressed other NK markers, IL-2Rβ and CD161, and these phenotypes corresponded to those of NKPs.[31,32] Interestingly, they expressed CD83 (Figure 3*B*), but they did not express other dendritic cell markers such as CD80, CD86, and CD209 (data not shown). It was also an intriguing finding that a fraction of these cells expressed integrin α_E, known as an IEL marker (Figure 3*B*).[33]

With the surface antigen expression patterns, the lin⁻ c-kit⁺ cells in the LPMCs were reminiscent of T/NKPs. On the other hand, the lin⁺ c-kit^{dim} subset likely may be a group of cells that already have begun differentiation into NK cells.

The lin⁻ c-kit⁺ Subset Has Abundant mRNA for Id2, PU.1, SpiB1, and Lymphotoxin

To confirm further the immature nature and differentiation potential of the lin⁻ c-kit⁺ cells, we examined the mRNA expression of several transcription factors essential for HSC differentiation. PU.1 and SpiB1 play important roles in HSC differentiation[34,35] and Id2 is essential for NK cell development.[36] The lin⁻ c-kit⁺ cells had much more abundant expression of Id2, PU.1, and SpiB1 mRNA compared with thymocytes (Figure 4). Note that mRNA of these transcription factors was decreased in the lin⁺ c-kit^{dim} cells, suggesting that they were at a later stage of differentiation than the lin⁻ c-kit⁺ population.

In addition to these transcription factors, we analyzed lymphotoxin α and β mRNA transcripts. Lymphotoxin α and β are reported to be critical for both NK cell development[37] and fulfillment of LTi functions.[38] The lin⁻ c-kit⁺ cells also had much more abundant mRNA transcripts of lymphotoxin α and β compared with thymocytes (Figure 4).

We next examined RAG-1, RAG-2, and pTα mRNA, which are essential for early T-cell differentiation. Although these transcripts were detectable in the lin⁻ c-kit⁺ cells, their expressions were far lower than that in thymocytes (Figure 4). These results indicate that the lin⁻ c-kit⁺ cells have an immature nature and the potential to differentiate into NK cells.

The lin⁻ c-kit⁺ LPMCs Are Committed to NK Cell Lineage In Vitro

Based on the results obtained so far, we assumed that the lin⁻ c-kit⁺ cells in the human adult intestine were a subset very close to T/NKPs. To confirm the differentiation capacity of the lin⁻ c-kit⁺ cells, we cultured LPMCs after depletion of both CD3⁺ and CD56⁺ cells without any exogenous stimuli. This culture method can maintain the interaction of the c-kit⁺ cells with the surrounding cells, via cell–cell contact or humoral soluble factors, which may be essential for the differentiation process. At the beginning of the culture period, we confirmed that the lin⁺ c-kit^{dim} subset was excluded completely because of CD3⁺ and CD56⁺ cell depletion (Figure 5*A*). However, surprisingly, c-kit^{dim} cells expressing lineage markers emerged and increased during the culture period (Figure 5*A* and *B*). Conversely, the lin⁻ c-kit⁺ cells gradually decreased. These lin⁺ c-kit^{dim} cells must have developed from the lin⁻ c-kit⁺ subset because the c-kit^{dim} cells did not emerge from the sorted c-kit⁻ population during 72 hours of culture (Supplementary Figure 1; supplementary material online at www.gastrojournal.org).

The c-kit⁺ cells became larger in size and had more granularity as they developed (Figure 5*C*). Most of the c-kit^{dim} cells up-regulated NK cell markers such as CD56, CD161, and IL-2Rβ, and they also expressed integrin α_E (Figure 5*C*, *D*, and *E*, Table 1). However, they did not express activated NK cell markers CD80 and CD86 (Table 1).[39] Meanwhile, very few CD3⁺ cells could be detected among the lin⁺ c-kit^{dim} populations (Figure 5*C* and *D*). Furthermore, the CD56⁺ cells in the newly emerging lin⁺ c-kit^{dim} cells contained cytotoxic granules (Figure 5*F*) and they could exert cytotoxicity against K562 cells (Figure 5*G*). They also were able to produce IFN-γ and TNF-α (Figure 5*H*). According to some previous studies, it takes up to 2 weeks for NK cell development from HSC in vitro culture.[40] Therefore, the lin⁻ c-kit⁺ subset likely included the population that already committed to NK cell lineage.

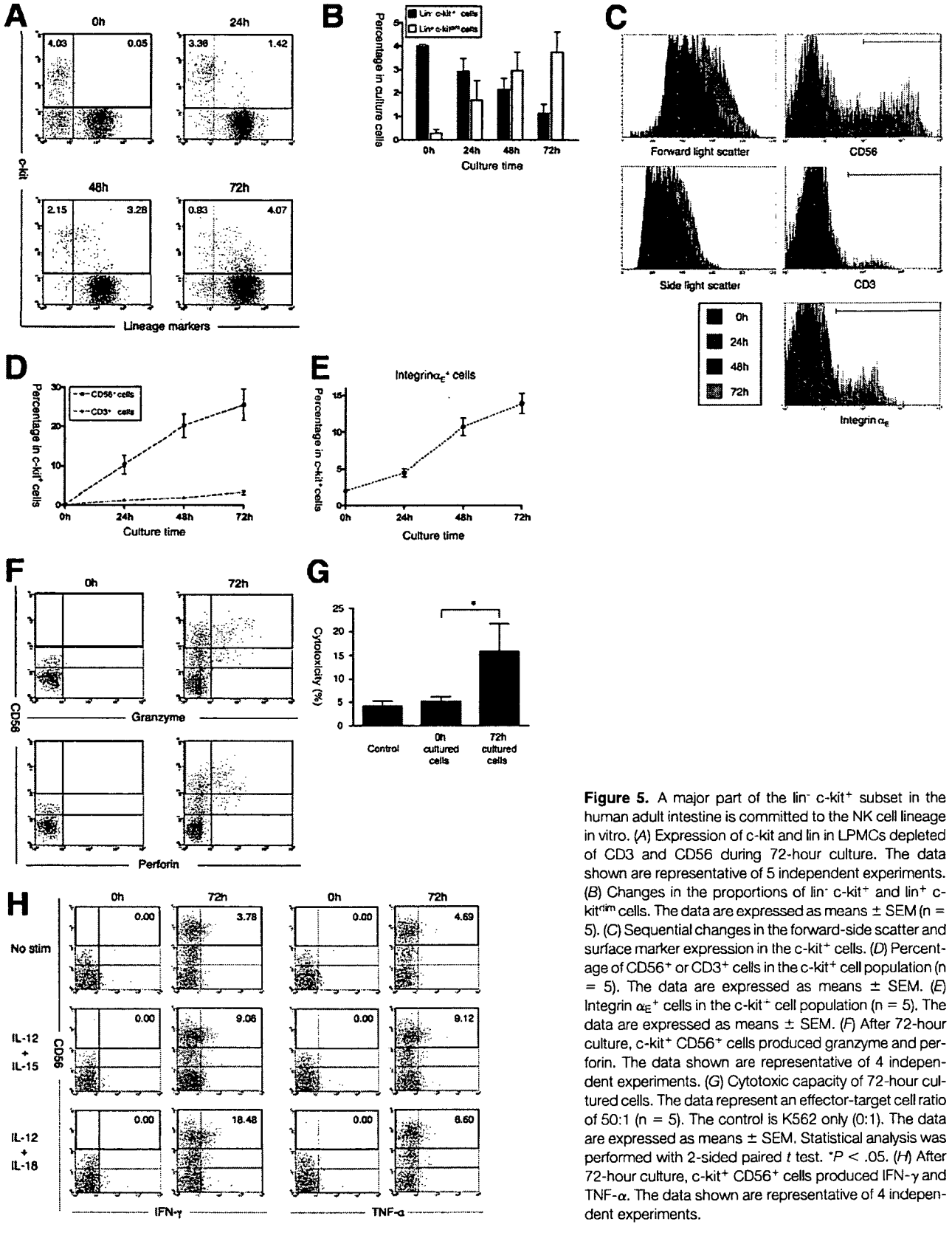


Figure 5. A major part of the lin$^-$ c-kit$^+$ subset in the human adult intestine is committed to the NK cell lineage in vitro. (*A*) Expression of c-kit and lin in LPMCs depleted of CD3 and CD56 during 72-hour culture. The data shown are representative of 5 independent experiments. (*B*) Changes in the proportions of lin$^-$ c-kit$^+$ and lin$^+$ c-kit^{dim} cells. The data are expressed as means ± SEM (n = 5). (*C*) Sequential changes in the forward-side scatter and surface marker expression in the c-kit$^+$ cells. (*D*) Percentage of CD56$^+$ or CD3$^+$ cells in the c-kit$^+$ cell population (n = 5). The data are expressed as means ± SEM. (*E*) Integrin α_E^+ cells in the c-kit$^+$ cell population (n = 5). The data are expressed as means ± SEM. (*F*) After 72-hour culture, c-kit$^+$ CD56$^+$ cells produced granzyme and perforin. The data shown are representative of 4 independent experiments. (*G*) Cytotoxic capacity of 72-hour cultured cells. The data represent an effector-target cell ratio of 50:1 (n = 5). The control is K562 only (0:1). The data are expressed as means ± SEM. Statistical analysis was performed with 2-sided paired *t* test. $^*P < .05$. (*H*) After 72-hour culture, c-kit$^+$ CD56$^+$ cells produced IFN-γ and TNF-α. The data shown are representative of 4 independent experiments.

Table 1. Phenotypes of the Newly Emerging c-kit^{dim} Cells After 72-Hour In Vitro Culture

Cell surface markers	Cultured cells (72 h)
CD34	–
CD38	+++
CD33	++
CD2	+++
CD7	++
CD11b	+
CD11c	+
CD161	+++
CD94	+
NKG2D	+
CD158a/b	–
NKB1	–
L-selectin	–
CD80	–
CD83	+
CD86	–
CD209	–
IL-2Rα	++
IL-2Rβ	++
IL-3Rα	–/+
IL-7Rα	+++
IL-18Rα	+++
CCR7	+
CXCR3	+
CXCR5	–
CX3CR1	–
Integrin α_4	+++
Integrin β_7	+
Integrin α_E	+

–, 0%–5%; –/+, 5%–10%; +, 10%–50%; ++, 50%–75%; +++, 75%–100%.

When only isolated lin$^-$ c-kit$^+$ cells were rendered to in vitro culture, they lost viability with or without exogenous human cytokines such as IL-2 and/or IL-15. Then we performed transfer experiments in which RAG-2–deficient mice received isolated human lin$^-$ c-kit$^+$ LPMCs. Very surprisingly, 3 weeks after transplantation we could detect human lin$^-$ c-kit$^+$ cells exclusively in murine LPMCs but not in IELs or splenic lymphocytes (Figure 6*A*). The absence of CD56 expression indicated that they did not differentiate into NK cells in murine intestine. However, human CD56$^+$ cells emerged when murine LPMCs containing human lin$^-$ c-kit$^+$ cells were cultured with exogenous IL-2 and/or IL-15 (Figure 6*B*).

These results suggested that a large proportion of human LP lin$^-$ c-kit$^+$ cells were the NK cell precursors. Given that newly emerging lin$^+$ c-kit^{dim} cells from lin$^-$ c-kit$^+$ subtype shared a very similar phenotype with the lin$^+$ c-kit^{dim} cells that actually reside in human LPMCs (Figure 3B and Table 1), this differentiation process may actually occur in the human intestine.

LPMCs and IELs Contain Functional NK Cells

The results thus far have indicated that the lin$^-$ c-kit$^+$ cells in the intestine have the potential to differentiate into NK cells. There have been only a few reports on NK cells in the human intestine,[41,42] therefore, we examined CD56 expression on LPMCs and IELs. It revealed that a considerable number of CD3$^-$ CD56$^+$ cells certainly exist in LPMCs (Figure 7*A*). These CD3$^-$ CD56$^+$ cells, which are referred to as LPNKs, were larger and had more granular morphology than lamina propria T cells (Supplementary Figure 2; supplementary material online at www.gastrojournal.org). CD3$^-$ CD56$^+$ cells also were found in IELs, which are referred to as intraepithelial NK cells (IENKs) (Figure 7*A*).[42] We next compared surface marker expression among the lin$^-$ c-kit$^+$, lin$^+$ c-kit^{dim}, and intestinal NK cells (LPNKs and IENKs) (Figure 7*B*). NK cell markers such as CD94, CD161, and NKG2D increased in the following order: lin$^-$ c-kit$^+$ cells < lin$^+$ c-kit^{dim} cells < intestinal NK cells, which is consistent with CD56 up-regulation on lin$^+$ c-kit^{dim} cells. Conversely, immature cell markers such as c-kit, IL-7Rα, and CD33 were decreased in the same order. These changes in surface marker expression also were observed during differentiation of lin$^-$ c-kit$^+$ cells into lin$^+$ c-kit^{dim} cells (Table 1). Taken together, these results would support the hypothesis that lin$^-$ c-kit$^+$ cells differentiate into intestinal NK cells via lin$^+$ c-kit^{dim} cells, possibly at the site of the intestine.

It is known that PBNK cells can be divided into 2 subsets according to the intensity of CD56 expression: CD56^{dim} and CD56^{bright} NK cells.[43,44] Although CD56 expression on the intestinal NK cells was not as high as on CD56^{bright} NK cells, the intestinal NK cells were reminiscent of CD56^{bright} NK cells owing to the lack of CD16, CX3CR1, and killer cell inhibitory receptor (CD158a/b, NKB1) expression (Figure 7*B*).[45,46] Moreover, both the intestinal NK cells and the peripheral CD56^{bright} NK cells expressed CD33, whereas the peripheral CD56^{dim} NK cells did not (Figure 7*B*).

To confirm that the intestinal NK cells harbor NK functions, cytotoxic molecules were examined. Both LPNKs and IENKs contained granzyme and perforin; and they were equipped with cytotoxic function, although the activity was weaker than that in peripheral CD56^{dim} NK cells (Figure 7*C* and *D*). Both LPNKs and IENKs also produced IFN-γ and TNF-α at a level comparable with peripheral CD56^{bright} NK cells (Figure 7*E–H*). With these phenotypes (ie, lower expression of granzyme and perforin and higher IFN-γ and TNF-α production), intestinal NK cells are reminiscent of peripheral CD56^{bright} NK cells. However, they clearly were discriminated from peripheral CD56^{bright} NK cells in terms of CD83 and integrin α_E expression (Figure 7*B*). These unique characteristics of intestinal NK cells may support the idea that they may have an original in situ differentiation system independent from PBNKs.

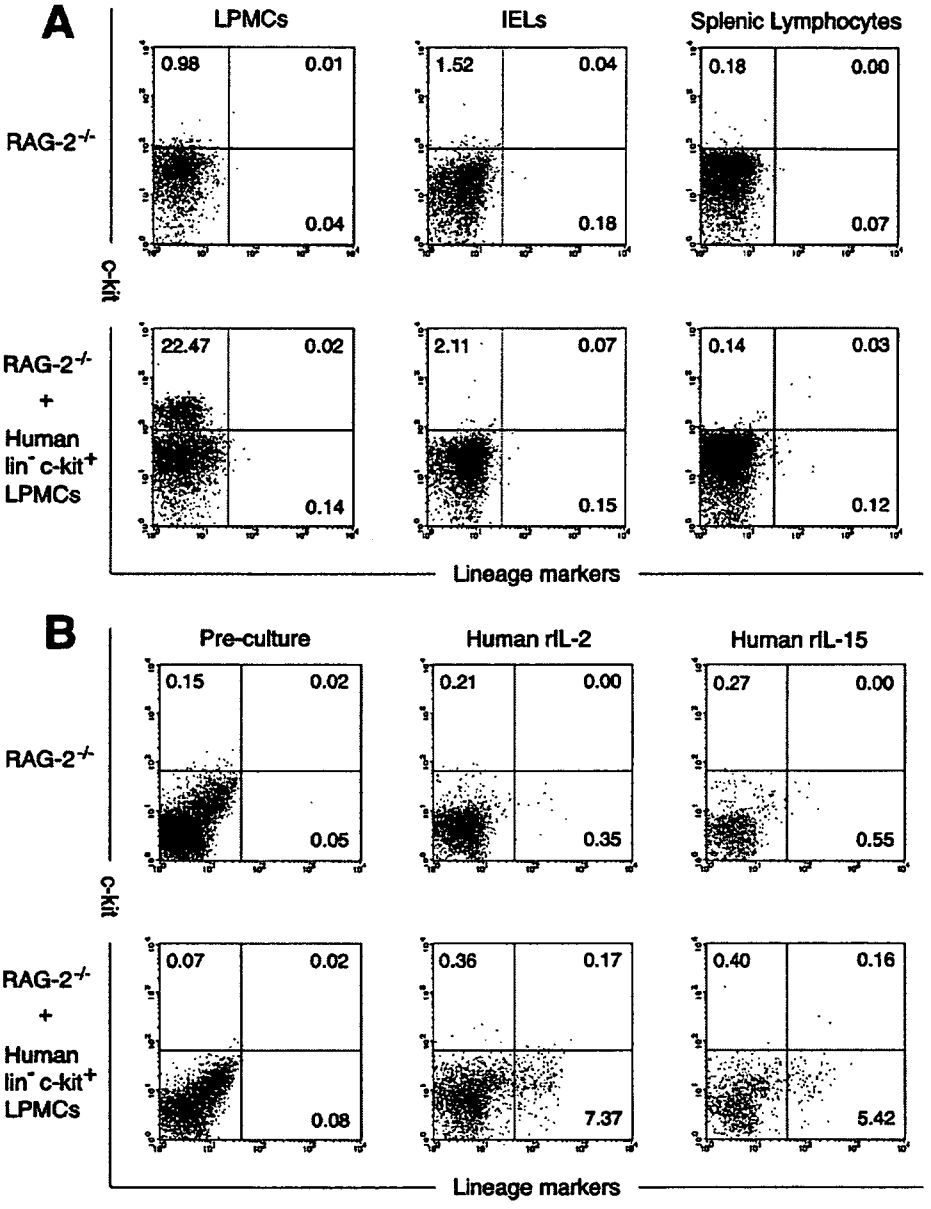
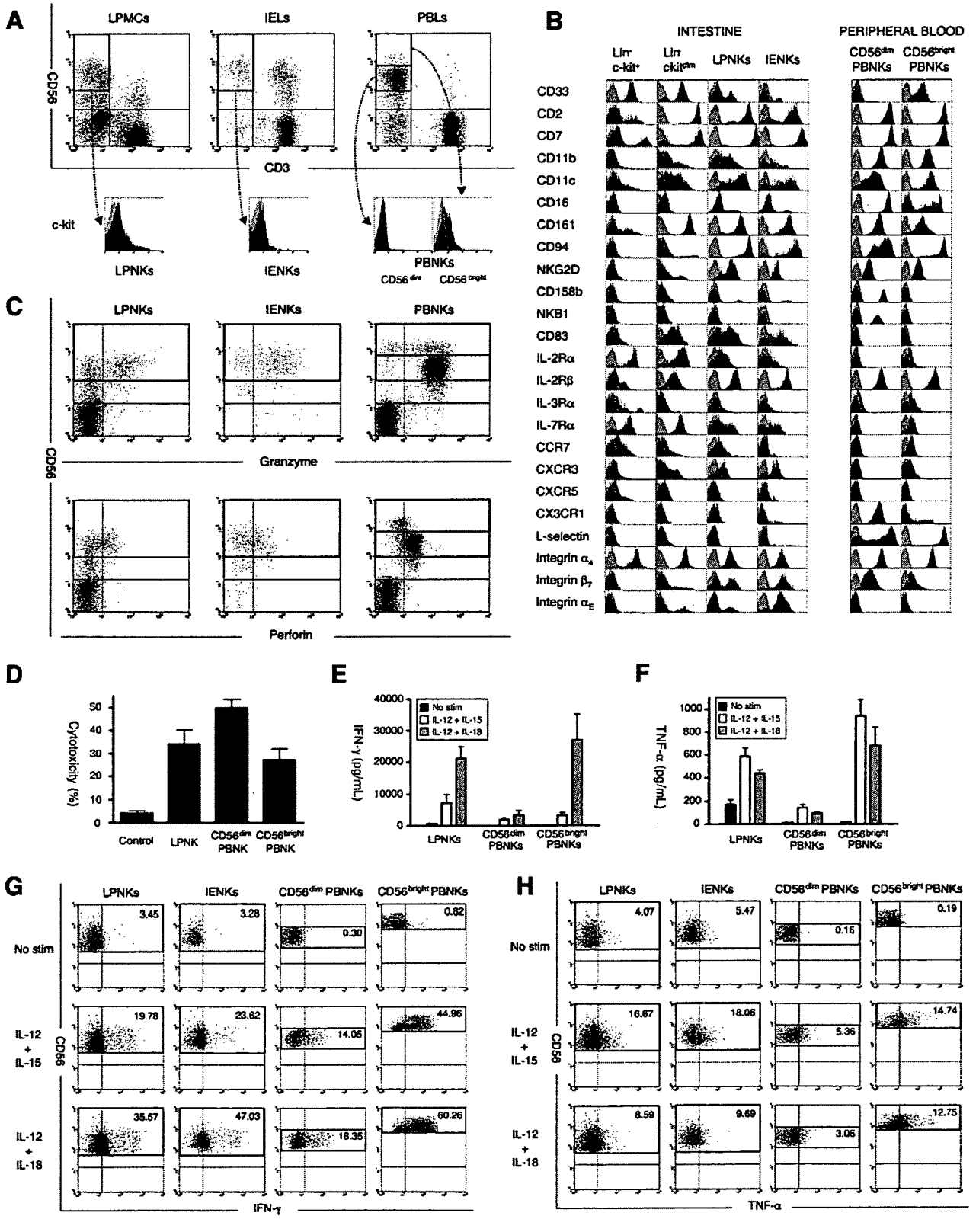


Figure 6. Human lin⁻ c-kit⁺ LPMCs after the transfer into recipient mice could differentiate into NK cells. (*A*) LPMCs, IELs, and splenic lymphocytes isolated from the RAG$^{-/-}$ mice transplanted with human lin⁻ c-kit⁺ LPMCs were stained by lineage markers and CD117 monoclonal antibody and were analyzed by fluorescence-activated cell sorter. The data shown are representative of 2 independent experiments. (*B*) Isolated LPMCs were cultured with human recombinant IL-2 (rIL-2) or rIL-15 for 7 days. The cultured cells were analyzed for expression of human CD3 and CD56. The data shown are representative of 2 independent experiments.

Intestinal NK Cells Are Increased in CD, Reflecting Accelerated Differentiation of lin⁻ c-kit⁺ Cells Into NK Cells

The involvement of NK cells in intestinal inflammation has not been well documented. Therefore, we examined intestinal NK cells in inflammatory bowel disease, a chronic inflammatory condition that consists of 2 major forms: CD and UC.[47] Flow cytometry revealed that both LPNKs and IENKs were increased in the samples from CD but not from UC patients, compared with

Figure 7. Analysis of surface antigen expression and function of intestinal NK cells. (*A*) LPMCs, IELs, and PBLs were stained for CD3 and CD56. CD3⁻ CD56⁺ cells were examined further for c-kit expression. (*B*) Expression of various surface markers, including NK cell markers, was compared among lin⁻ c-kit⁺ and lin⁺ c-kit^{dim} cells, LPNKs, IENKs, and PBNKs. The data shown are representative of more than 4 cases for each surface marker. (*C*) Production of granzyme, perforin from LPNKs, IENKs, and PBNKs were determined with intracellular staining. The data shown are representative of 4 independent experiments. (*D*) Cytotoxicity of freshly isolated LPNKs (n = 5) and PBNKs (n = 4). Effector-target ratio was 10:1. The data were expressed as means ± SEM. (*E*) IFN-γ and (*F*) TNF-α production from LPNKs (n = 5) and PBNKs (n = 4) after 72-hour culture with indicated cytokines. The data were expressed as means ± SEM. (*G*) IFN-γ or (*H*) TNF-α production in LPNKs, IENKs, and PBNKs after 8 hours of cytokine stimulation were detected using the Cytokine Secretion Assay Cell Detection Kit (Miltenyi Biotec). The data shown are representative of 4 independent experiments.



BASIC-ALIMENTARY TRACT

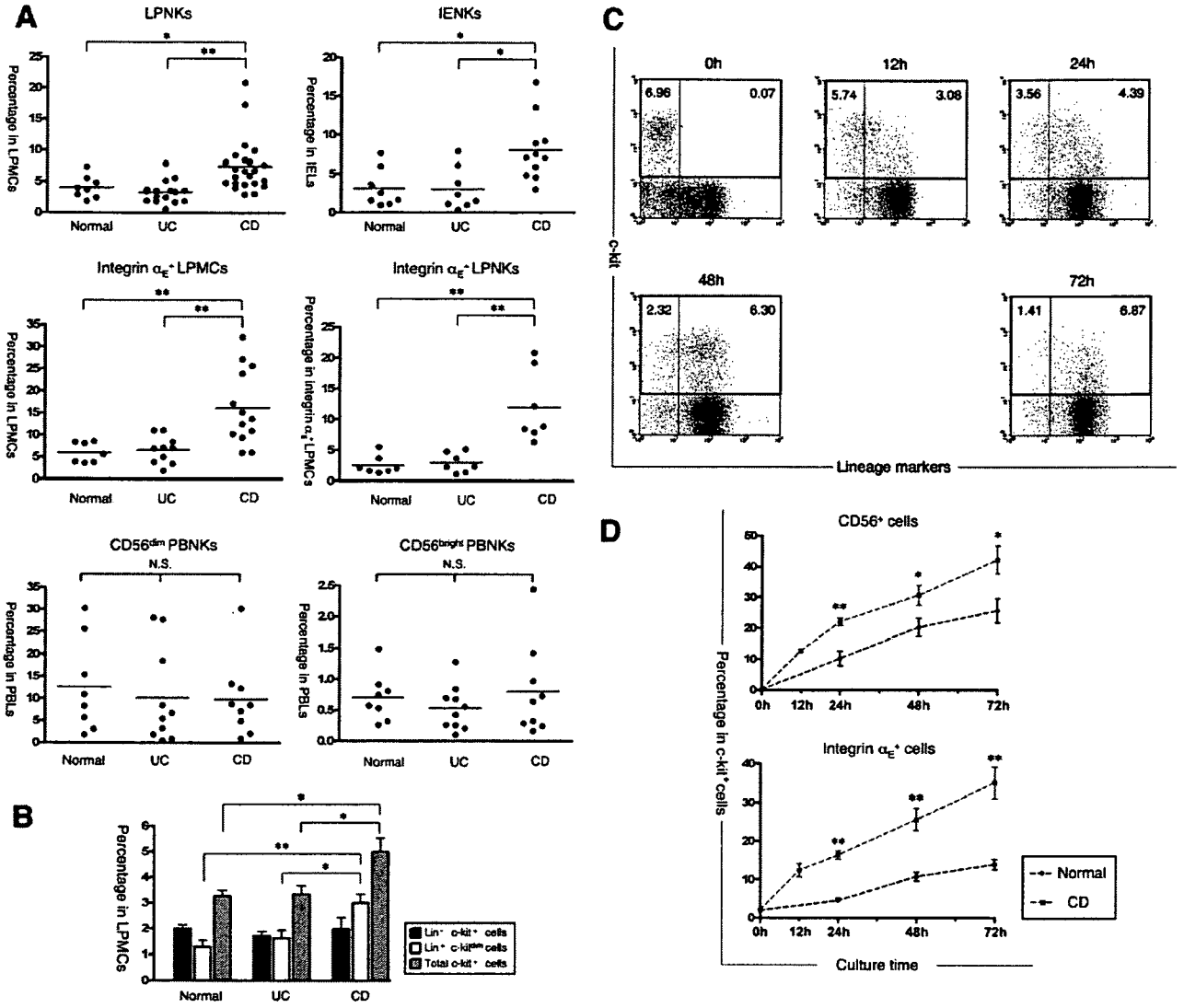


Figure 8. The c-kit^{dim} cells, LPNKs, and IENKs are increased in CD. (*A*) Percentage of NK cells among LPMCs (normal, n = 10; UC, n = 17; CD, n = 23) or IELs (normal, n = 8; UC, n = 8; CD, n = 11) (upper panels). Percentage of integrin $\alpha_E\beta_7^+$ cells among LPMCs (normal, n = 7; UC, n = 10; CD, n = 13) or LPNKs (normal, n = 7; UC, n = 7; CD, n = 7) (middle panels). Percentage of CD56^{dim} CD16$^+$ and CD56^{bright} CD16– NK cells among PBLs (normal, n = 8; UC, n = 10; CD, n = 9) (bottom panels). Statistical analysis was performed with the Kruskal–Wallis 1-way analysis of variance, and the Bonferroni–Dunn test for multiple comparisons. $^*P < .05$, $^{**}P < .01$. (*B*) Percentage of total c-kit$^+$, lin$^-$ c-kit$^+$, or lin$^+$ c-kit^{dim} cells among LPMCs (normal, n = 10; UC, n = 11; CD, n = 15). Results are expressed as means ± SEM. Statistical analysis was performed with the Kruskal–Wallis 1-way analysis of variance, and the Bonferoni–Dunn test for multiple comparisons. $^*P < .05$, $^{**}P < .01$. (*C*) LPMCs depleted of CD3 and CD56, obtained from CD patients, were cultured for 72 hours and analyzed for expression of c-kit and lin. The data shown are representative of 7 independent experiments. (*D*) The line graph shows the time–course changes for CD56+ or integrin α_E^+ cells in c-kit$^+$ LPMCs from normal controls (n = 5) or CD patients (n = 7). The data are expressed as means ± SEM for the percentage of CD56$^+$ or integrin α_E^+ cells among the c-kit$^+$ cells. Statistical analysis was performed with a 2-sided Mann–Whitney *U* test. $^*P < .05$, $^{**}P < .01$.

normal controls (Figure 8*A*). In contrast, the frequency of NK cells, both for CD56^{dim} and CD56^{bright}, was similar in peripheral blood among the 3 groups (Figure 8*A*). Furthermore, although lin$^-$ c-kit$^+$ cells existed with similar frequency, the lin$^+$ c-kit^{dim} cells were increased significantly in CD compared with UC or normal controls (Figure 8*B*). Then we repeated the in vitro culture experiments shown in Figure 5 and found that more lin$^+$ c-kit^{dim} cells were detected in CD samples at each time point (Figure 8*C*), and the majority of these cells expressed CD56 and integrin α_E (Figure 8*D*). Taken together, the increase of LPNKs and IENKs in CD patients could have been owing to accelerated differentiation from lin$^-$ c-kit$^+$ cells. Given that the intestinal NK cells can strongly produce IFN-γ and TNF-α, which is a key cytokine in the pathogenesis of CD, these increased intestinal NK cells may play a pathogenic role in chronic inflammation in CD.

Discussion

The sites of NK cell development in adults are understood poorly.[40,48] Although T/NKPs have been identified only in fetal tissues, the bone marrow is presumed to be the main site of NK cell generation in adults.[40,48] In this study, we have shown that lin$^-$ c-kit$^+$ cells in human adult intestine could differentiate into c-kit^{dim} cells, which express CD56 during in vitro culture, suggesting that these cells are NK cell precursors. Moreover, further analysis showed that in vitro differentiated c-kit^{dim} CD56$^+$ cells seemed to correspond to c-kit^{dim} CD56$^+$ cells actually present in human adult intestine. Combined together, adult intestine may have unique NK cell differentiation system in which lin$^-$ c-kit$^+$ NK precursors undergo in situ differentiation via c-kit^{dim} cells.

The newly discovered c-kit$^+$ cells in the human adult intestine also express CD34, another marker for HSCs or immune precursor cells.[17,28] In addition to c-kit and CD34, the intestinal immune precursors expressed CD38^{dim}, CD44, CD45RA, and Thy-1, the phenotypes of which correspond to those of common lymphoid progenitors or T/NKPs.[49] Furthermore, they had abundant mRNA transcripts for Id2, PU.1, SpiB1, and lymphotoxin, all of which are essential for HSC differentiation or NK cell development.

In the murine intestine, c-kit–expressing cells form small clusters named CP.[8] It has been reported that CP cells have the potential to differentiate mainly into extrathymic T cells in the intraepithelial space.[10] Interestingly, the intestinal c-kit$^+$ immune precursor cell expression level of RAG mRNA was very low, and the RAG expression level was similar to CP cells, which was reported previously by Oida et al.[50] Recent studies have shown that CD3$^-$ CD7$^+$ cells in the human fetal intestine express pTα mRNA[13] and give rise to CD3$^+$ T cells in vitro and in vivo, using SCID mice with engrafted human fetal intestine.[14] The intestinal c-kit$^+$ immune precursor cells are also CD3$^-$ CD7$^+$ and express RAG-1, RAG-2, and pTα mRNA. These results imply that the immune precursor cells in adult intestine include a subset similar or identical to the CD3$^-$ CD7$^+$ cells in the fetal intestine and they may differentiate into T cells in unusual environment, such as lymphopenia. However, in that they do not form aggregates and are much more committed to NK cells rather than T cells, they should be distinguished from the murine CP cells that differentiate into intraepithelial T cells. On the other hand, a recent study showed that c-kit$^+$ cells in CP represent LTi in adult mice, which organize isolated lymphoid follicles.[12] Furthermore, because LTi and T/NKPs have similar expression patterns of surface antigens and transcription factors,[51,52] they are considered to be subsets that are related closely to each other. Given that intestinal immune precursor cells also are similar to LTi in terms of surface antigen expression and transcriptional profile, it is possible that they contain an adult LTi subset.

Little information is available about intestinal NK cells. An earlier article reported on lymphokine activated killer activity in human LPMCs,[53] although they failed to identify NK cells in LPMCs, possibly because of the lack of suitable NK cell markers at that time. Some recent reports showed that human LPMCs and IELs contain NK cells capable of killing tumor cells and producing several cytokines, such as IFN-γ and TNF-α.[41,43] In this study, we intensively examined the intestinal NK cells to verify the hypothesis that they develop in situ from the immune precursor cells in intestinal lamina propria. In terms of NK cell markers, expression of CD56, as well as CD94, CD161, and NKG2D, was lowest in the c-kit$^+$ cells, and inversely highest in LPNKs/IENKs. In contrast, immature cell markers such as c-kit, IL-7Rα, and CD33 were highest in the intestinal immune precursor cells. Furthermore, these changes in surface marker expression also were observed during in vitro differentiation of lin$^-$ c-kit$^+$ cells into c-kit^{dim} cells. Collectively, these results support the idea that intestinal immune precursor cells can give rise to intestinal NK cells via c-kit^{dim} NKP-like cells.

PBNKs can be classified into 2 subsets. One subset is the conventional CD56^{dim} NK cells and the other is the CD56^{bright} NK cells.[43,44] Absence of CD16 expression is also a characteristic feature of the CD56^{bright} NK cells. Although CD56 expression of the intestinal NK cells was not as high as the peripheral CD56^{bright} NK cells, absence of CD16 expression indicates a similarity between the intestinal NK cells and the peripheral CD56^{bright} NK cells. In addition, we found that both the intestinal NK cells, especially LPNKs, and peripheral CD56^{bright} NK cells expressed CD33. Although CD33 is a myeloid lineage marker, it is reported that CD33$^+$ CD34$^+$ HSCs can give rise to CD16$^-$ NK cells in vitro.[54] Given that most intestinal immune precursor cells express CD33, and that intestinal NK cells are CD33$^+$ CD16$^-$, it is reasonable to assume that the intestinal NK cells may originate from the immune precursor cells. Furthermore, CD33 is reported to be expressed on CD56^{bright} NK cells in umbilical cord blood[55] and on T/NKPs in the fetal thymus.[29] Although the origin of CD56^{bright} NK cells still is controversial, it recently was reported that the peripheral CD56^{bright} NK cells differentiate in the lymph nodes, unlike conventional CD56^{dim} NK cells.[56] CD33$^+$ CD16$^-$ may be a phenotype that characterizes NK cells developing outside the bone marrow, such as lymph nodes, the thymus, and maybe the intestine.

The pathophysiologic contribution of intestinal NK cells to inflammatory bowel disease has yet to be elucidated. An interesting recent report suggested that the intestinal NK cells maintain homeostasis of intestinal mucosal immune system in mice. However, their roles have not been resolved in human beings.[57] We found that the differentiation of the intestinal immune precursor cells into NK cells was accelerated in CD, resulting in an increase in the number of intestinal NK cells in CD

compared with UC or normal controls. According to the previous report, $CD56^{bright}$ NK cells also are enriched at inflammatory sites, such as arthritis, infectious pleuritis, and bacterial peritonitis.[58] CD is regarded as a typical T helper type 1 response (Th1) disease driven by excessive IFN-γ production from dysregulated CD4 T cells infiltrating the inflamed tissue. However, given that NK cells constitute a considerable proportion of LPMCs or IELs (about 8%) and can highly produce IFN-γ, intestinal NK cells may contribute to the pathogenesis of CD. Overexpression of IFN-γ in CD may modulate intestinal NK cell differentiation because it was reported that this cytokine accelerated differentiation of human HSCs.[59]

In summary, we have identified c-kit$^+$ immune precursor cells in the human adult intestine for the first time. We also have shown that these cells are committed mainly to the NK cell lineage. Because this intestinal NK cell differentiation system may contribute to the pathophysiology of CD, further clarification of the role of intestinal NK cells will help to better understand the gut immune system and may lead to new therapeutic strategies against CD.

Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.05.017.

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Clinical Significance of Serum p53 Antibodies in Patients with Ulcerative Colitis and Its Carcinogenesis

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Background: For early detection of ulcerative colitis (UC)-associated colorectal cancer (CRC), surveillance colonoscopy is recommended in UC patients at high risk. However, poor acceptability deteriorates its effectiveness and a suitable marker for selecting patients at high risk is needed. Here we evaluated clinical usefulness of the measurement of anti-p53 antibodies (Abs) by enzyme-linked immunosorbent assay (ELISA) using sera samples from UC patients.

Methods: Sera from 286 patients with UC, 82 patients with sporadic CRC, and 63 healthy controls (HC) were obtained. Serum anti-p53 antibodies were detected with ELISA. Immunohistochemical detection was also performed in patients who developed dysplasia or CRC.

Results: Serum p53 Ab was positive in 15.0% of UC, while it was positive only in 1.6% of HCs. In sporadic CRCs, 52.4% of 82 patients were positive. In UC patients with disease duration equal to or longer than 8 years, positivity of serum p53 Ab was significantly higher than those in patients with shorter duration. Eight of 13 (61.5%) UC patients with CRC or dysplasia were positive for serum p53 Abs, which was significantly higher than that in patients without neoplasia. All UC patients with CRC were positive for p53 staining, while 2 were negative for serum p53 Ab. Finally, levels of serum p53 Ab had fallen in 4 patients with CRC we could monitor after surgery.

Conclusions: This study revealed that p53 Ab developed in the progression of UC-associated CRC but not in all patients with neoplasia, suggesting that serological detection of p53 Abs by ELISA is not suitable in primarily selecting patients at high risk; however, it is helpful in salvaging patients who drop from a surveillance program.

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Key Words: ulcerative colitis, colorectal cancer, p53, enzyme-linked immunosorbent assay, surveillance

Ulcerative colitis (UC) is an inflammatory bowel disease of unknown etiology characterized by periods of remission and acute episodes of relapse that accompany severe inflammation in colonic mucosa.[1] The risk for colorectal cancer (CRC) increases in patients with chronic UC after 10 years and the cumulative CRC rate is 2% after 10 years, 8% after 20 years, and 18% after 30 years of the disease,[2] while it was reported to be relatively low in Japan, 0.5%, 4.1%, and 6.1%, respectively.[3] Risk factors for CRC in patients with UC include disease duration, early onset, extensive disease, complication of primary sclerosing cholangitis, severity of inflammation, and family history of sporadic CRC.[4,5] Since the development of CRC accounts for poor prognosis in patients with UC, a surveillance program using colonoscopy with random biopsies in patients with long-standing UC is currently recommended.[6,7]

Cancer surveillance is based on the hypothesis that repeated testing of a high-risk population will identify patients who have cancer or who have a potential risk for the development of cancer. Dysplasia in UC mucosa is not recognized only as a precursor of cancer, but an indicator for the simultaneous presence of cancer.[8–10] Dysplasia is usually patchy and develops from flat mucosa, and therefore it may be missed even with multiple biopsies. Random colonic mucosal biopsies have been recommended in surveillance colonoscopy; however, the number of biopsy specimens required is large and the cost of this procedure is very high.[11,12] Although the number of patients subject to surveillance is increasing year by year, all of such patients do not enroll in a surveillance program. Poor acceptability for surveillance colonoscopy and low patient compliance deteriorate the effectiveness of surveillance programs.[13] Moreover, patients who developed CRC out of a surveillance program had poor prognoses.[3] Therefore, a suitable marker for selecting patients

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TABLE 1. Characteristics of Study Populations

	HC (n = 63)	UC (n = 286)	CRC (n = 82)
Sex (M:F)	23:40	136:150	56:26
Age (mean ± SD, range)	52.9 ± 17.8 (25–88)	40.3 ± 14.6 (14–85)	65.5 ± 11.2 (31–91)

HC, healthy control; UC, ulcerative colitis; CRC, sporadic colorectal cancer; M, male; F, female; SD, standard deviation.

at high risk of developing CRC in UC, who would be strongly recommended for surveillance colonoscopy, is needed, even though it must be ideal that all UC patients with longer duration and extensive disease could take surveillance colonoscopy every year.

Molecular mechanisms underlying UC-associated neoplasia have been studied for years, but understanding of these mechanisms remains incomplete. It is likely that an accumulation of multiple genetic alterations leads to dysplasia and cancer in UC in a manner similar to that of sporadic CRC.[14] However, biological features differ between UC-associated CRC and sporadic CRC. Mutations in the *APC* gene and *ras* proto-oncogenes are not consistently present in UC-associated CRC in contrast to sporadic CRC resulting from an adenoma-carcinoma sequence pathway.[15–19] Conversely, mutation of the *p53* tumor suppressor gene is a frequent occurrence in early dysplastic UC lesions and precedes *p53* loss of heterogeneity.[20,21] Mutations of the *p53* gene are associated with, and likely precede, dysplasia and cancer,[22] whereas mutations within the *p53* gene in sporadic CRC are thought to occur at the adenoma-carcinoma transition.[23] Inactivation of the *p53* gene is usually caused by missense point mutations, most of which modify conformation and stability of p53 protein and lead to its accumulation in the nuclei of tumor cells. Although the most accurate procedure for analysis of p53 status is DNA sequencing, immunohistochemical analysis has been widely employed to screening p53 alterations in clinical and research fields. *p53* gene mutations with detection of the mutated protein products using immunohistochemistry offer promising possibilities as a test complementary to dysplasia for use in cancer surveillance programs.[24] Meanwhile, it has been shown that patients with various types of neoplasia have p53 antibodies (Abs) in their sera.[25,26] Recently, an approach for diagnosis of p53 alterations has been developed, which consists of detection of p53 Abs in sera from patients affected by a wide variety of cancers by enzyme-linked immunosorbent assay (ELISA).[27–29] Serological detection of p53 Abs is easier to perform and to repeat, does not require tumor material, and is of potential interest for monitoring patients with cancer.

We previously reported that interferon-inducible gene family 1-8U expression was significantly higher in UC-associated CRC tissues than in mucosa with mild inflammation in the same UC patients[30] and that UC-associated cancer or dysplasia had a higher percentage of microsatellite instability than nonneoplastic UC mucosa.[31] Although these findings could be good markers for distinguishing UC-associated CRC from nonneoplastic tissue, we consider that these are not suitable markers for selecting patients at high risk for the development of CRC in the clinical setting. In the present study we attempt to evaluate the clinical usefulness of measurement of anti-p53 Abs by ELISA using sera samples from UC patients with or without neoplasia.

PATIENTS AND METHODS

Study Population

This study was carried out according to the ethical standards of the institution's human investigation committee and the Helsinki Declaration of 2004 and was approved by the ethical committee at Keio University Hospital. All patients and healthy controls (HCs) were enrolled at Keio University Hospital between 2002 and 2005 and gave informed consent.

Blood samples were obtained by venipuncture from 286 patients with UC and 82 patients with sporadic CRC as positive disease control. UC patients with CRC who had undergone surgery were excluded. The diagnosis of UC was according to accepted criteria.[32,33] At the time of diagnosis, we excluded infectious colitis, radiation colitis, ischemic colitis, Crohn's disease, and intestinal Behçet's disease. All the patients with sporadic CRC were at advanced stage and treated surgically. Blood samples were also obtained from 63 HCs who were defined as subjects never diagnosed to have any cancer or UC by yearly medical examinations including fecal occult blood test, even though total colonoscopy was not performed in every HC subject.

Out of 286 UC patients, 136 (47.6%) were male and 150 (52.4%) were female, with a mean age of 40.3 years (range, 14–85); in patients with sporadic CRC, 56 (68.3%) were male and 26 (31.7%) were female, with a mean age of 65.5 years (range, 31–91); and in HCs, 22 (34.9%) were male and 41 (65.1%) were female, with a mean age of 49.9 years (range, 25–83) (Table 1).

In UC patients, smoking habits and family history of CRC were reviewed. Clinicopathological features of UC such as duration of the disease, extension of the disease, clinical disease activity (Truelove-Witts criteria[34]) at the time of

sample collection, extraintestinal complications, and medications were collected from the medical records. No patients were known to have previously had any other malignant or autoimmune disease.

All patients with UC had undergone routine colonoscopy or barium enema study within 1 year before blood sampling. We performed surveillance colonoscopy with random biopsies in addition to targeted biopsies for UC patients with long-standing and extensive colitis; however, only 7.9% of patients received surveillance colonoscopy every year in our institute. The study population included patients with UC not subjected to a surveillance program, and therefore a considerable ratio of patients with UC did not have so-called surveillance colonoscopy.

Among 286 UC patients, endoscopic and histological examinations revealed that 8 patients had CRC, 1 had high-grade dysplasia (HGD), and 4 had low-grade dysplasias (LGD) at the colorectum. The degree of dysplasia was diagnosed according to previously published criteria.[10] UC-associated CRC was staged according to the fifth edition of the American Joint Committee on Cancer staging system.[35] No other patients with UC were revealed to have any neoplasms at the colorectum and following up these patients after blood sampling revealed that none of these developed CRC or dysplasia. Those with UC-associated CRC who underwent surgery were subjected to repeated analyses of serum anti-p53 Abs after surgery.

Serum Samples

Whole blood was centrifuged at 1200*g* for 10 minutes and serum was stored in aliquots at −80°C until assayed.

ELISA for Anti-p53 Abs

ELISA for detection of anti-p53 Abs in serum was carried out with a commercially available ELISA kit (DIANOVA, Oncogene Research Products, Cambridge, MA). This ELISA utilizes microtiter plates precoated with recombinant human wildtype p53 protein. All samples were assayed in duplicate. Briefly, 100 μL per well of calibrators, negative control, or 1:100 diluted samples were added to a microtiter plate and incubated for 1 hour at room temperature (RT). Following incubation the plate was washed to rid it of any unbound material and 100 μL of a peroxidase-conjugated goat antihuman antibody, which bound any captured human p53 antibody, was added to the microplate and incubated for 1 hour at RT. After washing, 100 μL of chromogenic substrate tetramethylbenzidine was added to the wells. Enzymatic reaction was stopped after 30 minutes with 50 μL of 2 M HCl stop solution. Color reaction was measured immediately by absorption at 450 nm using a spectrophotometer. A calibration curve was constructed from specific signals of standards and from the levels of Abs indicated on standard vials.

For determination of the cutoff line we evaluated serum anti-p53 Abs of 63 HCs and defined 5.854 U/100 μL, mean value plus 2 standard deviation (SD), as the cutoff line for the Japanese population. Therefore, we judged positive for serum anti-p53 Ab in the present study when the anti-p53 Ab level was higher than 5.854 U/100 μL.

Immunohistochemistry

For those who were revealed to have dysplasia or CRC with colonoscopy, we also evaluated p53 status with immunohistochemistry using biopsied samples. Briefly, paraffin-embedded tissue samples were cut into serial sections 6 μm thick, placed on coated slides, and deparaffinized through a series of xylene and ethanol. Slides were then incubated with anti-p53 primary antibodies (DO-7; DAKO, Glostrup, Denmark), followed by biotin-conjugated goat antibody against mouse as secondary antibody (E0433; DAKO). The following steps were done using a standard ABC method (Elite ABC kit, Vectastain, Vector Laboratories, Burlingame, CA). 3,3-Diaminobenzidine was used as substrate for the peroxidase enzyme reaction (Vectastain). All slides were counterstained with hematoxylin and observed under a microscope (CH40; Olympus, Japan).

Statistical Analysis

Data are expressed as mean ± SD. A Mann-Whitney *U*-test was used for comparison of 2 groups. A chi-square test was used to evaluate the relationship between categorical variables. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics of Patients with UC

Clinical characteristics of UC patients are summarized in Table 2. Routine colonoscopy or barium enema study was negative for dysplasia and CRC except in 13 patients who were shown to have dysplasia or CRC. None of the rest of the patients with UC have developed CRC or dysplasia since then.

Positivity of Serum p53 Abs

Among 63 HCs, titer of serum p53 Abs ranged from 0.00 U/100 μL to 6.76 U/100 μL and only 1 subject (1.6%) was considered positive for serum p53 Abs. In sporadic CRCs, titer of serum p53 Abs ranged from 0.00 U/100 μL to 444.19 U/100 μL and 43 of 82 patients (52.4%) were positive for serum p53 Abs, which was significantly higher than those in the HC group ($P < 0.0001$). Among 286 UC patients, serum p53 Ab levels ranged from 0.00 U/100 μL to 44.40 U/100 μL and 43 patients (15.0%) were positive for serum p53 Abs (Table 3). Although there was no significant difference in serum p53 Ab titers between the UC and HC groups, positivity of serum p53 Abs in the UC group was significantly

TABLE 2. Association Between Patient Characteristics and Serum p53 Ab Positivity

Characteristics	Patients No.	p53 Abs-positive No.	(%)	*P* Value
Sex				
Male	136	21	15.4	0.870
Female	150	22	14.7	
Smoking habits				
Nonsmokers	216	34	15.7	0.066
Smokers	25	0	0	
Ex-smokers	28	6	21.4	
(Unknown	17	3	17.6)	
CRC family history				
Positive	15	2	13.3	>0.99
Negative	271	41	15.1	
Age				
<37	138	22	15.9	0.370
≥37	148	21	14.2	

CRC, sporadic colorectal cancer.

higher than that in the HC group ($P = 0.0014$). Even if UC patients complicated with CRC were excluded, positivity of serum p53 Abs in the UC group (13.3%) was significantly higher than that in the HC group ($P = 0.0063$).

Serum p53 Abs and Clinicopathological Features in UC Patients

Characteristics of UC patients and numbers and percentages of p53 Abs-positive subjects are described in Table 4. No significant differences were found between p53-positive and sex, age, extent of disease, or clinical disease activity at blood sampling. However, 30 (20.8%) of 144 patients with disease duration equal to or longer than 8 years were positive for serum p53 Abs, while only 13 (9.2%) of 142 patients with durations less than 8 years were positive ($P = 0.0076$). If UC patients complicated with CRC were excluded, the difference between the 2 groups was not significant ($P = 0.053$). As for extent of disease, 8 (11.9%) of 67 patients with proctitis or proctosigmoiditis, 13 (15.5%) of 84 patients with left-sided colitis, and 21 (15.8%) of 133 patients with pan-colitis were positive for serum p53 Abs ($P = 0.7439$, not significant). In patients with disease duration equal to or longer than 8 years, there was a weak correlation between p53-positive and extent of disease (2 of 25 [8.0%] for proctitis or proctosigmoiditis, 9 of 50 [18.0%] for left-sided colitis, 18 of 68 [26.5%] for pan-colitis [$P = 0.1435$]).

In 286 UC patients we evaluated, endoscopic and histological examinations revealed 8 CRC, 1 HGD, and 4 LGD. Among these patients with neoplasms, 6 (75.0%) out of 8 patients with CRC and 2 (40.0%) out of 5 patients with dysplasia were positive for serum p53 Abs. Positivity of serum p53 Abs in UC patients who developed CRC was significantly higher compared with UC patients without CRC ($P = 0.0002$). The p53 Ab level of UC patients with CRC ranged from 1.5 U/100 μL to 44.4 U/100 μL, which was significantly higher than in patients without CRC ($P < 0.0001$; Fig. 1). The positivity of serum p53 Abs in UC patients with CRC (75.0%) was higher than that in sporadic CRC patients (52.4%), but it was not significant ($P = 0.2828$). The positive predictive value of positive p53 Ab for CRC in UC patients we evaluated was calculated at 14.0% (6/43).

Serum p53 Abs and p53 Overexpression by Immunohistochemistry

In all patients who had CRC or dysplasia, we evaluated p53 overexpression in tumor cells by immunohistochemistry using biopsied or surgical specimens. The results of immunohistochemical analysis of p53 are shown in Table 5. All UC patients with CRC were positive for p53 staining, while 2 were negative for serum p53 Ab. Of these 2 patients, 1 had far advanced CRC metastasizing to the liver.

We also evaluated p53 overexpression by immunohistochemistry in patients without neoplasms whose serum p53 levels were positive. However, all the results of p53 staining were negative (data not shown).

Changes of Serum p53 Ab Levels After Resection of Tumor

To investigate whether changes in serum p53 Ab levels reflect clinical course, we additionally monitored serum p53 Ab levels at several timepoints after surgery in 4 surgically treated patients with CRC whose preoperative serum p53 Abs were positive. In all 4 patients the level of serum p53 Ab had

TABLE 3. Positivity of Serum p53 Abs in Each Study Population

Population	Subject No.	p53 Abs-positive No.	(%)	*P* Value Against HC
HC	63	1	1.6	—
UC	286	43	15.0	0.0014
UC without CRC	278	37	13.3	0.0063
UC with dysplasia	5	2	40.0	<0.0001
UC with CRC	8	6	75.0[a,b]	<0.0001
Sporadic CRC	82	43	52.4	<0.0001

HC, healthy control; UC, ulcerative colitis; CRC, sporadic colorectal cancer.
[a] $P = 0.0002$ against UC without CRC.
[b] $P = 0.2828$ against sporadic CRC.

TABLE 4. Association Between Clinicopathologic Features of UC and Serum p53 Ab Positivity

Characteristics	Patients No.	p53 Abs-positive No.	(%)	*P* Value
Clinical disease activity				
Mild	183	31	16.9	0.390
Moderate	99	12	12.1	
Severe	4	0	0	
Extent of disease				
Proctitis/PS	67	21	11.9	0.744
Left-sided colitis	84	13	15.5	
Pan-colitis	133	8	15.8	
(Others	2	1	50.0	
Extent of disease in patients with duration ≥8 years				
Proctitis/PS	25	2	8.0	0.144
Left-sided colitis	50	9	18.0	
Pan-colitis	68	18	26.5	
Duration of disease				
<8 years	142	13	9.2	0.008
≥8 years	144	30	20.8	
Duration of disease in patients without CRC				
<8 years	140	13	9.2	0.053
≥8 years	138	24	17.4	
Extraintestinal signs				
Positive	21	4	19.0	0.534
Negative	265	39	14.7	

CRC, sporadic colorectal cancer; PS, proctosigmoiditis.
Clinical disease activity was defined by Truelove-Witts criteria.

fallen 4 to 9 months after surgery, and had fallen to within the normal range in 2 patients.

DISCUSSION

There is a need for new biomarkers for early detection, diagnosis, and prognosis to improve the outcome of UC-associated CRCs. In the framework of dysplasia-carcinoma sequence in UC in which mutations in the *p53* gene are the early event,[21] immunohistochemical detection of mutated p53 protein has been widely employed, and its usefulness in CRC surveillance for UC and association of abnormal staining with increased CRC-related mortality in patients with UC were demonstrated.[22,36]

In the present study, serum anti-p53 autoantibody was evaluated as a possible screening tool and we demonstrated that 75.0% of patients with CRC and 40.0% of patients with dysplasia had p53 Abs, while only 12.8% of patients without neoplasia had p53 Abs in the UC population. This is the first report to show a prevalence of serum p53 Abs in UC-associated neoplastic lesions. This indirectly raised the hypothesis that p53 antibodies may be associated with progression to cancer based on the fact that mutation and loss of heterozy-

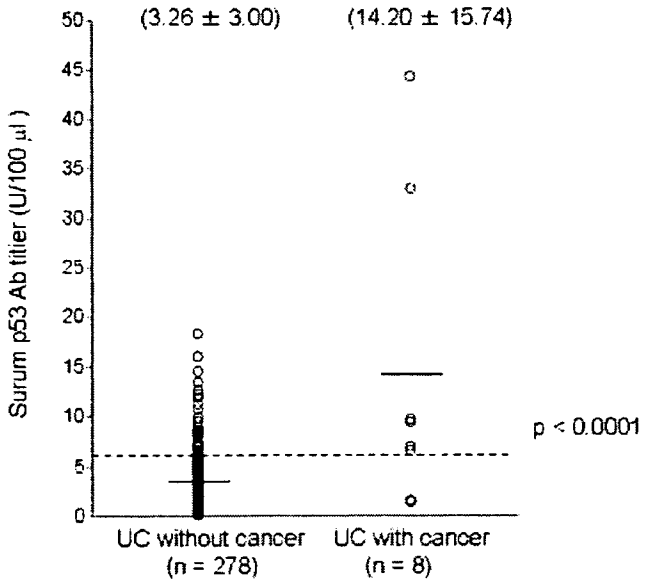

FIGURE 1. Serum p53 Ab titer in ulcerative colitis (UC) patients with colorectal cancer and UC patients without cancer. The p53 Ab levels of patients with cancer ranged from 1.5 U/100 μL to 44.4 U/100 μL, significantly higher than those of patients without cancer ($P < 0.001$).

TABLE 5. Clinicopathologic Characteristics of UC Patients with Colorectal Cancer or Dysplasia

Patient	Age	Sex	Duration (y)	Extent	Histology	Stage	p53 Ab	p53 Staining
CA1	52	F	4	Left	Well-diff	IV	–	+++
CA2	33	M	15	Total	Well-diff	II	+	+
CA3	32	M	7	Total	Well-diff	I	–	+++
CA4	65	M	11	Total	Well-diff	0	+	+
CA5	60	M	22	Left	Well-diff	I	+	++
CA6	30	M	13	Total	Well-diff	II	+	+
CA7	37	F	10	Left	Por+Muc	IV	+	++
CA8	51	F	19	Total	Well-diff	I	+	+
HGD	47	M	22	Left	HGD	n.a.	–	–
LGD1	38	F	15	Total	LGD	n.a.	–	++
LGD2	41	F	17	Total	LGD	n.a.	+	+
LGD3	65	M	10	Left	LGD	n.a.	–	+
LGD4	50	F	25	Total	LGD	n.a.	+	–

CA, cancer; HGD, high-grade dysplasia; LGD, low-grade dysplasia; Total, pan-colitis; Left, left-sided colitis; Well-diff, well-differentiated adenocarcinoma; Por, poorly differentiated adenocarcinoma; Muc, mucinous adenocarcinoma; n.a., not applicable.

gosity (LOH) of the *p53* gene occurred in association with carcinogenesis in UC and that p53 Abs reflected the accumulation of mutated p53 protein. To date, Hammel et al[37] reported 1 p53 Ab-positive UC-associated CRC in their study of 54 CRCs, the remaining 53 patients being sporadic ones. Previous studies reported high sensitivity and specificity of p53 Abs in patients with various types of cancers.[38] In the present study we had considerably high sensitivity of p53 Abs in UC patients with CRC, although a small number of patients was analyzed. One patient with p53 Ab-negative UC-associated CRC with liver metastasis in our study actually showed positive staining for p53 in tumor tissue. Tang et al[39] reported that the frequency of low p53 Ab levels was significantly increased in Stage IV patients with metastasis because the immune system and humoral response was suppressed in these patients, which could account for our seronegative case. By lowering the cutoff value of p53 Ab titer, the positivity, and in turn, the sensitivity for CRC could increase, while the specificity and negative predictive value would decrease. Considering the existence of p53-negative UC-associated CRCs that indicate better prognoses,[36] such a cutoff value makes it impossible that the sensitivity would be 100%. In the present study the positivity in sporadic CRCs was 52.4%, which is a little higher compared with the positivity in the literature, indicating that this cutoff value would be appropriate.

The specificity of p53 Ab has been a matter of debate. Most of commercially available ELISA kits utilize wildtype p53 recombinant protein for trapping serum p53 Abs. p53 protein epitopes recognized by p53 Abs are localized in amino- and carboxy-termini of the protein, similar to those found in animals immunized with wildtype p53 protein. Hence, the immune response to p53 is directed against immunodominant epitopes unrelated to mutational hot spots. It has also been suggested that rather than mutation type per se, p53 protein accumulation due to prolonged half-life by the mutation might be a common linkage to p53 Ab production.[40] Interestingly, p53 Abs have also been found in patients having a tumor that do not present evidence of p53 protein accumulation (as seen in 1 of our patients with LGD) because of a frameshift/stop/splice mutation or in the absence of mutation.[41] The presence of p53 Ab might be due to modification in p53 antigen processing or presentation to the immune system without protein accumulation. Finally, a decrease of p53 Ab levels after resection of the tumor in the present study could support specificity of the Ab for p53 protein accumulated tumor cells, which is also demonstrated by other studies.[37,42]

p53 Abs are detected in various types of cancers, such as colorectal, breast, lung, and pancreatic cancers[38] and the frequency of p53 Abs in patients with neoplasms is usually correlated with the frequency of *p53* mutations in tumor tissue, and most patients with p53 Abs exhibit an accumulation of p53 in their neoplastic cells.[29] Therefore, serological analysis of p53 can be used as a complementary procedure with molecular and immunohistochemical methods, since it does not require tumor tissues and can be easily used for follow-up of patients with p53 alterations. In sporadic CRCs, several studies found an association between p53 Abs and short survival[43–45] and these may reflect that mutation of the *p53* gene in the remaining allele in addition to allelic loss of p53 appears to be a relatively common event in the final stages of tumorigenesis in the adenoma-carcinoma sequence. On the other hand, *p53* mutation appears to be an early event