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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 γδ 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-γ 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-α/TNF-α 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, γδ 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 demonstrate 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-1β transfectants and blocked the killing of Rae-1β 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: FcγR

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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 μl ip of PBS containing 3×10^5 cells of $CD4^+CD45RB^{high}$ T cells. These mice were then administered with 250 μg ip of anti-NKG2D MAb in 250 μl 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 μm) 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 μm) 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 FcγR-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 μg of total RNA were used for complementary DNA synthesis in 14 μl of reaction volume by using random primers. One μl of complementary DNA was amplified with 12.5 μl of SYBR Green PCR master mix (Qiagen, Hilden, Germany) in a 25-μl reaction volume. Sense and antisense primers for the amplification of each gene were as follows: sense H60, 5′-GTGTGATGACGATTTGTTGAG-3′ and antisense H60, 5′-ATTGATGGATTCTGGGCCATA-3′; sense Mult-1, 5′-CTCATAGGAACAGCATGA-3′ and an-

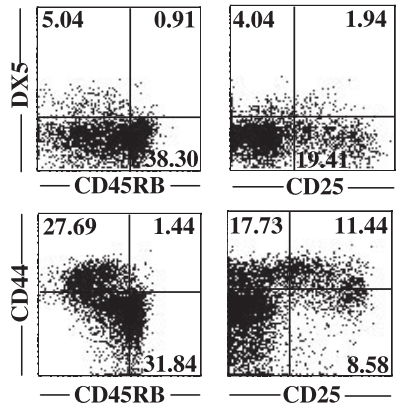

Fig. 1. $DX5^+$ NKT cells do not reside in splenic $CD4^+CD45RB^{high}$ cell population. Phenotypic characterization of splenic $CD4^+CD45RB^{high}$ cells and $CD4^+CD25^-$ cells in terms of expression of DX5 and CD44. Data represent FACS profiles from 3 independent experiments.

tisense Mult-1 5′-TCCTGTGAAATGTTTGTC-3′; sense isoforms of retinoic acid early transcript 1 (Rae-1) molecules (Rae-1α∼δ), 5′-ATAATGGATCCATGGCCAAGGCAGCAGTGACCAA-3′ and antisense Rae-1α∼δ, 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 (Applied 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 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) precoated with 5 μg/ml of hamster anti-mouse CD3ε MAb (145-2C11, BD Pharmingen) and 2 μg/ml of

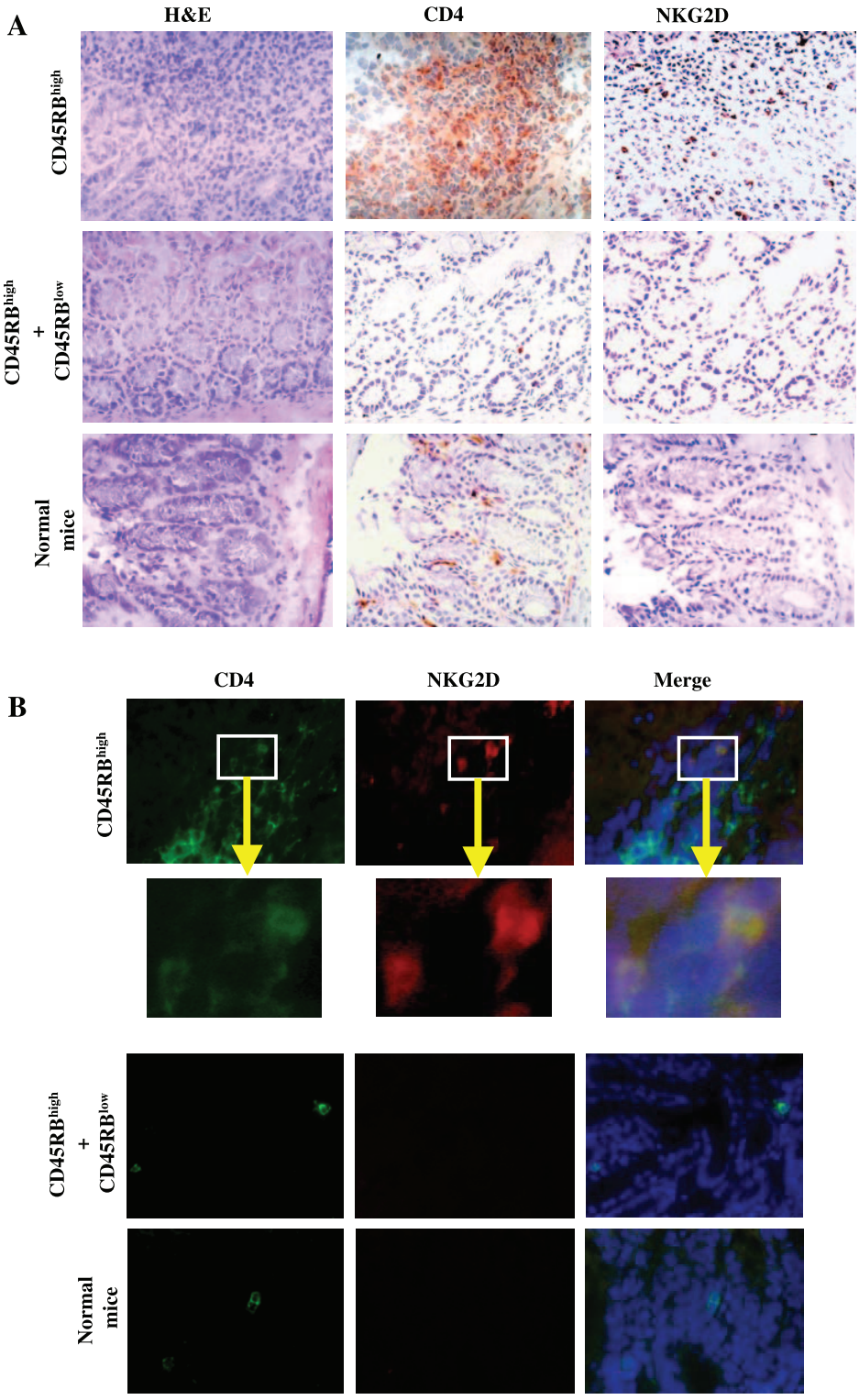

Fig. 2. Expression of NKG2D by colitic LP $CD4^+$ T cells. *A*: frozen serial sections of the colons from colitic SCID mice transferred with $CD4^+CD45RB^{high}$ T cells, noncolitic SCID mice transferred with a mixture of $CD4^+CD45RB^{high}$ T cells and $CD4^+CD45RB^{low}$ T cells, and age-matched normal 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 αβ$^+$ 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 populations 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. 2*A*, *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. 2*A*, *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 2*A* shows that CD4$^+$ T cells were markedly increased in the inflamed mucosa of colitic SCID mice transferred with CD4$^+$CD45RB^{high} T cells (Fig. 2*A*, *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. 2*A*, *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. 2*A*, *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. 2*B*, 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-

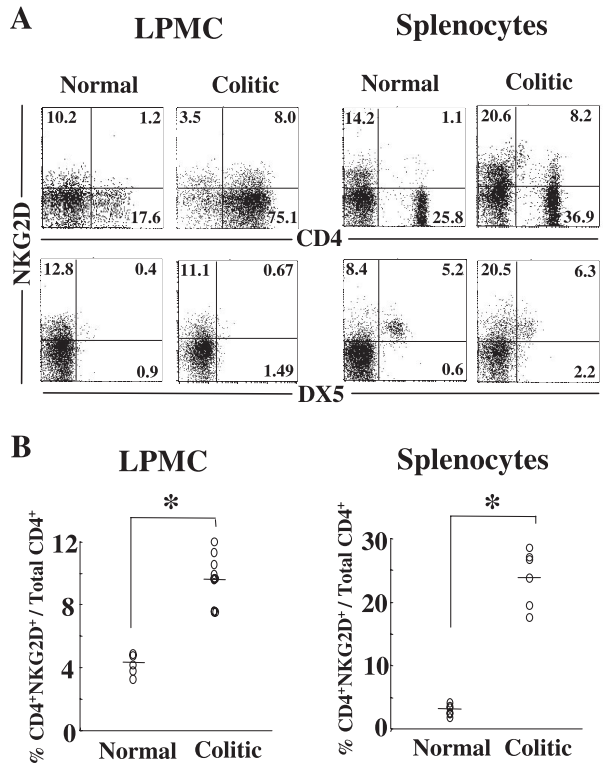

Fig. 3. NKG2D is expressed on lamina propria (LP) and splenic CD4$^+$ T cells in colitic mice. Expression of NKG2D on splenic and LP CD4$^+$ and DX5$^+$ cells in colitic SCID mice (6 wk after transfer) and age-matched normal BALB/c mice. *A*: freshly isolated cells from colitic mice and normal BALB/c 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*. Percentages 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. *$P < 0.05$. LPMC, 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. 3*A*). 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. 4*A*). This was statistically confirmed by assessing the mean fluorescence intensity (MFI) of NKG2D expression (Fig. 4*B*, *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.

Expression 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. 5*A*). 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. 5*A*). Because of the limitation of recovered cell number of immune cells from normal colon samples, we isolated IECs 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. 5*B*, H60 mRNA was expressed in every population, but was significantly increased in $CD11c^+$ cells compared with that in other populations (Fig. 5*B*, *top*). Mult-1 mRNA was expressed exclusively in $CD11c^+$ cells (Fig. 5*B*, *middle*). Rae-1 mRNA in $CD11c^+$ cells was significantly increased compared with that in IECs and $CD4^+$ T cell populations, indicating that the major population expressing NKG2D ligands is $CD11c^+$ dendritic cells rather than IECs in colitic mice.

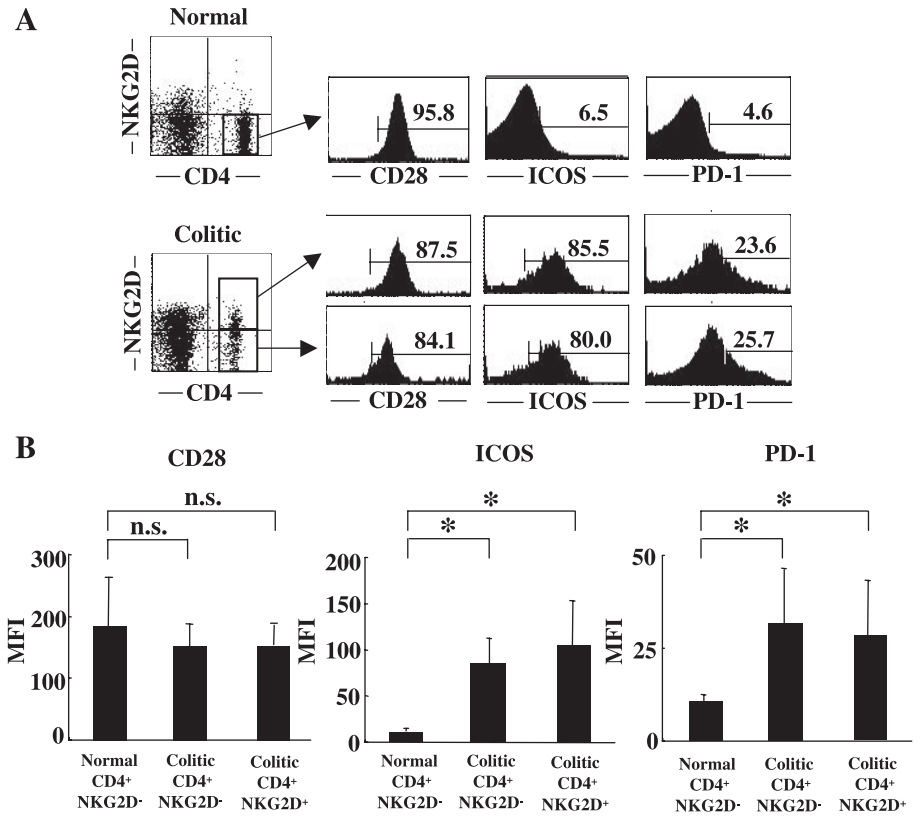

Fig. 4. Correlation between NKG2D molecule and other costimulatory molecules. *A*: expression of CD28, inducible T-cell costimulator (ICOS), and PD-1 on $CD4^+NKG2D^-$ and $CD4^+NKG2D^+$ subpopulations of splenic T cells obtained from control mice or colitic mice. Thick histograms represent staining 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.

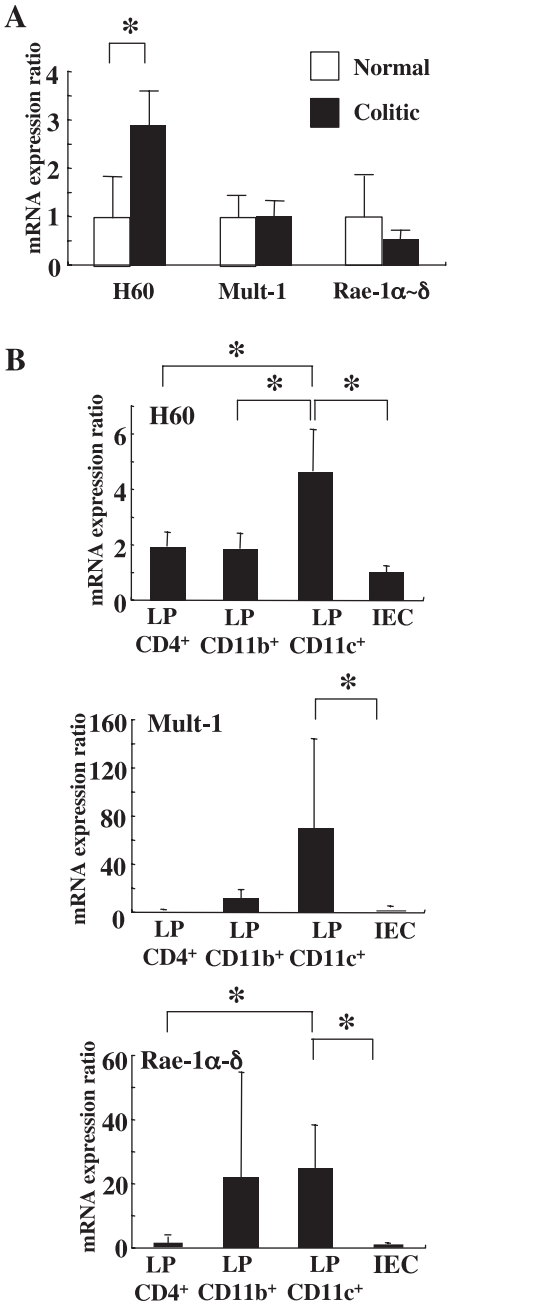

Fig. 5. Expression of H60, Mult-1, and Rae-1 mRNA in colonic samples. *A*: expression of H60, Mult-1, Rae-1, and G3PDH mRNA was determined by quantitative real-time RT-PCR using 3 whole colon samples each from normal and colitic mice. Relative mRNA expression of each ligand in colitic colon is compared 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-PCR using 3 samples each from colitic LP $CD4^+$, $CD11b^+$, $CD11c^+$ cells, and intestinal epithelial cells (IECs). Expression of H60 mRNA in colitic LP $CD11c^+$ cells was significantly increased compared with other populations. Expression of Mult-1 mRNA and Rae-1 mRNA in colitic LP $CD11c^+$ cells was also increased compared with that in colitic LP $CD4^+$, $CD11b^+$ 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. 6*A*, 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. 6*A*). 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. 6*B*). 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. 6*B*). A comprehensive assessment of colitis by clinical scores showed a clear difference between control IgG-treated mice and anti-NKG2D MAb-treated mice (Fig. 6*C*).

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. 6*D*). 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. 6*D*). 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. 6*F*). 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. 6*G*, LP $CD4^+$ cells from anti-NKG2D MAb-treated mice produced significantly less IFN-γ 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.

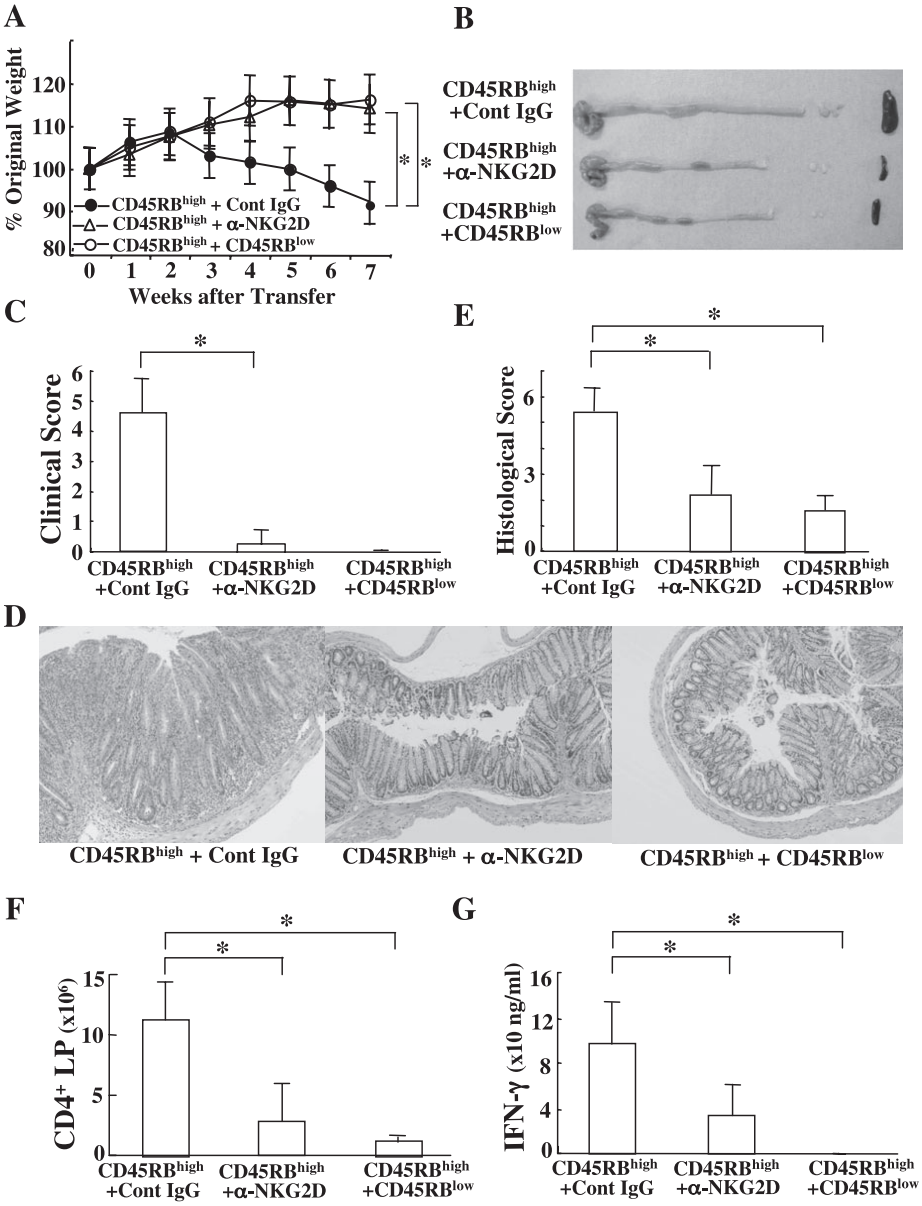

Fig. 6. Preventive effect of anti-NKG2D MAb on the development of colitis. The recipient mice were administered with anti-NKG2D MAb or control hamster IgG for 7 wk starting from the time of $CD4^+CD45RB^{high}$ T cell transfer. Other mice were transferred with $CD4^+CD45RB^{high}$ T cells and $CD4^+CD45RB^{low}$ 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-γ production by LP $CD4^+$ T cells. Isolated LP $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 MAbs for 48 h. IFN-γ 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. Expectedly, 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 IECs 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 IECs by NKG2D-MICA interaction. Furthermore, Allez and colleagues (1) have very recently reported that MICA and NKG2D expression is significantly increased on IECs 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 IECs, interestingly, we found that NKG2D ligands were mainly expressed in $CD11c^+$ dendritic cells rather than IECs 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 IECs 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 inflammation such as Crohn's disease.

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