Blockade of NKG2D signaling prevents the development of murine CD4\(^+\) T cell-mediated colitis


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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. Further more, 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 leucocyte 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).
(CD16/CDCD32)-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 (RMP1-20); 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^high MAb (16A) and sorted into the population. Phenotypic characterization of splenic CD4⁺ T cells was achieved by flow cytometry. Splenic mononuclear cells were then transferred with a mixture of 3×10⁵ CD4⁺CD45RB^high T cells and 3×10⁵ 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, 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 3×10⁵ CD4⁺CD45RB^high T cells and 3×10⁵ CD4⁺CD45RB^low T cells.

Histological examination. Tissue sections 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 Ca²⁺- and Mg²⁺-free Hank’s balanced salt solution containing 1 mM dithiothreitol (Sigma-Aldrich) for 30 min each to remove mucus. The supernatants containing intraepithelial cells and epithelial cells wereremoved. 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. Spleen 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 FcyR-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 CyChromosome 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 system (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’-GTGTTGATGACGATT-TGTGAG-3’ and antisense H60, 5’-ATTGATGGATTCTTGGGC-CATA-3’; sense Mult-1, 5’-CTCATAGGAACAGCAGTCA-3’ and antisense Mult-1, 5’-GTTGAACGTTGGGCGGCTC-3’; sense H61, 5’-ACTTCCAGATTCTTGC-3’ and antisense H61, 5’-GTTGAACCAAGCAGTCA-3’; sense H62, 5’-ACTTCCAGATTTCTTGCGG-3’ and antisense H62, 5’-GTTGAACCAAGCAGTCA-3’; sense H63, 5’-ACTTCCAGATTTCTTGCGG-3’ and antisense H63, 5’-GTTGAACCAAGCAGTCA-3’; sense H64, 5’-ACTTCCAGATTTCTTGCGG-3’ and antisense H64, 5’-GTTGAACCAAGCAGTCA-3’. The amplification efficiency of each gene was confirmed by generating a standard curve. The reaction volume was adjusted to a total of 20 μl with water.

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′-ATAATGGATCCATGGCCAAGGCAGTGACCAA-3′ and antisense Rae-1α~δ, 5′-ATATTATAGCGGCCGCTCACATG-CAAATGCAAATAAT-3′; and sense glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5′-TGAAAGTCGCTGTTGACGGATTGCGG-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 × 10⁵) were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO₂ 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...
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\textsuperscript{high} T cell-transferred SCID mice. To investigate whether interactions between NK2GD 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\textsuperscript{+}CD45RB\textsuperscript{high} T cells of normal BALB/c mice (27), where high refers to the brightest staining 30% fluorescence intensity of CD4\textsuperscript{+} T cells. In this colitis model, CD8\textsuperscript{+} T cells and B cells are absent as we transferred only CD4\textsuperscript{+}CD45RB\textsuperscript{high} T cells into SCID mice lacking both T and B cells. However, it should be noted that CD4\textsuperscript{+} donor population contains a small number of CD4\textsuperscript{+} NKT cells that can express inducible NK2GD in addition to a majority of conventional CD4\textsuperscript{+} T cells (Fig. 2A, middle). We then performed three-color flow cytometry analysis of the cells that were prepared for transfer. As shown in Fig. 1, a small number of DX5\textsuperscript{+} cells were surely observed in the CD4\textsuperscript{+}CD45RB\textsuperscript{low-moderate} population, but not in the CD4\textsuperscript{+}CD45RB\textsuperscript{high} population. Furthermore, we found that CD4\textsuperscript{+}CD25\textsuperscript{low} cells, which are another population of donor cells commonly used to induce colitis in SCID mice (17), contained substantial number of DX5\textsuperscript{+} cells (Fig. 1, top). Furthermore, almost all CD4\textsuperscript{+}CD45RB\textsuperscript{high} T cells had CD44\textsuperscript{low} (low refers to the dullest 30% CD4\textsuperscript{+} T cells, and moderate refers to the intermediate staining populations between the previous two populations) naive phenotype, whereas CD4\textsuperscript{+}CD25\textsuperscript{high} T cells contained substantial numbers of CD44\textsuperscript{high} memory T cells along with CD44\textsuperscript{low} naive T cells (Fig. 1, bottom). Thus we decided to use CD4\textsuperscript{+}CD45RB\textsuperscript{high} T cells as donor cells to assess the possible role of NK2GD expression by conventional CD4\textsuperscript{+} T cells in the development of colitis, so as to exclude the involvement of NKT cells.

After adoptive transfer of CD4\textsuperscript{+}CD45RB\textsuperscript{high} cells, the recipient mice manifested weight loss from 3 wk after transfer and clinical symptoms of colitis such as diarrhea with increased mucous in the stool, anorectal prolapse, and hunched posture by 6 wk (data not shown). The colons from these mice were enlarged and had a greatly thickened wall due to severe colonic inflammation (Fig. 2A, left). In contrast, when transferred with a mixture of CD4\textsuperscript{+}CD45RB\textsuperscript{high} and CD4\textsuperscript{+}CD45RB\textsuperscript{low} cells, the recipient mice did not develop colitis at all as well as control BALB/c mice (Fig. 2A, left). We then examined the expression of CD4 and NK2GD in this model by immunohistochemistry. Colonic samples were obtained from colitic SCID mice transferred with CD4\textsuperscript{+}CD45RB\textsuperscript{high} cells, noncolitic SCID mice transferred with CD4\textsuperscript{+}CD45RB\textsuperscript{high} and CD4\textsuperscript{+}CD45RB\textsuperscript{low} cells at 6 wk after the transfer, and age-matched BALB/c mice as a control. Figure 2A shows that CD4\textsuperscript{+} T cells were markedly increased in the inflamed mucosa of colitic SCID mice transferred with CD4\textsuperscript{+}CD45RB\textsuperscript{high} T cells (Fig. 2A, middle), but not in noncolitic SCID mice transferred with CD4\textsuperscript{+}CD45RB\textsuperscript{high} and CD4\textsuperscript{+}CD45RB\textsuperscript{low} T cells or normal mice. The majority of CD4\textsuperscript{+} 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 NK2GD\textsuperscript{+} cells were scattered within the location of CD4\textsuperscript{+} T cells (Fig. 2A, right), suggesting that NK2GD was expressed by a part of colitic CD4\textsuperscript{+} LP T cells. In contrast, a small number of CD4\textsuperscript{+} T cell were indeed found in the LP of noncolitic SCID mice transferred with CD4\textsuperscript{+}CD45RB\textsuperscript{high} and CD4\textsuperscript{+}CD45RB\textsuperscript{low} T cells and in normal mice, but expression of NK2GD could never be detected on these normal LP CD4\textsuperscript{+} T cells (Fig. 2A, middle and right). To further confirm that NK2GD is expressed on CD4\textsuperscript{+} T cells in colitic LP, we performed a double-staining experiment by fluorescent immunostaining. As shown in Fig. 2B, NK2GD was surely expressed by a part of CD4\textsuperscript{+} T cells in colitic mice. In contrast, a small number of CD4\textsuperscript{+} T cell showed scattered distribution in the LP of noncolitic SCID or normal BALB/c mice, but NK2GD was not detected in these mice.

To further confirm the expression of NK2GD on CD4\textsuperscript{+} T cells in colitic mice, we next performed two-color flow cytometry.
etry analysis. In the colitic samples, NKG2D expression on CD4+ T cells was significantly increased compared with that in normal mice (Fig. 3, A and B). Although it has been reported that murine NKG2D molecule is usually expressed on DX5+ NK or NKT cells (23, 25), DX5+ cells could not be detected on cells isolated from colonic tissues through conventional DTT/collagenase treatment (Fig. 3A). In the spleen of colitic mice, a significantly increased proportion of CD4+ T cells coexpressed NKG2D, which was not observed in normal mice (Fig. 3, A and B). Interestingly, we detected DX5+ cells in the spleen of both colitic and normal mice, where we found that almost all DX5+ cells expressed NKG2D with high intensity (Fig. 3, A and B).

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

Expression of NKG2D ligands in the colon of CD4+CD45RBhigh T cell-transferred SCID mice. To clarify the expression of NKG2D ligands in the colon, we next conducted quantitative real-time PCR analysis using whole colonic tissues from colitic mice or age-matched BALB/c mice. This quantitative PCR analysis revealed that mRNA expression of H60 was significantly increased in colitic colon samples compared with that in normal colon samples (Fig. 5A). In contrast, mRNA expression of Mult-1 and Rae-1 was not significant in colitic colon samples compared with that in normal colon samples (Fig. 5A). Because of the limitation of recovered cell number of immune cells from normal colon samples, we isolated 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. 5B, H60 mRNA was expressed in every population, but was significantly increased in CD11c+ cells compared with that in other populations (Fig. 5B, top). Mult-1 mRNA was expressed exclusively in CD11c+ cells (Fig. 5B, middle). Rae-1 mRNA in CD11c+ cells was significantly increased compared with that in IECs and CD4+ T cell populations, indicating that the major population expressing NKG2D ligands is CD11c+ dendritic cells rather than IECs in colitic mouse.

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.
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 (HM2G2D) were administered to the recipient SCID mice transferred with CD4+CD45RBlow T cells started from the day of transfer and then three times a week for 7 wk. As shown in Fig. 6A, control IgG-treated mice manifested progressive weight loss (wasting disease) from 3 wk after transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 5–6 wk after transfer. In contrast, anti-NKG2D MAb-treated mice appeared healthy with gradual increase of body weight, and no diarrhea was observed throughout the whole period of observation (Fig. 6A). At 7 wk after transfer, the colon of control IgG-treated mice was enlarged and had a greatly thickened wall, which was not observed in the anti-NKG2D MAb-treated mice or mice transferred with CD4+CD45RBlow and CD4+CD45RBhigh T cells (Fig. 6B). In addition, enlargement of the spleen and mesenteric lymph nodes were also evident in control IgG-treated mice, but not in anti-NKG2D MAb-treated mice (Fig. 6B). A comprehensive assessment of colitis by clinical scores showed a clear difference between control IgG-treated mice and anti-NKG2D MAb-treated mice (Fig. 6C).

Histological examination showed prominent epithelial hyperplasia with glandular elongation with a massive infiltration of mononuclear cells in the LP in the colon of control IgG-treated mice (Fig. 6D). In contrast, inflammation was mostly abrogated and only few mononuclear cells were observed in the LP of the colon from anti-NKG2D MAb-treated mice and in mice transferred with CD4+CD45RBlow and CD4+CD45RBhigh T cells (Fig. 6D). This difference was also confirmed by histological scoring of multiple colon sections, which was 5.2 ± 1.1 in the control rat IgG-treated mice, 2.2 ± 0.8 in anti-NKG2D MAb-treated mice, and 1.6 ± 0.6 in mice transferred with CD4+CD45RBlow and CD4+CD45RBhigh T cells (P = 0.01) (Fig. 6E). 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+CD45RBlow and CD4+CD45RBhigh T cells compared with control IgG-treated mice (Fig. 6F). Furthermore, the number of CD4+ splenocytes from control IgG-treated mice was significantly increased compared with that from age-matched normal BALB/c mice (data not shown). In contrast, the number of CD4+ splenocytes from anti-NKG2D MAb-treated mice was significantly less than that from control IgG-treated mice (data not shown).

We also examined the cytokine production by LP CD4+ cells of control IgG- or anti-NKG2D MAb-treated mice. As shown in Fig. 6G, LP CD4+ cells from anti-NKG2D MAb-treated mice produced significantly less IFN-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.
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+CD45RBhigh 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+CD25high T cells, and treatment by a neutralizing anti-NKG2D MAb (CX5) prevents the development of colitis. Very recently, their results are quite similar to ours, but it is noteworthy that normal splenic CD4+CD25high T cells that are a distinct cell population from CD4+CD25low regulatory T cells, include DX5+CD44highCD45RBlow CD4+ NKT cells along with CD44high memory and CD44low 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+CD45RBhigh T cells as donor cells, which were characterized to have a cell-surface phenotype of DX5 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.
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 NKG2D ligands were also expressed in colonic tissues in both samples by quantitative real-time RT-PCR analysis that this issue.

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 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.

**REFERENCES**


**GRANTS**

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