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The Development of Colitogenic CD4\(^+\) T Cells Is Regulated by IL-7 in Collaboration with NK Cell Function in a Murine Model of Colitis

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We previously reported that IL-7\(^{-/-}\)/RAG\(^{-/-}\) mice receiving naive T cells failed to induce colitis. Such abrogation of colitis may be associated with not only incomplete T cell maintenance due to the lack of IL-7, but also with the induction of colitogenic CD4\(^+\) T cell apoptosis at an early stage of colitis development. Moreover, NK cells may be associated with the suppression of pathogenic T cells in vivo, and they may induce apoptosis of CD4\(^+\) T cells. To further investigate these roles of NK cells, RAG\(^{-/-}\) and IL-7\(^{-/-}\)/RAG\(^{-/-}\) mice that had received naïve T cells were depleted of NK cells using anti-asialo GM1 and anti-NK1.1 Abs. NK cell depletion at an early stage, but not at a later stage during colitogenic effector memory T cell (TEM) development, resulted in exacerbated colitis in recipient mice even in the absence of IL-7. Increased CD44\(^+\)/CD62L\(^-\) TEM and unique CD44\(^+\)/CD62L\(^-\) T cell subsets were observed in the T cell-reconstituted RAG\(^{-/-}\) recipients when NK cells were depleted, although Fas, DR5, and IL-7R expressions in this subset differed from those in the CD44\(^+\)/CD62L\(^-\) TEM subset. NK cell characteristics were the same in the presence or absence of IL-7 in vitro and in vivo. These results suggest that NK cells suppress colitis severity in T cell-reconstituted RAG\(^{-/-}\) and IL-7\(^{-/-}\)/RAG\(^{-/-}\) recipient mice through targeting of colitogenic CD44\(^+\)/CD44\(^+\)/CD62L\(^-\) TEM and, possibly, of the newly observed CD44\(^+\)/CD44\(^+\)/CD62L\(^-\) subset present at the early stage of T cell development. The Journal of Immunology, 2012, 188: 2524–2536.

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recipient mice (13, 14). Additionally, our recent studies suggested that effector CD4+ T cells were able to induce colitis even in IL-7+/− RAG−/− mice that were parabiosed with colitic RAG−/− mice that had been injected with naive T cells 6 wk previously (15). Moreover, a deparabiosed IL-7+/− RAG−/− mouse, which was surgically separated from T cell-receiving RAG−/− IL-7+/− RAG−/− parabionts 6 wk after the initial surgery, still maintained chronic colitis for at least another 12 wk (16). These results suggested that the abrogation of colitis in the T cell-reconstituted IL-7+/− RAG−/− mice may be associated with not only incomplete T cell maintenance due to the lack of IL-7, but also with another mechanism by which the colitogenic CD4+ T cell development is suppressed.

It is known that NK cells are responsible for innate immune responses, including the depletion of tumor cells or cells infected with various kinds of viruses (17). Additionally, NK cells induce inflammation in tissues by the production of proinflammatory cytokines such as IFN-γ (18, 19). In contrast, NK cells are also known to be critical for anti-inflammatory effects in the context of autoimmune diseases (20, 21). It has been reported that NK cells abrogate disease severity of experimental autoimmune encephalomyelitis (EAE) due to the suppression of pathogenic T cells (22, 23). It has also been reported that depletion of NK cells results in enhanced severity of a chronic colitis model (24). However, the mechanisms by which NK cells regulate inflammation in this colitis model have not been well described. In this regard, we hypothesized that the abrogation of colitogenic T cell development that we observed in naive T cell-receiving IL-7+/− RAG−/− mice is associated with the effect of NK cells. We therefore focused our analysis of this phenomenon on NK cells.

**Materials and Methods**

**Animals**

Wild-type (WT) C57BL/6 mice were purchased from JapanCLEA (Tokyo, Japan). Rag-deficient (RAG−/−) mice on a C57BL/6 background were obtained from Taconic (Hudson, NY) and the Central Laboratories for Experimental Animals (Kanagawa, Japan). IL-7−/− mice were provided by Dr. R. Zamoyska (National Institute for Medical Research, London, U.K.) and were intercrossed with Rag−/− to generate IL-7−/− RAG−/− mice. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and recipients were used between 8 and 16 wk age. All animal experiments were approved by the Animal Review Board of Tokyo Medical and Dental University and were performed in accordance with institutional guidelines.

**Abs**

The following mAbs and reagents were obtained from BD Pharmingen (San Jose, CA): anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD27 (LG.3A10), anti-CD28 (37.51), anti-CD43 (S7), anti-CD44 (IM7), anti-CD45RB (16A), anti-CD51 (RMV-7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD94 (18d5), anti-CD95, anti-CD178 (MFL4), anti-CD244.2 (2B4 Bio allotyping), anti-Ly-49C (5E6), anti-Ly-49D (4E5), anti-Ly-49F (HBF-719), anti-Ly-49G2 (4D11), anti-IL-17α (A7R34), anti-NK1.1 (PK136), and streptavidin. Biotin-conjugated anti-mouse NKG2A/C/E, biotin-conjugated anti-mouse IL-7ε (A7R34), FITC-conjugated anti-mouse pan-NK cells (CD49b), and FITC-conjugated anti-mouse CD3ε (145-2C11) mAbs were purchased from eBioscience (San Diego, CA).

**Flow cytometry (FACS)**

To detect the cell surface expression of a variety of molecules, isolated mononuclear cells from individual organs including spleen (SPL), mesenteric lymph node (MLN), and colonic lamina propria (LP) were analyzed by FACS using standard staining methods. Briefly, the cells were suspended in PBS containing 2% FBS, which was used as the suspension fluid for subsequent staining, preincubated with an FcγR-blocking mAb (anti-CD16/32, 2.4G2; BD Biosciences) for 15 min to prevent nonspecific binding by the secondary Ab, and washed with suspension fluid followed by staining with specific FITC-, PE-, PerCP-, allophycocyanin-, or biotin-labeled Abs for 20 min on ice. Standard two-, three-, or four-color flow cytometric analyses were performed using the FACS Calibur (Becton Dickinson, Sunnyvale, CA) with appropriate software (CellQuest; BD Biosciences). Background fluorescence was also assessed by staining with control irrelevant isotype-matched mAbs.

**NK cell depletion in vivo**

The anti-asialo GM1 (ASGM1) polyclonal Ab was obtained from Wako Chemicals (Osaka, Japan) and reconstituted according to the manufacturer’s specifications. The anti-NK1.1 mAb was affinity purified from the culture supernatant of a hybridoma clone, PK136, obtained from the American Type Culture Collection (Manassas, VA). For effective depletion of NK cells in vivo, either the anti-ASGM1 polyclonal Ab (0.25 mg/mouse) or anti-NK1.1 mAb (0.5 mg/mouse) was injected i.p. into mice (25) at the indicated time points in each experiment. The same amount of rabbit Ig (Rockland Immunochemicals, Gibertsville, PA) or mouse IgG2a (Medical & Biological Laboratories, Nagoya, Japan) were used as the controls, respectively, for some of experiments. Effective (>95%) depletion of NK cells in vivo was confirmed by FACS analysis of single cells derived from individual organs such as SPL, MLN, and colonic LP.

**Purification of naive T cell subsets and induction of colitis**

For naive T cell purification, splenic mononuclear cells were obtained from WT mice and CD4+ T cells were isolated using anti-CD4 (L3T4) MACS magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Enriched CD4+ T cells (94–97% pure as estimated by FACS) were then labeled with PerCP- or allophycocyanin-conjugated anti-CD4, PE- or allophycocyanin-conjugated anti-CD44, and FITC-conjugated anti-CD62L. Subpopulations of CD4+ cells were generated by three-color sorting on a FACS Aria (Becton Dickinson). All populations were 98.0% pure on reanalysis. To induce an animal model of chronic colitis, 5 × 106 CD4+CD44−CD62L− (naive) T cells were adoptively transferred i.p. into 8- to 12-wk-old RAG−/− or IL-7−/− RAG−/− recipient mice as previously described (2, 3, 10).

**Isolation of LP lymphocytes**

LP lymphocytes (LPL) were isolated from healthy or colitic mice as previously described (10). Briefly, RAG−/− IL-7−/− RAG−/− recipient mice were sacrificed 6–12 wk after injection of naive T cells to induce colitis. The entire length of the colon was removed, opened longitudinally, washed with PBS, and cut into small pieces. The dissected tissues were incubated with Ca2+-, Mg2+-free HBSS containing 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 45 min to remove mucus, and the epithelial layer was then treated with 3.0 mg/ml collagenase (Roche Diagnostics, Mannheim, Germany) or 1.0 mg/ml DNase ( Worthington Biomedical, Freehold, NJ) for 2 h. The cells were pelleted, washed twice with PBS, and then were subjected to density gradient centrifugation using 40–75% isos-tonic Percoll (Amersham Biotech, Piscataway, NJ) solution diluted with HBSS. Isolated whole LP mononuclear cells were subjected to FACS to analyze each lymphocyte subset. In some experiments, such LP mononuclear cells were further labeled with allophycocyanin-conjugated anti-CD4 and FITC-conjugated anti-CD3 to isolate colitogenic CD4+ T cell subsets by FACS Aria. All populations were 98.0% pure on reanalysis. Isolated LP CD4+ T cells were subjected to cytokine production and cytotoxicity assays.

**Determination of clinical score of colitis**

The clinical score of colitis was determined using previously described methods (26) with minor modifications and was assessed by trained individuals blind to the treatment group. Briefly, initial body weight and wasting, hunching over, piloerection, diarrhea, and blood in the stool or per rectum of the T cell-receiving RAG−/− or IL-7−/− RAG−/− recipient mice were assessed when sacrificed. For wasting, weight loss of <20% from baseline was assigned 0 points and weight loss of >20% was assigned 1 point. For hunched over appearance, no obvious hunching was assigned 0 point, and extensive hunching was assigned as 1 point. For colon thickening, normal features were assigned 0 points, mild thickening was assigned 1 point, moderate thickening was assigned 2 points, and severe thickening was assigned 3 points. For stool consistency, 0 points were assigned to well-formed pellets, 1 point to pasty and semifomed stools that did not adhere to the anus, and 2 points to liquid stools that did adhere to the anus. An additional point was added if gross blood was noted. The scores of these parameters were added, resulting in a total clinical score ranging from 0 (healthy) to 8 (maximal colitis activity).
Histopathological examination of colitis

Mice receiving naive T cells were sacrificed 6 or 12 wk after the T cell transfer, and colonic specimens taken from proximal, middle, and distal colons were subjected to histopathological assessment. For this assessment, tissue samples were fixed in 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. The H&E-stained sections were the standard without prior knowledge of the type of donors, recipients, and treatments. The degree of inflammation in the colon was graded according to a modification of the previously described scoring system (26, 27). Briefly, for mucosal damage, 0 points were assigned to normal appearance, 1 point to discrete lymphoepithelial lesions, 2 points to diffuse crypt elongation, and 3 points to extensive crypt elongation or mucosal erosion-ulceration. For cell infiltration the points assigned were: 0, to normal, or presence of occasional leukocytes; 1, to widely scattered leukocytes or focal aggregates of leukocytes; 2, to confluence of leukocytes extending into the submucosa with focal effacement of the muscularis; 3, to transmural extension of leukocyte infiltration. For crypt abscess, the assigned points were: 0, to no crypt abscess; 1, to the presence of crypt abscess. The cumulative degree of these parameters was calculated as a total histological score ranging from 0 (no change) to 21 (extensive cell infiltration and tissue damage).

ELISA

To measure cytokine production, 1 × 10⁴ LP CD4⁺ T cells were cultured in 200 μl RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 50 μM penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), 10 mM HEPES, 1% nonessential amino acids, and 2 μM 2-ME (Life Technologies Invitrogen, Carlsbad, CA), termed complete RPMI 1640, in the presence of 5 ng/ml plate-bound anti-CD3ε (145-2C11) and 2 μg/ml plate-bound anti-CD28 (37.51) in flat-bottom 96-well plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere incubator containing 5% CO₂. Culture supernatants were re-collected after 24 h and analyzed for the production of cytokines such as IFN-γ, TNF, and IL-17.

Isolation of NK cells and cytotoxicity assay

Splenic cell suspensions were prepared from RAG⁻/⁻ or IL-7−/− RAG⁻/⁻ mice and treated with NH₄Cl buffer to remove erythrocytes. The NK cell population was then labeled with FITC-conjugated anti-DX5 (CD49b) and isolated for use as effector cells in the cytotoxicity assay by sorting on a FACSSaria. The purity of isolated NK cells was 98.0% on reanalysis. To measure cytotoxicity and 5 × 10⁴ NK cells were cultured in 200 μl RPMI 1640 supplemented with 10% FBS, 50 μM penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich), 10 mM HEPES, 1% nonessential amino acids, and 2 μM 2-ME (Life Technologies Invitrogen, Carlsbad, CA), termed complete RPMI 1640, in the presence of 5 μg/ml plate-bound anti-CD3ε (145-2C11) and 2 μg/ml plate-bound anti-CD28 (37.51) in flat-bottom 96-well plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere incubator containing 5% CO₂ for 48 h. Culture supernatants were removed and analyzed for the production of cytokines such as IFN-γ, TNF, and IL-17.

Statistical analysis

The results are expressed as the means ± SEM. Statistical significance was determined using the nonparametric Mann–Whitney U test, and differences were considered to be statistically significant when p < 0.05.

Results

NK cell depletion induces the early onset of colitis in naive T cell-transferred RAG⁻/⁻ mice

It has been reported that NK cells suppress the severity of inflammatory diseases such as EAE and colitis (22, 24). NK cells were depleted in the latter colitis study by injection of anti-NK1.1 or anti-ASGM1 Abs, or by the use of a perforin-deficient animal. That study suggested that NK cells may possibly have cytolytic activity for colitogenic CD4⁺ TEm in this model since knockout of the perforin gene resulted in exacerbation of disease severity. However, it is unclear which stage in the development of colitis is affected by NK cells. Therefore, we first assessed the effect of NK cell depletion at different time points in the development of chronic colitis.

To examine the effect of NK cells in the development of chronic inflammation in the colon, an animal model of colitis was induced by adoptive transfer of CD4⁺CD62L⁺D44⁺ (naive) T cells derived from WT SP into RAG⁻/⁻ recipient mice (2, 3). NK cells were depleted by i.p. injection of the anti-ASGM1 Ab (or vehicle control [PBS]) every other day for 12 wk starting from the day before naive T cell transfer (Fig. 1A). Additionally, some groups were injected with the anti-ASGM1 Ab for 4 wk followed by vehicle control for 8 wk (Fig. 1A), or with the vehicle control for 4 wk followed by 8 wk anti-ASGM1 Ab (Supplemental Fig. 1). Mice injected with the anti-ASGM1 Ab for 12 wk, or for just the first 4 wk, started to show wasting earlier than the vehicle control group that was injected for 12 wk (Fig. 1B). Alternatively, mice injected with vehicle control for 4 wk followed by 8 wk anti-ASGM1 Ab showed a similar wasting curve to that of mice injected with vehicle control for 12 wk (data not shown), suggesting that NK cell depletion at the later stage of colitis induction does not affect the severity of colitis.

However, there was no significant difference in clinical scores between these groups 12 wk after the T cell transfer (Fig. 1C), and all mouse groups showed a similar degree of colitis with thickening and shortening of the colon as well as splenomegaly when sacrificed (Fig. 1D). Consistent with this finding, microscopic evaluation of each group showed similar histopathological features such as wall thickening of the colon, infiltration mainly by mononuclear cells, crypt abscesses, crypt elongation, a decrease in goblet cells, and epithelial damage (Fig. 1E, 1F). Moreover, the production of proinflammatory cytokines by colonic LP T cells isolated from each group was similar (Fig. 1G).

However, there was concern that anti-ASGM1 Ab treatment at an early stage may affect the colitis severity in the RAG⁻/⁻ mice receiving naive T cells, since the groups with the Ab treatment at an early stage for 4 wk and 12 wk started to exhibit wasting earlier than the control group without the Ab treatment (Fig. 1B). Therefore, we examined these mice at a relatively early time and, interestingly, we found that the Ab-treated group showed significantly more severe colitis in clinical and histological scores compared with the control group 6 wk after the T cell transfer (Fig. 2). These data indicate that NK cell depletion affects the early stage of colitis development.

CD62L⁺CD44⁺ and CD62L⁺CD44⁻ T cell subsets are increased by NK cell depletion in naive T cell-reconstituted RAG⁻/⁻ recipient mice

Because the exacerbation at an early stage of colitis development was observed following NK cell depletion, we assessed the number of CD4⁺ T cells in SPL and MLN of naive T cell-receiving RAG⁻/⁻ mice treated with or without the anti-ASGM1 Ab. As seen in Fig. 3A and 3B, increased numbers of T cells were detected, especially...
in the SPL, within a week after naive T cell injection. Moreover, treatment with the anti-ASGM1 Ab revealed a significantly increased number of T cells in SPL and MLN (Fig. 3A, 3B). Thus, we next determined the development of TEM in these mice by assessment of the expression levels of CD62L and CD44 on T cells. From day 1 to day 3, most T cells still expressed CD62L, but not CD44, regardless of anti-ASGM1 Ab treatment. Interestingly, a CD62L$$^+$$CD44$$^+$$ subset had appeared in both SPL and MLN by day 5 after treatment with anti-ASGM1 Ab (Fig. 3C–F). This unique T cell subset was significantly increased in the naive T cell-receiving RAG$$^−$$/− recipients injected with either vehicle control for 12 wk (Ctrl, top), anti-ASGM1 Ab for 4 wk and then control for 8 wk (0–4 wk, middle), or anti-ASGM1 Ab for 12 wk (0–12 wk, bottom). Representative features from each group are shown. Data are expressed as means ± SEM from four mice. (G) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (left), TNF (middle), and IL-17 (right) in the culture supernatant were measured using ELISA. Data are indicated as means ± SEM from four samples.

FIGURE 1. NK cell depletion at the early stage of colitis induction in RAG$$^−$$/− mice results in wasting disease. (A) Protocol for NK cell depletion in a chronic colitis setting. RAG$$^−$$/− mice were injected with either 0.25 mg/mouse anti-ASGM1 Ab (0–12 wk) or vehicle control (Ctrl) every second day for 12 wk from the day before adoptive transfer of naive T cells, or were injected with anti-ASGM1 Ab for 4 wk followed by vehicle control injection for 8 wk (0–4 wk). (B) Wasting, as defined by percentage of initial body weight, in RAG$$^−$$/− mice induced colitis. Mice were injected with naive T cells and either vehicle control for 12 wk (Ctrl, C), anti-ASGM1 Ab for 12 wk (0–12 wk, Δ), or anti-ASGM1 Ab for 4 wk followed by 8 wk vehicle control (0–4 wk, □). The non-T cell-injected control group is also shown (RAG$$^−$$/−, cross). Data are expressed as means ± SEM from four mice. *p < 0.05. (C) Clinical scores of each group are shown. Data are expressed as means ± SEM from four mice. (D) Gross appearance of colons (left) and SP (right) from naive T cell-transferred RAG$$^−$$/− recipients injected with either vehicle control for 12 wk (Ctrl, top), anti-ASGM1 Ab for 4 wk and then control for 8 wk (0–4 wk, middle), or anti-ASGM1 Ab for 12 wk (0–12 wk, bottom). Representative features from four experiments are shown. (E) Histological feature of colons from naive T cell-transferred RAG$$^−$$/− recipients injected with control for 12 wk (Ctrl, left), anti-ASGM1 Ab for 4 wk and then control for 8 wk (0–4 wk, middle), or anti-ASGM1 Ab for 12 wk (0–12 wk, right). Representative features from each group are shown. (F) Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. (G) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (left), TNF (middle), and IL-17 (right) in the culture supernatant were measured using ELISA. Data are indicated as means ± SEM from four samples.
CD44− cells. Most CD62L−CD44− cells showed a similar IL-7R/CD127 expression level to the other subsets; however, some cells within this subset showed a lower expression of the IL-7R as seen in Fig. 4 (arrow). These results indicate that the mechanism by which NK cells suppress CD62L−CD44− T cells may be different from that by which they suppress TEM, which is due to NK cell-induced apoptosis via Fas and/or DR5.

The lack of IL-7 does not affect the cytotoxic activity of NK cells

Because we have previously observed the upregulated annexin V and downregulated Bcl-2 expressions in the CD4+ T cells transferred into IL-7−/−RAG−/− recipients (10), we speculated that the ability of NK cells to suppress the T cells could be affected by the presence or absence of IL-7. We therefore performed a cytotoxicity assay to test this hypothesis. As expected, NK cells (effector) had negligible cytotoxicity toward CD62L−CD44− naive T cells (target) derived from WT SP (T:E ratio, 1:5) regardless of whether rIL-7 was present (Fig. 5C). These data suggest that the cytotoxicity is decreased when the number of target T cells exceeds the capacities of effector NK cells to suppress T cells. However, it was still unclear whether the cytotoxic ability of NK cells could be modulated during its development in vivo in the presence or absence of IL-7. Therefore, the cytotoxic ability of NK cells derived from RAG−/− and IL-7−/−RAG−/− mice was examined. As seen in Fig. 5D, there was little mortality of CD62L−CD44− naive T cells alone, and this mortality was unaffected even if coincubated with NK cells derived from either RAG−/− or IL-7−/−RAG−/− mice (T:E ratio, 1:5). The mortality of CD62L−CD44− TEM derived was elevated compared with that of TEM alone when coincubated with NK cells derived from RAG−/− mice and was similar to that following coincubation with NK cells derived from IL-7−/−RAG−/− mice (T:E ratio, 1:5; Fig. 5E).

Additionally, the expression levels of NK receptors (30) that reflect the function of NK cells (Fig. 5F), as well as the levels of CD11b and CD27 that determine the differentiation status of NK cells (31) (Fig. 5G), were not altered in NK cells derived from IL-7−/−RAG−/− mice, compared with those from RAG−/− mice.

To further demonstrate that there are no differences of NK cell functions between RAG−/− and IL-7−/−RAG−/−, we also measured the cytotoxic activities of these cells against YAC-1 cells using the [51Cr] release assay, as well as the production of IFN-γ from these cells. As seen in Fig. 5H and 5I, neither the cytotoxicities against YAC-1 cells nor IFN-γ production of NK cells was modified in the IL-7−/−RAG−/− mice when compared with RAG−/− mice.
RAG⁻/⁻ mice. These results confirm that a lack of IL-7 does not affect the cytotoxic activity of NK cells either in vitro or in vivo.

**K cell depletion elicits severe colitis in naive T cell-transferred IL-7⁻/⁻ RAG⁻/⁻ recipient mice**

We previously reported that the development of colitis is abrogated by a lack of IL-7. Given that NK cells can suppress T cells in vitro and in vivo independently of IL-7, we next assessed the influence of NK cells on colitis in the context of IL-7 deficiency in vivo. IL-7⁻/⁻ RAG⁻/⁻ mice were injected i.p. with naive T cells with or without anti-ASGM1 Ab treatment, and colitis was monitored after 12 wk (Fig. 6A). As previously observed, the induction of colitis was completely abrogated in vehicle control-injected IL-7⁻/⁻ RAG⁻/⁻ mice, as shown by clinical and histological scores and cytokine production from colonic LP lymphocytes, although the presence of occasional leukocytes was observed in colonic tissues (Fig. 6B-E). However, when anti-ASGM1 Ab was injected, IL-7⁻/⁻ RAG⁻/⁻ mice showed elicitation of colitis and similar severity of clinical phenotypes, such as wasting and diarrhea, as did the groups of RAG⁻/⁻ recipients with or without anti-ASGM1 Ab treatment (Fig. 6B). Consistent with these findings, a significant deterioration in histological findings, such as mucosal damage, cell infl-
NK cells may target the CD62L<sup>+</sup>CD44<sup>+</sup> subset by a different mechanism from that by which they target the CD62L<sup>+</sup>CD44<sup>+</sup> subset. RAG<sup>−/−</sup> mice preinjected with anti-ASGM1 Ab were sacrificed 5 d after naive T cell transfer. Isolated splenocytes were stained with anti-CD62L, anti-CD44, and either anti-Fas, anti-DR5, anti-Qa1, or anti-CD127 Abs (open histograms) or isotype-matched control (filled histograms) and were then subjected to FACS. The populations within the appropriate gate on forward scatter and side scatter and either CD62L<sup>−</sup>CD44<sup>−</sup> (naive, R1), CD62L<sup>−</sup>CD44<sup>−</sup> (R2) or CD62L<sup>−</sup>CD44<sup>−</sup> (TEM, R3) were analyzed. Representative data from three experiments are shown.

**FIGURE 4.** NK cells may target the CD62L<sup>−</sup>CD44<sup>−</sup> subset by a different mechanism from that by which they target the CD62L<sup>−</sup>CD44<sup>−</sup> subset. RAG<sup>−/−</sup> mice preinjected with anti-ASGM1 Ab were sacrificed 5 d after naive T cell transfer. Isolated splenocytes were stained with anti-CD62L, anti-CD44, and either anti-Fas, anti-DR5, anti-Qa1, or anti-CD127 Abs (open histograms) or isotype-matched control (filled histograms) and were then subjected to FACS. The populations within the appropriate gate on forward scatter and side scatter and either CD62L<sup>−</sup>CD44<sup>−</sup> (naive, R1), CD62L<sup>−</sup>CD44<sup>−</sup> (R2) or CD62L<sup>−</sup>CD44<sup>−</sup> (TEM, R3) were analyzed. Representative data from three experiments are shown.
FIGURE 5. Cytotoxic activity of NK cells is not affected in the presence or absence of IL-7. (A–C) Splenic NK cells were isolated from WT mice by FACS sorting. Either CD4+CD62L−CD442 naive T cells isolated from WT SP (A) or CD4+CD62L−CD44+ T EM from colonic LP in RAG−/− mice that received naive T cells 12 wk previously (B and C) were stained with PKH2 and cocultured as target (T) cells with the isolated NK cells as effector (E) cells, in the presence or absence of IL-7 for 4 h. Cells were then harvested and stained with PI. The PKH2 and PI double-positive population is assumed to represent dead target cells (28). The mortality of target cells was calculated as the ratio of dead PKH2+ cells. (A) T:E ratio, 1:5, with or without rIL-7; (B) T:E ratio, 1:5, with or without rIL-7; (C) T:E ratio, 1:2.5, 1:1.25, or 1:0.625, without rIL-7. Control (CD4+ T cells alone) is also shown as a negative control. Data are expressed as means ± SEM from three experiments. *p < 0.001. (D and E) Splenic NK cells were isolated from either RAG−/− or IL-7−/− RAG1−/− mice by FACS sorting. Either the CD62L+CD442 naive T (D) or the CD62L−CD44+ T EM (E) subset was stained with PKH2 and cocultured for 4 h with splenic NK cells derived from either RAG−/− or IL-7−/−RAG1−/− mice. Cells were then stained with PI and subjected to the cytotoxic assay described above. Data are expressed as means ± SEM from three experiments. *p < 0.001. (F) Splenic NK cells were isolated from RAG−/− and IL-7−/− RAG−/− mice, and the expression of each NK receptor on these cells was assessed by FACS. The numbers indicate the percentage of cells positive for each NK receptor in the NK1.1-positive population. (G) Splenic NK cells isolated from either RAG−/− or IL-7−/− RAG−/− mice were stained with anti-CD11b and anti-CD27 Abs and were then subjected to FACS to evaluate their differentiation status. The numbers indicate the quadrant percentages of each differentiation status in the NK1.1-positive population. (H) Splenic NK cells were isolated from RAG−/− (open) and IL-7−/− RAG−/− (filled) mice by FACS sorting. YAC-1 cells were labeled with Na2[51Cr]O4 and cocultured as target (T) cells with the isolated NK cells as (Figure legend continues).
beginning (Fig. 9F), although there was no significant difference in either clinical or histological scores between these groups (Fig. 9B, 9E). These results suggest that NK cell depletion at the early stage, but not the late stage, of TEM development is critical for the induction of colitis in IL-7−/−RAG−/− recipient mice.

Discussion

We previously reported that adoptively transferred WT naive T cells injected into IL-7−/−RAG−/− mice interestingly failed to induce colitis (10). However, it is known that IL-7 is not required for the in vitro differentiation of naive T cells into Th1 or Th17 cells (12). We therefore speculated that the reason why the IL-7−/−RAG−/− mice that received naive T cells failed to maintain colitogenic CD4+ TEM may be associated not only with a lack of IL-7, but also with another mechanism that involves suppression of the primary stage of TEM development in the recipients. We previously reported that apoptosis is preferentially induced in CD4+ T cells when IL-7 is lacking in vivo. Thus, increased numbers of annexin V+CD4+ T cells were observed in IL-7−/−RAG−/− recipient mice, into which these T cells had been adoptively transferred, compared with CD4+ T cells in RAG−/− recipient mice (10). These data suggested that T cell suppression via apoptosis is a mechanism by which colitis is abrogated in IL-7−/−RAG−/− recipient mice. We therefore determined whether NK cells, which are known to induce apoptosis in CD4+ T cells, may play a role in such T cell suppression.

Several reports have suggested that NK cells suppress the inflammation caused by autoimmune responses not only in animal models such as EAE and collagen-induced arthritis, but also in clinical samples from patients with multiple sclerosis and systemic lupus erythematosus in humans (20–22, 28, 32, 33). For example, depletion of NK cells using Abs against NK1.1 or ASGM1 results in disease exacerbation in the EAE model (22, 28). Additionally, it has also been reported that NK cell depletion exacerbates an animal model of colitis, although the details underlying the mechanism have not been elucidated (24).

In the present study, NK cells were depleted in the naive T cell adoptively transferred colitis model to analyze the role of NK cells in this model. RAG−/− and IL-7−/−RAG−/− mice that had received naive T cells were depleted of NK cells using an anti-ASGM1 (Figs. 1, 2, 6, 9, Supplemental Figs. 1, 2). However, it was of concern that ASGM1 may be expressed not only in NK cells but also in some subsets of T cells and macrophages when activated (34). Therefore, we also administered anti-NK1.1 Ab

**FIGURE 6.** NK cell depletion with anti-ASGM1 Ab in naive T cell-receiving IL-7−/−RAG−/− mice, as well as in RAG−/− recipients, results in the development of colitis. (A) Protocol for NK cell depletion in a colitis setting. Naive T cell-receiving RAG−/− and IL-7−/−RAG−/− mice were injected with either anti-ASGM1 Ab (0–12 wk) or vehicle control (Ctrl) every second day for 12 wk starting from the day before adoptive transfer of naive T cells. (B) Clinical scores of each group are shown. Data are expressed as means ± SEM from three experiments. (C) Histological features of colons from naive T cell-transferred RAG−/− and IL-7−/−RAG−/− recipients injected with either vehicle control (Ctrl) or anti-ASGM1 for 12 wk (0–12 wk). Representative features from four experiments are shown. (D) Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. *p < 0.05. (E) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (left), TNF (middle), and IL-17 (right) in the culture supernatant are measured by ELISA. Data are indicated as means ± SEM from four samples. *p < 0.05, **p < 0.01.
using another experimental approach to confirm that the phenotypes shown in this model were induced by NK cell depletion (Fig. 8). Note that administration of anti-ASGM1 without T cell reconstitution to the IL-7^−/− RAG^−/− mice does not trigger any inflammation in the colon (Supplemental Fig. 2). Also note that the appropriate controls, such as the same amount of rabbit Ig as a control for anti-ASGM1 polyclonal Ab and mouse IgG2a as an isotype-matched control for anti-NK1.1 (PK136), respectively, do not induce colitis in the recipients either (Figs. 2, 8, Supplemental Fig. 2). Interestingly, NK cell depletion at an early stage during colitis induction resulted in exacerbated colitis in the recipient, even in IL-7^−/− RAG^−/− recipient mice, in association with increased clinical and histological scores as well as upregulated cytokine production by colonic LP T cells. We observed strong

**FIGURE 7.** Colitogenic T EM are induced in naive T cell-receiving IL-7^−/− RAG^−/− by NK cell depletion. (A) Absolute numbers of CD4^+ T cells are shown. CD4^+ SPL (left) or colonic LPL (right) were isolated from naive T cell-receiving RAG^−/− and IL-7^−/− RAG^−/− mice injected with either vehicle control (∼) or anti-ASGM1 Ab (+) for 12 wk. Data are expressed as means ± SEM from five mice. *p < 0.001. (B and C) Isolated SPL (B) or colonic LPL (C) were stained with anti-CD4 and either anti-CD44, anti-CD127/IL-7Rα, anti-CD69, or anti-NK1.1 Abs and were then subjected to FACS analysis. Representative data from four experiments are shown.

**FIGURE 8.** NK cell depletion with anti-NK1.1 Ab in naive T cell-receiving IL-7^−/− RAG^−/− mice results in the elicitation of colitis. (A) Protocol for NK cell depletion in a chronic colitis setting. IL-7^−/− RAG^−/− mice receiving naive T cells were injected with either 0.5 mg/mouse anti-NK1.1 Ab or isotype control every second day for 12 wk. (B) Clinical scores of each group are shown. Data are expressed as means ± SEM from five mice. *p < 0.001. (C) Histological feature of colons from naive T cell-transferred IL-7^−/− RAG^−/− recipients injected with isotype control (∼, top) or anti-NK1.1 Ab (+, bottom). Representative features from each group are shown. (D) Histological scores of each group are shown. Data are expressed as means ± SEM from five mice. *p < 0.001. (E) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (left) and TNF-α (right) in the culture supernatant were measured by ELISA. Data are indicated as means ± SEM from five samples. *p < 0.05, **p < 0.001.
infiltration in colonic tissues ∼4 wk after the adoptive transfer into RAG−/− recipients (10). We therefore compared the effect of NK cell depletion by treatment with an anti-ASGM1 Ab at early (0–4 wk) or late stages (4–12 wk) after naive T cell transfer to treatment over the entire 12-wk period (0–12 wk) after transfer. Ab treatment at the early stage and over the entire 12 wk resulted in a similar degree of colitis exacerbation whereas Ab treatment at the late stage did not exacerbate colitis (Figs. 1, 9). Such exacerbation of colitis occurred relatively latent in the presence of IL-7 in the RAG−/− compared with the IL-7−/− RAG−/− recipients when sacrificed at 12 wk after T cell transfer (Figs. 6, 7). However, the difference of colitis severity in the RAG−/− recipients with or without Ab treatment was interestingly remarkable when sacrificed at 6 wk after T cell receiving (Fig. 2). These results imply that NK cell function is critical for colitogenic T cell suppression at the early stage of colitis development.

Because the CD4+CD44+CD62L− colitogenic T EM in the recipients were suggested to be suppressed at the early stage by NK cells (Figs. 1, 2, 9), we further analyzed the effect of NK cells on the development of CD4+ T cells within a week after recon-

FIGURE 9. NK cell depletion at the early stage, but not at a late stage, in naive T cell-receiving IL-7−/− RAG−/− mice results in the elicitation of massive colitis. (A) Protocol for NK cell depletion in a setting of chronic colitis. IL-7−/− RAG−/− mice were injected with either vehicle control (Ctrl) or anti-ASGM1 Ab (0–12 wk) for 12 wk, anti-ASGM1 Ab for 4 wk followed by vehicle control for 8 wk (0–4 wk), or vehicle control for 4 wk followed by anti-ASGM1 Ab for 8 wk (4–12 wk). (B) Clinical scores of each group are shown. Data are expressed as means ± SEM from four mice. *p < 0.05, **p < 0.005. (C) Gross appearance of colons (left) and SP (right) from naive T cell-transferred IL-7−/− RAG−/− recipients injected with either vehicle control for 12 wk (Ctrl), anti-ASGM1 for 4 wk and then vehicle control for 8 wk (0–4 wk), vehicle control for 4 wk and then anti-ASGM1 Ab for 8 wk (4–12 wk), or anti-ASGM1 Ab for 12 wk (0–12 wk). Representative features from four experiments are shown. (D) Histological feature of colons from naive T cell-transferred IL-7−/− RAG−/− recipients injected with either control for 12 wk (Ctrl), anti-ASGM1 for 4 wk and then control for 8 wk (0–4 wk), vehicle control for 4 wk and then anti-ASGM1 Ab for 8 wk (4–12 wk), or anti-ASGM1 for 12 wk (0–12 wk). Representative features of each group are shown. (E) Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. *p < 0.05, **p < 0.01. (F) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (left), TNF (middle), and IL-17 (right) in the culture supernatant were measured by ELISA. Data are indicated as means ± SEM from four samples. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.
stitution into the RAG−/− recipients (Fig. 3). The number of CD4+ T cells in SPL and MLN was significantly increased 5–7 d after the transfer when NK cells were depleted compared with the control (Fig. 3A, 3B). Additionally, the significant increase of the CD44+CD62L− TEM subset was observed at this point when NK cells were depleted. CD44+CD44+CD62L− colitogenic TEM are suggested to be susceptible to cell death when they are activated. We therefore analyzed the expression of several markers characteristic of NK cell targets on the CD44+CD62L− TEM subset, such as Fas, DR5, and Qa-1, which are the specific receptors or ligand for Fas ligand, TRAIL, and NKG2A, respectively (Fig. 4). As expected, this T cell subset expresses high levels of Fas and DR5, thereby making them susceptible to apoptosis (20). Additionally, these T cells also express some but not a significant level of Qa-1, which induces inhibitory signaling in NK cells via NKG2A. These data indicate that NK cells may suppress CD44+CD44+CD62L− colitogenic TEM via apoptosis, and consistent with our previous observation of downregulated Bcl-2 and upregulated annexin V in CD44+ T cells by the lack of IL-7 in vivo (10).

Furthermore, we also observed an increased unique T cell subset, CD44+CD62L−, when NK cells were depleted (Fig. 3C–E). We were able to observe these cells in the SPL and MLN within 2 wk after T cell transfer into RAG mice, and subsequently they were not detectable afterward (Fig. 7B, 7C). The fact that the CD44+CD62L− T cell subset was only observed at the beginning of colitogenic T cell development would suggest that this interesting population may be associated with the importance of early stage at the pathogenic T cell development in this chronic colitis model. This T cell subset, which is distinct from CD44+CD62L− TEM, is likely to be a second target of NK cells. However, the expressions of Fas and DR5 are lower on these cells compared with those of the CD44+CD62L− TEM (Fig. 4). The expression of Qa-1 in CD44+CD62L− is not greatly different from that of the CD44+CD62L− subset. This phenotype of the CD44+CD62L− subset does not suggest that it is a target of NK cells. However, a recent report showed that CD44 expression on Th1 cells is required to prevent apoptosis via Fas signaling (35). Thus, the CD44+CD62L− subset may be susceptible to apoptosis, since these cells still express some level of Fas on their surface. This may be one of the reasons why early stage of T cell development in this colitis model is targeted by NK cells. Additionally, this possibility may be a potential reason why Th1 cells fail to survive when transferred into IL-7−/− RAG−/− mice. It is also possible that NK cells may regulate CD44+CD62L− and CD44+CD62L− cells by different mechanisms. Analysis of IL-7R expression levels of the CD44+CD62L− subset revealed two distinct populations: IL-7Rα− and IL-7Rα+ (indicated with an arrow in Fig. 4). The IL-7Rα− population in this subset could potentially arise due to transient downregulation of IL-7R expression during differentiation. Unfortunately, the scarcity of these cells prohibited their further analysis and characterization. However, these cells still need to be further studied.

Our recent studies suggested that IL-7−/− RAG−/− mice were able to induce colitis when parabiosed with colitic RAG−/− recipient mice that had received naive T cells 6 wk previously (15). Moreover, deparfiosed IL-7−/− RAG−/− mice, which were surgically separated from T cell-receiving RAG−/−–IL-7−/− RAG−/− parabiosis 6 wk after the initial surgery, still maintained chronic colitis for at least another 12 wk (16). The latter finding is similar to our present observation that IL-7−/− RAG−/− recipient mice, which had been depleted of NK cells at an early stage during induction, showed elicited colitis, even after completion of the anti-ASGM1 Ab treatment (Fig. 9). However, the mechanism by which the colitogenic T cells are maintained in the IL-7−/− RAG−/− mice after the establishment of massive colitis is still unclear. One potential interpretation is that the pathogenic T cells can continue to proliferate, resulting in induction of colitis when the T cell number exceeds the capacity of the NK cells to suppress the T cells. A second possibility is based on the recent report that IL-17 inhibits NK cell-suppressive ability (36). It has been suggested that the increased IL-17 production from T cells that occurs when the severity of the colitis increases may affect NK cell function. The latter possibility is supported by one of our observations that NK cell depletion starting at the late stage of colitis development failed to exacerbate colitis (Supplemental Fig. 1).

We observed that the characteristics of NK cells are not modified by the lack of IL-7 in RAG−/− mice (Fig. 5F). This observation is consistent with a previous report by Vossenrich et al. (37) showing that the lack of IL-7 does not affect the growth, phenotype, or effector functions of NK cells in vivo, although IL-7 had been reported to influence NK cell differentiation. Consistent with this, we also observed that the differentiation of NK cells, which is characterized by the expression of CD11b and CD27 (31), is not altered in the same mice (Fig. 5G). Additionally, there is no significant difference between NK cells derived from RAG−/− and IL-7−/− RAG−/− mice in terms of their cytotoxic activities against the target cells such as T cells and YAC-1 cells (Fig. 5D, 5E, 5H) as well as the production of IFN-γ (Fig. 5I). These data indicate that the dramatic difference in the severity of colitis between IL-7−/− RAG−/− and RAG−/− recipients following NK depletion is not caused by a difference in NK function between NK cells derived from RAG−/− and IL-7−/− RAG−/− mice.

The IL-7−/− RAG−/− recipient mice that received naive T cells failed to induce colitis even though the cytotoxicity of NK cells was not altered. One potential explanation of this result is that the susceptibility of T cells to apoptosis is increased in these mice. It has been reported by others that the expression of Bcl-2, an anti-apoptotic molecule, in T cells is downregulated in IL-7−/− mice (38, 39). We have also reported that Bcl-2 expression is downregulated in T cells injected into IL-7−/− RAG−/− recipient mice (10). A second explanation is based on our previous report that IL-7 contributes to the expansion of colitogenic T cells (39). Thus, these data suggest that colitogenic T cells are not able to survive in the mice due to their reduced expansion and increased susceptibility to apoptosis at the early stage of colitis development.

In this study, we demonstrate NK cell-mediated regulation of T cell development, which is associated with the pathogenesis of chronic colitis. Although the detailed mechanism still remains to be elucidated, an insight into such a mechanism is significant for understanding the regulation of mucosal immune responses.

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Disclosures
The authors have no financial conflicts of interest.

References
NK CELLS REGULATE COLITOGENIC T CELL DEVELOPMENT


Supplementary Figure 1. NK cell depletion at the early stage, but not late stage, of colitis induction in RAG<sup>−/−</sup> mice results in slightly exacerbated colitis.

(A) Protocol for NK cell depletion in a chronic colitis setting. RAG<sup>−/−</sup> mice were injected with either 0.25 mg/mouse of anti-ASGM1 Ab (0-12 wk) or vehicle control (Ctrl) every second day for 12 weeks from the day before adoptive transfer of naïve T cells, or were injected with the vehicle control for 4 weeks followed by 8 weeks of anti-ASGM1 Ab. (B) Clinical scores of each group are shown. Data are expressed as means ± SEM from 4 mice. NS, not significant. (C) Gross appearance of colons (left) and spleens (right) from naïve T cell-transferred RAG<sup>−/−</sup> recipients injected with either vehicle control for 12 weeks (Ctrl, top), control for 4 weeks and then anti-ASGM1 Ab for 8 weeks (4-12 wk, middle) or anti-ASGM1 Ab for 12 weeks (0-12 wk, bottom). Representative features from 4 experiments are shown. (D) Histological feature of colons from naïve T cell-transferred RAG<sup>−/−</sup> recipients injected with control for 12 weeks (Ctrl, left), control for 4 weeks and then anti-ASGM1 Ab for 8 weeks (4-12 wk, middle) or anti-ASGM1 Ab for 12 weeks (0-12 wk, right). Representative features from each group are shown. (E) Histological scores of each group are shown. Data are expressed as means ± SEM from 4 mice. (F) Cytokine production by lamina propria (LP) T cells from each group is shown. Concentrations of IFN-g (left), TNF
(middle) and IL-17 (right) in the culture supernatant were measured using ELISA. Data are indicated as means ± SEM from 4 samples.

**Supplementary Figure 2.** Anti-asialo GM1 antibody does not induce colitis in IL-7−/− RAG−/− mice.

(A) Protocol for NK cell depletion in a chronic colitis setting. IL-7−/− RAG−/− mice were injected with either 0.25 mg/mouse of anti-ASGM1 Ab or isotype control every second day for 12 weeks with or without adoptive transfer of naïve T cells. (B) Clinical scores of each group are shown. Data are expressed as means ± SEM from 4 mice. *, p<0.005. (C) Histological feature of colons from naïve T cell-transferred IL-7−/− RAG−/− recipients injected with anti-ASGM1 Ab alone (left), isotype control with naïve T cells (middle) or anti-ASGM1 Ab with naïve T cells (right). Representative features from each group are shown. (D) Histological scores of each group are shown. Data are expressed as means ± SEM from 4 mice. *, p<0.01.
Supplementary Figure 1