Role of natural killer T cells in the mouse colitis-associated colon cancer model

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Running Title

Role of NKT cells in colitis-associated colon cancer

Summary Sentence

Invariant NKT cells may inhibit tumor progression and inflammation in the mouse.

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Summary

Invariant natural killer T (iNKT) cells are considered innate-like lymphocytes, and regulate the immunity against inflammation and tumorigenesis. However, the impact of iNKT cells in inflammation-associated tumorigenesis remains unclear. In this study, we examined the physiological role of iNKT cells in a mouse colitis-associated colorectal cancer model. C57BL/6 (B6) and J18 NKT cell-deficient KO (KO) mice were used. Colitis-associated colorectal cancer was induced by azoxymethane (AOM) and dextran sodium sulfate (DSS). The resulting inflammation and tumors were examined. The surface markers of mononuclear cells from the liver and the colon were assessed by FACS. The levels of IL-13 from the colon were measured by ELISA. α-galactosylceramide (GC), or its close analog OCH, was administered intraperitoneally on the first day of each cycle of DSS-administration. In the AOM/DSS model, hepatic iNKT cells were significantly decreased. In KO mice there were significantly greater numbers of colon tumors and more severe inflammation than in B6 mice. FACS analysis revealed that the population of NK1.1+ T cells (non-invariant NKT cells) in the colon was increased when compared to B6 mice. The secretion of IL-13 was increased in the colon of KO mice after AOM/DSS. The number of colon tumors was significantly decreased in the GC-treated group compared to the control group. GC-treatment significantly inhibited IL-13 secretion from the colonic mononuclear cells and the number of colonic NK1.1+ T cells was significantly decreased. These results suggest that iNKT cells may play a critical role in
the prevention of tumor progression and inflammation in the AOM/DSS model.

Keywords: colitis-associated colon cancer; invariant NKT; IL-13; α-galactosylceramide, OCH

Introduction

Patients with chronic inflammatory bowel diseases such as ulcerative colitis (UC) and Crohn’s disease are at increased risk of developing colorectal cancer (1,2), indicating that chronic intestinal inflammation is a major risk for colorectal cancer (CRC) (3-5). Indeed, patients with long-standing, extensive UC have a higher risk of CRC than normal individuals (~16.5% at 30 years) after initial diagnosis (1). It has long been noted that cancer arises from regions of chronic inflammation, and the inflammatory cells and cytokines of the immune system found in tumors are more likely to contribute to tumor growth and progression.

Recent studies have suggested an immunosurveillance function for a unique subpopulation of T cells that play an important role in innate immunity called invariant natural killer T (iNKT) cells. iNKT cells are a unique subset of T cells characterized by the expression of both natural killer (NK) receptors and an invariant antigen receptor (T cell receptor; TCR) -chain that is encoded by V 14-J 18 gene segments in mice and V 24-J 18 segments in humans (6). Since the invariant V 14 antigen receptor is exclusively utilized by V NKT cells, and not by conventional T cells, the invariant V 14/V 24 antigen receptors are exquisite markers for iNKT cells. Both mouse and human invariant antigen receptors recognize a glycolipid antigen, -galactosylceramide (GC), which is derived from a marine sponge, and presented by a monomorphic MHC-like molecule, CD1d (7). Stimulation of NKT cells with GC or using antibodies against the TCR results in a vigorous response marked by
proliferation, expression of activation molecules, increased cytotoxic activity, and secretion of various cytokines such as TNF- and interleukin-2 (IL-2), as well as the Th1 cytokine, INF-, and the Th2 cytokine, interleukin-4 (IL-4) (8). iNKT cells regulate viral and bacterial infections, regulate autoimmune disease and provide anti-tumor immunity (9).

The role of iNKT cells in mucosal immunity has been elucidated by several investigations (10,11). In the intestine, invariant and non-invariant NKT cells can be found in the lamina propria of both mice and humans (12, 13). Activation of iNKT cells may contribute to induction of oral tolerance (14) and protection from intestinal infections in mice (15, 16). Furthermore, activation of iNKT cells may play a pivotal role in the protection or exaggeration of murine IBD models (17-20). Therefore, iNKT cells may have several regulatory functions in maintaining intestinal homeostasis. However, the role of iNKT cells in intestinal inflammation-related tumorigenesis is still unclear. Here, we investigated the anti-tumor immune response of iNKT cells in a mouse colitis-associated colorectal cancer model, induced by combined treatment with azoxymethane (AOM) and dextran sodium sulfate (DSS). Specifically, our data show that tumor progression in the colon is highly dependent on the absence of iNKT cells. Our experiments revealed that the combined treatment with AOM and DSS induced the increase in IL-13-positive NK1.1+T cells, especially in iNKT cell-deficient mice, suggesting that both the inflammation and the tumor could be controlled by the existence of iNKT cells. Moreover, activation of invariant NKT cells by GC prevents the growth of tumors in this model in association with decreased IL-13 secretion from the colon.
Materials and Methods

Mice

Specific pathogen-free C57BL/6(B6) mice were purchased from CLEA Japan (Tokyo, Japan). J 281-deficient (V 14NKT cell-deficient [KO]) mice in a B6 background were generated, as described previously (21). All mice were housed under specific pathogen-free conditions in microisolator cages in the animal facility at Hiroshima University, and only male mice (9 to 11 wk of age) were used.

The mouse model for colitis-related colon carcinogenesis

The mice were injected intraperitoneally (i.p.) with 9.6 mg/kg AOM (Sigma). After 7 days, 2.5% dextran sodium sulfate (DSS) (ICN, MW 5000kDa) was given in the drinking water for 5 days, followed by 16 days of regular water. This cycle was repeated three times and the mice were sacrificed 16 days after the last cycle (22). In order to investigate the preventive role of lipid antigen activation of iNKT cells on tumor growth, α-galactosylceramide (GC) or its close analog OCH, kindly provided by Dr Sachiko Miyake (National Institute of Neuroscience, NCNP), was administered intraperitoneally at the concentration of 4 μg/mouse on the first day of each cycle of DSS-administration. For control groups, 200 μl PBS was administered intraperitoneally.
**Assessment of the severity of colitis**

Body weight was measured every other day. Day 0 was determined as the day of first administration of DSS. The differences in body weight from day 0 were represented on a scale as % body weight.

Colonic tissues were removed and opened longitudinally. The length of the colon was measured after exclusion of the cecum and prior to division for histology. The tissues then were rolled concentrically and embedded in paraffin.

Sections were stained with hematoxylin-eosin. Colitis was scored according to the following morphological criteria described previously (23); Grade 0, normal appearance; Grade 1, mild infiltration of inflammatory cells into the lamina propria mucosa with either no erosion or only shallow erosion; Grade 2, deep erosion with marked inflammation, often including crypt abscess formation.

**Tumor examination**

The colon tumors were detected by the method described by Magnuson et al (24). Formalin fixed colons were stained with 0.5%(W/V) methylene blue for 30 min, and then placed on glass slides with the luminal side up and viewed under a light microscope at 5-30 times magnification (25). The location, number, and size of tumors were determined in each mouse. We counted tumors of 0.5 mm or more, which could be observed under a stereomicroscope, with tumors diagnosed according to our definition, using a methylene blue stain.
Isolation of mononuclear cells from colonic lamina propria, liver, and mesenteric lymph nodes

Colonic lamina propria lymphocytes (LPLs) were isolated as described previously (26). Briefly, nonadherent mesenteric tissues were removed and the entire length of the intestine was opened longitudinally and washed with PBS solution. The dissected mucosa was incubated with Ca\(^{++}\)Mg\(^{++}\)-free Hanks’ balanced salt solution containing 1mM ethylenediaminetetraacetic acid (Sigma, St Louis, MO) for 15 minutes with vortexing. Specimens were washed with Hanks’ balanced solution and then were incubated in 150U/mL collagenase (Wako Chemical Co) in RPMI 1640 medium for 1.5 hours at 37°C with stirring. The cells were suspended in 44% Percoll (Sigma) underlaid with 66% isotonic Percoll and were centrifuged for 20 minutes at 2200 revolutions per minute (rpm) at room temperature. Cells at the interface were collected and washed twice with cold PBS. Tumor infiltrating lymphocytes (TIL) were purified from the tumors by the same method as for LPLs.

Hepatic mononuclear cells (MNCs) were purified from the liver, which was mashed in Ca\(^{++}\)Mg\(^{++}\)-free Hanks’, and then passed through mesh. The resulting cell suspension was centrifuged and the pellet was washed twice with Hanks’ solution, after which MNCs were isolated using 44%/66% Percoll density gradient centrifugation (at room temperature, 2200 rpm 20 min) (27). After the mesenteric lymph nodes (MLNs) were removed, they were mashed in Ca\(^{++}\)Mg\(^{++}\)-free Hanks’, and then passed through mesh. The resulting cells were washed once with Hanks’ solution.
Flow cytometric analysis of mononuclear cells

For flow cytometry analysis, a single cell suspension (4×10^5 or 10^6 cells) was prepared and stained with fluorescently labeled antibodies (Abs). All Abs were purchased from BD Pharmingen, unless otherwise indicated. The Abs used were NK1.1 (NKR-P1B and NKR-P1C), CD3 (17A2), CD4 (RM4-5), CD8 (PNIM2780), CD25 (PNIM2795) and were labeled directly with one of the following fluorescent tags; FITC, PE, or APC.

iNKT cells were detected by using αGalCer-loaded CD1d:Ig fusion protein, labeled with PE-anti mouse IgG1(A85-1). Intracellular staining for IL-13 was done using a Cytofix/Cytoperm Plus Fixation/permeabilization Kit (BD) and biotinylated anti-IL-13 Ab (R & D Systems) (28). All samples were analyzed on a FACS Calibur using Cell Quest software.

Cytokine analysis

Colonic LPLs and mononuclear cells of MLNs were purified, transferred to 96-well plates (5×10^5 cells per well), and cultured for 48 hours in medium containing 500ng/mL phorbol myristate acetate (PMA) (Sigma) and 50ng/mL ionomycin (Sigma), 2.5mg/mL peptidoglycan from Staphylococcus aureus (LPS) (BioChemika) or 2μl/mL purified anti-mouse CD3e (BD Pharmingen). After 48 hours, supernatants were harvested and assayed for cytokines. IFN-, IL-4 and TNF- were measured with OptEIA Kits (BD, San Jose, CA). IL13 was measured with an IL-13 DuoSet (R & D System). All samples were analyzed in duplicate.
Statistical Analysis

Data were analyzed with the Japanese version of Stat-View software (Hulinks, Tokyo, Japan) on a Macintosh Computer (Apple Computer, Cupertino, CA). The data are expressed as the mean ±SD. The data were analyzed using an unpaired t-test or ANOVA analysis. Differences were considered to be statistically significant at P<0.05.

Results

Selective decrease in hepatic invariant NKT cells in the AOM/DSS model

To investigate the role of iNKT cells in the pathogenesis of the AOM/DSS-induced tumor model, the population of liver NKT cells was assessed by FACS. Fig 1A shows the representative FACS pattern of liver NKT cells in B6 mice. NKT cells were detected by staining either with anti-NK1.1 antibody or GalCer-loaded CD1d-fusion protein. The population of NK1.1+CD3+ lymphocytes decreased from 39.39% to 14.86% after AOM/DSS treatment. Similarly, CD1d-restricted T cells detected with the fusion protein decreased from 9.87% to 5.96% after treatment with AOM/DSS. When we examined 10 naïve mice and 10 AOM/DSS treated mice, the population of liver NK1.1+T cells or CD1d-restricted T cells was significantly decreased after AOM/DSS treatment (Fig1B). In subpopulation analysis, CD4+ but not CD8’NK1.1’T cells were significantly decreased (Fig1C). In contrast, there was no difference in the population of NK1.1 CD3’T cells (conventional T cells), nor the NK1.1’CD3’ cells (NK cells) (Fig1D). These data suggest that liver iNKT cells were selectively decreased in the AOM/DSS model.
Increased tumor incidence in iNKT cell-deficient mice

In order to investigate the functional role of iNKT cells in this colon carcinogenesis model, B6 and iNKT cell-deficient mice were treated with AOM/DSS. As shown in Fig2A, KO mice demonstrated an increased number of tumors compared to B6 mice, although in both mouse strains the tumors developed mainly in the distal colon.

When we counted the tumors in each mouse strain, KO mice had significantly more tumors than B6 mice (Fig2B). In KO mice the tumors were larger in size and spreading to the middle of the colon (Fig2C). The pathology of tumors in B6 mice was of a well-differentiated adenocarcinoma, restricted to the mucosa (Fig2D). There was no difference in the pathology of tumors between these mice. No metastasis was found in the liver of either B6 or KO mice. These data suggest that iNKT cells may inhibit the progression of colon tumors induced by AOM/DSS.

Exaggeration of intestinal inflammation in iNKT cell-deficient mice

The degree of intestinal inflammation was assessed in B6 mice and KO mice. As shown in Fig3A, body weight loss was more evident in KO mice than in B6 mice. The length of the colon, which is inversely correlated with the degree of inflammation, was significantly shorter in KO mice than in B6 mice after 3 cycles of DSS (Fig3B). Histological analysis of colons, scored according to the morphological criteria, also demonstrated that inflammation in KO mice was significantly more severe than in B6 mice in the distal colon (Fig3C). These data suggest that NKT cell deficiency may result in the exaggeration of colonic inflammation.
Lymphocyte subpopulations in the AOM/DSS model

We then turned our attention to the lymphocyte phenotype in colons. Using FACS, we first investigated how the frequency of colonic effector CD4$^+$ cells was affected. As shown in Figure 4A, AOM/DSS treatment increased the population of CD25$^+$CD4$^+$ cells in KO but not in B6 mice. When we compared these mice after AOM/DSS treatment, KO mice showed a higher population of CD25$^+$CD4$^+$ cells in the tumor and non-tumor lesions in the colon than B6 mice. These results suggest that a deficiency of iNKT cells may allow the expansion of effector T cells.

Next, we examined the percentage of immunosurveillance cells in the colon. After AOM/DSS treatment, there was no obvious change between B6 and KO mice in the frequencies of NK cells and CD8 cells in the lamina propria (Fig 4B). In contrast, the population of NK1.1$^+$T cells of colon lymphocytes was also increased significantly in KO mice in comparison to B6 mice after AOM/DSS treatment (Fig 4C).

Cytokine analysis in the AOM/DSS model

For cytokine analysis, there was no difference in the secretion of IFN-γ and TNF-α from the mononuclear cells in MLNs. In contrast, IL-13 secretion of MLNs was increased in KO mice after AOM/DSS treatment (Fig 5A). IL-13 secretion was also significantly increased in the colon of KO mice after AOM/DSS treatment (Fig 5B).

Intracellular cytokine analysis also demonstrated that the population of IL-13-positive CD3$^+$ cells of KO mice was significantly increased in both the MLNs and colon after the AOM/DSS treatment (Fig 5C). As shown in Fig 5D, 90% of IL-13-producing cells were NK1.1$^+$ T cells.
Decrease in tumor incidence by *in vivo* activated iNKT cells

iNKT cells were *in vivo* activated with GC or OCH to examine the role of iNKT cells on the inhibition of tumor incidence in this model. The cytokine production assay in the mononuclear cells from MLNs revealed that the Th1/Th2 ratio was significantly increased in the GC group compared to the PBS or OCH groups (Figure 6A, B). As shown in Figure 6C, the number of tumors in the GC group was significantly decreased compared to the control group. The degree of colonic inflammation was also decreased in the GC group (Figure 6D). GC treatment reduced the population of colonic NK1.1$^+$ T cells (Figure 6E). For cytokine analysis, IL-13 secretion from colonic lymphocytes was significantly decreased in GC-treated mice after AOM/DSS treatment (Fig6F). The treatment with OCH, a weaker agonist of iNKT cells, also reduced the number of tumors, although the difference was not statistically significant compared with the PBS group. These results suggest that activated iNKT cells may inhibit the tumor growth associated with colonic inflammation.

**Discussion**

Here we have shown that the absence of iNKT cells exaggerated both inflammation and tumorigenesis of the colon following induction by AOM/DSS. iNKT cell-deficient mice had a higher frequency of IL-13-positive NK1.1$^+$T cells than wild type mice in the colon in this model. Activation of iNKT cells by GC reduced the tumor incidence in this model. These results suggest that iNKT cells may play a critical role in protection against colitis and cancer.
induced by AOM/DSS treatment in mice.

An anti-tumor immune response of iNKT cells has been demonstrated in some mouse tumor models. iNKT cells activated by GC directly kill cancer cells by inducing cell-death with perforin, FasL, or TRAIL, and indirectly kill tumors by activating various types of immunosurveillance cells such as NK cells or CD8+ CTLs (29). However, the notion that iNKT cells regulate cancer rejection is based largely on tumor transplant models and the direct role of iNKT cells on colon cancer is still lacking.

iNKT cells are also involved in the pathogenesis of intestinal inflammation. Saubermann et al. reported that GC treatment ameliorates DSS-induced colitis, suggesting that iNKT cells have a protective role in DSS-induced colitis (17). We have reported that OCH, an analog of GC that exerts Th2-predominant immune responses (30), prevents DSS-induced colitis and the protection is associated with increased production of IL-4 and IL-10 (18). Therefore, iNKT cells could induce IL-4 or IL-10, which are anti-inflammatory cytokines that prevent intestinal inflammation.

However, the role of iNKT cells in inflammation-related tumorigenesis is still unclear. In this study, we investigated the anti-tumor immune response of iNKT cells in a mouse colitis-associated colorectal cancer model. We used a novel mouse model for colitis-related colon carcinogenesis using AOM and DSS (22). The AOM carcinogen induces genomic instability and mutation of oncogenes in the epithelia, whereas repeated DSS administration causes chronic inflammation, which greatly enhances the incidence of AOM-induced tumors (31).
First, we examined how the population of iNKT cells was affected after AOM/DSS administration. We checked liver iNKT cells because these cells are abundant in the liver (32). Although there were no change in the population of NK1.1<sup>–</sup>CD3<sup>+</sup> conventional T cells and NK1.1<sup>+</sup>CD3<sup>−</sup>NK cells, the population of liver NK1.1<sup>+</sup>T cells or CD1d-restricted T cells was significantly decreased in the liver after AOM/DSS treatment. These data suggest that iNKT cells may play a critical role in this model. The exact mechanism by which iNKT cells are decreased in the AOM/DSS model remains unknown. A potential mechanism that might explain reduced NKT cells in different models of hepatic pathologies has been presented recently (33). IL-12 is important to decrease hepatic iNKT cells in hepatosteatosis. Further investigations are needed to clarify the mechanism of NKT cell reduction in the AOM/DSS model.

Next, we assessed the role of iNKT cells in colon tumors and inflammation induced by AOM/DSS by using iNKT cell-deficient (KO) mice. Interestingly, KO mice demonstrated an increased number and size of tumors. However, there was no difference in the pathology of tumors between B6 mice and KO mice. The colonic inflammation in KO mice was also more severe than in B6 mice. Colon tumor progression depends on the degree of intestinal inflammation in this model because we have previously shown that the lower concentration of DSS (1.5%) resulted in the progression of smaller tumors. When the KO mice were subjected to AOM injection alone, colon tumors were not developed (data not shown). This evidence suggests that iNKT cells may inhibit the progression of colon tumors by ameliorating the colon inflammation induced by DSS.
To examine the anti-inflammatory role of iNKT cells in this model, we analyzed the population of MNCs in the colon. First, we wanted to see the difference in the activation state of these lymphocytes. There was no difference in the population of CD25^+CD4^+ cells between naïve B6 and KO mice. After AOM/DSS treatment, however, these populations increased in KO mice. When we compared B6 and KO mice after AOM/DSS treatment, KO mice showed higher populations of CD25^+CD4^+ cells than B6 mice. The presence of iNKT cells may be required for inhibiting the expansion of effector T cells, although the mechanism is under investigation.

We then examined the populations of immunosurveillance cells in the colon. There were no differences in the populations of CD8^+T cells and NK cells between B6 and KO mice after AOM/DSS treatment. This did not necessarily imply that iNKT cells provide tumor reduction independently of tumor immunosurveillance cells in this model because the necessary functional assay was not performed.

In contrast, the population of NK1.1^+T cells was significantly increased in the colon of KO mice after AOM/DSS. Moreover, the secretion of IL-13, but not IFN-γ and TNF-α, was significantly increased in the colons of KO mice. Intracellular cytokine analysis demonstrated that the population of IL-13-producing NK1.1^+T cells was also significantly increased in KO mice. The lack of iNKT cells can be associated with increased IL-13-producing NK1.1^+T cells.

IL-13 has a central role in mediating the suppressive activity of NKT cells in tumor immunosurveillance (34-37). The role of IL-13 in the pathogenesis of UC has been discussed
in several publications. IL-13 produced by non-invariant NKT cells has been shown to increase in UC (13), and IL-13 was involved in the disruption of intestinal epithelial barrier function associated with UC (38). Furthermore, IL-13Ralpha2 was elevated in intestinal epithelial cells from UC or colorectal cancer and initiates the MAPK pathway (39). IL-13 secreted from lamina propria lymphocytes in the colon of UC patients may be one of the causes of increasing inflammation and tumorigenesis.

NKT cells are at least divided into four population groups (11). The first two populations are CD1d restricted and are named type I and type II. Type I NKT cells are so-called invariant CD1d-restricted NKT cells, which provide anti–tumor and anti-inflammatory immunity. Type II NKT cells are non-invariant CD1d restricted NKT cells, which could promote tumor development (40, 41). A third group has a canonical TCR (Vα7.2i TCR, homologous Vα19i in humans) and these cells are known as mucosal-associated invariant T (MAIT) cells (42). MAIT cells are restricted by MR1 (43), a nonpolymorphic MHC class I-like molecule and rapidly produce effector cytokines like IL-4, IL-5, IL-10, IL-17, and IFN-γ after activation via MR1 (44, 45). The fourth group is more heterogeneous because it combines all other T cells that express NK receptors. This group of NKT cells expresses CD4 or CD8 and is restricted by classical MHC class I or class II molecules. The majority of NKT cells in the mouse intestine belong to this group (46). In our study, it was suggested that the NK1.1+ T cells, which increased in the colon of KO mice and have a potential to produce IL-13 after AOM/DSS, may behave like type II NKT cells. Further investigations are needed to elucidate the origin and characteristics of IL-13+NK1.1+T cells in the mouse colon.
It is unclear whether the interaction between these subsets of NKT cells occurs naturally in vivo. Ambrosino et al showed that the functions of type I NKT cells were suppressed by type II NKT cell activation in vivo and in vitro in tumor models (41). It is possible that type I NKT cells may inhibit the expansion of type II NKT cells, because both types of NKT cells were CD1d-restricted and competition for CD1d binding would occur. The interactions between type I and the other groups of NKT cells in the intestine remains to be elucidated.

Finally, we showed that stimulation of iNKT cells by the lipid antigen, GC, prevents tumor growth in the AOM/DSS model. Miyamoto et al demonstrated that the IFN-γ/IL-4 ratio is increased by GC treatment and decreased by OCH treatment in mouse serum and spleen (30). Therefore, both OCH and GC have a similar effect of activating NKT cells. However, GC favors a Th1 shift of NKT cells, while OCH has Th2 skewing activity. We also found that the mononuclear cells in MLNs had increased the IFN-γ/IL-4 ratio in vivo after GC treatment, but not OCH treatment. OCH also reduced the incidence of tumors but the effect was weaker than with GC. The effect of tumor reduction was associated with the degree of inhibition in IL-13-producing NK1.1+ T cells because GC treatment reduced NK1.1+ T cells in the colon more efficiently than OCH treatment. Targeting IL-13-producing NK1.1+ T cells, therefore, may be an alternative treatment strategy against colitis-associated colorectal cancer.

In conclusion, we showed that a deficiency in iNKT cells led to increased numbers of tumors and resulted in more severe colitis in an AOM/DSS colorectal cancer model. Furthermore, activation of iNKT cells by GC resulted in a decrease in the incidence of tumors in this model, probably due to the inhibition of IL-13-producing NK1.1+ T cells. These findings strengthen the hypothesis that iNKT cells are involved in protection against tumors in colitis-associated
cancer. In this work we have shed light on critical functions of iNKT cells in the colon, and this opens up new preventive and/or therapeutic avenues for the control of tumor surveillance in IBD and CRC patients.

**References**


10. van Dieren JM, van der Woude CJ, Kuipers EJ, Escher JC, Samsom JN, Blumberg RS,


33. Kremer M, Thomas E, Milton RJ et al. Kupffer cell and interleukin-12-dependent loss of


**Figure legends**

**Figure 1**

Phenotypic characterization of lymphocytes freshly isolated from liver in B6 mice after AOM/DSS treatment

(A) Representative FACS pattern of liver iNKT cells by gated lymphocytes in B6 mice after AOM/DSS treatment. NKT cells of liver were detected by staining either with anti-NK1.1 antibody or GalCer-loaded CD1d-fusion protein. Numbers denote the percentages of NK1.1<sup>+</sup>T and CD1d-restricted T cells indicated by the black line.

(B) The population of lymphocytes in liver of 10 naïve mice and 10 AOM/DSS treated mice were examined. The population of NK1.1<sup>+</sup>CD3<sup>+</sup> cells and CD1d-restricted T cells from gated lymphocytes is shown. *p<0.005, **p<0.05, AOM/DSS (-) vs (+). (C) The population of CD4 or CD8 among NK1.1<sup>+</sup>T cells is shown. *p<0.005, **p<0.05, AOM/DSS (-) vs (+). (D) The population of NK1.1<sup>-</sup>CD3<sup>+</sup> T cells and NK1.1<sup>+</sup>CD3<sup>-</sup> cells.

**Figure 2**

Absence of iNKT cells increases colon tumorigenesis

(A) Representative macroscopic images of colons from B6 and KO mice. (B) Numbers of tumors per colon from B6 (n=7) and KO (n=10) mice. Horizontal bars indicate median values. *p<0.005, B6 vs KO. (C) The location and the size of tumors. *p<0.05, B6 vs KO. (D) Histology of representative colon tumors using Hematoxylin and Eosin staining. The pathology of the tumors was of well-differentiated adenocarcinoma, restricted to the mucosa. The right panel shows histological analysis indicated by the black line at higher magnification.
The absence of iNKT cells increases the severity of colitis

(A) Percentage body weight change was compared between B6 (n=7) and KO (n=10) mice in chronic DSS treatment. Weight loss was more evident in KO mice than in B6 mice. *p<0.05, **p<0.005, ***p<0.0005, B6 vs KO.

(B) The length of the colon was measured. The colons of KO mice were significantly shorter than those of B6 mice after AOM/DSS. *p<0.05, B6 vs KO.

(C) Histological analysis demonstrated that colonic inflammation of KO mice in the distal part of the colon was significantly more severe than in B6 mice. *p<0.05, B6 vs KO. The data shown in panels (A), (B), (C) are representative of two independent experiments.

The lymphocyte phenotype in the colon

(A) Percentages of CD3+ cells that express both CD25 and CD4. “Colon” indicates the lymphocytes from tumor-free lesions. “Tumor” indicates the lymphocytes from tumors. Mean values and SD were calculated from at least nine mice of each group. *p<0.05, **p<0.005, B6 vs KO. (B) Percentages of lymphocytes that express NK1.1 or both CD3 and CD8. (C) Percentages of lymphocytes that express both CD3 and NK1.1. NK1.1+CD3+T cells of colon lymphocytes were increased significantly in KO mice in comparison to B6 mice after AOM/DSS treatment. *p<0.05, **p<0.005, B6 vs KO.
Figure 5

Cytokine production of the MLN or colon

(A) The mononuclear cells from MLNs were stimulated with either LPS (left panel) or PMA and ionomycin (right panel) for 48 hours. The culture supernatants were analyzed for the production of IFN-γ (top), TNF-α (center), and IL-13 (bottom) by ELISA. Mean values and SD were calculated from at least seven mice of each group. One experiment representative of two independent experiments with similar results is shown. (B) The mononuclear cells from colon were stimulated with PMA and ionomycin for 48 hours. The culture supernatants were analyzed for the production of IL-13 by ELISA. “Colon” indicates the lymphocytes from tumor-free lesion. “Tumor” indicates the lymphocytes from tumors. One experiment representative of two independent experiments with similar results is shown. *p<0.05, **p<0.005, B6 vs KO. (C) Percentages of lymphocytes that express both CD3 and IL-13 in MLNs and colon following intracellular cytokine analysis. *p<0.05, **p<0.005, B6 vs KO. (D) Representative FACS pattern of intracellular cytokine analysis of MLN lymphocytes from KO mice. The numbers indicate the percentage of IL-13+ T cells that express NK1.1 and CD3.

Figure 6. Activation of iNKT cells decreases the tumor incidence in the AOM/DSS model

(A) Cytokine production from the mononuclear cells in MLNs. B6 mice were injected with either GC or OCH and sacrificed after 24 hours. The mononuclear cells from MLNs were purified and incubated with PMA and ionomycin for 48 hours. The culture supernatants were analyzed for the production of IFN-γ (top) and IL-4 (bottom). Mean values and SD were
calculated from at least nine mice of each group. (B) The Th1/Th2 ratio (IFN- /IL-4) was determined by each concentration of cytokines.

(C) (Upper panel) Macroscopic examination of colon tumors. The number of tumors in the GC group was decreased compared to the PBS or OCH groups. (Lower panel) Numbers of tumors per colon from B6 mice treated with GC or OCH. The mice in the GC group had significantly fewer tumors than those in the PBS group (p<0.05). The mice in the OCH group had fewer tumors than those in the PBS group, although the results were not always statistically significant. The experiment was repeated twice with similar results. Horizontal bars indicate median values (n=7-10).

(D) The length of the colons of B6 mice treated with GC or OCH in the AOM/DSS model. The results of two independent experiments are shown. Mean values and SD were calculated from at least seven mice of each group. *p<0.05, **p<0.0005, GC group vs PBS or OCH groups.

(E) Percentages of lymphocytes that express both CD3 and NK1.1. The population of NK1.1+T cells in colonic LPLs was significantly decreased in the GC group. Mean values and SD were calculated from at least three mice of each group. *p<0.05, the GC group vs the PBS groups.

(F) The mononuclear cells from colons of the GC or OCH groups were stimulated with PMA and ionomycin for 48 hours. The culture supernatants were analyzed for the production of IL-13 by ELISA. Mean values and SD were calculated from four mice of each group. *p<0.0005, GC group vs PBS or OCH groups.
Fig. 1 (A)
Fig. 1 (B)

NK1.1<sup>+</sup>CD3<sup>+</sup>

CD1d<sup>+</sup>CD3<sup>+</sup>

<table>
<thead>
<tr>
<th>(%)</th>
<th>AOM/DSS</th>
<th>+</th>
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<tbody>
<tr>
<td>40</td>
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<table>
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<th>(%)</th>
<th>AOM/DSS</th>
<th>+</th>
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<tr>
<td>8</td>
<td><img src="chart2.png" alt="Bar Chart" /></td>
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**Significant difference:** *p < 0.05, **p < 0.01*
Fig. 1 (C)
Fig. 1 (D)

NK1.1⁺CD3⁺

NK1.1⁺CD3⁻
Fig. 2 (C)
Fig. 2 (D)

Well-differentiated adenocarcinoma (m)
Fig. 3 (B)
Fig. 3 (C)
Fig. 4(A)

CD25^+CD4^+ cells

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>KO</th>
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<tbody>
<tr>
<td>control</td>
<td>10</td>
<td>15</td>
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<tr>
<td>colon</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>tumor</td>
<td>15</td>
<td>20</td>
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</table>

AOM/DSS: —, +, ++
Fig. 4 (B)

CD3-NK1.1\(^+\) cells

CD3\(^+\)CD8\(^+\) cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>Colon</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>AOM/DSS -</td>
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<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
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<tr>
<td>AOM/DSS +</td>
<td>12</td>
<td>10</td>
<td>8</td>
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</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>Colon</th>
<th>Tumor</th>
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</thead>
<tbody>
<tr>
<td>AOM/DSS -</td>
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<td>1.5</td>
<td>&lt; 0.5</td>
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<tr>
<td>AOM/DSS +</td>
<td>2.5</td>
<td>2</td>
<td>1.5</td>
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</tbody>
</table>

B6 KO

KÖ
Fig. 4 (C)

NK1.1^+ T cells

B6
KO

AOM/DSS
control
colon
+ +
tumor

%
Fig. 5 (A)

Stimulation with LPS

IFN-γ

(pg/ml)

AOM/DSS

B6

KO

Stimulation with PMA+Homonycin

TNF-α

(pg/ml)

AOM/DSS

B6

KO

IL-13

(pg/ml)

AOM/DSS

B6

KO
Fig. 5 (B)

[Graph showing IL-13 concentration levels in control, colon, and tumor conditions with B6 and KO groups compared.]

- B6
- KO
Fig. 5 (C)

IL13+ cells in MLN

IL13+ cells in colon

<table>
<thead>
<tr>
<th>Condition</th>
<th>B6</th>
<th>KO</th>
</tr>
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<tbody>
<tr>
<td>AOM/DSS -</td>
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<tr>
<td>AOM/DSS +</td>
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<tr>
<td>Tumor</td>
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</tbody>
</table>

* p < 0.05
** p < 0.01
Fig. 5 (D)
Fig. 6 (C)

PBS  GC  OCH

Experiment 1

Experiment 2

number of tumors

PBS  GC  OCH  PBS  GC  OCH
Fig. 6 (E)

![Graph showing population of colonic NK1.1^T cells with controls and treatments.](image)

- PBS (-)
- PBS (+)
- GC (+)
- OCH (+)

* denotes statistical significance.
Fig. 6 (F)

![Graph showing IL-13 concentration for PBS, GC, and OCH groups with statistical significance indicated by asterisks.](image)