Ascites Specific Inhibition of CD1d-Mediated Activation of Natural Killer T Cells

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Abstract

Purpose: Natural killer T (NKT) cells recognize lipid antigen presented by CD1 molecules. NKT cells can both directly, through cytotoxicity, and indirectly, through activation of other effector cells, mediate antitumor immunity. It has been shown, however, that tumor-associated lipids are frequently shed into the tumor microenvironment, which can mediate immunosuppressive activity. Given that ovarian cancer – associated ascites has been reported to have increased levels of gangliosides, we examined the effect of tumor-associated and other ascites on CD1d-mediated antigen presentation to NKT cells.

Experimental Design: To investigate the effects of ascites on NKT cell activation, we pretreated CD1d-expressing cells with the ascites and measured their ability to stimulate cytokine production in NKT cells. To determine whether antigen processing or editing was necessary, CD1d-immunoglobulin – based artificial antigen presenting cells (aAPC) were also incubated with ascites. In addition, to examine specificity, we analyzed whether ascites fluid could influence the activation of classic $CD8^+$ T cells.

Results: Pretreatment of CD1d-expressing cells with ascites from the majority of patients inhibited the ability of the cells to stimulate/activate NKT cells in a dose-dependent manner. Ascites treatment also partially blocked the ability of α-galactosylceramide – loaded CD1d-immunoglobulin – based aAPC to activate NKT cells. In addition, our data show that treatment with ascites does not inhibit HLA-A2 – mediated activation of classic $CD8^+$ T cells.

Conclusions: Together, these data suggest that ovarian and other cancers may have developed immune evasion mechanisms specifically targeting the CD1/NKT cell system.

In the United States, ovarian cancer ranks fifth as a cause of cancer-related deaths among females. In fact, it has the highest mortality rate among gynecologic tumors. Unfortunately, the majority of cases are diagnosed at an advanced stage. Despite optimal cytoreductive surgery and primary chemotherapy, 5-year survival rates of patients with advanced ovarian cancer remain <30% (1, 2). Therefore, novel approaches to treatment are essential for effective, durable therapy.

Recent studies have focused on the role of adaptive immunity in epithelial ovarian cancer (3, 4). In fact, the presence or absence of specific T cell subsets has been correlated to survival. Zhang et al. (5) have shown that the presence of intratumoral T cells in epithelial ovarian cancer was associated with improved survival. Conversely, work by Curiel et al. (6) has shown that tumor infiltration by regulatory T cells ($CD4^+CD25^+$ T cells) is indicative of reduced survival in epithelial ovarian cancer. Lastly, a study by Sato et al. (7) suggests that intraepithelial $CD8^+$ T cells are associated with favorable prognosis in ovarian cancer. Although several groups have reported immune responses against ovarian cancer (3, 4), these studies involving antigen processing and presentation have historically focused on antigen presentation by the MHC class I molecules to classic $CD8^+$ T cells. However, unlike MHC class I molecules that present peptide antigens to T cells, CD1d molecules primarily present nonpeptidic ligands (mainly lipid antigens) to a subpopulation of T cells called natural killer T (NKT) cells (reviewed in ref. 8).

Following activation, NKT cells rapidly secrete both Th1 and Th2 cytokines and can mediate cytolytic activity (9). Classic NKT cells express a restricted T cell receptor that recognizes lipids in the context of CD1d. The majority of these NKT cells express an invariant T cell receptor α chain rearrangement: Vα14Jα18 in mice and Vα24Jα18 in humans, which is associated with Vβ chains of limited diversity (10 – 13), and are now referred to as canonical or invariant NKT cells. Other CD1d-specific T cells expressing different T cell receptor α chains are called noncanonical or nonclassic NKT cells.

NKT cells are important in regulating immune responses to infection and autoimmune diseases, as well as to tumors (14, 15). In fact, circulating numbers of NKT cells are markedly decreased in cancer patients (14, 16). Moreover, NKT cells are reduced in both function and number, regardless of cancer type

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Received 6/9/08; revised 8/4/08; accepted 8/6/08.

Grant support: HERA Foundation (T.J. Webb), 2004 Gynecologic Cancer Foundation/Ann Schreiber Ovarian Cancer Research Grant (R.L. Giuntoli, II), and NIH grants AI 44129, AI 29575, and CA 108835 (J.P. Schneck) and T32 AI07247-24 (T.J. Webb).

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doi:10.1158/1078-0432.CCR-08-1468

Translational Relevance

Natural killer T (NKT) cells are a unique subset of T cells that display markers characteristic of both natural killer (NK) and T cells. Circulating numbers of NKT cells are markedly decreased in patients with melanoma, prostate cancer, and numerous other cancers. In fact, activation of these cells through treatment with a synthetic glycolipid is currently being used in clinical trials. However, it is thought that the antitumor effects mediated by these cells are being hampered because the number and function of NKT cells are reduced in cancer patients. Tumor-associated glycolipids are frequently shed into the tumor microenvironment and can mediate immunosuppressive activity. These data suggest that one mechanism that tumors may have developed to evade immune detection is by shedding inhibitory lipids. In this study we found that ascites treatment specifically inhibits NKT cell activation. This study should provide a better understanding of NKT cell biology in terms of its role in anticancer immunity. This work has the potential to affect the development of new tests correlating NKT cell biology with patient outcomes, and may result in novel treatments that prevent disease progression.

(16). However, whereas numerous studies have examined the role of NKT cells in cancer in general, the specific role of these cells in the pathogenesis of ovarian cancer has yet to be elucidated.

It has been reported that ovarian malignancies may alter the local environment resulting in a suppression of the immune system (17). Many advanced ovarian cancer patients have a local accumulation of fluid called ascites that contains cellular components of the immune system such as lymphocytes and regulatory factors such as cytokines. Moreover, patients with advanced ovarian cancer have been reported to have higher levels of gangliosides in their plasma and ascites compared with the plasma samples of controls (18). Because NKT cells have been reported to recognize gangliosides in the context of CD1d (19, 20), we investigated whether ascites treatment of CDd1-expressing cells could alter their ability to activate NKT cells.

In addition, our group has developed noncellular, bead-based, artificial antigen presenting cells (aAPC), made by coupling HLA-immunoglobulin, along with other costimulatory molecules, onto magnetic beads. These aAPC have been shown to effectively expand cytomegalovirus (CMV)- and MART-1–specific CTL (21). In this study we developed CD1d-immunoglobulin–based aAPC. It has been reported that soluble forms of CD1d molecules loaded with lipid antigen are directly able to activate NKT cells (22–24). Because the engagement of the T cell receptor by the CD1d-antigen complexes is a fundamental requirement for NKT cell activation, ^{antigen}CD1d-immunoglobulin complexes offer a method to assess NKT cell activation in the absence of antigen processing or other cellular factors.

In this study, we sought to determine if treatment with ovarian cancer–associated ascites altered CD1d-mediated activation of NKT cells. Here, we report that treatment of CD1d-expressing cells with ascites from ovarian cancer patients and others abrogated their ability to activate both canonical and noncanonical NKT cells. This is the first report that ascites specifically inhibits the CD1d/NKT cell system.

Materials and Methods

Patient samples. Ovarian cancer–associated ascites and peripheral blood mononuclear cells were collected by the Kelly Gynecologic Oncology Service at Johns Hopkins Medical Institutions from patients undergoing primary cytoreductive surgery. Ascites was also collected from patients with hepatitis. All donors gave written informed consent before enrolling in the study. The Institutional Review Board of Johns Hopkins Medical Institutions approved this investigation.

Cell lines. Murine L cells transfected with vector alone or the wild-type *cd1d1* cDNA in pcDNA3.1-neo (Invitrogen; LCD1dwt) were kindly provided by R.R. Brutkiewicz (Indiana University School of Medicine, Indianapolis, Indiana; ref. 25), and were cultured in DMEM, supplemented with 2 mmol/L L-glutamine (BioWhittaker), 10% fetal bovine serum (FBS; HyClone), and ciprofloxacin (Serologicals Proteins). TAP (transporter associated with antigen processing)-deficient 174CEM.T2 (T2) cells were maintained in Iscove's modified Dulbecco's medium, with the same supplements described above.

NKT cells. The Vα14$^+$ NKT cell hybridoma cell lines DN32.D3 (26, 27), N38-2C12, N38-2H4, N38-3C3, and the CD1d1-specific NKT cell hybridoma N37-1A12 (Vα5$^+$) have all been described (28) and were cultured in Iscove's modified Dulbecco's medium supplemented with 5% FBS and 2 mmol/L L-glutamine. For primary mouse NKT cells, liver mononuclear cells were isolated as described previously (29), then the cells were stained with antibodies against CD3 and NK1.1 (Pharmingen), and sorted using a FACSAria. Primary human NKT cells were isolated from peripheral blood mononuclear cells using the CD3$^+$CD56$^+$ isolation kit (Miltenyi).

Generation of aAPC. The preparation of CD1d-immunoglobulin–based aAPC was done according to the previously described method (21). Briefly, to conjugate hCD1d-immunoglobulin dimer molecules to beads, 50 μg of hCD1d-immunoglobulin (Pharmingen) were added to 0.5 mL of epoxy beads (Dynabeads, M-450, Expoxy, 4×10^8 beads/mL; Dynal) in sterile 0.1 mol/L borate buffer, pH 7.0 to 7.4. The bead protein mixture was mixed with rotation and incubated for 24 h at 4°C. Then the beads were washed and the hCD1d molecules were loaded with lipid antigenα-galactosylceramide (α-GalCer; 5 μg/mL in 1 mL PBS containing 5×10^7 beads; Axxora, LLC).

Generation of CMV-specific CTL. CMV-specific CTL were generated using peptide-pulsed HLA-A2-immunoglobulin–based aAPC, as previously described (21).

Treatment of cells with tumor associated ascites. The ascites was cleared of cellular debris by centrifugation at $250 \times g$ for 10 min. The clarified supernatants were then stored at -20°C. For experiments, the supernatants were thawed at 4°C overnight, then L-vector, L-CDd1wt, CMV-peptide pulsed T2 cells, or aAPC were incubated with ascites (2-5 $\times 10^6$ cells per mL of ascites) for 4 h at 37°C. The cells were subsequently washed thrice with 10 mL PBS, resuspended in Iscove's modified Dulbecco's medium, supplemented with 5% FBS and 2 mmol/L L-glutamine and cocultured with or without the indicated NKT hybridomas for 20 to 24 h at 37°C. The cocultures with the primary T and NKT cells were incubated for 72 or 48 h, respectively. Cytokine release [interleukin-2 (IL-2), granulocyte macrophage colony-stimulating factor, or IFN-γ] was measured as an indication of NKT/T cell activation and was measured by standard sandwich ELISA.

Results

Pretreatment with tumor associated ascites inhibits CD1d-mediated activation of NKT cells. Ascites from a panel of ovarian cancer patients was used to treat CD1d expressing cells

Table 1. Panel of ascites samples

Code	Diagnosis	Histology	DN32.D3*	N37-1A12*	N38-2C12*
OC-4	Ovarian cancer	High-grade serous carcinoma	-	++	+++
OC-6	Ovarian cancer	High-grade serous carcinoma	++	+++	-
OC-18	Ovarian cancer	High-grade serous carcinoma	+++	++	++
OC-23	Ovarian cancer	High-grade serous carcinoma	++	-	++
OC-34	Ovarian cancer	High-grade clear cell carcinoma	+++	++++	++++
OC-36	Fallopian tube carcinoma	High-grade serous carcinoma	++++	++++	++
OC-37	Ovarian cancer	Mixed clear cell and low grade serous carcinoma	-	++	++
OC-38	Fallopian tube carcinoma	Moderately differentiated serous carcinoma	++++	++++	++++
OC-40	Ovarian cancer	High-grade serous and clear cell carcinoma	++++	++	+++
OC-46	Ovarian cancer	Atypical proliferative (borderline) serous tumor	++	-	++
OC-47	Ovarian cancer	MMMT, carcinosarcoma	++++	++++	++++
OC-48	Ovarian cancer	Well-differentiated endometrioid carcinoma	-	-	++
OC-56	Ovarian cancer	High-grade serous carcinoma	++++	++++	++++
OC-57	Primary peritoneal cancer	High-grade serous carcinoma	++++	++++	N.D.
OC-58	Ovarian cancer	High-grade serous carcinoma	-	-	N.D.
OC-59	Ovarian cancer	High-grade serous carcinoma	++++	++++	N.D.
OC-60	Ovarian cancer	High-grade serous carcinoma	++++	-	N.D.
OC-62	Ovarian cancer	High-grade serous carcinoma	-	+++	N.D.
OC-63	Ovarian cancer	High-grade adenocarcinoma	++	++	N.D.
OC-64	Uterine cancer	High-grade serous carcinoma	+++	++++	N.D.
OC-65	Ovarian cancer	High-grade serous carcinoma	+	++++	N.D.
OC-66	Ovarian cancer	High-grade serous carcinoma	++++	++++	N.D.
OC-67	Ovarian cancer	High-grade serous carcinoma	++++	+	N.D.
OC-68	Ovarian cancer	High-grade endometrioid carcinoma	++	+	N.D.
OC-69	Ovarian cancer	High-grade serous carcinoma	++	+	N.D.
LP3	Hepatitis C		-	-	N.D.
LP4	Hepatitis C		++++	++++	N.D.
LP5	Hepatitis C		-	-	N.D.
LP6	Hepatitis C		+++	++	N.D.
LP7	Hepatitis C		-	-	N.D.
LP8	Hepatitis C		+	++	N.D.

Abbreviation: N.D., not determined.
*Percent maximum inhibition of IL-2 production is shown as follows: -, no inhibition; +, <10%; ++, 10 to 30%; +++, 31 to 50%; and ++++, >51%.

(LCD1dwt), and the effect on CD1d-mediated antigen presentation was assessed (Table 1). Following treatment with ascites, LCD1dwt cells were washed extensively and cocultured with NKT cells. We evaluated the ability of the pretreated CD1d-expressing cells to stimulate the NKT cells by measuring the IL-2 released in the coculture supernatants by ELISA. We found that the ability of the CD1d-expressing cells to induce NKT cell cytokine production was reduced by 10% to >95% following treatment with the majority of ascites samples examined, as shown in Table 1 and Fig. 1A. Interestingly, we found that antigen presentation to both canonical ($V\alpha14^+$) DN32.D3, N38-3C3, N38-2C12, and N38-2H4, and noncanonical ($V\alpha5^+$) N37-1A12 NKT cell hybridomas was inhibited by pretreatment with ascites (Fig. 1A).

Next, we sought to examine whether nonmalignant ascites also contained immunosuppressive properties. Thus, we obtained portal hypertension ascites from patients with hepatitis C. As shown in Table 1, we found that half of the ascites (3 of 6) did not inhibit the ability of the CD1d-expressing cells to stimulate the NKT cells. In fact, treatment with only one sample from the patients with hepatitis C resulted in >50% inhibition. Treatment with nonmalignant ascites can have an effect on CD1d-mediated NKT cell activation; therefore, it will require many more samples to clearly establish if nonmalignant ascites can regularly alter CD1d-mediated NKT cell activation.

To examine the specificity of inhibition, we analyzed the ability of NKT cells to produce other cytokines. NKT cell hybridomas have been reported to secrete granulocyte macrophage colony-stimulating factors (30), which is important for their maturation and effector functions (31, 32). We found that production of granulocyte macrophage colony-stimulating factor was also suppressed by the majority of the ascites samples tested, as shown with samples OC-60 and OC-63 (Fig. 1B). Ascites treatment of stimulatory cells not only inhibited NKT hybridoma cell lines, but even more importantly also inhibited the activation of primary mouse NKT cells (Fig. 1C) and primary human NKT cells (Fig. 1D). Taken together, these data show that ovarian cancer–associated ascites can profoundly affect the ability of APC to activate NKT cells.

Pretreatment with serum does not inhibit CD1d-mediated activation of NKT cells. Numerous reports have shown that cancer patients have a reduction in NKT cell number and function. As we have found that pretreatment with ovarian cancer associated–ascites can reduce the stimulatory capacity of CD1d molecules, it is possible that this effect is not limited locally to the ascites. Therefore, to further analyze if the observed inhibitory effect is limited to the ascites or if it is a more global effect, we analyzed the inhibitory capacity of matched serum samples. As shown in Fig. 2, pretreatment with serum from patients or controls (human AB serum and FBS)

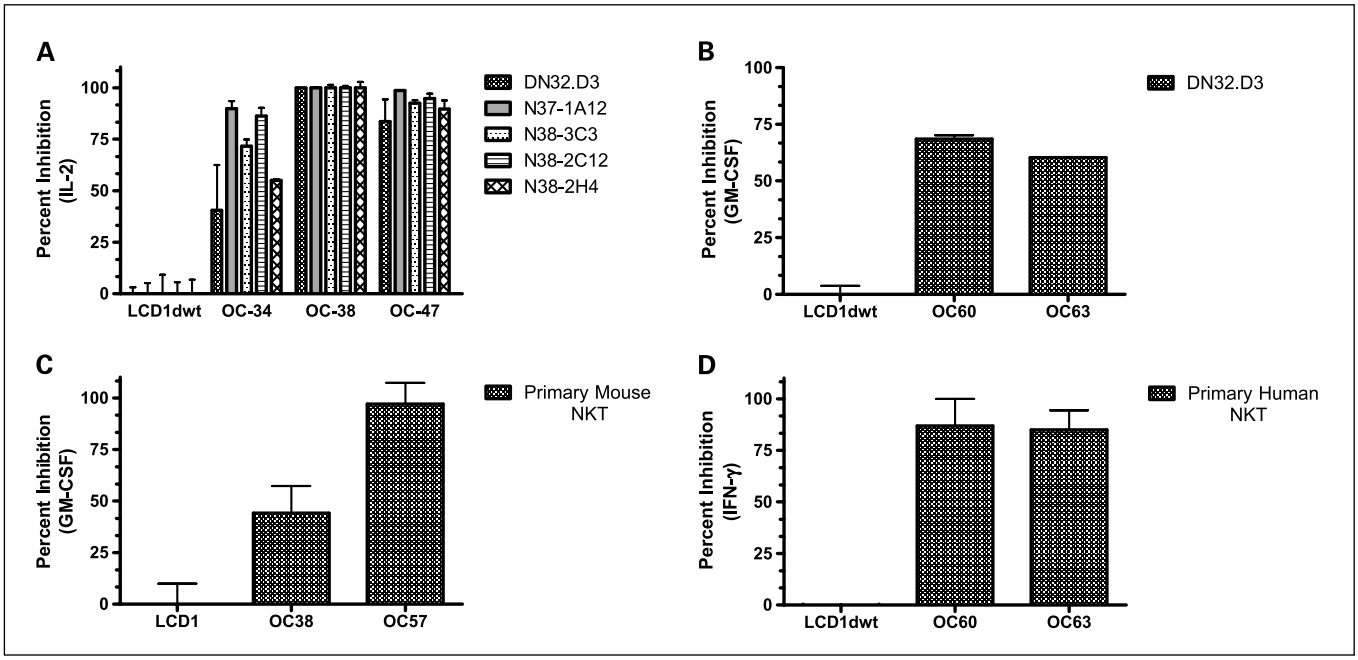

Fig. 1. Treatment with ascites from ovarian cancer patients inhibits CD1d-mediated antigen presentation. *A,* LCD1dwt cells were treated with media or ascites from the indicated patients for 4 h, washed extensively, and then cocultured with a panel of NKT cell hybridomas, DN32.D3, N37-1A12, N38-3C3, N38-2C12, and N38-2H4. IL-2 was measured by standard cytokine ELISA, and data are shown as percent inhibition, normalized to cells treated with culture media as 100% or the maximum level of stimulation. The results are representative of more than 15 experiments, in which samples from 3 to 12 patients were tested in each experiment and each sample repeated at least twice. *B,* the ascites-treated LCD1d1wt cells were cocultured with the $V\alpha14^+$ NKT cell hybridomas, DN32.D3, N38-2H4; primary mouse NKT cells (*C*); or primary human NKT cells (*D*). Recognition of CD1d was assessed by measuring cytokine production in the supernatants by ELISA. NKT cells were cocultured in media alone or with L cells transfected with vector alone as controls. Spontaneous cytokine release and background levels of cytokine production (as measured by coculturing NKT cells with L cells containing vector alone) were subtracted before calculating percent inhibition.

did not result in decreased stimulation/activation of NKT cells. Moreover, we found that serum pretreatment in some cases even augmented the ability of the APC to induce cytokine production from the NKT cells.

Tumor-associated ascites inhibits presentation of α-GalCer by aAPC. We developed bead-based CD1d-expressing aAPC (Fig. 3A). The system is based on directly coupling CD1d-immunoglobulin to magnetic beads and using them to stimulate the NKT cell hybridomas. Using this cell-free tool we can load CD1d-aAPC with α-GalCer, a potent stimulator of NKT cells, and determine if antigen processing or editing is necessary for the ascites-mediated inhibition. After establishing the system, the α-GalCer–loaded aAPC were treated with media or ascites for 4 hours, washed extensively, and cocultured with α-GalCer–specific NKT cells. Ascites treatment inhibited α-GalCer presentation by aAPC whereas mock treatment with media did not affect the aAPC-mediated NKT cell activation (Fig. 3B). Thus, these data show that antigen processing is not necessary for the ascites inhibition. Moreover, the inhibition is not a simply an issue of decreased cell viability.

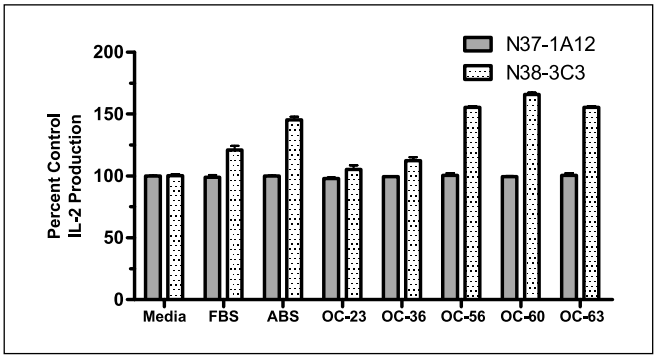

Fig. 2. Treatment with serum from ovarian cancer patient does not inhibit CD1d-mediated antigen presentation. LCD1dwt cells were treated with 1 mL media containing 5% FBS (*Media*), FBS, human AB serum (*ABS*), or serum from the indicated patients for 3 to 4 h, washed extensively, and then cocultured with N37-1A12 and N38-3C3 NKT cell hybridomas. IL-2 was measured by standard cytokine ELISA, and data are shown as percent control, normalized to cells treated with culture media as 100% or the maximum level of stimulation.

The inhibitory effect mediated by ascites treatment is CD1d specific. We next examined whether the immunosuppressive effect of ascites was limited to NKT cell activation or if treatment with ascites fluid also inhibited MHC class I–mediated activation of $CD8^+$ CTL. To address this question, we investigated whether ascites fluid could inhibit the activation of HLA-A2–restricted CMV-specific CTL. In this study, we used pretreated peptide-loaded target cells (T2) to stimulate CMV-specific $CD8^+$ T cells and measured IFN-γ release. It is possible that slight alterations in antigen presentation could be masked in the presence of strong stimuli. Therefore, to better monitor the effect of treating the target cells with ascites, we set up several effector to target cell ratios. As shown in Fig. 4A, ascites pretreatment did not inhibit activation of classic $CD8^+$ T cells at any ratio examined. In parallel, CD1d-expressing cells were treated with the same ascites samples and their ability to stimulate NKT cells was assessed (Fig. 4B). Whereas there was no effect observed in IFN-γ production by classic CMV-specific CTL, pretreatment with the same ascites samples resulted in an almost complete abrogation of cytokine production by NKT

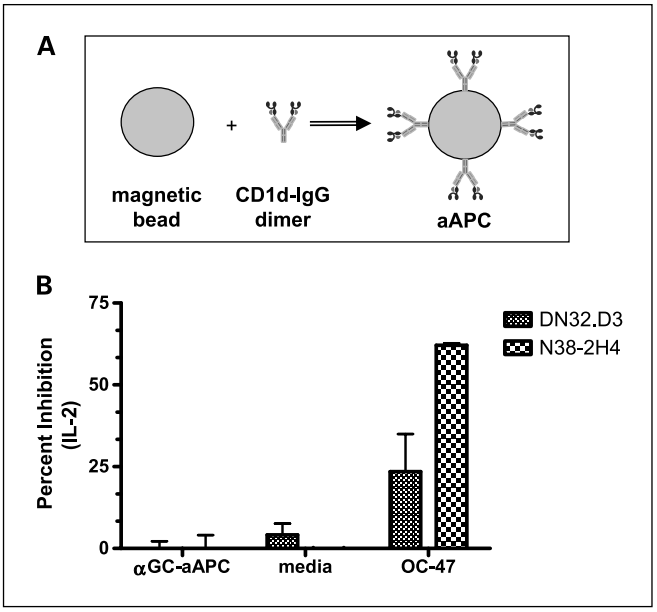

Fig. 3. *A,* generation of CD1d-based aAPC. The aAPC were made by coupling CD1d-immunoglobulin and anti-CD28 antibodies to magnetic beads. In this system, CD1d-immunoglobulin is used to provide the cognate antigen-specific signal through the T cell receptors. *B,* ascites treatment of α-GalCer – loaded aAPC inhibits antigen presentation by CD1d molecules. α-GalCer – loaded aAPC were incubated for 4 h with either media or ascites from patients (a representative patient [OC-47] is shown). The aAPC (1×10^5 cells per well) were washed extensively and then cocultured with the $V\alpha14^+$ NKT cell hybridomas, DN32.D3 (5×10^4) or N38-2H4 (1×10^5). NKT cell recognition of CD1d1 was assessed by measuring IL-2 production in the supernatants by ELISA.

cells. Taken together, these data show that inhibition of antigen presentation by tumor associated – ascites is CD1d specific.

Pretreatment with ascites results in a dose-dependent inhibition of NKT cell activation. Further analysis of the ability of ascites to mediate inhibition of antigen presentation by CD1d was necessary to characterize the effect. We diluted the ascites with cell culture media and treated the LCD1dwt stimulator cells for 4 hours, washed them extensively, and cocultured them with NKT cells as described above. As shown in Fig. 5A to D, increasing concentrations of ascites resulted in a concomitant reduction in NKT cell activation, as measured by cytokine release, in all NKT cell hybridomas examined. Even when the APC were treated with 25% ascites fluid or less, the blocking effect on NKT cell activation was still observed. Although all of the NKT cell lines are CD1d-restricted, they vary in their antigen specificity. Namely, the noncanonical NKT cell hybridoma N37-1A12 does not recognize α-GalCer in the context of CD1d molecules; its activation, however, is similarly reduced in a dose-dependent manner. Interestingly, the dose curves are markedly different for each cell line. In addition, the ascites was passed through 40-μm filters to further characterize its inhibitory effects. There was no change its ability to block CD1d-mediated activation of NKT cells (data not shown). Collectively, these data show that ovarian cancer – associated ascites treatment can block CD1d-mediated activation of NKT cells.

Discussion

In this study we have found that treatment of CD1d-expressing stimulator cells with ovarian cancer associated – ascites inhibited CD1d-mediated activation of NKT cells *in vitro*. This inhibition resulted in decreased production of multiple cytokines, IL-2, granulocyte macrophage colony-stimulating factor (in murine NKT cells; Fig. 1A-C) and IFN-γ (in primary human NKT cells; Fig. 1D). Interestingly, we found that this effect was limited to the ascites, as it was not found in the following treatment with serum-matched samples from the same patients (Fig. 2).

Ascites treatment of α-GalCer – loaded aAPC impaired their ability to activate NKT cells, which further suggests that the inhibition is independent of antigen processing (Fig. 3). Notably, in this study we used human CD1d-immunoglobulin dimers to generate aAPC. Thus, our study shows an inhibitory effect of ascites fluid on both mouse CD1d molecules (expressed on LCD1d1wt cells) and human CD1d molecules (immobilized on aAPC). Moreover, because α-GalCer has been well-characterized as a high-affinity ligand and potent stimulator of NKT cells, the level of inhibition observed following treatment with ovarian cancer associated – ascites suggests that the putative inhibitory ligand has a very high affinity for CD1d.

Other groups have implicated a role for the shedding of glycolipids as a method of tumor cell evasion from immune detection (33). Whereas inhibition of CD1d-mediated antigen presentation by a glycosphingolipid (gangliotriaosylceramide) shedding from a murine lymphoma has been previously reported (25), the inhibition mediated by gangliotriaosylceramide was specific to the invariant, canonical NKT cell

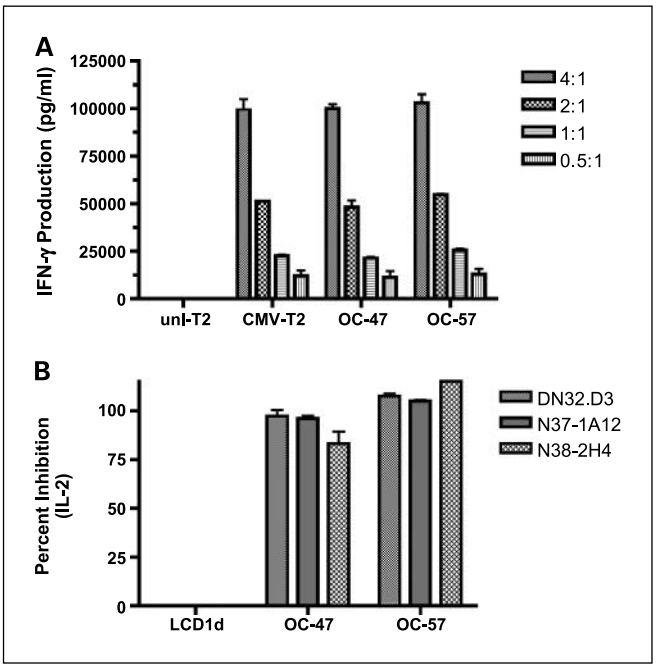

Fig. 4. Treatment with ovarian cancer associated ascites has no effect on HLA-A2-mediated antigen presentation. *A,* T2 cells were pulsed with CMV peptide, treated with media (CMV-T2) or ascites (OC47 and OC57) for 4 h, washed, and cocultured with CMV-specific $CD8^+$ T cells (1×10^5 cells per well) at the indicated ratios. IFN-γ released was measured by ELISA. *B,* LCD1d1wt cells were treated with ascites and then cocultured with the $V\alpha14^+$ NKT cell hybridomas, DN32.D3, N38-2H4, and the $V\alpha14^-$ NKT cell hybridoma, N37-1A12. NKT cell recognition of CD1d was assessed by measuring IL-2 production in the supernatants by ELISA. The results are representative of three experiments with similar results.

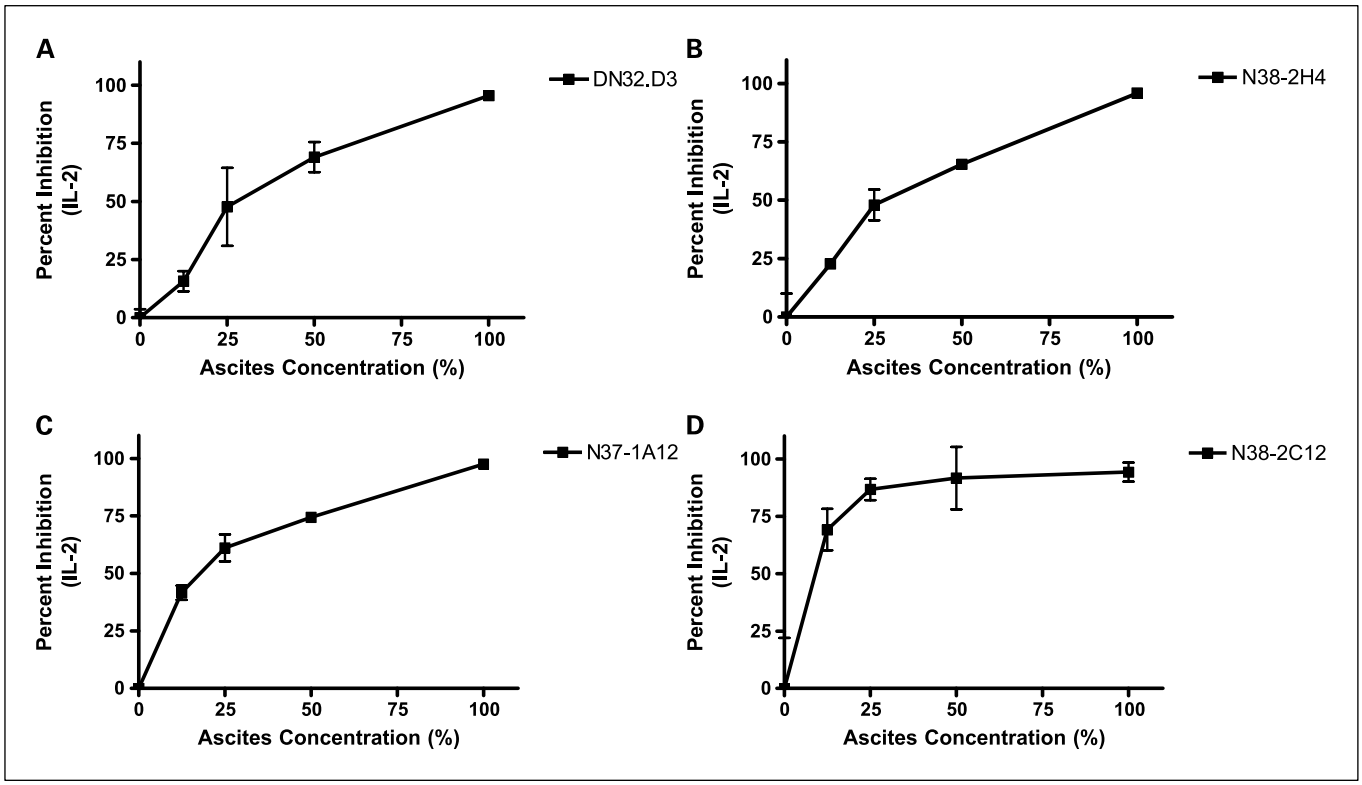

Fig. 5. NKT cell recognition of CD1d1 molecules is inhibited by treatment with ascites. LCD1d1wt cells were incubated for 4 h in the presence or absence of the indicated concentrations of ascites from patient OC-47. The cells were then cocultured with the Vα14$^+$ NKT cell hybridomas (*A*) DN32.D3 and (*B*) N38-2H4, and (*C*) Vα14$^-$ NKT cell hybridoma N37-1A12, and the Vα14$^+$ NKT cell hybridoma (*D*) N38-2C12. NKT cell recognition of CD1d1 was assessed by measuring IL-2 production in the supernatants by ELISA. The results are representative of two experiments with patient OC-47 ascites. Similar results were obtained when the experiment was done with ascites from another patient (OC-40).

population. In our study we have found dose-dependent inhibition of CD1d-mediated antigen presentation to both canonical and noncanonical NKT cells following pretreatment with ovarian tumor–associated ascites (Fig. 5A-D). Whereas CD1d molecules are known to traffic through endocytic compartments to present endogenous antigen to canonical NKT cells (34), presentation to noncanonical NKT cells (i.e., N37-1A12) is not dependent on antigen processing (35); this study suggests that treatment with ascites does not interfere with antigen processing, but rather may result in alterations in the expression of functional antigen-CD1d complexes on the cell surface. Our data using the α-GalCer–loaded aAPC (Fig. 3B) strongly support this hypothesis. Moreover, our findings in Fig. 5A to D are intriguing because the dose curves are markedly different for each NKT cell line examined. These data suggest that the discovery of natural ligands for NKT cells may reveal subtle differences in their antigen specificities that are not obvious with the potent agonist, α-GalCer.

Furthermore, the α-GalCer–loaded aAPC data (Fig. 3B), along with experiments showing that antigen presentation by HLA-A2 to classic T cells was not affected (Fig. 4), confirm that the reduction in antigen presentation is not due to the overall toxicity of the ascites or death of the treated stimulator cells. It is known that ovarian cancer patient ascites is rich in gangliosides (18), some of which have been previously shown to be presented by CD1d molecules (19, 20). We hypothesize that the mechanism of inhibition is the result of the binding of one or more gangliosides to the hydrophobic groove of CD1d, which blocks, covers, or competes with the binding of the endogenous ligand. This would explain the reduction in antigen presentation to all NKT cells examined (but not classic CTL) and account for the reversal of NKT cell activation using ascites-treated α-GalCer–loaded aAPC (Fig. 3B). It will be interesting to determine if ovarian cancer associated–ascites contains a specific family of gangliosides which mediate the inhibitory effects observed. Studies to identify the described inhibitory agent in the ascites are currently ongoing in the laboratory.

To our knowledge this is the first report showing that human tumor ascites specifically suppresses the CD1d/NKT system. Here, we present evidence which show that ascites, from cancer patients and possibly others, contain inhibitory substances (conceivably lipid antigens) that block CD1d-mediated activation of NKT cells. Thus, the presence of activated NKT cells may be a critical prognostic factor for ovarian cancer and more importantly, restoration of their function could be an effective therapeutic strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Joan Glick Bieler for critical reading of the manuscript, and Drew Bennett and Aaron Seyla for technical assistance.

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Clin Cancer Res 2008;14:7652-7658.