Enhanced Tumor Metastasis in Response to Blockade of the Chemokine Receptor CXCR6 Is Overcome by NKT Cell Activation

Robyn Cullen, Elitza Germanov, Takeshi Shimaoka and Brent Johnston

*J Immunol* 2009; 183:5807-5815; Prepublished online 7 October 2009; doi: 10.4049/jimmunol.0803520

http://www.jimmunol.org/content/183/9/5807

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/10/06/jimmunol.080352.0.DC1

**References**

This article cites 59 articles, 40 of which you can access for free at: http://www.jimmunol.org/content/183/9/5807.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Enhanced Tumor Metastasis in Response to Blockade of the Chemokine Receptor CXCR6 Is Overcome by NKT Cell Activation

Robyn Cullen, Elitza Germanov, Takeshi Shimaoka, and Brent Johnston

Invariant NKT (iNKT) cells can induce potent antitumor responses in vivo. However, the mechanisms that regulate the effects of iNKT cells are unclear. The chemokine receptor CXCR6, and its ligand CXCL16, have been shown to play critical roles in iNKT cell homeostasis and activation. Thus we investigated the role of CXCR6 in protection against experimental metastasis of B16-F10 melanoma (B16) and Lewis lung carcinoma (LLC) cells to the liver and lungs. Wild-type and CXCR6−/− mice exhibited no differences in tumor cell metastasis to the lungs. However, metastasis of LLC and B16 tumor cells to the liver was enhanced in CXCR6−/− mice. Liver metastasis was also increased in wild-type mice treated with a CXCL16 neutralizing Ab. As Ab treatments did not alter iNKT cell numbers, this implicates a direct role for CXCR6/CXCL16 in regulating antitumor immunity. Cytokine production, liver and lung metastasis were inhibited significantly in both wild-type and CXCR6−/− mice treated with glycolipids. Single doses of α-GalCer, α-C-GalCer, or OCH were sufficient to prevent liver metastasis and subsequent doses failed to elicit optimal cytokine responses. Our findings implicate a role for CXCR6 in natural immunosurveillance against liver metastasis. However, CXCR6 deficiency could be overcome by systemic iNKT cell activation, demonstrating that even suboptimal iNKT cell activation can protect against metastasis.

Received for publication October 20, 2008. Accepted for publication August 28, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by a grant from the Canadian Cancer Society (to B.J.) by the National Cancer Institute of Canada. B.J. holds the Canada Research Chair in Inflammation and Immunity, R.C. is the recipient of a Cancer Research Training Program Award with funding from the Dalhousie Cancer Research Program. E.G. is the recipient of a studentship from the Nova Scotia Health Research Foundation.

R.C. and E.G. contributed equally to this work.

Address correspondence and reprint requests to Dr. Brent Johnston, Department of Microbiology & Immunology, Dalhousie University, 5850 College Street, Halifax, Nova Scotia B3H 1X3, Canada. E-mail address: Brent.Johnston@Dal.ca

Abbreviations used in this paper: iNKT, invariant NKT cell; α-GalCer, α-galactosylceramide; DC, dendritic cell; LLC, Lewis lung carcinoma.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/852.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803520
CXCL16-deficient mice did not exhibit enhanced susceptibility to s.c. B16-F10 (B16) melanoma, but were unable to suppress development of the tumor mass upon stimulation with α-galactosylceramide (α-GalCer) (32). In contrast, the role of CXCR6 and CXCL16 in controlling metastasis is unknown. In this study, we examined the involvement of CXCR6 and CXCL16 in the control of B16 melanoma and Lewis lung carcinoma (LLC) tumor cell metastasis to the liver and lungs. Wild-type and CXCR6−/− mice were equally susceptible to tumor metastasis in the lungs. However, CXCR6−/− and CXCR6−/− mice were more susceptible to liver metastasis. It is possible that this resulted from the reduction of liver iNKT cells in these mice. However, wild-type mice treated with a neutralizing CXCL16 Ab exhibited increased susceptibility to metastasis in the absence of iNKT cell depletion, implicating a distinct functional role for CXCR6 and CXCL16 in tumor control. Despite the increased sensitivity of CXCR6−/− mice to tumor metastasis, we found that α-GalCer analogs mediated equivalent protection of wild-type and CXCR6−/− mice against B16 and LLC tumor metastasis. This demonstrates that systemic iNKT cell activation can overcome localized deficiencies in iNKT cell number and function to provide potent antitumor responses.

Materials and Methods

Mice

CXCR6 knockout (CXCR6−/−) mice containing an enhanced GFP replacement were obtained from Dr. D. Littman (New York University Medical Center, New York, NY) (34) and backcrossed on the C57BL/6 background for 10 generations. iNKT cell-deficient Jα18−/− mice were generated in the laboratory of Dr. M. Taniguchi (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan) (15). Additional C57BL/6 wild-type mice were purchased from The Jackson Laboratory. Mice were used in experiments at 8–12 wk of age. Animals were maintained in the Carleton Animal Care Facility at Dalhousie University. Experimental protocols were approved by the University Committee on Laboratory Animals in accordance with the guidelines of the Canadian Council on Animal Care.

Cell lines

B16 melanoma and LLC cells (American Type Culture Collection) were cultured at 37°C, 6% CO2 in DMEM (containing glucose and l-glutamine) supplemented with 1% penicillin/streptomycin and 10% FBS (Invitrogen). Cells were collected in the logarithmic growth phase for use in metastasis experiments.

Tumor metastasis

For experimental liver metastasis, B16 cells (1, 2.5, or 5 × 10⁴ in 50 μL of HBSS) or LLC cells (1 or 5 × 10⁵ in 50 μL of HBSS) were inoculated aseptically into the spleen through a small incision in the peritoneum (15, 35). For experimental lung metastasis, B16 cells (1 or 2.5 × 10⁵ in 50 μL of HBSS) were inoculated i.v. via the lateral tail vein (9, 15, 36). Mice were sacrificed on day 14 (some mice in the highest dose treatment groups were sacrificed as early as day 11 due to morbidity). Organs were weighed and tumor burden was assessed by counting surface tumor nodules and determining the area of tumor coverage with the aid of a dissecting microscope (model S6D; Leica Microsystems). Images were obtained using a Qimaging MicroPublisher 3.3 digital CCD camera and QCapture (v.2.8.1 software; Quorum Technologies). With the highest doses of tumor cells, individual tumor nodules could not be differentiated. A tumor coverage score was obtained by assessing the relative percentage of tumor coverage on both the anterior and posterior surfaces of the liver using Simple PCI image analysis software (Nikon). The liver weights of wild-type and CXCR6−/− mice were normalized to the liver weights of healthy age-matched controls. No differences were observed in the tumor metastasis of B16 and LLC in C57BL/6J mice and wild-type mice generated from the breeding of CXCR6−/− mice (data not shown).

Anti-CXCL16 treatment

To functionally block CXCL16-CXCR6 interactions, wild-type mice were treated i.p. daily with 200 μg of a neutralizing anti-CXCL16 Ab (33) or rat IgG isotype control (Sigma–Aldrich). Ab treatments were started one day before inoculation with 2.5 × 10⁵ B16 cells and continued for 11 days following tumor cell inoculation. Mice were sacrificed on day 12 and tumor burden was assessed in the liver.

Lymphocyte isolation and staining

Lymphocytes were isolated from the spleen, thymus, and bone marrow (femur and tibia) by mechanical dispersion through wire mesh, followed by erythrocyte lysis with ammonium chloride buffer. Liver lymphocytes were obtained by mechanical dispersion of the tissue through a wire mesh followed by centrifugation through a 35% Percoll gradient (GE Healthcare Bio-Sciences) and erythrocyte lysis. To examine the frequency and phenotype of iNKT cells, isolated cells were stained with allophycocyanin-labeled CD1d tetramers loaded with the α-GalCer analog PBS57 (National Institutes of Health Tetramer Core Facility, Emory Vaccine Center at Yerkes, Atlanta, GA), plus combinations of the following mAbs (BD Biosciences): PE-labeled anti-TCR-β (clone H57-597), FITC- or PerCP-labeled anti-CD4 (clone RM4-5), or PerCP-labeled anti-NK1.1 (clone PK136). Flow cytometry was performed using a two laser FACSCalibur with BD CellQuest Pro software (BD Biosciences). Isotype matched control Abs and unloaded CD1d tetramers were used to establish placement of gates and quadrants.

iNKT cell activation

To systematically activate iNKT cells, mice were treated on days 1, 5, and 9 following tumor inoculation with i.p. injections of 0.4 or 4.0 μg of α-GalCer ((2S,3S,4R)-1-O-(α-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol; Alexis Biochemicals), α-C-GalCer ((2S,3S,4R)-1-O-(α-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol; National Institutes of Health Tetramer Core Facility, Atlanta, GA), or OCH ((2S,3R,4S)-1-O-(α-D-galactopyranosyl)-2-tetraacetylamino-4,3,4-diol; National Institutes of Health Tetramer Core Facility, Atlanta, GA). Some groups of mice only received glycolipid treatments on day 1 following tumor inoculation. Control animals were treated with vehicle (0.9% saline containing 0.5% Tween 20). Mice were sacrificed after 14 days or when exhibiting signs of morbidity (as early as 11 days), and tumor burden was assessed.

Cytokine analysis

Blood was obtained from mice by submandibular venopuncture at 0, 2, 6, 24, and 48 h following i.p. administration of α-GalCer, α-C-GalCer, or OCH. Samples were centrifuged and serum was frozen at −20°C. Cytokine levels were measured in the serum samples using Flex Set Cytometric Bead Array assays for IL-2, IL-4, IL-12, IFN-γ, and TNF-α (BD Biosciences). Bead data was acquired using a two laser BD FACSCalibur cytometer and analyzed using FCAP Array (v.1.0.1 software; Soft Flow).

Statistical analysis

Data are expressed as mean ± SEM unless otherwise stated. A nonparametric two-tailed Mann-Whitney U test was used to compare between two data groups. Comparisons between more than two data groups were made using a Kruskal-Wallis nonparametric ANOVA with Dunn’s posttest. Statistical significance was set at p < 0.05.

Results

CXCR6 deficiency enhances tumor metastasis to the liver

CXCR6−/− mice are known to have reduced numbers of mature iNKT cells in the liver and lungs (30, 31), allowing us to test whether CXCR6 or normal iNKT cell localization are required to protect these tissues from tumor metastasis. B16 melanoma cells were inoculated into the spleens of wild-type and CXCR6−/− mice, leading to preferential liver metastasis via the portal circulation (35). Mice were sacrificed 14 days later and tumor burden in the liver was assessed by determining the number of tumor nodules, area of tumor coverage, and liver weights. Compared with wild-type mice, CXCR6−/− mice had 2- to 3-fold more tumor nodules in the liver when 1 or 2.5 × 10⁵ B16 tumor cells were inoculated into the spleen (Fig. 1A). Tumor nodule counts could not be reliably obtained for the 5 × 10⁵ dose of tumor cells due to difficulty in distinguishing individual tumor nodules. The surface area of the liver covered by B16 tumors paralleled the tumor nodule counts in a dose dependent manner and was significantly increased in CXCR6−/− mice at all doses of tumor cells tested.
The increased tumor burden in CXCR6⁻/⁻ mice was also reflected by a greater increase in liver weights in CXCR6⁻/⁻ vs wild-type animals inoculated with 2.5 or 5 × 10⁵ B16 tumor cells (Fig. 1C).

As CXCR6⁻/⁻ mice have an intermediate number of liver iNKT cells (31), we examined B16 metastasis in these animals to determine whether the degree of metastasis correlates with the number of iNKT cells. The level of tumor metastasis in CXCR6⁺/⁺ mice was intermediate between wild-type and CXCR6⁻/⁻ mice (Fig. 1D), suggesting that the number of iNKT cells could be an important factor in preventing the establishment of metastatic foci in the liver.

Crowe et al. (37) demonstrated that the CD4⁺ subset of liver iNKT cells is better than the CD4⁻ subset at controlling metastasis. To rule out a preferential depletion of this subset in CXCR6⁻/⁻ mice, we examined the ratio of CD4⁺ and CD4⁻ iNKT cells in wild-type and CXCR6⁻/⁻ mice (CXCR6⁺/⁺, 71.4 ± 2.6% CD4⁺ iNKT vs CXCR6⁻/⁻, 56.3 ± 4.5% CD4⁺ iNKT; p < 0.05). Although the total number of iNKT cells in the liver of CXCR6⁻/⁻ mice is reduced by 75–85% (31), there appears to be a greater depletion of CD4⁺ vs CD4⁻ iNKT cells. Therefore, the increased metastasis in CXCR6⁻/⁻ mice cannot be explained simply by a preferential depletion of CD4⁺ iNKT cells.

To establish that the enhanced metastasis observed in CXCR6⁻/⁻ mice occurs with other types of tumor cells, we also examined experimental liver metastasis with LLC cells. In comparison to wild-type mice, CXCR6⁻/⁻ mice displayed a 7-fold increase in the number of tumor nodules when inoculated with 1 × 10⁵ LLC tumor cells (Fig. 2A), and the surface area of the liver covered by LLC tumors was higher in CXCR6⁻/⁻ mice inoculated with 1 or 5 × 10⁵ LLC tumor cells (Fig. 2B). The increase in liver weight in CXCR6⁻/⁻ mice inoculated with 5 × 10⁵ LLC tumor
cells was significantly greater than the increase in wild-type mice (Fig. 2C). Collectively, these results implicate a role for CXCR6 sufficiency in the immunosurveillance and control of liver metastasis by different types of tumors.

**Neutralization of CXCL16 enhances tumor metastasis to the liver**

As CXCR6$^{-/-}$ mice exhibit a reduced number of iNKT cells in the liver and lungs (30, 31), it was unclear whether enhanced liver metastasis was due to the absence of CXCR6 function or a reduction in liver iNKT cells. We have shown previously that a neutralizing anti-CXCL16 Ab had little effect on the number of resident iNKT cells in the liver, only affecting recent thymic emigrants (31). Therefore, we treated wild-type mice with a CXCL16 neutralizing Ab or isotype control starting 1 day before inoculation with $2.5 \times 10^5$ B16 melanoma cells. Daily Ab treatments were continued until mice were sacrificed on day 12 to assess tumor burden in the liver. Mice treated with anti-CXCL16 Ab exhibited a 3-fold increase in tumor burden in the liver (increased number of tumor nodules and increased tumor coverage) compared with mice treated with the isotype control Ab (Fig. 3, A and B). However, liver weights of anti-CXCL16 treated mice did not increase significantly compared with their isotype treated counterparts (144.6 ± 37.6% vs 113.2 ± 8.2% of untreated control liver weight, $p = 0.065$). To confirm that Ab treatment did not deplete iNKT cells, we examined iNKT cell numbers after 12 days of treatment with anti-CXCL16 or isotype control Abs. The frequency and total number of iNKT cells in the liver of anti-CXCL16-treated mice was not significantly altered (Fig. 3, C and D). Similarly, the ratios of CD4$^+$ and NK1.1$^+$ iNKT cells were not altered by anti-CXCL16 treatments (Fig. 3C). No reductions were observed in the frequency or total number of iNKT cells in the spleen, thymus, and bone marrow (data not shown). These results implicate a functional role for CXCR6/CXCL16 in tumor immunosurveillance that is independent from the number of iNKT cells resident in the liver.

**CXCR6 deficiency does not alter tumor metastasis to the lungs**

To determine whether CXCR6$^{-/-}$ mice were more susceptible to lung metastasis, we induced experimental lung metastasis by injecting tumor cells via the tail vein (9, 15, 36). Mice were sacrificed 14 days later to assess tumor burden in the lungs. No significant differences in the number of tumor nodules or the area of tumor coverage were observed between wild-type and CXCR6$^{-/-}$ mice inoculated with 1 or $2.5 \times 10^5$ B16 tumor cells (Fig. 4, A and B). In contrast to the liver, these results demonstrate that CXCR6-deficiency and the resulting reduction in lung iNKT cells do not enhance susceptibility of the lung to tumor metastasis.

**NKT cell activation is defective in CXCR6$^{-/-}$ mice**

Previous studies have shown that experimental metastasis can be significantly reduced in mice by activating iNKT cells with the glycolipids $\alpha$-GalCer or $\alpha$-C-GalCer (9, 16, 17, 20, 21, 38, 39). This response is dependent on the production of IFN-$\gamma$ and IL-12 (9, 20, 21, 38). We examined serum cytokine levels in wild-type, CXCR6$^{-/-}$, and Jo18$^{-/-}$ (iNKT cell-deficient) mice following stimulation with the glycolipids $\alpha$-GalCer (induces Th1 and Th2 cytokines) (8, 10, 31, 38), $\alpha$-C-GalCer (induces a polarized Th1 response) (38, 39), and OCH (induces a polarized Th2 response) (40, 41). Consistent with literature reports, treatment of wild-type mice with $\alpha$-GalCer 1 day after B16 inoculation induced increases in serum levels of IL-2, IL-4, IL-13, IL-12, IFN-$\gamma$, and TNF-$\alpha$ (Fig. 5). In contrast, $\alpha$-C-GalCer induced IL-12 and high levels of IFN-$\gamma$, but failed to induce increases in the serum concentrations of other cytokines (Fig. 5). Treatment with OCH induced higher levels of IL-4 and IL-13, but lower levels of IFN-$\gamma$, consistent with a polarization toward Th2 (Fig. 5). In contrast to wild-type mice, CXCR6$^{-/-}$ mice exhibited reduced levels of most serum cytokines in response to $\alpha$-GalCer, $\alpha$-C-GalCer, and OCH, consistent with a role for CXCR6 in iNKT cell activation (31, 32). As IL-12 is produced largely by activated DCs, the reduction in serum IL-12 is consistent with a role for CXCR6 in cross-talk between iNKT cells and DCs. Serum cytokine levels did not increase in iNKT cell deficient Jo18$^{-/-}$ mice treated with $\alpha$-GalCer, $\alpha$-C-GalCer, or OCH (Fig. 5), confirming that the biological responses to these glycolipid Ags are dependent on the activation of iNKT cells. Similar to CXCR6$^{-/-}$ mice, serum cytokine responses to $\alpha$-GalCer were also impaired in...
Multiple doses of glycolipid Ags induce iNKT cell hyporesponsiveness

Although many studies have used multiple treatments with glycolipid Ags to prevent tumor metastasis (16, 21, 32, 37), other reports have demonstrated that iNKT cells become hyporesponsive to repeated stimulations with α-GalCer (9, 42–44). It is unclear whether hyporesponsiveness also occurs following α-C-GalCer and OCH treatments, and whether this would impact their anti-metastatic activities. In contrast to the cytokine burst elicited by the first glycolipid treatment 1 day after intrasplenic B16 injection (Fig. 5), we observed no increases in serum cytokines following subsequent treatments with α-GalCer or α-C-GalCer (Fig. 7). The second and third treatments with OCH induced low levels of IL-2, IL-4, and TNF-α, but no IFN-γ (Fig. 7), consistent with the ability of repeated doses to attenuate Th1/Th17-mediated autoimmune disease (40, 41). α-GalCer, α-C-GalCer, and OCH all induced hyporesponsiveness despite differences in their processing, stability, and affinity for CD1d (39, 45–47). However, IFN-γ responses appeared to be more sensitive to multiple glycolipid treatments as OCH still induced low levels of other cytokines. Consistent with these observations, single doses of glycolipid Ags given 1 day after tumor cell inoculation (Fig. 8) were as effective as multiple doses (Fig. 6) in preventing B16 tumor metastasis to the liver of wild-type and CXCR6−/− mice. The subsequent Th2 bias in animals treated with multiple doses of OCH (Fig. 7) did not alter the protection afforded by the initial OCH stimulation (Fig. 6 vs Fig. 8).

Discussion

iNKT cell activation has been shown to enhance clearance of tumor cells (9, 15–19, 21, 38, 39), whereas iNKT cell deficiency renders mice more susceptible to tumor development and metastasis (15, 18). Similarly, tumor burden in cancer patients has been associated with a reduced number of circulating iNKT cells or defects in iNKT cell function (48–52), consistent with a role for iNKT cells in antitumor immunity. Although relatively little is known about the mechanisms by which iNKT cells mediate natural tumor immunosurveillance, it is believed that iNKT cells can recognize endogenous glycolipids expressed by activated DCs or tumor derived glycolipid Ags (2, 18, 53, 54). In this study, we assessed whether tumor immunosurveillance by iNKT cells is impaired in CXCR6-deficient mice. Tumor metastasis to the liver, but not the lung, was increased in CXCR6−/− mice. However, this could be overcome by systemic iNKT cell activation with glycolipid Ags, despite defects in the cytokine responses mediated by CXCR6−/− iNKT cells.

We found that CXCR6−/− mice had enhanced susceptibility to experimental liver metastasis with two different tumor cell lines (Figs. 1 and 2), implicating a role for CXCR6 in natural antitumor immunity. However, we and others have shown that CXCR6 and CXCL16 mediate localization of iNKT cells to the liver and lungs under homeostatic conditions (30–32). Therefore, we reasoned that the defect in tumor clearance could be due to the reduction in iNKT cell accumulation. The decreased number of liver iNKT cells in CXCR6−/− mice translates into a 3-fold reduction in the “visititation” rate of iNKT cells to individual hepatocytes (30). As sinusoidal patrolling by iNKT cells likely contributes to tumor immunosurveillance, the reduction in iNKT cells in the sinusoids of CXCR6−/− mice may reduce the frequency of tumor cell recognition and clearance following intrasplenic inoculation of B16 or LLC cells. However, blockade of CXCL16 in wild-type animals also increased tumor metastasis. This treatment did not reduce the number of resident iNKT cells in the liver, suggesting that the interaction between CXCR6 and CXCL16 is indeed important for
the activation of functional iNKT cell tumor responses, and not just the accumulation of iNKT cells. As CXCL16 is expressed as a transmembrane protein on DCs, it is likely that CXCR6 and CXCL16 mediate a costimulatory interaction between iNKT cells and DCs. This is consistent with the impaired intracellular cytokine responses of CXCR6<sup>−/−</sup>iNKT cells stimulated in vivo with α-GalCer, α-C-GalCer, or OCH (4 µg/mouse). Serum cytokine levels were examined by cytometric bead array assay 0, 2, 6, 24, and 48 h after glycolipid administration. *, p < 0.05 compared with untreated (0 h). †, p < 0.05 compared with CXCR6<sup>+/+</sup> mice.

FIGURE 5. Serum cytokine levels following administration of glycolipid Ags. On day 0, wild-type (CXCR6<sup>+/+</sup>), CXCR6<sup>−/−</sup>, and Jα18<sup>−/−</sup> (iNKT cell-deficient) mice were inoculated in the spleen with 2.5 × 10<sup>5</sup> B16 melanoma cells. On day 1, mice received an i.p. injection of α-GalCer, α-C-GalCer, or OCH (4 µg/mouse). Serum cytokine levels were examined by cytometric bead array assay 0, 2, 6, 24, and 48 h after glycolipid administration. *, p < 0.05 compared with untreated (0 h). †, p < 0.05 compared with CXCR6<sup>+/+</sup> mice.

Interestingly, the role for CXCR6 in mediating protection from tumor metastasis extends to the liver but not the lungs (Fig. 4), despite our previous finding that mature iNKT cells are reduced in both the liver and lungs of CXCR6<sup>−/−</sup> mice (31). As there are significantly fewer iNKT cells in the lungs compared with the liver, the smaller iNKT cell population may not contribute as significantly to natural immunosurveillance. Alternatively, lung iNKT cells may not function as efficiently in antitumor immunity as iNKT cell subsets from the liver. Consistent with this hypothesis, Crowe et al. (37) demonstrated that liver iNKT cells were 2- to 3-fold more potent than spleen or thymic iNKT cells in mediating α-GalCer-dependent antitumor immunity. It is also possible that there could be differences in the amount of soluble vs transmembrane CXCL16 in the lung, which could modulate costimulation-dependent activation of lung iNKT cells. The ability of α-GalCer treatments to protect the lung from tumor metastasis is likely related to systemic
cytokine production rather than specific activation of lung iNKT cells. Consistent with this, α-GalCer treatment was equally effective in preventing tumor metastasis to the lungs of wild-type and CXCR6<sup>−/−</sup> mice (Fig. S3).<sup>5</sup>

Although iNKT cells express high levels of CXCR6 and play an important role in the early antitumor response, we cannot exclude a role for other CXCR6<sup>+</sup> cell types in longer term control of tumors. CXCR6 is expressed preferentially on activated and Th1 polarized lymphocytes (55, 56), and mediates migration of these cells in response to soluble CXCL16 (24, 34, 55). It is possible that CXCL16/CXCR6 signaling could be involved in polarizing Th1 cells to inflamed livers in a model of graft vs host disease (57). Similarly, CXCL16 Abs inhibited IFN-γ production from activated T cells in mice with experimental autoimmune encephalomyelitis (58), and IFN-γ responses were reduced in CXCL16<sup>−/−</sup> mice following a secondary stimulation with Propionibacterium acnes (32). These findings support the notion that CXCL16/CXCR6 signaling could be involved in polarizing Th1 antitumor responses or mediating homing of effector cells.

α-GalCer-mediated activation is associated with increases in serum IFN-γ and IL-12, which are critical for tumor clearance (9, 20, 21, 38). As iNKT cell activation and cytokine responses are impaired in CXCR6<sup>−/−</sup> mice (31), we hypothesized that glycolipid Ags would be less effective in preventing tumor metastasis in these animals. However, treatment of CXCR6<sup>−/−</sup> mice with α-GalCer, α-C-GalCer, or OCH resulted in a significant reduction in tumor burden, indistinguishable from the protection observed in wild-type mice (Fig. 6). One possibility is that systemic activation of iNKT cells in CXCR6<sup>−/−</sup> mice induces effector responses that exceed a minimum threshold required to prevent tumor establishment. Consistent with this notion, the blunted cytokine responses in CXCR6<sup>−/−</sup> mice were significantly increased compared with αGalCer (Fig. 5). Despite the reduction in liver and lung iNKT cells, iNKT cell numbers are normal or increased in other tissues of CXCR6<sup>−/−</sup> mice (31). Therefore, there is an available pool of iNKT cells that can respond to systemic activation signals, albeit at suboptimal levels. These results reveal that the level of cytokines generated by iNKT cell activation in wild-type mice is in excess of what is required to mediate protection from tumor metastasis. This suggests that reduced activity of iNKT cells in cancer patients should not preclude the development of therapies targeting function of these cells.

Compared with α-GalCer, the C-glycoside α-C-GalCer has been shown to elicit a more potent antitumor response against B16 lung metastasis (38). Our study is the first to examine α-C-GalCer in liver metastasis, and we observed similar tumor inhibition with 0.4- and 4.0-μg doses of α-C-GalCer (Fig. 6 and supplemental Fig. S2). Surprisingly, OCH at both doses was also protective against...
FIGURE 8. Reduction in tumor metastasis by a single dose of glycolipid Ag, CXCR6+/−, CXCR6−/−, and Jα18−/− (iNKT cell deficient) mice were inoculated with 2.5 × 10^6 B16 tumor cells. α-Ga-Cer, α-Ca-GalCer, OCH, or saline control were administered i.p. (4 μg/mouse) on day 1 after tumor inoculation. Metastasis to the liver (percentage of tumor coverage) was examined at day 14 (n = 5–7 per group). *p < 0.05 compared with wild-type mice. †p < 0.05 compared with saline control.

Acknowledgments

We thank Erin Chamberlain for excellent technical assistance with the cytokine arrays and tumor harvesting. We also thank Dr. Limnea Veinotie for helpful comments on the manuscript.

Disclosures

The authors have no financial conflict of interest.

References


26. Lee, P. T., K. Benlagha, L. Teyton, and A. Bendelac. 2002. Distinct functional subsets are generated in OCH-treated mice was similar to CXCR6+/− mice. That a low level of IFN-γ in CXCR6+/H11002 as iNKT-derived IL-13 has been implicated previously in the effects of glycolipids that induce a more complete Th2 polarization may not be present in sufficient numbers to provide efficient immunosurveillance. However, unstimulated iNKT cells restricted. These data suggest that the reduced activity of stimulated iNKT cells in CXCR6+/H11002 may be suboptimal activation of CXCR6+/− iNKT cells by exogenous CXCR6 cell-activating glycolipids, these compounds provided effective protection from tumor metastasis. These results suggest that a threshold level of iNKT cell activity is required and sufficient to prevent tumor metastasis.


