Vα24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages

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Tumor infiltration with Vα24-invariant NKT cells (NKTs) associates with favorable outcome in neuroblastoma and other cancers. Although NKTs can be directly cytotoxic against CD1d$^+$ cells, the majority of human tumors are CD1d$^-$. Therefore, the role of NKTs in cancer remains largely unknown. Here, we demonstrate that CD68$^+$ tumor-associated monocytes/macrophages (TAMs) represented the majority of CD1d-expressing cells in primary human neuroblastomas. TAMs stimulated neuroblastoma growth in human cell lines and their xenografts in NOD/SCID mice via IL-6 production. Indeed, TAMs produced IL-6 in primary tumors and in the BM of patients with metastatic neuroblastoma. Gene expression analysis using TaqMan low-density arrays of 129 primary human neuroblastomas without *MYCN* amplification revealed that high-level expression of TAM-specific genes (*CD14*, *CD16*, *IL6*, *IL6R*, and *TGFB1*) was associated with poor 5-year event-free survival. While NKTs were not cytotoxic against neuroblastoma cells, they effectively killed monocytes pulsed with tumor cell lysate. The killing of monocytes was CD1d restricted because it was inhibited by a CD1d-specific mAb. Cotransfer of human monocytes and NKTs to tumor-bearing NOD/SCID mice decreased monocyte number at the tumor site and suppressed tumor growth compared with mice transferred with monocytes alone. Thus, killing of TAMs reveals what we believe to be a novel mechanism of NKT antitumor activity that relates to the disease outcome.

Introduction

Vα24-invariant NKT cells (NKTs) are an evolutionarily conserved sub-lineage of T cells characterized by reactivity to self- and microbial-derived glycolipids presented by a monomorphic HLA class-I–like molecule, CD1d. NKTs may serve as an early source of cytokines that provide initial signals for other cells of the immune system to initiate innate and adaptive responses (1, 2). The antitumor potential of NKTs has been demonstrated in numerous models of cancer (3, 4). Selective decrease of NKT number and/or functional activity has been reported in patients with diverse types of cancer (5–7), and a recent study demonstrated that low levels of circulating NKTs predicted a poor clinical outcome in patients with head and neck squamous cell carcinoma (8). We previously demonstrated that NKTs infiltrated primary tumors in a subset of patients with stage 4 neuroblastomas without *MYCN* amplification, which are known to have a better prognosis than *MYCN*-amplified tumors (9, 10). The phenomenon was then extended to colorectal cancers, in which NKT infiltration in primary tumors served as an independent factor of favorable outcome (11). These findings suggest that NKTs may exert an antitumor activity at the tumor site via a yet-unknown mechanism.

Neuroblastoma is a biologically heterogeneous tumor of neural crest origin that represents the second most common solid tumor in children (12). Of the 45% of patients have high-risk disease at diagnosis, only 40% survive despite intensive myeloablative chemotherapy, BM transplantation, and retinoic acid therapy (13). About one-third of high-risk neuroblastomas have genomic amplification of the *MYCN* oncogene (14), which activates transcription of genes involved in cell cycle progression (15) and represses those that induce cell differentiation (16). We recently reported that *MYCN*-amplified tumors lack NKTs because of *MYCN*-mediated transcriptional repression of *CCL2* (17), a chemokine that is required for NKT migration to neuroblastoma (9). However, two-thirds of metastatic, high-risk neuroblastomas lack *MYCN* amplification, and the sources of growth promotion in these tumors are not well defined.

Neuroblastoma cells (like the majority of examined human solid tumors) are CD1d$^-$ and therefore cannot be a direct target for NKT cytotoxicity (18). A number of studies in mouse models demonstrated that NKTs mediate tumor immunosurveillance (3) and anti-metastatic activity of α-galactosylceramide (αGalCer) indirectly via activation of NK cell cytotoxicity (18–21). Consistent with the observations in mice, we previously reported that human NKTs, upon antigen recognition, could activate NK cell cytotoxicity against neuroblastoma cells (18). However, it is well established that NK cells poorly localize to the established tumors in humans (22) and have been found at extremely low levels in primary neuroblastomas (23). This suggests that, although NK cells may cooperate with NKTs to control the tumor initiation or the metastatic process, they are not likely to mediate NKT function in the established tumor.

An alternative target for NKTs in the established tumor could be CD1d$^+$ cells of the tumor stroma. We recently demonstrated

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: EFS, event-free survival; αGalCer, α-galactosylceramide; NKT, Vα24-invariant NKT cell; TAM, tumor-associated monocyte/macrophage; TLDA, TaqMan low density array.

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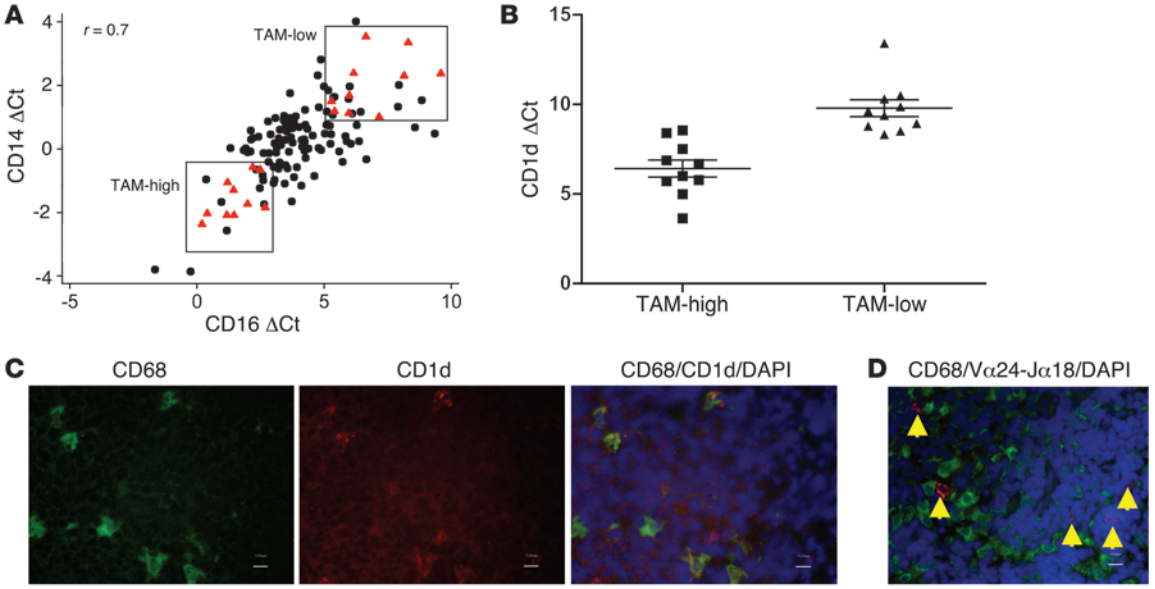

Figure 1
TAMs express CD1d in primary neuroblastoma. (**A**) RNA expression of monocyte/macrophage markers was quantified in primary stage 4 *MYCN* non-amplified neuroblastomas (n = 129) as a part of multi-gene TLDA analysis and plotted as ΔCt values. Samples with the highest and lowest levels of both gene expressions (n = 10 per group, triangles) were labeled as TAM-high (low Ct values) and TAM-low (high Ct values) and used in **B** for quantification of CD1d RNA expression by TaqMan RT-PCR. Data are mean ± SD; $P < 0.001$, by unpaired t test. (**C**) Frozen 8-μm sections were stained with antibodies against CD68 (green) and CD1d (red) and with DAPI (blue) for nucleated cells. Digital images of a microscopic field of tumor tissue (×40 magnification; scale bars: 10 μm) are representative of 5 TAM-high tumors (4–6 fields per tumor). (**D**) Staining was performed on sections from the same tissues and in the same manner as described in **C**, except that anti-CD1d was replaced with anti-NKT TCR 6B11 mAb. Yellow arrows indicate the locations of NKTs.

that monocytes were the only CD1d$^+$ subset of human leukocytes that colocalized with NKTs to neuroblastoma xenografts in NOD/SCID mice (17), suggesting that NKTs may interact with monocytes and their progenies in the tumor microenvironment. The accumulating evidence indicates that tumor-associated monocytes/macrophages (TAMs) contribute to tumor progression in various types of cancer (24–26). However, the role of TAMs in neuroblastoma has not been determined. In this study, we investigated the role of TAMs in neuroblastoma progression and tested a hypothesis that these cells could be a direct target for NKTs. We demonstrate that CD1d$^+$ TAMs promote neuroblastoma growth via IL-6 production and that expression of monocyte/macrophage markers, CD14/CD16, and IL-6 or IL-6R inversely correlates with long-term disease-free survival in patients with stage 4 *MYCN*–non-amplified neuroblastoma. Furthermore, TAMs cross-present neuroblastoma-derived endogenous CD1d ligand(s) and can be specifically recognized and killed by NKTs that may explain the association of NKT infiltration with favorable outcome in neuroblastoma and other types of cancer.

Results

TAMs are the dominant CD1d$^+$ subset in neuroblastoma. Since NKTs are strictly CD1d restricted and neuroblastoma cells do not express CD1d, we hypothesized that function of tumor-infiltrating NKTs may depend on CD1d$^+$ cells of the tumor stroma. Because monocytes express functional CD1d (27) and co-migrate with NKTs to neuroblastoma-derived CCL2 in *MYCN* non-amplified tumors (17), we examined whether CD1d expression in primary stage 4 *MYCN* non-amplified neuroblastomas correlated with monocyte/macrophage markers CD14 and CD16. TaqMan RT-PCR revealed a strong correlation between CD14 and CD16 (r = 0.7, $P < 0.001$; Figure 1A), and CD1d expression was significantly higher in CD14^{hi}CD16^{hi} (TAM-high) compared with CD14^{lo}CD16^{lo} (TAM-low) tumors ($P < 0.001$, by t test; Figure 1B). To directly examine whether TAMs express CD1d, we stained primary tumors for CD1d and a pan-macrophage marker, CD68. The representative immunofluorescent images in Figure 1C demonstrate that CD68$^+$ TAMs indeed expressed CD1d, albeit at a low level. The low level of CD1d expression on TAMs is consistent with the previously observed low levels of CD1d expression on monocyte lineage cells that nevertheless was associated with potent antigen-presenting function (27). Finally, by costaining the same tumor tissues for CD68 and the Vα24Jα18-invariant α-chain of NKT TCR, we found that NKTs were often in direct contact with or close proximity of TAMs (Figure 1D). Therefore, monocytes/macrophages are the dominant cell population that can potentially be recognized by NKTs at the tumor site.

Monocytes promote neuroblastoma growth via IL-6 production. Multiple studies have found that monocytes and their progeny can play a dual role in tumor immunity (25). However, the role of these cells in neuroblastoma and other pediatric cancers has not been defined. To test whether monocytes have a potential to directly affect neuroblastoma cell survival or proliferation, we cocultured CHLA-255 neuroblastoma cells with freshly isolated (by negative magnetic separation) peripheral blood monocytes. Figure 2A shows that monocytes (CD45^{hi}) stimulated proliferation of CHLA-

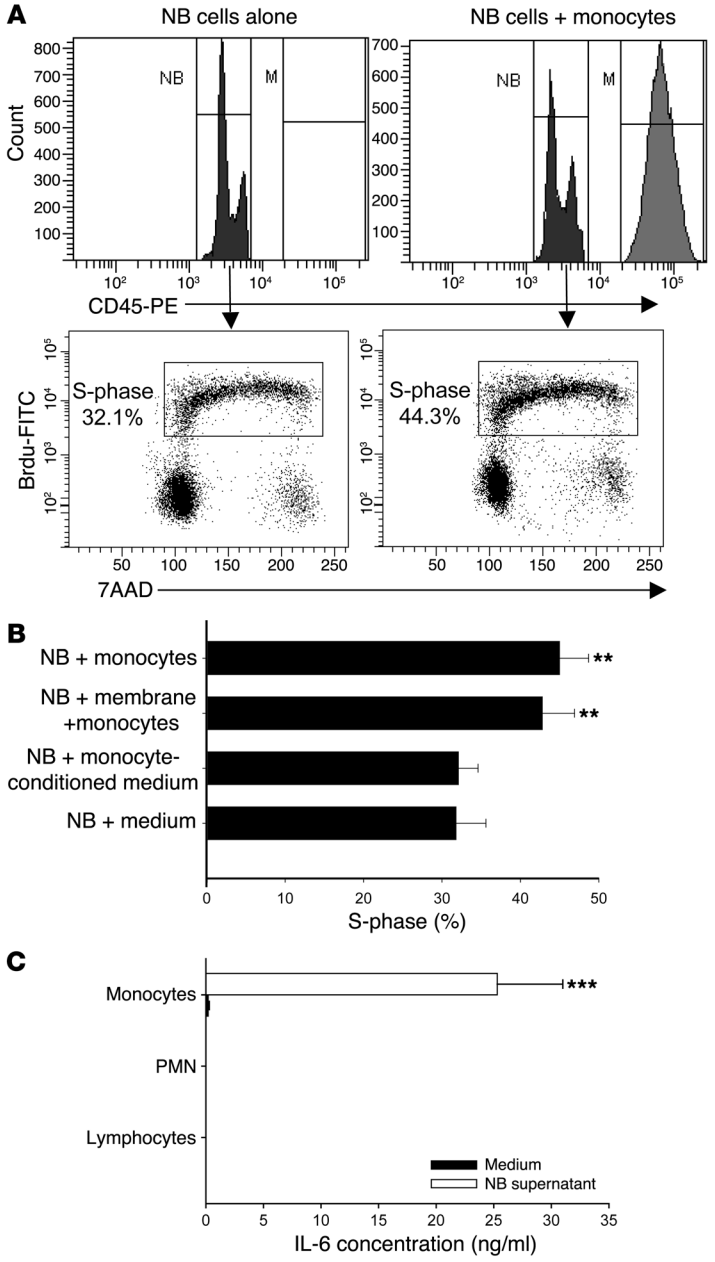

Figure 2

Monocytes promote neuroblastoma growth via IL-6 production. (**A**) CHLA-255 neuroblastoma cells (NB) were cultured alone (left) or mixed with monocytes (1:1 ratio; right) for 24 hours. For the last 40 minutes of culture, cells were pulsed with BrdU and, after fixation and permeabilization, were stained with FITC-conjugated anti-BrdU, 7AAD, and PE-conjugated anti-CD45. Neuroblastoma cells were identified by gating on $CD45^{lo}$ events (top panels), followed by analysis of cell cycle distribution in the gated population (bottom panels). (**B**) The S-phase percentage was quantified in the CHLA-255 cells described in **A** after culture in the conditions indicated on *y* axis and compared with neuroblastoma cells plus medium. Results are mean ± SD from 4 experiments. $^{**}P < 0.01$, by unpaired *t* test. (**C**) PBMCs were separated from polymorphonuclear cells (PMNs) by gradient centrifugation of blood leukocytes. Monocytes were isolated from PBMCs by negative selection, and lymphocytes were isolated from PBMCs by monocyte depletion, using the Dynal Monocyte Negative Isolation kit. Cells (50,000 per well) were cultured in non-adherent 96-well plates for 24 hours in the presence of CHLA-255 cell–conditioned or medium control. The concentration of IL-6 in the supernatants was quantified by CBAPlex. Results are mean ± SD from 8 separate experiments using leukocytes from different healthy individuals. $^{***}P < 0.001$ compared with medium, by unpaired *t* test.

255 neuroblastoma cells ($CD45^{lo}$) after 24 hours of coculture, as shown by a 12.2% increase of BrdU incorporation in neuroblastoma cells ($P = 0.01$). To examine whether this effect was contact dependent, we separated neuroblastoma cells and monocytes by a 0.4-μm-pore semi-permeable membrane in dual-chamber plates. The separation did not significantly decrease the growth-promoting effect of monocytes, although conditioned medium from monocytes alone failed to promote neuroblastoma cell proliferation (Figure 2B), indicating that the effect was mediated by soluble factors derived from both tumor cells and monocytes. Two studies recently demonstrated that galectin-3-binding protein (G3BP), secreted by neuroblastoma cells, induced IL-6 production in BM stromal cells (28), which in turn stimulated bone invasion of neuroblastoma cells (29). Since human monocytes/macrophages are known to express functional galectin-3, the receptor for G3BP (30), we examined whether monocytes can also be induced to produce IL-6 by neuroblastoma cell–conditioned medium. Indeed, Figure 2C shows that a supernatant from CHLA-255 (as well as SKN-BE2, data not shown) cells selectively induced IL-6 production in monocytes, but not in other leukocyte subsets. This activity was blocked by 70% after G3BP immunodepletion and restored by rhG3BP (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI37869DS1).

Next, we examined whether IL-6 is required for the growth-promoting effect of monocytes in coculture with neuroblastoma cells. Figure 3A demonstrates that the addition of a neutralizing anti–IL-6 mAb reduced by half the growth-promoting effect of monocytes on CHLA-255 cells, while rhIL-6 alone increased the tumor cell S-phase from 30.2% ± 2.7% to 39.7% ± 3.1% ($P = 0.02$). Finally, we used a subcutaneous neuroblastoma model in NOD/SCID mice to examine whether monocytes enhance neuroblastoma growth in vivo. Luciferase-transduced CHLA-255 (CHLA-255/luc) neuroblastoma cells were injected alone (in the left flank) or with freshly isolated human monocytes at 1:1 ratio (in the right flank). The tumor growth was monitored by bioluminescent imaging. As shown on Figure 3B, monocyte-containing xenografts grew significantly faster, resulting in a 4-fold increase of the luminescent signal by day 28 compared with the xenografts containing tumor cells alone ($P < 0.001$). Importantly, the tumor-promoting effect of monocytes was blocked in half by twice weekly injections of an anti–IL-6–neutralizing mAb ($P < 0.001$). Conversely, co-injection of CHLA-255/luc cells with IL-6–transduced CHLA-255/IL-6 as a paracrine source of IL-6 resulted in 2-fold increased luminescence compared with CHLA-255/luc alone ($P < 0.001$). Thus, we determined that monocytes promote the growth of neuroblastoma cells in vitro and in vivo. This effect at least in part depends on IL-6, which is produced by monocytes in response to neuroblastoma-derived G3BP and possibly other soluble factor(s).

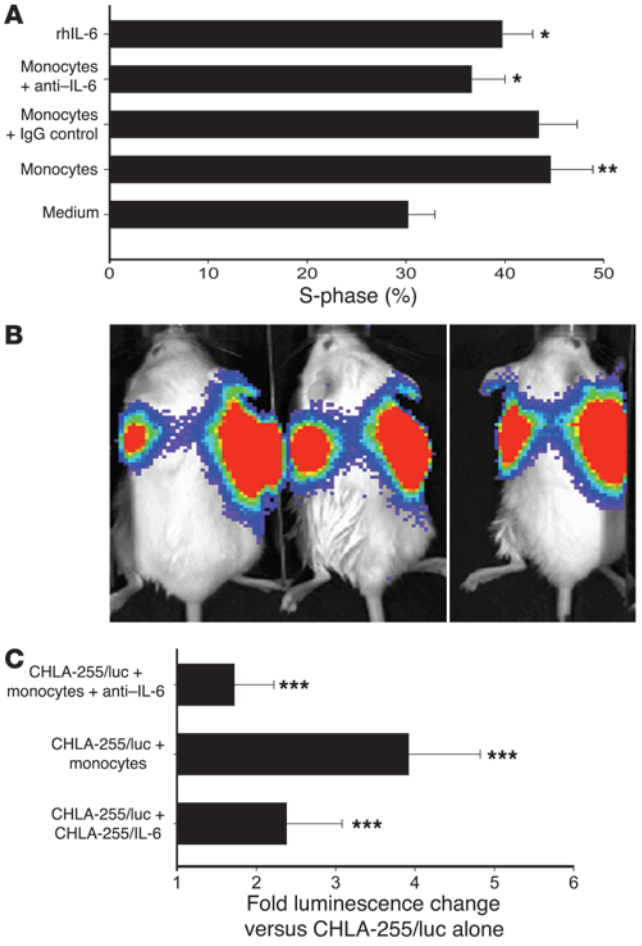

Figure 3
IL-6 is required for the tumor growth–promoting effect of monocytes. (**A**) CHLA-255 neuroblastoma cells were cocultured with freshly isolated monocytes alone or in the presence of anti–IL-6–neutralizing or isotype control mAb. In the absence of monocytes, tumor cells were cultured with medium alone or with rhIL-6 (10 ng/ml) as negative and positive controls, respectively. The rate of S-phase was measured by FACS (BrdU incorporation), as in Figure 2A. Data are mean ± SD from 3 experiments. (**B**) Luciferase-transduced CHLA-255/luc neuroblastoma cells were subcutaneously injected alone or with freshly isolated monocytes in the left or right flanks of NOD/SCID mice. Shown are bioluminescent images (day 28 after tumor implantation) of 3 representative mice (10 mice per group, 2 experiments). Bioluminescence intensity was proportional to the tumor size and increased on the color scale from blue to red. (**C**) As described in **B**, the group of mice receiving CHLA-255/luc plus monocytes in the right flank were compared with 2 other groups in the same experiments (10 mice per group): one group received the same injections of CHLA-255/luc and monocytes and was treated with anti–hIL-6–neutralizing mAb (monocytes + anti–IL-6); the other group was co-injected with IL-6–transduced CHLA-255/IL-6 and CHLA-255/luc cells in the right flank. The tumor size in the right flanks was expressed as fold increase compared with the left flank (CHLA-255/luc alone) of each mouse. Results are mean ± SD from 2 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, by 1-way ANOVA.

TAMs produce IL-6, and expression of TAM genes in primary tumors is associated with poor outcome. Our data suggested that, through IL-6 production, tumor-infiltrating monocytes/macrophages could promote tumor growth and contribute to the aggressive clinical behavior in neuroblastoma patients. To examine whether TAMs indeed produce IL-6 at the tumor site, we stained untreated primary tumors from patients with metastatic neuroblastoma for CD68 and IL-6. Figure 4A demonstrates that TAMs represented the main source of IL-6 in these tumors. Next, we performed expression analyses of CD14, CD16, IL-6, IL-6Rα (p80), and other immune-related genes (Table 1) in primary tumor specimens from 129 untreated patients with stage 4 *MYCN* non-amplified neuroblastomas using TaqMan low-density arrays (TLDAs). The first principal component of expression of CD14, CD16, IL6 IL6R, and TGF-β1 was significantly associated with event-free survival (EFS; $P = 0.001$, by Cox regression). Five-year EFS was 60% ± 6.1% and 27% ± 5.7% for patients with expression values below and above the median, respectively. Figure 4B demonstrates that higher expression of each of these 5 genes individually was significantly associated with poor outcome. Notably, TGF-β can be produced by both tumor cells and TAMs and is known to induce or upregulate CD16 expression in human monocytes or macrophages (31, 32). Consistent with those findings, we found a correlation between TGF-β and CD16 in primary neuroblastomas ($r = 0.58$, $P < 0.001$; data not shown). IL-10, another immune-inhibitory cytokine, which is typically expressed in M2 macrophages and TAMs (25, 33), showed a strong trend toward association with poor outcome ($P = 0.064$).

Table 1 shows the results of expression analysis of other genes that represented major effector and regulatory elements of the tumor immunity. The previously reported association of NKT Vα24-invariant TCR expression with increased 5-year EFS in 98 primary neuroblastomas (9) was evident in a smaller subset of *MYCN* non-amplified tumors (the same subjects as in Figure 1B) when controlled for CD1d ($P < 0.035$, $n = 20$, by Cox regression permutation test). In contrast, markers for other T cells and their subsets (CD3, CD8, CD4, FOXP3, IFN-γ, IL-13, and IL-17) did not associate with the outcome ($P > 0.05$; Table 1). Thus, high expression of multiple genes associated with monocytes/macrophages in primary *MYCN* non-amplified neuroblastomas correlates with a poor clinical outcome, whereas high expression of the invariant TCR of NKTs correlates with a good outcome.

Myelomonocytic cells are a major source of IL-6 in metastatic BM. BM, the place of origin for monocytes and other myelomonocytic TAM precursors (34), is the most common site of metastasis and relapse in neuroblastoma and many other types of cancer (35). Myelomonocytic cells are known to be the dominant subset of $CD1d^{+}$ cells in human BM (36), and we recently reported that NKTs preferentially localize to metastatic BM in patients with *MYCN* non-amplified neuroblastoma (17). Here, we examined whether monocytes and/or their precursors contribute to IL-6 production in primary metastatic BM. Freshly isolated mononuclear cells from primary BM specimens were cultured for 4 hours in the presence of brefeldin A to allow intracellular accumulation of produced cytokines followed by multicolor flow cytometry analysis to identify the following subpopulations: $CD45^{-}GD2^{+}CD56^{+}$ (neuroblastoma cells), $CD45^{+}CD33^{+}CD14^{+}$ (myelomonocytic cells), and $IL\text{-}6^{+}$ (IL-6–producing cells). Figure 5A demonstrates that less than 0.5% of $CD45^{+}$ cells from patients without detectable BM metastases produced IL-6. In contrast, in patients with 10% or more neuroblastoma cells in BM, IL-6 producers constituted 3.4% ± 2.2% of $CD45^{+}$ cells ($P < 0.01$). Interestingly, 95%–99% of $IL\text{-}6^{+}$ cells belonged to the $CD45^{+}CD33^{+}CD14^{+}$ myelomonocytic

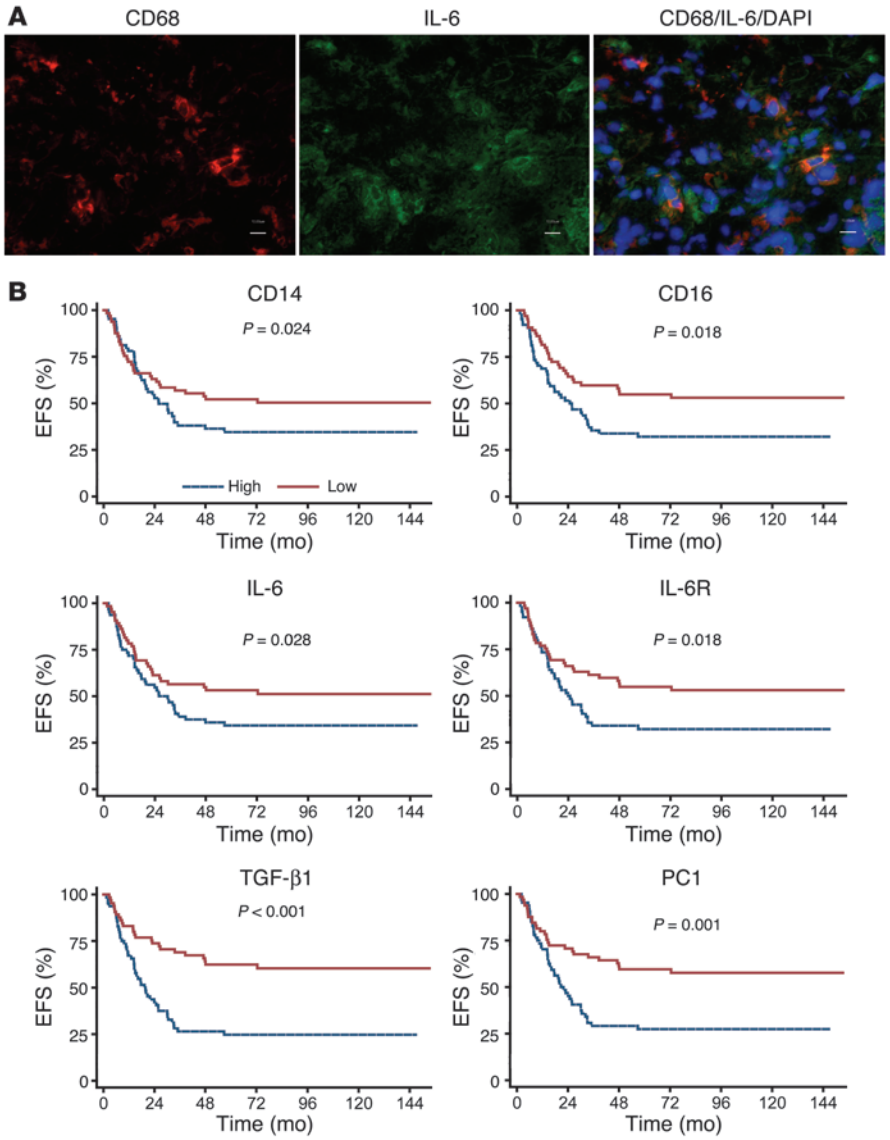

Figure 4
TAMs produce IL-6, and expression of TAM genes in primary tumors associates with poor outcome. (**A**) Frozen 8-μm sections were stained with antibodies against CD68 (red) and IL-6 (green) and with DAPI for nucleated cells (blue). Digital images of a microscopic field of tumor tissue (magnification, ×40; scale bars: 10 μm) are representative of 5 analyzed TAM-high tumors, as described in Figure 1 (4–6 fields per tumor). (**B**) Cox regression analysis on ranked expression values was performed to investigate the association between TAM-associated gene expression and EFS in 129 patients with *MYCN* non-amplified neuroblastoma. Plots show Kaplan-Meier survival curves for the indicated genes and the first principal component of these genes when subjects were divided by gene expression, as determined by TaqMan PCR, above and below the median values. *P* values are from the Cox regression analysis (Table 1).

population regardless of the tumor cell presence in BM (Figure 5A). When gated on the $CD33^+CD14^+$ subset of BM cells, only 4.9% ± 2.7% produced IL-6 in tumor-free BM, whereas 48.8% ± 8.3% ($P < 0.001$) of the cells were $IL\text{-}6^+$ in metastatic BM (Figure 5A).

In addition to intracellular staining, we quantified IL-6 production from primary BM mononuclear cells after overnight culture in ultra-low attachment plates by CBAPlex assay. Figure 5B demonstrates that, on average, mononuclear cells from BM with metastases produced nearly 10-fold more IL-6 compared with those from BM without metastases ($P < 0.001$). Furthermore, overnight incubation with CHLA-255 neuroblastoma cell–conditioned medium induced IL-6 production in the mononuclear cells from metastasis-free BM (Figure 5B). Thus, metastatic neuroblastoma cells in BM create a microenvironment enriched by myelomonocytic cells, which are induced to secrete large amounts of IL-6, a cytokine that promotes neuroblastoma growth and has been linked to bone invasion in neuroblastoma (29) and other types of cancer (37).

NKTs selectively kill monocytes cross-presenting neuroblastoma-derived glycolipids. Collectively, our data from in vitro and in vivo experimental systems as well as from primary and metastatic tumor specimens demonstrate that TAMs or myelomonocytic BM cells represent a major source of IL-6 that contributes to tumor growth and is associated with poor clinical outcome in *MYCN* non-amplified neuroblastoma. As these IL-6–producing TAMs also represent the majority of $CD1d^+$ cells and frequently colocalize with NKTs in the tumor microenvironment (Figure 1, C and D), we asked whether the observed positive association between tumor-infiltrating NKTs and a favorable outcome could be explained by their specific interaction with TAMs. To test this hypothesis, we examined whether tumor-derived antigens from lysed neuroblastoma cells could be cross-presented by monocytes to NKTs. First, we determined that the tumor lysate alone was not toxic for PBMCs at up to a 50% concentration (1:1 volume/volume dilution in medium; data not shown). When PBMCs were pulsed with 25% tumor lysate, NKTs (more than 99% pure $V\alpha24J\alpha18^+$ TCR cells, Supplemental Figure 2A) killed up to 90% of monocytes that exceeded NKT cytotoxicity against monocytes pulsed with a potent agonist, αGalCer, in the positive control (Figure 6A). At the same time, no

Table 1
Association of gene expression with 5-year EFS

Gene	5-year EFS, % below/above median expression level	*P* value
TAM-associated genes (*n* = 129)[A]		
CD14	52/35	0.028
CD16	55/32	0.001
IL10	53/34	0.064
IL6	53/34	0.024
IL6RA	55/32	0.018
TGFB1	62/25	<0.001
Prin. Comp.[B]	60/27	0.001
Other immune-related genes (*n* = 38)[A]		
CD3Z	63/46	0.555
CD8A	58/52	0.962
GNLY	53/57	0.613
IFNG	68/41	0.434
TBX21	58/51	0.501
CD4	58/52	0.417
CD40LG	58/52	0.284
FOXP3	63/46	0.089
IL13	56/53	0.185
IL15	58/51	0.455
IL17	57/53	0.600
IL8	62/47	0.065
CCL2	68/42	0.080
VEGF	51/58	0.430
CD1d/NKT genes (*n* = 20)[A]		
CD1D	50/20	0.045[C]
NKT	30/40	0.035[C]

[A]The initial TLDA analysis used 20 immune-related genes on 38 tumors; 6 genes (all relates to TAM) that showed significant associations with EFS were tested on an additional 91 specimens, to the total of 129. *P* values were based on Cox regression analysis on ranked expression values. [B]First principal component of *CD14*, *CD16*, *IL6*, *IL6RA*, and *TGFB1*. [C]RT-PCR for Vα24Jα18 TCR and CD1d was performed outside TLDA using 10 tumors with $CD14^{hi}CD16^{hi}$ expression and 10 tumors with $CD14^{lo}CD16^{lo}$ expression, as described in Figure 1, A and B. *P* values were based on a permutation test of Cox regression, with NKT controlled for CD1d and vice versa.

significant NKT cytotoxicity was observed when monocytes were pulsed with a lysate prepared from normal PBMCs using the same conditions as for the tumor lysate. Interestingly, while selectively killing monocytes, NKTs spared B cells, which also express CD1d. This could be explained by the fact that the level of CD1d expression on human B cells is 3.5 ± 0.3-fold lower than on monocytes ($P < 0.001$; Supplemental Figure 2B). In addition, B cells may less effectively cross-present tumor-derived ligands compared with monocytes, and this should be addressed by future studies.

To determine whether NKT cytotoxicity is CD1d restricted, we repeated the cytotoxicity experiment with isolated (negative selection) monocytes (more than 95% pure $CD14/CD1d^+$ cells; Supplemental Figure 2C) in the presence of isotype control or anti-CD1d–blocking mAb. Figure 6B demonstrates that anti-CD1d mAb significantly inhibited NKT cytotoxicity, indicating that it was CD1d restricted. The cytotoxicity was dose dependent with the minimum effective effector/target ratio as low as 0.25:1, while no killing of neuroblastoma cells was detectable at a 5:1 ratio (Supplemental Figure 2D). In parallel with inducing NKT cytotoxicity, lysate-pulsed monocytes also induced IFN-γ production that was similarly inhibited by anti-CD1d mAb (Figure 6C). Since TAMs generally have an M2-polarized phenotype (25), we tested whether M1 or M2 polarization (in vitro treatment with IFN-γ or IL-4, respectively) affects CD1d expression and susceptibility to NKTs. In accordance with a previous report (27), none of the cytokines affected CD1d expression in monocytes, nor did they increase NKT-mediated killing (Supplemental Figure 3). We also did not detect a significant difference in the levels of IL-6 production by polarized monocytes in response to neuroblastoma cell–conditioned medium (data not shown).

Next, we examined whether the CD1d-presented ligands were derived from tumor cell lysate or generated in monocytes upon stimulation with the lysate. Since the majority of known NKT ligands are glycosphingolipids (38, 39), we used N-butyl-deoxygalactonojirimycin (NB-DGJ), an inhibitor of glucosylceramide synthase (an enzyme required for the first step of glycosphingolipid biosynthesis) (40, 41) to pretreat tumor cells before obtaining tumor lysate or to treat monocytes in the course of pulsing with the tumor lysate. The inhibitor alone was not toxic either for tumor cells or monocytes (data not shown). Figure 6D demonstrates that pretreatment of tumor cells with NB-DGJ abrogated NKT IFN-γ production in response to lysate-pulsed monocytes, while no effect was observed when monocytes or a monocyte-NKT mixture was treated with the same inhibitor. These data indicate that, in the presence of tumor lysate, monocytes cross-present to NKTs the yet-unknown glycolipid(s) derived from neuroblastoma cells instead of generating new ligands themselves. Therefore, monocytes/macrophages can cross-present tumor-derived glycolipids and induce NKT cytotoxicity in a CD1d-restricted and ligand-dependent manner.

Adoptively transferred NKTs reduce the number of tumor-infiltrating monocytes in tumor xenografts and inhibit their growth-promoting effect. The observed in vitro NKT cytotoxicity against monocytes, which cross-present tumor-derived antigens, suggests that the killing of tumor-promoting monocytes by NKTs could occur at the tumor site. To test this hypothesis, we designed an in vivo experiment in which NOD/SCID mice with subcutaneous xenografts of CHLA-255 neuroblastoma cells were infused with human monocyte-enriched PBMCs alone or with NKTs (1:5 ratio) intravenously. After 48 hours mice were sacrificed, and the frequency of human $CD45^+CD14^+$ monocytes was quantified by flow cytometry in the tumor xenografts after gating out dead ($DAPI^+$) and human NKT ($6B11^+$) cells (Figure 7A). The frequency of monocytes was decreased about 3-fold in tumors of mice receiving monocytes with NKTs compared with those receiving monocytes alone ($P < 0.001$). Pretreatment of mice with an anti-hCD1d mAb before transfer of monocytes and NKTs significantly increased the frequency of monocytes in tumors. These results are consistent with the ability of NKTs to eliminate TAMs.

To examine whether NKTs suppress the growth-promoting effect of monocytes in neuroblastoma xenografts, we injected CHLA-255/luc cells alone or with freshly isolated monocytes in the right or left flanks, respectively, of the same mice (Figure 7B). The tumor growth in this control group was compared with mice that, in addition, received NKTs in both flanks without or with anti-hCD1d mAb (Figure 7B). In Figure 7B, the bar graph demonstrates that co-injection of NKTs at a 6:1 NKT/tumor cell ratio (the maximum NKT dose in this study) led to strong inhibition of monocyte-mediated growth enhancement of neuroblastoma xenografts ($P < 0.01$). The antitumor effect of NKTs was CD1d restricted, since it was inhibited by an anti-CD1d mAb

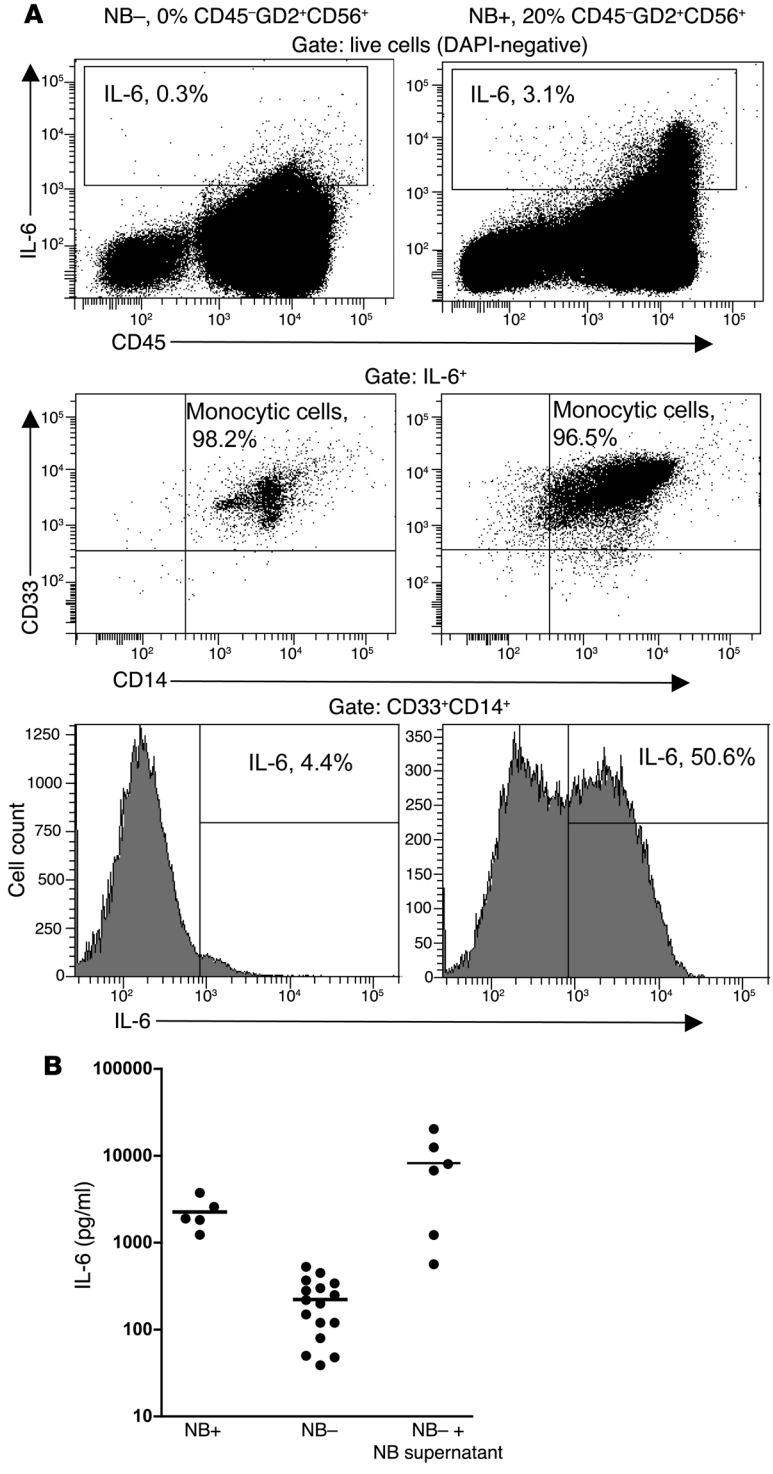

Figure 5
Myelomonocytic cells are a major source of IL-6 in BM with neuroblastoma metastases. (**A**) Freshly isolated BM mononuclear cells from stage 4 neuroblastoma patients were analyzed for the presence of neuroblastoma cells by GD2 and CD56 (NCAM) co-expression and are shown as NB– (left) or NB+ (right) examples. Without any in vitro stimulation, cells were cultured in the presence of brefeldin A for 4 hours, followed by staining for the indicated surface markers and intracellular IL-6. The frequencies of IL-6–producing cells among all live cells (top), of monocytic cells among IL-6–producing cells (middle), and of IL-6–producing cells among monocytic cells (bottom) are shown. Representative plots from 16 NB– and 5 NB+ BM mononuclear cells. (**B**) As in **A**, NB+ and NB– BM morphonuclear cells were cultured overnight. In addition, 7 of 16 NB– BM morphonuclear cells were cultured in the presence of a CHLA-255 neuroblastoma cell–conditioned medium. IL-6 concentration in the supernatants was determined by CBAPlex. Horizontal bars indicate the mean values. $P < 0.001$ compared with NB– mononuclear cells cultured in nonconditioned medium, by 1-way ANOVA.

($P < 0.05$). Importantly, NKTs did not have antitumor activity against tumors that were grown in the absence of monocytes ($P > 0.05$; Figure 7B). Figure 7C demonstrates that NKTs inhibited the tumor-promoting effect of monocytes in a dose-dependent manner. Significant antitumor activity was observed at NKT/monocyte ratios above 1:1 ($P < 0.01$), at which NKTs were cytotoxic in vitro (Supplemental Figure 2D). Therefore, we concluded that NKTs may indirectly suppress tumor growth by killing tumor-promoting TAMs.

Discussion

This study reveals what we believe is a novel mechanism of NKT antitumor activity via direct cytotoxicity against growth-promoting monocytes/macrophages in neuroblastoma. Even though tumor cells in neuroblastoma are CD1d$^-$ and cannot be directly targeted by NKTs, we found that CD1d is expressed by TAMs. We demonstrate that these cells are a major source of a tumor growth–promoting cytokine, IL-6, in primary and metastatic neuroblastoma and that high expression of TAM genes including IL-6 is associated with poor long-term EFS in patients with stage 4 *MYCN* non-amplified tumors. Importantly, TAMs can cross-present neuroblastoma-derived glycolipids and be killed by NKTs in a CD1d-restricted manner that results in suppression of tumor growth. This provides an explanation for our previous observation that NKT infiltration into *MYCN* non-amplified neuroblastomas is associated with a favorable outcome (9).

We found that CD1d expression in primary neuroblastoma tumors was mostly limited to TAMs and that tumor-infiltrating NKTs were often in direct contact with or close proximity to TAMs. This suggested that TAMs, rather than tumor cells themselves, could be specifically (via TCR) recognized by CD1d-restricted NKTs. Accumulated evidence indicates that this hypothesis is applicable not only to neuroblastoma but to the broad range of human tumors. Although NKTs can be directly cytotoxic against CD1d$^+$ tumors cells, only a few types of leukemia (36, 42) and 1 type of solid tumor (glioma) (43) have been reported to express CD1d in humans. In contrast, the majority of murine tumor cell lines are CD1d$^+$ (44). However, in the absence of strong exogenous ligands such as αGalCer, NKTs have little or no cytotoxic activity even against CD1d$^+$ human or murine tumor cells (36, 44). This suggests that in order to overcome NKT-mediated immune surveillance, progressing tumors may lose CD1d expression and/or

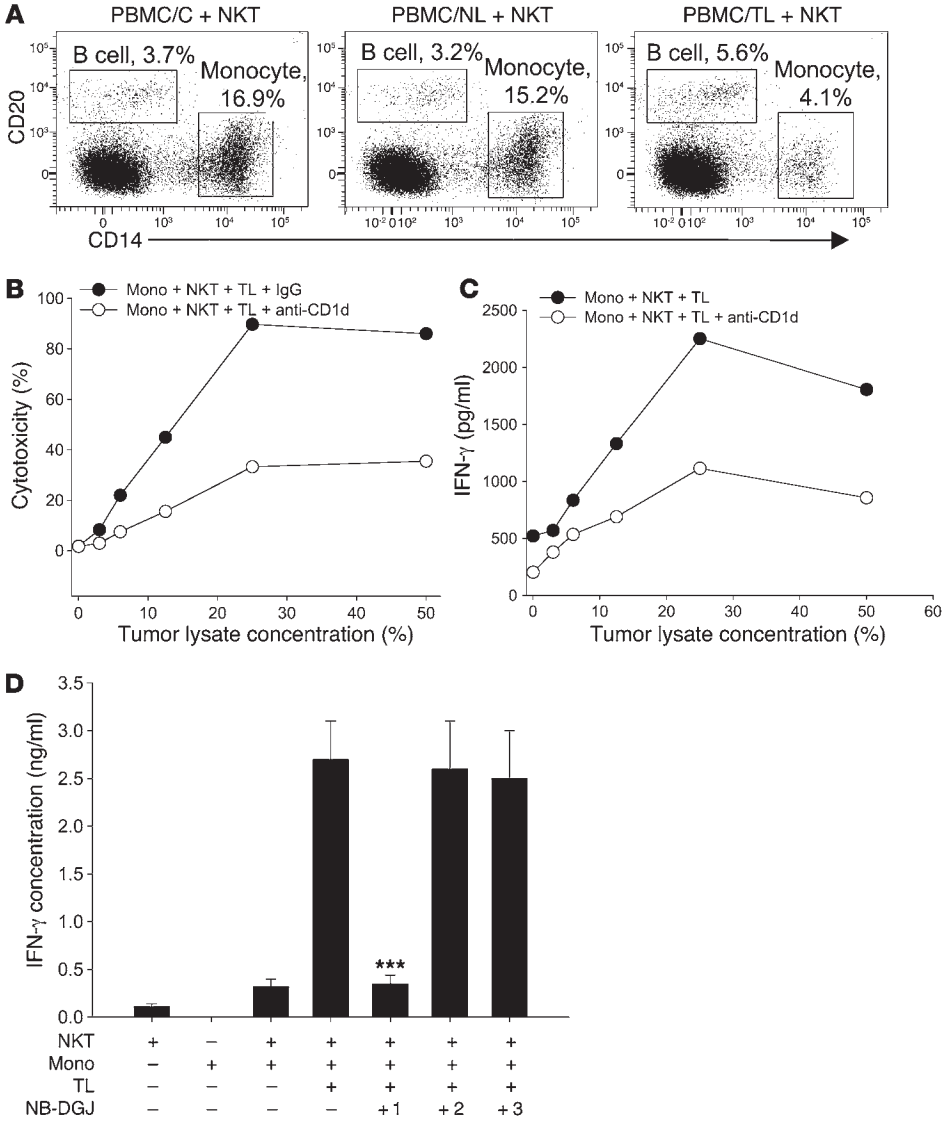

Figure 6

NKTs selectively kill monocytes cross-presenting neuroblastoma-derived glycolipids. (**A**) PBMCs were cultured overnight with medium control (PBMC/C), tumor cell lysate (PBMC/TL), or lysate of normal PBMCs (PBMC/NL). Cells were stained with Calcein-AM (retained in viable cells) and incubated for another 4 hours with NKT (5:1 effector/target ratio), followed by FACS for CD14 (monocytes) and CD20 (B cells) frequency in surviving PBMCs (gated on Calcein-AM–high; data not shown). Shown are representative plots from 1 of 4 experiments. (**B** and **C**) NKT cytotoxicity (**B**; 4-hour coculture) and IFN-γ production (**C**; 16-hour coculture, CBAPlex) was measured after monocyte pulsing with a range of tumor cell lysate dilutions in the presence of isotype-matching control or anti-CD1d–blocking mAb. Results are mean values from 1 representative of 3 experiments. (**D**) NKTs were incubated alone, with monocytes, or with tumor cell lysate–pulsed (TL-pulsed) monocytes. In addition, N-butyl-deoxygalactonojirimycin (NB-DGJ; 50 μM) was added to tumor cells for 24 hours before tumor cell lysate preparation (+1), in overnight culture of monocytes with tumor cell lysate (+2), and in monocyte/tumor cell lysate plus NKT coculture (+3). Production of IFN-γ was measured by CBAPlex. Data are presented as mean ± SD from 3 experiments. ***$P < 0.001$.

repress synthesis of strongly agonistic ligands for NKT TCR. In that respect, CD1d$^-$ neuroblastoma is a typical solid tumor, the cells of which cannot be directly recognized and killed by NKTs.

TAMs are often the most abundant inflammatory cells in the tumor microenvironment (24, 45). They derive from circulating monocytic precursors (34) that are highly plastic cells and can acquire a range of functional differentiation programs in response to microenvironmental signals (46). Our in vitro experiments demonstrated that coculture of monocytes with neuroblastoma cells (or their supernatants) induced monocytes to produce large amounts of IL-6, which in turn enhanced neuroblastoma cell proliferation. Consistent with that, coculture of monocytes with neuroblastoma cells resulted in the increased proliferation rate of the latter that was inhibited by an anti–IL-6–neutralizing mAb. Furthermore, the growth of neuroblastoma xenografts in NOD/SCID mice was enhanced in the presence of monocytes or paracrine IL-6 in the tumor microenvironment, and the tumor-promoting effect of monocytes was inhibited by an anti–IL-6 mAb. Taken together, these in vitro and in vivo experimental results indicate that neuroblastoma cells have the potential to direct monocyte differentiation toward TAMs that promote tumor growth at least in part via IL-6 secretion. We also demonstrated that in patients with BM metastases, myelomonocytic cells are a major source of IL-6, which is essential for bone invasion and further disease progression in neuroblastoma (29) as well as other types of cancer such as multiple myeloma, breast cancer, and prostate cancer (37). In another study that was recently reported, we further investigated the mechanism by which IL-6 promotes growth and induces drug resistance in neuroblastoma cells (47). Furthermore, Egler et al. recently demonstrated that IL-6 could be of clinical significance in neuroblastoma, as high serum IL-6 levels in patients at diagnosis were associated with poor outcome (48). However, the main source of IL-6 in neuroblastoma patients had not been identified in the previous studies. Our results unambiguously demonstrate that TAMs and their myelomonocytic precursors are the dominant source of IL-6 in primary neuroblastoma and metastatic BM, respectively.

Although the association of high frequency of TAMs with poor prognosis has been well documented in many types of cancer in adults (25, 49), little is known about the role of TAM-mediated innate inflammation in pediatric tumors. The presence of strong

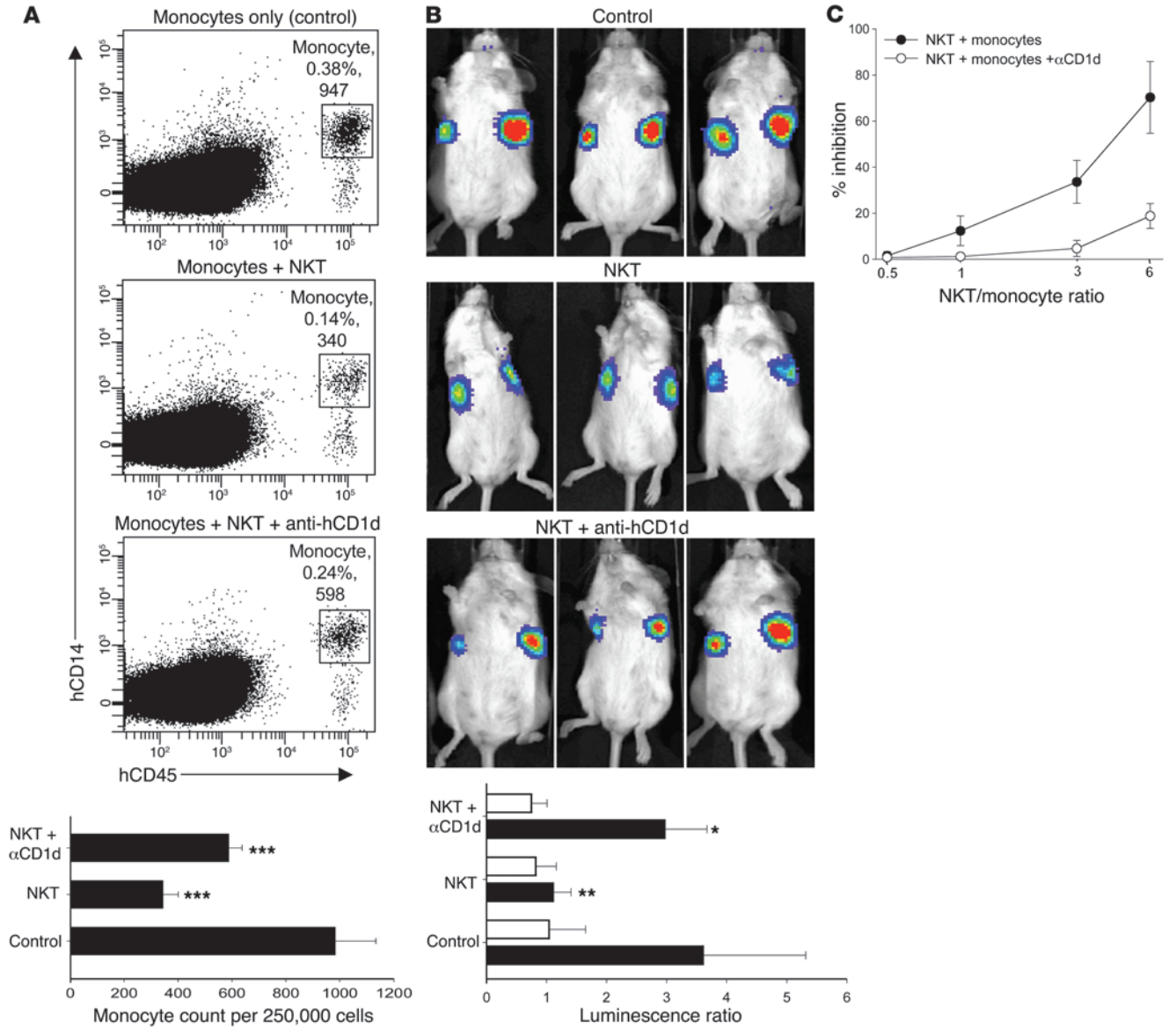

Figure 7

Adoptively transferred NKTs reduce the number of tumor-infiltrating monocytes in tumor xenografts. (**A**) CHLA-255 neuroblastoma cells were injected subcutaneously into NOD/SCID mice. When tumors reached approximately 0.5 cm in diameter, mice with similar tumor sizes were divided into 3 groups, which received intravenous monocyte-enriched human PBMCs alone (top), PBMCs plus NKTs (middle), and PBMCs plus NKTs and anti-hCD1d–blocking mAb (intraperitoneally). Mice were sacrificed 48 hours after adoptive cell transfer, and monocyte frequency was examined by FACS in single-cell suspensions prepared from tumor xenografts. Dead cells and NKTs were gated as DAPI-positive and PE-6B11–positive events (data not shown). Monocytes were identified as $hCD45^+CD14^+$ events among 250,000 live non-NKTs per sample. Data in bar graphs represent mean ± SD from 6–8 mice per group. ***$P < 0.001$, by 1-way ANOVA. (**B**) Top: CHLA-255/luc neuroblastoma cells were subcutaneously injected alone or with freshly isolated monocytes in the right or left flanks of mice (top panel). Other groups received NKTs in both flanks at a 6:1 NKT/tumor cell ratio without (middle panel) or with anti-CD1d51.1 mAb (bottom panel; 10 μg per site). Shown are bioluminescent front images (taken 21 days after tumor implantation) of 3 representative of 5 mice per group. Bottom: Black bars represent mean ± SD of left flank/right flank luminescence ratios in these mice. The white bars represent mean ± SD of ratios of the right flank luminescence of mice in each group relative to the median right flank luminescence in the control group. *$P < 0.05$, 1-way ANOVA. **$P < 0.01$, by paired *t* test. (**C**) The experimental setup was the same as described in **B**, with groups of mice receiving discrete numbers of NKTs as indicated on the *x* axis. Results were expressed as the percentage of inhibition of monocyte-mediated tumor growth enhancement.

oncogenes in pediatric tumors is often sufficient to explain aggressive tumor growth via cell-intrinsic mechanisms (50). For example, in *MYCN*-amplified neuroblastomas, *MYCN* is sufficient to drive uncontrolled proliferation of neuroblastoma cells while repressing CCL2 expression (17), the main chemoattractant for monocytes (51). However, about 70% of high-risk neuroblastomas lack *MYCN* amplification. We found that those *MYCN* non-amplified tumors that were infiltrated with TAMs expressed high levels

of IL-6. By analyzing the expression of immune/inflammatory genes in the primary tumors, we found that high expression of CD14, CD16, IL-6, and IL-6Rα was significantly associated with decreased 5-year EFS. In addition, the expression of TGF-β1 and IL-10, 2 major immunosuppressive cytokines known to be produced by TAMs (26), also correlated with poor outcome. Overall, our data indicate that in patients with *MYCN* non-amplified high-risk neuroblastoma, TAMs provide a growth-supportive microenvironment that facilitates tumor progression and that relates to the disease outcome.

We demonstrate here that neuroblastoma cells contain agonistic glycosphingolipid ligand(s) for NKTs that can be cross-presented by CD1d on monocytic cells, leading to potent NKT-mediated cytotoxicity against the antigen-presenting cells. Adoptive transfer experiments in NOD/SCID mice showed that human NKTs were able to kill monocytic cells in human neuroblastoma xenografts, resulting in inhibition of monocyte-mediated enhancement of tumor growth. These data provide strong in vivo experimental evidence that the observed phenomenon can be operative in patients with cancer. Moreover, some common conditions associated with tumor growth may facilitate TAM-mediated cross-presentation of tumor-derived ligands. For example, TAMs preferentially localize to the necrotic regions of tumors (52, 53), where tumor-derived intracellular glycolipids become available for cross-presentation. The same hypoxic areas are a rich source of chemokines such as CCL2 and CXCL12 (54) that promote migration of both TAMs and effector-memory T cells, including NKTs (55). This, in addition to specific TCR recognition, increases the chances of NKT contact with TAMs in the tumor microenvironment. Based on the presented findings, we propose what we believe is a novel mechanism of NKT-mediated antitumor activity, in which NKTs and monocytes colocalize to the tumor site in response to tumor-derived chemokines, followed by CD1d-restricted cross-presentation of tumor-derived glycolipid antigens to NKTs and killing of the monocytic cells. Since monocytes and their TAM progenies promote tumor growth and associate with poor outcome, killing of these cells by NKTs provides an explanation for the positive association of NKTs with a favorable outcome in neuroblastoma and other types of cancer.

The proposed mechanism of NKT antitumor function likely coexists with other modes that have been observed in mouse models of cancer. A number of reports have demonstrated that, whether or not NKTs kill tumor cells directly, they can activate both innate and adaptive antitumor immune responses via interactions with DC, NK, T, and B cells (3, 56–58). De Santo et al. recently reported that NKTs reduce the immunosuppressive activity of influenza A virus–induced myeloid-derived suppressor cells (MDSCs) in mice and humans (59). As in our study, the effect was CD1d dependent. Since TAMs are widely recognized as a major subset of MDSCs (26), data from both studies support the concept that interactions between NKTs and $CD1d^+$ myelomonocytic cells play important roles both in antiviral and antitumor immunity. The effect of NKTs in influenza did not require killing of MDSCs and was realized via CD40-mediated reduction of ARG1 and NOS2 activity that led to restoration of antiviral T cell response. Such a mechanism could enhance antitumor T cell response as well. However, our results demonstrate that expression of neither CD8 nor IFN-γ in primary neuroblastoma relates to the disease outcome. Although IFN-γ plays a critical role in tumor immunity in the "elimination" phase proposed in the cancer immunoediting theory (60), tumor cells in clinically apparent cancers are at the "escape" phase. They consist of genetically selected variants that are no longer effectively controlled by either NK or T cells. Unlike tumor cells, TAMs are genetically stable and may remain subject to regulation even in the advanced tumors. Therefore, NKT-mediated killing or suppression of TAMs may represent one of a few protective barriers that are operative in the immune system of cancer patients.

Clinical trials with αGalCer-pulsed DCs in cancer patients demonstrated sustained in vivo expansion of NKTs and activation of other cells of the immune system in a Th1-like manner (56, 61). Recent phase I trials also demonstrated that ex vivo–expanded NKTs can be safely used for adoptive transfer in cancer patients (62, 63). However, unlike in murine models, objective antitumor responses have not been observed in cancer patients. Data presented in this report suggest that NKT antitumor efficacy could be increased by sensitizing TAMs to NKT cytotoxicity. The latter requires additional studies that would aim to identify the NKT ligands produced by tumor cells, the mechanism of their cross-presentation by TAM CD1d, and the regulation of CD1d expression. We also must acknowledge that, due to production of both Th1 and Th2 cytokines, NKTs may play a dual role in tumor immunity. If TAMs are not killed by NKTs, they could further activate TAM tumor-promoting programs upon stimulation with NKT-derived IFN-γ and IL-13. Indeed, activated NKTs abundantly produce both of these cytokines, and the combined signaling from their receptors has been shown to be essential for TAM functional differentiation (26, 64). One strategy of avoiding such a scenario would be the use of synthetic NKT ligands that have been designed to polarize NKT response toward Th1, such as C-glysosidic analogs of αGalCer (65, 66). Another approach is to redirect NKT cytotoxicity against tumor cells using bispecific fusion proteins, as this redirection has recently been demonstrated with CD1d fusion to HER2-specific scFv in a B16 melanoma model (67). NKTs themselves could be genetically engineered to express chimeric antigen receptors against tumor-associated antigens to enable their dual reactivity against both tumor cells and tumor-supportive TAMs. Thus, NKT-based immunotherapies could be designed to target both tumor cells and a tumor-supportive microenvironment.

Methods

Human neuroblastoma specimens. Primary untreated stage 4 neuroblastomas (n = 129) were obtained from patients (38 of whom were less than 18 months old) enrolled in the Children's Cancer Group studies CCG-3881 and CCG-3891 (68). Specimens were snap-frozen within 2 hours of surgery in foil or after embedding in OCT and were maintained at –80°C. Clinical data came from participating institutions and were managed in the COG Statistics and Data Center according to standard operating procedures. Twenty BM specimens were from newly diagnosed patients with stage 4 neuroblastoma who were enrolled in Children's Cancer Group studies for neuroblastoma. Seven patients had BM metastases with at least 10% tumor cells in BM detected by immunocytology (69). Informed consent was obtained in accordance with institutional review board–approved policies and procedures for research dealing with human specimens. The studies on human subjects and specimens were reviewed and approved by the Institutional Review Board of the Childrens Hospital Los Angeles.

RNA isolation and TaqMan real-time RT-PCR and low-density arrays. Total RNA from frozen tumor sections was isolated using TRIzol (Invitrogen). The RNA quality was assessed with gel electrophoresis prior to reverse transcription into cDNA using M-MLV reverse transcriptase with oligo dT priming (Invitrogen). All the samples were run on the 2100 Bioana-

lyzer (Agilent), and samples with RNA integrity numbers less than 6.5 were excluded from the analysis if a repeat RNA isolation did not improve the RNA quality. Quantitative RT-PCR was performed with TaqMan gene expression assays using the ABI 7900 Sequence Detection System (Applied Biosystems). Briefly, the probes and primers were ordered from Applied Biosystems Custom TaqMan Gene Expression Assays (Table 1); those for Vα24-Jα18 TCR have been previously described (9). The TLDA microfluidic cards were loaded with 50 μl of TaqMan universal master mix and 50 μl (500 ng) of cDNA per channel of the microfluidic card. The relative change in gene expression was calculated using the ΔCt method using the mean of 3 housekeeping genes (*GAPDH*, *HPRT1*, and *SDHA*) as the control. A quantitative comparative Ct analysis was performed using the ABI PRISM 7900 Sequence Detection System and PE Biosystems software. Standard TaqMan real-time RT-PCR assays in 96-well plates were performed for Vα24-Jα18–invariant TCRα of NKTs (9) and for CD1d using the same housekeeping genes and analysis method as for TLDA-based assays.

Immunofluorescent microscopy. Frozen 8-μm sections were brought to room temperature and fixed in 75% acetone/25% ethanol solution for 5 minutes. Slides were then blocked with 5% donkey serum supplemented with 1% BSA in 0.1% Triton X-100 for 60 minutes. Sections were stained with 1:50 dilution of rabbit anti-human CD68 and 1:100 dilution of mouse anti-human CD1d (Santa Cruz Biotechnology Inc.) or anti–Vα24-Jα18–invariant TCRα 6B11 mAb in 2% donkey serum supplemented with 1% BSA in TBS Tween-20 (TBST) overnight at 4°C. For fluorescent visualization of Ab reactions, secondary antibodies were Alexa Fluor 488–conjugated anti-rabbit Ab (Invitrogen; 1:2000) and Cy3-conjugated anti-mouse Ab (Jackson ImmunoResearch Laboratories Inc.; 1:800) and diluted in 1% BSA with TBST. Slides were incubated with secondary antibodies for 60 minutes at room temperature. Sections were then washed in TBST, followed by TBS, and counterstained with DAPI (Vector Laboratories). Fluorescent images of tumor sections were acquired on a Zeiss Axiovert 200 M with a ×40 Plan Neofluar objective with Zeiss filter sets: 49 (DAPI), 38 (GFP), and 43 (Texas Red) using IPLab 4.0 software (Scanalytics Inc.).

Cell lines, plasmids, clones, and gene transduction. CHLA-255, CHLA-255/luc, and SK-N-BE(2) neuroblastoma cell lines were established and maintained as previously described (17, 70). To generate CHLA-255/IL-6, we transduced CHLA-255 cells with human IL-6 cDNA using a ViraPower T-REx lentiviral expression system (Invitrogen) as previously described (47).

Monocyte isolation, coculture experiments, and cell proliferation assay. PBMCs were purchased from Astarte Biologics or freshly isolated by gradient centrifugation (18) from discarded leukocyte filters obtained during platelet collection from unidentified healthy adults at the Children's Hospital Los Angeles Blood Collection Center. Monocytes were isolated by negative selection using Dynal Monocyte Negative Isolation Kit (Invitrogen) according to the manufacturer's instructions. To prevent monocyte activation by adhesion to plastic, all in vitro experiments were performed in Ultra Low Attachment Plates (Corning). We also found that GIBCO Dialyzed FBS (Invitrogen) had to be used to prevent monocyte activation in a short-term culture. In coculture experiments, neuroblastoma cells were added directly to monocytes or to the inserts separated by 0.4-μm membrane (Costar; Corning) from monocytes. For the cell cycle analysis, cells were pulsed with BrdU for 40 minutes before being harvested, followed by fixation, permeabilization, and staining with FITC anti-BrdU mAb and 7-amino actinomycin D (7AAD) (BD Biosciences) according to the manufacturer's instructions. In order to discriminate between neuroblastoma cells and monocytes (and any contaminating blood cells), PerCP-conjugated anti-CD45 mAb was added. Although some neuroblastoma cells expressed low-level CD45 intracellular, they were clearly discriminated from $CD45^{hi}$ monocytes. Cells in the S-phase of the cell cycle were identified by flow cytometry as BrdU-positive events.

Cytometric bead array analysis. Cytokines were detected with the CBAPlex beads (BD Biosciences) according to the manufacturer's manual and as previously described (18).

Flow cytometry (FACS analysis). To analyze the phenotype and frequency of IL-6–producing cells in human BM, primary BM mononuclear cells, which were prepared by density centrifugation, were cultured for 4 hours with brefeldin A, followed by staining with mAbs against surface antigens (PerCP–anti-CD45, PE–anti-CD33, and APC–Cy7-CD14), followed by fixation and permeabilization with a Perm/Fix kit (BD Biosciences) and staining for intracellular IL-6 with FITC-conjugated anti–IL-6. In a separate set, the same BM samples were analyzed for the frequency of metastatic NB cells by staining with FITC-conjugated anti-GD2 (custom synthesis), APC-conjugated anti-CD56, and PerCP-conjugated anti-CD45 (BD Biosciences). Fluorochrome- and isotype-matching mAbs suggested by BD Biosciences were used as negative controls.

To analyze the human leukocytes infiltrating tumor xenografts in NOD/SCID mice, reactivity to mouse and human Fcγ receptors was blocked by 5-minute pre-incubation with mouse Fc-block (1:1,000; BD Biosciences) and polyclonal human IgG (2 mg/ml; Sigma-Aldrich), respectively. Dead cells were excluded by staining with DAPI. The following set of antibodies was used to identify tumor-infiltrating leukocytes: PerCP-conjugated anti–hCD45, PE-conjugated NKT 6B11, and APC-conjugated Cy7-CD14. For each sample, 250,000 live (DAPI-negative) events were collected. The analysis was performed on an LSR II 4-laser flow cytometer (BD Biosciences) using BD Biosciences FACSDiva v. 6.0 and FlowJo v. 7.2.5 software (Tree Star Inc.).

In vitro cytotoxicity assay. Freshly isolated PBMCs were incubated overnight with different dilutions of tumor cell lysate obtained by 5 rapid freeze-thaw cycles of neuroblastoma cells of 10^7 CHLA-255 neuroblastoma cells in 1 ml of AIM-V Medium (Invitrogen) followed by low-speed centrifugation (18 *g* for 10 minutes) to remove the cell debris. Corresponding volume of AIM-V medium with or 100 ng/ml αGalCer (Alexis Biochemicals) were used as negative and positive controls, respectively. In the final 30 minutes, 1 μg/ml Calcein-AM was added to the culture, followed by 2 washing cycles. For the next 4 hours, NKTs were added at a 5:1 effector/target ratio. At the end of culture, cells were stained with APC-conjugated anti-CD20 (BD Biosciences) and APC-conjugated Cy7–anti-CD14 mAbs, followed by flow cytometry analysis. Live target cells (PBMCs) were gated as Calcein-AM-bright events, as previously described (71). Within this gate, the frequency of monocytes and B cells was analyzed as $CD14^+$ and $CD20^+$ events, respectively. Where indicated in the figure legends, NKT cytotoxicity was tested against negatively sorted monocytes.

In vivo experiments. Four-week-old female NOD/SCID mice were purchased from The Jackson Lab. CHLA-255/luc cells alone or with freshly isolated human monocytes or CHLA-255/IL-6 neuroblastoma cells were injected subcutaneously. Tumor growth was indirectly assessed by weekly bioluminescent imaging (at the Small Animal Imaging Core facility, Childrens Hospital Los Angeles). Where indicated, mice were injected intraperitoneally with neutralizing anti–hIL-6 Ab, AB-206 (100 μg/mouse; R&D Systems), or anti-hCD1d42.1 or anti-hCD1d51.1 mAb (40 μg/mouse; provided by S. Porcelli, Albert Einstein College of Medicine, New York, New York, USA). Some animals received intravenous injections of human PBMCs (2×10^8 total cells) enriched for monocytes by 40-minute adhesion to plastic dishes (65.3% ± 8.4% $CD14^+$ cells, as determined by FACS). Where indicated, mice also received ex vivo–expanded human NKTs (18) (10^8 cells per animal). Before injection into animals, NKTs had been cultured with 50 U/ml IL-2 and IL-15 (Peprotech) for 7–10 days without TCR stimulation, to achieve the resting phase when their trafficking pattern more closely resembled that of primary NKTs, as described previously (9). Mice were sacrificed after 48 hours, and cell suspensions prepared from tumors

were analyzed by multicolor flow cytometry as described in "Flow cytometry." As indicated in Figure 7, NKTs were co-injected with CHLA-255/luc cells and monocytes, and their antitumor efficacy was determined by bioluminescent imaging. Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Childrens Hospital Los Angeles.

Statistics. For in vitro and in vivo experiments, comparisons between 2 groups were done using the 2-sided unpaired Student's *t* test or 1-way ANOVA with the Tukey-Kramer post-hoc comparison of group means.

EFS was defined as the minimum interval from the date of diagnosis to the date of tumor recurrence, progression, second malignancies, death, or the last follow-up. EFS percentage at 5 years was estimated using the product-limit estimate with Greenwood standard errors. The effect of gene expression on EFS was tested using Cox regression analysis on ranked expression values. All *P* values reported are 2 sided. *P* values for a small sample size ($n = 20$) were computed based on a permutation test as indicated in the Table 1 legend.

Statistical computations were performed using GraphPad Prism 4.0 software and STATA 9.0 (StataCorp LP). Differences were considered significant when the *P* value was less than 0.05.

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