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Immune evasion mediated by tumor-derived lactate dehydrogenase induction of NKG2D ligands on myeloid cells in glioblastoma patients

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Contributed by Lewis L. Lanier, July 23, 2014 (sent for review May 21, 2014)

Myeloid cells are key regulators of the tumor microenvironment, governing local immune responses. Here we report that tumor-infiltrating myeloid cells and circulating monocytes in patients with glioblastoma multiforme (GBM) express ligands for activating the Natural killer group 2, member D (NKG2D) receptor, which cause down-regulation of NKG2D on natural killer (NK) cells. Tumor-infiltrating NK cells isolated from GBM patients fail to lyse NKG2D ligand-expressing tumor cells. We demonstrate that lactate dehydrogenase (LDH) isoform 5 secreted by glioblastoma cells induces NKG2D ligands on monocytes isolated from healthy individuals. Furthermore, sera from GBM patients contain elevated amounts of LDH, which correlate with expression of NKG2D ligands on their autologous circulating monocytes. NKG2D ligands also are present on circulating monocytes isolated from patients with breast, prostate, and hepatitis C virus-induced hepatocellular carcinomas. Together, these findings reveal a previously unidentified immune evasion strategy whereby tumors produce soluble factors that induce NKG2D ligands on myeloid cells, subverting antitumor immune responses.

LDH5 | glioma | cancer | tumor evasion

The therapeutic options for patients with high-grade glioma currently are limited to chemotherapy and radiation, which cause devastating side effects (1). Immunotherapy is an appealing treatment that can specifically target tumor cells; however, the immunosuppression found in cancer patients remains a significant challenge to clinical efficacy. An immunosuppressive tumor microenvironment may be one of the greatest obstacles to overcome, because many clinical studies demonstrate specific antitumor immune responses ex vivo but without improved clinical outcomes. The discordance between biological activity and clinical efficacy in patients treated with immunotherapy may be caused by tumor-mediated immunosuppression. Tumor cells can directly inhibit immune responses in the tumor microenvironment through the secretion of anti-inflammatory cytokines or recruitment of immunosuppressive cell populations (2, 3).

Natural killer (NK) cells are a central mediator of innate immunity that can impede tumor growth through the process of immune surveillance (4). NK cell activation occurs through ligation of specific cell-surface receptors, such the Natural killer group 2, member D (NKG2D) receptor (5), resulting in the production of proinflammatory cytokines such as IFN-γ and the release of cytotoxic granules that cause tumor cell lysis (6). There are eight ligands for NKG2D [e.g., MHC class I polypeptide-related sequence A (MICA), MHC class I polypeptide-related sequence B (MICB), and UL16-binding proteins 1–6 (ULBP1-6)], which can be expressed by any type of cell under stress (7). The importance of NKG2D in immune surveillance of tumors is highlighted by the observation that NKG2D-deficient mice are more susceptible to the development of oncogene-induced tumors (8). We previously reported that patients with glioblastoma multiforme (GBM) express low amounts of NKG2D on their NK cells, compared with healthy controls, and this reduced expression is caused, in part, by TGF-β–mediated down-regulation of NKG2D (9).

The myeloid cell lineage includes circulating monocytes in the peripheral blood and macrophages in tissues. Within tumors, tumor-infiltrating myeloid cells (TIMCs) can acquire immunosuppressive functions (10). Although myeloid-derived suppressor cells (MDSCs) have been documented to impair immune responses, Nausch et al. (11) reported that the expression of the NKG2D ligand Rae-1 on MDSCs in a mouse lymphoma model system activated, rather than suppressed, the host NK cells and potentiated tumor elimination (11).

Here we report that, surprisingly, NKG2D ligands are expressed on the cell surface of both the tumor-infiltrating myeloid cells and circulating monocytes in patients with GBM. Interactions between NKG2D ligand-bearing myeloid cells and NK cells down-modulate NKG2D on NK cells and impair their antitumor activity. Further, we demonstrate that lactate dehydrogenase isoform 5 (LDH5) secreted by glioblastoma cells induces the transcription and expression of NKG2D ligands in monocytes from healthy individuals and that sera from GBM patients contain elevated levels of LDH, correlating with NKG2D ligand expression on their myeloid cells.

Significance

This study describes a mechanism of immune escape in which glioblastoma cells produce a soluble protein, lactate dehydrogenase 5 (LDH5), that induces the expression of Natural killer group 2, member D (NKG2D) ligands on the surface of healthy myeloid cells. Expression of NKG2D ligands by myeloid cells causes the down-modulation of the activating NKG2D receptor on natural killer cells, thereby preventing their recognition of NKG2D ligand-bearing tumors and impairing their ability to attack and eliminate tumors. The discovery that monocytes in patients with several types of cancer also express NKG2D ligands suggests that LDH5 production by tumors may represent a common mechanism of immune evasion. Blocking LDH5 might improve recognition of tumors in cancer patients.

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Conflict of interest statement: L.L.L., C.A.C., and A.T.P. and the University of California, San Francisco have licensed intellectual property rights regarding NKG2D for commercial applications.

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Results

We reported previously that primary tumor cells in GBM patients express NKG2D ligands (9), even though the frequency of NK cells in the tumor is similar to that in peripheral blood (Fig. S1*A*). However, the level of NKG2D receptor on these NK cells was lower than observed in healthy control individuals (Fig. S1*B*). Myeloid cells were the most prevalent leukocytes infiltrating tumors in GBM patients (Fig. S1*C*); 57.6% (n = 18) of CD45$^+$ cells among GBM tumor-infiltrating leukocytes were MHC class II$^+$, CD11b^{high} myeloid cells compared with 28.28% (n = 13) in patients with benign intracranial meningioma (MNG) (Fig. S1*C*). In GBM patients, tumor-infiltrating myeloid cells (CD11b^{high} macrophages and CD11b^{low} CNS-resident microglia) and circulating monocytes in the peripheral blood expressed at least two of the NKG2D ligands, MICB and ULBP-1 (n = 33), which were not detected on myeloid cells in MNG patients (n = 16) (Fig. 1 *A* and *B*). Patients with newly diagnosed GBM who were analyzed before receiving any therapy consistently expressed MICB and ULBP-1 on circulating monocytes, indicating that expression was independent of surgery, chemotherapy, radiation, or steroid treatment (Fig. S2, n = 4). Transcriptional analysis revealed cell-intrinsic expression of MICB and ULBP-1 in GBM patients' circulating monocytes (n = 19) (Fig. 1*C*), excluding the possibility that soluble NKG2D ligands were passively acquired from the patients' sera (12). Of the eight NKG2D ligands evaluated by quantitative RT-PCR (qRT-PCR), only ULBP-1 and MICB were consistently expressed by monocytes and TIMCs in all GBM patients (Fig. 1*C*). Acid washing of the patients' monocytes removed β2-microglobulin, a surface protein lacking a membrane anchor, but did not remove MICB or ULBP-1, confirming that these NKG2D ligands are expressed endogenously (Fig. S3).

Given their expression on circulating monocytes, we hypothesized that the NKG2D ligands were induced by tumor-derived soluble factors that were acting systemically in the patients. By using cell-free supernatant or Transwell assays, we determined that soluble products derived from the U87 glioma cell line induced expression of MICB and ULBP-1 on primary monocytes from healthy blood donors (Fig. 1*D*). Heat denaturation (Fig. 1*E*) and size-exclusion dialysis (Fig. 1*F*) revealed that a heat labile factor of >10 kDa induced MICB and ULBP-1.

By using an unbiased protein purification screening strategy to determine the factor(s) responsible for in vitro induction of MICB and ULBP-1 transcription in healthy monocytes, we identified LDH5 as sufficient to induce NKG2D ligand expression in monocytes (Fig. 2 *A* and *B*). LDH is a tetrameric metabolic enzyme that binds pyruvate and promotes ATP production in resource-deprived environments. There are five isoforms of LDH, consisting of different ratios of α and β subunits, with varied tissue distribution. Consistent with prior reports of correlations between elevated LDH5 in sera of cancer patients and poor prognoses (13–16), our findings indicate that LDH5 may promote immune escape of tumor cells by inducing NKG2D ligands on host myeloid cells, thereby subverting the antitumor activity. Glioma cell lines U87, U251, and SF767 transcribe LDH−A and LDH−B (Fig. 2*C*), suggesting that they can make all five isoforms of LDH. These glioma cell lines secrete enzymatically active LDH into their supernatants (Fig. 2*D*) that is sufficient to induce MICB and ULBP-1 mRNA in healthy monocytes (Fig. 2*E*).

We evaluated NKG2D ligand mRNA expression following treatment with purified, native LDH5 and U87 supernatant in the presence of sodium oxamate, a pyruvate analog that blocks LDH enzymatic activity. Sodium oxamate significantly reduced the ability of U87 supernatant to induce NKG2D ligand mRNA expression in monocytes (Fig. 3*A*). NKG2D ligand induction required LDH5 enzymatic activity and the presence of its substrate pyruvate (Fig. 3*B*). Using purified, native LDH5 isolated from human liver, we found that concentrations of enzymatically

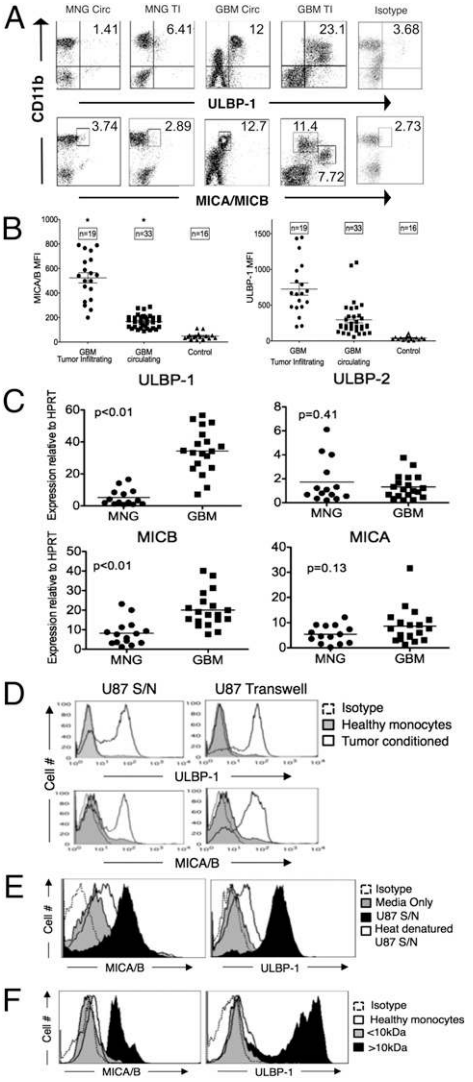

Fig. 1. Tumor-derived proteins induce expression of NKG2D ligands MICA/B and ULBP-1 on circulating and tumor-infiltrating myeloid cells. (*A*) Circulating PBMCs (Circ) and tumor-infiltrating leukocytes (TI) were isolated from patients with recurrent GBM (n = 33) or MNG (n = 14) and stained with fluorochrome-conjugated antibodies to CD45, CD11b, MICA/B, and ULBP-1. After gating on CD45$^+$ cells, CD11b$^+$ cells were evaluated for their expression of ULBP-1 (*Upper*) and MICA/B (*Lower*). (*B*) Mean fluorescence intensity (MFI) of cells stained for MICA and/or MICB (MICA/B) (*Left*) and ULBP-1 (*Right*) on CD45$^+$, CD11b$^+$ cells in CD11b^{hi} and CD11b^{lo} GBM TIMCs (n = 19) and circulating monocytes in GBM patients (n = 33) and healthy control subjects (n = 16). (*C*) mRNA from CD14$^+$ monocytes isolated from MNG (n = 14) or GBM (n = 19) was reverse transcribed and analyzed by qPCR for MICA, MICB, ULBP-1, and ULBP-2. (*D*) U87 cell-free supernatant (*Left*) was cultured with CD14-selected healthy monocytes for 48 h, and expression of MICA/B and ULBP-1 proteins was analyzed using flow cytometry. Ten thousand U87 cells were cultured in a Transwell system with CD14-selected healthy monocytes for 48 h. Monocytes then were analyzed for MICA/B and ULBP-1 expression using flow cytometry. The dashed open histograms represent isotype-matched control IgG staining, shaded histograms represent monocytes cultured in the absence of U87 supernatant (*Left*) or U87 cells (*Right*), and open histograms represent monocytes cultured with U87 supernatant (*Left*) or U87 cells (*Right*). (*E*) U87 cell supernatant was either added directly to CD14-selected healthy monocytes or heated to 95 °C for 10 min to denature proteins. Forty-eight hours later, monocytes were analyzed by flow cytometry for MICA/B and ULBP-1. (*F*) U87 cell supernatant was dialyzed to remove molecules <10 kDa, and fractions were added to healthy monocytes. Forty-eight hours later, monocytes were analyzed using flow cytometry for protein expression of MICA/B and ULBP-1.

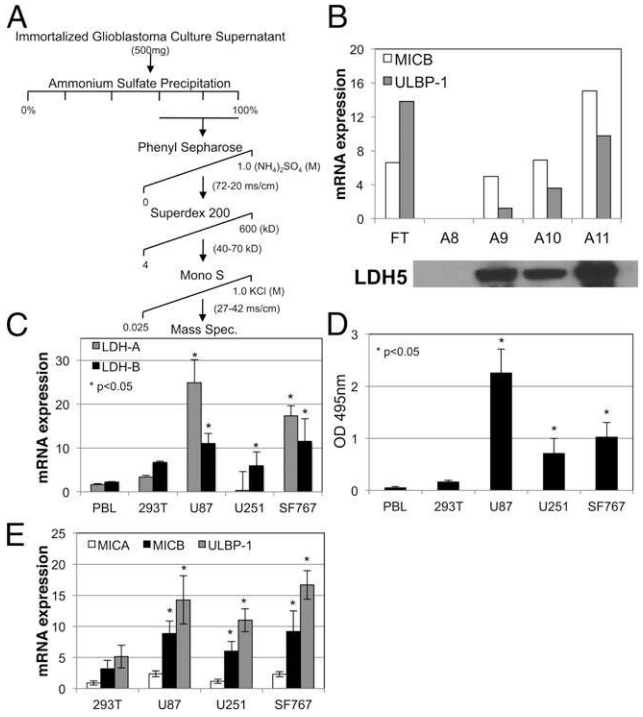

Fig. 2. Biochemical purification of tumor-derived LDH and expression in glioma cell lines. (*A*) Protein purification strategy. Five hundred milligrams of U87 glioblastoma cell line supernatant were subjected to ammonium sulfate precipitation. Active fractions were passed over a phenyl Sepharose column; then active fractions were subjected to size exclusion (Superdex 200), followed by ion exchange (MonoS) before MS analysis. (*B*) After separation by chromatography, fractions were dialyzed into PBS and cultured with healthy monocytes for 18 h. Whole-cell mRNA was reverse transcribed, and induction of MICB and ULBP-1 was analyzed relative to medium-alone controls. Western blot analysis for LDH5 was performed on 10 μg of total protein to confirm the presence of LDH5 in the active fractions. (*C*) mRNA isolated from PBMCs of healthy donors and 293T cells (as negative controls) and from U87, U251, and SF767 glioma cell lines were analyzed for expression of LDH-A and LDH-B by qPCR. (*D*) A 1:10 dilution of fresh, unfrozen glioblastoma cell line supernatant was analyzed for total LDH enzymatic activity and expressed as OD_{495} nm. (*E*) Supernatants from 293T cells and U87, U251, and SF767 glioma cell lines were cultured on monocytes from healthy donors. Eighteen hours later, mRNA was isolated, reverse transcribed, and evaluated for ULBP-1 and MICB mRNA expression relative to monocytes cultured in medium alone. $*P < 0.05$, $**P < 0.01$.

active LDH5 as low as 156 U/L significantly induced gene expression of MICB (7.85-fold induction) and ULBP-1 (13.26-fold induction) in monocytes compared with medium alone [healthy individuals have LDH5 serum concentrations of <150 U/mL (17)]. Higher concentrations of LDH5 (up to 625 U/L) induced a maximal increase in the amount of NKG2D ligand transcription. Interestingly, concentrations above 625 U/L induced less NKG2D ligand transcription, despite consistent cell viability, suggesting that high LDH5 concentrations may exhaust substrate or inhibit NKG2D ligand transcription (Fig. 3*C*)**.** LDH1, an isoform containing only β subunits, induced transcription of NKG2D ligands in monocytes, although greater concentrations of LHD1 were needed, and the amount of ligands expressed never reached that observed with LDH5 treatment (Fig. 3*D*). Transfection of 293T cells with cDNA expression vectors encoding LDH-A, LDH-B, or both LDH-A and LDH-B induced the transcription of MICB and ULBP-1 in healthy monocytes, and NKG2D ligand transcription increased if both subunits were present, potentially producing all five isoforms of LDH (Fig. 3*E*) and thus supporting redundant activity of one or more isoforms of LDH.

Having confirmed NKG2D ligand expression on the circulating monocytes in GBM patients and having identified a key tumor-derived enzyme responsible for transcriptional induction of NKG2D ligands on healthy monocytes, we considered the functional consequences of NKG2D ligand expression by monocytes. Because freshly isolated NK cells from the GBM patients demonstrate low expression of NKG2D and impaired NKG2D-dependent function (9) (Fig. S4), the patients' NK cells were cultured overnight in IL-2 to restore NKG2D expression and function and then were cocultured with autologous NKG2D ligand-bearing monocytes. As shown in Fig. 4*A*, coculture of the IL-2–activated NK cells with autologous NKG2D ligand-bearing monocytes triggered degranulation of 75.3% of NK cells and induced IFN-γ production by 20.2% of the degranulating NK cells. In contrast, NKG2D ligand-negative monocytes from MNG patients did not induce degranulation and cytokine production when cocultured with autologous IL-2–activated NK cells. Similar results were obtained when purified circulating monocytes from GBM and MNG patients were cocultured with the NK cell line, NKL (Fig. 4*A*). Antibody

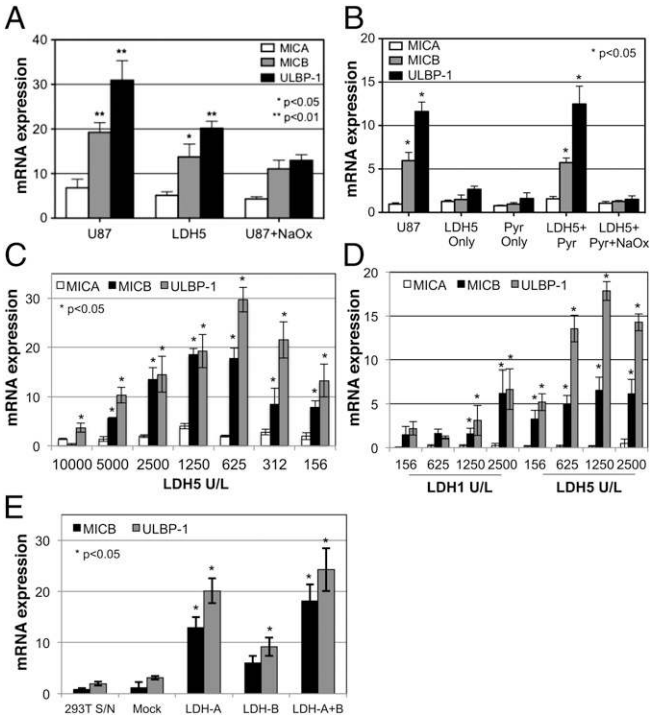

Fig. 3. Validation of LDH induction of NKG2D ligands in monocytes. (*A*) Six hundred twenty-five U/L of purified, native LDH5, fresh U87 supernatant, or fresh U87 supernatant in the presence of 100 mM sodium oxamate (NaOx) were cultured with healthy monocytes. Eighteen hours later, mRNA was isolated and analyzed for MICA, MICB, and ULBP-1 by qPCR. Values represent induction relative to monocytes cultured in medium alone. (*B*) Fresh U87 supernatant, 625 U/L of purified, native LDH5, LDH5 + 0.5 mM pyruvate (Pyr), or LDH5 + 0.5 mM pyruvate and 100 mM NaOx were cultured with healthy monocytes. Eighteen hours later, mRNA was isolated and analyzed for MICA, MICB, and ULBP-1 by qPCR. Values represent induction relative to healthy monocytes cultured in medium alone. (*C*) Healthy monocytes were cultured with native LDH5 isolated from liver cells. Eighteen hours later, MICB (gray bars) and ULBP-1 (black bars) were measured by qPCR. (*D*) Purified native LDH5 or LDH1 was cultured with monocytes from healthy donors. Eighteen hours later, MICB and ULBP-1 were measured by qPCR. Values represent induction relative to monocytes cultured in medium alone. (*E*) 293T cells were transiently transfected with a pcDNA3.1 (–) vector encoding LDH-A or LDH-B, or both constructs. Supernatant then was cultured with CD14-positively selected healthy monocytes. Eighteen hours later, MICB and ULBP-1 were measured by qPCR. Values represent induction relative to monocytes cultured in medium alone. $*P < 0.05$, $**P < 0.01$.

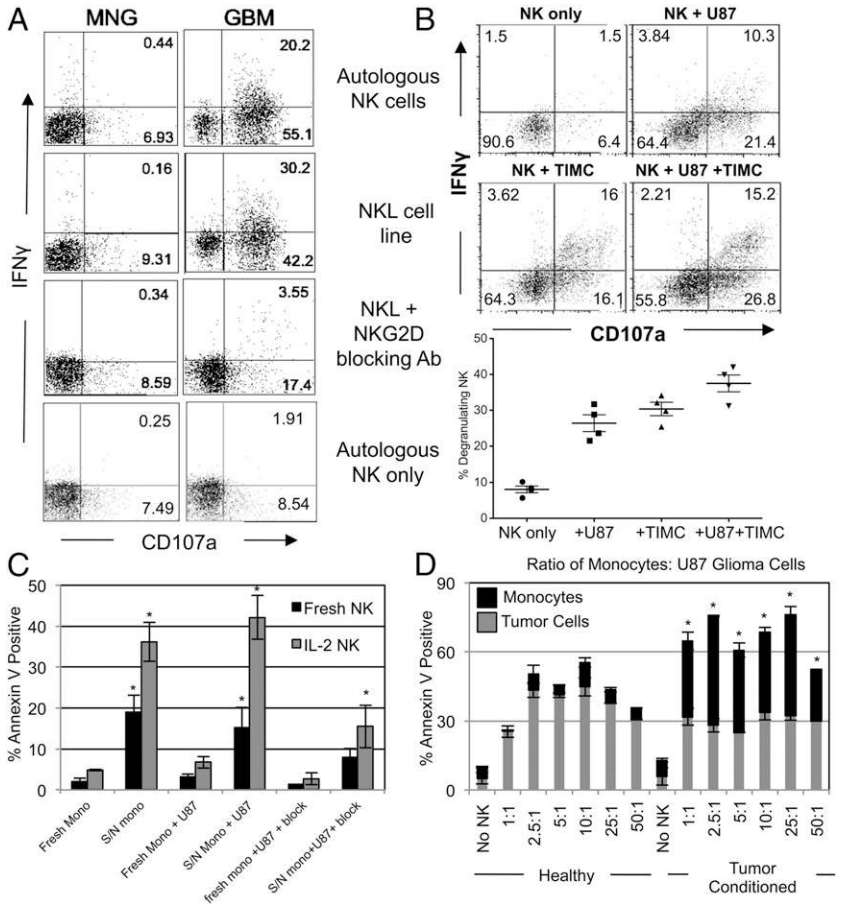

Fig. 4. Circulating monocytes and tumor-associated NKG2D ligand-expressing myeloid cells activate NK cells. (*A*) NKG2D ligand-expressing monocytes induced NK cell degranulation and cytokine production in a NKG2D-dependent fashion. Circulating monocytes were selected from patients with MNG or GBM and cocultured for 3 h with autologous IL-2–activated NK cells (*Top*), the NKL cell line (*Middle Rows*), or NKL cells plus a NKG2D-neutralizing antibody (*Bottom*) in the presence of PE-conjugated anti-CD107a and brefeldin A. Cells then were stained with CD56, CD11b, and IFN-γ. Plots represent CD56-gated populations. (*B*) IL-2–activated circulating NK cells from GBM patients were cultured at a 1:1 ratio with U87 tumor cell targets, autologous TIMCs, or both for 18 h in the presence of phycoerythrin-conjugated anti-CD107a and brefeldin A. (*Upper*) Cells were analyzed by flow cytometry for degranulation and IFN-γ production. (*Lower*) Summary of degranulation of circulating IL-2–activated NK cells in GBM patients ($n = 4$). (*C*) Freshly isolated or NK cells cultured for 24 h in 500 U/mL IL-2 isolated from healthy donors were cultured 1:1 with autologous healthy monocytes treated with control medium or with healthy monocytes pretreated for 24 h with U87 cell-free supernatant to induce NKG2D ligands (S/N mono) or were cultured 1:1:1 with healthy monocytes treated with control medium or with U87 supernatant and U87 tumor cells, and in the presence or absence of NKG2D blocking antibody. Target cell death was analyzed by staining with fluorochrome-conjugated annexin V. (*D*) Monocytes treated with control medium or U87 supernatant-treated monocytes (tumor-conditioned) isolated from healthy donors were cocultured with U87 tumor cells and autologous IL-2–activated NK cells for 18 h at the indicated ratios. Target apoptosis was measured using annexin V staining of either U87 cells (gray bars) or monocytes (black bars). $^*P < 0.05$.

blocking of NKG2D significantly decreased cytokine production and degranulation of NKL cells induced by NKG2D ligand-bearing monocytes derived from GBM patients, although degranulation over background in the NKG2D-blocked NK cells suggests the participation of additional activating receptor–ligand interactions (Fig. 4*A*).

IL-2–activated NK cells from GBM patients also responded against autologous TIMCs. In four GBM patients analyzed, 30.26% (SD ± 3.75%) of IL-2–activated peripheral blood NK cells degranulated in response to autologous TIMCs, compared with 26.4% (SD ± 4.68%) that degranulated in response to U87 tumor cells used as a positive control (Fig. 4*B*, *Upper*). The addition of both U87 cells and GBM TIMCs increased the percentage of degranulating NK cells (Fig. 4*B*, *Lower*), indicating that TIMCs expressing NKG2D ligand do not prevent NK cell activation.

We tested the ability of IL-2–activated NK cells from healthy blood donors to induce apoptosis of autologous monocytes before and after exposure to U87 glioblastoma cell supernatant to induce NKG2D ligands. NK cells induced apoptosis of autologous monocytes only after exposure to U87 supernatant, which contained LDH. This induction was reduced significantly in the presence of a NKG2D-blocking antibody (Fig. 4*C*). The addition of NKG2D ligand-expressing monocytes to cocultures of NK and U87 tumor cells demonstrated that autologous NKG2D ligand-bearing monocytes were killed more readily than U87 tumor cells and significantly reduced the percentage of apoptotic U87 cells (Fig. 4*D*).

Because LDH5 activity is lost after freezing, we measured the LDH activity in sera that was freshly isolated from four GBM patients. The amount of LDH measured in the patients' sera was greater than in supernatants harvested from the U87 tumor cell line used as control (Fig. 5*A*). Importantly, when cultured with healthy monocytes, the GBM patient sera induced transcription of MICB (6.37- to 14.54-fold over medium alone) and ULBP-1 (10.34- to 22.32-fold over medium alone) (Fig. 5*B*), suggesting that the LDH in the patients' sera is able to induce NKG2D ligand expression on healthy monocytes. In longitudinal studies, we observed that the mean fluorescent intensity of ULBP-1 staining of monocytes isolated from all GBM patients declined after tumor resection (Fig. 5 *C* and *D*); interestingly, however, in four of the six patients MICB, which was expressed in lower amounts, remained unchanged. In studies examining circulating monocytes in patients with other cancers, we detected NKG2D ligands on subsets of patients with hepatocellular carcinoma ($n = 34$), prostate cancer ($n = 14$), or breast cancer ($n = 27$) (Fig. 5*E*).

Discussion

Understanding the tumor microenvironment in patients has the potential to improve experimental therapy design. In patients, the impact of the tumor microenvironment on the immune system is highly complex, and ex vivo observations are the result of the collective influence of many cell types and the proteins they produce. Our in vitro study identifies a previously unidentified mechanism that may contribute to tumor immune escape in patients with GBM based on the observation that circulating monocytes express the activating ligands for the NK cell receptor, NKG2D. Although the relationship between tumor-derived LDH5 and NKG2D ligand expression on circulating monocytes in vivo will be only one of many consequences of the tumor on immune responses, we present several previously unreported findings that may provide insight into tumor immune escape in patients. Specifically, our data demonstrate: (*i*) expression

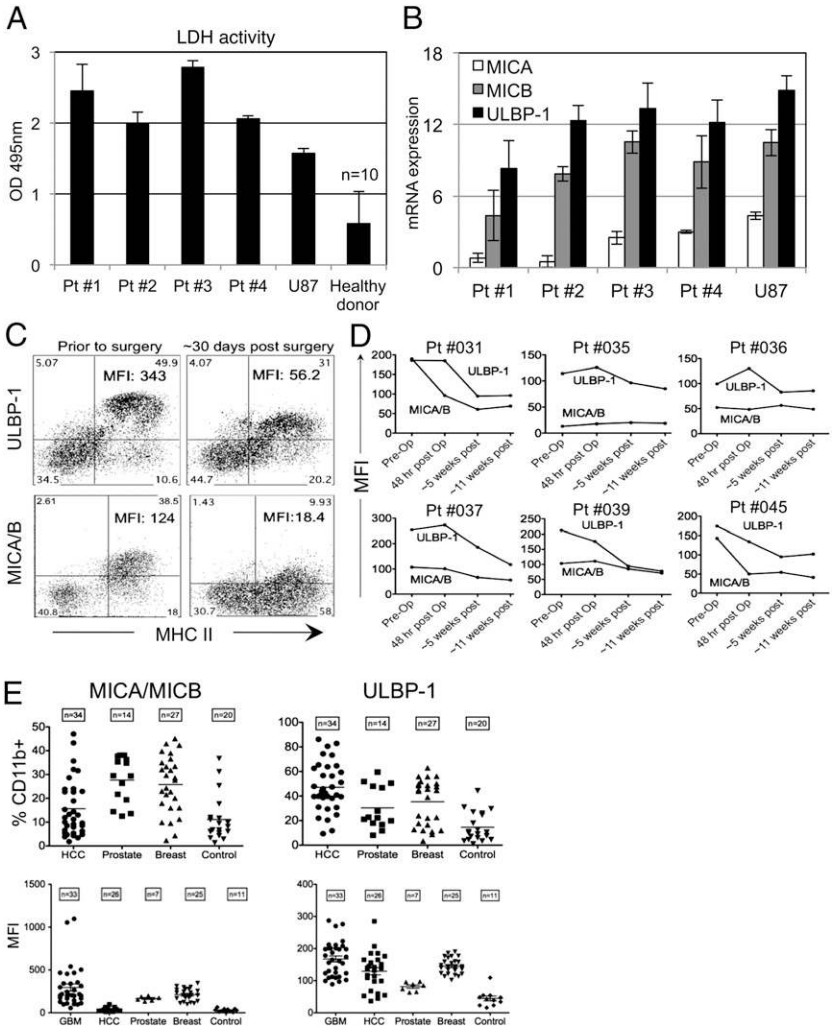

Fig. 5. Freshly isolated GBM patient sera contain active LDH and induce NKG2D ligands, which decrease following a reduction in tumor burden. (*A*) Freshly isolated (nonfrozen) sera from four patients with GBM and healthy donors ($n = 10$) were analyzed for total active LDH isoenzymes and analyzed for colorimetric change at OD_{495} nm. (*B*) Monocytes isolated from a healthy donor were incubated with freshly isolated (nonfrozen) sera from four patients with GBM or with U87-conditioned supernatant. Eighteen hours later MICA, MICB, and ULBP-1 were analyzed by qPCR. mRNA expression is shown relative to monocytes cultured in medium alone. (*C*) Flow cytometry analysis of MICA/B and ULBP-1 surface protein expression on circulating monocytes ($CD45^+$, $CD11b^+$, HLA-DR^+) from a patient before surgical resection of tumor and 33 d after gross total tumor resection. (*D*) Longitudinal analysis of MICA/B and ULBP-1 expression using flow cytometry on circulating monocytes in recurrent GBM patients following tumor resection. (*E*) PBMCs were isolated from patients with hepatocellular carcinoma (HCC), prostate cancer, or breast cancer or from healthy control subjects. $CD45^+$ $CD11b^+$ monocytes were analyzed for MICA/B and ULBP-1 expression by flow cytometry and displayed as percent positive relative to isotype-matched Ig controls (*Upper*). Patients with a percentage of MICB and ULBP-1 expressing monocytes greater than the mean percentage of control patients were then analyzed for mean fluorescence intensity of MICB and ULBP-1 expressing monocytes (*Lower*).

of two ligands for NKG2D, MICB and ULBP-1, on circulating monocytes, a finding that extends to subsets of patients with breast, prostate, and HCV-induced hepatocellular carcinoma; (*ii*) NK cell degranulation in response to autologous NKG2D ligand-bearing monocytes and tumor-infiltrating myeloid cells; (*iii*) identification of an extracellular, tumor-derived, metabolic enzyme that is sufficient to induce transcription of MICB and ULBP-1; and (*iv*) in a small cohort of patients with recurrent GBM, a decrease in the amount of NKG2D ligand expression on circulating monocytes within 5 wk of surgical reduction of the tumor, suggesting that NKG2D ligand expression is dependent on the presence of a tumor mass.

Our finding that LDH induces NKG2D ligands on myeloid cells represents one of many mechanisms that tumors may use to disrupt immune surveillance dependent on the NKG2D pathway. For example, previous studies by our group and others demonstrate that TGF-β can decrease NKG2D expression on NK cells in vitro (9, 18, 19) and therefore may contribute to decreased NKG2D expression on circulating NK cells in patients. Additional soluble tumor-derived proteins in addition to LDH may induce NKG2D ligand expression on myeloid cells. Our biochemical purification strategy suggests that other, as yet unidentified, factors can induce NKG2D ligand expression on monocytes from healthy donors, and previous studies have described NKG2D ligand expression following DNA damage, viral infection, and heat shock (5).

The NKG2D pathway serves an important role in host defense against viral pathogens and cancer. Viruses have evolved specific mechanisms to evade recognition by NKG2D-bearing NK cells and T cells. For example, mouse and human cytomegalovirus possess several genes encoding viral proteins that target and degrade NKG2D ligands before they are displayed on the surface of infected cells (7). Similarly, the E3/19K protein encoded by adenovirus retains MICA and MICB within the cytoplasm of infected cells (20), leading to their degradation, and cowpox and monkeypox viruses produce a soluble antagonist of NKG2D (21). Prior studies have reported that tumors also may evade detection by NKG2D by releasing NKG2D ligand-containing exosomes (22) or by secreting high amounts of TGF-β, which can block transcription of NKG2D receptor in T cells and NK cells (9, 23). Here, we report another mechanism whereby tumors can evade NKG2D-dependent immunity, which works systemically. In prior studies in which soluble NKG2D ligands have been detected in the sera of cancer patients, it has been assumed that the source of these ligands is the shedding of the proteins or exosomes by the tumor cells themselves. Our unexpected finding is that these NKG2D ligands might be derived from host immune cells rather than the tumors. Prior studies have reported that cancer patients with elevated amounts of LDH in sera have a poor prognosis (13–15, 24). Although this poor prognosis might simply reflect larger tumor burdens, the secretion of LDH also might contribute to the immune evasion of these cancers by its induction of NKG2D ligands on host myeloid cells. In a recent

paper, Husain et al. (25) have demonstrated that transfection of mouse tumor cells to overexpress LDH resulted in more aggressive tumor growth; however, in this study there was no evidence for induction of NKG2D ligands by host cells or for any modulation of the NKG2D receptor on the host's NK cells, implying another mode of action. Indeed, we have observed no direct effect of LDH or its product lactate on NKG2D expression or function using human NK cell effectors.

We hypothesize that the induction of MICB and ULBP-1 on host myeloid cells, both locally and systemically, by tumor-derived LDH provides a mechanism to subvert NK cell and $CD8^+$ T-cell responses against the tumor. We and others have demonstrated that chronic exposure of NK cells to NKG2D ligand-bearing cells in vivo and in vitro results in down-modulation of the NKG2D receptor on NK cells and inactivation of the NKG2D pathway (7, 26–30). Down-modulation of NKG2D on NK cells is mediated much more efficiently by cell surface-expressed NKG2D ligands than by soluble NKG2D ligands, likely because of the clustering and cross-linking of the NKG2D receptor by the cell membrane-associated NKG2D ligands. The ability of tumor-derived LDH to induce MICB and ULBP-1 on host myeloid cells systemically, as evidenced by detection of NKG2D ligand-bearing monocytes in the circulation of GBM patients in whom the tumor remains localized in the CNS, might down modulate NKG2D on NK cells even before their migration into the tumor site. Studies currently are underway to determine how LDH induces MICB and ULBP-1 on monocytes and whether the detection of NKG2D ligands on circulating monocytes in cancer patients will provide a useful biomarker for disease stage or relapse. Collectively, these findings reveal a previously unidentified mechanism of immune evasion by tumors and provide a potentially important target for therapeutic interventions.

Methods

Details of patient samples, flow cytometry, functional assays, PCR analysis, and statistical evaluation are presented in *SI Methods*. Primer sequences are listed in Table S1. Reagents, vendors, and protocols are included in *SI Methods*. Details of patient samples, flow cytometry, functional assays, PCR analysis, statistical evaluation are presented in *SI Methods*. Primer sequences are listed in Table S1. Additional questions pertaining to methods, protocols and reagents are available upon request.

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Supporting Information

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SI Methods

Patient Samples. Tumor tissue and blood were collected from patients with glioblastoma multiforme (GBM) during surgical resection. Blood also was collected from patients free of malignant glioma tumor burden during procedures such as meningioma resection. Meningioma-resected patients were used as negative controls for two reasons. First, because blood was collected from both patient populations at the time of surgery, all patients had been subjected to a standard algorithm of preoperative management including, but not limited to, antibiotic administration, a bolus of dexamethasone, and mannitol, as well as other medications including anticonvulsant drugs. Additionally, healthy controls are unavailable because, the patients must undergo intracranial surgery to obtain tumor-infiltrating lymphocytes. All patients gave informed consent for sample acquisition under the University of California San Francisco (UCSF) Internal Review Board (IRB)-approved Brain Tumor Research Center protocol CHR #10–01271. Longitudinal GBM patient samples were obtained from patients on a Phase I/II clinical trial for autologous heat shock protein vaccination that was approved by the UCSF IRB and was done in accordance with the Declaration of Helsinki and guidelines for Good Clinical Practice. The clinical trial is registered at www.clinicaltrials.gov. Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained from leukocyte reduction Pall filters (Blood Centers of the Pacific) and processed as described below.

Tumor-Infiltrating and Circulating Mononuclear Cell Isolation. PBMCs were isolated from whole blood by a Ficoll–Paque Plus density gradient centrifugation (GE HealthCare). Tumor-infiltrating lymphocytes were isolated from tissue samples by mincing tumor samples and treating with 1 mg/mL collagenase D (Sigma-Aldrich) in PBS for 30 min followed by Percoll density gradient centrifugation (Sigma-Aldrich) as previously described (1).

Cell Selections. Monocytes and natural killer (NK) cells were isolated using NK cell- or monocyte-positive selection kits according to the manufacturer's instructions (StemCell Technologies, Inc.).

Cell Lines. 293T and the glioma cell lines U87, SF767, and U251 were obtained from the UCSF Brain Tumor Research Center. Cell lines were cultured in DMEM H-21 with 10% (vol/vol) FBS and 1% penicillin and streptomycin.

NKG2D Ligand Induction on Freshly Isolated Monocytes from Healthy Donors. U87 tumor-conditioned medium was generated by culturing U87 cells in complete RPMI medium (RPMI-1640, 25 mM Hepes, 2.0 g/L $NaHCO_3$ supplemented with 2% (vol/vol) FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, and 10 mM nonessential amino acids) for 48 h when tumor cells were confluent. The U87-conditioned medium was diluted 1:1 with fresh complete RPMI medium to replenish nutrients and was used in subsequent assays for culturing with freshly isolated peripheral blood monocytes for 18–72 h.

Purification and Identification of Natural Killer Group 2, Member D Ligand-Inducing Factor. The Natural killer group 2, member D (NKG2D) ligand-inducing factor present in U87-conditioned medium was identified by chromatographic separations and assaying fractions for their ability to induce transcription of MICB and ULBP-1 when cultured with monocytes isolated from healthy donors. Briefly, 12 L of U87 medium supernatant was concentrated 10-fold using Amicon Ultra-15 protein concentrators with a 10-kDA restriction (Millipore). Proteins then were precipitated with 60–100% saturated ammonium sulfate. Active fractions were purified further on a Phenyl Sepharose column (GE Healthcare) by loading in 1 M ammonium sulfate and eluting with a 100 mM Na_2HSO_4–1 M Na_2HSO_4 gradient. The NKG2D ligand-inducing activity eluted in the range of 72–20 ms/cm and was purified further on a Superdex-200 column (GE Healthcare) and eluted in the range of 40–70 kDa. The activity-containing fraction was run over a MonoS column (GE Healthcare) using a binding buffer of 25 mM KCl, 50 mM sodium acetate, pH 5.0, and the elution buffer, 1 M KCl, 50 mM sodium acetate, pH 5.0. The activity eluted between 27 and 42 ms/cm. The purified activity-retaining fraction was analyzed in-gel and in-solution by MS-MS by the UCSF Mass Spectrometry Facility and the National Bio-Organic Biomedical Mass Spectrometry Resource Center.

Flow Cytometry. Cells were stained for 25 min on ice with monoclonal antibodies and washed in PBS with 2% (wt/vol) BSA. FITC-conjugated anti–ULBP-1, phycoerythrin (PE)-conjugated anti-MICA/B, PE-Cy7–conjugated anti-CD56, FITC-conjugated anti-CD3, allophycocyanin (APC)-conjugated anti-NKG2D, PE-Cy7–conjugated anti-CD14, APC-conjugated anti–HLA-DR, Pacific Blue-conjugated anti-CD11b, APC-conjugated anti–IFN-γ, FITC-conjugated anti-granzyme B, PE-conjugated anti-perforin, and APC- or PE-conjugated anti-CD107a were purchased from BD Pharmingen, R&D Systems, Inc., or eBioscience. Samples were analyzed using a BD FACSCalibur with Cell Quest Pro software, and data were analyzed using FlowJo software (TreeStar). Fluorochrome-conjugated isotype-matched control Igs were used to detect specific staining of cell populations.

Quantitative PCR. Whole-cell mRNA was extracted by using the RNeasy Mini Kit (Qiagen) and then was converted to cDNA using random hexamer priming and SuperScript III reverse transcriptase (100–250 ng RNA per reaction) (Invitrogen). PCR amplification was performed with SYBR Green master mix (5–10 ng cDNA per reaction) (Applied Biosystems) using an iQ5 Real-Time PCR thermal cycler (Bio-Rad). Reactions were performed in duplicate. Cycle threshold (Ct) values of NKG2D ligands were normalized to expression levels of hypoxanthine phosphoribosyltransferase and displayed as relative expression units. Primer sequences are listed in Table S1.

NK Cell Functional Assays. Freshly isolated NK cells were used in assays immediately after selection or were activated overnight with 500 U/mL recombinant IL-2 (National Cancer Institute Biological Resources Branch). Unless otherwise indicated, NK cells were cultured at a 1:1 ratio with monocytes or glioma tumor cells in the presence of PE-conjugated anti-CD107a for 3–18 h. Intracellular protein expression of cytokines and cytolytic granules was measured by adding brefeldin A to cultures 2–6 h before the end of the assay, and subsequently cells were fixed using 1% paraformaldehyde in PBS and then were stained for intracellular IFN-γ, perforin, and/or granzyme B. Target cell apoptosis was measured by staining with antibodies, listed above to distinguish tumor cells, NK cells, and monocytes. Tumor cells were defined as $CD45^-$, MHC class I^+ cells; monocytes were defined as $CD45^+$ $CD14^+$ MHC class II^+ cells; and NK cells were defined as $CD45^+$, $CD3^-$, $CD56^+$ cells. Cells were stained for extracellular annexin V using Annexin Staining buffer (BD Pharmingen) to detect apoptotic cells.

Lactate Dehydrogenase Assays. Induction of NKG2D ligands was evaluated by using purified native lactate dehydrogenase (LDH) isoenzyme 5 (MyBioSource or Abcam) at the indicated concentrations, expressed in enzymatically active units per liter. Monocytes were cultured for 24 h in RPMI complete medium.

LDH Inhibition. U87 glioma cells were washed and cultured for 24 h in the presence of 20 mM sodium oxamate (Sigma-Aldrich). Sodium oxamate then was dialyzed from the supernatant, and supernatant was added to monocytes from healthy donors for 24 h. Cells were analyzed for induction of MHC class I polypeptide-related sequence A (MICA), MHC class I polypeptide-related sequence B (MICB), and UL16-binding protein 1 (ULBP1) mRNA.

LDH Expression. *LDH-A* and *LDH-B* cDNA were amplified from RNA isolated from U87 cells using the following primer sequences: LDH-A 5′: CGGCCAGAATTCCGCCACCATGGCAACTCTAAAGGATCAGCTGA (5′ Eco RI site); LDH-A 3′: CGCCGACTCGAGTAA TAAAATTGCAGCTCCTTTTGGATC (3′ Xho I site); LDH-B 5′: CGCCGAGGTACCCGCCACCATGGCAACTCTTAAGGAAAAACTC (5′ Kpn I site); LDH-B 3′: CGCCGAGAATTCTAATCACAGGTCTTTTAGGTCCTTCTG (3′ Eco RI site). Restriction-digested cDNA was cloned into pcDNA3.1^{+} vector, and plasmids were amplified in Top10 competent *Escherichia coli*. Sequence analysis identified positive clones, and plasmid DNA was purified using a Qiagen MaxiPrep kit. One microgram of DNA was transiently transfected into 293T cells using Lipofectamine 2000. Seventy-two hours later, supernatant was analyzed to confirm LDH activity and assayed for NKG2D ligand induction on monocytes isolated from healthy donors.

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LDH Activity Assay. Supernatants and patient sera were analyzed for LDH enzymatic activity using a commercially available colorimetric assay according to the manufacturer's instructions (Sigma-Aldrich). Standard curves were generated using purified native LDH5.

Acquisition of PBMCs from Patients with Other Solid Tumors. Five milliliters of peripheral blood was collected from (*i*) breast cancer patients using the IRB-approved protocol UCSF CHR #H8409-27022–05 in collaboration with Michael Campbell; (*ii*) prostate cancer patients in collaboration with Larry Fong and John Kurhanewicz (IRB approval UCSF CHR #H7579-03002); and (*iii*) patients with hepatocellular carcinoma in collaboration with Stewart Cooper from the Ibrahim El-Hefni Liver Biorepository at California Pacific Medical Center [California Pacific Medical Center (CPMC) IRB approval CPMC #25.117 and 27.102].

Statistical Analysis. Data shown in all figures were collected from at least two independent experiments performed in triplicate. Representative raw experimental data are shown to clarify gating strategy and are summarized in Excel format. Determinations of statistical significance were made based on integrated experimental data. Statistical significance was determined by using a two-tailed Student *t* test with $P < 0.05$ as determined by analysis in Prism GraphPad software or Excel. Error bars represent ± SD. Quantitative PCR was analyzed using the Pfaffl method, and statistical significance was determined using a nonparametric Wilcoxon two-group test.

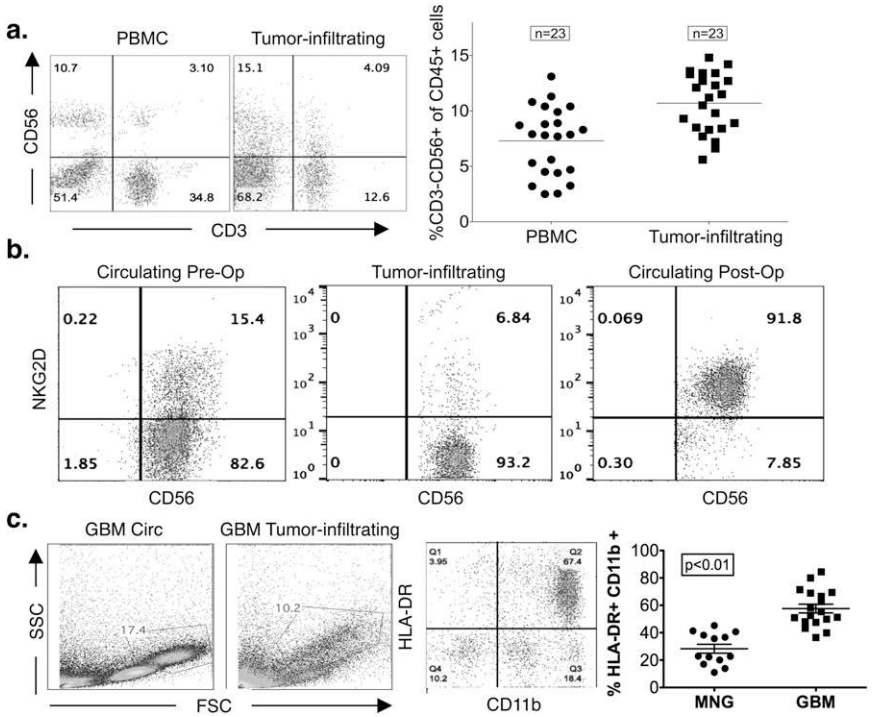

Fig. S1. Innate immune cell infiltration of glioblastoma tissue. (*A*) Following collagenase digestion of freshly isolated tumor tissue, circulating PBMCs and tumor-infiltrating leukocytes were gated, stained for CD45, CD3, and CD56, and analyzed by flow cytometry. Plots represent CD45-gated cells. (*B*) $CD3^{-}CD56^{+}$ PBMCs and tumor-infiltrating lymphocytes isolated from a representative GBM patient were stained for NKG2D before surgery and 34 d following a >90% reduction in tumor burden. (*C*) Tumor-infiltrating lymphocytes were isolated from patients with meningioma (MNG, $n = 13$) or GBM ($n = 18$) and were stained for CD45, HLA-DR, and CD11b and analyzed by flow cytometry. Dot plots represent $CD45^{+}$-gated cells.

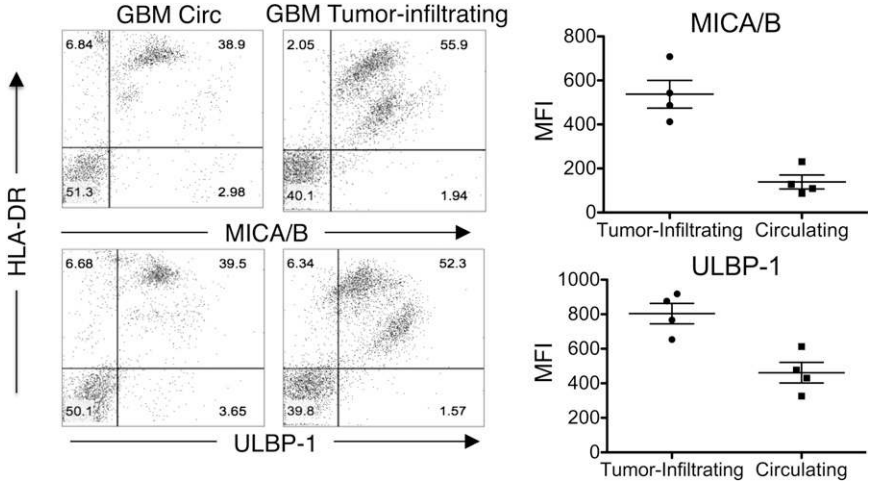

Fig. S2. Circulating and tumor-infiltrating myeloid cells isolated from patients with newly diagnosed GBM express NKG2D ligands, independently of steroids, chemotherapy, and radiation. Tumor-infiltrating myeloid cells and circulating monocytes were isolated from patients before surgical or therapeutic interventions ($n = 4$) and were analyzed for the expression of MICA/B (*Upper*) and ULBP-1 (*Lower*). Dot plots represent gating on $CD45^{+}CD11b^{+}$ cells.

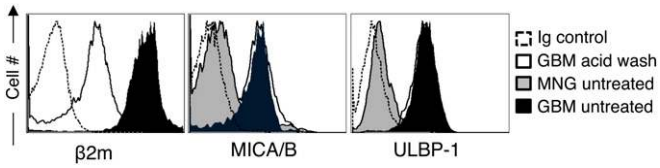

Fig. S3. NKG2D ligands are not passively acquired by monocytes. Monocytes were isolated from patients with GBM ($n = 2$), treated with HCl-acidified medium (pH 3.5) for 5 min, and then washed. Monocytes then were stained with antibodies for CD45, CD11b, HLA-DR, and β2-microglobulin (a protein component of the MHC class I complex that is removed during acid washing) (*Left*), MICA/B (*Center*), and ULBP-1 (*Right*) and were analyzed by flow cytometry.

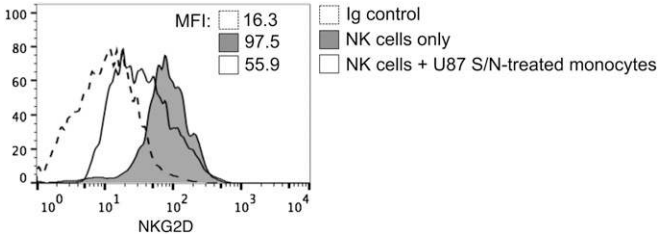

Fig. S4. NK cells from healthy donors have decreased NKG2D expression following coculture with autologous NKG2D ligand-expressing monocytes. Monocytes from healthy donors were isolated by $CD14^+$ cell selection using antibody-coated magnetic beads and were cultured with U87 supernatant for 48 h to induce NKG2D ligand expression. Autologous peripheral blood NK cells were enriched using negative selection and cultured overnight with IL-2 (500 U/mL). NK cells were cultured alone (shaded histogram) or with monocytes treated with U87-conditioned medium (open histogram) for 24 h, and NK cells were stained for CD3, CD56, and NKG2D. Histograms represent cells gated on $CD3^-CD56^+$ cells relative to isotype-matched control Ig staining (dashed histogram).

Table S1. Primers used in this study

Gene	Forward primer	Reverse primer
ULBP-1	TGCAGGCCAGGATGTCTTGT	CATCCCTGTTCTTCTCCCACTTC
ULBP-2	CAGAGCAACTGCGTGACATT	GGCCACAACCTTGTCATTCT
ULBP-3	GGATTTCACACCCAGTGGAC	GCCTCTTCTTCCTGTGCATC
ULBP-4	GGCTCAGGGAATTCTTAGGG	CATTTTGCCACCAGACACAG
ULBP-5	CAAGACAGTCACACCCGTCA	AAGCCATCCTGTGCAGTCTC
ULBP-6	TGACATCACCGTCATCCCTA	TGCTCACAAGACATCCTTGC
MICA	ACAATGCCCCAGTCCTCCAGA	ATTTTAGATATCGCCGTAGTTCCT
MICB	TGAGCCCCACAGTCTTCGTTA	CCTGCGTTTCTGCCTGTCATA