Meningiomas are the most common adult primary tumor of the central nervous system, but there are no known effective medical therapies for recurrent meningioma, particularly for WHO grade II and III tumors. Meningiomas arise from the meninges, located outside the blood-brain barrier, and therefore may be directly targeted by antibody-mediated immunotherapy. We found that PD-L1 was highly expressed in multiple human malignant meningioma cell lines and patient tumor samples. PD-L1 was targeted with the anti-PD-L1 antibody avelumab and directed natural killer cells to mediate antibody-dependent cellular cytotoxicity (ADCC) of PD-L1-expressing meningioma tumors both in vitro and in vivo. ADCC of meningioma cells was significantly increased in target cells that upregulated PD-L1 expression and, conversely, abrogated in tumor cells that were depleted of PD-L1. Additionally, the high-affinity natural killer cell line, haNK, outperformed healthy donor NK cells in meningioma ADCC. Together, these data support a clinical trial designed to target PD-L1 with avelumab and haNK cells, potentially offering a novel immunotherapeutic approach for patients with malignant meningioma.
Efficient ADCC- killing of meningioma by avelumab and a high-affinity natural killer cell line, haNK

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Abstract

Meningiomas are the most common adult primary tumor of the central nervous system, but there are no known effective medical therapies for recurrent meningioma, particularly for WHO grade II and III tumors. Meningiomas arise from the meninges, located outside the blood-brain barrier, and therefore may be directly targeted by antibody-mediated immunotherapy. We found that PD-L1 was highly expressed in multiple human malignant meningioma cell lines and patient tumor samples. PD-L1 was targeted with the anti-PD-L1 antibody avelumab and directed natural killer cells to mediate antibody-dependent cellular cytotoxicity (ADCC) of PD-L1-expressing meningioma tumors both in vitro and in vivo. ADCC of meningioma cells was significantly increased in target cells that upregulated PD-L1 expression and, conversely, abrogated in tumor cells that were depleted of PD-L1. Additionally, the high-affinity natural killer cell line, haNK, outperformed healthy donor NK cells in meningioma ADCC. Together, these data support a clinical trial designed to target PD-L1 with avelumab and haNK cells, potentially offering a novel immunotherapeutic approach for patients with malignant meningioma.
Introduction

Meningiomas are the most common primary tumor of the CNS (36.1%), occurring in 7.61 per 100,000 individuals (1). Most cases are low-grade (WHO grade I), and durable responses or cures can be achieved with surgery alone. However, atypical (WHO grade II) and anaplastic (WHO grade III) meningiomas frequently recur following resection, and recurrent tumors are often more aggressive (2, 3). The 5-year overall survival rate for patients with anaplastic meningioma is 35%-61% (4-6), and no effective treatment options are available for patients with these aggressive tumors.

Immunotherapy has become an important option for patients with solid tumors such as melanoma (7). However, anti-tumor immunity is hindered by PD-L1 on immune-suppressing cells and tumors. Antibody-mediated PD-L1 blockade has shown clinical benefit in locally advanced or metastatic bladder cancer, metastatic non-small-cell lung cancer (NSCLC), melanoma, renal-cell cancer, and ovarian cancer (8-11). Avelumab, a fully human anti-PD-L1 IgG1 antibody, has shown clinical efficacy against solid tumors and NSCLC (12, 13). In addition to blocking the immune-suppressing function of PD-L1, avelumab can mediate antibody-dependent cellular cytotoxicity (ADCC) of tumor cells by cytotoxic T cells and natural killer (NK) cells (14, 15).

Tumor killing by ADCC is induced when CD16 on cytotoxic effector cells, such as NK cells, ligates the Fc portion of target-bound antibody. NK cells are potent killers that contain large amounts of perforin and granzymes within cytotoxic granules. In addition to direct tumor cell lysis, NK cells can release cytokines, including interferon gamma (IFNγ), that further enhance the adaptive immune response. Yet, NK function and activity vary greatly among individuals.
due, in part, to genetic polymorphisms of Fc receptors that modulate NK activation. Patient NK cells that express the high affinity CD16 valine (V) allele (V/V), experience better clinical outcomes than those with the lower affinity V/phenylalanine (F) and F/F genotypes. Reports vary as to what percentage of the population is of the V/V genotype, but most studies report approximately 10-20% (16-20).

The activated NK line (aNK) NK-92 (21-23) has been shown to mediate ADCC in vivo. This parental line was engineered to express the high-affinity (ha) Fc receptor (V/V), enhancing cytotoxic activity and generating the haNK cell line. In addition, haNK cells were engineered to express IL-2 and granzyme B. As with healthy donor NK cells, haNK cells do not require recipient matching, making these cells an attractive and continuous source of off-the-shelf cytotoxic mediators in immunotherapy.

PD-L1 has been detected on meningioma cells from surgical resections (24, 25). Here, we demonstrate that patient-derived malignant cell lines constitutively express high levels of PD-L1 under basal conditions compared to low-grade meningioma and other cancer cell lines. The PD-L1-specific antibody, avelumab, directed meningioma cell lysis by healthy donor NK cells. Tumor killing was mediated by ADCC, as it was prevented by CD16 blockade and enhanced with healthy donor NK cells expressing the high-affinity CD16 receptor (haNK). The engineered aNK cell line, haNK, lysed meningioma cells more effectively than healthy donor NK cells when cultured with avelumab. Tumor cell killing was further enhanced with biologically relevant conditions of IFNγ exposure or hypoxia that upregulated PD-L1 surface expression and was abrogated when PD-L1 was deleted from tumor lines. These findings translated into a survival benefit in vivo using an orthotopic skull-based model or subcutaneously implanted meningioma
cells and treating with avelumab and haNK. Our studies suggest that an immunotherapy using avelumab-directed ADCC against malignant meningioma may offer a novel treatment approach for this cancer with no currently available effective treatment.

**Results**

Basal PD-L1 expression was quantified by flow cytometry on two prostate cancer, two breast cancer, two lung carcinoma, four chordoma, three low-grade meningioma and five malignant meningioma cell lines (Fig. 1A-1B). PD-L1 expression was highest on malignant meningioma cell lines and two-fold greater than that of the next highest cell line, HCC4006. Meningioma expression was next confirmed in tumor blocks from patients with meningioma. Diffuse PD-L1 positive cells were detected in lower grade samples whereas clusters of PD-L1 positive cells and increased overall positive staining on tumor cells was detected in higher grade specimens (Fig. 1C). PD-L1 was previously used to target chordoma tumor cell lines for ADCC by using NK cells and the anti-PD-L1 antibody avelumab (14). In Fujii, et al, stronger PD-L1 expression on chordoma tumor cells correlated with greater susceptibility to avelumab-directed ADCC of other tumor cells by peripheral blood mononuclear cells (PBMC) and NK cells (15).

Because the average PD-L1 intensity of meningioma was over 10-fold greater than in chordoma, its potential to target meningioma for ADCC with avelumab was further investigated.

The PD-1/PD-L1 axis has been reported to be potentially suppressive to NK cell function (26-29). We examined healthy donor unactivated NK cells or HaNK cells for the surface expression of PD-L1 (CD274), PD1 (CD279), and additional suppressive markers NKG2/KLRD1 (CD94), and KIR2DL1/2/3 (CD158), as well as B7-1 (CD80), (Supplemental Table 1). For 4 healthy
donors, the NK cell expression of PD-L1 ranged from 3% to 21%. HaNK cells had a surface expression of 60%. We hypothesized that ADCC mediated by the anti-PD-L1 IgG1 avelumab would ameliorate potential suppression (30). Avelumab or healthy donor NK cells as single agents induced minimal meningioma cell lysis as measured by indium release from tumor cells. However, the combination of avelumab with healthy donor NK cells lysed IOMM, GAR, and CH-157 cell lines with increasing effector to target ratios (Fig. 2A). Importantly, these NK cells were not preactivated with cytokines. The degree of lysis was dependent on the donor utilized and potentially affected by the expression of inhibitory ligands on a tumor cell. We interrogated IOMM cells for surface expression of molecules potentially inhibitory to NK cells: PD1 (CD279), PD-L1 (CD274), HLA-ABC, MIC A/B, and CEACAM1 (CD66 a/c/e), (Supplemental Table 1B). These cells expressed no PD-1, MIC A/B, or CEACAM1, however, they expressed substantial levels of PD-L1 and HLA-ABC. Despite this, NK cells were able to effectively lyse this tumor in the presence of avelumab (Fig. 1A).

To confirm that meningioma cells were lysed by NK cells via ADCC, a CD16 (FcγRIIIa) blocking antibody was used to prevent CD16 on NK cells from binding the Fc portion of avelumab. Blocking CD16 resulted in an 80% decrease in meningioma lysis, indicating that cell lysis was due to ADCC (Fig. 2B).

A single nucleotide polymorphism (SNP) of CD16 at position 158 impacts the affinity of CD16 for the Fc region of an antibody. NK cells from individuals with a valine (V) at position 158 (FcγRIIIa-158V) bind the Fc region of IgG1 with greater affinity than those with phenylalanine (F) at this position (FcγRIIIa-158F) (31). A stronger binding affinity of the Fc receptor on cytotoxic cells translates into a higher magnitude of ADCC response toward antibody-coated target cells.
This has clinical implications, as FcγRIIIa-158V/V expression correlates with longer progression-free survival for patients receiving cetuximab or trastuzumab (16, 19). In all meningioma lines tested, NK cells from V/V donors mediated greater ADCC than NK cells from F/F donors with fold increases of 1.74 (IOMM), 1.58 (CH-157), 1.28 (GAR), 1.26 (JEN), and 1.24 (SAM) for V/V relative to F/F (Fig. 2C).

Healthy donor NK cells were next compared to the haNK cell line for cytotoxic activity against IOMM meningioma cells. The haNK cell line was engineered from the non-Hodgkin lymphoma NK-92 cell line to express the high-affinity CD16 receptor and secrete high levels of IL-2. These modifications confer advantages of IL-2-mediated haNK survival and enhanced ADCC function via the high-avidity CD16 receptor. The haNK cell line lysed malignant meningioma cells when incubated with avelumab in vitro and was more effective at ADCC compared to healthy donor NK cells (Fig. 2D). Therefore, haNK cells were preferentially selected for further investigation.

Increased surface expression of PD-L1 was previously shown to enhance the efficacy of avelumab-directed ADCC against chordoma, a cancer found in the skull base and spine (14). PD-L1 is upregulated by hypoxia and IFNγ (32, 33), which are associated with advanced grade in meningioma (34). Meningioma cell lines cultured under hypoxia upregulated total PD-L1 protein compared to basal levels (Fig. 3A). Similarly, surface PD-L1 and total PD-L1 protein were increased when cells were cultured in the presence of IFNγ (Fig. 3B-3C). As with chordoma, ADCC was increased when meningioma cells were cultured under biologically relevant conditions that upregulated PD-L1 (Fig. 3D-3E). These data indicate that avelumab-mediated
ADCC by haNK cells may be increased in vivo in meningioma tumors that upregulate PD-L1 during hypoxia or IFNγ exposure.

The ability of haNK + avelumab to mediate ADCC was next investigated in vivo using IOMM meningioma cells that were stably transduced with GFP-luciferase for longitudinal imaging, detection of viable tumor, and assessment of tumor burden (Fig. 4A). Athymic mice implanted with IOMM meningioma in a skull-base orthotopic model had a median survival of 21 days with vehicle treatment, similar to avelumab treatment alone (median survival of 20 days) (Fig. 4B). Mice treated with haNK demonstrated a survival benefit (median survival of 27.5 days), and this was further enhanced in mice treated with avelumab and haNK cells (median survival of 29 days). The combination of avelumab + haNK also extended survival of mice bearing orthotopic, intracranial malignant meningioma tumors compared to avelumab alone. However, the rapid time to euthanasia in this intracranial model restricted the available window to evaluate treatment efficacy. Therefore, a subcutaneous IOMM meningioma model was employed. As in patients and in the orthotopic skull-based model, subcutaneously implanted IOMM tumors lack a blood-brain barrier (35), and therefore avelumab antibody may enter the tumor microenvironment. However, the subcutaneous model circumvents the short survival window of the skull-base murine model due to limited intracranial space and permits quantification of tumor volume.

Mice implanted with subcutaneous IOMM tumors and treated with the combination of avelumab and haNK cells had significantly smaller tumors than any other treatment cohort (Fig. 4C-4E). Cytotoxic immune cells were detectable in vehicle and combination avelumab + haNK treated groups, as shown by Granzyme B (GZMB) expression, likely due to HaNK cells or
infiltrating endogenous NK cells from athymic nude mice (Fig. 4F). However, tumors from mice in the combination avelumab + haNK cohort contained larger necrotic regions compared to tumors from mice in the vehicle cohort (Fig. 4G). Taken together, the combination of avelumab + haNK extended survival in both an orthotopic skull-base and a subcutaneous meningioma model in conjunction with increased cytotoxic immune infiltration and tumor necrosis. These data indicate that avelumab + haNK target meningioma for ADCC in vivo.

PD-L1 was next selectively deleted from IOMM meningioma cells to address target specificity. Exon 3 of CD274, which encodes PD-L1 protein, was targeted using the CRISPR/Cas9 system. Four variants of CD274 deletion were identified (Fig. 5A). PD-L1 negative cells were sorted by flow cytometry, and the efficiency of the sgRNA was examined using a SURVEYOR mutation detection assay (Fig. 5B). Loss of PD-L1 protein was confirmed by Western blotting and flow cytometry (Fig. 5C-5D, Supplemental Table 1B). Specificity of avelumab-directed ADCC was first demonstrated in vitro, as IOMM-CD274−/− cells were not lysed above background when incubated with avelumab and haNK cells (Fig. 5E). Further, in vivo subcutaneous tumor growth and final tumor volume were not significantly impacted by haNK or the combination avelumab + haNK in mice bearing IOMM-CD274−/− tumors (Fig. 5F-5H). PD-L1 deletion was maintained in IOMM-CD274−/− tumors grown in vivo (Fig. 5I). These data indicate that PD-L1 expression is specifically targeted by avelumab and is necessary to mediate ADCC of meningioma by haNK cells.
Discussion

PD-L1 is expressed by multiple types of tumors, enabling immune evasion (36-39). Similar to other tumors, PD-L1 expression in meningioma increases with grade and inversely correlates with immune infiltration (24). Antibodies that block PD-L1 have shown clinical benefit by preventing PD-L1 from binding the inhibitory receptor PD-1 on immune cells (11, 40, 41). Most PD-L1 blocking antibodies are unable to mediate ADCC due to design (durvalumab and atezolizumab are Fc-deficient IgG1) or antibody isotype (BMS-936559 is IgG4) (11, 41, 42). Yet, PD-L1 makes an attractive target for cytotoxic therapies due to high expression on tumor cells. Avelumab is a fully human IgG1 antibody that can both mediate ADCC and block the immunosuppressive PD-1:PD-L1 interaction. Although immune cells may also express PD-L1, avelumab does not deplete PD-L1-expressing immune subsets (43). Further, avelumab has shown promising efficacy in patients with various solid tumors and those with NSCLC (12, 13).

PD-L1 has been reported to be expressed on meningioma (24, 25). In our studies (Fig. 1C) one patient for each Grade 1-2, and 2 patients of Grade 3 meningioma were stained, and PD-L1 expression appeared to increase with tumor Grade. This confirms and extends the observations of Johnson et al., (25) who reported that overall PD-L1 expression in 58 FFPE meningiomas was 7%, and generally increased by grade: Grade 1, 3% PD-L1; Grade 6% expressing; and Grade 3, 18% expressing PD-L1. In addition, Du et al, (24) reported markedly greater levels of PD-L1 associated with meningioma: Grade 1, 40% PD-L1; Grade 60% expressing; and Grade 3, 70% expressing PD-L1. While Johnson et al. (25) suggested that these differences in overall expression could be due to the anti-PD-L1 antibody utilized, Gaule et. al., (44) directly compared the clone utilized by Johnson (IHC22C3) and found to yield concordant
results with the antibody used in our studies (SP142). Importantly, while PD-L1 expression in other tumor types has been correlated with monotherapy antibody responses, it is not known what expression levels of PD-L1 are sufficient to be targeted by an ADCC-enabling antibody.

In several tumor types, PD-L1 transcription is increased during biologically-relevant conditions of hypoxia (via HIF-1α) or interferon response (via IRF1) (33, 45). Despite high basal levels, we found that meningioma tumor cells further upregulate PD-L1 under these conditions and this enhanced tumor killing by the combination avelumab + haNK. Thus, targeting PD-L1 for ADCC may be particularly suited for conditions found in tumors from patients with malignant meningioma.

The degree of PD-L1 expression on target cells and the antibody binding affinity of cytotoxic effector cells contribute independently to ADCC efficacy (14). The haNK cell line provides an off-the-shelf source of NK cells that express the high affinity CD16 receptor to mediate ADCC. This may be particularly germane for heavily treated patients with malignant meningioma who do not possess high numbers of NK cells.

Activated NK cells release immune inflammatory factors, including IFNγ that may tip the balance toward anti-tumor immunity by activating host immunity. High grade meningiomas have an increased mutation burden compared to low grade meningioma and are predicted to have a significantly larger neoantigen load that may increase immunoreactivity (46). A high neoantigen load combined with strong immune infiltration (24) support the application of an immunotherapy-based therapeutic approach. Further, because meningioma treatment options include radiation, which may further increase neoantigen presentation, and minimal exposure
to immunosuppressive chemotherapy, patients may have a sufficient immune repertoire to mount a robust anti-tumor response.

Here, we demonstrate that avelumab can target meningioma tumors for ADCC using avelumab and healthy donor NK cells. ADCC is further enhanced by haNK cells, which have recently demonstrated safety in humans. These considerations, in addition to location of meningiomas outside the blood-brain barrier, support the design of a clinical trial using avelumab to target PD-L1-expressing meningioma tumors for ADCC by endogenous NK or haNK cells. This would potentially offer a novel therapeutic option for this challenging cancer with few treatment options and no proven effective chemotherapy.
Methods

Study design

Murine xenograft studies were designed to evaluate the effect of treatment among experimental groups. Power analyses were performed \textit{a priori} with a two-tailed, two-sample \(T\) test. Power was set to 0.8 and \(\alpha\) of 0.05 to detect differences between group means of twice the observed standard deviation (SD). Mice were randomized based on tumor luminescence prior to treatment. Primary endpoints were survival and tumor-generated luminescence as a correlate for tumor size.

Cell culture and reagents

Meningioma cell lines IOMM-Lee, SAM, JEN, GAR, and CH-157 were provided by Dr. Randy Jensen (University of Utah). Chordoma lines JHC7, UM-Chor1, U-CH1, and U-CH2 were obtained from the Chordoma Foundation (Durham, NC). Prostate cancer lines (LnCAP and PC3), breast cancer lines (MDA-MB-231 and MCF-7), and lung carcinoma lines (H460 and HCC4006) were obtained from ATCC. Growth medium was DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Healthy donor PBMC were obtained from the NIH Clinical Center Blood Bank (protocol 99-CC-0618) and cryopreserved until use. NK cells were purified from cryopreserved healthy donor PBMC using a negative selection NK cell isolation kit (StemCell Technologies or Miltenyi Biotech). Samples were genotyped for CD16 polymorphisms as described previously (14). haNK cells were provided by NantKwest (Culver City, CA) through a Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI), National Institutes of Health (NIH). haNK cells were cultured in phenol-free X-Vivo-10.
medium (Lonza, Walkersville, MD) with 5% heat-inactivated human AB serum (Omega Scientific, Tarzana, CA). Cells were incubated under 95% relative humidity at 5% CO₂. For hypoxia experiments, cells were cultured in Heracell VIOS 160i CO₂ incubators (Thermo Scientific) with 5% CO₂ and either 5% or 1% oxygen. For IFNγ treatment, cells were treated with 10ng/mL recombinant human IFNγ (Peprotech) for 48 hours. Avelumab and the appropriate IgG₁ isotype control were obtained from EMD Serono (a business of Merck Healthcare KGaA) as part of a CRADA with the NCI. CD16 was blocked by incubating effector cells with anti-CD16 (clone B73.1; eBioscience, San Diego, CA) prior to ADCC assays.

**CRISPR knockout**

The sgRNA was designed according to the DNA sequence of CD274 exon 3 through [http://crispr.mit.edu](http://crispr.mit.edu). Targeting sequence TGA ACT GAC ATG TC was cloned into the PX459 plasmid (Addgene #62988) and transfected into IOMM meningioma cells using Lipofectamine 3000 (Thermo Fisher). The following primer sequences were utilized: sgCD274.F (CACCGTGAACATGAACTGACATGTC); sgCD274.R (AAACGACATGTCAGTTCATGTTCAC); Surveyor.CD274.F (GGGGTACAAGAGCAGTGTTGT); and Surveyor.CD274.R(TGGTTTGGATGAATGGAGGTTGA). IOMM-CD274⁻/⁻ cells were sorted by flow cytometry and identified by the absence of PD-L1 surface staining. The knockout efficiency was confirmed using Surveyor assay, Sanger sequencing, Western blotting, and flow cytometry.

**Antibody-dependent cellular cytotoxicity assay**
ADCC assays were performed as previously described (14). In brief, trypsinized target cells were pulsed with $^{111}$In for 20 minutes at 37°C, and then washed to remove unbound $^{111}$In. Targets were incubated with avelumab or isotype antibody (2µg/mL) and allowed to adhere in a 96-well round-bottom plate at 2,000 targets/well. Healthy donor NK or haNK effector cells were added at the indicated E:T ratio, the plates were briefly centrifuged, and the ADCC assay was run for 4 hours at 37°C. Supernatants were harvested and $^{111}$In was quantified using a WIZARD2 Automatic Gamma Counter (PerkinElmer, Waltham, MA). Spontaneous $^{111}$In release was quantified from supernatants of target cells incubated without effectors. Maximum $^{111}$In release was quantified from supernatants of target cells incubated with 0.05% Triton X-100. ADCC lysis was calculated as: Percent lysis = \[
\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{complete cpm} - \text{spontaneous cpm}} \times 100.
\] ADCC assays were performed in RPMI 1640 medium supplemented with 10% FPB, 1% PSG, 1% sodium pyruvate, 1% NEAA, and 15mM HEPES.

**Flow cytometry**

Single cell suspensions were washed, resuspended in PBS/BSA 1%, and counted using a Cellometer Auto 2000 cell counter (Nexcelom). Viability was determined with trypan blue counterstaining or fixable viability dye eFluor780 (eBioscience) in PBS. Single cell suspensions were washed, resuspended in PBS/BSA 1%, and counted using a Cellometer Auto 2000 cell counter (Nexcelom). Viability was determined with trypan blue counterstaining.

Prior to antibody staining, cell suspensions were incubated with human fc block (Catalog #564219, BD Biosciences) for 10 minutes. Surface staining was performed in PBS/BSA 1% for 30 minutes on ice with CD274-APC or CD74-PE (Clone 29E.2A3; Catalog #329708,BioLegend);
CD279-APC (Clone EH12.2H7, Catalog#329908, Biolegend); CD80-APC (Clone 2D10, Catalog#305220, Biolegend); CD158-APC (Clone HP-MA4, Catalog# 339510, Biolegend); CD94-APC (Clone DX22, Catalog# 305508, Biolegend); CD66a/c/e (Clone ASL-32, Catalog #342308, Biolegend); MIC A/B-APC (Clone 6D4, Catalog# 320908, Biolegend); HLA-ABC-APC (Clone W6/32, Catalog# 311410, Biolegend). Corresponding isotypes were used for negative controls. Cells were fixed with Cytofix Fixation Buffer (Catalog# 554655, BD Biosciences) for 15 minutes followed by washing with PBS/BSA 1% before sample acquisition. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo version 10.5.3 (Tree Star).

**Western blots**

Tumor cells were lysed using RIPA buffer with a protease inhibitor cocktail (EMD Millipore). Lysates were separated by SDS-PAGE (BioRad) and transferred to PVDF membranes. Membranes were probed for PD-L1 (E1L3N) and β-actin (8H10D10) from Cell Signaling Technology.

**Mice and tumor lines**

Female athymic nude mice, 6-8 weeks old, were purchased from Charles River (Frederick, MD) and maintained in pathogen-free microinsulator cages. For orthotopic meningioma models, 1*10^4 IOMM meningioma cells were injected as single-cell suspensions at the skull base. Avelumab (200µg/mouse) and haNK cells (1*10^7 cells/mouse) were injected intravenously (IV) on M-W-F for 2 weeks. Prior to in vitro or in vivo use, haNK cells were lethally
irradiated (10Gy) to block cellular proliferation. Importantly, irradiation did not impair IL-2 production or cytotoxic function (22, 47). For subcutaneous models, 5*10^6 IOMM meningioma cells were injected subcutaneously on the flank. Tumor lines were stably transduced with firefly-luciferase, permitting luminescence reading as a proxy for tumor size. Mice were injected with 3mg luciferin (GoldBio) and images were analyzed with Living Image software (Xenogen Corporation). Flank tumor models were measured with calipers, and tumor volume was calculated with an ellipsoid formula: (length*width^2*π)/6. All studies were conducted on protocols approved by the NCI Animal Care and Use Committee.

**Immunohistochemistry**

5-µm serial sections were prepared from formalin-fixed, paraffin-embedded xenograft tissues. Immunohistochemistry staining for granzyme B (NB100-684, Novus Biologicals) and cleaved caspase-3 (Asp175, Cell Signaling) were performed by HistoServe, Inc., using standard procedure. In brief, tissue sections were deparaffinized and rehydrated in xylene and ethanol, respectively. Sections were then subjected to heat-induced antigen retrieval at 95°C for 20 minutes and incubated with primary antibody overnight at 4°C. HRP-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody were incubated with the specimens and developed by DAB substrate. Immunohistochemistry staining for PD-L1 (SP142, 1:100, Spring Bioscience) of formalin-fixed, paraffin-embedded meningioma tissue blocks were performed on the automated Leica Bond-Max (Leica Biosystems, Buffalo Grove, IL) according to the manufacturer’s instructions. Sections were stained alongside a positive control of normal human placenta (HuFPT080, US Biomax, Inc.). Tissue sections underwent citrate antigen
retrieval for 20 minutes at 98°C prior to antibody staining. Images were captured using Nikon Eclipse CIE light microscope. A standard hematoxylin and eosin (H&E) staining was used for histopathological evaluation.

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism 7.0 software. Data are expressed as mean +/- standard deviation or the standard error of the mean (SEM) as indicated. Statistical significance was evaluated by Student’s t-test, ANOVA, or unequal variance (Welch) ANOVA. This has now been clarified in the Materials and Methods section. P-values < 0.05 were considered significant.

**Author contributions**

AJG designed research studies, conducted experiments, acquired and analyzed data, and wrote the manuscript. SH conducted experiments, acquired and analyzed data, and contributed to writing the manuscript. MP, HS, WZ, JL, VS, YL, JJ, XC, and RF conducted experiments and acquired and analyzed data. RJ and DG provided reagents. JS provided reagents and edited the manuscript. MRG designed research studies and edited the manuscript. EKN provided reagents and edited the manuscript. CY conducted experiments, analyzed data, provided reagents, and edited the manuscript. JWH designed research studies, analyzed data, provided reagents, and edited the manuscript. DMP designed research studies and analyzed data.
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Figure 1.
PD-L1 is highly expressed in malignant meningioma

A

B

C

Grade I

Grade II

Grade III
Fig. 1: PD-L1 is highly expressed on malignant meningioma compared to other tumor cell lines.

(A) Representative flow cytometry plots of the indicated cell lines. PD-L1 expression is shown on singlet-gated, viable cells. The fluorescence minus one (FMO) control stain without PD-L1 antibody is shown for each cell line in gray. (B) PD-L1 intensity was normalized by subtracting the geometric mean fluorescence intensity (gMFI) of PD-L1 for the FMO sample from the PD-L1 gMFI of the matched stained sample. Each symbol represents an individual sample; multiple symbols represent separate runs. (C) PD-L1 immunohistochemistry of a meningioma tumor block from a patient with either grade I (first column), grade II (middle column), or grade III (last column) tumors. Two areas are shown for each tumor under 40x magnification. Scale bar = 100µm.
Figure 2: Avelumab-mediated ADCC is dependent upon Fc receptor binding
**Fig. 2: ADCC of meningioma is mediated by Fc receptor binding, and ADCC is increased with high-avidity Fc receptors.**

(A) ADCC assay of malignant meningioma lines by healthy donor NK cells. Meningioma lines were incubated with avelumab alone, healthy donor NK cells + isotype antibody, or NK cells + avelumab. The ratio of NK to meningioma cells is indicated. Each sample was plated in triplicate. Data were analyzed with a two-way ANOVA, *, p<0.05. Shown is representative data from 3 repeats utilizing different healthy donors. (B) ADCC of IOMM incubated with avelumab or healthy donor NK cells with isotype, avelumab, or avelumab with α-CD16 (Fc-blocking) antibody. Data were analyzed with a two-way ANOVA. (C) ADCC assay of five meningioma lines by NK cells from healthy donors with the F/F or V/V polymorphism of the CD16 receptor (E:T of 10:1). Data were analyzed with unpaired Student’s T test comparing NK + isotype with NK + avelumab conditions. (D) Lysis of IOMM meningioma by healthy donor NK cells versus haNK cells in the presence of avelumab. Samples were plated in triplicate. Data were analyzed with one-way ANOVA.
Figure 3: PD-L1 upregulation by hypoxia and IFNγ enhances avelumab-mediated ADCC

A

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B

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<th>IFNγ:</th>
<th>CH-157</th>
<th>GAR</th>
<th>SAM</th>
<th>IOMM</th>
<th>JEN</th>
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<td>PD-L1</td>
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<td>β-actin</td>
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C

CH-157, GAR, SAM, IOMM, JEN

PD-L1 expression

untreated & IFNγ

D

Monogama cells (%)

20% oxygen

1% oxygen

p = 0.0014

E

Monogama cells (%)

Vehicle & IFNγ

p = 0.0015
Fig. 3: PD-L1 upregulated by interferon gamma and hypoxia enhance meningioma lysis by haNK cells.

(A) Total PD-L1 protein was detected by Western blotting in five meningioma cell lines cultured under 20%, 5%, or 1% oxygen. (B) Western analysis of PD-L1 protein from the indicated meningioma lines cultured with or without IFNγ treatment. (C) Surface PD-L1 was measured by flow cytometry for the indicated cell lines. Cells were untreated (filled gray histogram) or treated with IFNγ (dashed line). (D) ADCC assay of IOMM cells incubated with isotype or avelumab antibody and haNK cells under 20% or 1% oxygen (E:T of 25:1). (E) ADCC assay of IOMM cells cultured with vehicle or IFNγ for 2 days before incubating with isotype or avelumab antibody and haNK cells (E:T of 25:1). Each condition was plated in triplicate, and data were analyzed with a two-way ANOVA.
Figure 4: Avelumab + haNK extends survival in preclinical meningioma model

A

B

C

D

E

F

G

H&E
**Fig. 4: Avelumab and haNK treatment extends survival in meningioma tumor-bearing mice.**

**(A-B)** Female athymic nude mice were implanted with IOMM meningioma cells in a skull-base model. Mice were randomized to the indicated cohorts with 9-10 mice per group. Treatment commenced four days following tumor implantation. Mice received a total of 7 treatments. **(A)** Luminescence from GFP-luciferase-expressing IOMM tumor cells was quantified weekly. Each line represents an individual mouse. Arrows indicate commencement of treatment. **(B)** Kaplan-Meier survival analysis of tumor-bearing mice. IC, intracranial. **(C-E)** IOMM meningioma cells were implanted subcutaneously. Mice were randomized based on tumor volume and treated as in A-B. Treatment commenced 17 days following tumor implantation, and mice received a total of 6 treatments. Each cohort contained 9-10 mice. **(C)** Mean tumor volume with SEM is shown for each cohort. **(D)** Tumor volume on day 32 following tumor implantation. Each symbol represents an individual mouse, and data are displayed with mean and standard deviation. Data were analyzed using an unpaired T test with Welch's correction. **(E)** Subcutaneous tumors on day 32 of tumor growth. **(F)** Granzyme B (GZM B) was detected by immunohistochemistry in subcutaneous meningioma tumors on day 24 of tumor growth. Vehicle-treated (left, \( n = 2 \)) or combination-treated (right, \( n = 2 \)) are shown at 20x magnification. Scale bar = 200µm. **(G)** Hematoxylin and eosin (H&E) stain of serial sections from tumor samples in (F). Scale bar = 200µm.
Figure 5
PD-L1 is necessary for hNK+avelumab-mediated ADCC

A. Homo sapiens CD274 exon 2

B. CD274 sgRNA

C. PD-L1

D. PD-L1

E. % VLD

F. Tumor Volume (mm³) vs. Tumor growth (days)

G. Tumor Volume (mm³) vs. Tumor growth (days)

H. iOMM WT iOMM PD-L1 KO

I. Immunohistochemistry
**Fig. 5: PD-L1 is necessary for avelumab-directed, haNK-mediated ADCC of meningioma.**

(A) Schematic illustration for CRISPR/Cas9 knock out strategy. Guide RNA (green) was designed to target the genetic sequence of CD274 exon 3. The protospacer adjacent motif (PAM) is shown in orange. Genotypes of resultant clone variants 1 to 4 are shown. (B) Surveyor assay shows successful cleavage of CD274 exon 3. Arrows indicate DNA fragments resulting from CRISPR targeting. (C) Western blot of parental IOMM and CD274−/− IOMM meningioma cell lysates. GAPDH was used as a loading control. (D) Flow cytometry histogram of parental (WT) and CD274−/− (IOMM PD-L1 KO) IOMM cells stained with PD-L1 (Blue histograms), or isotype control (red histograms). (E) ADCC assay of parental or CD274−/− IOMM cells exposed to the indicated conditions. Each condition was run in triplicate. Data were analyzed with an unpaired T test. (F-H) CD274−/− IOMM meningioma cells were implanted subcutaneously. Female athymic nude mice were randomized based on tumor volume into the indicated cohorts with n = 10 mice per group. Treatment commenced on day 16 following tumor implantation. Mice received a total of 8 treatments. (F) Mean tumor volume with SEM is shown for each cohort. (G) Tumor volume on day 36 following tumor implantation. Each symbol represents an individual mouse, and data are displayed with mean and standard deviation. Data were analyzed using an ANOVA test with Welch’s correction. (H) Subcutaneous tumors on day 36 of tumor growth. (I) PD-L1 IHC of an IOMM-CD274−/− subcutaneous xenograft tumor (top) and normal human placenta (bottom; positive control). Images were taken with 20x (left) or 40x (right) magnification.