Reduction of MDSCs with All-trans Retinoic Acid Improves CAR Therapy Efficacy for Sarcomas

Adrienne H. Long1,2, Steven L. Highfill1, Yongzhi Cui1, Jillian P. Smith1, Alec J. Walker1, Sneha Ramakrishna1, Rana El-Etrify3, Susana Galli3, Maria G. Tsokos3, Rimas J. Orentas, and Crystal L. Mackall1,4

Abstract

Genetically engineered T cells expressing CD19-specific chimeric antigen receptors (CAR) have shown impressive activity against B-cell malignancies, and preliminary results suggest that T cells expressing a first-generation disialoganglioside (GD2)-specific CAR can also provide clinical benefit in patients with neuroblastoma. We sought to assess the potential of GD2-CAR therapies to treat pediatric sarcomas. We observed that 18 of 18 (100%) of osteosarcomas, 2 of 15 (13%) of rhabdomyosarcomas, and 7 of 35 (20%) of Ewing sarcomas that 18 of 18 (100%) of osteosarcomas, 2 of 15 (13%) of rhabdomyosarcomas, and 7 of 35 (20%) of Ewing sarcomas expressing GD2. T cells engineered to express a third-generation GD2-CAR incorporating the 14g2a-scFv with the CD28, OX40, and CD3ζ signaling domains (14g2a.CD28.OX40.Cζ) mediated efficient and comparable lysis of both GD2+ sarcoma and neuroblastoma cell lines in vitro. However, in xenograft models, GD2-CAR T cells had no antitumor effect against GD2+ sarcoma, despite effectively controlling GD2+ neuroblastoma. We observed that pediatric sarcoma xenografts, but not neuroblastoma xenografts, induced large populations of monocytic and granulocytic murine myeloid-derived suppressor cells (MDSC) that inhibited human CAR T-cell responses in vitro. Treatment of sarcoma-bearing mice with all-trans retinoic acid (ATRA) largely eradicated monocytic MDSCs and diminished the suppressive capacity of granulocytic MDSCs. Combined therapy using GD2-CAR T cells plus ATRA significantly improved antitumor efficacy against sarcoma xenografts. We conclude that retinoids provide a clinically accessible class of agents capable of diminishing the suppressive effects of MDSCs, and that co-administration of retinoids may enhance the efficacy of CAR therapies targeting solid tumors.


Introduction

Chimeric antigen receptors (CAR) are synthetic immune receptors that link an antigen-binding domain, commonly a mAb-derived single-chain variable fragment (scFv), to T-cell signaling domains (1, 2). T cells genetically engineered to express CARs mediate non–MHC-restricted, tumor-specific killing and provide new opportunities for the adoptive immunotherapy of cancer. This novel class of therapeutics has demonstrated impressive antileukemic activity against B-cell hematologic malignancies (3–10), but has not yet shown clear evidence for efficacy against solid tumors. Studies using CARs targeting the α-folate receptor on ovarian cancer (11) or carboxy-anhydrase-IX on renal cell carcinoma (12) showed no objective responses in patients. CARs targeting the disialoganglioside GD2 on neuroblastoma showed modest antitumor activity in clinical trials, with 3 of 11 patients treated with active disease achieving a complete remission (13, 14).

The basis for the limited clinical activity of CARs against solid tumors observed thus far is likely multifactorial. The GD2-specific CAR utilized in the study noted above incorporated only the CD3ζ signaling domain (i.e., a first-generation CAR) and did not incorporate additional costimulatory domains that have since been demonstrated to enhance CAR expansion and persistence (8). Most current CARs under study incorporate one or two costimulatory domains in addition to CD3ζ. We have demonstrated that CAR potency can be diminished via T-cell exhaustion induced by tonic, antigen-independent CAR signaling (15). This phenomenon is present in CARs incorporating the 14g2a scFv, though the effects are diminished by anti-exhaustion signaling provided by TNFR family member costimulatory receptors. Finally, solid tumors evade immune responses via an immunosuppressive microenvironment, which may pose a key barrier to the efficacy of CAR therapies tested thus far (16).

Myeloid-derived suppressor cells (MDSC) have emerged as important contributors to solid-tumor immune evasion. MDSCs are a heterogeneous population of immature myeloid cells that are expanded by and recruited to tumors. MDSCs...
dampen T-cell responses through numerous mechanisms, including depletion of essential nutrients via production of arginase I, NOxS, and indoleamine 2,3 dioxygenase, as well as production of suppressive reactive oxygen species, nitrosylation of chemokines, and expression of PD-L1 (17–20). The impact of MDSCs on CAR therapies has not been systematically studied to date.

GD2 is a validated cell-surface antigen expressed ubiquitously in neuroblastoma. In addition to the promising early clinical results seen with first-generation GD2-specific CAR T cells, clinical studies have also demonstrated improved survival in patients with high-risk neuroblastoma when mAbs to GD2 are administered in combination with standard therapy (21). GD2 expression has been reported in sarcomas (22–24). We sought to evaluate GD2 expression on the three most common pediatric sarcomas [osteosarcoma, rhabdomyosarcoma (RMS), and Ewing sarcoma] and to assess whether a third-generation GD2-CAR incorporating GD28 and OX40 costimulatory domains (GD2-CAR.OX40.28) could mediate antitumor activity in preclinical models of neuroblastoma and pediatric sarcoma. Here, we report high GD2 expression on a majority of pediatric osteosarcomas and that a third-generation GD2-CAR effectively lysed GD2+ osteosarcoma and neuroblastoma cell lines in vitro. In vivo, however, GD2-CAR T cells mediated potent antitumor activity against neuroblastoma xenografts, but only minimal activity against osteosarcoma xenografts. The lack of antitumor activity was associated with sizable expansions of CD11b+Ly6G+ murine MDSCs that suppress human T cells. Treatment of animals with all-trans retinoic acid (ATRA) diminished the number and suppressive functionality of MDSCs, and co-administration of ATRA with GD2-CAR T-cell therapy resulted in enhanced antitumor effects against sarcoma xenografts in vivo, compared with either agent alone.

Materials and Methods

Mice
Immunocompromised NSG mice (NOD.Cg-Prkdccid Il2rgtm1Wá Ê Il6stfltm1Shl N) were purchased from The Jackson Laboratory. All studies were conducted according to NCI Animal Care and Use Committee approved protocols.

Cells and culture conditions
All cell lines used in this study were obtained between 2006 and 2014, authenticated by STR fingerprinting in 2014 (DDC Medical), and validated to be Mycoplasma free by PCR. The 143b, G292, and MG63 lines were provided by C. Khanna (NCI, NIH); EW8, RMS559, and RH30 lines by L. Helman (NCI, NIH); Kelly and LAN5 lines by C. Thiele (NCI, NIH); 293GP line by the Surgery Branch, NCI, NIH; and a PG13-based magnetic selection from spleens of tumor-bearing mice 15 to 20 days after tumor inoculation. Isolated MDSCs were then isolated using CD11b or Ly6G magnetic selection from spleens of tumor-bearing mice 15 to 20 days after tumor inoculation. Isolated MDSCs were then cultured in AIM-V media with r-human IL2 (aldesleukin; Prometheus).

CAR retroviral transduction of T cells
Retroviral supernatant for the third-generation GD2-CAR (SGF.iCasp9.2A.14g2a.CD28.OX40.28) was harvested from the producer cell clone. Retroviral supernatants of the previously described GD2-specific CARs incorporating either CD28 or 4-1BB (MSGV.14g2a.CD28, C or MSGV.14g2a.4-1BB, C) were produced by transient transfection of the 293GP cell line using the RD114 envelope protein (15). Cryopreserved PBMCs were thawed and activated using αCD3/αCD28 beads (Invitrogen) at a 3:1 bead:cell ratio for 48 hours. Activated human lymphocytes were added at 106 cells per well. Transduction was repeated the following day, and cells were expanded in AIM-V media with 300 IU/mL of rIL2.

Lytic assay
The ability of CAR T cells to lyse target cell lines in vitro was evaluated using a 51Cr release assay. Target cell lines were labeled with 51Cr and incubated with GD2-CAR-transduced effector T cells at varying effector-to-target (E:T) ratios. After 8 hours of coincubation at 37°C, supernatant was removed and the amount of 51Cr released into the media was assessed using a Top Count reader. Lysis was calculated as follows: % lysis = [(experimental lysis spontaneous lysis)/(maximum lysis spontaneous lysis)] × 100%, where maximal lysis was induced by incubation in a 2% Triton X solution.

Chemokine production assay
G-CSF production by tumor cell lines was evaluated within supernatants by ELISA (R&D Systems). ILS production by tumor cell lines was evaluated with the MesoScale Discovery Pro-Infamatory multi-array 96-well system.

MDSC suppression assays
Where indicated, mice received surgically implanted subcutaneous ATRA pellets (21-day timed release; 5 mg; Innovative Research of America) or sham surgeries 1 day prior to tumor inoculation. MDSCs were then isolated via CD11b or Ly6G magnetic selection from spleens of tumor-bearing mice 15 to 20 days after tumor inoculation. Isolated MDSCs were then cultured in AIM-V media at varying MDSC:T-cell ratios and incubated overnight. For flow-based proliferation assays, human T cells were pre-labeled with CellTrace Violet or CSFE (Invitrogen). Following overnight incubation, either human αCD3/αCD28-coated stimulatory beads (1:1) or the GD2-CAR anti-idiotype 1A7 antibody (0.5 μg/mL; Biological Research Branch of NCI) and a cross-linking anti-mouse F(ab’)2 (2.5 μg/mL, Jackson Immunoresearch) were added to the coculture to serve as a proliferation stimulus.
5 additional days of incubation, cells were harvested and proliferation evaluated by either flow cytometry (percentage of divided calculated using FlowJo 9 software, Treestar) or direct cell count measurements. All MDSC suppression assays were conducted in custom RPMI 1640–direct cell count measurements. All MDSC suppression assays were calculated using FlowJo 9 software, Treestar) or adjacent lung tissue and alveolar RMS (penicillin, 100 50 mmol/L 2-mercaptoethanol, 10 mmol/L HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L sodium pyruvate.

Flow cytometry and analysis

GD2 expression on tumor cell lines was performed using the anti-GD2 (clone 14g2a; BD PharMingen) and compared with isotype controls. T-cell transduction was measured using the 14g2a anti-idiotypic clone 1A7 (Biological Research Branch of NCI) conjugated to Dylight 650 or 488 (Pierce Protein Biology Products). T-cell phenotypes were identified using the following antibodies to human CD4 (clone OKT4; eBioscience), human CD8 (clone RPA-T8; eBioscience), human CD45 (clone H130; eBioscience), human PD-1 (clone eBio105; eBioscience), human TIM-3 (clone F35-2E2; eBioscience), and human LAG-3 (clone 3DS223H; eBioscience). MDSCs were evaluated using antibodies to Ly6C (clone HK1.4; eBioscience), Ly6G (clone 1A8; eBioscience), and CD11b (clone M1/70; eBioscience). All antibodies were used per manufacturer's recommendations. Live/dead cells were distinguished with Fixable Viability Dye eFluor 780 or 506 (eBioscience). Flow cytometry was done using FACS Fortessa with FACS Diva software (BD Biosciences) and analyzed by FlowJo 9 software (Treestar).

Immunohistochemistry

Frozen tissue samples were obtained from surgical samples at the time of clinically indicated tumor resection or biopsy after informed consent. Sections were sliced at 5-μm thickness, fixed in cold acetone for 10 minutes, dried at room temperature for 5 minutes, and rehydrated in PBS for 10 minutes. Samples were quenched with endogenous peroxidase block (DAKO) for 10 minutes, washed for 5 minutes, and incubated for 60 minutes at 37°C with mouse anti-human GD2 (14g2a; Millipore) diluted in DAKO’s antibody diluent with background reducing components to a 1:100 concentration. After washing, sections were incubated for 30 minutes in anti-mouse (biotinylated goat anti-mouse IgG; Vector) at a concentration of 2.5 μg/mL. Sections were washed with DAKO wash buffer, incubated in DAKO peroxidase substrate solution for 5 minutes, and washed in PBS. The reaction was developed by a 2-to-5-minute incubation with the 3’-3’ diaminobenzidine chromogenic solution (Vector). Slides were then counterstained with hematoxylin, dehydrated with a series of alcohol solutions (50%–100%), followed by three changes of xylene and mounted with Cyto-seal XVI (Thermo Scientific). Analysis was performed using standard microscopy.

Tumor models and treatment

Xenograft studies were performed using NSG mice 6 to 12 weeks of age. Mice were inoculated with 5 × 10⁵ or 10⁵ 143b, EW8, or Kelly cells either periosteal to the tibia or subcutaneously on day 0. Where noted, tumors were injected with Matrigel (Corning) diluted 1:1 in PBS. Human GD2-CAR–transduced or Mock T cells were adoptively transferred 3 to 5 days later, as indicated in individual experiments. Mice received cytokine support of 1 μg of IL7 (Cytheris Inc.) and 5 μg of M25 antibody (anti-IL7; Immunex) intraperitoneally three times per week following T-cell transfer (29). All-trans retinoic acid pellets (21-day timed release; 5 mg; Innovative Research of America) were surgically implanted subcutaneously on day ~1. Sham surgeries were performed on controls.

Graphs and statistical analysis

Graphs were generated using Graphpad Prism software. Graphs represent mean values ± standard error of the mean. P values were calculated using the Students t test for comparing like groups, repeated-measures ANOVA for tumor growth curves, or log-rank statistics for survival analyses. P < 0.05 was considered statistically significant and is illustrated with an asterisk (*). Deaths of mice occurring before the establishment of tumor were censored from survival analysis.

Results

GD2 expressed on human pediatric sarcomas

To evaluate GD2 as a potential target for CAR T-cell therapies in pediatric sarcoma, we first analyzed GD2 expression on tissue samples. Frozen tissue sections from 68 pediatric sarcomas, obtained at the time of clinically indicated biopsy or resection, were analyzed via immunohistochemistry for GD2 expression (Fig. 1 and Table 1; Supplementary Table S1). We observed that 100% of osteosarcoma samples expressed GD2 (n = 18 samples from 17 patients). In 15 of 18 of the osteosarcoma samples (5/8 primary osteosarcomas and 10/10 metastatic osteosarcomas) expression was deemed high, based upon GD2 expression in >60% of tumor cells. Among RMS tissues studied (n = 14 alveolar RMS and n = 1 embryonal RMS), two samples were positive for GD2, and both demonstrated high-level expression. Among Ewing sarcoma tissues studied (n = 35), seven were positive for GD2, but none showed high expression (Table 1). These results confirm that GD2 is a potential target for immunotherapy in patients with osteosarcoma and suggest that GD2 could serve as a target in subsets of patients with RMS and Ewing sarcoma.
GD2-CAR T cells efficiently lyse GD2⁺ sarcoma and neuroblastoma cell lines in vitro

We next sought to evaluate the capacity of T cells expressing a third-generation GD2-CAR (14g2a.CD28.OX40.) (25–28) to kill GD2⁺ human sarcoma cell lines in vitro, compared with killing of GD2⁺ neuroblastoma cell lines. GD2 was expressed on 2 of 2 neuroblastoma cell lines tested (Kelly, LAN5), 3 of 3 osteosarcoma cell lines tested (143b, MG63, and G292), 1 of 2 RMS cell lines tested (RMS559⁺, RH30⁻), and 1 of 1 Ewing sarcoma cell line tested (EW8; Fig. 2A). Surface expression was similar between the 143b osteosarcoma and Kelly neuroblastoma, and therefore these lines were chosen for further comparative studies aimed at assessing the relative efficacy of GD2-CAR T cells in targeting sarcoma versus neuroblastoma.

Human T cells were transduced with the GD2-CAR, with consistently ~70% transduction efficiencies at day 7 after activation for both CD4⁺ and CD8⁺ T cells (Fig. 2B). CD4:CD8 ratios within both the GD2-CAR and Mock T-cell products were typically ~3:1 (Fig. 2C). ⁵¹Cr release assays demonstrated equivalent lysis of the 143b osteosarcoma cell line and the Kelly neuroblastoma line (Fig. 2D), and GD2-CAR T cells were able to lyse all GD2⁺ cell lines tested (Supplementary Fig. S1). Very little lysis of the GD2-negative line RH30 was observed, indicating high specificity of T cells expressing the GD2-CAR. Thus, we conclude that T cells expressing the GD2-CAR have comparable efficacy against GD2⁺ sarcoma versus neuroblastoma tumor lines in vitro.

Table 1. Summary of GD2 expression on pediatric sarcomas

<table>
<thead>
<tr>
<th>Samples analyzed</th>
<th>Number (%) of samples expressing GD2</th>
<th>Number (%) of samples GD2hi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteosarcoma (n = 18)</td>
<td>18 (100%)</td>
<td>15 (83%)</td>
</tr>
<tr>
<td>RMS (n = 15)</td>
<td>2 (13%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Ewing sarcoma family of tumors (n = 35)</td>
<td>7 (20%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

NOTE: GD2hi represents GD2 expression in >60% of tumor cells.

Figure 2. GD2-CAR T cells effectively lyse GD2⁺ sarcoma cell lines in vitro. A, Flow-cytometric analysis of GD2 expression on human osteosarcoma (143b, G292, and MG63), Ewing sarcoma (EW8), RMS (RMS559 and RH30), and neuroblastoma (Kelly and LAN5) cell lines grown in vitro. Numerous sarcoma cell lines demonstrated strong GD2 surface expression. B, Representative transduction efficiency of GD2-CAR T cells, evaluated on day 7 following initial activation. Transduction efficiency measured by staining with clone 1A7, an anti-idiotypic antibody specific for the 14g2a antibody. C, Representative CD4:CD8 ratios of GD2-CAR and Mock T-cell products, evaluated on day 7 following initial activation. D, Lysis of target cells measured by ⁵¹Cr release. GD2-CAR T cells equivalently lyse the GD2⁺ 143b and Kelly cell lines, but do not lyse the GD2-negative cell line RH30.
Poor in vivo efficacy associated with differential induction of MDSCs

We next compared the in vivo efficacy of GD2-CAR T cells against the 143b osteosarcoma line versus the Kelly neuroblastoma line in a xenograft model using immunodeficient NOD-SCID-γc−/− (NSG) mice. Despite lysing 143b tumor cells in vitro, GD2-CAR T cells showed minimal antitumor effect against 143b osteosarcomas in vivo (Fig. 3A) and did not improve survival (Fig. 3B). Similar results were also seen against EW8 xenografts (Supplementary Fig. S2A and S2B). This poor in vivo efficacy could not be fully attributed to T-cell exhaustion induced by antigen-independent signaling of GD2-specific CARs (15), because the same GD2-CAR T cells provided a significant antitumor benefit against Kelly neuroblastoma tumors in vivo (Fig. 3C and D).

All of the studies presented thus far were conducted with a third-generation GD2-CAR incorporating both CD28 and OX40.

Figure 3.
GD2-CAR has poor in vivo efficacy against the 143b osteosarcoma cell line in a xenograft model that is not fully attributable to T-cell exhaustion. A, tumor growth curves and (B) survival of NSG mice inoculated with 10⁶ 143b cells periosteally on day 0, followed by adoptive transfer of 10⁷ untransduced-Mock or GD2-CAR T cells on day 3. GD2-CAR T cells have no antitumor effect against 143b. n = 5 mice/group. C, tumor growth curves and (D) survival of NSG mice inoculated with 10⁶ Kelly cells subcutaneously with Matrigel on day 0, followed by adoptive transfer of 10⁷ untransduced-Mock or GD2-CAR T cells on day 3. GD2-CAR T cells prevent outgrowth of Kelly tumors, leading to enhanced overall survival. n = 5 mice/group. E, exhaustion marker expression on T cells transduced with GD2-specific CARs incorporating different costimulatory domains (GD2-CAR, a third-generation CAR with CD28 and OX40; a second-generation CAR with CD28; and a second-generation CAR with 4-1BB) 9 days following initial activation. The third-generation GD2-CAR T cells have an intermediate expression of exhaustion markers compared with the second-generation CD28 CAR and the 4-1BB CAR. F, tumor growth curves of NSG mice inoculated with 10⁶ Kelly cells subcutaneously with Matrigel on day 0, followed by adoptive transfer of 10⁷ untransduced-Mock or GD2-specific CAR T cells on day 3. The third-generation GD2-CAR T cells had an intermediate efficacy compared with the second-generation CD28 CAR and the 4-1BB CAR. n = 10 mice/group.
The third-generation CAR demonstrates intermediate exhaustion and functionality, with less exhaustion and improved in vitro activity compared with a second-generation GD2-specific CAR incorporating only CD28 (Fig. 3E and F), but increased exhaustion and decreased functionality compared with a second-generation CAR incorporating 4-1BB. These findings are consistent with those of previous reports that CD28 signaling contributes to exhaustion, whereas TNFR family member signaling ameliorates exhaustion (15).

The inability to fully attribute poor in vivo potency to T-cell exhaustion led us to question whether immunosuppressive properties of sarcomas that manifest in vivo, rather than CAR-intrinsic, properties could be inhibiting GD2-CAR T-cell therapies. We have observed that granulocytic MDSCs are primary mediators of immune suppression in murine models of sarcoma (30). Therefore, we assessed whether tumor-induced MDSCs could also be responsible for the poor efficacy of GD2-CAR T-cell therapies against sarcomas.

We monitored the blood of NSG mice inoculated with sarcomas (143b and EW8) or neuroblastomas (Kelly) for the expansion of myeloid populations, compared with non-tumor-bearing mice. NSG mice bearing 143b and EW8 tumors showed an increase in CD11b+Ly6G+ and CD11b+Ly6C+ myeloid cell populations, compared with non-tumor-bearing controls (Fig. 4A and B and Supplementary Figs. S2C–S2E and S3A). We observed granulocytic CD11b+Ly6G+Ly6C+ cells to be the major MDSC subset (>90% of CD11b+ cells) in the blood, spleen, and tumor compartments of sarcoma-bearing mice. Despite bearing similarly sized tumors, we evaluated 143b, EW8, and Kelly cell culture supernatants for the presence of growth factors and chemokines reported to influence MDSC expansion and migration. Associated with high level MDSC expansion in vivo, we observed that 143b cells produce greater quantities of G-CSF and IL8 compared with Kelly cells in vitro (Supplementary Fig. S3C). Such factors have been implicated in MDSC induction and expansion (30). However, IL8 was only modestly elevated in EW8-bearing mice, suggesting that additional mechanisms may also be involved (17, 33).

To assess whether the murine MDSC population could suppress the function of human T cells utilized in this xenograft model, we conducted coculture suppression assays with murine CD11b+ and human T cells. Briefly, splenic CD11b+ cells were isolated from sarcoma-bearing mice and were cultured at varying ratios with human T cells stimulated either through endogenous receptors with mCD3/mCD28 beads or through the GD2-CAR with a crosslinking anti-idiotypic antibody. After 4 to 5 days of in vitro culture, we assessed proliferation of human cells. Murray CD11b+ myeloid cells induced by human sarcoma xenografts inhibited human T-cell proliferative responses in a dose-dependent manner, when T cells were stimulated either through the native TCR (Fig. 4E and F and Supplementary Fig. S2D and S2E) or through the GD2-CAR (Fig. 4G and Supplementary Fig. S3D and S3E). Therefore, human sarcomas implanted into immunodeficient mice expanded MDSCs that were capable of suppressing human CAR T cells.

### ATRA reduces monocytic MDSCs and diminishes granulocytic MDSC suppression

ATRA is a clinically approved drug that induces immature myeloid cells to differentiate into a nonsuppressive subtype (34–36). Therefore, we tested whether ATRA treatment altered the quantity or suppressive phenotype of 143b-induced MDSCs. NSG mice were implanted subcutaneously with a 21-day time-released ATRA pellet prior to the injection of 143b tumor cells, and the peripheral blood MDSCs were monitored (Fig. 5A and B). We observed that ATRA treatment led to a significant reduction in the number of monocytic CD11b+Ly6C+ cells in the peripheral blood. Though the size of the granulocytic CD11b+Ly6G+ population was not consistently reduced, granulocytic MDSCs from ATRA-treated tumor-bearing mice had diminished suppressive potency compared with those from untreated tumor-bearing mice (Fig. 5C–E). Similar trends were also observed regarding the numbers of MDSCs present within tumors of mice treated with and

**Figure 4.** Human sarcoma implanted in NSG mice induces expansion of host MDSCs. A, representative flow cytometry plots of peripheral blood, evaluating presence of CD11bLy6G+ or CD11bLy6C+ MDSCs in mice inoculated with 106 143b osteosarcoma peritumorally or 106 Kelly cells subcutaneously with Matrigel. Evaluated day 28 after tumor inoculation. B, cumulative data from A, quantifying absolute number of CD11bLy6G+ (left) and CD11bLy6C+ (right) cells per 50 μL of blood. 143b tumors induce significant expansion of both CD11bLy6G+ and CD11bLy6C+ populations, compared with non-tumor controls or Kelly tumor-bearing mice with similarly sized tumors. C, absolute number of CD11bLy6G+ (left) and CD11bLy6C+ (right) cells per 143b tumor-bearing mice. 143b tumors contain significant CD11bLy6G+ populations, compared with Kelly tumors. D, impact of tumor location and Matrigel on induction of CD11bLy6G+ (left) and CD11bLy6C+ (right) cells in blood. NSG mice were inoculated with 106 143b (red) or Kelly (blue) tumor cells. MDSCs were quantified in the blood 20 days after tumor injection and plotted as a function of tumor size. Tumors were placed subcutaneously (SC, square) or periostally (POS, circle), with (filled) or without (open) Matrigel (MG). R2 and P value statistics were calculated from linear regression of pooled 143b and Kelly data. E, cell trace violet dye dilution of human T cells following coinoculation with murine CD11b+ MDSCs in vitro. Human hCD3/eCD28 beads were added as a proliferative stimulus. Flow cytometry was performed 4 days later. hCD3/eCD28 bead–induced proliferation without MDSCs in coculture shown in black. CD11b+ MDSCs isolated from 143b tumor-bearing mice suppress human T-cell proliferation in a dose-dependent manner (red). F, quantification of T-cell proliferation in E, measured as a percentage of T cells divided. G, absolute cell counts of GD2-CAR T cells (9 days after initial activation) cocultured with murine CD11b+ MDSCs derived from 143b tumor-bearing mice. GD2-CAR T cells were stimulated via the CAR with the anti-idiotypic IA7 antibody (0.5 μg/mL) and a cross-linking anti-mouse F(ab)2 (2.5 μg/mL). Cell counts from cultures were performed 5 days after CAR stimulation. CD11b+ MDSCs isolated from 143b tumor-bearing mice suppress human CAR T-cell proliferation in a dose-dependent manner.
ATRA treatment reduces number and suppressive capacity of murine MDSCs induced by sarcoma in NSG mice. **A**, representative flow cytometry plots of peripheral blood, evaluating presence of CD11b^+Ly6G^+ or CD11b^+Ly6C^+ MDSCs in mice inoculated with 10^6 143b osteosarcoma periosteally on day 0, treated with or without ATRA sustained-release subcutaneous pellets on day 1. Evaluated day 15 after tumor inoculation. **B**, cumulative data from **A**, showing the absolute number of CD11b^+Ly6C^+ (top) and CD11b^+Ly6G^+ (bottom) cells per 50 μL of blood. **C**, Flow-based proliferation analysis. **D**, MDSC:T-Cell ratio: 1:2, 1:4, 1:8, 1:16. **E**, CD4^+ T-Cell proliferation (% divided) and CD8^+ T-Cell proliferation (% divided) with different MDSC:T-Cell ratios.
without ATRA (Supplementary Fig. S3F). Thus, we observe that ATRA treatment of tumor-bearing mice dramatically reduces the quantity of monocytic MDSCs and diminishes the suppressive potency of granulocytic MDSCs in xenograft models of sarcoma.

**ATRA treatment enhances efficacy of GD2-CAR T cells against sarcoma in vivo**

We next asked whether inhibiting the suppressive capabilities of MDSCs through ATRA treatment improved the efficacy of GD2-CAR T cells against tumors. Mice were implanted with sustained-release ATRA pellets day −1 prior to inoculation with sarcoma cells, and subsequently treated with GD2-CAR T cells. Treating tumor-bearing mice with both ATRA and GD2-CAR T cells led to an enhanced antitumor effect, with delayed tumor outgrowth and improved survival (Fig. 6A and B and Supplementary Fig. S2A and S2B) compared with animals receiving ATRA plus Mock T cells. We could detect no additional benefit of ATRA to GD2-CAR treatments in animals bearing neuroblastoma (Supplementary Fig. S4A).

Further suggesting that ATRA-mediated modulation of MDSCs led to enhanced T-cell reactivity, we observed that 143b tumor–bearing mice with GD2-CAR T cells and ATRA had a higher frequency of CD8<sup>+</sup> CAR<sup>+</sup> T cells in the peripheral blood compared with animals that did not receive ATRA (Fig. 6C). Similar increases in GD2-CAR were not observed in Kelly tumor–bearing mice, which did not induce MDSC expansion and thus would not be expected to have enhanced T-cell reactivity following ATRA treatment (Supplementary Fig. S4B). The improved antitumor efficacy of GD2-CAR T cells in ATRA treated mice could not be attributed to changes in growth kinetics of the 143b tumor, because we saw no difference in the tumor growth between animals receiving Mock T cells alone versus those receiving Mock T cells + ATRA (Fig. 6A). Finally, we found no evidence that ATRA treatment increased GD2 antigen expression on tumors upon exposure either in vitro or in vivo (Supplementary Fig. S5). Together, these results suggest that ATRA may improve efficacy of the GD2-CAR against sarcoma tumors through its effects on MDSCs.

In addition to causing maturation of MDSCs, ATRA has also been reported to increase the frequency of regulatory T cells (37, 38). Such a secondary effect of ATRA could theoretically inhibit the efficacy of CAR T-cell therapies. Therefore, we evaluated whether ATRA treatment led to an increased incidence of FOXP3<sup>+</sup> T cells in vivo in this model system. Analysis of peripheral blood 21 days after T-cell injection demonstrated no detectable FOXP3 cells in either ATRA-treated or nontreated mice (Supplementary Fig. S6). Taken together, we conclude that ATRA treatment enhances GD2-CAR T-cell efficacy against sarcomas that induce MDSCs, without causing an increase in regulatory T-cells.

**Discussion**

Recent clinical trials have demonstrated impressive activity of CAR T cells targeting CD19 on hematologic malignancies (3–10). However, similar results have not yet been observed using CARs to target antigens on other malignancies, including solid tumors (11–14). Despite this, we were encouraged by preliminary results suggesting clinical benefit of a first-generation GD2-specific CAR in patients with neuroblastoma and sought to assess the efficacy of a costimulation-endowed CAR against pediatric sarcomas. We assessed GD2 expression on three common histologies and confirmed that it is expressed on essentially all osteosarcomas, with a sizable fraction of primary and metastatic tumors expressing a high level (22, 39, 40). Our results confirm those from Kailayangiri and colleagues demonstrating GD2 on Ewing sarcoma cell lines, although we did not observe significant expression of GD2 on Ewing sarcoma tissues (23). Information on GD2 expression in RMS is limited (41), and our data suggest that it may be a relevant target for a minority of patients. Together, these results indicated that GD2 is a rational target for the developing of CAR-based therapies for some pediatric sarcomas.

Using a third-generation GD2-CAR that incorporated the CD28 and OX40 costimulatory domains, we observed equivalent in vitro killing of GD2–expressing cells, regardless of histology. However, we observed poor in vivo activity of GD2-CAR T cells against the 143b osteosarcoma cell line, despite strong in vivo activity against the Kelly neuroblastoma cell line. Granulocytic MDSCs are a primary mediator of immune evasion in murine models of sarcoma (30), so we examined whether similar MDSCs were contributing to an immunosuppressive microenvironment that inhibits in vivo efficacy of GD2-CAR T cells against sarcomas. However, whether human tumors induce murine MDSCs in xenograft models of cancer, and whether such cells can suppress human T cells, has not been reported. Our data demonstrate that murine MDSCs can be expanded by human tumors in xenograft models of cancer, and that such murine MDSCs can suppress human T cells. The observation that MDSC-mediated T-cell suppression can cross species is consistent with a sizable literature that implicates nutrient depletion as a major mechanism of MDSC suppression (17–20). Our findings also suggest a possible role for G-CSF and IL8 in the recruitment of murine MDSCs, two factors known to influence MDSC expansion and migration. IL8 was only modestly elevated in EW8 bearing mice, consistent with previous evidence that such populations can be induced by a variety of cytokines and/or chemokines (17, 33). The mechanism by which ATRA alters MDSC recruitment is likely complex and may differ depending on the model. Overall, the identification that murine MDSCs contribute to an immunosuppressive microenvironment within human xenograft tumors is an important finding that will likely affect future evaluation of immunotherapies within xenograft models.
Our results are also consistent with increasing evidence that MDSC populations are expanded in patients with sarcoma (30, 42–44). We recently reported that human sarcoma patients have increased numbers of granulocytic MDSCs within unfractionated blood compared with healthy controls, and increased serum levels of cytokines responsible for MDSC expansion/migration (30). Fibrocyte MDSCs and hematopoietic stem/progenitor cells that develop into MDSCs are expanded in patients with pediatric sarcomas (42, 43), and we reported that expanded populations of monocytic and granulocytic cells adversely affected the efficiency with which GD2-CAR T cells could be generated from apheresis products obtained from children and young adults with osteosarcoma and neuroblastoma (44). Though our present study highlights sarcoma-induced expansion of MDSCs and illustrates differential amounts of expansion between sarcoma cell lines and one neuroblastoma cell line, the absence of MDSCs may not be generalizable to other neuroblastoma models or neuroblastoma patients. Indeed, studies have suggested a clinical significance of tumor-associated myeloid cells in neuroblastoma in some settings (45, 46). Thus, further work is needed to fully understand how pediatric tumors differentially induce MDSCs.

Sarcoma-induced MDSCs were associated with poor CAR T-cell efficacy against xenografts in vivo, implicating the immunosuppressive solid tumor microenvironment as a modulator of CAR T-cell efficacy. We found this by directly measuring MDSC inhibition of T-cell responses that had been triggered through CAR receptor signaling. Consistent with our findings, a reduction in MDSCs is associated with the enhanced efficacy of both VEGFR2-CAR T cells upon constitutive IL12 expression (47) and of ErbB2-CAR T cells upon anti-PD1 therapy (48). The CARS in both of these reports had the CD28 costimulatory domain in common with our GD2-CAR, but one was a third-generation CAR that included 4-1BB (VEGFR2.CD28.4-1BB.ζ) and the other a second-generation with only CD28 (ErbB2.CD28.ζ). We chose to focus our evaluation on the third-generation GD2-CAR with CD28 and OX40 costimulatory domains given its current use in clinical trials (NCT02107963). However, it is possible that different costimulatory domains may enhance susceptibility to MDSC suppression and remains a focus of our ongoing work.

ATRA is one of the few clinically available drugs that modulate MDSCs in both animal models and in human patients (35, 36). It mediates potent myeloid cell differentiating effects in the setting of acute promyelocytic leukemia and is well tolerated in this setting with cytotoxic chemotherapy (49), suggesting that it could be integrated into immunotherapeutic regimens for cancer. Use of our sarcoma xenograft models found that cotreatment with ATRA and GD2-CAR...
MDSCs Limit GD2-CAR T-cell Therapy

T cells led to enhanced antitumor effects compared with either single agent alone, which is likely mediated through a decreased number of monocytic MDSCs and potentially also via a reduction in the suppressive capacity of granulocytic MDSCs induced by sarcomas. The diminished in the suppressive capacity of granulocytic MDSC was approximately 50%, which may explain why we observed only a modest improvement in survival. It will be of interest to determine if this survival enhancement is improved further when treating subtypes of tumors that preferentially expand monocytic MDSC. Such effects of ATRA may be due to an increased expression of glutathione synthase in MDSCs (50), leading to higher glutathione and neutralization of reactive oxygen species (ROS), which play a role in T-cell suppression and prevent MDSC differentiation. Though ATRA can also induce FOXP3+ T regulatory cells in vivo, we found no such evidence in our model system with adoptively transferred, activated human CAR T cells. Thus, retinoids provide a clinically accessible class of agents capable of modulating MDSCs that may enhance the efficacy of CAR therapies targeting solid tumors.

In conclusion, we identify GD2 as a promising target for CAR T-cell therapies against pediatric sarcoma. Though xenograft models suggest that sarcoma-induced MDSCs are capable of inhibiting CAR T-cell efficacy in vivo, we identify ATRA as an effective therapy in diminishing the suppressive capacity of MDSCs and enhancing the in vivo efficacy of GD2-CAR T-cell against sarcoma. Our findings demonstrate the need for enhanced understanding of the immunosuppressive mechanisms that may limit immunotherapies against solid tumors and suggest that MDSCs may play a role in preventing CAR efficacy against solid tumors in patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Authors’ Contributions
Conception and design: A.H. Long, S.L. Highfill, Y. Cui, J.P. Smith, R.J. Orentas, C.L. Mackall
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.H. Long, S.L. Highfill, Y. Cui, J.P. Smith, A.J. Walker, S. Ramakrishna, R. El-Etriby, S. Galli, M.G. Tsokos, R.J. Orentas
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.H. Long, S.L. Highfill, Y. Cui, J.P. Smith, A.J. Walker, S. Ramakrishna, S. Galli, M.G. Tsokos, C.L. Mackall
Writing, review, and/or revision of the manuscript: A.H. Long, S.L. Highfill, Y. Cui, A.J. Walker, S. Ramakrishna, M.G. Tsokos, R.J. Orentas, C.L. Mackall
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.L. Highfill
Study supervision: S.L. Highfill, C.L. Mackall
Other (immunohistochemical staining/pathology): R. El-Etriby

Grant Support
This research was supported by the Intramural Research Program of the NIH, NCI (to all the authors) and an NIH T32 training grant awarded to Northwestern University (T32GM080152; A.H. Long). Research supported by a Stand Up To Cancer – St. Baldrick’s Pediatric Dream Team Translational Research Grant (SU2C-AAIR-DT1113. Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research (A.H. Long, J.P. Smith, Y. Cui, A.J. Walker, R.J. Orentas, and C.L. Mackall).

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

Received September 8, 2015; revised July 22, 2016; accepted July 29, 2016; published OnlineFirst August 22, 2016.


Downloaded from cancerimmunolres.aacrjournals.org on January 9, 2021. © 2016 American Association for Cancer Research.
28. Martin CE, van Leeuwen EAMM, Im SJ, Roopenian DC, Sung Y-C, Surt CD. IL-7–anti–IL-7 mAb complexes augment cytokine potency in mice through association with IgG-Fc and by competition with IL-7R. Blood 2013;121:4624–29.
29. Martin CE, van Leeuwen EAMM, Im SJ, Roopenian DC, Sung Y-C, Surt CD. IL-7–anti–IL-7 mAb complexes augment cytokine potency in mice through association with IgG-Fc and by competition with IL-7R. Blood 2013;121:4624–29.
34. Martin CE, van Leeuwen EAMM, Im SJ, Roopenian DC, Sung Y-C, Surt CD. IL-7–anti–IL-7 mAb complexes augment cytokine potency in mice through association with IgG-Fc and by competition with IL-7R. Blood 2013;121:4624–29.
Reduction of MDSCs with All-trans Retinoic Acid Improves CAR Therapy Efficacy for Sarcomas
Adrienne H. Long, Steven L. Highfill, Yongzhi Cui, et al.

Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-15-0230

Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2016/08/20/2326-6066.CIR-15-0230.DC1

This article cites 50 articles, 21 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/4/10/869.full#ref-list-1

This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/4/10/869.full#related-urls

Sign up to receive free email-alerts related to this article or journal.
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/4/10/869.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.