Cross-talk between T Cells and Hematopoietic Stem Cells during Adoptive Cellular Therapy for Malignant Glioma

Tyler J. Wildes, Adam Grippin, Kyle A. Dyson, Brandon M. Wummer, David J. Damiani, Rebecca S. Abraham, Catherine T. Flores, and Duane A. Mitchell

Abstract

Purpose: Adoptive T-cell immunotherapy (ACT) has emerged as a viable therapeutic option for both peripheral and central nervous system (CNS) tumors. In peripheral cancers, optimal efficacy of ACT is reliant on dendritic cells (DCs) in the tumor microenvironment. However, the CNS is largely devoid of resident migratory DCs to function as antigen-presenting cells during immunotherapy. Herein, we demonstrate that cellular interactions between adoptively transferred tumor-reactive T cells and bone marrow–derived hematopoietic stem and progenitor cells (HSPCs) lead to the generation of potent intratumoral DCs within the CNS compartment.

Experimental Design: We evaluated HSPC differentiation during ACT in vivo in glioma-bearing hosts and HSPC proliferation and differentiation in vitro using a T-cell coculture system. We utilized FACS, ELISAs, and gene expression profiling to study the phenotype and function of HSPC-derived cells ex vivo and in vivo. To demonstrate the impact of HSPC differentiation and function on antitumor efficacy, we performed survival experiments.

Results: Transfer of HSPCs with concomitant ACT led to the production of activated CD86+CD11c+MHCI+ cells consistent with DC phenotype and function within the brain tumor microenvironment. These intratumoral DCs largely supplanted abundant host myeloid-derived suppressor cells. We determined that during ACT, HSPC-derived cells in gliomas rely on T-cell–released IFNγ to differentiate into DCs, activate T cells, and reject intracranial tumors.

Conclusions: Our data support the use of HSPCs as a novel cellular therapy. Although DC vaccines induce robust immune responses in the periphery, our data demonstrate that HSPC transfer uniquely generates intratumoral DCs that potentiate T-cell responses and promote glioma rejection in situ. Clin Cancer Res; 24(16); 3955–66. ©2018 AACR.

Introduction

Median survival for malignant gliomas is near 15 to 20 months with chemotherapy, radiation, tumor-treating fields, and surgical resection (1, 2). To overcome the dearth of curative therapies, immunotherapeutic strategies including adoptive T-cell immunotherapy (ACT) and dendritic cell (DC) vaccination have emerged that synergize with standard of care and promote tumor rejection (3–11). Recently, it has been demonstrated that the success of ACT in peripheral cancers is dependent on intratumoral DCs (12). However, few DCs have been found in the homeostatic brain as well as the brain tumor microenvironment (13–15). On the contrary, there is significant infiltration of protumor myeloid-derived suppressor cells (MDSCs) in brain tumors that diminish the success of therapies (16, 17). Our prior studies have demonstrated that intravenously administered hematopoietic stem and progenitor cells (HSPCs) traffic to sites of malignant glioma growth and significantly enhance the efficacy of ACT, at least in part, through the chemotactic attraction of tumor-specific lymphocytes into the tumor microenvironment (5). In this report, we demonstrate that HSPCs interface with tumor-reactive lymphocytes and differentiate into potent antigen-presenting cells (APC) of a DC phenotype, supplant intratumoral MDSCs, present captured tumor antigens leading to enhancement of intratumoral T-cell activation, and promote the immunologic rejection of invasive gliomas.

HSPCs have recently become characterized as significant immunomodulatory cells during infection, autoimmunity, and cancer (5, 6, 18–20). In the context of peripheral solid tumor-bearing hosts, several studies have characterized HSPCs as immunosuppressive, tumor-promoting, and as seeds for metastasis (17, 21–27). On the contrary, a few studies have described HSPC-mediated expansion and recruitment of T cells as a novel method of potentiating cytotoxic responses (5,6). Although there are conflicting reports on the immunologic role of HSPCs in peripheral cancers, HSPCs selectively migrate to malignant gliomas through the SDF-1–CXCR4 axis (28) and refrain from migration to organs including liver, lung, muscle, and spleen (29).
Translational Relevance

Malignant gliomas are notoriously recalcitrant and often overcome any attempts at engendering effective antitumor immunity. We have previously demonstrated hematopoietic stem and progenitor cells (HSPCs) play a novel and synergistic role in enhancing the effectiveness of adoptive T-cell therapy targeting invasive malignant gliomas. Herein, we demonstrate that adoptive cellular therapy incorporating HSPC transfer is effective against multiple preclinical brain tumor models (glioblastoma, diffuse intrinsic brain stem glioma, and group 3 medulloblastoma) and that HSPCs are driven by T-cell–released IFNγ to differentiate into potent intratumoral dendritic cells (DCs) in situ within the tumor microenvironment. These HSPC-derived DCs additionally supplant intratumoral host myeloid-derived suppressor cells, potentiate T-cell activation in situ, and drive intracranial tumor rejection. These findings unravel the mechanistic cross-talk between tumor-specific T cells and HSPCs within malignant gliomas during adoptive cellular therapy and point to the novel utilization of HSPCs in cellular immunotherapy.

In addition, HSPC migration to gliomas can be enhanced with standard therapies such as irradiation and temozolomide (30). However, the differentiation of HSPCs and the functions of their progeny in the brain tumor microenvironment remain unclear. Our goal here was to determine the role of HSPC progeny in glioma immunity in situ during adoptive cell transfer. To accomplish this, we studied HSPC differentiation in the brain tumor microenvironment, the function of HSPC-derived cells, and mechanisms of synergy between HSPCs and tumor-reactive T cells.

We therefore investigated HSPC differentiation and function in brain tumor-bearing hosts during ACT and host conditioning (4–11). Here, we demonstrate that HSPCs in the brain tumor microenvironment supplant host MDSCs and differentiate into CD86+/CD11c+MHCII+ activated DCs. This differentiation occurs through tumor-reactive T-cell–released cytokines including IFNγ and its signaling through IFNγ receptor (IFNγR) on HSPCs. Although activated DC vaccines are capable of induction of peripheral immune responses (3, 31), our data demonstrate that HSPC transfer uniquely leads to accumulation of intratumoral DCs in malignant gliomas and supplants immunosuppressive MDSCs within the tumor microenvironment. These findings have significant implications for ACT in the treatment of refractory brain tumors.

Materials and Methods

Mice

Five- to 8-week-old female C57BL/6 mice (The Jackson Laboratory, 000664), transgenic DsRed mice (The Jackson Laboratory, 006051), transgenic GREAT mice (The Jackson Laboratory, 017580), and IFNγR−/− mice (The Jackson Laboratory, 003288) were used for experiments. All investigators adhered to the "Guide for the Care and Use of Laboratory Animals" and the University of Florida Animal Care Services are fully accredited by the American Association for Accreditation of Laboratory Animal Care. All studies were approved by the Institutional Animal Care and Use Committee and are covered under protocol number 201607966.

RNA isolation

Total tumor RNA (tRNA) isolation from tumor cell lines was performed with RNasea Mini Kit (Qiagen, 74104) per the manufacturer’s protocol.

Tumor-reactive T cells

Tumor-reactive T cells were generated as previously described through ex vivo expansion with bone marrow–derived DCs (BMDC; ref. 5).

Tumor models

Tumor-bearing experiments were performed in syngeneic sex-matched C57BL/6 mice. The KR158B-luc glioma line (provided by Dr. Karlyne M. Reilly, NCI Rare Tumor Initiative, NIH) has been verified histologically as high-grade glioma, and gene expression analysis by RNA sequencing demonstrated appropriate haplotype background and expression of astrocytoma-associated genes. KR158B-luc cells (106) were implanted into the caudate nucleus by injecting 2 mm lateral to midline at the bregma suture and 3 mm deep (5, 32). Neural stem cell (NSC) tumor cells were generated through previously described in vitro culture of sorted granule neuron precursor cells (33). NSC medulloblastoma cells (1 × 107) were implanted into the cerebellum 1 mm lateral to the midline and 3 mm deep (33, 34). K2 brain stem glioma cells (provided by Dr. Oren Becher, Northwestern University, Northwestern Medicine Feinberg School of Medicine) were developed through previously described methods including an induced H3.3K27M mutation in the progenitor cells of the brainstem (35). K2 cells (1 × 105) were implanted into the brain stem of mice 1 mm caudal to the lambda suture on the midline and 3.5 mm deep. Tumors were injected with a stereotactic frame (Stoelting, 53311) and a 250 μL syringe (Hamilton, 81120) with a 25-gauge needle. All lines tested negative for mycoplasma contamination (IDEXX, September 26, 2017) and if passaged in vitro, were passaged less than 10 times after thawing.

HSPC transfer

HSPCs were freshly derived from bone marrow of naïve C57BL/6 mice. For in vivo tracking experiments, HSPCs were harvested from naïve DsRed mice. After red blood cell lysis, bone marrow was prepared for lineage depletion by MACS multistand with Lineage Depletion Kit and LS columns (Miltenyi Biotec, 130-090-858, 130-042-401, and 130-042-303).

Adoptive cellular therapy

Treatments of tumor-bearing mice began with 5 Gy nonmyeloablative (NMA) lymphodepletion or 9 Gy myeloablative (MA) by total body irradiation (TBI) with X-rays (X-RAD 320, Precision X-ray) 4 days after intracranial injection. On day 5 after intracranial tumor injection, mice received a single intravenous injection of 107 autologous ex vivo–expanded tRNA T cells ± the inclusion of 3.5 × 107 lin− HSPCs (Miltenyi Biotec, 130-090-858). Beginning day 6 after tumor injection, 2.5 × 107 tRNA-pulsed BMDC vaccines were injected intradermally posterior to the pinna weekly for three vaccines.
remained unclear how T cells and HSPCs were interacting within T-cell recruitment to the brain tumor microenvironment, it between DCs, T cells, and HSPCs (5). Although HSPCs promote

Brain tumor processing
Tumor resection extended to gross borders of tumor mass near the site of injection. Tumors were dissociated mechanically with a sterilized razor blade and chemically with papain (Worthington, NC9809987) for 30 minutes. Tumors were filtered with a 70-μm cell strainer (BD Biosciences, 08-771-2) prior to antibody incubation.

Flow cytometry
Flow cytometry was performed on the BD Biosciences FACS Canto-II. IFNγ release by T cells from transgenic GREAT mice was detected at FL-1 for yellow fluorescent protein (YFP) that is expressed under the IFNγ promoter. Cells from transgenic DsRed mice were detected at FL-2. FACS sorting was completed using the BD Biosciences FACS Aria II. Cells were prepared ex vivo as described above and suspended in 2% FBS (Seradigm, 97068-091) in PBS (Gibco, 10010-049). Antibodies were applied as per the manufacturer’s recommendation with isotype controls (Supplementary Table S1). Analysis and flow plots were generated with FlowJo version 10 [Tree Star] after omission of doublets and debris and were gated on size and granularity.

T-cell function assays and supernatant transfer system
In vitro experiments utilized restimulation assays including effector cells (T cells) and targets (pulsed DCs or tumor cell lines) that are cocultured in a 10:1 ratio in 96-well U-bottom plates in triplicate as a measure of T-cell activity. IFNγ Platinum ELISAs (Affymetrix, BM5606) were performed on acellular media that were harvested and frozen from the supernatants after 48 hours. The supernatant transfer system utilized the 10:1 ratio of T cells and DCs to generate supernatants. Anti-IFNγ (Affymetrix, 16-7331-85), anti-IFNγ (Affymetrix, 16-7311-85), and recombinant IFNγ [Thermo Fisher Scientific, 50-925-7] were used for HSPC differentiation cultures.

PCR array
Brain tumors were dissociated, RNA isolated with the RNeasy Kit, and analyzed with the RT² Profiler Array Cancer Inflammation and Immunity Cytokine PCRArray (Qiagen, PAMM-181ZD-12).

Statistical analysis
All experiments were analyzed in Prism 7 (GraphPad). The median survival for tumor-bearing animals is 25 to 42 days. Statistical analysis was performed using the Kaplan–Meier survival analysis, log-rank test, one-way ANOVA, unpaired t test, or unpaired Mann–Whitney rank test as appropriate. P values were calculated using GraphPad software (GraphPad Software, Inc., San Diego, CA, USA). Survival differences were considered statistically significant at P < 0.05. Animal studies were performed as six individual experiments.

Results
We have previously demonstrated that the success of HSPCs and ACT in malignant gliomas is reliant on cellular interactions between DCs, T cells, and HSPCs (5). Although HSPCs promote T-cell recruitment to the brain tumor microenvironment, it remained unclear how T cells and HSPCs were interacting within malignant gliomas, and what the cellular fates were of HSPC-derived progeny. Given previous reports of HSPC-mediated expansion of cytotoxic T cells (6), and the dependence of ACT on intratumoral DCs in peripheral malignancies (12), we hypothesized that HSPC-derived cells that migrate to brain tumors directly promote antitumor immunity in situ. We therefore studied the intratumoral differentiation of HSPCs and functional capacity of progeny to determine mechanisms of how HSPCs promote antitumor immunity.

We evaluated these interactions during ACT that uses tRNA-pulsed BMDCs to expand tumor-reactive lymphocytes for several orthotopic brain tumor models. Intracranially implanted Nf1+/−; p53+/− (KR158B-luc) malignant gliomas, CMyc-driven (NSC) medulloblastomas, and H3.K27M mutant (K2) brainstem gliomas were all responsive to the combination of adoptively transferred tumor-reactive lymphocytes, intradermal BMDC vaccination, and intravenously administered imiHSPC transfer after MA 9 Gy TBI. This HSPCs + ACT treatment platform extended median overall survival and promoted long-term cures in all aggressive brain tumor models (P < 0.0001; Fig. 1). To determine the mechanism of action, we studied KR158B-luc tumor-bearing hosts after 5 Gy NMA conditioning and ACT with and without syngeneic HSPC transfer from bone marrow of DsRed transgenic animals to track the fate of HSPC progeny. Similar to the adoptive therapies utilized in the melanoma setting (6, 7, 9), the antitumor efficacy of ACT in our model is greatest in the MA 9 Gy setting. However, we studied the NMA 5 Gy setting to offer the benefits of lymphodepletion while removing the requirement for bone marrow HSPC rescue when doing experiments with or without the use of wild-type (WT) HSPC transfer. This allowed for continuous investigation of a treatment platform with experimental flexibility.

We determined that intravenously administered HSPCs migrate to brain tumors, engraft, and persist within the tumor microenvironment 7 days posttransfer (Fig. 2A and B). During an initial phenotype of HSPC-derived cells in brain tumors, we discovered that a fraction of HSPC-derived cells upregulated the initial phenotype of HSPC-derived cells in brain tumors, we discovered that a fraction of HSPC-derived cells upregulated the unique capacity for HSPC tropism to malignant brain tumors and propensity to differentiate into intratumoral CD11c+ cells. Previous reports have attributed HSPC migration to gliomas to the CXCR4 chemotactic axis (28), and this has been corroborated in our model systems (not shown). To determine whether HSPCs migrate to gliomas as multipotent or lineage-restricted cells, we FACS-sorted DsRed+/− HSPC-derived cells from brain tumors and used the isolated cells to rescue nontumor-bearing animals treated with MA TBI (Fig. 2C). HSPC-derived cells isolated from brain tumor 3 hours after infusion were capable of rescuing 60% of myeloablated hosts, demonstrating that pluripotent stem cells within the HSPC transfer reach the brain tumor microenvironment shortly after injection and retain immunologic reconstitution capacity (WT HSPCs: 100% survival, 3 hours posttransfer HSPCs: 60% survival; not significant; Fig. 2D). However, only 20% of mice could be rescued from myeloablative therapy using HSPC-derived cells isolated from the tumor microenvironment 24 hours after transplant, demonstrating the
significant onset of lineage restriction (WT HSPCs: 100% survival, 24 hours posttransfer HSPCs: 20% survival; \( P = 0.0133 \); Fig. 2D).

We next investigated HSPC-derived cells in brain tumors during ACT (Fig. 3A). HSPC-derived cells persisted in brain tumors long after transfer, including near moribund endpoint 31 days after injection (Fig. 3B; Supplementary Fig. S1A). During engraftment in tumor, HSPCs supplanted host cells in the brain tumor microenvironment, and this displacement was facilitated by immunotherapeutic treatment. Specifically, when HSPCs are administered alone after 5 Gy, HSPCs drive a 57.5% decrease in host DsRed\(^{-}\) Ly-6G/6C\(^{+}\) MDSCs (−HSPCs: 13.92%; +HSPCs: 5.92%, \( P < 0.0001 \); Fig. 3C; Supplementary Fig. S1B). When administered during ACT, HSPCs drive an 87.7% decrease in host MDSCs, indicating that ACT facilitates this process (ACT alone: 24.18%; ACT + HSPCs: 2.98%, \( P < 0.0001 \); Fig. 3C; Supplementary Fig. S1C). To ensure that the reduction in intratumoral host MDSCs represented a true displacement and not altered expansion of other cellular components, we evaluated the absolute counts of MDSCs within the tumor microenvironment. We also discovered that nearly all Ly-6G/6C\(^{+}\) cells expressed CD11b\(^{+}\) and restricted the MDSC definition to CD11b\(^{+}\) Ly-6G/6C\(^{+}\) (36–39). HSPCs drove a 60% decrease in total host CD11b\(^{+}\) Ly-6G/6C\(^{+}\) MDSCs, whereas ACT + HSPCs drove a 77% decrease in total host CD11b\(^{+}\) Ly-6G/6C\(^{+}\) MDSCs (HSPCs, \( P = 0.036 \); ACT + HSPCs, \( P = 0.0015 \); Fig. 3D). To further define the intratumoral CD11b\(^{+}\) Ly-6G/6C\(^{+}\) population as an MDSC population (38), we analyzed their functional capacity to suppress T cells. To perform this, we evaluated FACS-sorted tumor-infiltrating host MDSCs in a T-cell stimulation assay measuring release of IFN\(\gamma\) by activated tumor-specific T cells cultured with tRNA-pulsed BMDCs. When tumor-derived host CD11b\(^{+}\) Ly-6G/6C\(^{+}\) cells were included in this assay, they caused a 3-fold suppression of IFN\(\gamma\) release by tumor-specific T cells (\( P < 0.0001 \); Fig. 3E). To characterize the MDSC population with a functionally suppressive molecule of import in the tumor microenvironment (40), we investigated the
expression of PD-L1 on host MDSCs. We determined that 85% of Ly-6G/6C+ cells express PD-L1 and that HSPC transfer supplants PD-L1 on host MDSCs. We determined that 85% of Ly-6G/6C+ cells express PD-L1 and that HSPC transfer supplants PD-L1 on host MDSCs. We determined that 85% of Ly-6G/6C+ cells express PD-L1 and that HSPC transfer supplants PD-L1 on host MDSCs.

We next evaluated the phenotype of HSPC-derived cells in brain tumors of ACT-treated animals. When HSPCs are administered in conjunction with ACT (3, 5), the HSPCs differentiate into CD11c+ MDSCs as well (5 Gy: 6.3%, 5 Gy + HSPC + ACT: 1.5%, P = 0.0079; Fig. 3F and G). These results demonstrate that tumor-infiltrating MDSCs are immuno-suppressive and that HSPC transfer can supplant these populations in brain tumors. This highlights an important pathway to reprogram the suppressive signature of the brain tumor microenvironment.

We next evaluated the phenotype of HSPC-derived cells in brain tumors of ACT-treated animals. When HSPCs are administered in conjunction with ACT (3, 5), the HSPCs differentiate into CD11c+CD11b+MHCII+ DCs in the tumor microenvironment with high expression of costimulatory activation marker CD86 and low expression of CD80 (HSPC-derived cells 21 days posttransfer:CD11c+: 83.7%, CD11b+: 84.5%, MHC II: 79.9%, CD86+: 76.2%, CD80+: 14.8%; Fig. 4A and B, Supplementary Fig. S1D, ref. 41). Detailed profiling indicates that HSPCs display little differentiation into CD3+ lymphocytes, c-kit+ stem cells, F4/80+ macrophages, PD-L1+ APCs, or Ly-6G/6C+ MDSCs and that HSPC-derived CD11c+ cells are largely negative for DC subtype markers CD8 and CD103 (Fig. 4A and B, Supplementary Fig. S1D). We also studied the durability of HSPC-derived DCs in brain tumors and determined they persist and outnumber the HSPC-derived MDSCs even until day 31 posttransfer when suboptimally treated animals are nearing moribund endpoint (Fig. 4C). Interestingly, the small population of HSPCs that do differentiate into MDSCs are less suppressive in a T-cell stimulation assay than host-derived MDSCs (HSPC-derived: 3108.6 pg/mL, host-derived: 1619.7 pg/mL IFNγ, P = 0.0159; Supplementary Fig. S1E). Given the lack of HSPC differentiation into MDSCs, during HSPC transfer with ACT, there remains a significant net loss of total MDSCs in the tumor microenvironment while HSPC-derived DCs engraft (Supplementary Fig. S1F).

To evaluate the function of HSPC-derived DCs, we FACs-sorted HSPC-derived CD11b+Ly-6G/6C− cells, a population of non-MDSC CD11b+ HSPC-derived cells (87.4% of HSPC-derived cells), from brain tumors of mice treated with ACT and DsRed+ HSPC transfer. We then cocultured HSPC-derived DCs with tumor-specific T cells in a T-cell restimulation assay and identified significant activation of polyclonal tumor-specific T cells with an 18-fold increase in IFNγ (P = 0.0117; Fig. 4D). This level of T-cell activation was comparable with direct recognition of KR158B-luc glioma cells by tumor-reactive lymphocytes in vivo, demonstrating efficient antigen capture and presentation by HSPC-derived cells isolated from brain tumors. To demonstrate the same function completely in vivo, we treated tumor-bearing animals with ACT alone or ACT with HSPCs. Tumor-reactive lymphocytes were generated from GREAT mice, whose cells express YFP under the control of the IFNγ promoter. Therefore, we could track the antigen-specific activation of tumor-reactive T cells by IFNγ reporter expression in vivo. Although ACT led to significant IFNγ...
Host immunity in brain tumors during HSPC engraftment. A, Experimental layout for remainder of in vivo experiments including ±HSPC transfer and ±ACT (TCs with BMDC vaccine) following 5 Gy nonmyeloablative total body irradiation. Experimental plan similar to plan in Fig. 1A but includes 5 Gy irradiation instead of 9 Gy to remove the requirement for HSPC transplant and DsRed<sup>+</sup> HSPCs are administered to allow tracking of HSPC-derived cells (DsRed<sup>+</sup>) or host-derived cells (DsRed<sup>−</sup>). B, Representative flow cytometry of brain tumors of animals harvested 17 and 31 days post-ACT and 350,000 HSPCs. The experiment was performed twice. C, Bar graphs represent flow cytometry of brain tumors of animals 21 days posttreatment. Groups include untreated (n = 14), 5 Gy alone (n = 10), 5 Gy + HSPC transfer (n = 22), 5 Gy + ACT (n = 8), or 5 Gy + ACT + HSPC (n = 24) transfer. Gated on all DsRed<sup>−</sup> host-derived cells. Data are pooled from >2 experiments and represent mean ± SEM. Data from individual experiments are separated in Supplementary Fig. S1. D, Bar graph represents absolute cell count flow cytometry of brain tumors of animals 21 days posttreatment with 5 Gy alone (n = 7), 5 Gy + HSPCs (n = 12), or 5 Gy + HSPCs + ACT (n = 15). Data are pooled from two experiments and represent mean ± SEM. Gated on all DsRed<sup>−</sup> host-derived cells. MDSC marker was narrowed from Ly-6G/6C<sup>+</sup> in Cto CD11b<sup>+</sup>Ly-6G/6C<sup>−</sup> double-positive MDSCs. E, FACS sorting of host-derived CD11b<sup>+</sup>Ly-6G/6C<sup>−</sup> cells from brain tumors of mice 21 days posttreatment with ACT without HSPCs. A total of 4 × 10<sup>7</sup> FACS-sorted host-derived MDSCs were introduced into a coculture restimulation assay with 4 × 10<sup>6</sup> tRNA-pulsed DCs and 4 × 10<sup>6</sup> tumor-reactive T cells. Forty-eight hours later, an IFNγ ELISA was performed on the supernatants to determine the degree to which host-derived MDSCs suppressed T-cell activation by DCs. The experiment was performed twice and data represent mean ± SD. F, Bar graph represents flow cytometry of brain tumors 21 days posttreatment with 5 Gy, 5 Gy + HSPCs, or 5 Gy + HSPCs + ACT. Data, mean ± SD of PD-L1<sup>+</sup>Ly-6G/6C<sup>−</sup> host MDSCs. PD-L1 is a suppressive molecule found on tumor-infiltrating MDSCs. G, Further phenotypic characterization of host MDSCs. Bar graph represents flow cytometry percent expression of PD-L1 on the host Ly-6G/6C<sup>−</sup> MDSC population in brain tumor. Data, mean ± SD, **P < 0.01, ***P < 0.001, ****P < 0.0001, by unpaired Student t test for in vitro studies (n = 3) and by Mann-Whitney t test for in vivo studies (n ≥ 5).
with ACT or HSPCs. We discovered that although host populations never expressed greater than 40% of either CD11c or MHC II, the HSPC-derived population expresses 84% CD11c and 80% MHC II only during ACT (Supplementary Fig. S2C). Flow cytometry also showed that ACT increased the percentage of HSPC-derived CD11c+ cells from 15% to 84% and MHC II+ cells from 5% to 80% (P = 0.0079; Supplementary Fig. S2C). When we performed absolute cell counts to verify this effect, ACT doubled the absolute number of total HSPC-derived cells, CD11c+, and CD86+ HSPC-derived cells in brain tumors (Fig. 4F). To further assess the impact of ACT on HSPCs, we determined by PCRarray that when ACT is combined with HSPCs, there is an upregulation of IFNγ signaling, IFN-responsive genes, and APC-released cytokines including Il12a and Il12b (fold upregulation-Ifng: 2.6-fold, If1: 2.05-fold, Myd88: 2.05-fold, Tnfsf10: 1.66-fold, Il12a: 5.51-fold, Il12b: 3.42-fold, Il6: 2.13-fold, Cxcl9: 1.65-fold, Cxcl5: 2.44-fold, Cxcl10: 1.88-fold; Supplementary Fig. S2D and S2E). This suggests a feedback loop in which activated T cells influence HSPC differentiation into DCs, while HSPC-derived DCs in turn promote T-cell activation.
Given the impact ACT had on HSPC engraftment and gene signatures, and the appearance that IFNγ was critical, we sought to verify the role of activated T-cell–released cytokines that drive HSPC differentiation (42,43). Therefore, we performed supernatant transfer onto HSPCs to determine the effects of soluble factors released by tumor-reactive T cells when cultured with BMDCs presenting cognate antigens (Fig. 5A). Supernatant transfers onto freshly isolated HSPCs included conditioned media containing acellular supernatants from 2-day cocultures of tRNA T cells with tRNA-pulsed BMDCs (hereafter referred to as TC + DC supernatants). After 3-day culture of HSPCs in the conditioned media, soluble factors caused a >100-fold increase in HSPC proliferation by CellTrace Violet (P = 0.0021; Fig. 5B and C). Importantly, TC + DC supernatants polarized HSPC differentiation in the proliferated populations toward DCs and away from MDSCs (MHC II⁺: 81.2%, CD11b⁻Ly-6G/6C⁺: 27.3%; Fig. 5B and C). Moreover, the largest CellTrace Violet peaks significantly correlated with decreased expression of MDSC markers and the increased expression of activated DC markers including MHC II coexpressed with CD11c and CD86 on HSPC-derived cells (Fig. 5B and C; Supplementary Fig. S3). On the contrary, supernatants from T cells that had not engaged their cognate antigens expressed by BMDC targets (T cells alone) polarized HSPC differentiation toward MDSCs (83%) and away from DCs (9%). This demonstrates that HSPCs differentiate into DCs in the presence of soluble factors released by activated T cells.

We then compared the phenotype and functional capacity of HSPC-derived cells generated by 3-day culture with BMDCs generated as previously described through a 9-day selection culture. This determined that HSPC-derived cells largely expressed...
a similar phenotype to BMDCs including high CD11c, CD11b, MHC II, and CD86 expression (Supplementary Fig. S4A). To confirm that these HSPC-derived cells were functionally DCs, we tested the capacity of these HSPC-derived cells to prime naïve T cells. To perform this, we harvested HSPC-derived DCs or BMDCs from culture, pulsed them with ttRNA, and used them to prime great mice. We then measured a significant and nearly equivalent increase in IFNγ-producing CD3+ cells and IFNγ release by CD3+ T cells in spleens of HSPC-derived DC and BMDC-primed hosts (%: P = 0.0079; MFI: P = 0.0317; Supplementary Fig. S4B). This indicated that although HSPC-derived DCs from brain tumors could activate tumor-reactive T cells, in vitro HSPC-derived DCs can also prime naïve T cells in the periphery at a level similar to traditional BMDCs.

To determine whether HSPC differentiation into DCs was dependent on T cells recognizing cognate antigen, supernatants were collected from either cocultures of KR158B-luc glioma cells generating soluble factors that promoted HSPC differentiation into DCs, or from culture, pulsed with ttRNA, and used to prime naïve T cells. To perform this, we harvested HSPC-derived DCs or BMDCs supernatant transfer onto HSPCs and determined that antigen-matched TC supernatant transfer onto HSPCs and determined that antigen-matched TC supernatants were significantly better at upregulating MHC II on HSPCs compared with the antigen-mismatched supernatants (B16F10OVA: 28% vs. ttRNA DC: 70%; P = 0.0002; Supplementary Fig. S5A). We also determined that T-cell recognition of KR158B-luc glioma cells generated soluble factors that promoted HSPC differentiation into DCs (Supplementary Fig. S5B). Overall, this indicates that T-cell-released cytokines significantly shift after T-cell activation with antigen-specific target cells to promote HSPC differentiation into DCs. In addition, this indicated that tumor-reactive T cells should likely release these cytokines into the tumor microenvironment in vivo. We hypothesized that IFNγ would likely be the key T-cell-released cytokine in this system given the preponderance of IFNγ upregulation in ACT-treated brain tumors (Supplementary Fig. S2B) and the described role for IFNγ in myelopoiesis, APC activation, and broad antitumor immunity (44–48). Therefore, we performed a series of in vitro experiments with selective inclusion or depletion of T-cell–released cytokines. We evaluated GM-CSF in parallel with IFNγ given its similarly important role in HSPC differentiation. We first demonstrated that T cells release IFNγ and GM-CSF after recognition of antigen-specific targets (P < 0.0001 for IFNγ and GM-CSF; Supplementary Fig. S6). We next performed GM-CSF and IFNγ antibody blockade during supernatant transfer. Antibody blockade caused a significant decrease in HSCP-derived DCs during supernatant transfer with 25 μg/mL anti-IFNγ (57% decrease) or 25 μg/mL anti–GM-CSF (77% decrease; Supplementary Fig. S7A). To confirm these results, we evaluated supernatant transfer with T cells from GM-CSF and IFNγ knockout animals. This generated functional T cells that produced a milieu of activated T-cell cytokines after antigenic stimulation, including TNFα, without the contribution of either IFNγ or GM-CSF (Supplementary Fig. S7B). When analyzing HSPCs cultured in IFNγ-deficient TC + DC supernatants, HSPCs proliferated more and significantly increased their differentiation into MDSCs (90% increase, P = 0.0001) and decreased differentiation into DCs (65% decrease, P < 0.0001) when compared with WT TC + DC supernatants (Fig. 5D; Supplementary Fig. S8). On the contrary, GM-CSF–deficient supernatant transfer mildly impaired HSPC proliferation and differentiation. This suggests a role for T-cell–released IFNγ in arresting HSPC proliferation for the selective differentiation into a DC instead of an MDSC. We then tested the impact of 1 μg of IFNγ on 3-day in vitro culture of HSPCs from WT or IFNγ receptor knockout (IFNγR−/−) mice. This demonstrated that HSPC-derived cells express 5-fold more MHC II when treated with 125 pg/mL IFNγ (P < 0.0001) and that this upregulation is abrogated when the HSPCs are from IFNγR−/− mice (P < 0.0001; Fig. 6A).

Having demonstrated the capacity for T-cell–derived IFNγ to drive HSPC differentiation in vitro in an antigen-dependent manner, and the effectiveness of ACT in increasing intratumoral IFNγ, we evaluated whether IFNγ signaling was critically involved in intratumoral HSPC differentiation. To do so, we analyzed the intratumoral differentiation of WT or IFNγR−/− HSPCs in mice treated with ACT. This demonstrated that IFNγR−/− HSPCs have a significantly impaired ability to differentiate into CD11c+ MHCII+ DCs (WT: 71.98%; IFNγR−/−: 44.27%; P = 0.0357; Fig. 6B, Supplementary Fig. S9). In addition, IFNγR−/− HSPC-derived CD11c+MHCII+ cells have an impaired ability to upregulate the CD86 costimulatory activation marker (32% decrease; WT: 68.9%; IFNγR−/−: 46.74%; P = 0.0159; Fig. 6C). Finally, IFNγR−/− HSPCs differentiated into CD11bLy-6c/G6C+ MDSCs 131% more than WT HSPCs (WT HSPCs: 3.6% MDSCs, IFNγR−/− HSPCs: 8.3% MDSCs; P = 0.0161; Supplementary Fig. S9).

Because removing IFNγ from HSPCs impaired the ability to generate intratumoral DCs, we hypothesized that IFNγ−/− HSPCs would have an impaired ability to prolong survival in conjunction with ACT in tumor-bearing animals. We utilized a 5 Gy model system because IFNγ−/− T cells did not significantly impair the ability to drive the generation of potent, tumor-reactive T cells. Through capitalizing on these mechanistic understandings, HSPC transfer and ACT hold significant potential as effective cellular immunotherapies for malignant brain tumors and potentially other solid malignancies.

Discussion

Although HSPCs possess the ability to differentiate into any hematopoietic cell, many reports demonstrate that HSPCs in solid cancer-bearing hosts are immunosuppressive (21–27). Specifically, they are polarized to differentiate into MDSCs and can home to the bone marrow, spleen, tumor site, and the peripheral blood to...
Impair immune responses and even promote metastases. Our study demonstrates that the combination of ACT and HSPC transfer can reprogram the typical immunosuppressive differentiation and function of HSPCs in solid cancers. Although we focused on the brain tumor microenvironment because HSPCs are tropic for this region, it will be interesting to investigate the cellular interplay in peripheral lymphoid organs and to see whether these findings can be translated to peripheral solid cancers to supplant host MDSCs, promote the differentiation of DCs, and thereby promote cytotoxic rejection of tumors.

Dogma indicates that the brain is immunoprivileged to protect neurons from immunologic targeting and manage critical skull pressures (15, 49); however, recent studies have demonstrated considerable immune activity in the brain, including the cytotoxic rejection of central nervous system (CNS) malignancies (4). Despite this success, the CNS has a lack of activated DCs in the brain at homeostasis (13–15). This diminishes sampling of tissues that are immune-protected and can delay or impair the stimulation of an inflammatory response in the brain. Although certain APCs can be activated in the brain during autoimmunity and infection, in the context of brain malignancies, brain immunoprivilege works in unison with the already immunosuppressive microenvironment of brain tumors to doubly impair APC activation and infiltration (17). This can manifest with a downregulation of MHC class I and II as well as costimulatory molecules including CD86 (14, 17).

Importantly, recent reports have indicated the success of ACT for peripheral cancers is reliant on intratumoral activated DCs (12). Our study provides a mechanism for counteracting the lack of activated DC infiltration in brain tumors. Through HSPC transfers and the simultaneous infusion of ACT, we can generate a significant population of DCs that promote cytotoxic responses. These HSPC-derived cells are particularly potent because of the CD86 costimulatory marker that enables the generation of a strong T-cell response (14, 50). Moreover, given the upregulation of IL12 in brain tumors treated with HSPCs and ACT, it will be interesting to investigate the role of HSPC-derived DC-released cytokines that promote immune cell development and antitumor immunity.

Our study suggests that during HSPC + ACT therapy, there is an intricate network of functions including chemotaxis, proliferation, differentiation, T-cell activation, and cytotoxic tumor destruction that requires further investigation. This future investigation will include investigation of other cell types, including NK cells, other
cytokines including type I IFNs (12), and attention to immunosuppressive gene signatures. Given the highly pliable nature of the microenvironment, we anticipate that there will be a collaborative set of alterations in the brain tumor microenvironment that extends beyond our current findings to yield an impairment of immunosuppression in favor of immune rejection. In addition, although we have demonstrated that HSPCs act as their own immunologic agent in promoting antitumor immunity, there is already a keen interest in using HSPCs as a carrier to intracranial tumors. It will be interesting to see whether HSPCs can be loaded as an immunomodulatory vehicle to further reprogram the brain tumor microenvironment.

Potential limitations to ACT and HSPC therapy could include HSPC differentiation into suppressive cells in the periphery, an exhaustion of HSPC-derived DCs near endpoint, tumor escape from immunoediting, or the potential for HSPC-derived DCs and T cells to have checkpoint blockade molecule interactions. In future studies it will be important to test the timing of tumor implantation and treatment. Ongoing studies evaluating mobilized HSPCs, synergism with checkpoint blockade, host conditioning with chemotherapy, overcoming tumor escape from T-cell immunotherapy, and endogenous tumor models will be instrumental to continue to improve our translation to the clinic.

Although brain tumors are largely resistant to DC infiltration and activation (13, 14, 49), we have demonstrated a unique mechanism of supplanting MDSCs while generating intraglomial DCs with potent immunologic functions. Importantly, this synergy combines a minimization of immunosuppression and concomitant immune activation. Taken together, these data imply that HSPCs are more than just stem or progenitor cells capable of rescuing bone marrow, but cells capable of synergistically enhancing in situ antitumor immunity in the immunosuppressive brain tumor microenvironment.

References

Disclosure of Potential Conflicts of Interest
D. A. Mitchell holds ownership interest (including patents) in OncoLogic, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: T. J. Wildes, C. T. Flores, D. A. Mitchell
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. J. Wildes, B. M. Wummer, D. J. Damiani, R. S. Abraham, C. T. Flores
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. J. Wildes, A. Grippin, K. A. Dyson, D. J. Damiani, C. T. Flores, D. A. Mitchell
Writing, review, and/or revision of the manuscript: T. J. Wildes, A. Grippin, K. A. Dyson, B. M. Wummer, C. T. Flores, D. A. Mitchell
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. J. Wildes
Study supervision: T. J. Wildes, C. T. Flores, D. A. Mitchell

Acknowledgments
This research was supported by the University of Florida Health Cancer Center Predoctoral Award (to T. Wildes), NCI R01 CA155563 (to D. Mitchell), American Brain Tumor Association Research Collaboration Grant (to C. Flores), Alex's Lemonade Stand Young Investigator Grant (to C. Flores), Florida Center for Brain Tumor Research Grant (to C. Flores), the Preston A. Wells, Jr. Endowment at the University of Florida, and University of Florida Clinical and Translational Sciences Award (5UL1TR001427-03).

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 November 21, 2017; revised March 26, 2018; accepted April 25, 2018; published first April 30, 2018.
42. Schurr CM, Rether C, Ochsenbein AF. Cytotoxic CD8\(^+\) T cells stimulate hematopoietic progenitors by promoting cytokine release from bone marrow mesenchymal stromal cells. Cell Stem Cell 2014;14:460–72.
48. Kalitski P, Schuettemaker JHN, Hilkens CMU, Wierenga EA, kapsenberg ML. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-\(\gamma\) and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. J Immunol 1999;162:3231–6.
Cross-talk between T Cells and Hematopoietic Stem Cells during Adoptive Cellular Therapy for Malignant Glioma

Tyler J. Wildes, Adam Grippin, Kyle A. Dyson, et al.

Clin Cancer Res 2018;24:3955-3966. Published OnlineFirst April 30, 2018.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-17-3061

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2018/04/28/1078-0432.CCR-17-3061.DC1

Cited articles
This article cites 50 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/24/16/3955.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/24/16/3955.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/24/16/3955.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.