ImmunoHorizons

RESEARCH ARTICLE

Innate Immunity

MR1 Tetramer–Based Artificial APCs Expand MAIT Cells from Human Peripheral Blood That Effectively Kill Glioblastoma Cells

Raj Priya and Randy R. Brutkiewicz

Department of Microbiology and Immunology, Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN

ABSTRACT

Immunotherapy for cancer treatment requires the activation of cytotoxic effector lymphocytes. Mucosal-associated invariant T (MAIT) cells are innate T cells that recognize the MHC class I–like molecule MR1. MAIT cells play an important role in the immune response against microbial infections and can directly kill tumor cells. Although MAIT cells can be expanded ex vivo, this method is time-consuming, expensive, and requires allogenic feeder layers. To overcome the limitations of conventional dendritic cell–based vaccines and ex vivo expansion of human T cells, an artificial APC (aAPC) approach to expand antitumor effector cells has several advantages. In this study, we explored an efficient in vitro method to amplify MR1-specific MAIT cells from human peripheral blood using aAPCs made by coating cell-sized latex beads with an Ag-loaded MR1 tetramer complex and anti-CD28 Ab. We further elucidated the cytotoxic potential of such expanded MAIT cells against three human glioblastoma multiforme (GBM) cell lines to explore their potential use as a novel immunotherapeutic tool, as the mostly lethal GBM poorly responds to conventional chemotherapy. When aAPCs were compared with the standard allogenic feeder layer–based approach for MAIT cell expansion, they were significantly more effective. Our results indicate that the aAPC-expanded MAIT cells remained functional, retained their original phenotype, secreted proinflammatory cytokines, and showed cytotoxicity against the GBM cell lines. Hence, MAIT cells have the potential to be a novel tool in immunotherapy approaches for the treatment of human GBM. *ImmunoHorizons*, 2021, 5: 500–511.

INTRODUCTION

Glioblastoma multiforme (GBM) is a devastating disease, the most common primary brain tumor and highly aggressive. Despite recent advances in modern surgery, chemotherapy, and radiotherapy for cancer in general, the survival time for GBM patients remains at 15–16 months and, therefore, urgently requires the development of new, alternative therapeutic approaches (1). Because of the brain being an immune-privileged site and the presence of the blood–brain barrier, even the most current advanced treatments are ineffective in the case of glioma. Earlier studies showing immune activation by brain tumor cells using dendritic cell–based vaccines (2–4) and the migration of tumor-specific activated cytotoxic T cells into the brain (5, 6) suggest that immunotherapy could potentially be a promising treatment option. Another type of T cell, a subpopulation of innate T cells called mucosal-associated invariant T (MAIT) cells also have the capacity to kill tumor cells. In humans, MAIT cells constitute 1–10% of blood T cells (7), 10–50% of liver T cells (8), and 2–4% of lung T cells (9). MAIT cells were originally shown to play a protective role in host defense against infectious diseases, including bacterial and viral infections (10–12). They recognize processed microbial vitamin B metabolites presented by the nonpolymorphic MHC class

Received for publication January 19, 2021. Accepted for publication June 1, 2021.

Address correspondence and reprint requests to: Dr. Randy R. Brutkiewicz, Department of Microbiology and Immunology, Stark Neurosciences Research Institute, Indiana University School of Medicine, Building R2, Room 302, 950 W. Walnut Street, Indianapolis, IN 46202. E-mail address: rbrutkie@iu.edu

This work was supported by National Institutes of Health Grant R01 CA161178 and the Indiana Spinal Cord Injury and Brain Injury Grant Program (to R.R.B.) as well as U54 DK106846, supporting the Indiana University School of Medicine's Cooperative Center of Excellence in Hematology.

Abbreviations used in this article: aAPC, artificial APC; GBM, glioblastoma multiforme; LDH, lactate dehydrogenase; MAIT, mucosal-associated invariant T; MFI, mean fluorescence intensity; MOI, multiplicity of infection; 5-OP-RU, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil; PFA, paraformaldehyde; qPCR, quantitative real-time PCR.

https://doi.org/10.4049/immunohorizons.2100003

ImmunoHorizons is published by The American Association of Immunologists, Inc.

I–like molecule MR1 (13–15). Apart from their role in infectious diseases, MAIT cells are proinflammatory innate T cells, able to secrete cytotoxic proteins that can directly kill tumor cells; hence, they are also potentially attractive anticancer effector cells (10, 16). MAIT cells have been shown to play both passive and active roles in antitumor immune responses or under conditions that actually promote tumor growth (16–22). Moreover, MAIT cells indirectly promote cytotoxicity by activating other antitumor effector cells (23). The potential use of MAIT cells as anticancer effector cells has several advantages: they recognize MR1 molecules, which are nonpolymorphic and highly conserved in humans and other mammalian species; they secrete proinflammatory cytokines that rapidly activate immune responses, possess an effector-memory phenotype, and secrete cytotoxic mediators such as perforin (12, 22, 24–26).

Despite the previously described role for MAIT cells in infectious diseases and certain cancers, the contribution of the MR1/MAIT cell axis in cancers of the CNS is less well known. Recently, we found MAIT cells in the normal mouse brain, as well as surface expression of MR1 on brain astrocytes and microglia (27). In the context of the MR1/MAIT cell axis in GBM, only one report described the detection of MR1 expression and MAIT cell TCR mRNA transcripts in a brain tumor (28). To the best of our knowledge, no follow-up studies have been pursued.

In the current study, we used the artificial APC (aAPC) approach to expand human peripheral blood MAIT cells and assess their functional capacity and ability to kill human GBM cell lines.

MATERIALS AND METHODS

Cells

The human glioma cell lines A172 and T98G were purchased from the American Type Culture Collection (Manassas, VA). The U251 human GBM line was kindly provided by Dr. P. Tolifon, National Institutes of Health (Bethesda, MD). All three GBM lines were cultured in DMEM (Lonza, Walkersville, MD) supplemented with 10% FBS, antibiotics, and 2 mM L-glutamine. THP-1 cells (a human monocytic leukemia cell line) were a kind gift from Dr. L. Pelus, Indiana University School of Medicine (Indianapolis, IN) and maintained in RPMI 1640 medium supplemented with 10% FBS.

Preparation of fixed Escherichia coli

Paraformaldehyde (PFA)–fixed *E. coli* were prepared as previously described (27, 29). For all experiments, the fixed *E. coli* were used at a multiplicity of infection (MOI) of 300 for 6 h to stimulate $MR1^+$ cells.

Abs and tetramers

Fluorochrome-conjugated anti-MR1 (clone 26.5), Vα7.2 (clone 3C10), CD161 (clone HP-3G10), CD4 (clone OKT4), CD8 (clone SK1), CD44 (clone IM7), Ki67 (clone 11F6), and PD-1 (clone EH12.2H7) were purchased from BioLegend. Allophycocyanin-conjugated, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU)–loaded human MR1 tetramers, and unconjugated 5-OP-RU–loaded MR1 tetramers were obtained from the National Institutes of Health Tetramer Facility. Fluorochrome-conjugated anti-CD3 (clone UCHT1) and CD152 (clone BNI3) were purchased from BD Biosciences. FITC-conjugated anti-CD107a (clone H4A3) was purchased from BD Pharmingen. Dead cells were excluded using the fixable LIVE/DEAD dye (Invitrogen).

PBMC isolation

PBMCs were isolated from deidentified buffy coats of healthy blood donors (Indiana Blood Center, Indianapolis, IN) by centrifugation on a Ficoll-Hypaque (GE Healthcare) density gradient. Aliquots of the isolated PBMCs were frozen and stored in liquid nitrogen until used. Before starting the experiments, frozen PBMC stocks were thawed and incubated overnight at 37°C in RPMI medium supplemented with 10% FBS.

Feeder layer–based expansion of MAIT cells from human PBMC

MAIT cells were expanded from human PBMCs and FACS sorted as described by us previously (29). Briefly, the cells were first stained with a PE-conjugated anti-Vα7.2 mAb and MACS sorted using anti-PE MicroBeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Next, the sorted cells were stained with an FITC-labeled mAb against human anti-CD161, and then $V\alpha7.2^+CD161^{++}$ cells were FACS sorted on FACSAria cell sorter (BD Biosciences). The sorted cells were cocultured with a gamma-irradiated feeder layer consisting of allogeneic PBMCs in the presence of PFA-fixed *E. coli* and human rIL-2 (PeproTech, Rocky Hill, NJ) for up to 21 d.

Preparation of aAPCs and control beads

The beads were prepared as previously reported (30) with some modifications. To prepare the aAPCs, 3.5-μm polystyrene beads (Invitrogen, Waltham, MA) were first coated with an anti-human CD28 mAb (CD28.2; 2 μg/5 × 10^7 beads; Sigma-Aldrich) for 12 h at 4°C on a rocker, followed by washing twice with PBS. Next, the beads were coated with 5-OP-RU–loaded MR1 tetramers for an additional 12 h at 4°C (2 μg/5 × 10^7 beads), also on a rocker. Excess unbound protein was removed by two 10-min washes in PBS. The prepared 5-OP-RU–MR1 tetramer/anti-CD28–conjugated aAPCs were stored in PBS at 4°C. The control beads were prepared in an identical fashion, but without the MR1 tetramers.

To confirm the conjugation of the MR1 tetramers and anti-CD28 to the latex bead, the beads were stained with mAbs against MR1 and anti-mouse IgG, respectively; flow cytometry analysis was then performed. To validate the functionality of the aAPCs, they were cocultured with PBMC-derived human primary MAIT cells (expanded using the feeder layer approach) for 48 h, and IFN-γ secretion into the supernatant

https://doi.org/10.4049/immunohorizons.2100003

was measured by ELISA. CD69 surface expression on the aAPC-activated MAIT cells was analyzed by flow cytometry.

Expansion of primary MAIT cells from PBMC using aAPCs

PBMCs (2.5×10^5 cells per well) were stimulated with aAPCs (2.5×10^5 beads per well) in vitro in 96-well plates containing RPMI medium supplemented with 10% FBS, antibiotics, 15 mM HEPES, and 55 μM β-mercaptoethanol (complete RPMI 1640). After 24 h at 37°C, human rIL-2 was added at a final concentration of 10 ng/ml (PeproTech). Fresh medium and IL-2 were replenished twice per week. On days 7, 14, and 21, the expanded cells were collected, counted, and plated into fresh wells at 2.5×10^5 cells per well, together with fresh 2.5×10^5 aAPCs per well in complete RPMI 1640 supplemented with IL-2. A small portion of cells at each timepoint was used to determine the percentage of MAIT cells by flow cytometry. After a total of three stimulations with aAPCs, the expanded cells were collected and washed, and the MAIT cells were electronically sorted.

FACS sorting and phenotypic characterization of expanded MAIT cells

The collected aAPC-expanded cells were blocked in human AB serum for 20 min on ice. The $CD3^+$ cells were positively selected using magnetic beads (Miltenyi Biotech), according to the manufacturer's instructions, by incubating the cells with anti-CD3, -CD161, and -Vα7.2 for 30 min on ice. The cells were then washed twice and sorted as $V\alpha7.2^+$ $CD161^{++}$ cells on an FACSAria cell sorter (BD Biosciences). The sorted MAIT cells were cultured in complete RPMI media containing IL-2. The cells were checked for purity and phenotypically characterized for effector/activation cell surface markers using flow cytometry. The Abs used were specific for the following: anti-CD4, CD8, CD44, PD-1, and CD152.

Assessment of MR1 expression by flow cytometry

The three human GBM lines were incubated for 6 h with fixed *E. coli* in 96-well plates at 37°C. The control cells received an equal volume of PBS. The cells were washed and stained with anti-MR1 mAb or isotype control for 30 min on ice, fixed, and then analyzed by flow cytometry for the expression of surface MR1.

Detection of MR1 expression by quantitative real-time PCR

To detect MR1 at the transcriptional level, RNA was isolated from the GBM cell lines (1×10^7 cells) using a commercial kit (RNeasy Mini Kit; QIAGEN). One microgram of RNA was converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out in 10-μl reaction volumes containing 5 μl of 2× TaqMan Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific), 1.0 μl of cDNAs, 0.5 μl of MR1 (Hs01042278) or GAPDH (Hs02786624) primers, and 3 μl of PCR grade water using the QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). As a calibrator, RNA from the $MR1^+$ human myeloid cell line THP-1 was used (31). The amount of MR1 mRNA, normalized to GAPDH and relative to the THP-1 cell line, was calculated with the $2^{-\Delta\Delta CT}$ method as described previously (32).

MAIT cell activation assay

The GBM cell lines were incubated with either fixed *E. coli* or PBS (control) in 96-well plates for 6 h at 37°C. The cells were then washed with PBS three times and cocultured with ex vivo–expanded MAIT cells overnight. In some wells, the anti-MR1 mAb or a mouse IgG2a isotype control mAb (20 μg/ml; MOPC-173; BioLegend) was added and incubated for 30 min prior to the addition of MAIT cells. The cells were stained with a LIVE/DEAD dye and anti-CD3, -CD69, and -Vα7.2 mAbs for 30 min on ice and subjected to flow cytometry analysis. The percentage of $CD69^+$ cells in the $Live^+CD3^+V\alpha7.2^+$ cell population was calculated.

Proliferation assay

GBM cells treated as above with fixed *E. coli* or PBS were cocultured with MAIT cells in complete media supplemented with IL-2 in 96-well plates for 5 d. The MAIT cells were washed and first stained with the LIVE/DEAD dye and anti-CD3 and -Vα7.2 mAbs for 30 min on ice, fixed/permeabilized, washed, and then incubated with an FITC-conjugated anti-Ki67 mAb on ice for 30 min. The data were acquired on an LSR II flow cytometer (Becton Dickenson). Gates were defined via isotype controls and fluorescence minus one staining. The percentage of $Ki67^+$ cells in the $LIVE^+CD3^+V\alpha7.2^+$ cell population was calculated using FlowJo software (BD Life Sciences).

MAIT cell degranulation assay

To assess MAIT cell degranulation, anti-CD107a mAb was added to the fixed *E. coli*/PBS–treated GBM/MAIT cell cocultures (1:1 effector to target cell ratio) for 12 h, with brefeldin A added for the last 3 h of culture. The cells were washed and then stained for the identification of CD107a on the surface of MAIT cells by flow cytometry. Unstimulated cells and an isotype control mAb were used to set the gates for determining the expression level of CD107a.

MAIT cell–mediated cytotoxicity assay

GBM cell lines treated with fixed *E. coli* or PBS were used as target cells. They were then cocultured with MAIT cells at different E:T ratios (1:1, 10:1, and 50:1) at 37°C overnight. The supernatant was collected, and the lactate dehydrogenase (LDH) released by lysed target cells into the supernatant was measured by the LDH-Cytox Assay Kit (BioLegend) as per the manufacturer's instructions. As a positive control, GBM/MAIT

cells treated with lysis buffer (maximum lysis) that was provided with the kit was used. The percentage of specific lysis was calculated using the following formula: Specific lysis = (A − C)/(B − C) × 100, where A is the average absorbance from triplicate sets of wells in which GBM/MAIT cells were cocultured together in the presence or absence of *E. coli*, B is the average absorbance from triplicate sets of wells in which GBM/MAIT cells were cultured in the presence of the lysis reagent, and C is the average absorbance from triplicate sets of wells of the medium control.

Detection of MAIT cell cytokine release

The GBM cell lines were treated with either fixed *E. coli* or PBS, followed by washing three times with PBS. The GBM cells and primary MAIT cells were then cocultured for 48 h (at a 1:1 effector/target cell ratio). To confirm MR1 dependence in Ag-specific cytokine secretion by MAIT cells, the anti-MR1 mAb or isotype control were added and incubated with the cells 30 min prior to the addition of MAIT cells. The concentrations of IFN-γ, TNF-α, and IL-17 in the coculture supernatants were determined by ELISA. As a control, the GBM cell lines and MAIT cells were cultured alone after stimulation with fixed *E. coli* or PBS.

Statistical analysis

The results were analyzed by an unpaired two-tailed Student *t* test using Prism software (version 6.05 for Windows; GraphPad). The displayed error bars in the graphs indicate the SD from the mean (mean ± SD). A p value <0.05 was considered significant.

RESULTS

Generation of MR1 tetramer–based aAPCs

We used a recently published protocol in which latex bead–based aAPCs allow for the rapid expansion of human MAIT cells in an Ag-dependent manner (30). Although T cells can be expanded using anti-CD3 and -CD28, this approach expands all of the T cells and is not specific for just MAIT cells. Our previously described allogenic feeder layer approach results in the proliferation of MAIT cells, but that proliferation is relatively slow (29). For the current study, we generated aAPCs by immobilizing Ag-loaded human MR1 tetramers and anti-human CD28 on cell-size latex beads (Fig. 1A). Control aAPCs consisted of beads on which only anti-CD28 mAbs are bound. To confirm that the MR1 tetramers and anti-CD28 were effectively immobilized on the surface of aAPC, the prepared aAPCs were stained with an anti-MR1 mAb and anti-mouse IgG1, respectively, and then analyzed by flow cytometry. The results indicated that both the MR1 tetramers and anti-CD28 mAb were efficiently bound to the surface of the latex beads (Fig. 1B). To assess the functional efficiency of the aAPCs, we cocultured primary MAIT cells with aAPCs or control beads for 48 h and measured the level of IFN-γ in the cell culture supernatants by ELISA (Fig. 1C). The aAPCs were able to induce the production of IFN-γ by the MAIT cells; very minimal IFN-γ was observed with MAIT cells cocultured with control beads. We further validated the functionality of the aAPCs by assessing the levels of the activation marker CD69 on the surface of the MAIT cells after a 12-h incubation by flow cytometry (Fig. 1D). aAPC-stimulated MAIT cells (but not those stimulated with control beads) upregulated CD69, further confirming that the aAPCs were functional.

Next, we asked whether the aAPCs were able to selectively induce the proliferation of MAIT cells present in whole PBMCs. In other words, can you expand MAIT cells from whole PBMCs that have been cocultured with aAPCs? For this, a flow cytometry analysis of PBMCs from normal blood donors was performed before and after a 5-d coculture with aAPCs or control beads in the presence of IL-2. Before the coculture with aAPCs, it was found that donor 1's MAIT cells made up 2.04% of the $CD3^+$ MR1 $tetramer^+$ PBMCs. After the 5-d coculture with the aAPCs, that level increased to 29.6% (Fig. 1E). MAIT cells from two other donors showed similar results (data not shown), suggesting that the frequency of MAIT cells increased by coculturing whole PBMCs with the aAPC.

Expansion kinetics of MAIT cells by MR1 tetramer–based aAPCs

To assess the kinetics of the aAPC-mediated expansion of MAIT cells, aAPCs were cocultured with PBMC from four healthy donors. In normal PBMCs, human MAIT cells range from 1 to 10%, with an average of ∼3–4% (7, 33). The percentages of total MAIT cells in the cultures were determined on days 7, 14, and 21 by flow cytometry. As shown in Fig. 2A, the level of MAIT cells in PBMCs from two representative donors progressively increased from an average of 31.55 and 56.6% on days 7 and 14, respectively, to a maximum of 83.8% on day 21. Thus, these results suggest that aAPCs are able to efficiently expand MAIT cells from the PBMCs of healthy human donors. Moreover, the percentages of MAIT cells defined as $CD3^+V\alpha7.2^+CD161^{++}$ (74.4 ± 7.5%) or $CD3^+$ MR1 $Tetramer^+$ cells (84 ± 2.7%) from the aAPC/PBMC cocultures were significantly higher in all four PBMC donors as compared with cocultures with control beads (1.1 ± 0.9 and 1.4 ± 0.3%, respectively; Fig. 2B). The percentage of $CD3^+$ MR1 $tetramer^+$ cells in the $V\alpha7.2^+CD161^{++}$ population and the percentage of $V\alpha7.2^+CD161^{++}$ cells in the $CD3^+$ MR1 $tetramer^+$ population were 85.5 ± 5 and 86 ± 7%, respectively (data not shown). Additionally, the mean fluorescence intensity (MFI) for Vα7.2, CD161, and the MAIT TCR (as measured by staining with the MR1 tetramer) was higher in the aAPC-expanded MAIT cells than in those cocultured with control beads (Fig. 2C). Furthermore, the percentage, as well as MFI, of aAPC-stimulated $CD3^+$ cells was also more elevated at all three timepoints (Fig. 2D). Cell viability over time was stable for the first 14 d of

https://doi.org/10.4049/immunohorizons.2100003

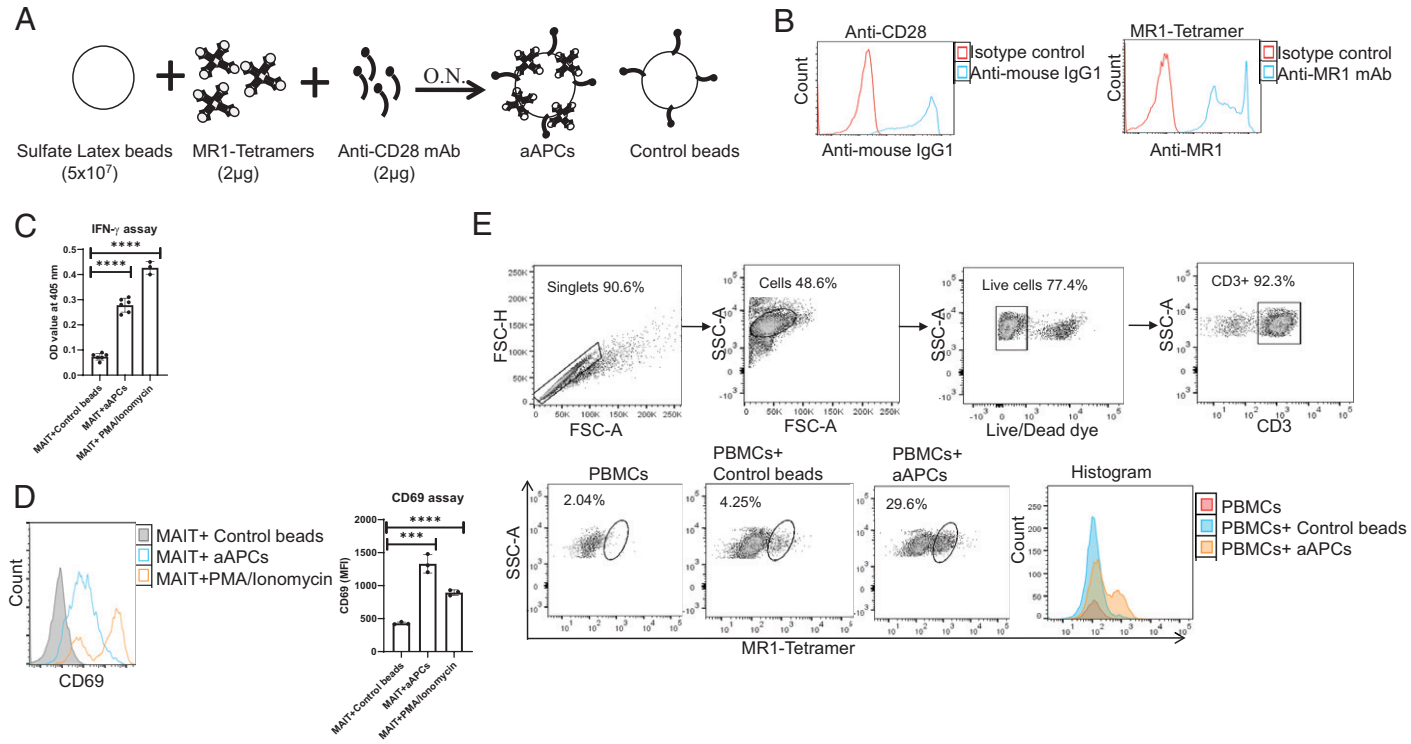

FIGURE 1. Preparation of MR1 tetramer + anti-CD28–based aAPCs for the expansion of human MAIT cells.

(**A**) Experimental design: latex beads were incubated with 5-OP-RU–loaded human MR1 tetramers and an anti-human CD28 mAb overnight (O.N.) at 4°C. Control beads are those on which only the anti-CD28 mAb is bound. (**B**) Binding of the anti-CD28 mAb and MR1 tetramers to the beads was confirmed by flow cytometry using mAbs against mouse IgG and MR1, respectively. (**C**) aAPCs (or control beads) were cocultured with primary human MAIT cells for 48 h, and the supernatant was analyzed for the presence of IFN-γ by ELISA. aAPC-expanded MAIT cells treated with PMA/ionomycin were used as a positive control. (**D**) Induction of CD69 expression on primary human MAIT cells by aAPCs. MAIT cells were cocultured O.N. with aAPCs, and CD69 expression was analyzed by flow cytometry. The histogram is a representative experiment illustrating aAPC-induced CD69 expression on MAIT cells. The bar graph represents the results of all three independent experiments combined. MAIT cells treated with PMA/ionomycin were used as a positive control. (**E**) Expansion of MAIT cells upon a 5-d coculture with aAPCs. The cells were stained with the MR1 tetramer and analyzed by flow cytometry. The data for (C) and (D) are representative of three to six independent experiments and expressed as the mean ± SD. ***$p < 0.001$, ****$p < 0.0001$, unpaired Student t test.

culture (~57 ± 2.7% on average), with a marked increase to over 92.5 ± 1.5% on day 21; this also reflected the fold increase in MAIT cells on day 21 (Fig. 2E). We also compared the MAIT cell fold expansion between the aAPC- and feeder layer–based approaches. The fold expansion of MAIT cells induced by aAPCs was significantly higher (60 ± 14) versus the feeder layer approach (26 ± 4; Fig. 2F). Additionally, the MAIT cell yield was dramatically higher by the aAPC-based method than the feeder layer technique ($4.4 \times 10^6 \pm 0.7 \times 10^6$ and $4.5 \times 10^4 \pm 1.2 \times 10^4$ cells, respectively (Fig. 2G). The purity of the expanded MAIT cells following these two expansion procedures was also different. Whereas the feeder layer approach resulted in an MAIT cell purity of 80 ± 3% after 21 d of culture, with the aAPC-based protocol, 96.3 ± 2% of the cells were MAIT cells (Fig. 3A). The expanded MAIT cells were phenotypically characterized as normal MAIT cells (i.e., $CD3^+CD4^-CD8^+CD44^+PD1^+CTLA4^-$ T cells; Fig. 3B). From this point, we electronically sorted the aAPC-expanded MAIT cells to a higher level of purity (e.g., 96.3%; Fig. 3A) and used these in the experiments described below.

Treatment with E. coli *induces the upregulation of surface MR1 on GBM cells*

MR1 expression has been detected on the surface of many human tumor cell lines, such as THP-1 (29). Whether glioma cells express MR1 has not yet been examined. Therefore, first, we determined the cell surface expression of MR1 on three commonly used human GBM cell lines by flow cytometry: A172, T98G, and U251. Steady-state surface MR1 glycoprotein levels were low to undetectable in all three lines (Fig. 4A). When we analyzed MR1 mRNA expression in these GBM lines using qPCR, it was evident that all three expressed the MR1 gene (Fig. 4C). We and others have shown that incubating cells with *E. coli* or several other microorganisms can increase cell surface expression of MR1 (29, 34, 35). Furthermore, there is evidence in

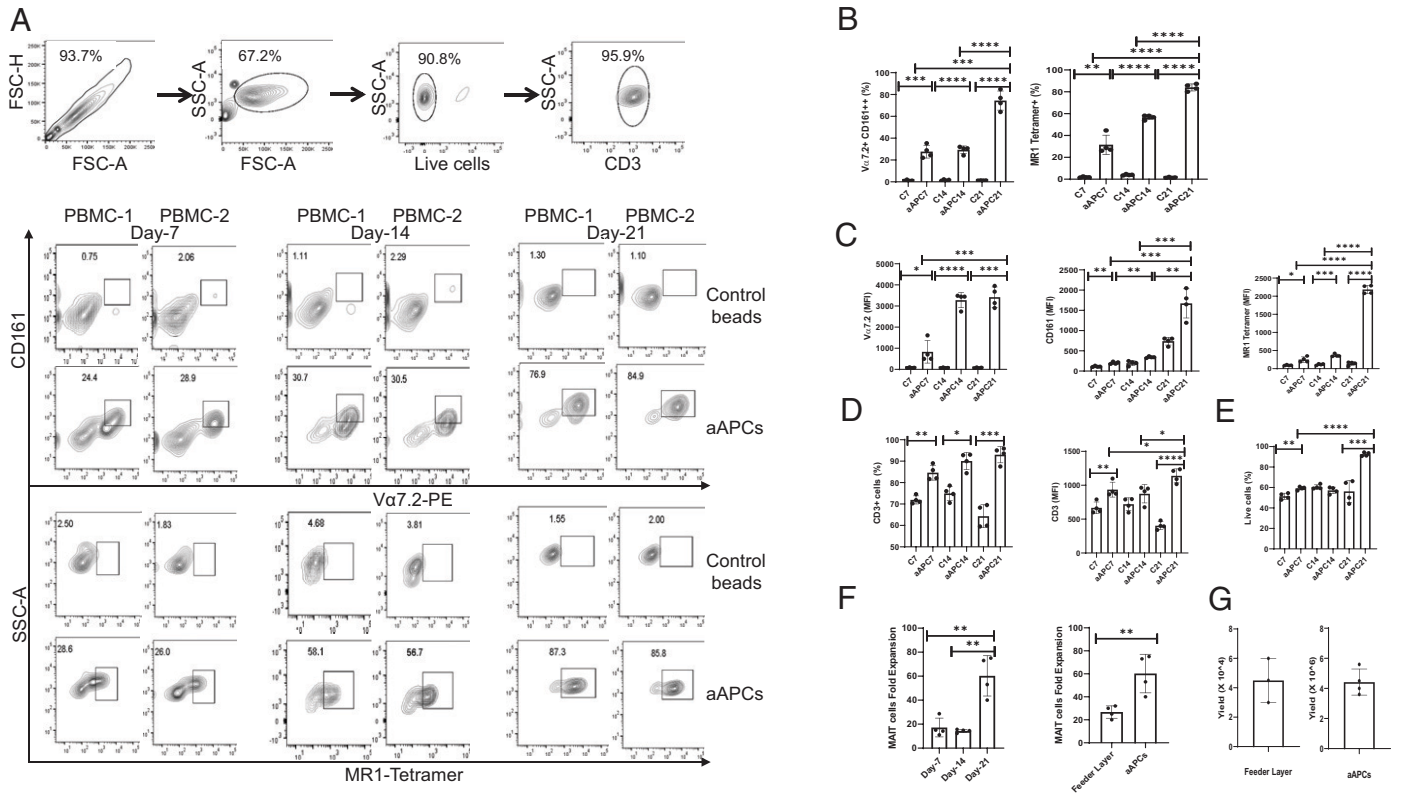

FIGURE 2. aAPCs effectively expand human MAIT cells from PBMCs.

(**A**) Kinetics of MAIT cell expansion by aAPCs. PBMCs from two different normal donors were cocultured with aAPCs (or control beads) at a ratio of 1:1 for 7, 14, and 21 d. MAIT cells were phenotypically identified by flow cytometry by their historical definitions as either $CD3^+$MR1 tetramer$^+$ or $CD3^+V\alpha7.2^+CD161^{++}$ cells. (**B**) The percentages of $CD3^+V\alpha7.2^+CD161^{++}$ and $CD3^+$MR1 tetramer$^+$ cells from four different normal donor PBMCs are shown. (**C**) The MFIs of anti-Vα7.2, anti-CD161, and MR1 tetramer staining on $CD3^+$ cells are shown. (**D**) The percentage of $CD3^+$ cells and MFI of CD3 surface expression in aAPC-expanded MAIT cells. (**E**) The percentage of live cells and fold expansion of aAPC-induced MAIT cells on days 7, 14, and 21 d of coculture. (**F**) Comparisons of fold expansion and total MAIT cell yield (**G**) between the feeder layer– and aAPC-based approaches. The data are representative of three independent experiments and expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p <$ 0.0001, unpaired Student *t* test.

some GBM patients that an infection postsurgery actually increases survival (36, 37). Thus, we next asked whether there is any change in MR1 expression on the GBM lines upon incubation with *E. coli*. We incubated the GBM cell lines with PFA-fixed *E. coli* at an MOI of 300 (or PBS as a negative control) for 6 h and then stained for cell surface expression of MR1 by flow cytometry. An increase in surface MR1 expression in all three GBM cell lines was evident, with significant increases in the percentage of MR1$^+$ GBM cells (Fig. 4A, 4B). Therefore, the results suggest that these GBM cell lines express endogenous MR1, which is upregulated upon exposure to *E. coli*.

Increased MAIT cell activation and proliferation induced by E. coli*–stimulated GBM cells*

We assessed the expression of the key T cell activation marker CD69 on MAIT cells after coculturing with either *E. coli*– or PBS-treated GBM cells, in the presence or absence of an anti-MR1 mAb. As shown in Fig. 5, the surface expression of CD69 increased substantially on MAIT cells after culturing with GBM cells preincubated with PFA-fixed *E. coli*. Coculturing MAIT cells with GBM lines that were not exposed to *E. coli* did not alter CD69 expression. The addition of an anti-MR1 mAb prevented the upregulation of CD69 on MAIT cells, indicating that the increase in CD69 expression was MR1 dependent. We next determined the ability of *E. coli*–incubated GBM lines to stimulate MAIT cell proliferation, as measured by intracellular Ki67 staining. It has been previously reported that in the GBM microenvironment, the tumor cells, by secreting a plethora of inhibitory factors, can inhibit effector T cell function and thereby promote tumor growth (38). In MAIT cells cocultured with *E. coli*–stimulated GBM cells, there was an increase in the percentage of those that were Ki67$^+$ (Fig. 6B–D). These data suggest that MAIT cells can efficiently proliferate upon

https://doi.org/10.4049/immunohorizons.2100003

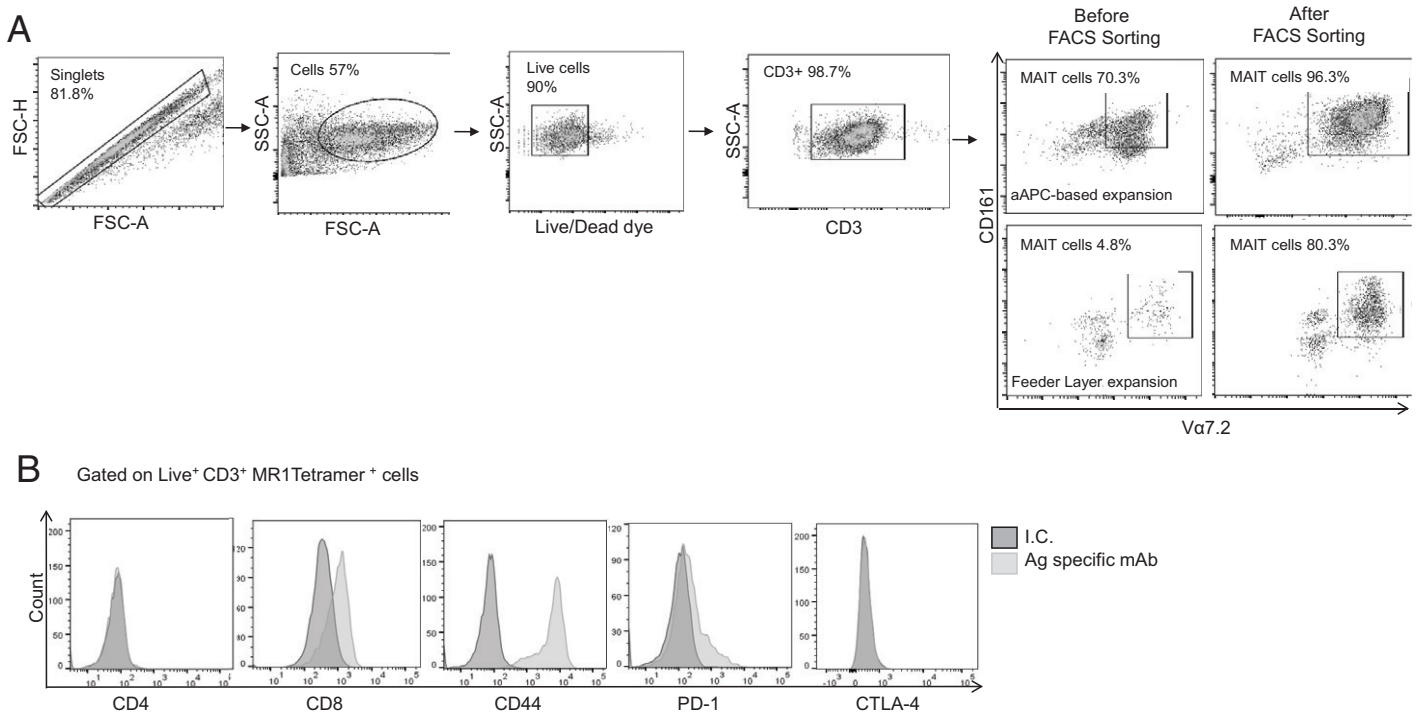

FIGURE 3. Phenotypic characterization of aAPC- and feeder layer–expanded human MAIT cells.

(**A**) The expanded human MAIT cells were stained with anti-CD161 and -Vα7.2 and analyzed by flow cytometry. The percentages of MAIT cells after expansion by both approaches and also after cell sorter–based purification were compared. (**B**) Live cell–gated $CD3^+$ MR1 $tetramer^+$ MAIT cells were stained with mAbs against CD4, CD8, CD44, PD-1, and CTLA-4 for cell surface phenotypic analysis by flow cytometry. I.C., isotype control.

recognition of GBM cells under conditions that increase MR1 Ag presentation.

MAIT cells directly kill glioma cells pretreated with E. coli

MAIT cells have the capacity to mediate cytotoxicity of target cells in much the same way as another innate T cell, NKT cells (39). One way to measure this ability is by measuring degranulation upon target cell engagement via the expression of CD107a on the MAIT cell surface. CD107a (LAMP-1) is an intracellular marker for late endosomes/lysosomes but is expressed on the surface of T cells and NK cells when their cytotoxic machinery is triggered; this can be detected using flow cytometry (40). Thus, we cocultured aAPC-expanded

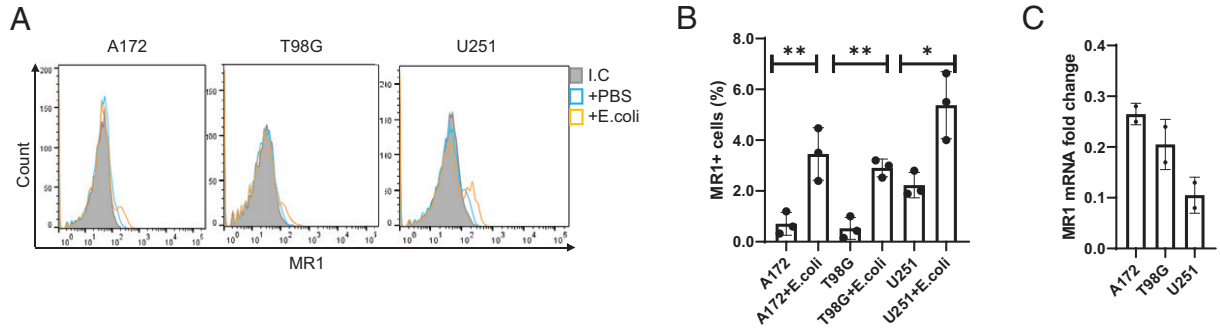

FIGURE 4. MR1 surface expression on human GBM cell lines pretreated with *E. coli*.

(**A**) The human GBM lines A172, T98G, and U251 were incubated with PBS or PFA-fixed *E. coli* (MOI = 300) for 6 h and then stained with an APC-labeled anti-MR1 mAb and analyzed by flow cytometry. Representative histograms (of three experiments performed) showing MR1 surface expression for each GBM line. (**B**) The percentage of $MR1^+$ cells in all three independent experiments is shown and expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$, by an unpaired Student *t* test. (**C**) qPCR analysis of MR1 gene expression in the human GBM cell lines relative to GAPDH, which served as an internal control. The data are presented as the mean ± SD from two independent cDNA samples, with three replicates in each sample.

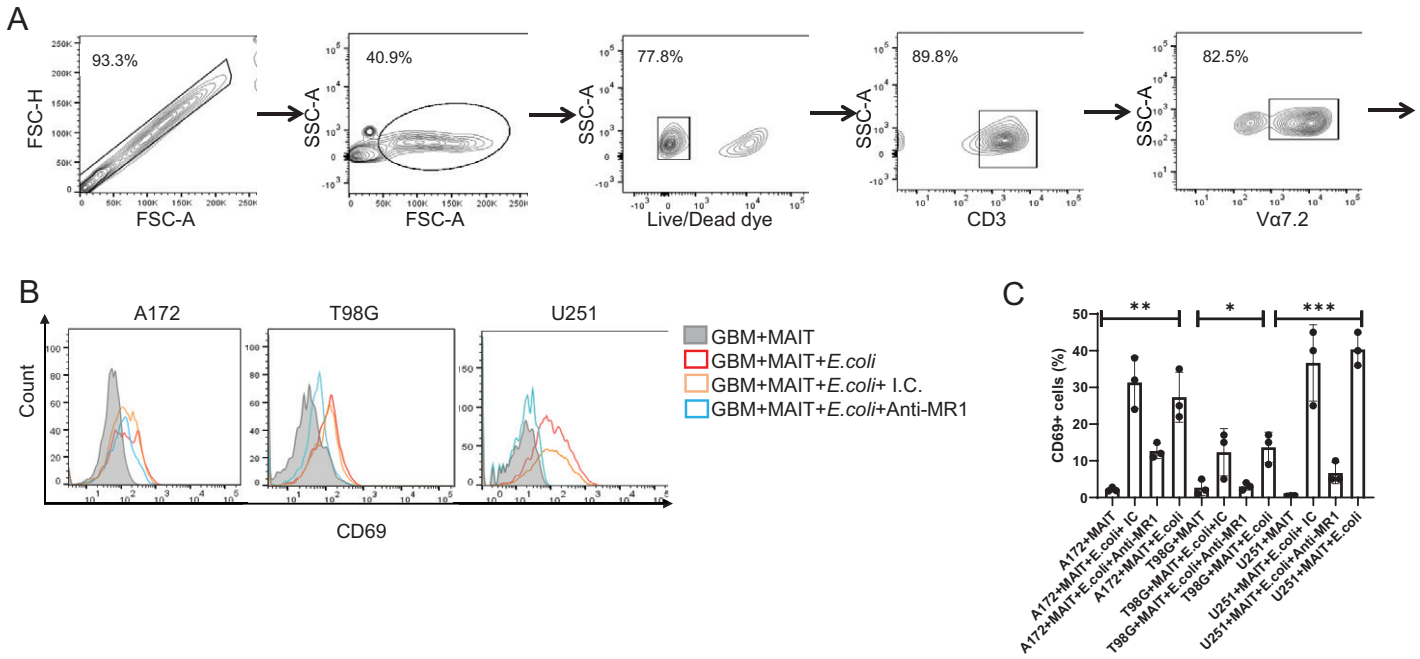

FIGURE 5. MAIT cell activation by *E. coli*–treated GBM cells.

(**A**) Flow cytometry gating strategy for aAPC-expanded human MAIT cells. (**B**) The three human GBM cell lines were treated with either fixed *E. coli* or PBS (control) and cocultured overnight with aAPC-generated MAIT cells. In some cases, an anti-MR1 or isotype control (I.C.) mAb was added and incubated for 30 min prior to the addition of the MAIT cells. Following staining with an anti-CD69 mAb, the level of CD69 on the MAIT cells was determined by flow cytometry and is indicated in the histograms. (**C**) The percentage of $CD69^+$ cells in the $CD3^+V\alpha7.2^+$ cell population was calculated, and the combined results for the three individual experiments are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, by an unpaired Student *t* test.

MAIT cells for 12 h with the three human GBM cell lines that were pretreated with *E. coli* or PBS for 6 h. We then stained for surface CD107a expression and performed a flow cytometry analysis. Fig. 6E and 6F shows the percentage of $CD107a^+$ cells on MAIT cells cultured with *E. coli*–stimulated GBM cells at an effector to target cell ratio of 1:1. There was a significant increase in surface CD107a expression on these MAIT cells relative to those cultured with PBS-treated GBM cells. In isolation, low numbers of MAIT cells expressed CD107a. Additionally, MAIT cells cocultured with *E. coli*–pretreated GBM cells secreted higher levels of IFN-γ and TNF-α compared with controls (Fig. 7). MAIT cells are known to also produce IL-17, particularly in inflammatory diseases (14). In cocultures of MAIT cells with fixed *E. coli*–treated GBM cells, there was no detectable IL-17 (Fig. 7). Therefore, MAIT cells secrete IFN-γ and TNF-α, but not IL-17, upon interaction with GBM cells presenting microbial Ags.

To determine whether MAIT cells can directly kill the GBM cells, we cocultured MAIT cells with GBM cells that were treated with either fixed *E. coli* or PBS at different effector to target cell (E:T) ratios and measured target cell killing by an LDH release assay. Although no significant difference in the release of LDH between the two groups was observed at the 1:1 and 10:1 E:T ratios, at the 50:1 E:T ratio, the level of released LDH into the supernatants was much higher than that observed in GBM cells treated with PBS. Therefore, these data suggest that MAIT cells can efficiently kill GBM cells in the presence of an MR1-presented Ag (Fig. 8).

DISCUSSION

In this study, we have used an MR1 tetramer–based aAPC approach for the induction and expansion of human MAIT cells as a means to measure their antitumor activity against GBM cell lines. We have previously reported that allogenic feeder layers stimulated with *E. coli* can be used to expand MAIT cells (29). Recently, the use of beads coated with MR1 tetramers has been used to expand MAIT cells (30); however, the kinetics of MAIT cell expansion, yield, and a comparison with the traditional approach had not been analyzed. Importantly, we showed in this study that this bead-based technique resulted in an expansion of PBMC-derived MAIT cells of ∼74-fold, as compared with the allogenic feeder layer procedure, in which the fold expansion was only 30.

The potential role of MAIT cells in the host response against cancers suggests the consideration of its use as a novel, promising immunotherapeutic tool; however, it is technically difficult to expand MAIT cells to obtain a sufficient number for clinical studies. In humans, although MAIT cells are readily found in the blood and have been shown to proliferate ex vivo

https://doi.org/10.4049/immunohorizons.2100003

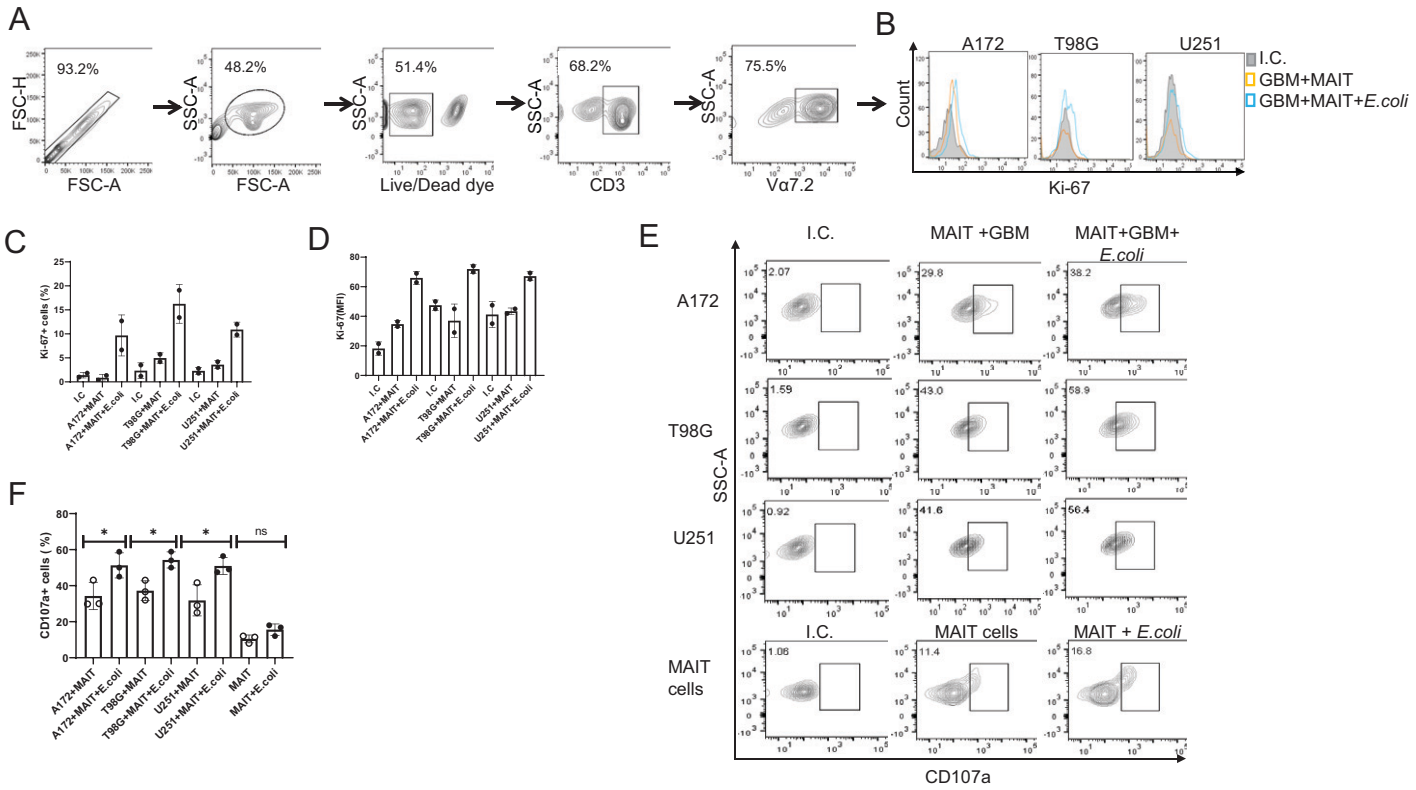

FIGURE 6. Human MAIT cell proliferation and degranulation induced by GBM cells pretreated with *E. coli*.

(**A**) GBM cells treated with PFA-fixed *E. coli* or PBS were cocultured with MAIT cells in the presence of IL-2 for 5 d. The cells were first stained with mAbs against CD3 and Vα7.2. They were then fixed/permeabilized and incubated with an anti–Ki-67 mAb. Analysis was by flow cytometry. (**B**) A representative result from the three GBM lines. (**C**) The percentage of Ki-67$^+$ MAIT cells. (**D**) The MFI of Ki-67 expression in MAIT cells. (C) and (D) are combined data from two independent experiments. (**E**) aAPC-generated MAIT cells were incubated for 12 h with the different GBM lines treated with fixed *E. coli* or PBS at an effector to target cell ratio of 1:1. The cells were then stained with mAbs against CD3, Vα7.2, and CD107a. The percentages of CD3$^+$Vα7.2$^+$ cells that are CD107a$^+$ from a representative experiment are shown. (**F**) Combined data from three independent experiments. The data are presented as the mean ± SD. *$p < 0.05$, by an unpaired Student *t* test. ns, not significant.

in presence of the riboflavin derivative 5-OP-RU (41, 42) or on a gamma-irradiated allogenic feeder layer preincubated with bacteria and in the presence of IL-2 (29), these approaches are associated with several drawbacks. First, the 5-OP-RU compound is very unstable and not available commercially; second, a feeder layer risks cell culture contamination; third, expanding MAIT cells requires a combination of cytokines; and fourth, under each of these culture conditions, MAIT cells proliferate slowly, resulting in a low yield. Hence, these approaches are prohibitively expensive and unattractive. With the development of MR1 tetramers for human MAIT cell detection via flow cytometry (33, 41), we sought to capitalize on this tool to generate bead-based aAPCs that can easily expand human MAIT cells in an Ag-specific manner. In fact, aAPCs have been used to expand another innate T cell subpopulation, NKT cells (43). The bead-based aAPC system is predicated on the principle that a T cell requires three signals for its induction and proliferation: Ag, costimulatory stimulus, and T cell–activating cytokines (44, 45). The bead-based aAPC preparation includes MR1 tetramers loaded with the ligand 5-OP-RU and an Ab against the costimulatory molecule CD28. Accordingly, in the current report, we prepared aAPCs for expanding MAIT cells from the peripheral blood of healthy human donors.

The expanded MAIT cells were functionally active and cytotoxic against GBM cells. GBM is a devastating disease in which the lifespan is limited to ∼15 mo (1), with no effective therapies currently available—this includes conventional immunotherapies. As MAIT cells have been demonstrated to show cytotoxic activity (25), we focused our efforts on whether aAPC-expanded human MAIT cells have the capacity to lyse GBM lines in an Ag-specific manner. The use of MAIT cells in GBM-targeting immunotherapy could be beneficial in a scenario in which conventional T cells have been found to be less effective, particularly in cases in which the downregulation of MHC class I molecules on the tumor cells has occurred (46). The cytotoxic effector function of MAIT cells requires microbial vitamin B–derived Ags to be presented by tumor-associated MR1. Although we used *E. coli* in this study, the use of

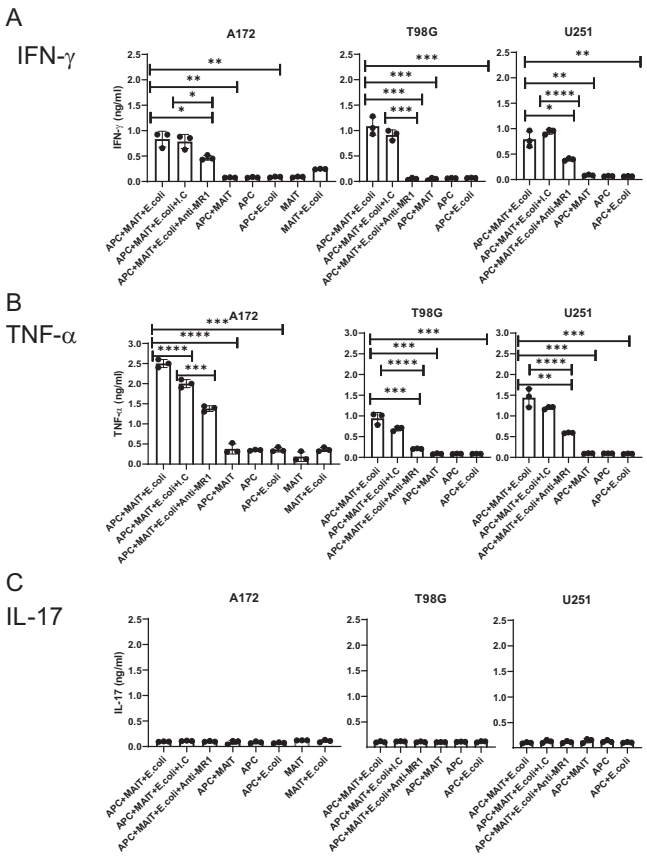

FIGURE 7. aAPC-expanded human MAIT cells produce IFN-γ and TNF-α (but not IL-17) upon coculture with *E. coli*–treated GBM cell lines.

The human GBM cell lines were treated with either fixed *E. coli* or PBS and cocultured with aAPC-expanded MAIT cells for 48 h at an effector to target cell ratio of 1:1. The supernatants were analyzed for the production of IFN-γ (**A**), TNF-α (**B**), and IL-17 (**C**) by ELISA. In some cases, an anti-MR1 or isotype control mAb (I.C.) was added 30 min prior to the addition of MAIT cells. The results for three independent experiments are shown and expressed as the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, by an unpaired Student *t* test.

nonpathogenic bacteria to provide MR1-presented Ags in the tumor microenvironment could be a promising approach in MAIT cell–mediated antitumor immunotherapy. Additionally, as MR1 is expressed by all cells at different levels, the ability of MAIT cells to predominantly target and kill tumor cells is an essential requirement. The generation of genetically modified bacteria expressing the riboflavin synthase gene under the control of a hypoxic promoter could be exploited to allow the synthesis of MAIT cell Ags in tumor cells only. This would help MAIT cells specifically target the tumor cells. Interestingly, Mehta and colleagues (47) demonstrated that actively motile bacteria expressing the tumor-suppressor protein p53 under the control of a hypoxia promoter, could migrate and localize to a glioblastoma tumor microenvironment effectively.

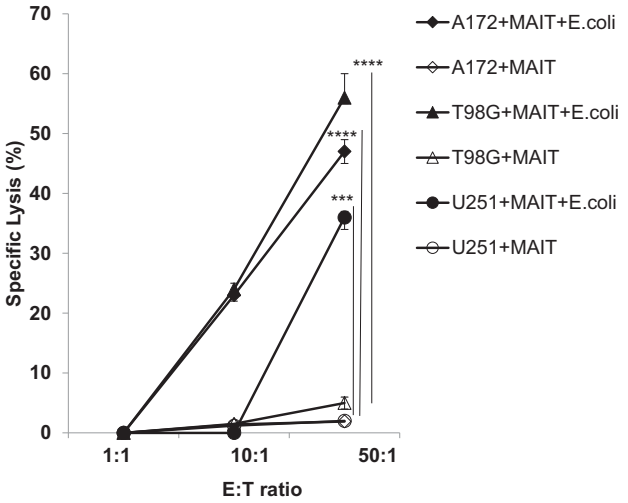

FIGURE 8. aAPC-expanded human MAIT cells directly kill *E. coli*–treated GBM cells.

The GBM cell lines treated with *E. coli* (or PBS) were cocultured with MAIT cells at different effector to target cell ratios (1:1, 10:1, and 50:1). The level of GBM killing was measured using an LDH-based assay and indicated as the percentage of specific lysis. The data are from two independent experiments and presented as the mean ± SD. $***p < 0.001$, $****p < 0.0001$, by an unpaired Student *t* test at an E:T ratio of 50:1.

In the current study, we found that MAIT cells efficiently interacted with the GBM cells, became activated, and secreted several proinflammatory cytokines while concomitantly killing the cancer cells. Moreover, compared with conventionally generated MAIT cells, the aAPC-expanded MAIT cells remained functional and stably retained their original phenotype. Thus, we propose that aAPC-generated MAIT cells can easily be used for adoptive antitumor immunotherapy. Considering the evidence that circulating MAIT cells are reduced in patients with a number of different cancers (22), it will be of interest to assess whether the same approach can be used to ex vivo expand this immune cell population isolated from these patients. In conclusion, the current study provides evidence that latex bead–based aAPCs can dramatically expand human MAIT cells from the peripheral blood, which demonstrate cytotoxicity against GBM cells and the production of proinflammatory cytokines in an MR1-dependent, Ag-specific manner. Future efforts could potentially couple this approach to checkpoint inhibitor or other contemporary modes of immunotherapy.

DISCLOSURES

The authors have no financial conflicts of interest.

ACKNOWLEDGMENTS

We thank Drs. L. Pelus and P. Tolifon for providing the THP-1 and U251 cells, respectively, as well as the National Institutes of Health

https://doi.org/10.4049/immunohorizons.2100003

Tetramer Core Facility for providing 5-OP-RU–loaded human MR1 tetramers. We also acknowledge the Flow Cytometry Resource Facility, Indiana University School of Medicine, for assistance. Finally, we express our gratitude for the critical help of Dr. J. Liu in the initial run ups of the experiments that ultimately became this article.

REFERENCES

1. Alexander, B. M., and T. F. Cloughesy. 2017. Adult glioblastoma. *J. Clin. Oncol.* 35: 2402–2409.
2. Kikuchi, T., Y. Akasaki, M. Irie, S. Homma, T. Abe, and T. Ohno. 2001. Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. *Cancer Immunol. Immunother.* 50: 337–344.
3. Yamanaka, R., T. Abe, N. Yajima, N. Tsuchiya, J. Homma, T. Kobayashi, M. Narita, M. Takahashi, and R. Tanaka. 2003. Vaccination of recurrent glioma patients with tumour lysate-pulsed dendritic cells elicits immune responses: results of a clinical phase I/II trial. *Br. J. Cancer* 89: 1172–1179.
4. Yu, J. S., C. J. Wheeler, P. M. Zeltzer, H. Ying, D. N. Finger, P. K. Lee, W. H. Yong, F. Incardona, R. C. Thompson, M. S. Riedinger, et al. 2001. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. *Cancer Res.* 61: 842–847.
5. Smolders, J., K. M. Heutinck, N. L. Fransen, E. B. M. Remmerswaal, P. Hombrink, I. J. M. Ten Berge, R. A. W. van Lier, I. Huitinga, and J. Hamann. 2018. Tissue-resident memory T cells populate the human brain. *Nat. Commun.* 9: 4593.
6. Stelma, F., A. de Niet, M. J. Sinnige, K. A. van Dort, K. P. J. M. van Gisbergen, J. Verheij, E. M. M. van Leeuwen, N. A. Kootstra, and H. W. Reesink. 2017. Human intrahepatic CD69+ CD8+ T cells have a tissue resident memory T cell phenotype with reduced cytolytic capacity. *Sci. Rep.* 7: 6172.
7. Dusseaux, M., E. Martin, N. Serriari, I. Péguillet, V. Premel, D. Louis, M. Milder, L. Le Bourhis, C. Soudais, E. Treiner, and O. Lantz. 2011. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* 117: 1250–1259.
8. Tang, X. Z., J. Jo, A. T. Tan, E. Sandalova, A. Chia, K. C. Tan, K. H. Lee, A. J. Gehring, G. De Libero, and A. Bertoletti. 2013. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J. Immunol.* 190: 3142–3152.
9. Hinks, T. S., J. C. Wallington, A. P. Williams, R. Djukanović, K. J. Staples, and T. M. Wilkinson. 2016. Steroid-induced deficiency of mucosal-associated invariant T cells in the chronic obstructive pulmonary disease lung. Implications for nontypeable *Haemophilus influenzae* infection. *Am. J. Respir. Crit. Care Med.* 194: 1208–1218.
10. Gold, M. C., S. Cerri, S. Smyk-Pearson, M. E. Cansler, T. M. Vogt, J. Delepine, E. Winata, G. M. Swarbrick, W. J. Chua, Y. Y. Yu, et al. 2010. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol.* 8: e1000407.
11. Loh, L., Z. Wang, S. Sant, M. Koutsakos, S. Jegaskanda, A. J. Corbett, L. Liu, D. P. Fairlie, J. Crowe, J. Rossjohn, et al. 2016. Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. *Proc. Natl. Acad. Sci. USA* 113: 10133–10138.
12. van Wilgenburg, B., L. Loh, Z. Chen, T. J. Pediongco, H. Wang, M. Shi, Z. Zhao, M. Koutsakos, S. Nüssing, S. Sant, et al. 2018. MAIT cells contribute to protection against lethal influenza infection in vivo. *Nat. Commun.* 9: 4706.
13. Karamooz, E., M. J. Harriff, and D. M. Lewinsohn. 2018. MR1-dependent antigen presentation. *Semin. Cell Dev. Biol.* 84: 58–64.
14. Keller, A. N., A. J. Corbett, J. M. Wubben, J. McCluskey, and J. Rossjohn. 2017. MAIT cells and MR1-antigen recognition. *Curr. Opin. Immunol.* 46: 66–74.
15. McWilliam, H. E., and J. A. Villadangos. 2018. MR1 antigen presentation to MAIT cells: new ligands, diverse pathways? *Curr. Opin. Immunol.* 52: 108–113.
16. Jeffery, H. C., B. van Wilgenburg, A. Kurioka, K. Parekh, K. Stirling, S. Roberts, E. E. Dutton, S. Hunter, D. Geh, M. K. Braitch, et al. 2016. Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *J. Hepatol.* 64: 1118–1127.
17. Duan, M., S. Goswami, J. Y. Shi, L. J. Wu, X. Y. Wang, J. Q. Ma, Z. Zhang, Y. Shi, L. J. Ma, S. Zhang, et al. 2019. Activated and exhausted MAIT cells foster disease progression and indicate poor outcome in hepatocellular carcinoma. *Clin. Cancer Res.* 25: 3304–3316.
18. Gherardin, N. A., L. Loh, L. Admojo, A. J. Davenport, K. Richardson, A. Rogers, P. K. Darcy, M. R. Jenkins, H. M. Prince, S. J. Harrison, et al. 2018. Enumeration, functional responses and cytotoxic capacity of MAIT cells in newly diagnosed and relapsed multiple myeloma. *Sci. Rep.* 8: 4159.
19. Huang, W. C., Y. C. Hsiao, C. C. Wu, Y. T. Hsu, and C. L. Chang. 2019. Less circulating mucosal-associated invariant T cells in patients with cervical cancer. *Taiwan. J. Obstet. Gynecol.* 58: 117–121.
20. Yan, J., S. Allen, E. McDonald, I. Das, J. Y. W. Mak, L. Liu, D. P. Fairlie, B. S. Meehan, Z. Chen, A. J. Corbett, et al. 2020. MAIT cells promote tumor initiation, growth, and metastases via tumor MR1. *Cancer Discov.* 10: 124–141.
21. Zabijak, L., C. Attencourt, C. Guignant, D. Chatelain, P. Marcelo, J. P. Marolleau, and E. Treiner. 2015. Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. *Cancer Immunol. Immunother.* 64: 1601–1608.
22. Won, E. J., J. K. Ju, Y. N. Cho, H. M. Jin, K. J. Park, T. J. Kim, Y. S. Kwon, H. J. Kee, J. C. Kim, S. J. Kee, and Y. W. Park. 2016. Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer. *Oncotarget* 7: 76274–76290.
23. Toubal, A., I. Nel, S. Lotersztajn, and A. Lehuen. 2019. Mucosal-associated invariant T cells and disease. *Nat. Rev. Immunol.* 19: 643–657.
24. Dias, J., M. J. Sobkowiak, J. K. Sandberg, and E. Leeansyah. 2016. Human MAIT-cell responses to *Escherichia coli*: activation, cytokine production, proliferation, and cytotoxicity. *J. Leukoc. Biol.* 100: 233–240.
25. Kurioka, A., J. E. Ussher, C. Cosgrove, C. Clough, J. R. Fergusson, K. Smith, Y. H. Kang, L. J. Walker, T. H. Hansen, C. B. Willberg, and P. Klenerman. 2015. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol.* 8: 429–440.
26. Slichter, C. K., A. McDavid, H. W. Miller, G. Finak, B. J. Seymour, J. P. McNevin, G. Diaz, J. L. Czartoski, M. J. McElrath, R. Gottardo, and M. Prlic. 2016. Distinct activation thresholds of human conventional and innate-like memory T cells. *JCI Insight* 1: e86292.
27. Priya, R., and R. R. Brutkiewicz. 2020. Brain astrocytes and microglia express functional MR1 molecules that present microbial antigens to mucosal-associated invariant T (MAIT) cells. *J. Neuroimmunol.* 349: 577428.
28. Peterfalvi, A., E. Gomori, T. Magyarlaki, J. Pal, M. Banati, A. Javorhazy, J. Szekeres-Bartho, L. Szereday, and Z. Illes. 2008. Invariant Valpha7.2-Jalpha33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells. *Int. Immunol.* 20: 1517–1525.
29. Liu, J., and R. R. Brutkiewicz. 2017. The Toll-like receptor 9 signalling pathway regulates MR1-mediated bacterial antigen presentation in B cells. *Immunology* 152: 232–242.
30. Liu, Y., W. Wang, X. Wu, and X. Weng. 2020. Detection, expansion, and isolation of human MAIT cells. *Methods Mol. Biol.* 2111: 285–293.

31. Ussher, J. E., B. van Wilgenburg, R. F. Hannaway, K. Ruustal, P. Phalora, A. Kurioka, T. H. Hansen, C. B. Willberg, R. E. Phillips, and P. Klenerman. 2016. TLR signaling in human antigen-presenting cells regulates MR1-dependent activation of MAIT cells. *Eur. J. Immunol.* 46: 1600–1614.
32. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 25: 402–408.
33. Gherardin, N. A., M. N. Souter, H. F. Koay, K. M. Mangas, T. Seemann, T. P. Stinear, S. B. Eckle, S. P. Berzins, Y. d'Udekem, I. E. Konstantinov, et al. 2018. Human blood MAIT cell subsets defined using MR1 tetramers. *Immunol. Cell Biol.* 96: 507–525.
34. McWilliam, H. E., S. B. Eckle, A. Theodossis, L. Liu, Z. Chen, J. M. Wubben, D. P. Fairlie, R. A. Strugnell, J. D. Mintern, J. McCluskey, et al. 2016. The intracellular pathway for the presentation of vitamin B-related antigens by the antigen-presenting molecule MR1. *Nat. Immunol.* 17: 531–537.
35. Salio, M., W. Awad, N. Veerapen, C. Gonzalez-Lopez, C. Kulicke, D. Waithe, A. W. J. Martens, D. M. Lewinsohn, J. V. Hobrath, L. R. Cox, et al. 2020. Ligand-dependent downregulation of MR1 cell surface expression. *Proc. Natl. Acad. Sci. USA* 117: 10465–10475.
36. De Bonis, P., A. Albanese, G. Lofrese, C. de Waure, A. Mangiola, B. L. Pettorini, A. Pompucci, M. Balducci, A. Fiorentino, L. Lauriola, et al. 2011. Postoperative infection may influence survival in patients with glioblastoma: simply a myth? *Neurosurgery* 69: 864–868.
37. Shah, A. H., I. Jusué-Torres, M. E. Ivan, R. J. Komotar, and N. Kasahara. 2018. Pathogens and glioma: a history of unexpected discoveries ushering in novel therapy. *J. Neurosurg.* 128: 1139–1146.
38. Wei, J., J. Barr, L. Y. Kong, Y. Wang, A. Wu, A. K. Sharma, J. Gumin, V. Henry, H. Colman, W. Priebe, et al. 2010. Glioblastoma cancer-initiating cells inhibit T-cell proliferation and effector responses by the signal transducers and activators of transcription 3 pathway. *Mol. Cancer Ther.* 9: 67–78.
39. Godfrey, D. I., J. Le Nours, D. M. Andrews, A. P. Uldrich, and J. Rossjohn. 2018. Unconventional T cell targets for cancer immunotherapy. *Immunity* 48: 453–473.
40. Lorenzo-Herrero, S., C. Sordo-Bahamonde, S. Gonzalez, and A. López-Soto. 2019. CD107a degranulation assay to evaluate immune cell antitumor activity. *Methods Mol. Biol.* 1884: 119–130.
41. Corbett, A. J., S. B. Eckle, R. W. Birkinshaw, L. Liu, O. Patel, J. Mahony, Z. Chen, R. Reantragoon, B. Meehan, H. Cao, et al. 2014. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* 509: 361–365.
42. Schmaler, M., A. Colone, J. Spagnuolo, M. Zimmermann, M. Lepore, A. Kalinichenko, S. Bhatia, F. Cottier, T. Rutishauser, N. Pavelka, et al. 2018. Modulation of bacterial metabolism by the microenvironment controls MAIT cell stimulation. *Mucosal Immunol.* 11: 1060–1070.
43. Webb, T. J., J. G. Bieler, J. P. Schneck, and M. Oelke. 2009. Ex vivo induction and expansion of natural killer T cells by CD1d1-Ig coated artificial antigen presenting cells. *J. Immunol. Methods* 346: 38–44.
44. Azuma, M. 2019. Co-signal molecules in T-cell activation : historical overview and perspective. *Adv. Exp. Med. Biol.* 1189: 3–23.
45. Corthay, A. 2006. A three-cell model for activation of naïve T helper cells. *Scand. J. Immunol.* 64: 93–96.
46. Reeves, E., and E. James. 2017. Antigen processing and immune regulation in the response to tumours. *Immunology* 150: 16–24.
47. Mehta, N., J. G. Lyon, K. Patil, N. Mokarram, C. Kim, and R. V. Bellamkonda. 2016. Bacterial carriers for glioblastoma therapy. *Mol. Ther. Oncolytics* 4: 1–17.