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Engineering immune cells with chimeric Ag receptors (CARs) is a promising technology in cancer immunotherapy. Besides classical cytotoxic $CD8^+$ T cells, innate cell types such as NK cells have also been used to generate CAR-T or CAR-NK cells. In this study, we devised an approach to program a nonclassical cytotoxic T cell subset called mucosal-associated invariant T (MAIT) cells into effective CAR-T cells against B cell lymphoma and breast cancer cells. Accordingly, we expressed anti-CD19 and anti-Her2 CARs in activated primary human MAIT cells and $CD8^+$ T cells, expanded them in vitro, and compared their cytotoxicity against tumor cell targets. We show upon activation through CARs that CAR-MAIT cells exhibit high levels of cytotoxicity toward target cells, comparable to $CD8^+$ CAR-T cells, but interestingly expressed lower levels of IFN-γ than conventional CAR $CD8^+$ T cells. Additionally, in the presence of vitamin B_2 metabolite 5-ARU (5-amino-4-D-ribitylaminouracil dihydrochloride), which is a conserved compound that activates MAIT cells through MHC class I–related (MR1) protein, MAIT cells killed MR1-expressing target breast cancer and B cell lymphoma cell lines in a dose-dependent manner. Thus, MAIT cells can be genetically edited as CAR-T cells or mobilized and expanded by MR1 ligands as an off-the-shelf novel approach to cell-based cancer immunotherapy strategies while being comparable to conventional methods in effectivity. ***The Journal of Immunology*****, 2022, 209: 1523–1531.**

Chimeric Ag receptor (CAR)–based approaches have been one of the most promising immunotherapies in the last decade. Most CAR immunotherapies are focused on the engineered conventional $CD8^+$ T cells against tumor targets, referred to as CAR-T cells (1). Although other cell types such as NK cells (2), γδ T cells (3), and macrophages (4) have also been tested as CAR-modified cells, different subtypes of the T cell family and innate cells remain to be tested to determine their effectiveness, durability, and safety for CAR immunotherapies.

Human mucosal-associated invariant T (MAIT) cells constitute a large subset of innate-like T cells that recognize conserved bacterial ligands from vitamin B metabolic pathways (B_2 [riboflavin] and B_9 [folate]) (5–7). Predominantly residing in tissue, MAIT cells account for 1–8% of peripheral T lymphocytes and up to 40% of tissue specific T cells (8–10). An invariant TCR α-chain (Vα7.2–Jα33) integrated with a narrow TCR β-chain repertoire is used to identify MAIT cells (10). MAIT cells recognize their specific Ags through MHC class I–related (MR1) protein, which can allow them to avoid attacking the host cells during a cell-based immunotherapy, contrary to conventional T cells, which use polymorphic MHC molecules for Ag recognition. In addition to Vα7.2 TCR, MAIT cells are also characterized by a high expression of CD161 and IL-18R surface markers, and thus can consequently be identified as TCR $Vα7.2^+$ $CD161^+$IL-$18Rα^+$ αβ T cells (10, 11).

MAIT cells are activated through vitamin B_2 metabolites presented on MR1 molecules on APCs. Upon activation, they secrete cytokines such as IFN-γ, TNF-α, and IL-17 and display cytotoxicity through perforin and granzyme expression (12–15). Several studies have reported that MAIT cells are abundant in the tumor microenvironment (16–18). Their tendency to naturally migrate to the tumor microenvironment can be exploited for immunotherapy, such as CAR engineering approaches.

In this study, we engineered MAIT cells with CARs to target CD19- and Her2-expressing cell lines. MAIT cells expressing anti-Her2 or anti-CD19 (herein named CAR-MAIT cells) showed high cytotoxicity to target cells and expressed low levels of proinflammatory cytokines such as IFN-γ. Additionally, CAR-MAIT cells were able to kill their targets in the presence of vitamin B_2 metabolites in a dose-dependent manner, which can be a method to mobilize them toward MR1-expressing tumor cells. Taken together, these findings suggest that MAIT cells can be a novel candidate cell type for cancer immunotherapies given their cytotoxic abilities that are comparable to conventional CAR-T cells, combined with their off-the-shelf generation potential.

Materials and Methods

PBMCs and T cell purification

Healthy adult blood was obtained from AllCells (Quincy, MA). PBMCs were isolated using Ficoll-Paque plus (GE Healthcare). $CD4^+$ T, $CD8^+$ T,

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Abbreviations used in this article: 5-ARU, 5-amino-4-D-ribitylaminouracil hydrochloride; CAR, chimeric Ag receptor; GzB, granzyme B; iNKT, invariant NKT; IRES, internal ribosome entry site; MAIT, mucosal-associated invariant T; MR1, MHC class I–related; scFv, single-chain variable fragment.

and CD19$^+$ B cells and CD14$^+$ monocytes were purified using Dynal CD4$^+$, CD8$^+$, and CD19$^+$ isolation kits (all from Invitrogen) and CD14 MicroBeads UltraPure (Miltenyi Biotec). To purify Vα7.2$^+$ cells, CD4$^-$ fraction of PBMCs was used. Cells were stained with Vα7.2 PE-conjugated Ab for 30 min at 4°C, washed with PBS containing 5% FBS, and then labeled with anti-PE MicroBeads (Miltenyi Biotec) for 15 min at 4°C. After washing, labeled cells were resuspended in PBS + 5% FBS, passed through a cell strainer, and separated on an autoMACS Pro separator for isolation of Vα7.2$^+$ and Vα7.2$^-$ cells. CD4$^+$ T, CD8$^+$ T, and CD19$^+$ B cells and CD14$^+$ monocytes were >99% pure and assessed by flow cytometry staining with respective Abs. Vα7.2$^+$ T cells were >95% pure and verified by flow cytometry. Verification of sorted MAIT cells is shown in Supplemental Fig. 4.

Designing CAR and MR1 overexpression constructs

CAR constructs consist of a CD8α signal peptide, a single-chain variable fragment (scFv) of a CD19 or Her2 Ab, a CD8 hinge domain, a CD8 transmembrane domain, a 4-1BB (CD137) intracellular domain, and a CD3ζ domain that were designed with SnapGene and synthesized via GenScript. The CD8a signal peptide, CD8 hinge, CD8 transmembrane domain, 4-1BB intracellular domain, and CD3ζ domain sequences were obtained from the Ensembl genome browser and codon optimized with SnapGene by removing the restriction enzyme recognition sites that are necessary for subsequent molecular cloning steps while preserving the amino acid sequences. Anti-CD19 and anti-Her2 scFv amino acid sequences were obtained from Addgene plasmids 79125 and 85424, respectively, reverse translated to DNA sequences, and codon optimized with SnapGene 5.2.4. The human MR1 transcript variant 1 (NM_001531) cDNA clone was purchased from OriGene. The constructs were then cloned into a lentiviral expression vector with a multiple cloning site separated from GFP reporter via an internal ribosome entry site (IRES).

Lentiviral production and titration

Cloned lentiviral constructs including anti-CD19 CAR, anti-Her2 CAR, and MR1-encoding vectors were cotransfected with the packaging plasmids VSVG (the envelope glycoprotein of vesicular stomatitis virus), pLP1, and pLP2 into 293 cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Viral supernatants were collected 24–48 h post-transfection, filtered through a 0.45-μm syringe filter (Millipore) to remove cellular debris, and concentrated with Lenti-X (Invitrogen) according to the manufacturer's protocol. Lentivirus supernatant stocks were aliquoted and stored at −80°C. To measure viral titers, virus preparations were serially diluted on Jurkat cells. At 72 h postinfection, GFP-positive cells were counted using flow cytometry and the number of cells transduced with virus supernatant was calculated as infectious units per milliliter. The cells were cultured in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FBS; Atlanta Biologicals, Lawrenceville, GA), 8% GlutaMAX (Life Technologies), 8% sodium pyruvate, 8% MEM vitamins, 8% MEM nonessential amino acid, and 1% penicillin/streptomycin (all from Corning cellgro) for 72 h (19). Then, 0.05% trypsin/0.53 mM EDTA (Corning cellgro) was used to detach adherent MDA-231 cells.

Engineering CAR-T cells and target tumor cell lines

CD8$^+$ T cells were activated using anti-CD3/CD28–coated beads (Invitrogen) at a 1:2 ratio (beads/cells) and infected with anti-CD19 CAR, anti-Her2 CAR, or empty lentivectors with a multiplicity of infection of 3. Cells were then expanded in complete RPMI 1640 medium supplemented with 10% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Corning cellgro), and 10 ng/ml IL-2 for 10–12 d and cultured at 37°C and 5% CO_2-supplemented incubators. For PBMC assays, cells were activated with 30 μM 5-amino-4-D-ribitylaminouracil dihydrochloride (5-ARU, Toronto Research Chemicals, North York, ON, Canada). IL-2 was added 72 h after their activation to avoid non-MAIT cells growth. Respective viruses were added 24 h after the activation. Cells were expanded for 10–12 d and cytotoxicity assays were performed following their expansion. In Fig. 1B, T2 cells were added to PBMC cultures on day 8 after activation and then cocultures were analyzed by flow cytometry 3 d later. To generate and expand pure primary MAIT cells, sorted Vα7.2$^+$ cells were cultured overnight in complete medium containing IL-7 + IL-15 (20 ng/ml). At the second day, cells were activated with 30 μM 5-ARU in the presence of autologous CD14$^+$ monocytes and CD19$^+$ primary B cells. IL-2 (20 ng/ml) was added the next day along with either anti-CD19 CAR lentivirus, anti-Her2 CAR lentivirus, or empty vector as a control. A portion of the activated MAIT cells were left noninfected and expanded for 3 wk and used in Fig. 3F. For MR1-overexpressing MDA-231, wild-type MDA-231 cells were transduced with a multiplicity of infection of 3 of MR1 overexpression lentivirus and proliferated. The infection levels were determined by GFP expression on flow cytometry analysis.

Staining and flow cytometry analysis

Cells were resuspended in staining buffer (PBS + 2% FBS) and incubated with fluorochrome-conjugated Abs for 30 min at 4°C. Identification of MAIT T cells was determined by staining with Vα7.2-PE and CD161-BV421 Abs (BioLegend). The CD4$^-$ population for calculating MAIT cell percentage in PBMCs was determined by gating out CD4$^+$ cells stained with CD4-BV605 Ab (BioLegend). CAR CD8$^+$ T cells were identified with CD3-allophycocyanin/Cy7 or CD8–Alexa Fluor 700 Abs (BioLegend). Activation of CAR-MAIT and CAR CD8$^+$ T cells were determined with CD25 staining using CD25-allophycocyanin Ab (BioLegend). CAR expression was determined with human Her2/ErbB2 protein, Fc tag (Acro) or human CD19 (20-291) protein, Fc tag, low endotoxin (super affinity) (Acro) followed by a secondary staining with allophycocyanin-conjugated anti-human IgG Fc Ab (BioLegend). For cytotoxicity assay analysis, T2, Nalm6, and primary B cells were labeled with PE/Cy7 anti-human CD19 Ab (BioLegend) and MDA-231 cells were stained with allophycocyanin-conjugated anti-human CD340 (erbB2/HER-2) Ab (BioLegend). MR1 expression on T2 cells was assessed with anti-MR1-PE (BioLegend). Samples were acquired on a BD FACSymphony A5 analyzer and data were analyzed using FlowJo (BD Biosciences).

Cytotoxicity assays

Following the expansion of effector cells for 10–12 d, the cells were analyzed for their GFP and CAR expressions. The E:T ratio was calculated based on the number of CAR-expressing cells. CAR-expressing cells were titrated from 1:1 to 1:128 with 2-fold dilutions while the target cell number was constant. For the primary MAIT cell cytotoxicity assay, 5-ARU was titrated from 30 to 0.003 μM with 3-fold reciprocal dilutions. Cytotoxicity assay conditions were analyzed with flow cytometry at 72 h of coincubation. The cells were first gated based on their forward and side scatter properties to exclude doublets, debris, and dead cells. Viability dye staining was included in the original experiments (Fig. 1D) and showed that forward and side scatter gating was excluding dead and dying cells. Target cells were identified with CD19 (primary B cells, T2, and Nalm6 lymphoma cell lines) and Her2 staining (MDA-231, breast cancer cell line), and effector cells were identified with CD3 staining and GFP expression from the CAR-GFP lentivector. Cytotoxicity for each E:T ratio condition was calculated based on the corresponding control condition with the following formula: [(percentage of target cells in control condition − percentage of target cells in experimental condition)/percentage of target cells in control condition] × 100.

Cytokine assay

Cell culture supernatants from cytotoxicity assays were collected 72 h after combining the effector and the target cells and were immediately stored at −80°C. Secreted proteins and cytokines, including IFN-γ, TNF-α, GM-CSF, and granzyme B (GzB) production, were measured using a Qbeads Plex screen assay (Essen BioScience, Ann Arbor, MI) according to the manufacturer's instructions and analyzed using iQue Screener PLUS (IntelliCyt, Albuquerque, NM).

Statistical analyses and reproducibility

All statistical analyses were performed using GraphPad Prism v8 software. A two-tailed unpaired *t* test was used to determine the statistical significances, and exact *p* values are reported ($p < 0.05$ was considered significant). Numbers of repeats for each experiment are described in the associated figure legends.

Data availability

The source data for the figures along with the Supplemental Figures are available upon request.

Results

Activation and cytotoxicity of anti-CD19 CAR-engineered MAIT cells

To engineer primary MAIT cells into CAR-MAIT cells, we first designed a lentiviral CAR construct composed of a CD8α signal peptide, an anti-CD19 Ab scFv, a CD8 hinge domain, a CD8 transmembrane domain, a 41BB costimulatory domain, and a CD3ζ domain. The CAR construct was designed to be coexpressed with a GFP marker via an IRES under an LTR promoter (Fig. 1A). To express the CAR construct and expand MAIT cells, we first activated MAIT cells by stimulating these cells in PBMCs with 5-ARU, a vitamin B_2 biosynthesis derivative. Within the PBMCs, MAIT cells were defined as CD4$^-$ or CD8$^+$ T cells expressing both TCR Vα7.2 and CD161

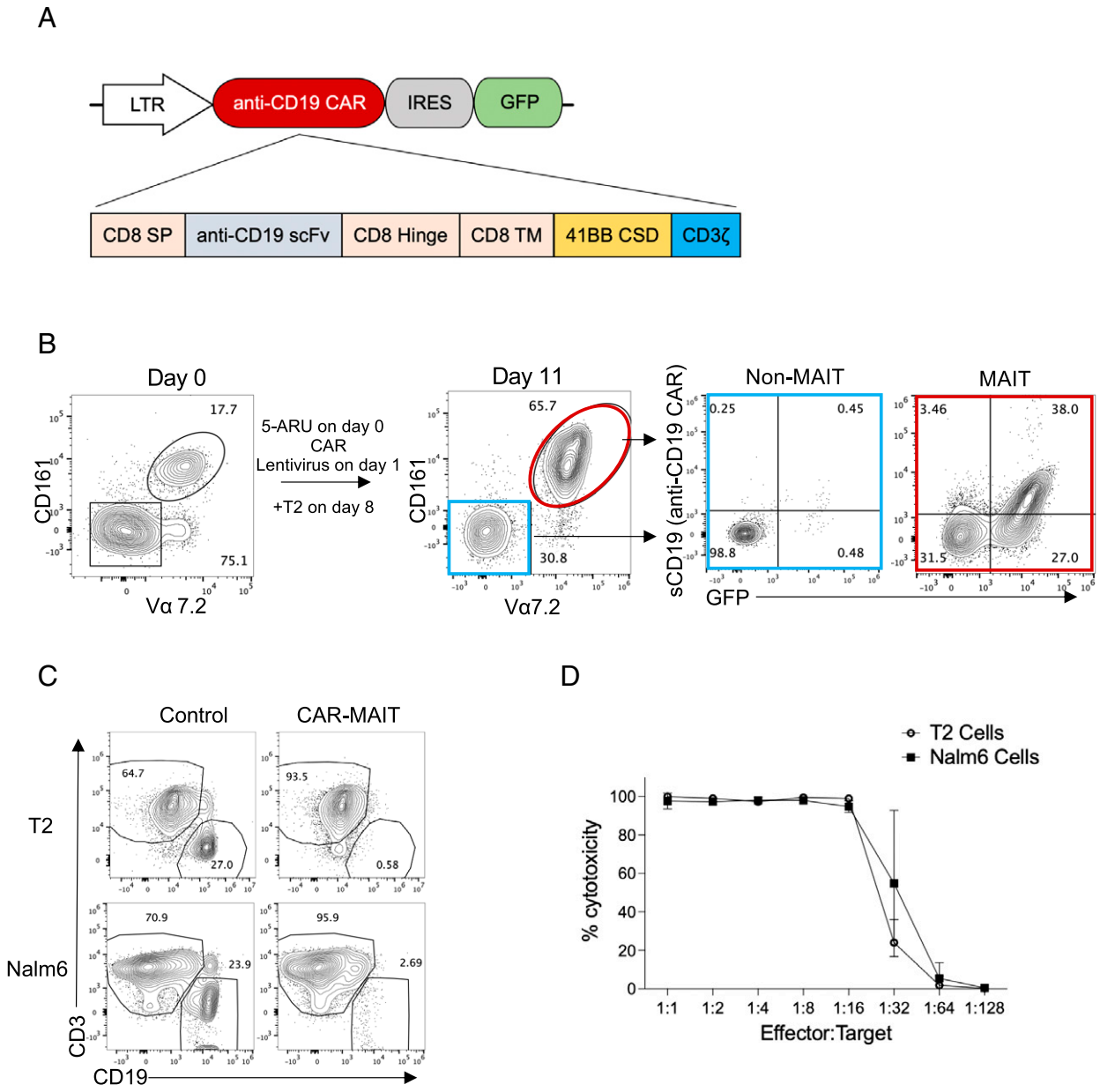

FIGURE 1. Engineering human primary MAIT cells into CAR-MAIT cells. (**A**) Schematic illustration of anti-CD19 CAR construct. A constitutive LTR promoter drives the anti-CD19 CAR and GFP genes that are separated by an internal ribosome entry site (IRES). The anti-CD19 CAR construct consists of a CD8α signal peptide, a single-chain variable fragment (scFv) of an anti-CD19 Ab, a CD8 hinge, a CD8 transmembrane domain, a 4-1BB (CD137) costimulatory domain, and a CD3ζ domain. (**B**) Flow cytometry plots demonstrate the proliferation and transduction of primary MAIT cells with an anti-CD19 CAR construct. Vα7.2^{+}CD161^{+} CD8^{+} T cells within PBMCs were activated with the riboflavin metabolite 5-amino-6-D-ribitylaminouracil (5-ARU), transduced with a lentivirus encoding CD19 targeting a CAR and GFP marker, and then target T2 cells were added on day 8 after activation and cultures were analyzed by flow cytometry 3 d later. Non-MAIT T cells are shown in blue boxes, and MAIT cells are indicated with a red circle and a red box. (**C**) Representative flow cytometry plots of CAR-MAIT cytotoxicity assays with CD19^{+} T2 and Nalm6 target cells. MAIT cells transduced with an empty lentivector were used as controls. Effector MAIT cells were identified with CD3 staining, and target cells were shown via CD19 staining. An effector (CAR-expressing T cells)-to-target ratio of 1:1 is shown. (**D**) Line graph representing CAR-MAIT cytotoxicity against Nalm6 and T2 cells. Circles and squares indicate T2 and Nalm6 cell cytotoxicity curves, respectively. Error bars represent 1 SD of mean value. The E:T ratio was calculated based on the number of effector cells expressing CARs. The E:T ratio was titrated from 1:1 to 1:128 with 2-fold reciprocal dilutions of effector cells. Th percent cytotoxicity was calculated based on the percentage of cell death in experimental conditions in relationship to control conditions. Each experiment was performed three times.

surface markers (20) (Fig. 1B). One day after stimulating MAIT cells in PBMCs with 5-ARU, cells were transduced with lentivirus encoding the CAR construct. The cells were cultured for 7 d in the presence of IL-2 and then T2 cells were added at 1:1 E:T ratio. Three days later cocultures were stained for CD8, CD161, TCR Vα7.2, and anti-CD19 CAR expression. After 11 d, activated MAIT cells were selectively expanded and therefore constituted most CD8^{+} T cells (Fig. 1B, Supplemental Fig. 1A). MAIT cells also selectively expressed anti-CD19 CAR compared with Vα7.2^{-} CD161^{-} non-MAIT cells, corresponding to the marker GFP levels (Fig. 1B).

After confirming CAR expression in MAIT cells, we tested their cytotoxic function using T2 and Nalm6 cell lines as target cells known to express CD19 on their surface (Supplemental Fig. 1B). Flow cytometry analysis 72 h after coculture revealed that anti-CD19 CAR-MAIT cells were able to kill their targets (Fig. 1C). A more

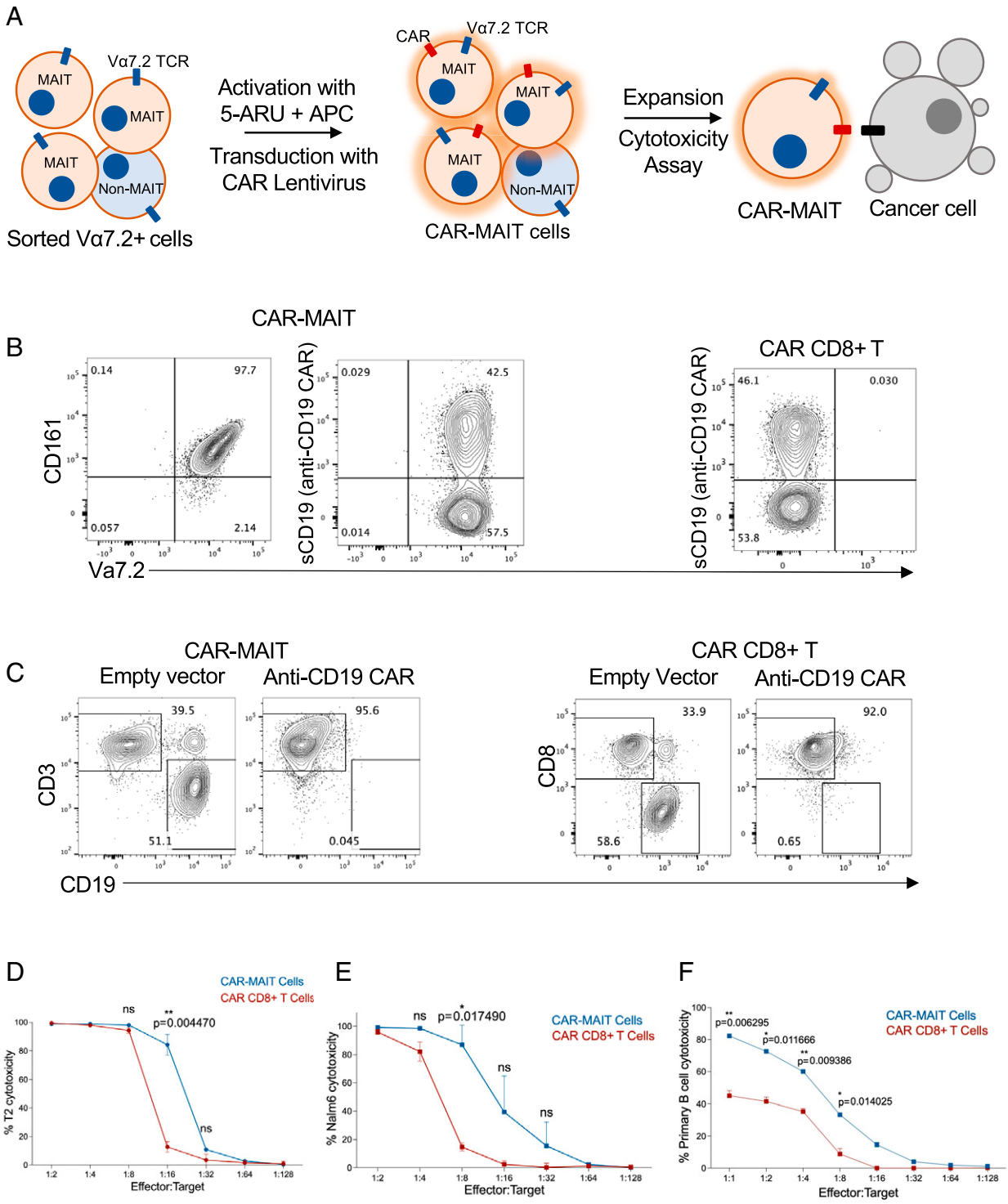

FIGURE 2. Development and generation of purified CAR-MAIT cells and cytotoxicity toward B cell lymphomas. (**A**) Schematic illustration demonstrating the sorting, engineering, and cytotoxicity assay strategy for CAR-MAIT cells. Vα7.2$^+$ cells were isolated from the CD4$^-$ PBMC population. Blue rectangles on the cell surface represent Vα7.2 TCRs. The cells with orange and blue cytoplasm show MAIT and non-MAIT cells, respectively. Sorted Vα7.2$^+$ cells were activated by adding the 5-ARU molecule in the presence of autologous MR1-expressing APCs. Red glow around the cells represents activation. Selectively activated MAIT cells were then transduced with CAR-encoding lentiviruses and expanded in IL-2 media. Red rectangles on the cell surface represent CARs. Finally, CAR-engineered MAIT cells were combined with target cells for cytotoxicity assays; the black rectangle on the cell surface represents CD19 protein. (**B**) Flow cytometry plots demonstrating CAR surface expression in CAR-MAIT cells and CAR CD8$^+$ T cells. Engineered CAR-T cells were stained with CD19-Fc fusion protein and then with an anti-Fc Ab to determine the expression levels of anti-CD19 CARs. (**C**) Representative flow cytometry plots of cytotoxicity assays in which CAR-MAIT cells and CAR CD8$^+$ T cells were combined with T2 cells at an E:T ratio of 1:1 and 1:2, respectively. The E:T ratio was calculated based on the number of effector cells expressing CARs. CAR-MAIT cells, CAR CD8$^+$ T cells, and the target cells were identified by the expression of CD3, CD8, and CD19, respectively. Empty vector–transduced MAIT and CD8$^+$ T cells were used as a control. (**D** and **E**). Line graphs of killing percentages from the cytotoxicity assays of CAR-MAIT cells and CAR CD8$^+$ T cells targeting T2 and Nalm6 cells. Red and blue lines represent CAR CD8$^+$ T and CAR-MAIT cells, respectively. The E:T ratio was titrated from 1:2 to 1:128 with 2-fold reciprocal dilutions *(Figure legend continues)*

detailed cytotoxic assay testing different E:T ratios showed that MAIT cells were highly cytotoxic, killing target cells up to an E:T ratio of 1:32 (Fig. 1D). Taken together, these findings demonstrate the specific killing of target cells by CAR-MAIT cells.

Expansion, isolation, and cytotoxicity of purified CAR-MAIT cells to target cancer cell lines

To further investigate the possibility of using MAIT cells as candidates for cancer immunotherapies, we developed a sorting and expansion strategy to generate purified CAR-MAIT cells for further analyses (Fig. 2A). Given that most MAIT cells do not express CD4 (21), we used the $CD4^-$ fraction of PBMCs to sort TCR $V\alpha7.2^+$ cells. MAIT-enriched $V\alpha7.2^+$ cells were then activated with 5-ARU in the presence of autologous $CD19^+$ B cells and $CD14^+$ monocytes as APCs and transduced with CAR-expressing lentiviruses. Cells were then expanded in the presence of IL-2 for 10–12 d (Fig. 2A).

We next sought to compare the cytotoxic function of CAR-MAIT cells and conventional CAR $CD8^+$ T cells. For this, we generated $CD8^+$ anti-CD19 CAR-T cells by lentivirus transduction upon CD3/CD28 activation. Both CAR-T cell subsets were then expanded for 10–12 d in IL-2–containing media. Staining for anti-CD19 CAR surface expression revealed that both cell types expressed CD19-CAR at comparable levels (Fig. 2B). The effector cells were then cocultured for 3 d with T2, Nalm6, or primary B cells, all of which express the target Ag CD19 on their surface (Supplemental Fig. 1B, 1C). Both conventional CAR $CD8^+$ T and CAR-MAIT cells almost completely killed the target cells, whereas no cytotoxicity was observed by cells expressing empty vector controls (Fig. 2C), at different E:T ratios (Fig. 2D, 2E). Interestingly, CAR-MAIT cells displayed slightly more cytotoxicity to T2 ($p = 0.0044$) and Nalm6 ($p = 0.0174$) than did conventional CAR $CD8^+$ T cells at the E:T ratio of 1:16 and 1:8, respectively (Fig. 2D, 2E). CAR-MAIT cells had more cytotoxicity also toward primary B cells compared with CAR CD8 T cells (Fig. 2F). A representative flow cytometry data plot of a primary B cell cytotoxicity assay is shown in Supplemental Fig. 2.

Cytotoxicity of CAR-MAIT cells against a breast cancer cell line

We next engineered anti-Her2 CAR-MAIT cells to test their efficiency against Her2-expressing cells, as some solid tumors have high Her2 expression (22). The anti-Her2 CAR cassette was constructed by switching the extracellular domain of anti-CD19 CAR with an anti-Her2 scFv as described in *Materials and Methods*. Anti-Her2 CAR-expressing conventional $CD8^+$ T cells were also generated to compare the cytotoxic features of both effector cells. Surface staining of anti-Her2 on CAR-MAIT and CAR $CD8^+$ T cells revealed that both engineered effector cell types expressed comparable levels of CAR, which correlated with the GFP reporter expression (Fig. 3A). Breast cancer cell line MDA-231 cells were stained with an anti-Her2 Ab to confirm its expression (Fig. 3B). We next determined the cytotoxicity of these CAR-engineered cells to the MDA-231 cells. Effector cells expressing anti-CD19 CAR were used as negative controls. Both anti-Her2 CAR-MAIT and anti-Her2 CAR $CD8^+$ T cells efficiently killed MDA-231 cells (Fig. 3C), and CAR-MAIT cells showed significantly higher cytotoxicity than conventional CAR-T cells at the E:T ratios of 1:1 ($p = 0.015$), 1:2 ($p = 0.0027$), 1:4 ($p = 0.007$), and 1:8 ($p = 0.03$) (Fig. 3D).

We also tested the cytotoxicity of noninfected primary MAIT cells against MR1-expressing cancer cells in the presence of 5-ARU. For this, we engineered MDA-231 cell line to overexpress MR1 and then used them as target cells in a cytotoxicity assay with primary MAIT cells in the presence of 5-ARU. Engineered MDA-231 cells were stained with an anti-MR1 Ab and MR1 overexpression was confirmed (Fig. 3E). Primary MAIT cells were purified, activated, and expanded as described in *Materials and Methods* without adding any lentivirus. After 3 wk of expansion in IL-2–containing media, noninfected MAIT cells were cocultured with wild-type or MR1-overexpressing MDA-231 cells in the presence of different concentrations of 5-ARU for 2 d. Flow cytometry analysis revealed that primary MAIT cells were able to kill MDA-231 cells overexpressing MR1 in the presence of different concentrations of 5-ARU, in a dose-dependent manner, whereas no cell death was observed in the wild-type MDA-231 condition at any concentration (Fig. 3F, Supplemental Fig. 3A). CD25 staining showed activation of MAIT cells in cocultures treated with 5-ARU (Supplemental Fig. 3B).

Cytokine secretion by activated CAR-MAIT and conventional CAR-T cells

We next determined the cytokine response of CAR-MAIT cells upon triggering CAR signaling. A multiplex flow-based immunoassay was used to measure IFN-γ, TNF, GzB, and GM-CSF levels in the supernatants of E:T cocultures at a ratio of 1:1. CAR-MAIT cells secreted significantly lower levels of IFN-γ than did CAR $CD8^+$ T cells in cytotoxicity assays targeting primary B cells ($p = 0.003$), T2 cells ($p = 0.006$), Nalm6 cells ($p = 0.002$), and MDA-231 cells ($p < 0.0001$) (Fig. 4A). However, TNF secretion by CAR-MAIT cells was significantly higher compared with CAR $CD8^+$ T cells against primary B ($p = 0.0009$) and T2 ($p = 0.0423$) cells, whereas it was lower against MDA-231 cells ($p = 0.0079$) (Fig. 4B). We did not observe a significant difference in TNF expression between the two effector cell types when targeting Nalm6 cells (Fig. 4B). Similar to IFN-γ, GzB and GM-CSF levels were also significantly lower in CAR-MAIT cytotoxicity conditions targeting primary B ($p = 0.0023$ and 0.0003, respectively), T2 ($p = 0.002$ and 0.0009, respectively), Nalm6 ($p = 0.0011$ and 0.0005, respectively) and MDA-231 cells ($p = 0.0367$ and 0.0002, respectively) (Fig. 4C, 4D).

Discussion

Cancer immunotherapies exploiting CARs show great promise in treating certain tumor types. However, there are major disadvantages such as a high risk of adverse effects like cytokine release syndrome, neurotoxicity, anaphylaxis, and graft-versus-host disease, lower efficacies against solid tumors compared with hematological malignancies, and also practical issues such as the need to use the patient's own T cells for each treatment, which increases the cost and prevents off-the-shelf treatment options (23, 24). To overcome some of these hurdles, we sought to develop a novel CAR-T cell with a nonclassical T cell subset, MAIT cells, against a variety of tumor types. In this study, we demonstrate highly efficient generation of CAR-MAIT cells, which display equivalent or higher cytotoxicity to target cells, compared with conventional CAR-T cells.

Conventional T cells recognize Ags in the context of MHC class I or MHC class II molecules on the presenting cells, which results in an allogeneic reaction when the cells derived from non-self donors

of effector cells. Percent cytotoxicity was calculated based on the percentage of cell death in experimental conditions in relationship to control conditions. (**F**) Line graph showing the percent cytotoxicity of CAR-MAIT and CAR $CD8^+$ T cells on primary B cells at ratios ranging from 1:2 to 1:128. Cytotoxicity assays were replicated twice with different donors. Error bars represent 1 SD of mean value. Two-tailed unpaired *t* tests were used to determine statistical significance. The numbers of replicates in (C) and (D) were three and two for (E).

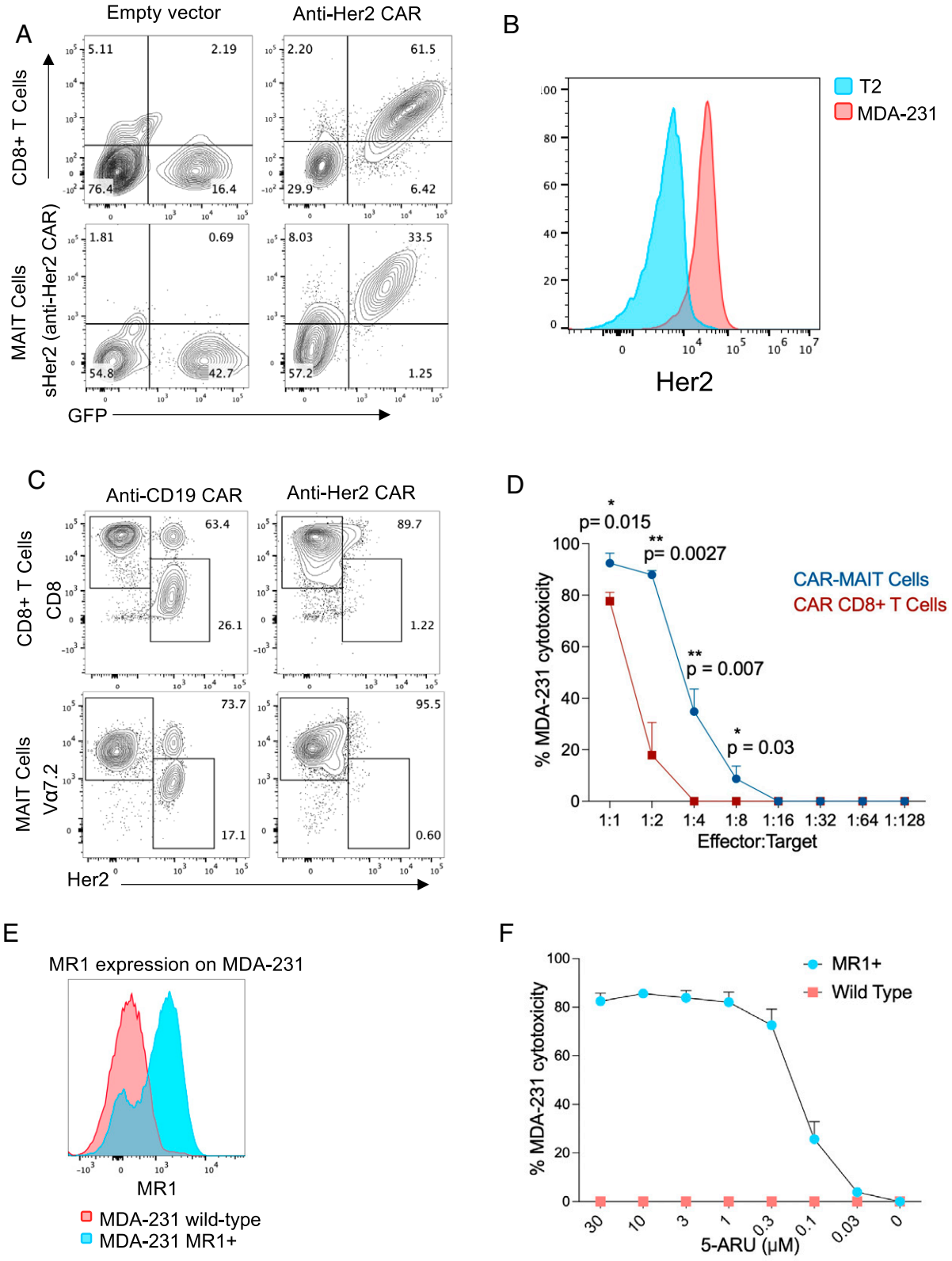

FIGURE 3. Cytotoxicity of MAIT cells against a solid tumor cell line, MDA-231. (**A**) Flow cytometry plots demonstrating the anti-Her2 CAR surface expression in CAR-MAIT cells and CAR $CD8^+$ T cells. Engineered CAR-T cells were stained with Her2-Fc fusion protein and an anti-Fc Ab to demonstrate the expression levels of anti-Her2 CARs. GFP lentivirus markers were also used to show the coexpression with anti-Her2 CAR in engineered T cells. (**B**) Flow cytometry overlays of MDA-231 cells stained with an anti-Her Ab. The red population in the histogram represents T2 cells as a control, whereas the blue population shows the presence of Her2 expression in anti-Her2–stained MDA-231 cells. (**C**) Representative flow cytometry plots of cytotoxicity assay using CAR-MAIT cells and CAR $CD8^+$ T cells as effector cells and $Her2^+$ MDA-231 as target cells. MAIT cells, $CD8^+$ T cells, and MDA-231 cells were identified with CD8, Vα7.2, and Her2 expression, respectively. The effector (CAR-expressing T cells)-to-target ratio of 1:1 is shown. (**D**) Line graph showing the percent cytotoxicity of CAR-MAIT cells and CAR $CD8^+$ T cells targeting MDA-231 cells. Red and blue lines represent CAR $CD8^+$ T and CAR-MAIT cells, respectively. Error bars represent 1 SD of mean value. The E:T ratio was calculated based on the number *(Figure legend continues)*

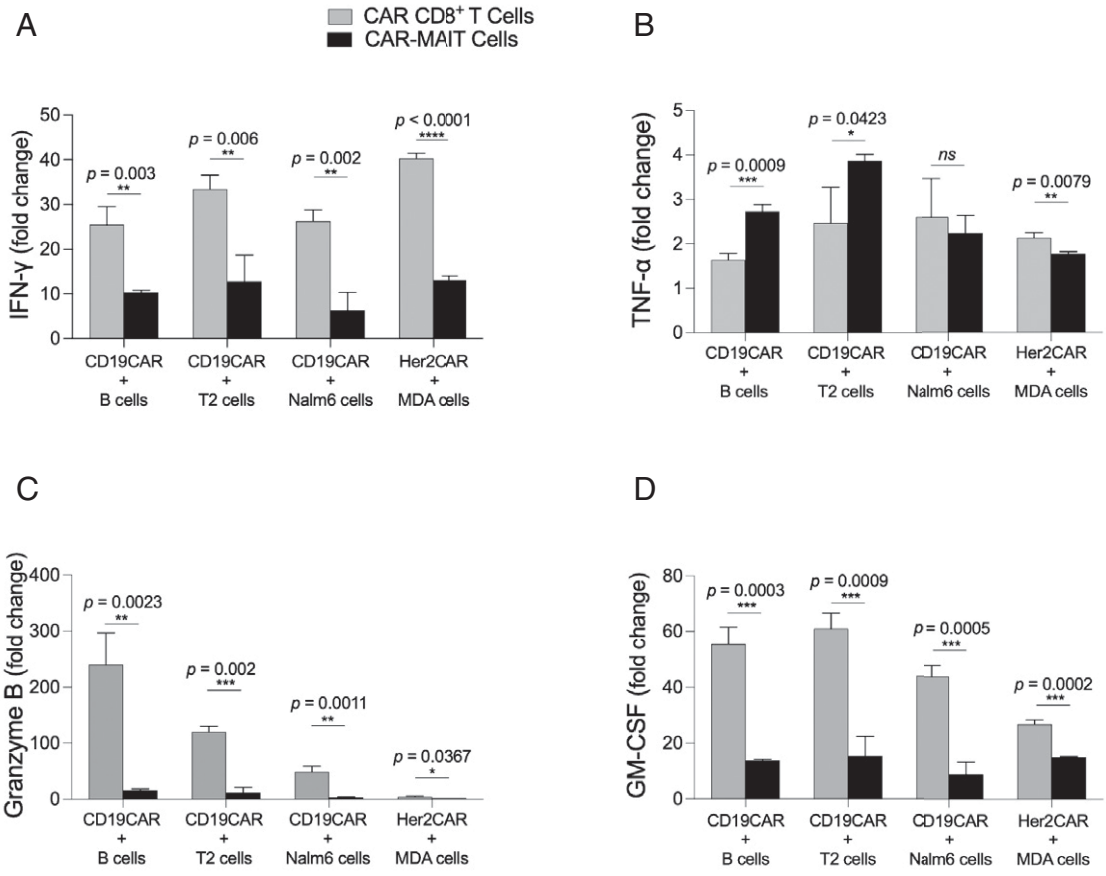

FIGURE 4. Cytokine secretion of CAR $CD8^+$ T and CAR-MAIT cells upon activation. (**A**–**D**) In the supernatants of cocultured CAR-MAIT and CAR $CD8^+$ T effector cells with primary B, T2, Nalm6, or MDA-231 target cells, shown in gray and black bars, respectively, the following cytokines were measured and expressed as fold change: (A) IFN-γ, (B) TNF, (C) granzyme B (GzB), and (D) GM-CSF. Fold changes were calculated based on the fold increase of experimental conditions compared with control conditions. Cytotoxicity assays were replicated twice with different donors. Error bars represent 1 SD of mean value. A two-tailed unpaired *t* test was used to determine statistical significance.

are used for treatment. Thus, current CAR therapies use the patient's own T cells that are separated by leukapheresis, engineered in vitro, and then transferred back to the same patient. This limitation to use only autologous cells prevents development of off-the-shelf cell-based therapies, which would greatly improve the process, making it a less expensive, faster, and more readily available treatment option.

Recently, invariant NKT (iNKT) cells are being used in adaptive immunotherapies to address this issue (25–28). iNKT cells are also increasingly studied for their distinct features, including their robust antitumor activity, their intrinsic nature of regulating other immune system members, and their susceptibility to engineering, namely the qualities they share with MAIT cells (29). Following their activation with αGalSer, iNKT cells were used in preclinical studies on patients with multiple myeloma, adenocarcinoma, renal cell carcinoma, non–small cell lung cancer, and large-cell carcinoma with encouraging results (1). Similar to iNKT cells, MAIT cells also do not employ the MHC class I molecule for Ag recognition and conceivably lack the allogeneic adverse reactions. The advantage of MAIT cells to iNKT cells is the abundance of MAIT cells in the human immune system compared with low frequencies of iNKT cells (30). In this regard, MAIT cells have the potential to be the main cell type in the future of adaptive off-the-shelf cancer immunotherapies.

To be able to generate engineered human MAIT cells and proliferate them to a degree at which they can be employed against target cells, we optimized an expansion protocol. The protocol we designed in our study for the MAIT cells using 5-ARU, similar to using αGalSer for iNKT cells, is of great importance in the field of cell-based immunotherapy because it allows us to engineer and make use of this distinct and abundant human T cell subtype. To our knowledge, this is the first study to demonstrate the feasibility of using CAR-engineered MAIT cells against specific targets.

Our key finding was the demonstration that MAIT cells engineered with CARs perform cytotoxicity against specific cancer targets. This

of effector cells expressing CARs. The E:T ratio was titrated from 1:2 to 1:128 by 2-fold reciprocal dilutions of effector cells. The percent cytotoxicity was calculated based on the percentage of cell death in experimental conditions in relationship to control conditions. A two-tailed unpaired *t* test was used to determine statistical significance. (**E**) Histogram overlay demonstrating the MR1 expression levels in wild-type MDA-231 cells and MR1-overexpressing MDA-231 cells. Red and blue populations indicate wild-type and MR1-overexpressing MDA-231 cells, respectively. (**F**) Noninfected MAIT cell cytotoxicity toward wild-type and MR1-overexpressing MDA-231 cells in the presence of different concentrations of 5-ARU. MAIT cells were purified, activated, and expanded as described in *Materials and Methods*. After 3 wk of expansion in IL-2–containing media, MAIT cells were cultured with wild-type (red squares) and MR1-overexpressing (blue circles) MDA-231 cells in the presence of 5-ARU. 5-ARU was titrated from 30 to 0.003 μM by 3-fold reciprocal dilutions. Cytotoxicity assays were replicated twice with different donors.

finding encouraged us to perform extensive cytotoxicity assays comparing CAR-MAIT cells with conventional CAR $CD8^+$ T cells. Remarkably, we found that CAR-MAIT cells show similar or, in some cases, significantly higher cytotoxicity compared with the conventional CAR $CD8^+$ T cells in our in vitro assays. In future studies, it will be important to address the efficacy and potential side effects of CAR-MAIT cells in in vivo experiments. For example, how specifically do CAR-MAIT cells target cancer cells compared with normal tissue expressing similar Ags? How long will they persist in vivo? Will they be effective in solid tumor microenvironments?

The second key finding in our study was that despite robust cytotoxic capacity, CAR-MAIT cells secrete significantly less IFN-γ, GzB, and GM-CSF compared with conventional CAR-T cells. Indeed, a major pitfall in cell-mediated adaptive immunotherapies is the severe adverse effects of T cell overactivation, which results in high proinflammatory cytokine secretion (31). This in turn can lead to cytokine release syndrome and neurologic toxicities, which are two of the biggest concerns after the infusion of engineered CAR T cells (32). Thus, lower cytokine secretion and high cytotoxic activity of CAR-MAIT cells compared with conventional CAR $CD8^+$ T cells could be of importance in developing safer and more effective immunotherapeutic effector cells. In fact, reducing GM-CSF has been demonstrated to decrease cytokine release syndrome and neuroinflammation but enhance CAR-T cell function (33). Reduced IFN-γ levels could mean that CAR-MAIT cells may be less potent at stimulating an endogenous antitumor response (34) and lead to worse prognosis or may be beneficial by promoting less inflammation in the tumor microenvironment compared with conventional CAR $CD8^+$ T cells. These are also some of the questions that need to be addressed via in vivo studies comparing conventional and MAIT CAR-T cells. Taken together, these findings could be particularly relevant for adaptive immunotherapies using lower doses of CAR-MAIT cells compared with $CD8^+$ CAR-T cells, which may result in better therapeutic efficiencies while minimizing adverse effects to normal cells or tissues.

Another key outcome in our study was engineering CAR-MAIT cells to recognize and kill target cells that express Her2 on their surface more efficiently than conventional CAR $CD8^+$ T cells. This result was critical because Her2 is one of the surface molecules highly expressed on several solid tumors (22). Additionally, it has been previously reported that primary MAIT cells can infiltrate tissue sites of chronic inflammation (35) and are abundant in the mucosal-associated and solid tumor microenvironment (16–18). Therefore, this migration capacity toward sites of inflammation can be of advantage in employing CAR-MAIT cells for solid tumor-targeting immunotherapies. It is also reported that MAIT cell accumulation in the tumor microenvironment is associated with poor survival in colorectal cancer patients (36). Engineering MAIT cells with CARs to recognize tumor cells in solid tissues can turn this caveat of MAIT cell infiltration into the tumor microenvironment to an advantage in a cell-based therapy approach described in this study.

In addition, we also showed that primary MAIT cells were able to kill MR1-overexpressing target cells in the presence of 5-ARU in a dose-dependent manner. Since MR1 can be expressed by several tumor types and because MAIT cells are found to be increased in some cancer niches such as primary colorectal lesions, hepatic metastatic lesions, or multiple myeloma (37), it may be possible to exploit their 5-ARU–mediated activation to unleash their antitumor activity. It is also conceivable to combine both MR1-restricted and specific CAR engineering to fine tune MAIT antitumor responses, especially in more challenging solid tumor microenvironments. It will be important in future studies to further develop these engineering approaches and test them in vitro in three-dimensional models of cancer and ensure that these cells can effectively infiltrate the tumor environment and are endowed with fail-safe mechanisms.

Future animal experiments will help to observe the in vivo behaviors of the CAR-MAIT T cells in detail and will shed light on this approach from different perspectives.

Development of CAR-MAIT cells and other innate-like T cells with unique feature sets can accelerate novel adaptive immune cell–based approaches in cancer immunotherapy and overcome some of the key challenges in the quest to use the immune system as an important cancer treatment.

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Disclosures

The authors have no financial conflicts of interest.

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