Lenalidomide Enhances the Function of CS1 Chimeric Antigen Receptor–Redirected T Cells Against Multiple Myeloma

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Abstract

Purpose: Multiple myeloma remains an incurable malignancy of plasma cells despite considerable advances in treatment. The purpose of the study was to develop novel chimeric antigen receptors (CAR) for the treatment of multiple myeloma and explore combinatorial therapy using CAR T cells and immunomodulatory drugs such as lenalidomide for increasing treatment efficacy.

Experimental Design: We redirected central memory T cells to express second-generation CAR-specific for CS1 and adoptively transferred them into multiple myeloma tumor-bearing mice to test their anti-multiple myeloma activity. CS1 CAR T cells were transduced and expanded in the presence of lenalidomide *in vitro*. The phenotype and effector function of CS1 CAR T cells treated with and without lenalidomide were compared. Finally, CS1 CAR T cells and lenalidomide were administered to treat multiple myeloma–bearing mice as combinatorial therapy.

Results: CS1 CAR T cells exhibited efficient antitumor activity when adoptively transferred into mice. Mechanistic studies indicated that the addition of lenalidomide during CS1 CAR T-cell expansion *in vitro* enhanced the immune functions of CS1 CAR T cells, including cytotoxicity, memory maintenance, Th1 cytokine production, and immune synapse formation. Furthermore, lenalidomide enhanced the antitumor activity and persistence of adoptively transferred CS1 CAR T cells *in vivo*.

Conclusions: The study demonstrates that lenalidomide improves the anti-multiple myeloma properties of CS1-directed CAR T cells and provides a basis for a planned clinical trial using the combination of lenalidomide with engineered T cells against CS1 in relapsed myeloma. *Clin Cancer Res; 24(1); 106–19. ©2017 AACR.*

Introduction

Dramatic clinical responses have been reported recently using Chimeric antigen receptor (CAR) T cells specific for the B-cell antigen CD19, in patients with chronic and acute lymphocytic leukemia (1, 2). Similar strategies have been explored in multiple myeloma using CD19 as well as other targets such as BCMA (3, 4). In our study, we examine the target CS1 and demonstrate that combining immunomodulatory drugs (IMiD) with adoptive cellular therapy (ACT) has the potential for synergistic effects via direct actions on the tumor cells together with IMiD stimulation of CAR T cells.

CS1 is a cell surface glycoprotein of the signaling lymphocyte activation molecule (SLAM) receptor family that is highly and selectively expressed on normal plasma cells and multiple myeloma cells, with lower expression on natural killer (NK) cells and no expression on other normal tissue (5). CS1 is highly expressed in over 95% of cases of multiple myeloma irrespective of cytogenetic abnormalities and throughout the disease process (5). The high expression of CS1 on multiple myeloma cells coupled with the lack of or low expression in normal and essential tissues makes CS1 a reasonable target for CAR T-cell therapy against multiple myeloma.

IMiDs, for example, lenalidomide, as a primary treatment modality for multiple myeloma not only yields direct anti-multiple myeloma effects but also costimulates T cells. Both *in vitro* and *in vivo* studies demonstrated that lenalidomide enhances tumor-specific Th1 cytokines such as IL2 and IFNγ (6, 7), preferentially stimulates CD8$^+$ T cells (8), and inhibits IL2-mediated generation of Tregs (9).

In the studies reported here, we investigated the effects of lenalidomide on CS1 CAR T cells *in vitro* and the anti-multiple myeloma activity in a xenograft mouse model.

Materials and Methods

Antibodies and flow cytometry

Fluorochrome-conjugated isotype controls against CD3, CD4, CD8, PD1, Tim3, CD28, CD27, CD62L, CD127, CD107a, IFNγ,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Chimeric antigen receptor (CAR)-directed T-cell therapy is a promising approach for hematologic malignancies, including multiple myeloma; however, uncertainties surround the optimal target as well as enhancing the persistence and expansion of engineered cells *in vivo*. This study demonstrated that CS1-specific CAR T cells mediated efficient effector function *in vitro* and anti-multiple myeloma activity *in vivo*. Furthermore, we explored further improving the function and persistence of these engineered CS1-specific T cells using a combinatorial therapy of CAR T cells with immunomodulatory drugs such as lenalidomide. We found that lenalidomide plays a costimulatory role in modulating CAR T cells and strengthens the antitumor activity and persistence of adoptively transferred CS1 CAR T cells against multiple myeloma *in vivo*. The rational combination of these immunotherapeutic regimens is an effective strategy for increasing efficacy against multiple myeloma and provides a generalizable approach for combining lenalidomide and CAR T cells.

TNFα, and IL2 and streptavidin-PE (SA-PE) were obtained from BD Biosciences. The antibody against CS1 was purchased from R&D Systems. Biotinylated erbitux was generated from cetuximab purchased from the City of Hope pharmacy. The antibody against EGFR was obtained from eBioscience. All mAbs were used according to the manufacturer's instructions. The percentage of cells in a region of flow cytometric analysis was calculated using FCS Express V4 (De Novo Software).

Lentivirus vector construction

The lentivirus CAR construct was modified from the previously described CD19-specific scFvFc:ζ chimeric immunoreceptor (10). The CS1 CAR contained a CS1-specific scFv (CS1R), a CD28z costimulatory domain, an IgG4-Fc spacer with a CH2 deletion to ensure enhanced potency and persistence (11), and a truncated human EGFR (huEGFRt), which includes a cetuximab (erbitux) binding domain. A T2A ribosome skip sequence links the codon-optimized CS1R:CD28:z sequence to the huEGFRt sequence, resulting in coordinate expression of both CS1R:CD28:z and EGFRt from a single transcript (CS1RCD28EGFRt; ref. 12). The CS1RCD28EGFRt DNA sequence (optimized by GeneArt) was then cloned into a self-inactivating (SIN) lentiviral vector, pHIV7 (gift from Jiing-Kuan Yee, City of Hope, Duarte, CA).

Cell lines

To generate LCL OKT3, allogeneic LCLs (EBV-transformed lymphoblastoid cell lines) were resuspended in nucleofection solution using the Amaxa Nucleofector kit T. OKT3-2A-Hygromycin_pEK plasmid was added to 5 mg/10^7 cells, the cells were electroporated using the Amaxa Nucleofector I, and the resultant cells were grown in RPMI1640 with 10% FCS containing 0.4 mg/mL hygromycin. MM.1s was cultured in RPMI1640 with 10% FCS, and KG1a was cultured in IMDM with 10% FCS. To generate firefly luciferase+GFP+MM.1S (fflucGFPMM.1S), MM.1S cells purchased from ATCC were transduced with lentiviral vector encoding eGFP-ffluc. Initial transduction efficiency was 50%, so the GFP cells were sorted by FACS for >98% purity.

U266B and MM.1R cells were purchased from ATCC. Banks of all cell lines were authenticated for the desired antigen/marker expression by flow cytometry prior to cryopreservation, and thawed cells were cultured for less than 6 months prior to use in assays. Bone marrow cells were obtained from patients with multiple myeloma under protocols approved by the City of Hope Institutional Review Board.

Generation of multiple myeloma–specific T cells

Leukapheresis products were obtained from healthy donors under protocols approved by the City of Hope Institutional Review Board. Central memory T cells (T_{CM}) defined as $CD45RO^+CD62L^+$ were isolated on Miltenyi autoMACS (Miltenyi Biotec Inc). Briefly, PBMCs were isolated by density gradient centrifugation over Ficoll-Paque (GE Healthcare) and incubated with anti-CD14 and anti-CD45RA microbeads (Miltenyi Biotec Inc) for 30 minutes at room temperature. $CD14^+$ and $CD45RA^+$ cells were then immediately depleted using the autoMACS. The unlabeled negative fraction of cells was labeled with biotinylated-DREG56 mAb (COHNMC CBG) followed by incubation with anti-biotin microbeads (Miltenyi Biotec Inc). The $CD62L^+$ T_{CM} cells were purified with positive selection on auotMACS. Immediately after selection, the T_{CM} cells were activated, transduced, and maintained as described previously (13). Cultures were supplemented with lenalidomide every 48 hours for 21 days before *in vitro* analysis.

Cytokine production

A total of 5×10^5 CS1 CAR T cells were cocultured overnight with 5×10^5 LCL OKT3, MM.1S, or KG1a cells in 96-well tissue culture plates. The supernatant was collected, and cytokines were measured with Bioplex cytokine analysis (10-plex kit; in triplicates) according to the manufacturer's instructions.

Intracellular cytokine staining

A total of 5×10^5 CS1 CAR T cells were activated overnight with 5×10^5 LCL OKT3, MM.1S, MM.1R, U266B or KG1a cells in 96-well tissue culture plates in the presence of Brefeldin A (BD Biosciences). The cell mixture was then stained using anti-CD8, anti-CD4, and biotinylated erbitux/streptavidin to analyze surface coexpression of CD8, CD4 and CAR, respectively. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences). After fixation, the T cells were stained with antibodies against IFNγ, TNFα and IL2. Cells were then analyzed using multicolor flow cytometry on MACSQuant (Miltenyi Biotec Inc.). In some experiments, cocultures were set up in the presence of CS1 protein (BioVision).

Degranulation

A total of 5×10^5 CS1 CAR T cells were cocultured with 2.5×10^5 LCL OKT3, MM.1S, MM.1R, U266B or KG1a cells in RPMI T-cell medium containing Golgi stop (BD Biosciences) and CD107a for 6 hours at 37°C. Degranulation was assessed using multicolor flow cytometry on MACSQuant. In some experiments, cocultures were set up in the presence of CS1 protein (BioVision).

Cytotoxicity assay

Four-hour luciferase-based cytotoxicity assays (LCA) were performed as described previously (14) using CS1 CAR T cells as effector cells and CS1-expressing MM.1S cells as targets.

Side population assay (Hoechst stain)

MM.1S cells were treated with different concentrations of lenalidomide (0, 1, and 10 µmol/L) for 48 hours. Following treatment, cells were washed and resuspended in RPMI at a concentration of 1×10^7 cells/mL. Hoechst dye (Sigma) was added to suspension to achieve a final concentration of 5 µg/mL. Cells were incubated 2 hours with gentle vortexing every 30 minutes. Cells were then spun down at 2,000 rpm for 2 minutes, and media were replaced with Hank balanced salt solution. Propidium iodide (5 µg/mL) was added, and cells were incubated for 5 minutes at 4°C (15, 16). Analysis was conducted on MACSQuant.

Immune synapse staining

In an 8-well chamber, 6×10^4 MM.1S tumor cells were labeled with CellTracker Blue (Life Technologies) to visually distinguish from T cells and seeded in each well. They were cocultured with the same amount of CS1 CAR T cells, which had been treated with different concentrations of lenalidomide. After 2-hour culture at 37°C and 10% CO_2, the coculture was washed with PBS three times and cells were fixed for 10 minutes with 4% paraformaldehyde (PFA) at 4°C, followed by air dry for 10 minutes, and another wash with PBS.

To detect CAR, the cells were blocked by 0.5% FCS PBS for 1 hour, then 1:150 diluted Fab antibody (goat anti mouse, Sigma, F4018) was added and incubated overnight at 4°C. After washing with 0.5% FCS PBS three times, the cells were stained with secondary antibody against goat (L+H) conjugated with Alexa488 for 1 hour at room temperature. The F-Actin Visualization Kit (Biochem Kit) was used according to the manufacturer's instructions. Briefly, the cells were washed and permeabilized for 5 minutes at room temperature. After one wash with washing buffer, cells were stained with 200 µL working stock Rhodamine Phalloidin and incubated for 30 minutes at room temperature in the dark. After 3 washes, slides were covered by mounting medium. Each sample was duplicated and stained. For FACS-based immune synapse formation analysis (17, 18), GFP+ MM.1S or GFP+ KG1a cells were cocultured with the same number of erbitux- streptavidin-PE (SA PE)–stained CS1 CAR T cells that had been treated with different concentrations of lenalidomide. After 30-minute incubation, cocultures were washed, and GFP and CAR double-positive cells were quantified with flow cytometry.

Quantitative image analysis of immune synapse

Images were taken under microscope (Zeiss observe II, ×40 oil). To quantify immune synapses, entire slides were scanned, and the area of F-actin and Fab conjugation was measured using software (Image propremier 9.2) by setting up the single staining F-actin, Fab, and CellTracker as background. The areas of cell conjugation (yellow–golden brown) were selected for the measurement as polarization of synapse. The exported areas ($pixel^2$) were further analyzed and converted to average $pixel^2$ for entire slides.

Sequencing library preparation, deep sequencing using Illumina HiSeq2500, and RNA-Seq data analysis

The CD8+CAR+ T cells treated with 0, 1, and 10 µmol/L of lenalidomide were purified by FACS after costaining with antibodies against EGFR and CD8. The total RNA was isolated using RNeasy Plus Micro and Mini Kits according to protocol. RNA integrity was verified with the Agilent 2100 Bioanalyzer. The RNA sequencing libraries were prepared using SMARTer Stranded Total RNA-Seq Pico input Kit (Clontech). Briefly, 10 ng of DNA-free total RNA was reverse transcribed by SMARTScribe Reverse Transcriptase with random priming. The enzyme's terminal transferase activity results in addition of a few nontemplated nucleotides to the 3′ end of the cDNA, creating an extended template to enable the reverse transcriptase to continue replicating to the end of the oligonucleotide. The first round of PCR amplification added full-length Illumina adapters, including barcodes. The ribosomal cDNA (originating from rRNA) was then cleaved by ZapR in the presence of the mammalian-specific R-Probes. The rRNA-depleted cDNA were enriched via a second round of PCR amplification using primers universal to all libraries. The sequencing libraries were validated with running Agilent bioanalyzer DNA high sensitivity Chip, and quantified using Qubit and qPCR. The library templates were prepared for sequencing using cBot cluster generation system with HiSeq PE Cluster Kit V4 (Illumina). Sequencing runs were performed in the paired end mode of 101 cycles of read1, 7 cycles of index read, and 101 cycles of read2 using HiSeq2500 platform with HiSeq SBS Kit V4 (Illumina). Real-time analysis (RTA) 2.2.38 software was used to process the image analysis and base calling.

qPCR analysis

The CD8+CAR+ T cells treated with 0, 1, or 10 µmol/L of lenalidomide were purified by FACS after costaining with antibodies against EGFR and CD8. The cells from three different donors were purified and analyzed. The cDNA was used for TaqMan qPCR (7500 FAST, Life Technology). The TaqMan probes used in this study are listed in Supplementary Table S1. The gene expression levels are presented as $2^{\Delta C_t}$.

Xenograft models

All mouse experiments were approved by the City of Hope Institutional Animal Care and Use Committee. Six- to 10-week-old NOD/*Scid* IL2RγC^{null} mice were injected intratibially on day 0 with 2×10^6 fflucGFP MM.1S cells. Five days later, mice were injected intravenously with dosed 1×10^6 CAR T cells or non-transduced mock cells. For experiments using lenalidomide, mice were administered 5-7.5 mg/kg of lenalidomide intraperitoneally daily for 30 days. Anesthetized mice were imaged using a Xenogen IVIS 100 series system (Xenogen). Photons from ffLuc+ tumor xenografts were quantified using the software program Living Image (Xenogen), and the bioluminescence signal was measured as total photon flux normalized for exposure time and surface area and expressed in units of photons per second per cm^2 per steradian. Human T-cell engraftment in peripheral blood, bone marrow, and spleen was determined by flow cytometry after staining with antibody against human CD45, CD8, and erbitux for CAR detection.

Statistical analysis

Analyses were performed using Prism (GraphPad Software Inc.). Log-rank (Mantel–Cox) test and Mann–Whitney test were used to ascertain the statistical significance of the *in vivo* data. The paired *t* test (two-tailed) and two-way ANOVA were used for the analysis of *in vitro* data.

Results

CS1 is highly expressed on multiple myeloma cells and primary multiple myeloma bone marrow cells

We conducted flow cytometry to characterize surface CS1 expression on multiple myeloma cells. Multiple myeloma cell line MM.1S cells are highly (70%–80%) CS1-positive. We also assessed antigen expression on bone marrow mononuclear cells from patients with newly diagnosed or relapsed multiple myeloma. Consistently, primary multiple myeloma cells across patients express high levels of CS1 (Fig. 1A).

Generation of T$_{CM}$-derived CS1 CAR T cells against multiple myeloma

We isolated T$_{CM}$ cells from healthy donors, followed by activation and transduction with a lentivirus (13) encoding second-generation CAR specific for CS1, containing CD28 as costimulatory molecule and EGFRt as a potential molecule for detection, selection, and in vivo ablation (ref. 12; Fig. 1B). Engineered CS1 CAR T cells (Fig. 1C) exhibited similar expansion in vitro (Fig. 1C), comparable with the nontransduced mock T cells.

CS1 CAR T cells mediate efficient effector function and cytolytic activity

To test the effector function of CS1 CAR T cells, we cocultured expanded CAR T cells with MM.1S cells for 6 hours for degranulation assay and cytokine secretion determination. Gated CS1 CAR T cells express CD107a as an indicator of degranulation and IFNγ upon antigen stimulation (Fig. 1D). We then cocultured CAR T cells with luciferase-expressing MM.1S target cells for 4 hours; specific lysis was analyzed after adding the substrate luciferin (Fig. 1E). CS1 CAR T cells exhibited specific and efficient killing of multiple myeloma cells.

CS1 CAR T cells exhibit efficient antitumor activity in vivo

A total dose of 1×10^6 CAR T cells specific for CS1 derived from T$_{CM}$ cells were intravenously transferred into MM.1S tumor-bearing mice. CS1 CAR T cells exhibited significantly higher antitumor activity as compared with mock T cells ($P = 0.03$; Fig. 1F and G).

To further study whether tumor relapse is due to the low dose of CAR T cells, we increased the CS1 CAR T-cell dose 3-fold (3×10^6) with the same tumor burden. Tumor recurrence was not delayed (Supplementary Fig. S1), which suggested that other mechanisms were at play in regards to disease relapse.

Soluble CS1 does not interfere with CS1 CAR function

Multiple myeloma cells also secrete soluble forms of CS1 and BCMA (19, 20). Other investigators have previously shown that CAR-expressing T cells were not blocked from recognizing target cells by soluble proteins, including BCMA (21). CS1 concentrations of >10 times the median CS1 levels found in the serum of patients with multiple myeloma did not block recognition of multiple myeloma cells by CS1 CAR T cells, as indicated by equivalent CD107a degranulation and IFNγ production (Supplementary Fig. S2A and S2B). Furthermore, we analyzed cytolytic activity by adding graded concentrations of CS1 protein during the 4-hour luciferase-based cytolytic assay (LCA). Again, soluble CS1 protein did not interfere with CS1 CAR–specific lysis (Supplementary Fig. S2C).

Lenalidomide exerts a costimulatory effect on CS1 CAR T cells in a dose-dependent fashion

Lenalidomide has known activity in the activation and expansion of normal T cells (22). We engineered and expanded CS1 CAR T cells in the presence of 0, 1, and 10 μmol/L lenalidomide. We found that lenalidomide preferentially expanded the CD8$^+$ CAR T-cell subset in a dose-dependent manner (Fig. 2A) along with increased CAR and total T-cell expansion, but decreased the proportion of CD4$^+$ T cells, irrespective of the different proportions of CD4$^+$ and CD8$^+$ T cells in the starting populations across different donors (Supplementary Figs. S3 and S4). The skew toward CD8$^+$ cells became more dramatic when cultures were extended for >3 weeks (Fig. 2A). CAR$^+$ T cells, especially CD8$^+$ CAR T cells, exhibited comparable levels of memory T-cell signatures such as CD62L, CD127, CD27, and CD28 in the presence and absence of lenalidomide and maintained a relatively minor exhaustive phenotype as indicated by low expression of PD1 and Tim3 among both lenalidomide-treated and untreated CAR T cells (Fig. 2B). Th1 cytokines are critically important for the induction of antitumor cellular immunity in vivo (23), and lenalidomide has been shown to polarize toward the Th1 phenotype (24). We also demonstrated that lenalidomide-treated CS1 CAR T cells exhibited higher Th1 cytokine production, including IFNγ and TNFα, as measured in both the supernatant of cocultures and cell-based intracellular cytokine analysis (Fig. 2C and D) in a dose-dependent manner. Interestingly, autocrine IL2, the major determinant of CAR T-cell potency and persistence, was greatly elevated in the CS1 CAR T cells treated with lenalidomide. However, the production of T-cell immunosuppressive Th2 cytokines (e.g., IL5, IL10, and IL4) was greatly reduced (Fig. 2C). Lenalidomide exhibited a costimulatory function similarly on CD8$^+$CAR$^+$ and CD4$^+$CAR$^+$ T cells (Figs. 2D). To prove these results are independent of multiple myeloma cell lines, we tested two other multiple myeloma cells, MM.1R and U266B, representing CS1 high and low expressers (Fig. 3A). Upon stimulation with targets, CS1 CAR T cells express CD107a and secrete IFNγ and TNFα against all the multiple myeloma cells tested. The effector functions are correlated with the CS1 expression levels on the targets (Fig. 3B). Consistently, lenalidomide-treated CS1 CAR T cells exhibited better effector function against different multiple myeloma targets in a dose-dependent manner (Fig. 3C).

Lenalidomide eradicates multiple myeloma–initiating cells without downregulating CS1 expression on multiple myeloma cells

We assayed the sensitivity of MM.1S cells to lenalidomide as well as the maintenance of surface CS1 during lenalidomide treatment. The overall growth of MM.1S cells was greatly inhibited by the addition of lenalidomide every other day in the culture, indicating that MM.1S cells are sensitive to lenalidomide in vitro (Supplementary Fig. S5A). We performed flow cytometry–based Hoechst staining to evaluate the existence of a multiple myeloma tumor-initiating population with stem-like features known as side population cells (SP). Forty-eight hours after treatment of CS1 CAR T cells, a dramatic decrease of SP was observed in the treated MM.1 S cells (Supplementary Fig. S5B). Meanwhile, lenalidomide did not downregulate CS1 expression on the MM.1S cells, assuring intact antigen for CAR T-cell targeting (Supplementary Fig. S5C).

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Figure 1.
T$_{CM}$-derived CS1 CAR T cells exhibit specific effector function and anti-MM activity *in vivo*. **A,** Multiple myeloma (MM) cell line MM.1S and bone marrow (BM) mononuclear cells from patients with multiple myeloma were stained with fluorochrome-conjugated isotype controls and antibodies specific to CS1 and CD38. The percentages of positive cells are presented after exclusion of dead cells with DAPI. Representative data of ten multiple myeloma patients' bone marrow are presented. **B,** Schematics of CS1 and lentiviral CAR constructs, composed of a CS1-specific scFv, IgG4 hinge region, and a CD28 costimulatory domain. The IgG4 hinge region was shortened by deleting the CH2 portion. The CAR sequence is followed by a T2A ribosomal skip sequence and the coding sequence for the EGFRt tracking/suicide gene. **C,** The selected central memory cells (T$_{CM}$) were transduced with the second-generation CS1 CAR following CD3/CD28 bead activation and expanded in the presence of rhIL2 (50 U/mL) and IL15 (0.5 ng/mL) for 3 weeks. CAR expression was defined by erbitux-biotin and streptavidin (SA)-PE staining. (*Continued on the following page.*)

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Lenalidomide improves immune synapse formation between CAR T cells and tumor cells

The term immunologic synapse (IS) refers to the organization of membrane proteins that occurs at the interface between the T cell and the target cells when contact is made during the effector phase (25). The enhancement of immune function by lenalidomide on CAR T cells may be related to increased immune synapse formation. We cocultured CAR T cells that had been treated with lenalidomide with target cells for 2 hours. The areas of cell conjugation and polarization between Fab-positive CAR T cells and CellTracker-labeled tumor cells were defined as immune synapses and were quantified using average pixel[2]. We also directly measured the numbers of conjugates as a result of immunologic synapse formation between CS1 CAR T cells and MM.1S target cells using flow cytometry (18). Consistently, immune synapse formation was found to be significantly increased with the lenalidomide-treated CS1 CAR T cells as compared with untreated CS1 CAR T cells (Fig. 4A–C; Supplementary Fig. S6A). No immune synapse formation was observed when CS1 CAR T cells were cocultured with KG1a or mock T cells were cocultured with MM.1S cells (Fig. 4D; Supplementary Fig. S6B and S6C). In concert with these findings, cytolytic activity was increased with lenalidomide-treated CAR T cells in a dose-dependent fashion, which became especially apparent when a low effector:target (E:T) ratio was used (Fig. 4E and F). These data support that enhanced immune synapse formation is associated with the improved cytolytic activity of CAR T cells.

Lenalidomide enhances transcriptional signatures of immune synapse and T-cell function

We sought to compare the transcriptome of the CAR T cells treated with and without lenalidomide. RNA-seq gene expression analysis was conducted with purified $CD8^+CAR^+$ T cells (>90%; Fig. 5A). More than 600 genes were differentially expressed (fold change > 2) among the lenalidomide-treated and untreated $CD8^+CAR^+$ T cells (Fig. 5B) in a dose-dependent manner (389 progressively upregulated and 276 downregulated). Full gene names are listed in Supplementary Tables S2 and S3. Among these genes, immune synapse–related genes such as cell junction and biological assembly–related genes are significantly increased with lenalidomide treatment ($P = 4.55 \times 10^{-4}$ - 2.08×10^{-7}; Fig. 5C; Supplementary Table 4). Moreover, lenalidomide causes global changes in gene transcripts that are characteristic of improved stem memory, homing, and effector function of CAR T cells. Genes associated with stem memory or less differentiated phenotypes, such as *IL7*, *ACTIN1*, *KIT*, *WNT5B*, *IL9*, and *MYB* (26, 27), are upregulated together with genes associated with enhanced effector function, such as *IRF4* (28) and *IFI44* (29). Lenalidomide-treated CS1 CAR T cells have elevated gene signatures that are related to better homing capacity such as integrins

ITGA4 and *ITGB3* (30), which all responded in a dose-dependent manner (Fig. 5D). In contrast, exposure to lenalidomide suppresses genes in CAR T cells that are associated with exhaustion such as *PDCD1*, *CD160* (31, 32), terminal differentiation genes such as *GZMK* and *GZMH*, *EOMES* (33, 34) and immune suppression signals such as *TGFBR3* and *IL10* (35; Fig. 5E). Collectively, these findings align with the changes noted above supporting that lenalidomide exerts a costimulatory effect on CS1 CAR T cells. To rule out donor-dependent factors, we purified $CD8^+CAR^+$ cells from three different donors and analyzed the expression of representative genes. Consistently, dose-dependent effects of lenalidomide on CAR T cells were observed (Fig. 5F–H).

Lenalidomide improves CS1-targeted anti-multiple myeloma activity and prolongs functional CAR T-cell persistence *in vivo*

We investigated the synergistic effects of CS1 CAR T cells and lenalidomide against multiple myeloma in our established xenograft mouse model (Fig. 6A). Although 5–7.5 mg/kg lenalidomide daily injection alone and mock T cells plus daily injection of lenalidomide did not induce tumor regression, a single CS1 CAR T-cell infusion in conjunction with lenalidomide induced complete tumor remission during lenalidomide treatment (30 days; Supplementary Fig. S7) and significantly prolonged survival ($P = 0.008$ and $P = 0.001$; Fig. 6B) as compared with the group treated with CS1 CAR T cells alone. Fifty days after CAR T-cell infusion, CAR T cells were detected only in the group of mice administered both CS1 and lenalidomide, but not in mice given CS1 CAR T cells alone, suggesting improved CAR T-cell persistence with lenalidomide (Fig. 6C). Upon further evaluation, we found that persisting CAR T cells in the group receiving lenalidomide and CS1 CAR combinatorial treatment were predominantly $CD8^+$, in contrast to the input CAR T cells that were $CD4^+$ dominant, which is consistent with the *in vitro* data showing enhanced $CD8^+CAR$ T-cell expansion (Fig. 6D).

We observed that mice treated with combining CAR T cells and daily injection of lenalidomide remain tumor free. However, relapse occurs once ceasing lenalidomide treatment for two weeks (Supplementary Fig. S7), suggesting that lenalidomide is required for durable tumor regression as it is used in the clinic as a long-term maintenance therapy. We further investigated whether the presence of lenalidomide is needed for sustained and enhanced CAR T-cell function *in vivo*. CS1 CAR T cells treated *ex vivo* with and without lenalidomide were injected into multiple myeloma–bearing mice without giving exogenous lenalidomide administration. Consistently, no improved antitumor activity was observed when mice were treated with the lenalidomide pre-treated CAR T-cell products as compared with untreated CS1 CAR T cells (Supplementary Fig. S8). To further support this scenario, we performed an experiment where we treated the CS1 CAR T cells with lenalidomide for two weeks. We then split the culture into

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(*Continued*.) Percentages of CAR+ cells are indicated in each quadrant on the basis of gating of cells stained with SA-PE alone. Nontransduced T_{CM} cells were used as control. Growth of total cell number was determined by Guava Viacount at different time points. **D,** Expanded CS1 CAR T cells were cocultured with MM.1S cells at a 1:1 ratio in medium containing Golgi plug and CD107a for six hours at 37°C. KG1a cells were used as negative stimulator. Degranulation was determined using multicolor flow cytometry. Percentages of CD107a+ and intracellular IFNγ+ cells from gated erbitux (CAR)+ T cells are presented. **E,** CS1 T cells as effector cells were cocultured with luciferase-expressing MM.1S as targets. After 4-hour incubation, luminescence flux was read following addition of substrate luciferin. LCL OKT3 were used as a positive control and myeloid leukemic cells KG1a as a negative control. Representative data from six different donors are presented in **C–E. F,** A total of 2×10^6 fflucGFP MM.1S cells were intratibially (i.t.) injected into NSG mice. Five days following tumor inoculation, mice were injected intravenously with dosed 1×10^6 CAR T cells or nontransduced mock cells. Tumor signals were monitored with Xenogen imaging once a week. **G,** The bioluminescence signal was measured as total photon flux normalized for exposure time and surface area and expressed in units of photons (p) per second per cm^2 per steradian (sr). Representative data from six separate *in vivo* experiments are presented ($N = 5$ mice per group in each experiment).
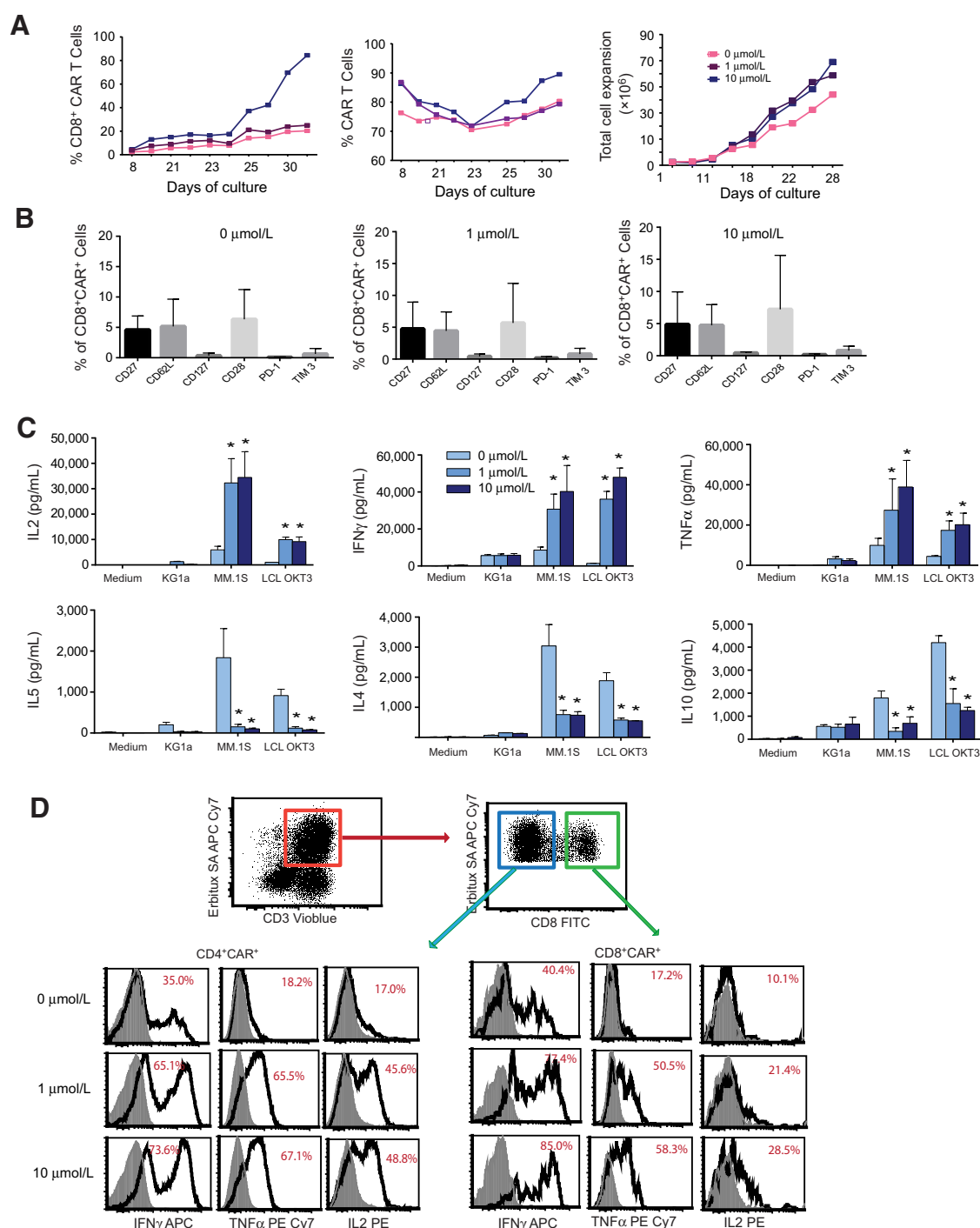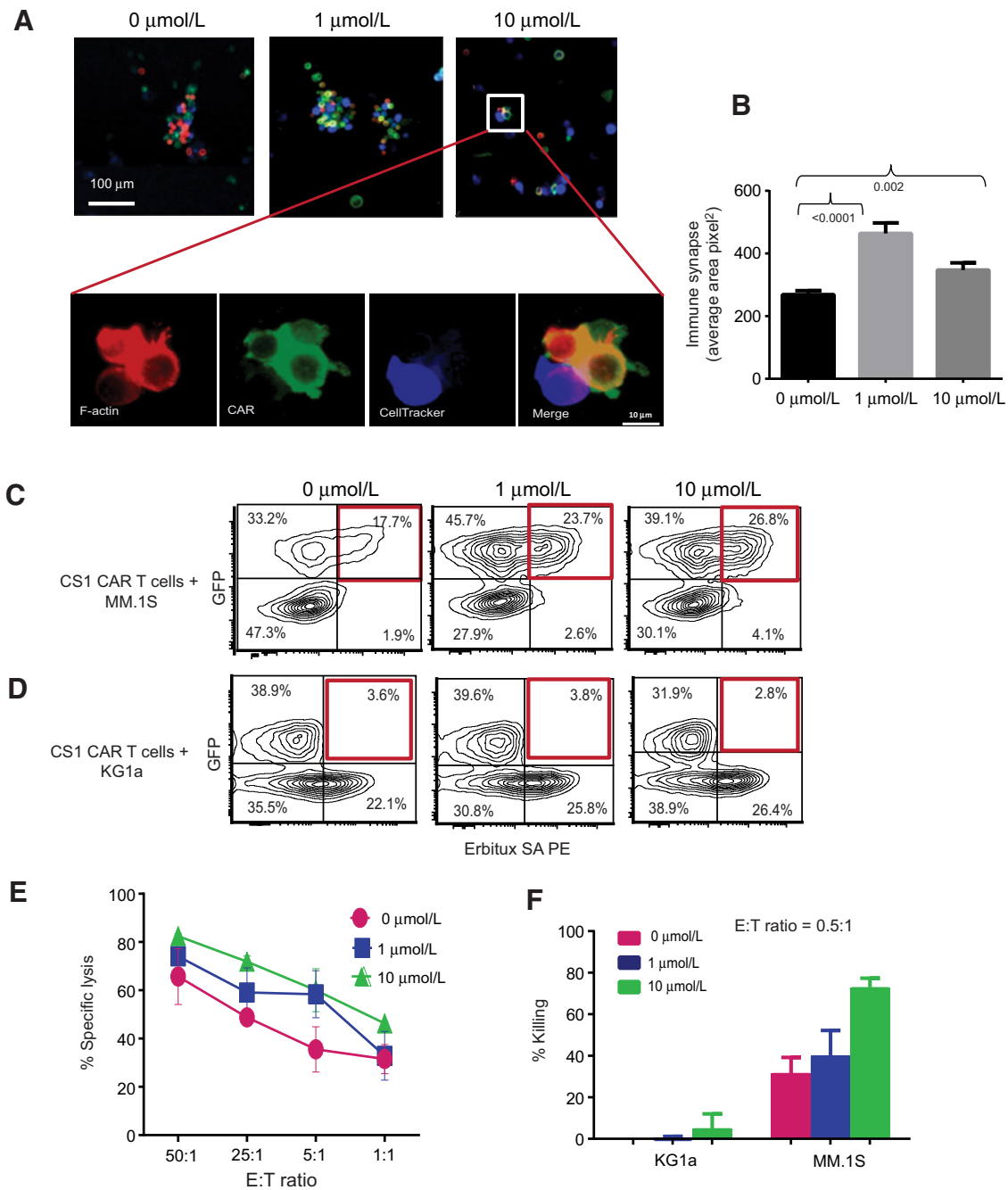
Figure 2.
Lenalidomide exerts a costimulatory effect on CS1 CAR T cells in a dose-dependent fashion. T_CM cells were activated and transduced with lentivirus encoding CS1 CAR and were expanded *in vitro* in the presence of 0, 1, and 10 μmol/L lenalidomide for 3-4 weeks. **A,** Growth of total cell number was determined by Guava Viacount at different time points. Cells were stained with antibodies against CD8 and EGFRt (CAR), and percentages of positive cells at different time points are presented. **B,** After 3 weeks of *in vitro* expansion, cells were labeled with indicated antibodies. Percentages of positive cells under CD8$^+$CAR$^+$ gating are presented. Means + SEM from three donors are presented. **C,** 5 × 10^5 CS1 CAR T cells treated with different concentrations of lenalidomide were cocultured overnight with 5 × 10^5 LCL OKT3, MM.1S, or KG1a cells. The supernatant was collected, and cytokines were measured with Bioplex cytokine analysis from triplicates. Two-way ANOVA test was used. Representative data of two donors are presented. **D,** A total of 5 × 10^5 CS1 CAR T cells were activated overnight with 5 × 10^5 MM.1S cells in the presence of Brefeldin A. The cell mixture was then stained using Erbitux and streptavidin followed intracellular staining with antibody against IFNγ, TNFα, and IL2. Cytokine positive cells under indicated gating are presented. Representative data from three different donors are presented.
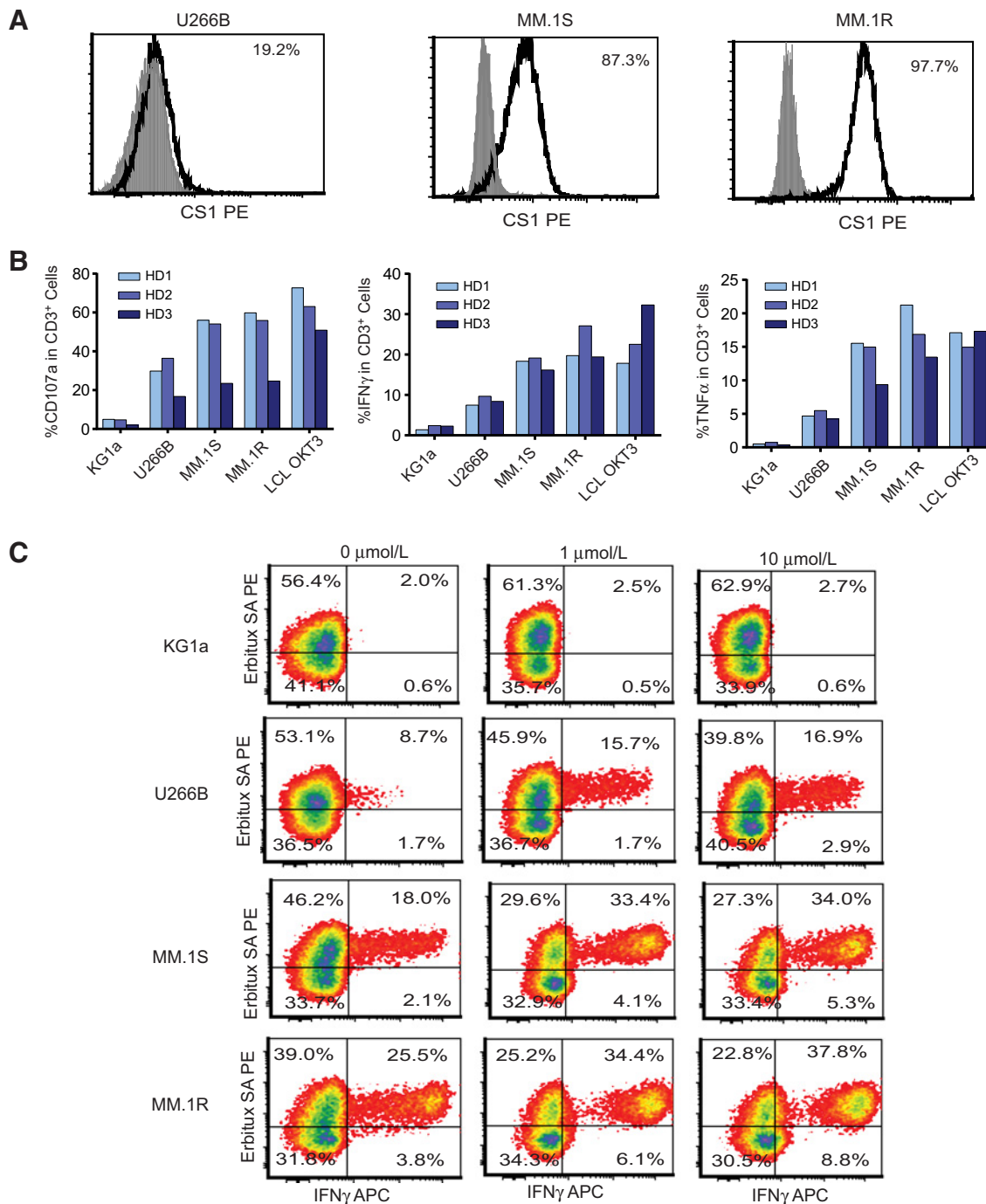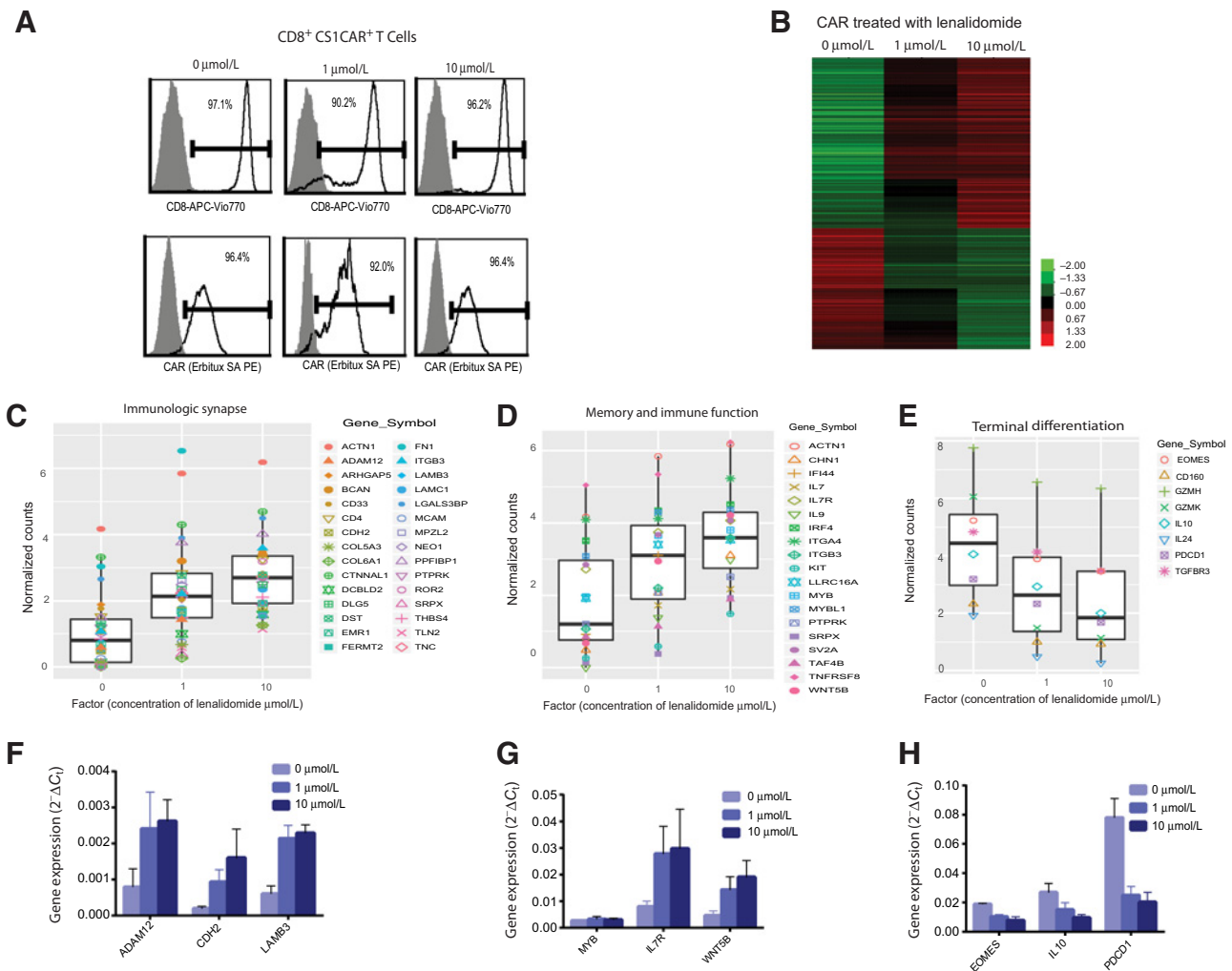
Figure 3.
Lenalidomide exerts a costimulatory effect on CS1 CAR T cells against different multiple myeloma (MM) cells. **A,** CS1 expression on U266B, MM.1S and MM.1R. Multiple myeloma cells were analyzed by staining with fluorochrome-conjugated isotype controls and antibodies specific to CS1. The percentages of positive cells over isotype controls (filled gray) are presented after exclusion of dead cells with DAPI. **B,** Expanded CS1 CAR T cells were cocultured with U266B, MM.1S, and MM.1R cells at a 1:1 ratio in medium containing Golgi plug and CD107a for six hours at 37°C. A total of 5×10^5 CS1 CAR T cells were activated overnight with 5×10^5 U266B, MM.1S and MM.1R cells in the presence of Brefeldin A. The cell mixture was then stained using Erbitux and streptavidin followed intracellular staining with antibody against IFNγ and TNFα. Percentages of CD107a-positive and cytokine-positive cells from the CD3+ population were determined using multicolor flow cytometry. KG1a cells were used as negative stimulator and LCL OKT3 cells as positive stimulator. Data from three different donors are presented. **C,** T_CM cells were activated and transduced with lentivirus encoding CS1 CAR and were expanded *in vitro* in the presence of 0, 1, and 10 μmol/L lenalidomide for 3–4 weeks. A total of 5×10^5 propagated CS1 CAR T cells were activated overnight with 5×10^5 U266B, MM.1S and MM.1R cells in the presence of Brefeldin A. The cell mixture was then stained using erbitux and streptavidin followed intracellular staining with antibody against IFNγ. Cytokine-positive cells under CD3 gating are presented. Representative data from three different donors are presented.

Figure 4.
Lenalidomide improves immune synapse formation between CAR T cells and tumor cells. Multiple myeloma (MM) tumor cells labeled with CellTracker Blue were cocultured with the same number of CS1 CAR T cells treated with different concentration of lenalidomide. After 2-hour incubation, cocultures were washed and fixed with 4% paraformaldehyde (PFA). To detect CAR, the cells were stained with Fab antibody followed by secondary antibody conjugated with Alexa 488. The F-actin was labeled in red. **A,** The images were taken under microscope. **B,** To quantify immune synapses, entire slides were scanned and the area of F-actin and Fab conjugation were measured using Image propremier 9.2 software by setting up single staining F-actin, Fab, and CellTracker as background. The areas (yellow–golden brown) of cell conjugation and polarization for synapse were selected for measurement. Statistical analysis was performed with two-tailed paired t test. P values are indicated. **C** and **D,** In FACS-based synapse analysis, GFP[+] MM.1S cells or GFP[+] KG1a were cocultured with the same number of erbitux SA-PE–stained CS1 CAR T cells that had been treated with different concentrations of lenalidomide. After 30-minute incubation, cocultures were washed, and GFP and CAR double–positive cells were quantified with flow cytometry. **E,** CS1 CAR T cells treated with different concentrations of lenalidomide were cocultured with luciferase-expressing MM.1S for 4 hours. Specific lysis was analyzed after adding substrate luciferin. Average from three replicates are depicted. **F,** CS1 CAR T cells treated with different concentrations of lenalidomide were cocultured with MM.1S at a E:T ratio of 0.5 to 1 for 24 hours. Percent killing was analyzed and calculated on the basis of flow cytometric analysis of the tumor cells remaining in the culture. Average killing from two separate experiments of two donors are presented.

Figure 5.
Lenalidomide enhances transcriptional signatures of immune synapse and T-cell function. **A,** CD8^{+}CS1 CAR^{+} T cells in expanded CS1 CAR T-cell population treated with different concentrations of lenalidomide were purified. Percentages of CD8^{+} and CAR^{+} cells are depicted. **B,** Differentially expressed genes that have RPKM fold change ≥ 2 between compared groups of indicated concentrations of lenalidomide are presented. Normalized counts for immune synapse (**C**), memory and immune function (**D**), and terminal differentiation genes in CAR T cells are presented (**E**). Reprehensive genes in purified CD8^{+}CAR^{+} T cells derived from three donors were analyzed using qRT-PCR. Means + SEMs of $2^{\Delta C_t}$ of immune synapse (**F**), memory and immune function (**G**), and terminal differentiation genes are presented (**H**).

two, one culture underwent continued lenalidomide treatment every other day for an additional two weeks and the other culture was no longer treated with lenalidomide. Consistently, in the culture with continued lenalidomide, we observed increased levels of CD107a and percentages of CD8^{+} T cells at the end of the experiment, compared with values of untreated cultures. We also noted increased intracellular IL2, which is a hallmark of T-cell persistence and function. However, discontinuation of lenalidomide resulted in decreased IL2 production and a lower percentage of CD8^{+} T cells, while CD107a levels remained the same. In addition, CAR T cells expressed higher levels of PD1 upon discontinuation of lenalidomide (Supplementary Fig. S9). Overall, our data indicated that the costimulatory effects of CAR T cells mediated by lenalidomide rely on the continuing activation of the T-cell signaling pathway. The role of lenalidomide in this context is therefore not an enhancement during manufacturing but rather a combinatorial agent that improves the function of these CAR T cells.

Discussion

In this study, we demonstrate that CS1 CAR T cells exhibit efficient anti-multiple myeloma activity *in vivo*. Second, we show that lenalidomide, in addition to its known antimyeloma effects, can also augment T-cell function and number. This in turns leads to a synergistic increase in the antimyeloma activity of CAR T cells through increased cytotoxicity and improved persistence of the engineered cells.

To validate the potential use of CS1 CAR T cells for multiple myeloma, we examined the effector function of CS1 CAR T cells against different multiple myeloma cells, varying in the levels of CS1 expression. Data demonstrated that CS1 CAR T cells exhibited
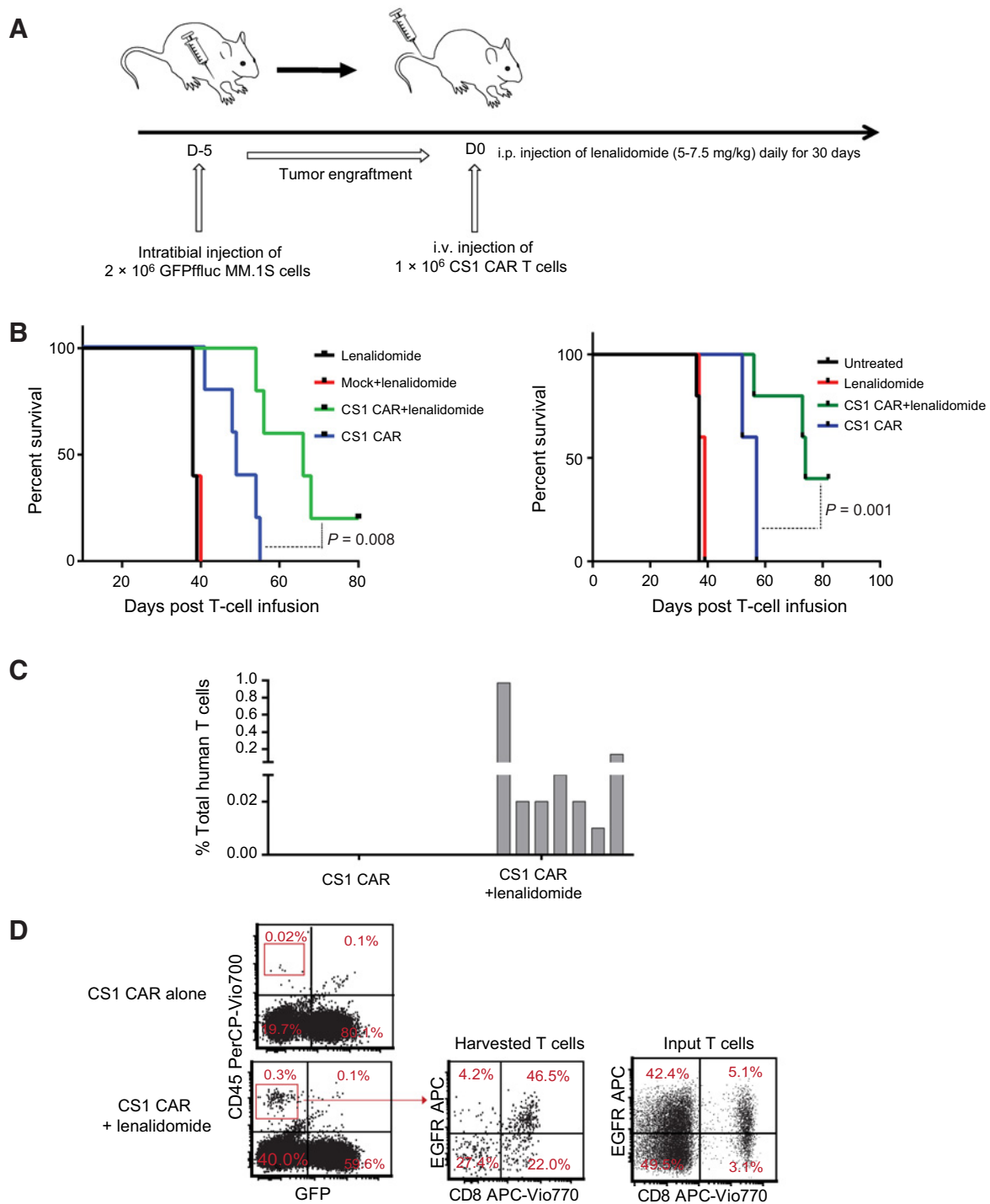
Figure 6.
Lenalidomide improves the anti-multiple myeloma (MM) activity of CS1 CAR T cells and prolongs functional CAR T-cell persistence *in vivo*. **A,** A total of 2×10^6 fflucGFP MM.1S cells were intratibially (i.t.) injected into NSG mice. Five days following tumor inoculation, mice received a single injection (i.v) of dosed 1×10^6 CAR T cells and daily intraperitoneal injections of 5–7.5 mg/kg lenalidomide for 30 days. Nontransduced mock cells were infused into control mice.
B, Kaplan–Meier survival curves are presented. N = 5 mice per group. Representative data from four separate experiments are presented. **C,** Fifty days post CAR T-cell infusion, human T cells harvested from peripheral blood, bone marrow, and spleen (3 mice per group) were analyzed with flow cytometry after staining with antibody against human CD45. Human CD45⁺GFP− cells are depicted. **D,** The harvested bone marrow cells were labeled with antibodies against CD45, CD8, and EGFR. Percent of positive cells in gated CD45⁺ population are depicted. Representative data of four in vivo experiments are presented.

antigen-dependent effector function as indicated by degranulation and cytokine production, supporting a selective targeting for multiple myeloma cells which express high CS1 on the surface. More interestingly, CS1 CAR T cells exhibited efficient effector function against MM.1R, which are resistant to dexamethasone because of mutant glucocorticoid receptor (36, 37). These data may indicate the effectiveness of CS1 CAR T cells on dexamethasone-resistant patients with multiple myeloma.

Although significant tumor remission was observed in the CS1 CAR T-cell–treated mice, tumor relapse eventually occurred. We first speculated that tumor antigen escape was the cause. Unexpectedly, recurrent tumors remained CS1-positive regardless of CS1 CAR T-cell treatment. We further evaluated CAR T-cell persistence in the tumor-bearing mice, as lack of CAR T-cell persistence is another contributor to failure of T-cell therapy, and greater T-cell persistence and survival are associated with high rates of complete remission. To our surprise, CAR T cells were detected in CS1 CAR T-cell–treated mice. Although these persisting CAR T cells had low expression of exhaustion markers such as PD1 and Tim3, the levels were too low to prevent tumor relapse (data not shown). These data highlight the need for a strategy that can improve CAR T-cell function and expansion/persistence to achieve durable tumor remissions.

Lenalidomide has been shown to promote immunologic memory in the T-cell populations of patients with multiple myeloma by resulting in an excess of memory T cells and decrease of terminally differentiated T cells (38). In agreement with others' observations (38), our data showed that lenalidomide preferentially expanded CD8^{+} CAR T cells, resulting in increased total cell expansion. The expanded CD8^{+} CAR T cells exhibited less differentiated phenotypes but maintained minimal exhaustive features as reported (39) and terminal differentiation genes, suggesting that lenalidomide improves memory CAR T-cell maintenance.

Lenalidomide exerts a costimulatory effect on T-cell responses that includes increased production of IL2 and IFNγ and inhibits production of anti-inflammatory cytokines (40, 41). Consistently, in our studies, CAR T cells treated with lenalidomide exhibited a strong capacity to produce Th1 cytokines upon stimulation with different CS1^{+} tumors such as U266B, MM.1S, and MM.1R; these cytokines are required for durable antitumor activity of T cells (42). In contrast, Th2 cytokines that hamper the induction of effective T-cell responses (42, 43) were downregulated, demonstrating that lenalidomide can favorably moderate the immune milieu. Furthermore, lenalidomide-treated CAR T cells exhibited increased cytolysis of multiple myeloma cells. These effects may be attributed to the enhanced immune synapse formation between the lenalidomide-treated CAR T cells and tumors as is supported by gene signatures enriching for immune synapse formation and is in accordance with previous studies (44, 45).

In the murine model, we chose a relatively low dose of lenalidomide to allow assessment of potential synergistic effects when combined with CAR T-cell treatment. In spite of the suboptimal dose of lenalidomide in the NSG model as compared with the doses used by others (46, 47), combination therapy completely eradicated tumor. Therefore, the combinatorial effects mainly reflect the impact of lenalidomide on CAR T cells. Patients treated with lenalidomide have shown faster CD8^{+} T-cell reconstitution compared with CD4^{+} cells (48). Consistently, CD8^{+} CAR T cells became dominant within the harvested human T cells from mice, which markedly differs from the CD4-dominant composition of input CAR T cells, further confirming our in vitro data showing

CD8^{+} CAR enrichment in the presence of lenalidomide. Pretreated CS1 CAR T cells exhibited similar antitumor activity to untreated CS1 CAR T cells in vivo, indicating the requirement of sustained lenalidomide to maintain the T-cell costimulatory pathway and obtain synergistic effects with CAR T cells.

We also noticed that recurrent tumors in mice that had received combination therapy had a different distribution pattern than tumors in mice treated with CS1 CAR T cells alone. Specifically, CS1 CAR T-cell–treated tumors were located mainly in the bone marrow; in contrast, mice with recurrent tumors from combination treatment had to be euthanized because of large extramedullary subcutaneous tumors where there were no CAR T cells detected (data not shown). Lenalidomide disrupts the microenvironmental interactions between multiple myeloma cells and bone marrow stromal cells (49), resulting in inhibition of tumor growth. Our data suggest that lenalidomide may impair bone marrow microenvironmental support for multiple myeloma growth, directing the relapsed tumor to seed preferentially in extramedullary tissues. Evidently, CS1 CAR T cells did not penetrate into the metastasized extramedullary tumors and faced the same barriers presented by other types of solid tumors (50).

In summary, this study provides novel mechanistic insights into CAR T-cell functional augmentation by lenalidomide in terms of in vivo antitumor activity and persistence. The synergistic effects could be the consequence of directly inhibiting tumor-initiating cells (SP) while enhancing the antitumor activity of CAR T cells. This study provides the basis for a planned clinical trial using the combination of lenalidomide with engineered T cells against CS1 in relapsed myeloma.

Disclosure of Potential Conflicts of Interest

Authors' Contributions

Conception and design: X. Wang, M. Walter, A.Y. Krishnan, S.J. Forman
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wang, R. Urak, C. Huynh, L. Lim, C.W. Wong, W.-C. Chang, M. Htut
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wang, M. Walter, R. Urak, C. Huynh, L. Lim, W.-C. Chang, S.H. Thomas, J.F. Sanchez, L. Yang, A.Y. Krishnan, S.J. Forman
Writing, review, and/or revision of the manuscript: X. Wang, M. Walter, R. Urak, C. Huynh, L. Lim, S.H. Thomas, J.F. Sanchez, F. Pichiorri, M. Htut, A.Y. Krishnan, S.J. Forman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Wang, A.Y. Krishnan
Study supervision: C.E. Brown

Acknowledgments

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