

# Anti-B-cell Maturation Antigen Chimeric Antigen Receptor T cell Function against Multiple Myeloma Is Enhanced in the Presence of Lenalidomide



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## Abstract

Anti-B-cell maturation antigen (BCMA) chimeric antigen receptor (CAR) T cells have shown promising clinical responses in patients with relapsed/refractory multiple myeloma. Lenalidomide, an immunomodulatory drug, potentiates T cell functionality, drives antimyeloma activity, and alters the suppressive microenvironment; these properties may effectively combine with anti-BCMA CAR T cells to enhance function. Using an anti-BCMA CAR T, we demonstrated that lenalidomide enhances CAR T cell function in a concentration-dependent manner. Lenalidomide increased CAR T effector cytokine production, particularly under low CAR stimulation or in the presence of inhibitory ligand programmed cell death 1 ligand 1. Notably, lenalidomide also enhanced CAR T cytokine production, cytolytic activity, and activation profile relative to untreated CAR T cells in chronic stimulation assays. This unique potentiation of both

short-term CAR T activity and long-term functionality during chronic stimulation prompted investigation of the molecular profile of lenalidomide-treated CAR T cells. Signatures from RNA sequencing and assay for transposase-accessible chromatin using sequencing indicated that pathways associated with T-helper 1 response, cytokine production, T cell activation, cell-cycle control, and cytoskeletal remodeling were altered with lenalidomide. Finally, study of lenalidomide and anti-BCMA CAR T cells in a murine, disseminated, multiple myeloma model indicated that lenalidomide increased CAR T cell counts in blood and significantly prolonged animal survival. In summary, preclinical studies demonstrated that lenalidomide potentiated CAR T activity *in vivo* in low-antigen or suppressive environments and delayed onset of functional exhaustion. These results support further investigation of lenalidomide and anti-BCMA CAR T cells in the clinic.

## Introduction

Despite improvements in the treatment of newly diagnosed multiple myeloma, it remains uncured, and nearly all patients relapse and become resistant to available treatments (1). On the basis of the encouraging activity of chimeric antigen receptor (CAR) T cells targeting CD19 in non-Hodgkin lymphoma, CAR T cells targeting plasma cells expressing B-cell maturation antigen (BCMA) have been developed for multiple myeloma (2). Although BCMA CAR T cells have shown promise in the clinic, an immunosuppressive myeloma tumor microenvironment, including programmed cell death 1 ligand 1 (PD-L1) expression (3), and the potential for activation-induced exhaustion

may limit the durable responses of CAR T cells in some patients (4).

Lenalidomide is an immunomodulatory drug indicated for the treatment of multiple myeloma (5); it has pleiotropic effects that directly impair primary tumor growth and modulate the immunosuppressive tumor microenvironment to help facilitate a more robust antitumor inflammatory response (6, 7). Studies have shown that lenalidomide can directly increase *in vitro* T cell function, even in heavily treated patients with progressive disease, irrespective of immunomodulatory drug refractory status (8). Lenalidomide has also been shown to increase acute *in vitro* and *in vivo* CAR T cell functionality in several model systems with varying CAR constructs and indications (9, 10). For example, lenalidomide in combination with CS1-directed CAR T cells with a CD28z endodomain was associated with increased secretion of T-helper (Th) 1-associated cytokines, decreased secretion of Th2-associated cytokines, and increased survival in tumor-bearing mice (10). Additional studies are required to refine lenalidomide's mechanism of action and investigate its application in a chronic CAR T stimulation setting in conditions of varying antigen density or in the presence of inhibitory ligands such as PD-L1.

CAR T cells have unique functional properties that can be altered by the characteristics of the single-chain variable fragment, choice of transmembrane and endodomain, and manufacturing process, all of which determine CAR T fitness during long-term stimulation (11). In this study, we sought to characterize a novel

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anti-BCMA CAR construct and determine short- and long-term effects of lenalidomide on CAR T function. In addition, immunomodulatory drugs have been shown to directly induce CD28 tyrosine phosphorylation (12), suggesting that these drugs impinge on costimulatory signaling pathways; however, the role of a 41BBz endodomain during immunomodulatory drug application is unclear. To understand the complex nature of lenalidomide's mechanism of action, we applied RNA sequencing (RNA-seq) and assay for transposase-accessible chromatin using sequencing (ATAC-seq) technology to determine whether these functional differences were associated with changes in the regulatory networks involved in T cell function and activation. Finally, we examined concurrent and delayed administration of lenalidomide with a subcurative dose of anti-BCMA CAR T injection to further identify potential clinical applications and dosing strategies.

## Materials and methods

### *In vitro* cytolytic, cytokine, and flow cytometry CAR T assessment

T cells obtained from peripheral blood samples from consenting healthy adult donors and a patient with multiple myeloma refractory to pomalidomide were transduced to express a construct containing an extracellular BCMA-binding single-chain variable fragment and intracellular 41BBz endodomain (65%–76% CAR<sup>+</sup>, Supplementary Fig. S1A; Supplementary Table S1; Supplementary Methods). Human materials used in this research were received by the researchers in a fully deidentified manner from commercial repositories or under unrelated IRB-approved clinical studies from adults who consented to testing of their donated samples for future research purposes. Cultures were established with an effector-to-target ratio of 0.3:1 or 1:1 with OPM-2 or RPMI-8226 multiple myeloma target cells. Cocultures with increased effector cell counts relative to target cell counts results in rapid clearance of BCMA<sup>+</sup> target cells. Therefore, E:T ratios of 1.0 and 0.3 were selected to ensure that more CAR T cells would receive an activating stimulus for evaluation with lenalidomide. Lenalidomide (Sigma) was titrated across and above the clinical maximum concentration (1.9  $\mu\text{mol/L}$  for a 25-mg oral dose in patients with multiple myeloma) to assess the functional range of the drug (13). Cell-free supernatants were collected after 24 hours. Experiments were performed 2 to 3 times in 4 donors.

Unless noted, anti-BCMA CAR T cells were stimulated with 50  $\mu\text{g/mL}$  BCMA beads for the time indicated at a bead:CAR<sup>+</sup> T-cell ratio of 1:1. For PD-L1 experiments, 50  $\mu\text{g/mL}$  PD-L1 or control immunoglobulin G was coupled along with 50  $\mu\text{g/mL}$  BCMA. For prestimulation experiments, after 7 days of incubation, cells were debeaded, washed, and cocultured with RPMI-8226-NuLight Red target cell lines in the presence of 1  $\mu\text{mol/L}$  lenalidomide or vehicle control. Experiments were performed twice in 3 donors.

For surface phenotype analysis, anti-BCMA CAR T cells were cultured with BCMA beads for 7 days, stained with a live/dead dye (Invitrogen; Thermo Fisher Scientific), BCMA-fractured crystallizable (Fc), antibodies for surrogate CAR marker, CD3, CD4, CD8, CD25, PD-1, TIM3, and LAG3 (BD Biosciences) and then analyzed on a Fortessa flow cytometer (BD Biosciences). For intracellular cytokine staining, cells were stimulated on BCMA beads for 24 hours, with protein transport cocktail (BD Bio-

sciences) added in the final 4 hours of incubation. Cells were then stained with live/dead dye and surface markers (surrogate CAR marker, CD3, CD4, CD8, CD25, PD-1), fixed/permeabilized (BioLegend), and stained for intracellular IL2, IFN $\gamma$ , and TNF $\alpha$  (BioLegend). Experiments were performed twice in 3 donors.

### Serial stimulations

Anti-BCMA CAR T cells were plated with irradiated MM1.S target cells at an effector-to-target ratio of 1:2 in the presence of lenalidomide (0.1  $\mu\text{mol/L}$ ). Every 3 to 4 days, CAR<sup>+</sup> cells were enumerated, phenotyped by flow cytometry, and replated with freshly thawed and irradiated MM1.S target cells and lenalidomide. Twenty-four hours following replating on days 5, 8, and 15, cell-free supernatant was assessed for cytokine levels by Meso Scale Discovery. Following incubation on day 4, a sample was stained with a live/dead dye and panel of surface markers (EGFR, CD3, CD4, CD8). Fluorescently labeled count-bright beads were added to each sample to get the absolute counts of the total CD3<sup>+</sup>, Erb<sup>+</sup>, BCMA<sup>+</sup>, and CAR<sup>+</sup> cells. Replating was maintained for 28 days or until the cell count was <50,000 cells. Experiments were performed 3 times in 5 donors.

### RNA-seq and ATAC-seq assays

Anti-BCMA CART cells were cultured in the presence or absence of BCMA beads for either 24 hours or 7 days with or without 1  $\mu\text{mol/L}$  lenalidomide. Strand-specific, poly-A-selected RNA-seq reads were trimmed, mapped to hg38, and quantified using ArrayStudio (OmicSoft). ATAC-seq was performed according to published methodology (See Supplementary Methods; ref. 14). Experiments were performed twice in 3 and 4 donors.

### *In vivo* OPM-2 tumor model

All animal studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee. NOD.Cg-Prkdc<sup>scid</sup>IL-2rg<sup>tm1Wjl</sup>/SzJ mice (NSG; The Jackson Laboratory) were injected intravenously with  $2 \times 10^6$  OPM-2/luciferase cells and allowed to engraft for 14 days prior to intravenous CART infusion. One day prior to or 14 days following injection with  $1 \times 10^6$  CART or mock control T cells, animals were dosed (intraperitoneally) daily for 50 days with 10 mg/kg lenalidomide in PBS. Blood was collected for quantitation of circulating CAR T cells and cells were stained with antibodies to exclude mouse-specific cells (H-2kd, TER-119, and muCD45) and analyzed by flow cytometry (See Supplementary Methods).

### Statistical analysis

Linear fixed-effect or mixed-effect models (15), a more flexible technique related to more traditional nested or repeated measures ANOVA methods, were used to assess the significance of lenalidomide treatments on cytolytic activity and cytokine production, with treatment, and time treated as fixed effects and animal and donor treated as random effects, nested with time when repeated measurements were derived from the same animal. *P* values were obtained by likelihood ratio tests comparing the full model with the effect of interest against the model without the effect of interest. Flow cytometry median fluorescence intensity values were log<sub>2</sub> transformed and bioluminescence values were log<sub>10</sub> transformed to better approximate normality. Survival analyses were performed (16) with log-rank testing to determine the significance of the effect of lenalidomide treatments on survival curves.

## Results

### Anti-BCMA CAR T function is enhanced by lenalidomide in the presence of BCMA-expressing myeloma cell lines

Cytolytic activity and cytokine production of lenalidomide-treated CAR T cells transduced with the anti-BCMA CAR were evaluated *in vitro* in the presence of BCMA-expressing multiple myeloma cell lines with varying sensitivity to lenalidomide (Supplementary Methods). Multiple donors were assessed to evaluate donor-dependent effects of lenalidomide, including CAR T product manufactured from an immunomodulatory drug-refractory patient. An additional scfv was tested in the presence of lenalidomide to confirm the effects were not binder-specific (Supplementary Fig. S1B). Increased anti-BCMA CAR T cytolytic activity against OPM-2 target cells titrated with increased concentrations of lenalidomide across all donors at the 0.3:1 E:T ratio ( $P = 6.2 \times 10^{-5}$ ; Fig. 1A). Donor 1 and 2 demonstrated the least efficient target cell killing for OPM-2 cells (Supplementary Fig. S1C) and had increased activity with lenalidomide at a 1:1 ratio (Supplementary Fig. S1D). In addition, all CAR T donors (baseline shown in Supplementary Fig. S1E) had significantly increased IFN $\gamma$ , IL2, and TNF $\alpha$  production in a lenalidomide concentration-dependent manner upon coculture with OPM-2 cells ( $P < 0.002$ ; Fig. 1B). The treatment effect of lenalidomide on CAR T cytolytic activity appeared to be donor dependent in coculture with RPMI-8226 (Fig. 1C), with the patient donor showing a significant increase in cytolytic activity ( $P = 1.9 \times 10^{-8}$ ). Notably, cytokine production by CAR T cells in RPMI-8226 coculture (baseline shown Supplementary Fig. S1F) was significantly increased across all donors and cytokines upon treatment with lenalidomide ( $P < 0.003$ ; Fig. 1D). Importantly, addition of lenalidomide did not alter BCMA target expression (Supplementary Fig. S2). These results demonstrated that when anti-BCMA CAR T cells were stimulated with 0.3:1 and 1:1 effector-to-target ratios, addition of lenalidomide increased effector functionality of CAR T cells across several metrics of CAR T function, including cytolytic activity and cytokine production.

### Lenalidomide potentiation of anti-BCMA CAR T cytokine expression is dependent on stimulation strength

The CAR T-intrinsic treatment effects of lenalidomide on cytokine production for the CD4 $^{+}$  and CD8 $^{+}$  CAR T populations were next evaluated in the absence of target cells. As lenalidomide can directly limit multiple myeloma cell viability, a CAR-specific stimulation reagent of recombinant human BCMA was used to assess the direct effect of lenalidomide on activated CAR T cells in the absence of BCMA-expressing target cells. To this end, recombinant human BCMA-labeled beads were developed to stimulate CAR T cells and provide the means to titrate both the magnitude of stimulation [low (5  $\mu\text{g}/\text{mL}$ ), medium (50  $\mu\text{g}/\text{mL}$ ), and high (200  $\mu\text{g}/\text{mL}$ )] and the concentration of lenalidomide (0.1 and 1.0  $\mu\text{mol}/\text{L}$ ). At a medium stimulation condition, we measured secreted cytokine production and observed a mean 200% increase in IL2 and TNF $\alpha$  concentrations compared with vehicle control, with donor-dependent increases in IFN $\gamma$  (Supplementary Fig. S3). Anti-BCMA CAR T cells activated with BCMA beads showed stimulation level-dependent effects, with the low, 5- $\mu\text{g}$  BCMA beads causing lower CAR T CD25 expression and intracellular IFN $\gamma$  cytokine staining compared with medium- (50  $\mu\text{g}$ ) and high- (200  $\mu\text{g}$ ) BCMA beads (Fig. 2A and B, left). Lenalidomide significantly ( $P < 0.05$ ) increased the percentage of IFN $\gamma^{+}$  and

TNF $\alpha^{+}$  intracellular staining at multiple stimulation levels for both CD4 $^{+}$  (Fig. 2A) and CD8 $^{+}$  (Fig. 2B) CAR T cells. In the absence of stimulation, lenalidomide had no effect on CAR T cytokine staining, indicating that cytokine enhancement provided by lenalidomide requires stimulation.

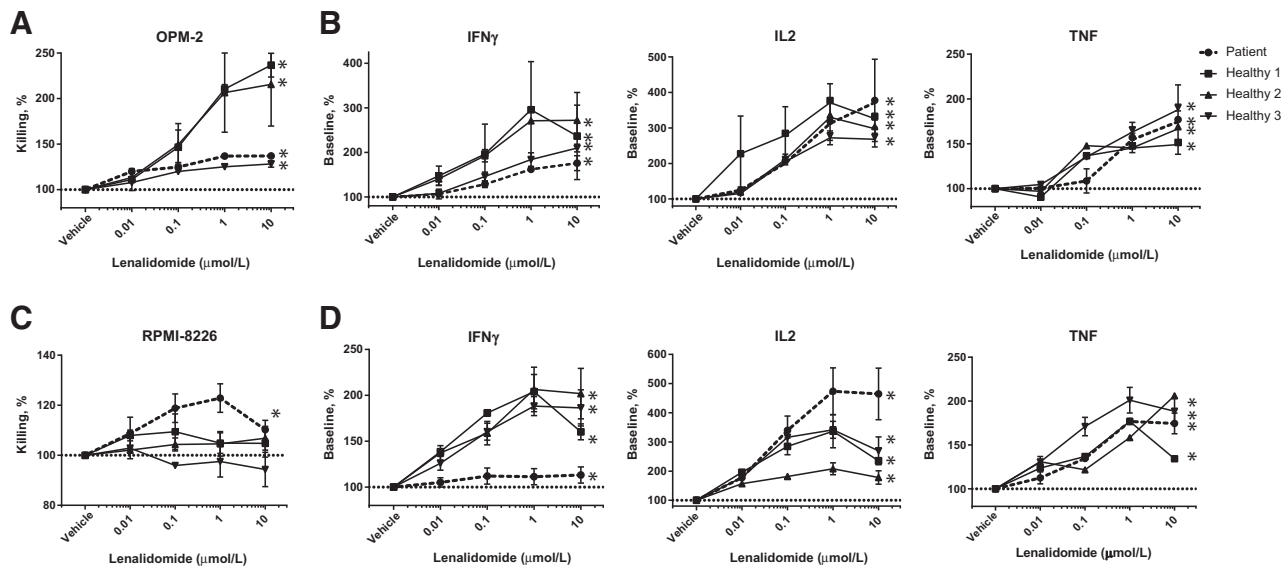
Inhibitory receptors can alter T cell receptor-mediated activation and limit the effector functionality of T cells (17). We explored whether the lenalidomide-induced potentiation of CAR T activation and cytokine production could override PD-L1-mediated inhibition. Evaluation of both healthy and patient donor CAR T cells demonstrated that addition of recombinant PD-L1 to recombinant BCMA beads significantly reduced IFN $\gamma$ , IL2, and TNF $\alpha$  levels ( $P < 0.006$ ; Fig. 2C and D). Importantly, lenalidomide treatment potentiated secreted cytokine levels beyond those from CAR T cells treated with vehicle in the presence of PD-L1 ( $P < 0.007$ ).

### Anti-BCMA CAR T function during serial and chronic stimulation was prolonged by lenalidomide

Previous studies indicate that performance in a serial stimulation assay may represent CAR T fitness and *in vivo* efficacy (18). Notably, after repeated stimulation with MM1.S target cells, lenalidomide increased CAR T expansion on average by 0.82 population doublings over 28 days across 5 donors relative to controls ( $P = 2.8 \times 10^{-8}$ ; Fig. 3A). Multiple samples at intermediate time points were collected to assess cytokine production during serial stimulation until a donor had insufficient cells to continue the assay in the vehicle-treated group. Increased cell counts were associated with a significant increase in IL2, IFN $\gamma$ , and TNF $\alpha$  production per cell in the media ( $P < 1.2 \times 10^{-9}$ ; Fig. 3B–D).

We also developed a novel long-term chronic stimulation assay designed to diminish anti-BCMA CAR T effector function. We observed a limited increase in BCMA CAR T cytolytic activity against RPMI-8226 cells in the presence of lenalidomide in an acute assay (Fig. 1); however, CAR T prestimulation appears to exhaust the cells and results in decreased functionality. Prestimulated CAR T cells showed decreased cytolytic activity ( $P = 2.1 \times 10^{-4}$ ) and IFN $\gamma$  cytokine production ( $P = 0.03$ ) compared with freshly thawed anti-BCMA CAR T cells (Fig. 4A), indicating that chronic prestimulation leads to functional impairment. CAR T cells were also prestimulated with BCMA beads with 1  $\mu\text{mol}/\text{L}$  lenalidomide prior to analysis of cytolytic activity and cytokine production. Notably, the presence of lenalidomide during the prestimulation period preserved cytolytic function ( $P = 0.04$ ), and a trend was observed toward increased cytokine production compared with cells exposed to vehicle during the prestimulation period (Fig. 4B–D).

The phenotype of anti-BCMA CAR T cells stimulated for 7 days with BCMA beads was assessed, and the addition of lenalidomide significantly increased CAR $^{+}$  viability of anti-BCMA CAR T material across 3 healthy donors ( $P = 0.04$ ; Fig. 4E). The addition of lenalidomide did not alter the total cell count across all donors (Fig. 4E) in this 7-day period, and no significant differences were observed in percentage CAR $^{+}$  between vehicle- and lenalidomide-treated CAR T cells or among CAR $^{+}$  cells in memory subtypes by classification with CD45RA or CD27 by CCR7 (Supplementary Fig. S4). Flow cytometric analysis across CAR T donors indicated that the addition of lenalidomide functionally altered the balance between activation and immunoregulatory markers by increasing the surface expression of TIM3 in the CD8 $^{+}$  population ( $P = 4.0 \times$



**Figure 1.**

Anti-BCMA CAR T cytolytic activity and cytokine production increased with lenalidomide in a concentration-dependent manner. Anti-BCMA CAR T materials from 3 healthy donors and 1 patient donor were assessed for cytokine production using 2 multiple myeloma cell lines. Cytolytic activity (**A** and **C**) and cytokine production were measured after 24 hours (**B** and **D**) against OPM-2 (**A** and **B**) and RPMI-8226 (**C** and **D**). Cultures were incubated at a ratio of 0.3:1 (effector to target) for cytolytic activity and 1:1 for cytokine production. Data were normalized to DMSO vehicle control; error bars, SEM. For all functional assessments, dose response modeling indicated a significant effect for lenalidomide for each donor and across all donors ( $P < 0.001$ ), except healthy donors for RPMI-8226 cytolytic assay. \*,  $P < 0.05$  for lenalidomide across each donor.

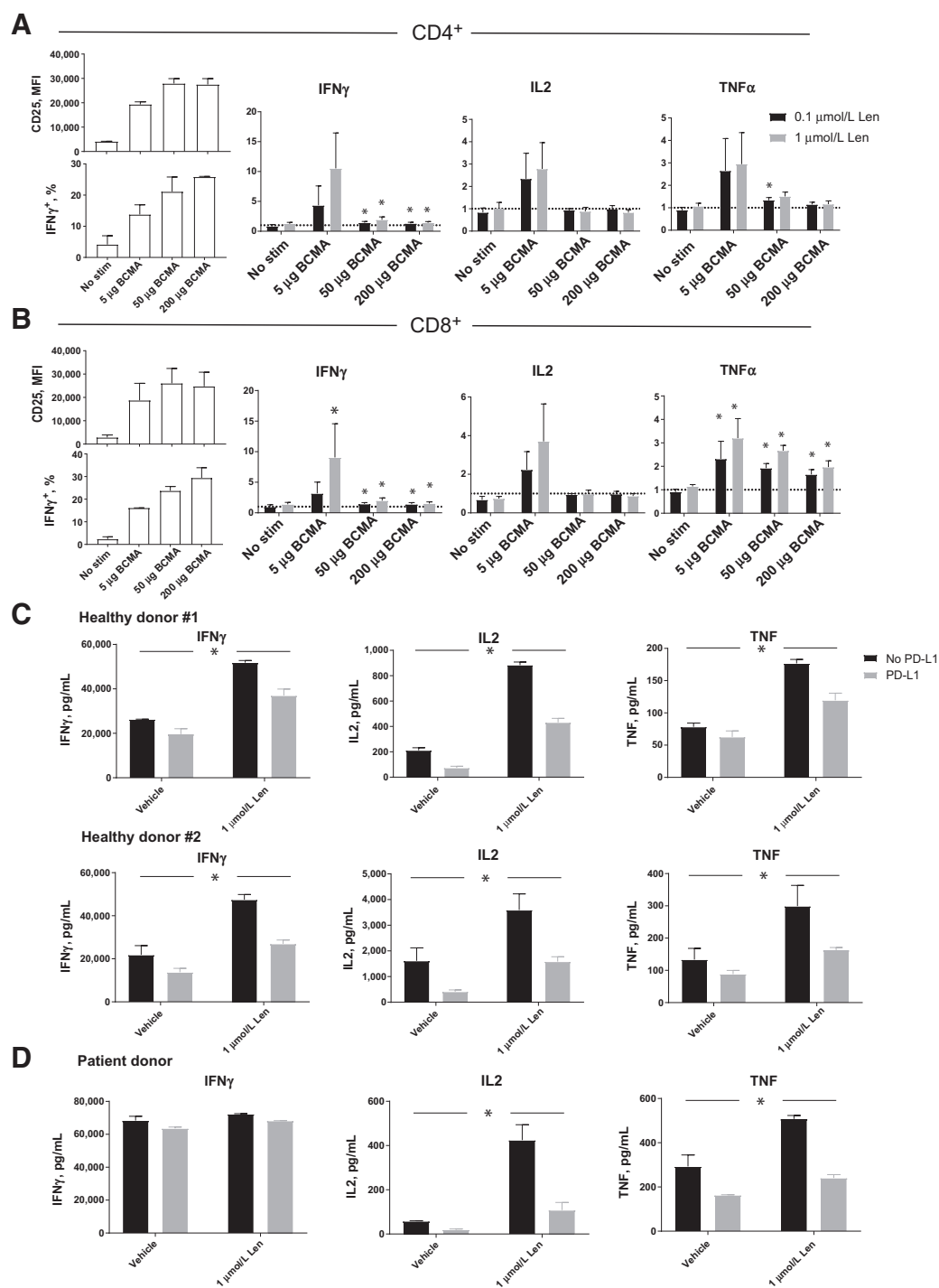
$10^{-4}$ ), with mixed effects on the  $CD4^+$  CAR $^+$  population (Fig. 4F). Across all donors and in both the  $CD4^+$  and  $CD8^+$  CAR $^+$  populations, lenalidomide increased CD25 ( $CD4^+$  and  $CD8^+$ ;  $P = 2.2 \times 10^{-16}$ ) and the percentage positive for LAG3 expression ( $CD8^+$   $P < 0.03$ ;  $CD4^+$   $P = 0.002$ ). Notably, a decrease in the percentage of PD-1 $^+$  cells was also observed in the  $CD4^+$  population ( $P = 0.04$ ), with 2 of 3 donors showing a decrease in the  $CD8^+$  population as well.

#### Anti-BCMA CAR T RNA-seq and ATAC-seq profiles were altered by lenalidomide after short-term and chronic stimulation

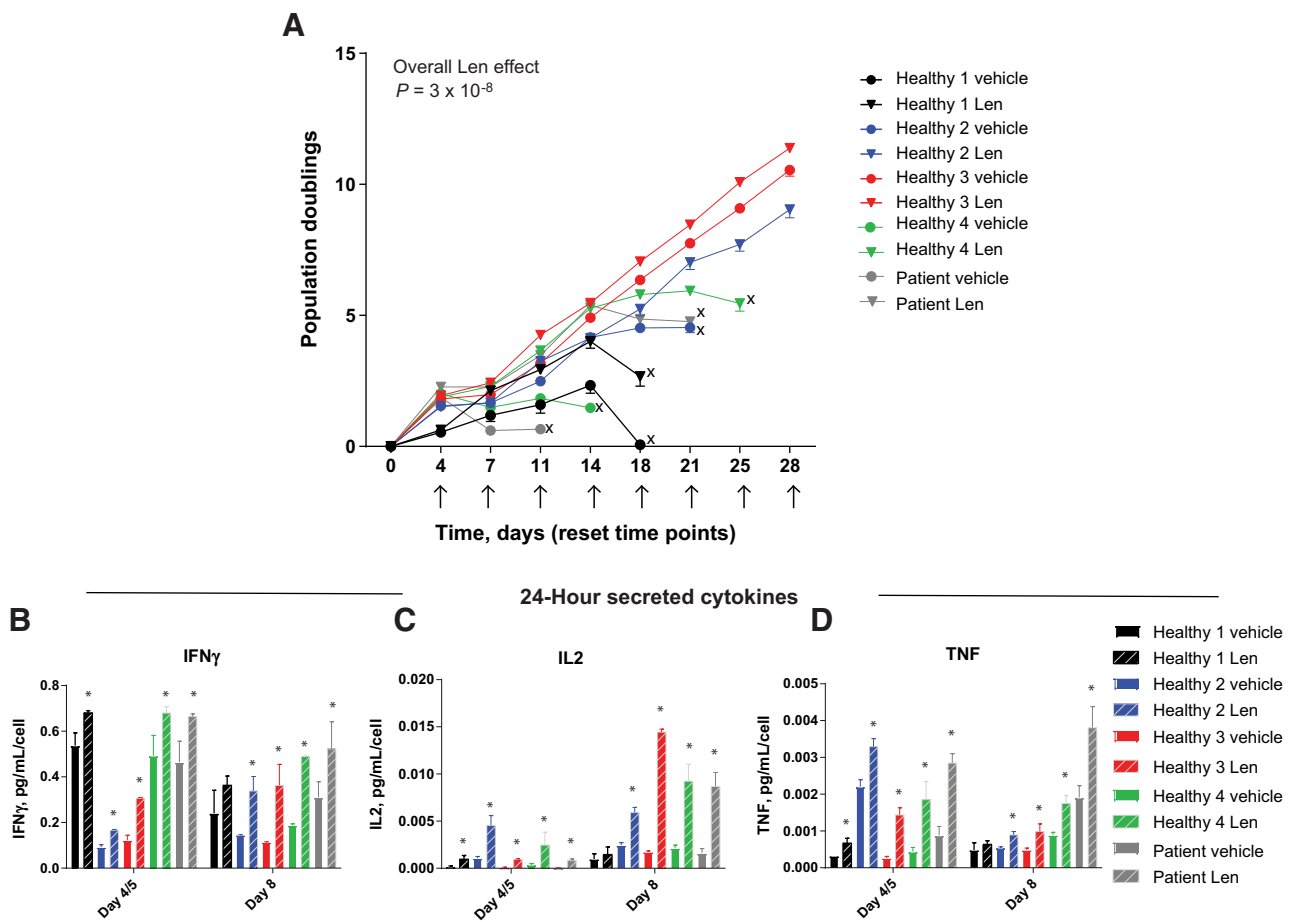
Because few phenotypic changes were noted by FACS following the addition of lenalidomide in the context of antigen-specific stimulation, we decided to employ unbiased transcriptomic and epigenomic analyses to further assess features that could underlie the enhanced functionality. Investigation of the molecular signature of lenalidomide-treated anti-BCMA CAR T cells was assessed following short-term (24-hour) or chronic (7-day) stimulation, as described above. Principal component analysis demonstrated clustering based on stimulation (stimulation or no stimulation) and time (24 hours or 7 days) for both the RNA-seq (Fig. 5A; GSE113281) and ATAC-seq (Fig. 5C; GSE113853) datasets. Next, we examined the role of lenalidomide after 24 hours or 7 days of stimulation after accounting for donor-to-donor variability. RNA-seq analysis showed alteration of a small set of genes (214) at 24 hours and a larger number of genes (583) changed after 7 days of stimulation in the presence of lenalidomide (Fig. 5B). Notably, ATAC-seq analysis revealed a limited set of chromatin accessibility changes associated with lenalidomide treatment after 24 hours of stimulation, with a dramatic change in profile and an increase in the number of sites with changes in chromatin

accessibility after 7 days of stimulation in the presence of lenalidomide (Fig. 5D). To further identify specific transcriptional changes associated with lenalidomide treatment, gene ontology analysis was applied to the RNA-seq dataset. Pathways associated with T cell chemotaxis (leukocyte extravasation, integrin, integrin-linked kinase, and C-X-C motif chemokine receptor 4-associated gene sets), intracellular signaling, and cytoskeleton (Rac/Rho/Cdc42) were upregulated in the presence of lenalidomide within 24 hours of stimulation compared with vehicle controls (Fig. 5E). These data support an increase in inducible costimulator (ICOS)-related signaling pathways, a finding that is in line with previous publications demonstrating an increase in ICOS and ICOS ligand in the  $CD3^+$  population of peripheral blood mononuclear cells treated with lenalidomide *ex vivo* (19). After 7 days of stimulation, lenalidomide upregulated pathways associated with Th1 T cell response and costimulation while decreasing Th2-associated gene signatures (Fig. 5F).

To determine whether chromatin accessibility correlated with the transcriptional changes observed during lenalidomide treatment, we integrated the ATAC-seq and RNA-seq data from our chronic 7-day analysis (Fig. 5G). Across donors, we observed a significant increase in chromatin accessibility across multiple loci, including those associated with IFN $\gamma$  and IL2RA (CD25), and these changes were correlated with a significant increase in transcription. Importantly, the upregulation of IFN $\gamma$  and CD25 were concordant with previous findings from chronic stimulation experiments where lenalidomide treatment resulted in significantly higher proportions of cells expressing these markers. We also observed a decrease in CD69 and CCR7 chromatin accessibility and gene transcription on lenalidomide treatment. Finally, we analyzed the ATAC-seq dataset for motif enrichment and



**Figure 2.** Anti-BCMA CAR T cytokine production was increased by lenalidomide (Len) within 24 hours and across a range of stimulation intensities. Analysis of CD25 and intracellular cytokine levels (left, white bars indicate baseline effects of bead stimulation) for healthy CAR T donors after 24 hours of BCMA bead stimulation (gated on transduced live CD3<sup>+</sup> CAR<sup>+</sup>) for CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) subsets. Gray bars demonstrate relative change for (Len) compared with vehicle alone. C and D, Analysis of effector cytokine production following CAR-specific stimulation on 50  $\mu$ g BCMA and 50  $\mu$ g PD-L1 beads for 24 hours in the presence of 1  $\mu$ mol/L Len. \*,  $P < 0.05$  effect of Len for each stimulation condition.



**Figure 3.**

Anti-BCMA CAR T cell count, cytokine production, and activation were increased by lenalidomide after repeated stimulation *in vitro*. **A**, Analysis of cell counts following serial stimulation in an MM1.S cell line in the presence or absence of 0.1  $\mu\text{mol/L}$  Len. Data represent population doublings across 5 donors; error bars, SDs across 3 technical replicates. Linear mixed effects modeling indicated a significant effect for Len for each donor across time ( $P = 2.8 \times 10^{-8}$ ). **B-D**, Analysis of bulk cytokine production 24 hours following serial stimulation replating at the indicated time points for 5 separate donors. Cytokine production was normalized to cell number at each reset to account for differences in cell replating density; error bars, SDs. Linear-mixed models indicated a significant effect for Len across donors and time points for IFN $\gamma$  ( $P < 2.9 \times 10^{-10}$ ), IL2 ( $P = 1.3 \times 10^{-13}$ ), and TNF $\alpha$  ( $P = 1.2 \times 10^{-9}$ ). \*,  $P < 0.05$  compared with vehicle for each donor.

observed enrichment for a number of motifs bound by multiple factors associated with T cell activation, including AP-1/Jun and nuclear factor  $\kappa\text{B}$  (Fig. 5H; ref. 20).

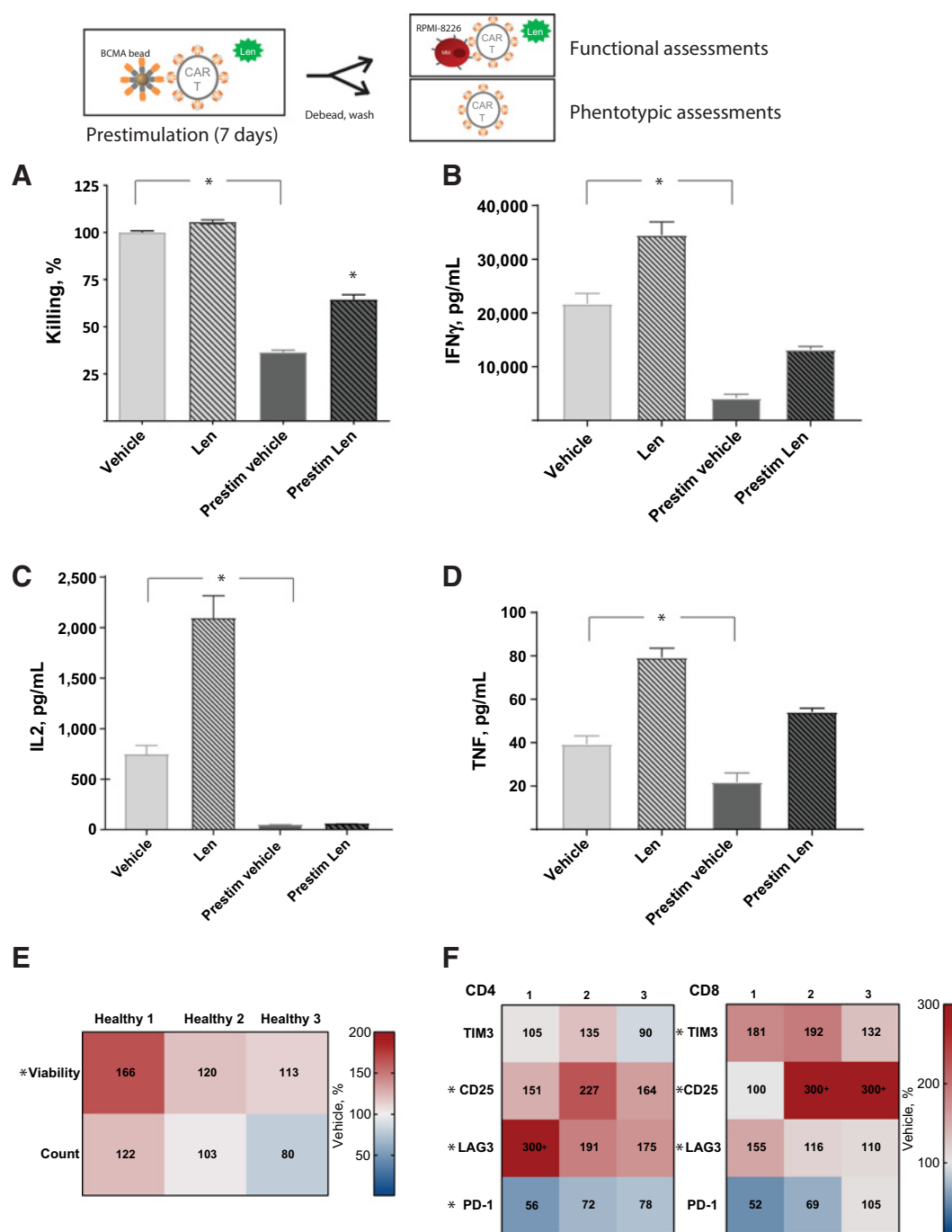
#### Subcurative dose of anti-BCMA CAR T demonstrated improved tumor clearance and survival *in vivo* when concurrently dosed with lenalidomide

Finally, to assess *in vivo* CAR T function by lenalidomide, mice implanted with OPM-2 tumors were dosed with a subcurative dose of anti-BCMA CAR T cells. Mice with established tumors were dosed daily with lenalidomide 1 day prior or 14 days following injection with a subcurative dose of  $1 \times 10^6$  anti-BCMA CAR T cells (Fig. 6A). The addition of concurrent lenalidomide led to a significant decrease in tumor burden for donor 1 ( $P = 0.02$ ) and increased survival for donor 1 ( $P = 0.057$ ) and donor 2 ( $P = 0.04$ ) compared with vehicle-treated animals injected with anti-BCMA CAR T alone (Fig. 6B-E). Animals on the concurrent lenalidomide dosing regimen also showed increased CAR T counts in the peripheral blood after 7 days ( $P = 7.3 \times 10^{-6}$ ) but not at later time points (Fig. 6F and G).

Lenalidomide had a small but significant mock CAR T effect on tumor burden for donor 1 alone ( $P = 0.003$ ). The addition of delayed dosing of lenalidomide did not improve tumor clearance and survival for either CAR T donor, suggesting that the benefit of this combinatorial approach relied on concurrent administration.

#### Discussion

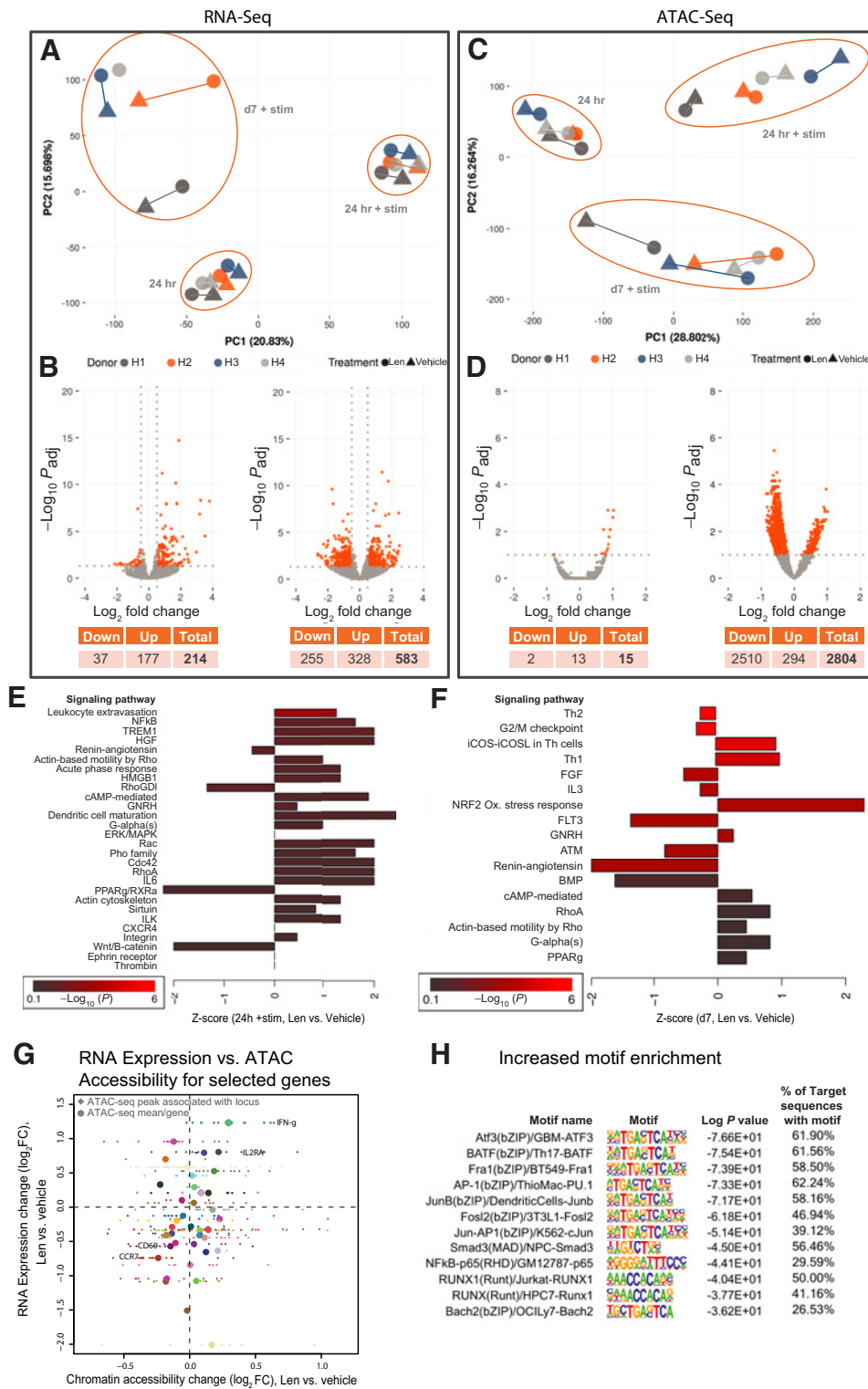
Our studies further explore the mechanism of action and applications of lenalidomide in combination with CAR T cells. In anti-BCMA CAR T cells with a 41BB/CD3z-containing endodomain, we observed a rapid increase in cytokine production following stimulation with either myeloma target cells or direct CAR stimulation via antigen-coated beads. Notably, lenalidomide increased cytokine production and activation across multiple assays at a clinically relevant concentration, indicating an increase in effector function. In addition, an immunomodulatory drug-refractory sample derived from a patient with multiple myeloma also demonstrated increased *in vitro* functionality in the presence of lenalidomide, indicating that refractory tumor



**Figure 4.**

Lenalidomide reduced functional exhaustion and altered surface phenotype of anti-BCMA CAR T cells. Cells were treated for 7 days on 50  $\mu$ g BCMA-coated beads in the presence or absence of 1  $\mu$ mol/L Len. **A–D**, Representative healthy donor–derived, freshly thawed anti-BCMA CAR T cells [vehicle (Veh), Len] or CAR T cells prestimulated with 7 days of BCMA bead stimulation and then cultured with RPMI-8226 cells to measure cytolytic activity (over 7 days; **A**) and cytokine production (24 hours; **B–D**). Percentage killing was normalized to anti-BCMA CAR T cells prestimulated on beads in the presence of vehicle. Prestimulated CAR T cells showed decreased cytolytic activity ( $P = 2.1 \times 10^{-4}$ ) and cytokine production ( $P = 0.03$  for IFN $\gamma$ ) compared with freshly thawed anti-BCMA CAR T cells. Len during the prestimulation period increased cytolytic function ( $P = 0.04$ ). Significance was determined using *t* tests from linear regression coefficients. Three anti-BCMA CAR T donors (each column) were assessed for overall viability (**E**) and cell count and by flow cytometry (**F**) for median fluorescence intensity (MFI; CD25 and TIM3) or percentage positive PD-1 and LAG3 on the surface of T cell markers in CD4<sup>+</sup> CAR<sup>+</sup> and CD8<sup>+</sup> CAR<sup>+</sup> subsets (gated on live CD3<sup>+</sup> cells). Values shown are percentage baseline (Veh) MFI, viability, or count. \*,  $P < 0.05$ .



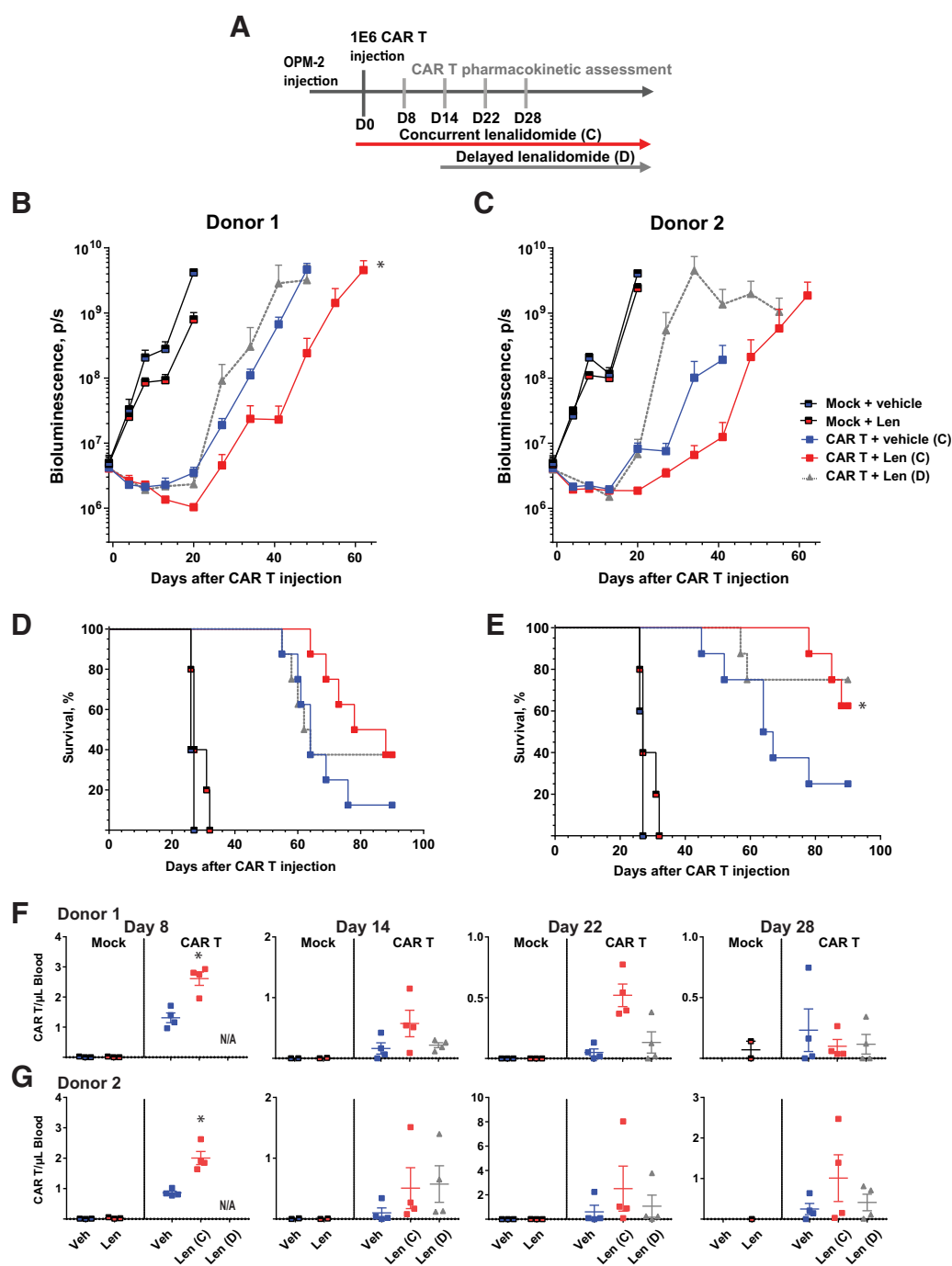


**Figure 5.**

Anti-BCMA CAR T RNA-seq and ATAC-seq profiles were altered by lenalidomide after short- and long-term stimulation. **A** and **C**, Principal component analysis of expression (**A**; RNA-seq) and chromatin accessibility peaks (**C**; ATAC-seq). **B** and **D**, Volcano plots of differentially expressed genes (**B**) or peaks  $\pm$  Len at 24 hours and 7 days (**D**). Directionality and significance of expression changes in selected, enriched biological pathways at 24 hours (**E**) and 7 days (**F**) in CAR T cells  $\pm$  1  $\mu$ M/L Len. **G**, RNA expression compared with chromatin accessibility changes for selected T cell loci. **H**, Top enriched motif predictions in ATAC-seq loci with increased accessibility along with their enrichment significance and prevalence at 7 days  $\pm$  1  $\mu$ M/L Len. FC, fold change; Ox, oxidative.

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**Figure 6.**

*In vivo* efficacy of subcurative dose of anti-BCMA CAR T and blood anti-BCMA CAR T count was altered by lenalidomide. **A**, Two Len dosing regimens, concurrent (C) or delayed (D) daily dosing, were tested in a disseminated NSG mouse OPM-2 tumor model with a single, subcurative dose of anti-BCMA CAR T cells from 2 separate donors (8 mice per group). **B–E**, Tumor bioluminescent measurement (**B** and **C**) and animal survival (**D** and **E**). Error bars, SEM. Concurrent Len led to a significant decrease in tumor burden for donor 1 ( $P = 0.02$ ) and increased survival (log-rank test) for donor 1 ( $P = 0.057$ ) and donor 2 ( $P = 0.04$ ) compared with vehicle (Veh)-treated animals injected with anti-BCMA CAR T alone. Linear mixed-effects models (accounting for repeated mouse measurements over time) were used to estimate treatment effects for the tumor burden analysis, and log-rank testing was used for significance testing for the survival analyses. **F** and **G**, Flow cytometric analysis of blood CAR T cells gated on CD45<sup>+</sup> CD3<sup>+</sup> CAR<sup>+</sup>. Error bars, SEM. Concurrent Len showed significantly increased CAR T expansion after 7 days *in vivo* ( $P = 7.3 \times 10^{-6}$ , *t* test). \*,  $P < 0.05$  for concurrent lenalidomide compared with vehicle control.

status may be independent of the effects of lenalidomide on CAR T cells. Previous studies demonstrated that increased T cell cytokine production associated with lenalidomide was partly due to the degradation of the transcription factors Ikaros and Aiolos by Cereblon (21). In addition, Ikaros has been shown to alter the threshold for activation of T cells downstream of T cell receptor and IL2 receptor signaling as well as protein kinase C, PI3K, and calcineurin signaling (22). Furthermore, our studies support the hypothesis of immunomodulatory drug–lowered activation threshold in functional assays controlling CAR stimulation. We also demonstrated that, in the presence of PD-L1 engagement, the addition of lenalidomide potentiated cytokine production beyond control levels, suggesting that this combinatorial approach may override suppressive inputs from the microenvironment to sustain antitumor functionality.

Chronic and serial stimulation assays may recapitulate the repeated stimulation to which a CAR T cell is exposed in patients' tumors and allow for examination of an exhaustion-like state of the CAR T cells. Our functional data support a prolonged period for CAR T cell cytolytic activity and cytokine production in the presence of lenalidomide. Prolonged lenalidomide treatment period increased IL2 across serial stimulation and chronic stimulation assays. The increased IL2 production over time may provide a mechanism to sustain T cell effector function over chronic stimulation; this mechanism agrees with previous studies demonstrating that low IL2 production by CAR T cells was associated with exhaustion (23). Exposure to lenalidomide in chronic or serial stimulation assays also resulted in increased TNF $\alpha$  and IFN $\gamma$ , increased viability, and decreased PD-1 expression on the CAR T cell surface. Notably, characterization of CD19-directed CAR T cells determined that the PD-1–negative CAR T population was associated with therapeutic response (24). These results were observed in the presence of increased TIM3 and LAG3, markers previously shown to be associated with T cell exhaustion (25). The duality of these exhaustion-associated markers may indicate that the addition of lenalidomide to CAR T cells leads to an alternative differentiation or activation state outside the canonical states of T cell differentiation. In other words, lenalidomide may affect these "exhaustion" signaling pathways independently of PD-1, or, alternatively, these markers are not indicative of functional exhaustion in these anti-BCMA CAR T cells. In support of these data, previous studies have shown that a dissociation between surface markers and functional assessment of exhaustion and molecular dissection of cell state may be more informative (26).

In addition to functional assays, the RNA- and ATAC-seq studies resulted in a number of insights into possible mechanisms for lenalidomide-induced increases in CAR T function. First, the number of transcriptional and chromatin accessibility changes associated with stimulation and time were predominant compared with the effects of lenalidomide, indicating a relatively subtle effect of lenalidomide on transcriptional networks. Second, the changes associated with lenalidomide were broad, including early changes in transcripts associated with cytoskeletal remodeling and chemotaxis. After chronic stimulation, a distinct transcriptional signature emerged that included a decrease in transcripts associated with the Th2 response, G<sub>2</sub>–M checkpoint, and ATM along with an increase in Th1, peroxisome proliferator-activated receptor  $\gamma$ , and actin cytoskeleton-associated genes. These effects may support a role for lenalidomide treatment and cell-cycle control and T cell activation (27). Previous studies have

also demonstrated the effects of immunomodulatory drugs on Th1- and Th2-associated signatures as well as changes in elements associated with cytoskeletal remodeling and T cell migration (10, 28). The demonstrated early alterations in cytokine production by lenalidomide may contribute to an altered T cell state that is able to simultaneously enhance aspects of both memory and effector function (29). Overall, these results suggest that additional factors beyond those previously reported are involved in the lenalidomide-induced prolongation of CAR T function, including possible changes in cell-cycle control.

The application of ATAC-seq provided further insights into potential mechanisms of action of lenalidomide. Although both stimulation and time were the predominant drivers of chromatin accessibility changes, lenalidomide treatment was associated with increases in chromatin accessibility in loci enriched in motifs associated with T cell activation and function after chronic stimulation. These epigenetic changes were coincident with the marked functional changes in CAR T cells incubated with lenalidomide. Alterations in chromatin accessibility signatures have been associated with T cell exhaustion and may be a more robust indicator of exhaustion compared with T cell surface ligand expression (30). These data demonstrated that chronic stimulation with lenalidomide resulted in increased chromatin accessibility and gene expression of IL2 and CD25 and decreased gene expression and chromatin accessibility of CCR7 and CD69. Previous studies suggested that CCR7-expressing cells produced higher levels of IL2 (31); however, the current study indicates that the IL2 pathway could be altered independently by lenalidomide, resulting in an alternative T cell state. CD69, a marker of T cell activation, has a NF- $\kappa$ B-responsive element that is required for the CD69 response to TNF $\alpha$  (32). The closing of CD69-associated chromatin and decrease in transcripts may be a reaction to sustained increases in TNF $\alpha$  production by CAR T cells cultured with lenalidomide, or it may be a T cell response to increased activation in the presence of lenalidomide. Lenalidomide-treated cells demonstrated increased transcription factor motif enrichment of T cell activation-associated factors, supporting the idea that these cells are exposed to sustained activation signaling. Overall, the lenalidomide-induced CAR T cell state has elements of both effector T cell function, including increased IFN $\gamma$  and TNF $\alpha$  production, and memory T cell function, including increased IL2 and long-term proliferation. Additional studies are underway to determine more about the functional consequences of this alternative CAR T cell state and the associated gene expression and epigenetic changes.

Finally, we observed an increase in function by a subcurative dose of anti-BCMA CAR T cells in the OPM-2 orthotopic animal model with the addition of lenalidomide. Early pharmacokinetic measurements indicated an increase in CAR T counts in the blood, which were associated with improved tumor control and survival. The combination of increased CAR T pharmacokinetic and increased functionality of CAR T cells, as observed in *in vitro* studies, may have led to improved control over tumor growth following a subcurative dose of CAR T cells. Because lenalidomide may also be applied in a delayed administration setting, possibly weeks after CAR T administration due to toxicity challenges with lymphodepletion and immunomodulatory drugs, we investigated the feasibility of delayed lenalidomide administration. Interestingly, the addition of lenalidomide following peak CAR T expansion at 14 days did not result in improved tumor clearance or survival. These results suggest

several possibilities. First, the CAR T cells may have been functionally exhausted at 14 days following injection and were unable to be enhanced by delayed lenalidomide administration. Second, the improved tumor clearance was observed because of early CAR T function and circulating numbers, and tumor clearance cannot be improved or rescued at such a delayed progression. Additional studies should be undertaken to determine whether a window for delayed administration of lenalidomide with CAR T cells exists that is more proximal to CAR T administration.

In summary, these studies provide novel insights into the mechanism of functional changes in CAR T cells following lenalidomide addition *in vitro* and *in vivo*. The changes associated with lenalidomide were intrinsic to anti-BCMA T cells because precise control of CAR stimulation alone in the presence of lenalidomide led to increased functionality, particularly at lower levels of stimulation. In addition, the transcriptional and epigenetic changes associated with lenalidomide treatment suggest that an alternative CART cell state of both enhanced memory and effector T cell functions is induced with long-term lenalidomide treatment. Overall, the administration of lenalidomide, a standard of care for patients with multiple myeloma, in combination with anti-BCMA CAR T in the clinic may be warranted on the basis of the potential for a combination of effects, including tumoricidal effects, a more permissive tumor microenvironment for CAR T function, and the observed intrinsic effects on CAR T function.

#### Disclosure of Potential Conflicts of Interest

M. Works has ownership interest (including patents) in Celgene. N. Soni has ownership interest (including patents) in Celgene. C. Hauskins has ownership interest (including patents) in Celgene. C. Sierra has ownership interest (including patents) in Celgene. A. Baturevych has ownership interest (including patents) in Celgene. J.C. Jones has ownership interest (including patents) in Celgene. W. Curtis has ownership interest (including patents) in Juno. P. Carlson has ownership interest (including patents) in Juno/Celgene stock. T.G. Johnstone is a senior data scientist at and has ownership interest (including patents) in Celgene. D. Kugler has ownership interest (including patents) in Celgene. R.J. Hause is an associate director at and has ownership interest (including patents) in Juno Therapeutics. L. Wimberly has ownership interest

(including patents) in Celgene. C.R. Clouser has ownership interest (including patents) in Celgene. H.K. Jessup has ownership interest (including patents) in Celgene. R.A. Salmon has ownership interest (including patents) in Juno Therapeutics, a Celgene company. M.O. Ports has ownership interest (including patents) in Celgene. No potential conflicts of interest were disclosed by the other authors.

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