Antagonism of IAPs Enhances CAR T-cell Efficacy

Jessica Michie, Paul A. Beavis, Andrew J. Freeman, Stephin J. Vervoort, Kelly M. Ramsbottom, Vignesh Narasimhan, Emily J. Lelliott, Najoua Lalaoui, Robert G. Ramsay, Ricky W. Johnstone, John Silke, Phillip K. Darcy, Ilia Voskoboinik, Conor J. Kearney, and Jane Oliaro

Abstract

Chimeric antigen receptor (CAR) T-cell therapy has proven successful in the treatment of hematological malignancies, notably acute lymphoblastic leukemia and B-cell lymphoma. However, the efficacy of CAR T cells against solid tumors is poor, likely due to tumor-associated immunosuppression. Here, we demonstrated that antagonizing the “inhibitor of apoptosis proteins” with the clinical smac-mimetic, birinapant, significantly enhanced the antitumor activity of CAR T cells in a tumor necrosis factor (TNF)-dependent manner. Enhanced tumor cell death occurred independently of the perforin-mediated granule exocytosis pathway, underscoring the cytotoxic potential of CAR T-cell–derived TNF. Combining CAR T-cell therapy with birinapant significantly reduced established tumor growth in vivo, where either therapy alone was relatively ineffective. Using patient biopsy-derived tumoroids, we demonstrated the synergistic potential of combining CAR T-cell therapy with smac-mimetics. Taken together, we identified CAR T-cell–derived TNF as a potent antitumor effector, which can be further harnessed by smac-mimetics.

Introduction

Adoptive cellular therapy using chimeric antigen receptor (CAR) T cells is showing great promise for the treatment of cancer (1, 2). However, although CAR T cells have been successful in treating hematological malignancies (2), results in the solid tumor setting have been less positive (3). One reason for the lack of efficacy of CAR T cells in solid tumors is the immunosuppressive microenvironment encountered (1). This includes the expression of ligands on tumor cells that bind to checkpoint receptors on the T cells and inhibit their function. For this reason, combining CAR T cells with additional therapies that target checkpoint receptor–ligand interactions may improve outcomes in solid cancers. PD-1 blockade, for example, has been shown to significantly enhance the efficacy of both CAR T-cell (4) and adoptive cell therapy (5). The efficacy of CAR T-cell therapy may also be enhanced by agents that sensitize tumor cells to T-cell effector mechanisms, including cytokines. Smac-mimetics (SM) are a new class of antitumor agents that can sensitize tumor cells to tumor necrosis factor (TNF)–mediated cell death (6). Because TNF is key to the antitumor effector function of cytotoxic T cells (7), this class of small-molecule drugs may also enhance CAR T-cell efficacy.

During apoptotic cell death, the mitochondrial protein Smac/Diablo binds to and antagonizes the major “inhibitor of apoptosis proteins” (IAPs). Because IAPs are often upregulated in human cancer and facilitate resistance to tumor cell death following therapy, small-molecule mimetics of Smac/Diablo (SMs) were developed (6, 8). Antagonism of IAPs also results in formation of the death-inducing signaling complex upon TNF stimulation, rendering the tumor cell sensitive to TNF-induced cell death via the extrinsic pathway (9). Thus, therapies that boost immunity, particularly in the context of elevated TNF secretion, are likely to synergize with SMs. Indeed, IAP antagonism can promote antitumor immunity in multiple myeloma (10) and glioblastoma (11) when combined with checkpoint inhibition, in part due to TNF secreted by immune cells such as CD8+ T cells.

Based on the positive results from preclinical studies (12, 13), several SMs have completed phase I/II trials in patients with advanced solid and hematological cancers, with the SM birinapant showing the most promise (8). Given the critical role of TNF in antitumor effector functions of cytotoxic T cells (7) and the ability of SMs to sensitize tumor cells to TNF-dependent apoptosis, we investigated the potential of this class of drugs to enhance CAR T-cell efficacy. We demonstrated that IAP antagonism by birinapant sensitized tumor cells to CAR T-cell–derived TNF, significantly enhancing the antitumor activity of CAR T-cell therapy both in vitro and in vivo. The results pave the way for a combination therapy that may improve the efficacy of adoptive cell therapy in solid malignancies.
Materials and Methods

Mice
All animal studies were performed in accordance with the NHMRC Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) and with approval from the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee. C57BL/6 human HER2 transgenic mice (14) and C57BL/6 perforin knockout mice (15) were bred in-house. C57BL/6 and C57BL/6 TNF knockout mice were obtained from the Walter and Eliza Hall Institute (Parkville, Victoria). All mice were housed in the Peter MacCallum Cancer Centre Animal Core Facility under specific pathogen-free conditions.

Human samples
Human PBMCs were isolated from healthy donor buffy coats supplied by Australian Red Cross Blood Service. Biopsy samples from 2 patients with HER2+ metastatic colorectal cancer were included in this study. All human biopsy studies were conducted in accordance with the Declaration of Helsinki under a protocol approved by the Peter MacCallum Cancer Centre Human Ethics Committee, which included informed written consent from all patients.

Antibodies, cytokines, and drugs
Neutralizing antibodies: mouse anti-TNF (BioLegend; clone MP6-XT22), human anti-TNF (BioLegend; clone MAb11). Enbrel (etanercept) was obtained from the Walter and Eliza Hall Institute of Medical Research (Parkville, Victoria). Antibodies for immunofluorescence: anti-TNF (BioLegend; clone MP6-XT22), IFNγ (eBioscience; clone XMG1.2), Tubulin (Rockland Immunochemicals; clone 600-401-880). Secondary antibodies conjugated to Alexa fluorophores and ProLong antifade with DAPI were purchased from Molecular Probes. For cell stimulation, anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) antibodies were purchased from Molecular Probes. For cell stimulation, anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) antibodies were purchased from BioLegend (clone 24D2). Birinapant was supplied by Medivir dissolved in 12% Captisol solution in water (vehicle).

Cell lines
The cell lines MC38 and E0771 were retrovirally transduced to express the human HER2 antigen under the control of the mouse stem cell virus LTR promoter as previously described (14). Mouse cell lines were cultured in DMEM medium and the human cell lines HeLa and AU565 in RPMI medium (Gibco), both supplemented with 10% FCS (Thermo Scientific), penicillin/streptomycin (Gibco), L-glutamine, nonessential amino acids, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium pyruvate, and [Calbiochem]). T cells were activated with anti-CD3 (0.5 μg/mL) and anti-CD28 (0.5 μg/mL) in the presence of IL2 (100 IU/mL) and IL7 (2 ng/mL) at a density of 5 × 10^6 cells/mL.

The generation of murine and human CAR T cells was done as previously described (14, 16). Briefly, retrovirus encoding a CAR composed of an extracellular scFv–anti-human HER2 fused to the transmembrane domains of CD28 and CD3ζ was transduced into activated T cells from either the spleen of mice or from human PBMCs in the presence of IL2 (100 IU/mL) and IL7 (2 ng/mL; PeproTech). The viral packaging GP.E86 cell line containing empty (control) LXSN or LXSN-anti-HER2 CAR retroviral vector was generated as described previously (17). The CAR construct used in this study was a second-generation CAR composed of an extracellular single-chain variable fragment (scFv) specific for human-HER2, a CD8 hinge region, transmembrane and intracellular CD28 and CD3ζ domains, and a c-Myc tag domain allowing for CAR T-cell FACS sorting (18). Primary mouse T cells were collected from splenocytes of C57BL/6 human-HER2 transgenic or wildtype C57BL/6 mice. T cells were retrovirally transduced as described previously (19). After transduction, T cells were maintained in supplemented RPMI media with IL2 (100 U/mL) and IL7 (2 ng/mL). Tnfζ or perforinζ CAR T cells were generated as above from C57BL/6 Tnfζ or perforinζ mice.

In vitro assays
CAR T-cell activity was measured using a standard chromium release assay as previously described (15). The percentage-specific killing was determined using the formula: (Sample 51Cr release - Spontaneous 51Cr release)/(Total 51Cr release - Spontaneous 51Cr release) × 100, and represented as a Michaelis–Menten kinetic trend. All assays were performed using triplicate wells. The rate of killing in the control is represented as 100% killing, and treatment groups were compared with this value. All killing assays were done over 18 hours unless otherwise specified. To generate relative killing bar graphs, relative killing at the E:T ratio that results in 50% maximal killing of the least cytotoxic condition was compared, using Michaelis–Menten kinetic trends, as done previously (15, 20). Cytokines were detected using a mouse inflammation CBA kit (BD Biosciences, 552364) as per manufacturer’s instructions and analyzed on a FACSVerse (BD Biosciences). All assays were analyzed using triplicate determinations.

Flow cytometry
Cells assayed via flow cytometry were plated out in V-bottom plates in 100 μL media before being pelleted by centrifugation at 1400 RPM for 4 minutes and resuspended in 100 μL FACs buffer (PBS plus 2% FCS). Cells were then stained with an anti-human HER2 antibody (clone 24D2; BioLegend) for 30 minutes on ice before being washed twice in FACs buffer, resuspended in 100 μL FACs buffer and transferred to bullet tubes for analysis. Analysis of samples was performed on a Fortessa X20 (BD Biosciences), and data were analyzed with FlowJo (Tree Star).

3’RNA sequencing (RNA-seq)
MC38 cells expressing human HER2 antigen and AU565 cells were left untreated or cocultured with CAR T cells at a 2:1 ratio at 37°C for 6 hours. Cells were washed thoroughly
with PBS to remove contaminating CAR T cells, and cells were lysed. Total RNA was isolated as per the kit manufacturer’s instructions (Nucleospin RNA extraction kit, Macherey-Nagel). Single-end 75-bp RNA-seq was performed in-house at the Peter MacCallum Cancer Centre Molecular Genomics Core on the NextSeqEution 500 (Illumina). Demultiplexing of the reads was performed using CASAVAv1.8.2, and low-quality reads Q < 30 were removed. Cutadapt (v1.9) was used to trim polyA-derived sequences and biased 3’ reads resulting from random hexamer priming. HISAT2 was used to map the resulting reads to the human reference genome. Read counting was performed using featureCounts; part of the subread package. Voom-LIMMA workflow was used to normalize data for differential gene expression. Gene set enrichment analysis was performed using GSEA2 (v3.0) for identification of enriched signatures obtained from the MSigDB Hallmarks data sets (7). Sequencing data have been deposited into the Gene-Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE124140.

Fixed and time-lapse microscopy

Microscopy of T-cell–tumor conjugates was done as previously described (15). The slides were imaged using a FV1000 confocal microscope (Olympus). CAR T cells selected for quantitation had a single contact site with one tumor cell, indicating a single synapse event. The percentage of T cells that were positive for the indicated cytokine in a synapse or not was quantitated manually by confocal microscopy. A minimum of 100 cells were counted in each condition.

For time-lapse imaging, CAR T cells were sorted for double-positive CD8 and CAR (anti-myc) cells and labeled with CellTrace Violet (CTV, Molecular Probes) as follows: 1 × 10^7 cells were stained with 5 μmol/L CTV in PBS for 20 minutes at 37°C and washed for 5 minutes with media. Cells were then pelleted and resuspended in prewarmed media for 10 minutes. Labeled T cells were then added to adherent targets in media containing propidium iodide (PI) and 1 μmol/L birinapant or vehicle control (Captisol). Chamber slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C, and constant CO2 concentration of 5% was infused using a gas incubation system with active gas mixer (“The Brick”; Ibid). Optical sections were acquired through sequential scans of Brightfield/DIC on a TCS SP5 confocal microscope (Leica Microsystems) using a ×40 (NA 0.85) air objective and Leica LAS AF software. Image analysis was performed using Meta-Morph Imaging Series 7 software (Universal Imaging).

Mouse experiments

C57BL/6 human HER2 transgenic mice were injected subcutaneously with 2.5 × 10^7 MC38-HER2 cells. At day 7 after tumor injection, mice were preconditioned with total-body irradiation (4 Gy) prior to the administration of 1 × 10^7 CAR T cells on days 7 and 8. Mice were also treated with 50,000 IU IL2 on days 0 to 4 after T-cell transfer. Mice were treated with either vehicle (Captisol) or birinapant (12 mg/kg per mouse) on days 0, 4, and 8 after T-cell transfer. Tumor growth was monitored approximately every second day using a caliper square to determine the product of 2 perpendicular tumor diameters. The mice were culled when the tumor size reached the ethical limit (150 mm²) and recorded for the survival plot.

Patient-derived tumoroids

Tumoroids were grown from tumor cells derived from colorectal patient biopsies. Tumor cells were plated on μ-Plate 36 Well (ibidi) by adding 20 μL of Matrigel/cell suspension per well, and placed to set in 37°C incubator for 30–60 minutes. Following incubation, 350 μL of OB media supplemented with 500 mmol/L A8301 (Torcis, Bioscience), 2 × 827 (Gibco, Thermo Scientific), epidermal growth factor (50 ng/mL; Sigma-Aldrich), gastrin (1 μg/mL; Sigma-Aldrich), 1 mmol/L N-acetyl Cysteine (Sigma-Aldrich), 5 μmol/L SB202190 (Sigma-Aldrich), 10 μmol/L SB431542 (Sigma-Aldrich), 10 μmol/L Y27632 (Sigma-Aldrich) was added to each well, and tumoroids were grown in hypoxic conditions (37°C, 5% O2, 5% CO2) for 5 to 7 days. Tumoroids were grown to >50 μm and checked for viability using a light microscope on the day of assay, before proceeding. Tumoroids were deemed suitable for coculture after 10 to 14 days after initial biopsy.

A total volume of 350 μL supplemented OB media was applied to the Matrigel-embedded tumoroids. PI (Sigma-Aldrich) was added to a final concentration of 2 μg/mL. CAR T cells/well (2.5 × 10^5) were then resuspended in this media. Wells were set up with the following conditions: tumoroids + vehicle control, tumoroids + CAR T cells/TNF (10 ng/mL), tumoroids + birinapant (1 μmol/L), tumoroids + CAR T cells/TNF + birinapant, CAR T cells + birinapant + TNF-neutralizing antibody (80 μg/mL). Tumoroids were incubated at 37°C, 5% CO2 for 24 hours, and then end-point images were acquired. U-Plate 96-well plates were mounted on a heated stage in a temperature-controlled chamber, maintained at 37°C, 5% CO2 (“The Brick”; Ibid). Using the cellsens software (Olympus), imaging of the plate was obtained on an UPLSAPO 4X (NA 0.16) air objective using a Hammamatsu ORCA-Flash 4.0 camera. Z-stack images were acquired by taking 25 sequential images (Z spacing 50 μm) of PI (emission 656 nm, exposure 368.644 ms) through the Matrigel, and Z stack images converted to one image in EFI format. Image quantitation was conducted using Meta-Morph Imaging Series 7 software. An identical region of interest was identified within the Matrigel, and integrated morphometry analysis was used to filter out individual T cells based on size. A threshold was applied to delineate PI-positive pixels, and integrated intensity was measured. Identical conditions were applied to compared images.

Statistical analyses

Statistical significance was determined using an unpaired Student t test, ANOVA, or log-rank (Mantel–Cox) test for mouse survival data using GraphPad Prism 7 software. Differences were considered significant when P < 0.05.

Results

CAR T cells produce cytokines upon tumor cell recognition

TNF is a key inflammatory cytokine produced by cytotoxic T cells upon target recognition that is critical for their antitumor effector function (7). Here, we generated mouse CAR T cells specific for the human HER2 antigen (Fig. 1A; ref. 21) and found that upon recognition of HER2-expressing MC38 colon carcinoma cells (MC38-HER2), TNF and interferon gamma (IFNγ) were key cytokines produced (Fig. 1B). Human HER2-directed CAR T cells also secreted TNF and IFNγ when exposed to HeLa cells that overexpressed HER2 (Supplementary Fig.
Figure 1. CAR T cells produce inflammatory cytokines upon tumor cell recognition. A, Schematic representation of the CAR T-cell. B, MC38-HER2 cells were exposed to CAR T cells at the indicated effector-to-target (E:T) ratio. After 4 hours, cytokines in supernatants were measured by cytometric bead array. C, Left, MC38-HER2 or parental cells were seeded in chamber slides, then overlaid with CAR T cells. After 2 hours, cells were fixed and stained as indicated, then visualized by confocal microscopy. Scale bar, 10 μm. Right, the percentage of CAR T cells that formed an immunologic synapse was quantitated by confocal microscopy (>100 cells). The percentage of CAR T cells positive for the indicated cytokines was quantitated by confocal microscopy (>100 cells). MC38-HER2 cells were left untreated or incubated with CAR T cells (in triplicate) at low effector-to-target ratio for 6 hours followed by (D) T7 RNA-seq of viable cells. Left, heat map showing significantly regulated genes following CAR T-cell treatment (P < 0.05). GSEA enrichment score plots from RNA-seq data. Right, log2 counts/million cells from RNA-seq data in D. Error bars, mean ± SEM of triplicate determinations. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 by an unpaired Student t test.
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S1A). Confocal microscopy of mouse CAR T cells cocultured with MC38-HER2 or parental MC38 cells (Fig. 1C, left) revealed an increase in immunological synapse formation between CAR T cells and MC38-HER2 tumor cells and a significantly increased number of synapses positive for TNF and IFNγ (Fig. 1C, right).

We also detected significant transcriptional changes in the MC38-HER2 tumor cells in response to CAR T-cell treatment (Fig. 1D, left), with GSEA analysis revealing significant gene enrichment in pathways relating to TNF signaling through NF-κB and the IFNγ response (Fig. 1D, center). Further analysis of the data confirmed upregulation of specific genes known to be induced by TNF and IFNγ signaling, including CXCL10, NFKB1A, IRF1, and TAPI (Fig. 1D, right). Transcriptional analysis of the human breast cancer line AU565 (which endogenously expresses HER2) cocultured with human CAR T cells also showed gene enrichment pathways relating to TNF signaling (Supplementary Fig. S1B). These data demonstrated that CAR T cells can produce the inflammatory cytokines, TNF and IFNγ, which elicits a transcriptional cytokine response in tumor cells.

Birinapant enhances tumor cell death in the presence of CAR T cells

Because the SM, birinapant, has previously been shown to enhance the death of tumor cells expressing a model antigen in the presence of transgenic OT-I T cells (15), we wanted to determine if birinapant could enhance the antitumor activity of CAR T cells to tumor cells expressing the tumor antigen HER2. Mouse CAR T cells were cocultured with MC38 or breast cancer E0771 tumor cells (both engineered to express human HER2), and human CAR T cells with HeLa or AU565 tumor cells (both endogenously express HER2), and cell death was measured using a chromium release killing assay. In all cell lines, a significant increase in cell death in the presence of birinapant compared with vehicle was observed (Fig. 2A). Birinapant did not induce cell death of the CAR T cells (Supplementary Fig. S1C), as has been previously observed for other T cells (15, 22).

To investigate the mechanism of this enhanced tumor cell death in the presence of birinapant, we monitored the interaction of mouse CAR T cells with MC38-HER2 cells by time-lapse imaging. We observed a significant increase in the death of MC38-HER2 cells, indicated by red fluorescence, in the birinapant-treated cocultures (Fig. 2B; Supplementary Movies S1–S2). Further analyses of the imaging revealed a significant increase in the percentage of cell death that occurred independently of direct CAR T-cell interaction, described as “bystander killing” (refs. 7, 15; Fig. 2B, right). To confirm this, we conducted a killing assay with a 50:50 mix of unlabeled MC38-HER2 and chromium-labeled MC38 parental cells. CAR T cells do not recognize non-HER2–expressing tumor cells (14), and therefore, any chromium release occurred independently of direct CAR T-cell interaction (Fig. 2C). These data demonstrated a significant increase in bystander killing in the presence of CAR T cells and birinapant (Fig. 2D).

Birinapant enhances CAR T-cell efficacy in a TNF-dependent manner

We next wanted to determine the mechanism by which birinapant enhances tumor cell death in the presence of CAR T cells. Given the ability of birinapant to sensitize cells to TNF-mediated cell death, we first added an anti-TNF–neutralizing antibody to the cocultures of both mouse and human CAR T cells and their cognate target tumor cells (Fig. 3A). In all cases, the addition of anti-TNF significantly reduced the ability of birinapant to enhance tumor cell death in the presence of CAR T cells.

To confirm this observation, we also utilized TNF-deficient (TNF−/−) CAR T cells, which had significantly decreased effector function compared with wild-type (WT) CAR T cells, an effect that was not ameliorated by the administration of birinapant (Fig. 3B). We detected no TNF production from the TNF−/− CAR T cells, as expected, but equivalent amounts of IFNγ were secreted between TNF−/− and WT CAR T cells (Fig. 3C). To confirm that birinapant did not enhance tumor cell death via the perforin-mediated granule exocytosis pathway, we repeated the assay with perforin−/− CAR T cells. Perforin−/− CAR T cells had decreased effector function compared with WT CAR T cells at 4 hours, and tumor cell death was unaffected by the addition of birinapant (Fig. 3D, left). However, over an 18-hour assay, tumor cell death in the presence of perforin−/− CAR T cells was increased, and this was further augmented in the presence of birinapant (Fig. 3D, center). This increase in cell death was significantly reduced by the addition of a TNF-neutralizing antibody (Fig. 3D, right), demonstrating that the increase in tumor cell death in the presence of CAR T cells and birinapant is perforin-independent and TNF-dependent.

Combination CAR T-cell therapy and birinapant enhances tumor control

Using a human-HER2 transgenic mouse model (14), we next investigated the combination therapy of CAR T cells and birinapant in vivo. MC38-HER2 tumor–bearing C57BL/6 transgenic human-HER2 mice were treated with 1 × 105 CAR T cells on days 0 and 1, and birinapant (12 mg/kg) on days 0, 4, and 8. Control mice established robust tumors, which were not cleared by CAR T-cell or birinapant therapy alone. However, the combination of CAR T-cell therapy and birinapant significantly reduced tumor growth, with complete tumor clearance observed in 50% mice. Long-term overall survival was significantly increased following the combination therapy (Fig. 4A), demonstrating that the addition of birinapant enhanced CAR T-cell therapy in vivo.

Birinapant enhances CAR T-cell efficacy using patient tumoroids

To determine if the combination of CAR T cells and birinapant would enhance tumor cell death in a human cancer, we utilized patient biopsy-derived colorectal tumoroids that expressed HER2 (Supplementary Fig. S2A). The tumoroids were not sensitive to TNF or birinapant alone, but the combination significantly enhanced tumor cell death, as indicated by red fluorescence (Fig. 4B; Supplementary Fig. S2B). To test the effect of birinapant on CAR T-cell efficacy using this model, human CAR T cells were applied to Matrigel-embedded tumoroids (Fig. 4C, top), which endogenously expressed HER2 (Fig. 4C, bottom). The addition of CAR T cells resulted in minimal tumoroid death, whereas the combination of CAR T cells and birinapant resulted in a significant increase in tumoroid death compared with CAR T-cell treatment alone (Fig. 4D). The addition of an anti-TNF–neutralizing antibody to the combination-
Figure 2.
IAP antagonism enhances tumor cell death in the presence of CAR T cells. A, Chromium release assay (18 hours) using mouse CAR T cells with E0771-HER2 and MC38-HER2 targets and human CAR T cells with HeLa-HER2 and AU565 targets (at the indicated E:T ratios) in the presence of vehicle control (Captisol) or birinapant (1 μmol/L). B, Left, MC38-HER2 cells were seeded in chamber slides, then overlaid with mouse CAR T cells (2:1 ratio) labeled with CTV in PI-containing media. Representative still images at the indicated time points are depicted (hr:min). Scale bar, 50 μm. Right, percentage PI-positive cells without direct CAR T-cell contact was manually quantitated by live imaging. The number of tumor cells that died throughout the duration of the movie (as measured by PI uptake) without contact with a T-cell was quantitated and presented in the graph. Quantitation data are pooled from 3 separate camera positions. C, Schematic of chromium release assay to quantify bystander killing. D, Relative killing of MC38 parental cells by bystander killing in the presence vehicle control (Captisol) or birinapant (1 μmol/L), as depicted in C. All relative killing data are pooled from 3 independent experiments. Error bars, mean ± SEM; *, P < 0.05; **, P < 0.01 by an unpaired Student t test.
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Figure 3. Birinapant enhances tumor cell death in the presence of CAR T cells in a TNF-dependent, perforin-independent manner. 

A, Chromium release assay (18 hours) using mouse CAR T cells with E0771-HER2 and MC38-HER2 targets and human CAR T cells with HeLa-HER2 and AU665 targets (at the indicated E:T ratio) in the presence of vehicle control (Captisol) or birinapant (1 μmol/L) and presence or absence of a TNF-neutralizing antibody (10 μg/mL). B, Chromium release assays using WT or Tnf−/− CAR T cells and MC38-HER2 cells as targets (at the indicated E:T ratio) in the presence or absence of vehicle control (Captisol) or birinapant (1 μmol/L). C, WT or Tnf−/− CAR T cells were exposed to MC38-HER2 cells at the indicated E:T ratio. After 4 hours, cytokines in supernatants were measured by CBA. D, Left, chromium release assay (4 hours) using WT or perforin−/− CAR T cells and MC38-HER2 cells as targets (at the indicated E:T ratio) in the presence of vehicle control (Captisol) or birinapant (1 μmol/L). Center, chromium release assay after 18 hours. Right, chromium release assay using MC38 HER2 cells and WT or perforin−/− CAR T cells (at the indicated E:T ratio) untreated (vehicle) in the presence or absence of anti-TNF-neutralizing antibody (10 μg/mL). Error bars, mean ± SEM of triplicate determinations from a representative of 2 independent experiments. *, P < 0.05 by an unpaired Student t-test.

treated tumoroids significantly reduced the amount of tumoroid death, suggesting that TNF-mediated bystander killing was the effector pathway of combination CAR T-cell and birinapant therapy, not direct T-cell contact (Supplementary Fig. S2C). Together, these data demonstrate that the addition of birinapant to CAR T-cell treatment can significantly enhance tumor cell
Birinapant enhances CAR T-cell therapy in vivo and in patient-derived tumoroids. C57BL/6 human HER2 transgenic mice were injected subcutaneously with MC38-HER2 cells (2.5 × 10⁶ cells/mouse) and tumors were allowed to establish for 7 days. Mice received total body irradiation (4 Gy) before adoptive transfer of anti-HER2 CAR T cells intravenously (1 × 10⁶ cells/mouse), and a total of 5 doses of intraperitoneal IL2 (50,000 IU/injection) over 5 consecutive days. Birinapant was administered at 12 mg/kg by intraperitoneal injection on days 0, 4, and 8 after CAR T-cell injection. **A**, Growth of MC38 tumors at indicated time points and tumor size at 12 days after treatment. Data are mean ± SEM of 14–15 mice/group, pooled from 2 independent experiments. **B**, Survival of mice to day 50 after treatment. Data are mean ± SEM of 14–15 mice/group, pooled from 2 independent experiments. **C**, P < 0.05 by ANOVA. Right, survival of mice to day 50 after treatment. Data are mean ± SEM of 14–15 mice/group, pooled from 2 independent experiments. **C**, P < 0.05; **C**, P < 0.01; **C**, P < 0.0001 by log-rank (Mantel–Cox) test. **B**, Fluorescent microscopy of patient-derived colorectal tumoroids embedded in Matrigel and administered vehicle control (Captisol) or soluble TNF (10 ng/mL) in PI-containing media. **C**, Top, schematic representation of CAR T-cell treatment of Matrigel-embedded tumoroids. Dashed lines, production of TNF by CAR T cells and bystander killing of tumoroids by TNF-mediated apoptosis. Bottom, tumoroids were dissociated into single-cell suspensions and analyzed for HER2 expression by flow cytometry compared with isotype control. **D**, Tumoroids were treated with vehicle control (Captisol) or CAR T cells in the presence or absence of birinapant (1 µmol/L), or in the presence of birinapant and a neutralizing TNF antibody in PI-containing media. Total fluorescent intensity was quantified using Meta-Morph software. Data are mean ± SEM from tumoroids derived from 2 individual patients in 3 independent experiments. **, P < 0.05; **, P < 0.01 by ANOVA.
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Discussion

Although the development of CAR T-cell therapy has been revolutionary within the field of immunotherapy, the clinical success has been limited to hematological cancers (2) and a handful of solid cancers (23, 24). A limited number of studies have reported combination therapies that enhance CAR T-cell therapy in solid cancers. Here, we demonstrated that the SM, birinapant, significantly enhanced the efficacy of CAR T cells in a solid tumor setting, in a TNF-dependent manner.

Birinapant is currently undergoing clinical trials as a single agent for a number of hematological and solid tumor cancers (25, 26). Many tumors upregulate IAPs (27) to facilitate resistance to apoptosis following standard chemotherapies (28). The degradation of IAPs by SMs results in the secretion of TNF, which can then induce apoptosis in an autocrine manner via the extrinsic pathway (6). Tumor cells that do not secrete TNF following IAP degradation can also be killed by the exogenous addition of TNF (29). We have previously shown the potential of cytotoxic lymphocytes as a source of TNF that can be utilized by SMs in vitro (15). Using a human-HER2 self-antigen mouse model, we demonstrated here that HER2+ MC38 tumor-bearing mice treated with CAR T cells and birinapant had a significant survival advantage compared with either monotherapy, with complete remission achieved in half the mice. Given the lack of robust responses to CAR T-cell therapy observed in this model (14), these data may have significant implications for previously difficult-to-treat solid malignancies.

In order to demonstrate the potency of this combination therapy on patient tissue, we utilized HER2+ patient-derived colorectal tumoroids embedded in Matrigel as a model of a solid tumor. We have previously uncovered a critical role of the TNF-mediated apoptotic pathway as a mechanism of inducing tumor cell death in the absence of direct T-cell contact (7, 15). We demonstrated that, even with low CAR T-cell penetration, in the presence of birinapant, CAR T-cell–derived TNF can penetrate through the Matrigel and trigger apoptosis of buried tumor cells through this ‘bystander’ effect (7, 15). This may therefore represent a therapy that overcomes the limitation of small numbers of CAR T cells reaching and penetrating the tumor efficiently, while allowing for potent killing of antigen-negative tumor cells by TNF. Capitalizing on the enhanced TNF produced by T cells following checkpoint blockade (15) may also provide a therapy by combining CAR T-cell administration along with birinapant and anti–PD-1 treatment. Given all 3 therapies are either in the clinic or currently being assessed in clinical trials, we expect there can be rapid translation of this therapy into patients.

Disclosure of Potential Conflicts of Interest

R.W. Johnstone reports receiving commercial research grants from Roche and AstraZeneca and is a consultant/advisory board member for MecRx. No potential conflicts of interest were disclosed by the authors.

Authors’ Contributions

Conception and design: J. Michie, C.J. Kearney, J. Oliaro


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Michie, P.A. Beavis, A.J. Freeman, K.M. Ramsbottom, V. Narasimhan, E.J. Lelliott, R.G. Ramsay, J. Silke, J. Oliaro


Writing, review, and/or revision of the manuscript: J. Michie, P.A. Beavis, V. Narasimhan, R.G. Ramsay, R.W. Johnstone, P.K. Darcy, C.J. Kearney, J. Oliaro

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Narasimhan, J. Silke

Study supervision: S.J. Vervoort, R.G. Ramsay, I. Voskoboinik, C.J. Kearney, J. Oliaro

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