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Expression of a Functional CCR2 Receptor Enhances Tumor Localization and Tumor Eradication by Retargeted Human T cells Expressing a Mesothelin-Specific Chimeric Antibody Receptor

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Abstract

Purpose: Adoptive T-cell immunotherapy with tumor infiltrating lymphocytes or genetically-modified T cells has yielded dramatic results in some cancers. However, T cells need to traffic properly into tumors to adequately exert therapeutic effects.

Experimental Design: The chemokine CCL2 was highly secreted by malignant pleural mesotheliomas (MPM; a planned tumor target), but the corresponding chemokine receptor (CCR2) was minimally expressed on activated human T cells transduced with a chimeric antibody receptor (CAR) directed to the MPM tumor antigen mesothelin (mesoCAR T cells). The chemokine receptor CCR2b was thus transduced into mesoCAR T cells using a lentiviral vector, and the modified T cells were used to treat established mesothelin-expressing tumors.

Results: CCR2b transduction led to CCL2-induced calcium flux and increased transmigration, as well as augmentation of *in vitro* T-cell killing ability. A single intravenous injection of 20 million mesoCAR + CCR2b T cells into immunodeficient mice bearing large, established tumors (without any adjunct therapy) resulted in a 12.5-fold increase in T-cell tumor infiltration by day 5 compared with mesoCAR T cells. This was associated with significantly increased antitumor activity.

Conclusions: CAR T cells bearing a functional chemokine receptor can overcome the inadequate tumor localization that limits conventional CAR targeting strategies and can significantly improve antitumor efficacy *in vivo*. *Clin Cancer Res; 17(14); 4719–30. ©2011 AACR.*

Introduction

Adoptive T-cell immunotherapy (ACT) denotes the transfer of T lymphocytes for the treatment of malignant or infectious diseases. This approach has resulted in dramatic clinical responses in different cancer types including melanoma and Epstein-Barr virus lymphomas (1–3). Classically, ACT involved the transfer of tumor infiltrating lymphocytes (TIL) that were expanded *ex vivo* from tumor biopsies. However, only 30%–40% of tumor biopsy specimens yield satisfactory T-cell populations, the expansion time required for patient therapy is long, and the approach has been primarily useful in malignant melanoma (4). One reason for this may be that the immunosuppressive environment of many tumors induces tolerance by the deletion or functional inactivation of TIL T-cell receptors (TCR; refs. 5–7).

A major advance in ACT has been the ability to rapidly generate large numbers of genetically redirected T cells that target specific tumor antigens by using peripheral blood lymphocytes. One approach is the insertion of recombinant TCRs (8, 9). Another approach has been the creation of "chimeric antibody receptors" (CAR; ref. 10). CARs are cell surface molecules in which the V_H and V_L regions of a monoclonal antibody are expressed as a single-chain variable fragment (scFv) and linked to the signal transduction domain of the CD3-zeta chain. More recent versions have added additional costimulatory domains, such as CD28 and 4–1BB, fused to the CD3-zeta chain. CAR-mediated ACT has several advantages over TCR-based ACT including high-affinity recognition of the tumor antigen, MHC-independent activity, and the ability to link additional signaling modules to one antigen recognition event to achieve optimal T-cell activation (11, 12). Our group has focused on introducing CARs by using lentiviral vectors (13) and has initiated a clinical trial by using this technology.

Although ACT seems to be one of the most robust forms of immunotherapy for the treatment of tumors, the strategy

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Translational Relevance

In this study, we have provided experimental evidence showing that matching the chemokine receptor expression on T cells that bear the mesothelin-reactive chimeric antibody receptor (CAR) to the chemokine secretion by the target tumor leads to enhanced T-cell homing to and activity against the tumor. We have further shown that with properly trafficking CAR T cells, it is possible to eradicate large, established tumors, with a single dose of T cells, and without adjunct cellular or cytokine therapy. These discoveries have important implications for optimizing adoptive T-cell immunegene therapy for malignant pleural mesothelioma, a cancer with poor survival that is still minimally affected by current treatment strategies, as well as other tumors.

requires further optimization to overcome some significant hurdles (11, 12). An overarching issue is safety, especially in light of lethal toxicities in recent attempts at applying ACT strategy clinically (reviewed in ref. 14). However, there are also a number of issues related to increasing the efficacy of the infused T cells including: (i) augmenting T-cell trafficking to tumor, (ii) increasing survival within tumor, and (iii) making sure that the T cells retain antitumor activity within the intratumoral milieu of immunosuppressive cytokines and cells (e.g., T-regulatory cells and myeloid-derived suppressor cells; ref. 15–20). In patients with metastatic melanoma, persistence of tumor antigen-specific T cells after adoptive transfer correlates with tumor regression (21).

T-cell trafficking involves a complex 4-step interaction between circulating lymphocytes and endothelial cells that requires initial T-cell attachment to and rolling on endothelium, T-cell activation on the endothelial surface, secondary adhesion, and T-cell extravasation (16). All 3 of these latter steps involve chemokines and chemokine receptors (CCR). T-cell trafficking can be enhanced through binding of the tumor-produced chemokines to the appropriate CCRs on the activated T cells injected. Secretion of chemokines from the tumor that do not match the expression of the appropriate CCRs on the T cells will result in suboptimal trafficking (22). Given that the adoptively transferred T cells are being genetically modified by insertion of optimized TCRs or CARs, a reasonable hypothesis is that additional modifications to change chemokine receptor expression could be advantageous.

Our group is developing ACT employing T cells transduced with lentiviral vectors encoding a chimeric antibody receptor recognizing the protein mesothelin (mesoCAR T cells). Mesothelin is a surface protein that is expressed at low levels on serosal cells (i.e., on the pleura, pericardium, peritoneum, and tunica vaginalis), but is highly expressed on a number of malignancies, including malignant pleural mesothelioma (MPM), ovarian cancer, and pancreatic cancer (23, 24). Because of the restricted, low basal levels of expression on nonmalignant tissue, and the ubiquitous overexpression on various tumor types, mesothelin seems to be an attractive tumor antigen target. Clinical trials are underway by using unmodified or exotoxin-conjugated anti-mesothelin antibodies and have shown safety and some efficacy (25–27). We have recently shown antitumor activity of adoptively transferred mesoCAR T cells in preclinical mouse models of MPM (28, 29) and have initiated a pilot and feasibility clinical trial by using mesoCAR T cells for patients with MPM. The hypothesis for this study is that optimization of this approach requires the CCR repertoire on the injected activated mesoCAR T cells to be "matched" to the chemokines most abundantly secreted by MPM tumors. Accordingly, we determined (i) which chemokines are consistently secreted at high levels by MPM tumor cells, (ii) which CCRs are expressed in T cells activated using clinically compliant conditions, (iii) if introduction of an appropriate "matching" CCR into T cells augments trafficking to tumor, (iv) and if enhanced trafficking translates into improved antitumor efficacy. Given the clinical prospects of treating MPM patients with extensive disease, our priority was to address these issues by using large, established tumors with only a single dose of CAR T cells, and without adjunct cytokine/cellular therapy.

Materials and Methods

Mice

NOD/severe combined immunodeficient/γ-chain knockout (NSG) mice were bred and maintained at the Wistar Institute Animal Facility. All mouse experiments were carried out in accordance with the Wistar Institute Animal Care and Use Committee guidelines and were approved by the University of Pennsylvania School of Medicine's IACUC.

Antibodies

Antibodies used in this study are outlined in Supplementary Figure S1.

Flow cytometry

Flow cytometric analysis (FACS) was conducted according to standard protocols by using a FACSCanto (Becton Dickinson) flow cytometer and analyzed with FACSDiva (Becton Dickinson) and FlowJo software (TreeStar).

Tumor cells

The M108 tumor cell line was established from a patient with MPM malignant pleural fluid and has been previously described (28). M108 cells naturally express mesothelin (Supplementary Fig. S2). M108 was grown in E^H media as described previously (30). MTSO human mesothelioma cells were purchased from the American Type Tissue Collection. mEM human mesothelioma cell line was acquired from an MPM surgical sample and was stably transduced to express mesothelin (mEM-meso; it is not uncommon for

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MPM tumor lines to lose mesothelin expression after long periods of *in vitro* culture).

Evaluating MPM tumor chemokine expression

Human MPM cell lines (previously described by Crisanti and colleagues; ref. 31) were cultured in R10 (L-glutamine supplemented RPMI-1640 medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate), and supernatant was collected. Fluids were run on a Bio-plex Multiplex Cyokine Assay. Supernatants or malignant pleural fluid samples from subjects with MPM were analyzed by ELISA for cytokine measurements by using the manufacturer's instructions.

Evaluating resting and activated T-cell CCR expression

CCR expression was measured by FACS in resting peripheral blood mononuclear cells (PBMC) acquired from the University of Pennsylvania's Human Immunology Core. Purified PBMC T cells were activated by magnetic beads coated with anti-CD3/anti-CD28 (protocol details outlined elsewhere; ref. 28). TILs were acquired from malignant MPM pleural effusion samples or MPM surgical biopsies and activated with high-dose IL-2 (Novartis; protocol details outlined elsewhere; ref. 4).

Lentivirus preparation

The human CCR2b construct was synthesized by Integrated DNA Technologies (Coralville) in the pIDT.SMART cloning plasmid and flanking 5′ *Bam*HI and 3′ *Sal*I restriction sites allowed subsequent subcloning into the lentiviral vector pELNS bearing the EF1α promoter (32). The mesoCAR construct contains the single-chain Fv domain of the anti-mesothelin antibody (SS1 scFv) and the CD3z and 4–1BB intracellular signaling domains, and has been previously shown to have good anti-mesothelioma tumor efficacy in an NSG MPM mouse model (28). Packaging of each plasmid into lentivirus has been previously described (33).

Transduction of mesoCAR and CCR2b into human T cells

Bulk CD4 and CD8 T cells isolated from PBMC were transduced with mesoCAR lentiviral vectors [at a multiplicity of infection (MOI) ∼3] 24 hours after the start of bead activation. The following day, the media with lentivirus was discarded by centrifugation, and T cells were split into 2 populations, with 1 receiving fresh R10 and the other receiving CCR2b lentiviral vector at an MOI ∼3. Cells were expanded for approximately 2 weeks and cryopreserved at −140°C in 90% FBS/10% dimethyl sulfoxide until use.

MesoCAR FACS detection

Cells were stained by using a biotinylated goat anti-mouse IgG recognizing the $F(ab')_2$ fragment (Jackson Immunoresearch) as the primary antibody and phycoerythrin-conjugated streptavidin (BD) as the secondary antibody.

Confirmation of CCR2b function by calcium flux analysis in the presence of CCL2

CCR2b-transduced T cells were analyzed by using a single cell assay as described (34). Briefly, cells were loaded with 4 μg/mL of Fluo-3 AM and 10 μg/mL of Fura Red AM at 37°C for 30 minutes. Probenecid (4 mmol/L) was added to prevent active secretion of loaded dyes from cells. Cells were then analyzed by exciting at 488 nm in the BD FACS Canto, and collecting Fluo-3 fluorescence from 515 to 535 nm and Fura Red fluorescence from 665 to 685 nm. After baseline measurements, CCL2 was added at 100 ng/mL and analysis was continued. Untransduced T cells were used as negative controls. Ionomycin was added at 1 μg/mL at the conclusion of analysis to confirm proper cell loading with the 2 calcium-sensitive probes.

Confirmation of CCR2b function by transwell migration in the presence of CCL2

Six hundred microliters of R10 media alone or with 100 ng/mL of CCL2 was placed in triplicate wells of a 24-well plate. Corning 0.5-μm polycarbonate membrane, 65-mm Transwell inserts (Corning 3421) were placed in the wells, and 100 μL of 5×10^6/mL transduced or untransduced T cells were placed in the top chamber. After incubation for 4 hours at 37°C and 5% CO_2, the number of T cells that migrated to the bottom chamber was quantified manually by using a hemocytometer. This was repeated by using M108-conditioned supernatant collected over 24 hours in the presence or absence of anti-CCL2 antibody (Centocor, Inc.).

In vitro testing of mesothelioma tumor cell killing by mesoCAR bearing T cells

M108 was stably transduced to express firefly luciferase (ffluc) and plated in a 96-well plate at 5,000 cells per well in triplicate. After overnight incubation at 37°C and 5% CO_2, T cells expressing either mesoCAR or mesoCAR + CCR2b were cocultured at a 20:1 effector/target ratio in the absence or presence of 100 ng/mL CCL2. After 4 hours of incubation at 37°C and 5% CO_2, the wells were washed, remaining tumor cells were lysed in BD luciferase cell culture lysis buffer, and luminescence was determined after addition of 100 μL of luciferin reagent (Promega E1501).

We also conducted a similar killing assay utilizing CCL2-secreting MPM tumor lines, mEM and mEM-meso, to show that antitumor activity was not induced by antigen-independent activation of T cells via chemokine–CCR interaction.

In vivo testing of T-cell trafficking and antitumor activity

Five million M108 cells in a solution of X-Vivo 15 media (Lonza) and 50% Matrigel (BD Biosciences) were subcutaneously injected in the flanks of NSG mice. After large tumors (200–300 mm^3) were established (usually after 4 weeks), the mice were grouped and received 1 of 3 treatments via tail vein: (i) 20 million untransduced bead-activated T cells, (ii) 20 million bead-activated T cells transduced with mesoCAR, or (iii) 20 million bead-activated T cells

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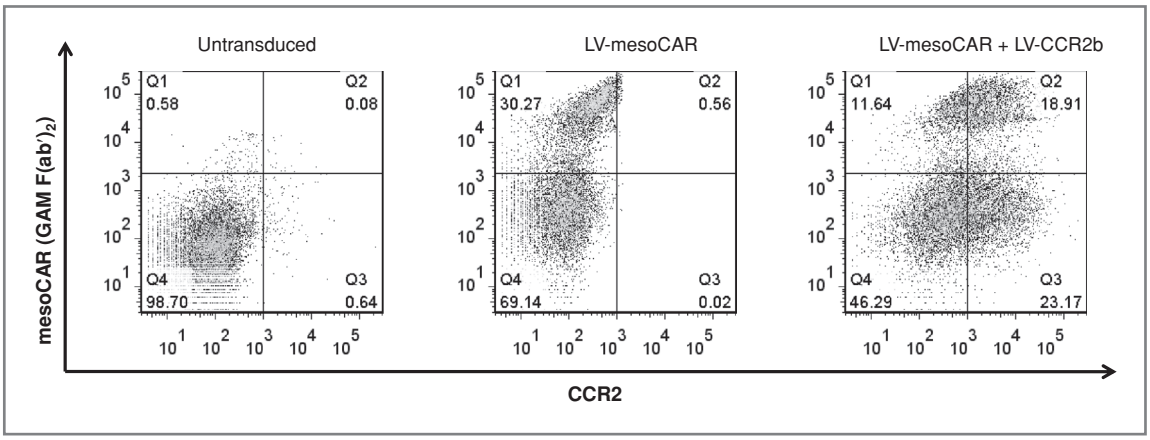

Figure 1. Expression of mesoCAR and CCR2b after lentivirus (LV) transduction. Bead-activated T cells (left), bead-activated, LV-mesoCAR transduced T cells (middle), or bead-activated, LV-mesoCAR and LV-CCR2b transduced T cells (right) were studied by using 2 color FACS analysis. Expression of the mesoCAR is shown on the *y*-axis. Expression of CCR2 is shown on the *x*-axis.

transduced with mesoCAR and CCR2b (mesoCAR + CCR2b; refer to Fig. 1 for transduction efficiencies.) Tumors were harvested 5 days after T-cell injection and digested in a solution of 1:2 DNAse/collagenase with rotation at 37°C. Digested tumor was then filtered through 70 μm nylon mesh cell strainers and washed twice with PBS + 1% FBS, with red blood cell lysis carried out with Pharm Lyse (BD Biosciences) if needed. Whole blood was also obtained by retroorbital bleeding into heparinized tubes and subjected to red blood cell lysis. One million cells were placed in standard FACS tubes and were stained for human CD45 or CD3 expression.

A pilot experiment was done to look at CCR2b-induced trafficking of T cells to tumor. Ten million bead-activated human T cells stably transduced via lentiviral vector to express firefly luciferase ± CCR2b were injected intravenously into NSG mice with large (200–300 mm^3) established CCL2-secreting MSTO flank tumors. By using *in vivo* IVIS bioluminescent imaging (Caliper LifeSciences) mice were imaged at multiple time points after injection to assess the fate of non-mesoCAR directed T cells when augmented with CCR2b.

Statistical analyses

For the *in vitro* killing assays, *in vivo* flank tumor studies, and *ex vivo* TIL analyses comparing difference between 2 groups, we used unpaired Student's *t*-tests. When comparing more than 2 groups, we used 1-sided ANOVA with appropriate post hoc testing. Differences were considered significant when $P < 0.05$. Data are presented as mean ± SEM.

Results

Chemokine production by MPM tumors

Our first goal was to assess which chemokines were produced consistently and at elevated levels by MPM tumors. Cell culture supernatants from 11 human MPM cell lines were analyzed by a multiplex cytokine assay. Of the 20 chemokines and cytokines examined, CCL2 was one of the most highly and uniformly expressed. Confirmation by using an ELISA, showed that the mean concentration of CCL2 was 3,500 ± 2,210 pg/mL/10^6 cells/24 hours, with 6 of the 11 lines secreting more than 500 pg/mL/10^6 cells/24 hours (Table 1; top). To confirm this observation in actual

Table 1. Supernatants from equal numbers of mesothelioma cell line cultures or pleural fluids from patients with mesothelioma were analyzed by ELISA for CCL2 concentrations (expressed in pg/mL)

	Cell line no.										
	H2502	**I-45**	**LRK**	**MSTO**	**M30**	**OK7**	**OK6**	**OK5**	**OK4**	**M108**	**mEMMESO1**
CCL2 (pg/mL/10^6 cells/24 h)	29	5,294	25,000	3,183	106	35	38	1,719	121	2,405	606
	Patient no.										
	101	**104**	**105**	**108**	**110**	**111**	**112**				
Pleural fluid CCL2 (pg/mL)	137	6,864	6,587	4,372	404	2,466	4,635				
Serum CCL2 (pg/mL)	ND	51	35	ND	129	AS	63				

Abbreviations: ND, nondetectable; AS, above standards.

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Table 2. CD4 and CD8 T cells from resting PBMCs, high-IL-2–stimulated TILs, anti-CD3/CD28 bead-activated isolated T cells were analyzed for CCR expression by FACs

	CXCR3 (%)	CCR2 (%)
CD4 T cells	CXCR3 (%)	CCR2 (%)
Resting	32.91 ± 7.32	5.43 ± 1.88
High IL-2	64.24 ± 8.72	10.11 ± 3.06
Anti-CD3/anti-CD28	82.72 ± 6.70	6.93 ± 0.44
CD8 T cells		
Resting	73.74 ± 2.46	5.03 ± 1.88
High IL-2	60.35 ± 12.00	13.32 ± 2.19
Anti-CD3/anti-CD28	97.35 ± 1.51	6.88 ± 1.01

NOTE: CXCR3 was highly expressed among all samples. CXCR1 and CCR2 were expressed at low levels (Numbers represent % of CD4 or CD8 T cells in each sample that were positive for CCR expression).

mesothelioma patients, CCL2 levels were measured by ELISA in pretreatment pleural fluids obtained from 7 MPM patients from an earlier clinical trial (35). The average concentration of pleural fluid CCL2 was 3,640 ± 1,030 pg/mL, with 5 samples showing greater than 2,400 pg/mL (Table 1; bottom). Serum levels were overall much lower.

Chemokine receptor expression on resting and activated T cells

We next analyzed which CCRs were expressed on activated T cells, with special attention to the receptor that binds to CCL2 (CCR2). Because different *in vitro* activation protocols can result in different CCR profiles (see, discussion), we com-pared expression levels on resting T cells from PBMCs, MPM TILs activated by high IL-2 supplementation (600 IU/mL), and bead-activated peripheral blood T cells (the method we are using in our clinical trials). FACS was conducted by gating on CD4 or CD8 positive lymphocytes (Table 2). Consistent with the literature (36–38), the most highly expressed CCR was CXCR3 (which binds CXCL9, 10, and 11), with expression as high as 83% of the bead-activated cells. In contrast, CCR2 was uniformly expressed at low levels among resting and activated T cells, although IL-2 stimulated TILs had higher expression levels than T cells activated by beads. In the bead-activated cells, on average, only about 7% of cells expressed CCR2. Under all conditions of activation, the intensity of CCR2 expression was low (representative FACS tracings are shown in Supplementary Fig. S3).

CCR2b can be successfully transduced into activated T cells

In light of the high CCL2 secretion by the MPM tumors but low CCR2 expression on the activated T cells, we hypothesized that T-cell trafficking to MPM could be augmented by genetically engineering T cells to express CCR2. Because CCR2b is the most dominant isoform of this receptor (39), a codon-optimized construct of CCR2b was generated and packaged into a lentivirus vector. Lentiviral vector transduction of T cells undergoing anti-CD3/anti-CD28 bead activation was achieved by using the lentiviral vector for mesoCAR alone or transduction by using both mesoCAR and CCR2b lentiviral vectors.

Compared with untransduced cells (Fig. 1; left), single mesoCAR transduction resulted in expression in approximately 30% of cells (Fig. 1; middle). FACS analysis post-transduction with both vectors revealed that approximately 12% of T cells expressed mesoCAR only, approximately 23% of cells expressed CCR2b only, and approximately 19% of T cells had robust expression of both mesoCAR and CCR2b (Fig. 1; right).

Functional activity of transduced CCR2b in activated T cells

Because CCR engagement by ligand induces an increase in intracellular calcium (40), we loaded transduced and untransduced T cells with the calcium-sensitive dyes, Fura Red and Fluo-3. Calcium flux was then monitored in real-time by using FACS. As shown in Figure 2A, addition of 100 ng/mL CCL2 (arrow) to untransduced T cells did not induce a calcium influx, whereas the subsequent addition of the calcium ionophore, ionomycin, (arrowhead), resulted in a large increase in the fluorescence ratio indicating calcium influx. In contrast, as shown in Figure 2B, addition of 100 ng/mL CCL2 (arrow) to CCR2b T cells resulted in a clear calcium spike (arrow), as did the subsequent addition of ionomycin (arrowhead), showing that the introduced CCR2b exhibited physiological signal transduction in T cells.

The chemotactic function of the transduced CCR2b was also tested. Figure 2C shows the results from transwell studies in which transduced or untransduced T cells were allowed to migrate through a 0.5 μm polycarbonate membrane toward media alone or media containing 100 ng/mL CCL2 in the lower well. Whereas CCL2 only increased the migration rate of mesoCAR T cells by 0.7% ± 2.7% compared with media alone, CCL2 induced a 45% ± 4.4% increase in migration of mesoCAR + CCR2b T cells compared with media alone ($P < 0.001$), indicating a robust functional enhancement of the CCR2b modified T cells.

The assay was repeated to evaluate migration of CCR2b T cells toward M108-conditioned supernatant (Fig. 2D). Relative to transwell migration of CCR2b T cells toward R10, supernatant from M108 induced over a 100% increase in transwell migration. Anti-CCL2 antibody caused complete inhibition of supernatant-induced migration, confirming the effect as being CCL2 dependent.

Effect of CCR2b transduction on T cell *in vitro* killing of M108

We next explored the susceptibility of M108 to mesoCAR and mesoCAR + CCR2b T cell-mediated lysis by using *in vitro* killing assays. As shown in Figure 3, when we added mesoCAR T cells to luciferase-labeled tumor cells at a

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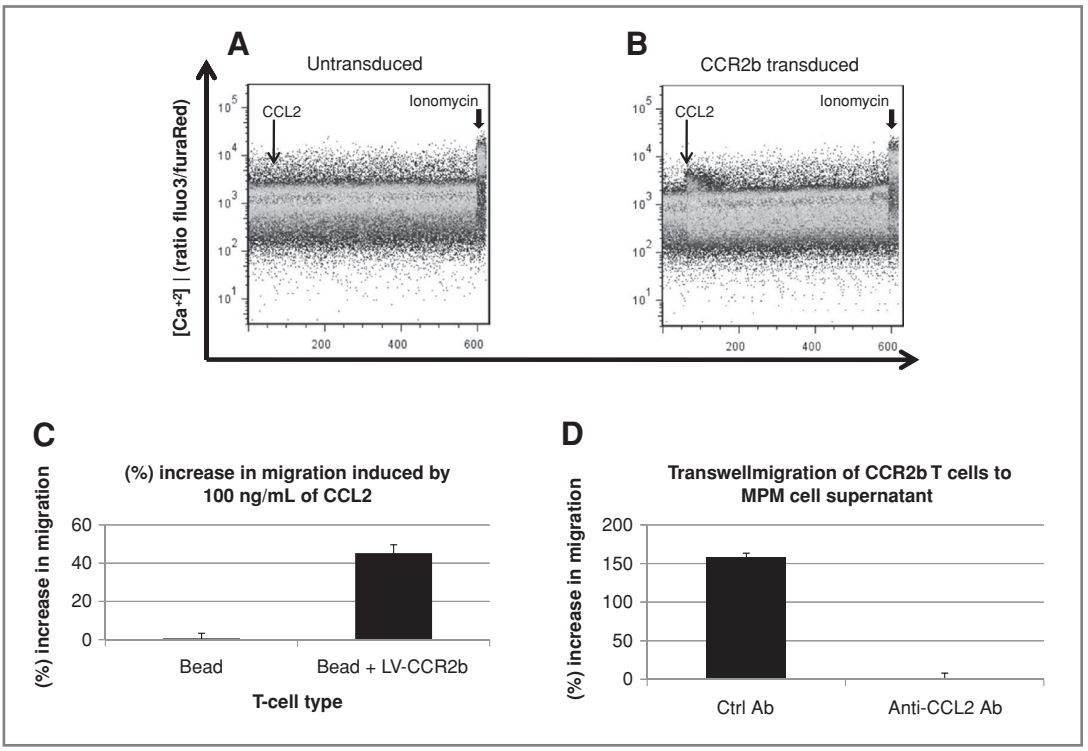

Figure 2. Ability of CCL2 to induce calcium flux and transwell migration in T cells. A and B, bead-activated T cells (A) or bead-activated, LV-CCR2b transduced T cells (B) were loaded with the calcium sensitive dyes (Fluo 3 AM/Fura Red AM) and studied by using FACS analysis over time. Cells were first exposed to 100 ng/mL of CCL2 (thin arrow) and then to ionomycin (arrowhead) to confirm equal loading. C, bead-activated, LV-CCR2b transduced T cells had 45% greater migration through 0.5 μm polycarbonate membranes toward R10 with 100 ng/mL of CCL2 than untransduced bead-activated T cells. D, M108 tumor supernatant also induced CCR2b T-cell migration in a CCL2 dependent manner. The addition of anti-CCL2 Ab blocked T-cell migration. Control Ab was added to media as a negative control.

constant ratio of 20 lymphocytes to 1 tumor cell and determined the number of live tumor cells after 4 hours, the T cells were able to kill 35.5% ± 1.9% of M108 cells. Interestingly, mesoCAR + CCR2b T cells were able to kill approximately 20% more M108 cells than mesoCAR T cells (55.2% ± 1.6% vs. 35.5% ± 1.9%, $P < 0.01$). Additionally, this effect was further augmented by approximately 13% when mesoCAR + CCR2b T cells were exposed to 100 ng/mL of CCL2 during coculture (55.2% ± 1.6% vs. 68.3% ± 3.2%, $P = 0.02$).

To address the question of whether interaction between CCL2 and CCR2b may nonspecifically activate T cells (which in turn could lead to potential "on-target/off-tumor" toxicity), we tested the ability of mesoCAR + CCR2b T cells to kill mesothelin-expressing versus non-mesothelin-expressing tumor targets in the presence of supplemental CCL2. As shown in Supplementary Figure S4, without tumor expression of mesothelin, the mesoCAR + CCR2b T cells were not able to kill tumor cells, even in the presence of CCL2.

Effect of CCR2b transduction on T-cell infiltration in NSG mice with M108 flank tumors

We next tested the ability of each type of activated T cell to traffic into established tumors. M108 cells were injected in the flanks of 4 groups (10 mice per group) of NSG mice, and tumors were allowed to grow to between 200 and 300 mm^3. Because this typically takes about 4 weeks, the tumors were well established, and previous work has

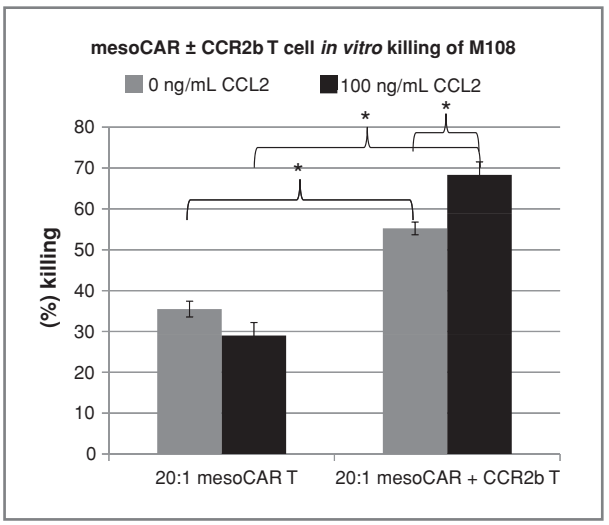

Figure 3. Effect of CCR2b on mesoCAR T-cell killing of M108 tumor cells. *In vitro* killing of tumor cells by T cells was assessed by coculturing mesoCAR ± CCR2b T cells with M108 tumor cells at 20:1 ratio for 4 hours in the presence and absence of CCL2 (*,$P < 0.05$).

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shown that the tumors are highly vascularized by this point (28). At this time, mice were injected with either (i) saline, (ii) 20 million untransduced, bead-activated T cells, (iii) 20 million bead-activated T cells transduced with mesoCAR alone, or (iv) 20 million bead-activated T cells transduced with both mesoCAR and CCR2b.

Five days after tail vein injection of T cells, 3 tumors in each group were harvested, digested, and subjected to FACS analysis (Fig. 4A). Because prior studies suggested that relative to the number of tumor cells, very few T cells traffic to tumor at this early time point, we chose to conduct FACS analysis of digested tumor instead of *in vivo* imaging. The abundance of human T cells present in the tumors injected with untransduced T cells (3,400 per million total cells) or with the mesoCAR T cells (4,100 per million total cells) was very low (~0.3%–0.4%) and not significantly different. In contrast, there was a large (>12.5-fold) and significant increase in the number of infiltrated T cells in the mesoCAR + CCR2b group (51,700 per million total cells = ~5.2%) compared with the mesoCAR group ($P = 0.02$) and/or the untransduced T cell group ($P = 0.02$).

Blood acquired via retroorbital bleeding from the 3 sacrificed animals was pooled and also subjected to FACS analysis (Fig. 4B). Mice in the untransduced group had 114,100 circulating T cells per million total cells, and mice in the mesoCAR T-cell group had 103,600 circulating T cells per million total cells. However, mice in the mesoCAR + CCR2b T-cell group had only 36,500 circulating T cells per million total cells. We interpret this to indicate that the increased TILs seen in the mesoCAR + CCR2b group tumors were because of enhanced trafficking and not enhanced systemic proliferation.

Effect of CCR2b transduction on antigen-independent T-cell trafficking to CCL2 secreting flank tumors

Although these data definitively show increased numbers of T cells within the tumors at day 5, it is difficult to say whether the increased numbers of T cells within the tumor were because of (i) increased trafficking, (ii) decreased apoptosis, (iii) increased proliferation, or (iv) increased retention in the tumor after the initial entry.

To explore these possibilities, we labeled T cells or CCR2b-bearing T cells with firefly luciferase and compared the ability of these cells to home to MPM tumor that made large amounts of CCL2 but did not express mesothelin (the MSTO cell line). As shown in Figure 4D, by 24 hours, both types of T cells initially trafficked to the liver, but as early as 24 hours after injection, the CCR2b T-cell injected mouse revealed T cells in the flank tumor, which continued to accumulate to a larger degree than in the control mouse. By the 3rd day, the control mouse had no T cells in the flank tumor, whereas some accumulation could be detected in the mouse injected with CCR2b T cells. By day 6, no T cells were detectable in either mouse by imaging. Because no antigen was provided and thus no T cell proliferation was induced, this study clearly shows increased trafficking of the CCR2b T cells to the tumor.

Effect of CCR2b transduction on antitumor activity in NSG mice with M108 flank tumors

In the same experiment described above (NSG; flank M108), we also observed significant differences in tumor growth over time (Fig. 4C). The tumors in the control and untransduced T-cell groups grew steadily at similar rates, reaching a size of approximately 500 mm^3 by day 22 after T-cell injection. In contrast, tumor size was significantly smaller ($P < 0.01$) in both the mesoCAR and mesoCAR + CCR2b groups than in the control or untransduced T-cell groups. Importantly, however, tumor size was significantly smaller in the mesoCAR + CCR2b compared with the mesoCAR alone group ($P < 0.01$). In fact, at day 22 post–T-cell transfer, virtually all of the tumors in mice injected with the single dose of mesoCAR + CCR2b T cells had completely regressed.

Thus, the presence of CCR2b in the mesoCAR T cells significantly increased the number of intratumoral T cells and significantly enhanced antitumor efficacy.

Discussion

This study showed that it was possible to increase the number of adoptively transferred T cells within large, well established, mesothelin-expressing tumors by matching the CCRs expressed on the activated T cells with the chemokines secreted by the tumor. This was accompanied by significantly enhanced antitumor efficacy, even in large established tumors.

Our study clearly showed increased numbers of CCR2b-expressing T cells within the tumor at 5 days, however, it is very difficult to say for certain whether the increased numbers of T cells within the tumor were because of (i) increased trafficking, (ii) decreased apoptosis, (iii) increased proliferation, or (iv) increased retention in the tumor after the initial entry. Although it is likely that all factors played a role, we do have some imaging data with a tumor that does not express mesothelin (and thus is unlikely to stimulate antigen-induced proliferation), showing increased trafficking of the CCR2b-expressing cells (Fig. 4D). The data showing decreased number of meso-CAR-CCR2b–expressing T cells in blood compared with untransduced or mesoCAR-transduced T cells also support the idea of increased trafficking.

There are a number of reasons to believe that increasing the number of T cells within a tumor (i.e., by improving T-cell trafficking) will be advantageous. The number of injected T cells that actually enter a tumor without targeting is extremely small (in our case ~0.3% of the tumor cells). After transduction with CCR2b, T-cell infiltration increased more than 12.5-fold, to represent approximately 5% of the tumor. Because adoptively transferred T cells must function within tumors (either through direct killing or activation of local immune cells), it seems desirable to have the largest possible number of T cells within the tumor. The rapid localization of T cells to tissues where their targeted antigen is highly expressed may also increase their ability to proliferate and thereby increase the effector to target ratio.

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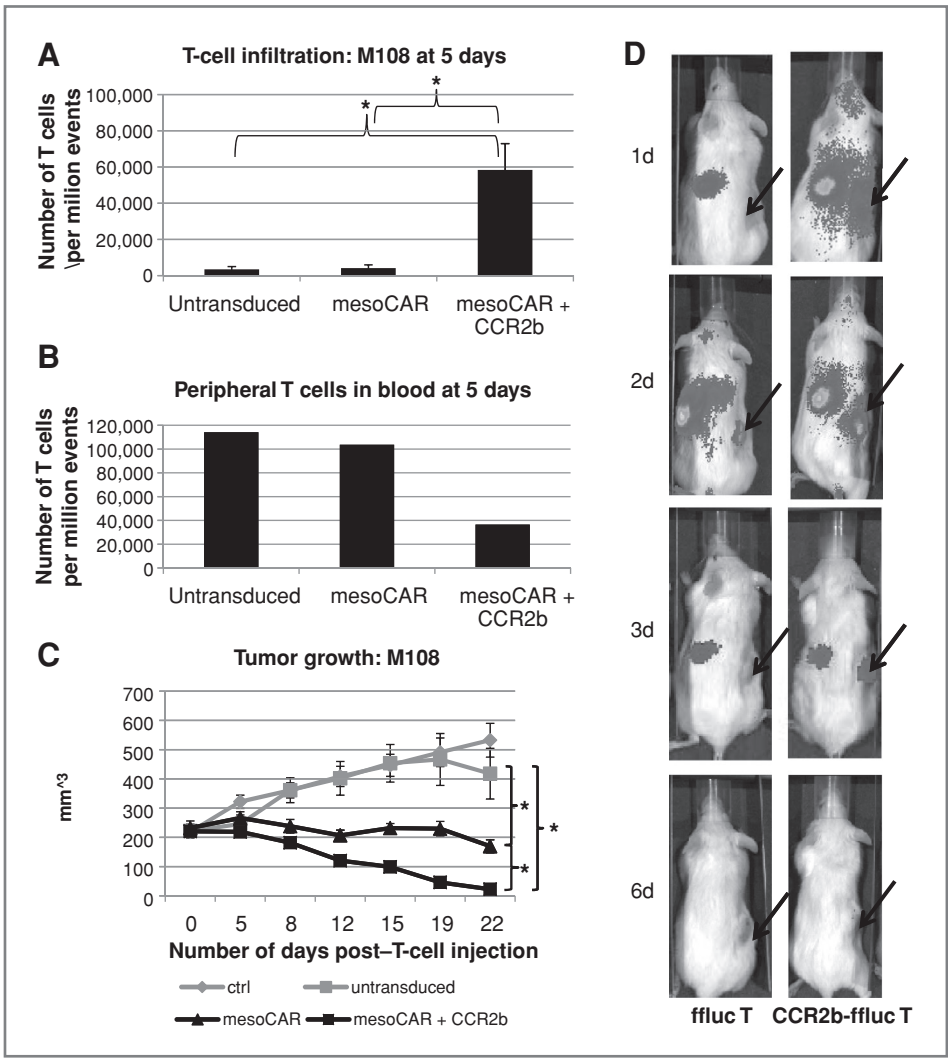

Figure 4. T-cell tumor trafficking and infiltration and tumor growth after intravenous injection of activated T cells. A and B, activated T cells were injected intravenously NSG mice with large established M108 tumors. Groups included no T cells (ctrl), bead-activated, but untransduced T cells (untransduced), bead-activated T cells transduced with mesoCAR (mesoCAR), and bead-activated T cells transduced with both mesoCAR and CCR2b (mesoCAR + CCR2b). Five days after T-cell injection, flank tumors (A) and pooled blood (B) from 3 mice in each group were subjected to FACS to determine the number of human T cells. There was a significantly increased number of T cells having infiltrated the tumor in the mesoCAR + CCR2b than the mesoCAR group (*, $P < 0.02$). C, M108 tumor size was measured over time and revealed significantly increased antitumor activity after injection of mesoCAR + CCR2b T cells compared with mesoCAR T cells. *, indicates statistically significant differences in mean tumor size at day 22 post–T-cell injection. Difference in mean flank tumor size in the mesoCAR and mesoCAR + CCR2b groups was statistically significant ($P = 0.003$). Tumor volume was calculated by using the following formula: volume = length × width × width/2. D, activated T cells were injected intravenously in NSG mice with large established MSTO tumors. Groups included bead-activated T cells transduced with firefly luciferase (ffluc T) and bead-activated T cells transduced with firefly luciferase and CCR2b (ffluc + CCR2b T). The first day after T-cell injection, the flank tumor of mice injected with ffluc + CCR2b T cells had T-cell infiltration as measured by IVIS bioluminescence imaging, whereas the ffluc T-cell mice did not. Flank tumor trafficking continued to be augmented in the ffluc + CCR2b T-cell group through day 3 postinjection. However, by day 6 postinjection, neither group had detectable bioluminescent imaging.

Others have shown that early involvement of tumor-reactive T cells is critical to effective ACT (41).

There may also be a number of important safety advantages to tumor targeting. Focusing T-cell activity to tumors could allow lower doses to be administered, thus increasing the therapeutic index, because the CAR cells will traffic to tumor instead of being distributed in the periphery where they could have deleterious "on-target/off-tumor" effects. This can be seen in Figure 4B, where CCR2b T cells were found in much lower number in the blood than the T cells lacking CCR2b. Minimizing "on-target/off-tumor" effects remains an important goal in light of case reports of fatalities resulting from CAR T-cell treatment toxicity (reviewed in ref. 14). In the example of mesothelin-targeted T cells, normal pericardial and peritoneal tissues express mesothelin, albeit at lower levels than tumor, and could be potential source of toxicity. Finally, it is possible that improving T-cell trafficking to tumors, where substantial

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antigen-induced local proliferation could occur, could lessen, if not eliminate, the need for the aggressive lymphodepletion regimens that are now being employed (42, 43). Lymphodepletion by using chemotherapy (e.g., cyclophosphamide and fludarabine) and whole-body irradiation presents substantial side effect risks and markedly increases the costs of current trials. Of course, this hypothesis remains speculative and will need to be further tested in appropriate animal models (see, below).

Our data show that T cells activated by engagement of their TCRs by using anti-CD3/anti-CD28 beads (the method we plan to use in future clinical trials) upregulate the expression of CCR7, CCR5 (data not shown), and most strongly, CXCR3 (from 15% to 49% of cells). Similar changes in CCRs after activation have been reported in the literature (44–46). We are focusing on the anti-CD3/CD28 bead activation protocol in our clinical trials because it has been our experience that feeder cells or artificial antigen presenting cells (aAPC), though able to sufficiently activate and expand T cells *in vitro*, compete for lentivirus vector, decreasing the overall resultant transduction efficiency in our final T cells. In addition, the quality control for the approval of feeder cells or aAPCs for use in clinical trial is more cumbersome and expensive than that for established artificial bead systems.

However, the type of stimulation used seems to make a difference in the CCR expression pattern observed (22, 47, 48). In the past, our group showed that more potent stimulation methods like anti-CD3/CD28 beads markedly downregulated CCR5, whereas less potent activation stimuli like PHA, Con-A, anti-CD3 Ab + IL-2, and plate-bound anti-CD3/CD28 antibodies did not completely downregulate CCR5 (50, 51). A similar situation seems to exist for CCR2. Brown and colleagues (22), reported that activation of PBMCs propagated with OKT3 antibody, irradiated allogeneic PBMC, EBV-transformed lymphoblastoid feeder cells, and 25 U/mL IL-2 (all relatively "mild" activation stimuli) resulted in T cells with high levels of CCR2 (see their Fig. 4C). We have also seen increased levels of CCR2 (20%–30% of cells) when we activate T cells by using modified K562 cells (49) comparable with the one used by Brown and colleagues. In contrast, activation with anti-CD3/CD28 beads markedly downregulated CCR2. Increased secretion of IL2 from T cells stimulated by the aAPCs (shown in Suhoski and colleagues) also likely plays a role based on our unpublished data showing that increasing concentrations of IL2 in the T-cell medium leads to upregulation of the CCR2 receptor. Brown and colleagues supplemented their T-cell growth cultures with 25 U/mL of IL2, whereas we did not.

The expression pattern of CCRs by using costimulated T cells indicates that these T cells would most likely be attracted to tumors expressing the chemokines CCL5 (binding to CCR5) or CXCL9, CXCL10, or CXCL11 (binding to CXCR3). However, when we examined a large panel of mesothelioma cell lines and pleural fluids from patients with malignant mesothelioma, we found that there was very little secretion of these chemokines (data not shown). Instead we observed substantial amounts of CCL2 expressed by most (but not all) cell lines and patient specimens. The other chemokine produced by many of the cell lines was CXCL8 (interleukin 8; data not shown). As the receptors for CXCL8 (CXCR1 and CXCR2) are not expressed on our T cells, we are also pursuing similar studies to introduce CXCR1 into T cells.

By introducing the properly matched CCR (i.e., CCR2b) into the T cells by using a lentiviral vector, we were able to achieve dual expression of the CAR and the CCR in almost one quarter of the cells (Fig. 1). This is similar to the results published by Di Stasi and colleagues (52) by using retroviral vectors for a CD30 CAR and the CXCR4 receptor. This relatively low percentage of doubly positive cells represents a technical limitation presented by the need for 2 independent lentiviral transductions, each with less than 100% efficiency. We did not purify these cells after activation, yet still saw impressive responses. We are now modifying our approach to use a biscistronic vector expressing both transgenes and are achieving higher dual expression levels and expect to further augment preclinical efficacy.

The concept of matching tumor-secreted chemokines with the appropriate CCR on the transfused, activated T cell, was first proposed by Kershaw and colleagues (47) who conducted *in vitro* studies matching secretion of the chemokine Groα (CXCL1) by melanoma cells with a retrovirally-transduced CXCR2 receptor in IL-2-activated T cells. Augmented migration of transduced T cells toward tumor supernatant was shown in a transwell assay, but no animal experiments were reported. The importance of chemokine/CCR matching was also shown by Brown and colleagues (22) in experiments showing that their activated T cells expressed CCR2 (unlike ours) and that these cells trafficked better to tumors from lymphoma, neuroblastoma, and melanoma cell lines that expressed high levels of CCL2. Subsequently, at least 3 groups have shown augmented trafficking and antitumor efficacy of TILs or CAR T cells in which chemokine receptor modification has been accomplished in models of Hodgkins lymphoma (by using a human CD30-CAR expressing CCR4; 52), neuroblastoma (by using a human GD2-CAR expressing CCR2b; 46), and melanoma (by using a mouse transgenic T-cell line expressing CXCR2; ref. 53). In these studies, expression and function of the transduced CCRs were confirmed *in vitro*, followed by demonstration of increased *in vivo* migration and a resultant slowing in tumor growth. However, in each of these studies, the modified T cells were introduced only 1 week or less from the time of xenograft tumor cell injection (thus before true "establishment" of the tumors), IL-2 was injected during the period of ACT, and multiple injections of T cells were given. Although IL-2 supplementation may promote survival of introduced T cells, there is now clear data showing that IL-2 also stimulates the expansion of Treg cells (54, 55)–clearly, an undesired effect, especially in MPM (56, 57). Additionally, Peng and colleagues (53) also injected peptide-pulsed dendritic cells and used flow cytometry-assisted sorting for CAR-positive T cells, whereas we did not. We feel that our study significantly extends these

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previous reports by (i) being the first report to show an enhancement of actual tumor regression (rather than just slowing of tumor growth), (ii) direct enumeration of the efficiency of CAR T-cell trafficking rather than by using estimates from bioimaging, (iii) showing that increased adoptive T-cell migration and antitumor activity are possible in a model of large established tumors, (iv) showing clear efficacy with only one dose of genetically modified T cells, and (v) showing that targeted 2nd generation CARs can be highly effective after intravenous injection without the need for additional adjuvant cytokine or cellular therapy. Interestingly, the *in vitro* data from this article suggests that CCL2–CCR2b interactions may actually augment T-cell effector function independent of its effects on trafficking, and studies to uncover this mechanism are planned.

Two potential limitations of this study relate to the animal model used. A subcutaneous tumor xenograft model was used to study MPM, a disease that normally occupies the pleural or peritoneal cavity. Flank tumors were used because the primary goal of this study was to study trafficking. We have previously used mesoCAR T cells to treat an orthotopic mesothelioma model and have shown this approach works well (29). However, because these animals have multiple small tumors studding the peritoneum, it would not have been possible to quantitatively study intratumoral trafficking. We plan to conduct future studies in peritoneal or pleural models focusing on efficacy. Although it is necessary to use NSG mice because of their unique ability to support human T cells, this model is highly artificial. First, these mice lack many normal components of the innate and acquired immune system making the tumor microenvironment abnormal. Second, there are species-specific interactions that cannot be duplicated in this model. For example, our mesoCAR T cells do not recognize mouse mesothelin, limiting our ability to assess toxicity. We have recently developed techniques to transfect the human mesoCAR into mouse T cells, enabling them to kill mouse tumors cells transduced to express human mesothelin. Although no model is perfect, we plan to use this "syngeneic system" to study important issues relating to lymphodepletion and the effects of a more normal tumor microenvironment.

One additional issue to consider would be sites other than MPM where CCR2 overexpressing T cells might accumulate, as this could determine potential toxicities. CCL2 can be produced by many cell types, including endothelial, fibroblast, epithelial, smooth muscle, mesangial, astrocytic, monocytic, and microglial cells (58–61), however, we were unable to find literature describing high levels of CCL2 being present in tissues during nondiseased states. Thus, it seems that CCL2 may not be primarily involved in normal homeostasis, but rather in pathologic processes (62). However, high levels of CCL2 have been reported in inflammatory diseases [e.g., rheumatoid arthritis, atherosclerosis (60, 63)], wound healing (64, 65), and malignant diseases [e.g., breast cancer, bladder cancer, gastric cancer (66–68)]. With regard to our mesoCAR + CCR2b T cells, it is thus possible that they could be recruited to sites of wound healing or inflammation, as well as to tumors. However, because of their very restricted antigen reactivity, they would only be potentially activated in the limited number of tissues expressing mesothelin at baseline (i.e., peritoneum, pericardium, and tunica vaginalis). For this reason, toxicity in those areas will thus be closely monitored in any clinical trials. This may be a more serious issue with CARs of different antigen specificity, depending on their potential off-tumor targets.

One of the goals of this study was to provide relevant preclinical data to support optimization of ongoing clinical trials. Our first target population for the mesoCAR T cells will be patients with MPM. This disease is highly associated with previous asbestos exposure and usually presents in the fifth to seventh decade of life with dyspnea, a pleural effusion, and nonpleuritic chest pain. Current therapies are inadequate, stimulating our group and others to pursue novel immune and immune-gene therapy approaches (24, 35, 69–72). As mentioned above, most MPM's express high levels of mesothelin (as do ovarian and pancreatic cancers). Once we have collected our baseline safety and efficacy clinical trial data, the findings from this study suggest that we can improve the approach employing tumor-targeting strategies by using CCR2b or potentially other CCRs. In addition, our data suggest that the efficacy of ACT, in general, might be improved in clinical trials by "personalizing" the injected T-cell chemokine receptor profile with the chemokines produced by the subject's own tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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