Pathogen-Boosted Adoptive Cell Transfer Therapy Induces Endogenous Antitumor Immunity through Antigen Spreading

Gang Xin1, Achia Khatun1,2, Paytsar Tophchyan1,2, Ryan Zander1, Peter J. Volberding1,2, Yao Chen1,2, Jian Shen1,2, Chunmei Fu3, Aimin Jiang3, William A. See4, and Weiguo Cui1,2

ABSTRACT

Loss of target antigens in tumor cells has become one of the major hurdles limiting the efficacy of adoptive cell therapy (ACT)-based immunotherapies. The optimal approach to overcome this challenge includes broadening the immune response from the initially targeted tumor-associated antigen (TAA) to other TAA s expressed in the tumor. To induce a more broadly targeted antitumor response, we utilized our previously developed Re-energized ACT (ReACT), which capitalizes on the synergistic effect of pathogen-based immunotherapy and ACT. In this study, we showed that ReACT induced a sufficient endogenous CD8+ T-cell response beyond the initial target to prevent the outgrowth of antigen loss variants in a B16-F10 melanoma model. Sequentially, selective depletion experiments revealed that Batf3-driven cDC1s were essential for the activation of endogenous tumor-specific CD8+ T cells. In ReACT-treated mice that eradicated tumors, we observed that endogenous CD8+ T cells differentiated into memory cells and facilitated the rejection of local and distal tumor rechallenge. By targeting one TAA with ReACT, we provided broader TAA coverage to counter antigen escape and generate a durable memory response against local relapse and metastasis.

See related Spotlight on p. 2

Introduction

Despite the unprecedented success of adoptive cell therapy (ACT), a considerable amount of patients become resistant to and relapse from the therapy after initial responses (1). Recurrence is frequently associated with the emergence of tumor cells that lose the initial ACT-targeted antigens, due to genomic instability and high mutation rates of malignant cells (2, 3). As antigen escape–related relapse has repeatedly been observed following monolithic antigen-targeted therapy, it has become one of the greatest obstacles for improving the clinical outcome of patients and expanding the spectrum of cancers treated with ACT. To overcome these issues, ACT-mediated immune responses must be broadened from the initial targeted antigen to other tumor-associated antigens (TAA; refs. 4, 5). Such a phenomenon, called antigen spreading, has been well-documented and positively correlates to the efficacy of immunotherapies, such as cancer vaccines and immune checkpoint blockade (6, 7).

Antigen spreading usually occurs following initial therapy-mediated tumor destruction, which leads to the release of secondary (i.e., non-targeted) TAAs (4, 5, 8). Subsequently, these antigens can be taken up by professional antigen-presenting cells, such as dendritic cells (DC), to induce T-cell responses. As a result, these broadly targeted antitumor immune responses may prevent the outgrowth of antigen escape variants and provide long-term protection against relapse. Despite these advantages, antigen spreading in ACT is rarely achieved at a meaningful level for therapeutic efficacy. This is likely associated with insufficient activation of Batf3-dependent conventional type 1 DCs (cDC1), which are required for the initial cross-priming of antitumor CD8+ T cells and effector T-cell recruitment into the tumor microenvironment (TME; ref. 9). Therefore, adequate activation of this DC subset is crucial to initiate the endogenous T-cell response against newly released TAA s (10).

In addition to primary tumor control, the ultimate goal of ACT is to generate memory T cells that protect against local relapse and distant metastasis. To avoid tumor growth at the site of the primary tumor, induction of a newly discovered subset of memory cells, known as tissue-resident memory (T RM) cells, is essential (11). T RM cells were first discovered in an infection model (12), where they provide first-line protection at the portal of pathogen entry during a secondary local infection (13, 14). A similar role of T RM cells in cancer has also been observed (15). In support of this notion, a few studies in a variety of human cancers have shown that increased tumor infiltration by T cells with a T RM cell-like phenotype correlates with improved overall survival in lung cancer (16, 17), breast cancer (18), bladder cancer (19), and ovarian cancer (20). More convincingly, local vaccines that induce melanoma-specific T RM cells potently suppress tumor growth in mucosal tissues compared with systemic immunizations, as observed in mouse models (16, 21). In contrast, preventing cancer metastasis to distal sites through the blood or lymph system may rely more on circulating memory cells. These cells, including central memory (T CM) or effector memory (TEM) cells, recirculate between the blood and lymphoid organs to continuously scan for previously encountered antigens (22). Consistent with this, the presence of circulating memory cells has been correlated with improved survival in patients with melanoma (23). Taken together, the optimal ACT should aim to generate both circulating and tissue-resident memory CD8+ T cells.

In an attempt to overcome antigen escape and prevent recurrence and metastasis, we utilized a newly developed approach that combines ACT with a pathogen-based vaccine, named Re-energized ACT
Xin et al.

Bone marrow chimeras and T-cell depletion

To generate Thy1.1 endogenous CD8+ T-cell bone marrow chimeras (BMC), CD8+/− mice were irradiated with a Gammacell 40 Exactor with two doses of 500 rad, each 4 hours apart. To obtain donor bone marrow (BM) from Thy1.1+/− mice, and CD51-c-diphertheria toxin receptor (DTR) mice were provided by Dr. Ainim Jiang from Roswell Park Comprehensive Cancer Center (Buffalo, NY). C57BL/6 Thy1.1+/− and Thy1.1 Pmel mice were obtained from The Jackson Laboratory. Pmel mice were crossed with C57BL/6 mice for at least four generations to generate Thy1.2/Thy1.2 Pmel mice. All in vivo experiments used 8- to 12-week-old females, and all animal procedures were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Tumor cell lines

All tumor cells were cultured in DMEM from Lonza supplemented with 10% Hyclone FBS (Thermo Fisher Scientific), glutamine (2 mmol/L; Corning), and penicillin/streptomycin (100 U/mL; Corning). For in vivo inoculation, tumor cells were passaged fewer than 5 times before each use. B16-F10 and E0771 were originally purchased from the ATCC and obtained from Dr. Susan Kaech (Salk Institute, San Diego, CA) in 2013, both of which have not been further authenticated and tested for Mycoplasma.

CRISPR-Cas9 system was used to generate a B16-gp100-KO cell line (25). Two gRNAs have been designed (5’CTGGCTGTGGG-GGCGCTCTGA’3’ and 5’CCACCCACACTACCTTCATA’3’) targeting the “EESGRNQDWL” immunogenic peptide on gp100 using benchling (https://benchling.com/crispr) and the CRISPR tool from the Feng Zhang lab, MIT (http://crispr.mit.edu/). Electroporation with a CRISPR/Cas9 ribonuclease-protein complex from IDT was performed following the protocol provided by IDT CRISPR genome editing (www.idtdna.com) using Amasca 4D Nucleofector system with program number DJ-110, specific for the B16-F10 cell line. Verification of CRISPR/Cas9 editing of gp100 in B16-F10 cell line was done by a Surveyor nuclease detection kit (IDT), according to the protocol (www.idtdna.com), using a single-cell clone following serial dilution.

The deletion of gp100 was further confirmed by in vitro killing assays using activated Pmel-1 TCR transgenic CD8+ T cells, which recognize the MHC class I (H-2D+)–restricted epitope of gp100. E0771-GP33 was transduced with LCMV-GP33-41 minigene under the control of actin promoter, which was kindly provided by Dr. Hanspeter Pircher (University of Freiburg, Germany). The expression of GP33 was verified by in vitro killing assays using activated P14 TCR transgenic CD8+ T cells, which recognize GP33. The B16-GP33 cell line was used because it enables monitoring of GP33 antigen–specific T-cell responses in tumor-bearing hosts using tetramer staining by flow cytometry.

Materials and Methods

Mice

C57BL/6 and C57BL/6 CD45.1/CD45.1 were purchased from NCI (Rockville, MD). Batf3−/− mice and CD11c-diphertheria toxin receptor (DTR) mice were transduced with the MSCV-IRES-GFP-OT-I vector (24) to generate Batf3-dependent DCs crucial for the induction of endogenous antitumor CD8+ T-cell responses. Endogenously activated CD8+ T cells could further differentiate into TREG cells and circulating memory T cells to provide optimal protection against local and systemic rechallenge. Overall, our study suggests that ReACT, although targeting only one tumor antigen, can mobilize endogenous T-cell responses against new TAs to overcome antigen escape and form long-term memory protection against cancer relapse and metastasis.

Bone marrow chimeras and T-cell depletion

To generate Thy1.1 endogenous CD8+ T-cell bone marrow chimeras (BMC), CD8+/− mice were irradiated with a Gammacell 40 Exactor with two doses of 500 rad, each 4 hours apart. To obtain donor bone marrow (BM) from Thy1.1+ mice and CD8+/− mice, femurs and tibiae were harvested, and BM was flushed out using RPMI with 10% Hyclone FBS (Thermo Fisher Scientific). Note that 3 × 106 cells consisting of a 30%/70% mixture of Thy1.1+ and CD8+/− BM cells were i.v. transferred to the irradiated recipients and allowed to engraft for 8 weeks. The reconstitution was confirmed by flow cytometry before the initiation of experiments. To deplete Thy1.1-expressing cells, anti-mouse Thy1.1 (BioXcell) was administered i.p. 6 to 9 days after tumor inoculation, which was also 1 day before ReACT. As control, another group of mice was injected with mouse IgG2a isotype control (BioXcell). All antibodies were administered every other day over the course of 2 weeks.

For CD11cDTR/WT and CD11cDTR/Batf3−/− mixed chimeras, 3 × 106 cells of a 1:1 mixture were injected into irradiated B6 mice and allowed to reconstitute for 8 weeks. For DC depletion, diphertheria toxin (DT; Sigma-Aldrich; 500 ng/dose/mouse) was injected i.p. 5 and 7 days after ReACT. Note that 1 × 106 P14 cells were transferred on day 6.
Pennsylvania School of Medicine, Philadelphia, PA) and provided by Dr. Susan Kaech. Tumor sizes were measured by caliper 3 times per week and expressed as tumor volume, calculated as [longest dimension \( \times \) (perpendicular dimension)]\(^2/2\). The mice with complete tumor eradication were classified as responders, whereas others that succumbed to tumor growth were classified as nonresponders. Once the tumor size exceeded 2,500 mm\(^3\), mice were euthanized. For the P14 transfer experiments, mice were sacrificed 8 days after ReACT for immune cell isolation.

In all recall experiments, mice that cleared primary tumors after ReACT and remained tumor-free for 1 or 2 months were used as memory mice, whereas age and gender-matched naïve mice were included as controls. For local rechallenge, mice were inoculated with \( 1 \times 10^3 \) E0771-GP33 tumor cells on the same flank where primary tumors (B16-F10) were eradicated. For systemic recall responses in the metastasis model, all mice were i.v. injected with \( 1 \times 10^3 \) B16-F10 cells and sacrificed 15 days later for harvesting lungs tissues, which were perfused with PBS and fixed by 10% formalin (Sigma-Aldrich). Subsequently, all tumor nodes in the lung were counted, and the images were acquired using a Zeiss Lumar V12 Stereoscope (Zeiss). For CD8 T-cell depletion, 500 ìg anti–mouse CD8 (clone 2.43, BioXcell) was administered i.p. twice weekly for 2 weeks after tumor rechallenge.

**Immune cell isolation and flow cytometry**

The tumors were dissected from mice 8 days after ReACT and minced into small pieces using scissors, followed by incubation with collagenase XI (0.7 mg/mL; Sigma-Aldrich), and bovine pancreatic DNase (30 mg/mL; Sigma-Aldrich) for 1 hour at 37°C. The tumors were then mashed against a 70 µm cell strainer to yield cell suspensions for isolating tumor-infiltrating lymphocytes (TIL) by Lymphocyte Cell Separation Medium (Cedarlane Labs). Subsequently, these single-cell suspensions were subjected to red blood cell lysis and filtration before staining for flow cytometry, as previously described (24). For blood cells, mice were bled from the facial vein, and immune cells were isolated by Histopaque (Sigma-Aldrich). Skins of the tumor-cleared area and distal area were dissected from memory mice and processed as described above. The spleens were mashed against a 70 µm cell strainer, followed by red blood cell lysis and filtration to generate single-cell suspensions for flow cytometry staining.

For Granzyme B staining, TILs were stained for surface markers before fixation and permeabilization (all buffers from Biologend). The permeabilized cells were then washed and stained with antibodies against granzyme B in permeabilization buffer. To assess the cytokine production of transferred P14 cells, \( 1 \times 10^6 \) TILs were stimulated with complete T-cell medium in the presence of GP33 peptide (1 µg/mL; Genscript), brefeldin A (Biologend), and IL2 (10 ng/mL; Peprotech) for 6 hours. The complete T-cell medium included RPMI with 10% heat-inactivated FBS (Hyclone), 10 mmol/L HEPES (Corning), 1% nonessential amino acids MEM (Corning), 1% sodium pyruvate (Corning), l-glutamine (2 mmol/L; Corning), penicillin/streptomycin (100 U/mL; Corning), and d-mercaptoethanol (Sigma-Aldrich). After incubation, surface markers were stained before fixation and permeabilization, followed by IFNγ staining. For IFR8 staining, TILs were fixed and permeabilized using the True-Nuclear Transcription Factor Buffer Set (Biologend) after surface staining. All antibodies and buffers are listed in Supplementary Table S1. All flow cytometry data were acquired on an LSRII (BD Biosciences) and analyzed by Flowjo (BD Biosciences).

**Enzyme-linked immunospot assay**

For enzyme-linked immunospot (ELISPOT) assays, an Immunospot mouse IFNγ ELISPOT Kit from CTL was used. Note that \( 1 \times 10^3 \) target cells (B16-F10 or B16-F10.G418\(^{res}\)) were treated with mouse IFNγ (500 U/mL; Peprotech) for 12 hours and then irradiated by Gammacell 1000 (120 Gy). Effector cells were CD8\(^+\) T cells isolated from spleen, draining lymph nodes (DLN), and lymph nodes (LN) from ReACT-treated mice that cleared tumors 1 month prior. Targets cells were seeded at 25,000 cells per well. Effector cells were seeded at 10\(^5\) cells per well. Plates were wrapped in foil, incubated for 24 hours, and then developed according to the manufacturer’s protocol. Plates were scanned using a CTL-ImmuNoSpot Plate Reader, and data were analyzed using CTL-ImmuNoSpot Software.

**Statistical analysis**

Graphs were generated and statistical analyses performed using GraphPad Prism version 5.02 (GraphPad Software). The overall tumor growth from endogenous CD8\(^+\) T-cell depletion was analyzed by two-way ANOVA, whereas the comparison of tumor-free mice after secondary challenge was determined by the log-rank (Mantel–Cox) test. For all other comparisons, t tests were used to determine the statistical significance (*, \( P < 0.05 \) and **, \( P < 0.01 \)).

**Results**

**ReACT induces an endogenous antitumor CD8\(^+\) T-cell response**

As we previously showed, ReACT (schematic shown in Supplementary Fig. S1A) sufficiently induces a strong antitumor CD8\(^+\) T-cell response and reverses the immunosuppressive TILs, which leads to complete tumor eradication in approximately 70% of B16-F10 tumor–bearing mice (24). Motivated by the robust efficacy of ReACT in primary tumor regression, we attempted to further explore the memory response in the current study. To this end, tumor-free survivors were inoculated with same tumor cells. The majority of mice (75%) were protected from rechallenge, indicating long-lasting adaptive immunity (Fig. 1A). Because ACT of tumor-reactive CD8\(^+\) T cells was part of the therapy, we reasoned that this protection was mediated by a CD8\(^+\) T-cell memory response. Indeed, depletion of CD8\(^+\) T cells in survivors before the tumor rechallenge almost completely abolished tumor rejection (Fig. 1A). In an attempt to characterize the presence of memory T cells, we failed to detect any adoptively transferred CD8\(^+\) T cells in spleen and LNs by flow cytometry (Fig. 1B). Taken together, these results raised at least two of the following possibilities: (i) The adoptively transferred CD8\(^+\) T cells formed memory cells, but they are too few to be detected, and (ii) ReACT might have elicited endogenous CD8\(^+\) T-cell responses against tumor cells, which formed long-lasting immunity following the primary tumor eradication.

To address this possibility, we first sought to evaluate the contribution of endogenous T cells in eliminating the primary tumor. To specifically deplete endogenous CD8\(^+\) T cells without affecting the transferred Pmel cells and other immune cells, we generated BMC mice. As illustrated in Fig. 1C, Thy1.1\(^+\) C57BL/6 (Thy1.1-B6) BM cells were mixed with Thy1.2\(^+\) Cbda\(^{–/–}\) BM cells at a 30%/70% ratio and transferred into irradiated Cbda\(^{–/–}\) mice. After reconstitution, we confirmed that all CD8\(^+\) T cells were Thy1.1\(^–\), whereas only 30% of all other T cells expressed Thy1.1 (Fig. 1D). The monoclonal Thy1.1 antibody was used to deplete all CD8\(^+\) T cells without affecting the majority of other immune cells (Fig. 1E and F; Supplementary Fig. S1B). Thy1.2\(^+\) Pmel cells were used as the cellular source of ReACT; therefore, they were spared from Thy1.1 depletion. BMC mice were inoculated with B16-F10 followed by ReACT, as previously...
described (24). The treatment of anti-Thy1.1 or IgG2a isotype control was started 1 day before ReACT therapy and sustained for a week. As expected, endogenous Thy1.1⁺ CD8⁺ T cells were effectively depleted without affecting the transferred Thy1.2⁺ Pmel cells (Fig. 1E and F). Consistent with previous results, ReACT induced robust tumor regression and eradication in around 70% of control isotype–treated mice.
whereas depletion of endogenous CD8^+ T cells resulted in a significantly reduced antitumor response. Although initial tumor regression was comparable between the two treatment groups until 10 days after therapy, eventually 5 of 7 Thy1.1-depleted mice relapsed and succumbed to the disease (Fig. 1G). Overall, these data suggested that ReACT mobilized the endogenous CD8^+ T-cell response, which played a vital role in eliminating B16-F10 tumors.

**ReACT prevents tumor antigen escape in vivo**

ReACT induced endogenous CD8^+ T-cell responses and prompted us to ask if we could use the ReACT strategy to overcome baseline antigen heterogeneity and antigen escape–mediated immune evasion by targeting a single tumor antigen. To test this idea on a B16-based melanoma tumor model with a controlled incidence of antigen escape, we generated a tumor variant that could not be recognized by a gp-100–specific TCR expressed by Pmel cells. Because it is well established that Pmel cells recognize the immunogenic epitope (EGSRNQDWL) on the melanoma antigen gp100 (26), we employed CRISPR/Cas9 technology to create a gp-100–null B16-F10 cell line. This new line had an insertion of one nucleotide at the beginning of this epitope, which induced a frame-shift mutation that altered the reading of subsequent codons and abrogated its expression. Based on this, the B16-gp100-KO tumor cell line was generated and sequenced to confirm the deletion (Supplementary Fig. S2A). Activated Pmel CD8^+ T cells could not elicit cytotoxic activity against B16-gp100-KO cells (Fig. 2A). Taken together, we successfully generated a tumor escape variant, which evaded tumor destruction mediated by transferred Pmel cells.

By inoculating mice with mixed parental B16-F10 with B16-gp100-KO cells at the indicated ratios (100%-0%, 95%-5%, 90%-10%, 80%-20%, or 0%-100%), we established tumors with various degrees of antigen escape. After 7 days, mice were treated with ReACT as previously described (24). Consistent with our previous observation, more than 70% of mice implanted with parental B16-F10 tumors cleared those tumors after ReACT (Fig. 2B). Complete and durable tumor regression was achieved in 60% of mice bearing tumors containing 5% of tumor escape variants (Fig. 2B), which suggested that ReACT induced protection against antigen loss–mediated tumor evasion. To further explore the efficacy of this protection, we increased the proportion of tumor escape variants (B16-gp100-KO) to 10% in the inoculation mixture. ReACT still induced pronounced tumor regression in these mice, and tumor eradication was achieved in 30% of mice (Fig. 2B). However, this tumor eradication rate was reduced to 10% in ReACT-treated mice when the inoculation mixture contained 20% B16-gp100-KO (Fig. 2B). As expected, ReACT failed to protect any mice when 100% B16-gp100-KO tumor cells were used (Fig. 2B). Collectively, these results indicated that ReACT could efficiently control antigen escape to a certain degree in the B16-F10 tumor model.

**Figure 2.**

**In vivo** antitumor activity of ReACT in the B16 tumor antigen escape model. A, B16-F10 and B16-F10^{gp100^+/-} cells were incubated with activated Pmel cells in the presence of Caspase 3/7 apoptosis green fluorescence detection reagent. The killing of tumor cell lines was measured in real-time by IncuCyte S3 Live-Cell Analysis System and shown in SEM among replicates. B, C57BL/6 mice were inoculated with WT B16-F10 or mixed (95% WT with 5% B16-gp100^KO), 90% WT with 10% B16-gp100^KO, and 80% WT with 20% B16-gp100^KO and 100% B16-gp100^KO. Seven to 10 days later, all mice were treated with ReACT, and tumor growth was monitored. Solid lines, nonresponders; dotted lines, responders. Data were compiled from two independent experiments; 10 mice/group. Significance was determined by two-way ANOVA.
ReACT elicits antigen spreading to expand the antitumor response

Next, we sought to characterize the ReACT-induced endogenous immune response, which potentially reacted to the new antigenic variants of tumor cells. For this study, we utilized B16-GP33 tumor cells, which are a derivative of the B16-F10 cell line modified to constitutively express GP\textsubscript{33-41}, a glycoprotein epitope from lymphocytic choriomeningitis virus. In this model, GP\textsubscript{33-41} tetramer\textsuperscript{+} cells were too few to be reliably detected in the tumor, P14 transgenic T cells, which express a TCR specific to GP\textsubscript{33-41}, were transferred into congenic mice to facilitate the detection of tumor-specific CD8\textsuperscript{+} T cells (Fig. 3A). These P14 chimera mice were inoculated with B16-GP33 tumors and received conventional ACT with Pmel cells, 

Intratumoral injection, or ReACT, respectively. Congenic markers were used to distinguish between P14 and Pmel cells, as well as CD8\textsuperscript{+} T cells from the hosts. Consistent with our previous report (24), ReACT significantly increased the accumulation and function of transferred Pmel (Thy1.1\textsuperscript{+}CD45.2\textsuperscript{+}) cells in contrast to conventional ACT (Fig. 3B and C).

Given the critical role of tumor-reactive T cells, other than Pmel cells, in the therapeutic effect against tumor escape, we next assessed the impact of ReACT on the quality and quantity of P14 cells. One week after treatment, we found that ReACT induced substantially greater intratumoral expansion of P14 cells than ACT or Listeria alone (Fig. 3B and C). Intratumoral injection of Listeria did elicit a stronger recruitment of P14 T cells than ACT, suggesting a bystander effect of bacterial infection–induced T-cell priming. Although P14 cells in Listeria-treated tumors released significantly higher IFNγ than the ACT-treated group in response to ex vivo restimulation with cognate GP\textsubscript{33-41} peptide, ReACT treatment provoked the strongest production of IFNγ among all treated groups (Fig. 3D and E). The enhanced effector function of P14 cells was further confirmed by ReACT–boosted granzyme B expression (Fig. 3F and G). Overall, our data suggested that ReACT induced antigen spreading, which was likely mediated by bacterial infection, and rendered the additional induction of CD8\textsuperscript{+} T-cell responses against tumor antigens not initially targeted by adoptively transferred T cells.

Batf3-dependent cDCs are required for ReACT-induced antigen spreading

An important component of our therapeutic strategy is bacterial infection, which is known to induce an inflammatory environment that bolsters DC-mediated cross-priming of T cells. Having established the critical role of antigen spreading in overcoming antigen escape during ReACT, we speculated that ReACT mobilized these CD8\textsuperscript{+} T cells by harnessing DC subsets that specialize in cross-presentation. To address this possibility, we first examined the effect of ReACT on the accumulation and activation of cDC1s by flow cytometry. Compared with ACT, ReACT significantly enriched the frequency of the tumor-residing CD11c\textsuperscript{+}CD26\textsuperscript{+}MHCIIC\textsuperscript{+}XCR1\textsuperscript{+}CD172a\textsuperscript{high} cDC1 population (Fig. 4A and B; Supplementary Fig. S3A), with heightened expression of classical cDC1 markers, such as CD11c, IRF8, and CD24 (Supplementary Fig. S3B; refs. 27, 28). Their expression of DC activation markers CD80 and CD40 was also enhanced by ReACT (Supplementary Fig. S3C).

Because cDC1s are essential for effector T-cell trafficking to the tumor and exhibit superior cross-presentation of cell-associated antigens, costimulation, and IL12 production over their counterpart DCs (10, 29), we hypothesized that cDC1s were required for ReACT-mediated antigen spreading. To test this, we utilized a mixed BMC model that can temporally deplete cDC1s to allow normal development of an antitumor immune response (Supplementary Fig. S4A). BMC mice were generated by mixing BM cells from transgenic mice expressing the DTR (under the control of a CD11c promoter) and Batf3-deficient mice at 1:1 ratio. The mixed BM cells were then transplanted into lethally irradiated C57BL/6 mice to generate CD11c\textsuperscript{DTR}/Batf3\textsuperscript{+}/– BMC mice. Because Batf3 is known to be essential for development of cDC1s (30), the reconstituted cDC1s from these BMC mice can only be derived from CD11c\textsuperscript{DTR} and are, therefore, subject to depletion by administering DT. To create control mice that preserved cDC1s after DT depletion, lethally irradiated C57BL/6 mice were grafted with BM cells from CD11c\textsuperscript{DTR} and WT mice to generate CD11c\textsuperscript{DTR}/WT chimeras.

After the successful reconstitution (Supplementary Fig. S4B), both CD11c\textsuperscript{DTR}/Batf3\textsuperscript{+}/– and CD11c\textsuperscript{DTR}/WT BMC mice were inoculated with B16-GP33 tumors, followed by ReACT treatment 1 week later. To determine the role for Batf3–lineage cDC1s in endogenous T-cell recruitment, depletion of DTR– DCs was performed 5 days and 7 days after ReACT in both groups of mice (Fig. 4C). One day after DT depletion, P14 CD8\textsuperscript{+} T cells were adoptively transferred intravenously to the mice, followed by assessment of recruitment to tumor 48 hours later (Fig. 4C). As expected, a significant reduction in cDC1s was observed in the tumors from Batf3\textsuperscript{+}/– reconstituted mice compared with WT reconstituted mice (Fig. 4D and E). Consequently, although Pmel cells remained unchanged (Fig. 4F and G; Supplementary Fig. S5A and S5B), the recruitment of P14 cells into tumors was almost abolished in CD11c\textsuperscript{DTR}/Batf3\textsuperscript{+}/– BMC mice (Fig. 4H). Collectively, these results indicated that tumor-residing cDC1s were required for ReACT–induced antigen spreading.

ReACT induces tumor-reactive CD8\textsuperscript{+} T cells to differentiate into T\textsubscript{RM} cells

Batf3-dependent cDC1s promote the generation of T\textsubscript{RM} cells (11, 31), which have been shown to provide optimal tumor protection. We previously showed that 60% of mice that cleared tumors after ReACT rejected a local rechallenge (24) and that this protection was dependent on CD8\textsuperscript{+} T cells (Fig. 1A and B). Next, we sought to distinguish the relative contribution of CD8\textsuperscript{+} T\textsubscript{RM} cells derived from transferred Pmel cells versus those recruited endogenously by ReACT. To test this, we characterized memory T-cell development in a model that tracks antigen-spreading CD8\textsuperscript{+} T cells (P14 cells) recognizing a distinct TAA (GP33). P14 T cells declined in the blood 2 weeks following treatment and underwent a similar contraction as the adoptively transferred Pmel cells (Fig. 5A). Consistent with data shown in Fig. 1B, 1 month after tumor eradication, we did not readily detect Pmel cells in circulation, whereas a small portion of P14 cells developed into circulating memory cells (Fig. 5A). P14 cells, but not Pmel cells, were also recovered in the lesion area of the skin where the tumor was eradicated (Fig. 5B and C). Because these antigen-specific T cells were found within the skin lesion, we further examined their expression of T\textsubscript{RM} markers. Almost all P14 cells from the local skin were CD62L\textsuperscript{lo} and high expression of CD103 and CD69 compared with splenic CD44\textsuperscript{+} CD8\textsuperscript{+} T cells (Fig. 5D and E), which is consistent with T\textsubscript{RM} phenotype.

Based on emerging evidence that highlights the important role of T\textsubscript{RM} cells in mediating optimal tumor protection (21, 32, 33), we next assessed whether P14 T\textsubscript{RM} cells were capable of providing protection against local recurrence. ReACT-treated mice that cleared tumors were rechallenged with an irrelevant E0771 breast tumor cell line expressing GP33 (the only shared antigen with the previously eradicated B16-
As a control group, naïve mice were inoculated with E0771-GP33 cells and successfully established tumors within 2 weeks. On the contrary, ReACT-treated mice not only significantly delayed tumor establishment, but also approximately 60% of mice achieved complete protection against rechallenge (Fig. 5F), suggesting successful formation of a memory CD8⁺ T-cell response against escape variants. These data demonstrate that ReACT-induced tumor-specific P14 cells differentiated into memory T cells, including TRM cells, which were the main contributors of long-term protection against local relapse.
ReACT offers long-term protection against systematic recall

Besides local T_{RM} cells, circulating memory T cells are also required for ideal antitumor immunity, especially long-term immune protection against distal metastasis (32). To investigate the impact of ReACT on memory cell formation in lymphoid organs, mice that cleared tumors after ReACT were sacrificed 1 month after tumor eradication. After stimulation with B16-F10 tumor cells, their splenic T cells produced significantly more IFNγ than naïve splenic T cells (Fig. 6A and B). The gp100-deleted B16-F10 cell line also could stimulate IFNγ production in splenic T cells from ReACT-treated mice, suggesting that ReACT induced a broader T-cell response to multiple tumor antigens. Next, we explored whether ReACT-induced circulating memory cells could confer protection against systemic metastasis in a well-established lung metastasis model (34). To do this, ReACT-treated (after eradication) or naïve mice were i.v. rechallenged with B16-F10 cells and sacrificed 15 days later for examination of the tumor foci in the lungs. As expected, mice with eradicated tumors after ReACT displayed a 30.7-fold reduction in number of lung tumor foci compared with naïve mice, as confirmed by histologic examination (Fig. 6C and D). This indicated that ReACT also induced the development of effector memory CD8+ T cells for systemic protection against metastases. Overall, we have shown that ReACT could expand the immune response beyond the initial target to

Figure 4.
Activation of endogenous tumor-reactive CD8+ T cells is dependent on BATF3-driven cDC1s. B16-F10 tumor-bearing mice were treated with either ACT or ReACT 7 to 10 days following tumor inoculation. One week after therapy, tumor samples were harvested and analyzed by flow cytometry for the cDC1 population. The frequency of XCR1highCD172alow cDC1s among Lin−CD11c+CD26+ MHCII+ cells is shown in representative flow plots (A) and cumulative dot graphs (B). C, CD11cDTR/WT (WT) and CD11cDTR/Batf3−/− (Batf3−/−) BMCs were generated and inoculated with B16-GP33 tumors, followed by ReACT. Five days after ReACT, DT was given to deplete DTR-expressing Dcs. The following day, 1 × 10^6 P14 T cells were transferred, and their accumulation within the tumor was assessed 8 days later by flow cytometry. The frequency of Lin−CD11c+CD26+ MHCII−XCR1−CD172a− cells is shown in representative flow plots (D) and a scatter plot (E). The frequency of GP33+ P14 and GFP+ Pmel CD8+ T cells in tumors is shown in representative flow plots (F) and scatter plots for Pmel (G) and P14 cells (H). Data represent pooled data from two independent experiments with n = 8 for WT and n = 9 for Batf3−/−. Mean ± SEM is shown. The significance was determined by t test (**, P ≤ 0.01; ***, P ≤ 0.001; n.s., not significant).
include new antigenic targets, which could provide protection against antigen escape and relapse.

Discussion

CAR T-cell therapy trials and other ACT studies demonstrate that antigen escape has emerged as a major issue (35–37), affecting the durability and efficacy of therapies. In this study, we showed that ReACT induced antigen spreading and expanded the antitumor response from one initial target antigen to multiple TAAs. As a result, this approach significantly prevented outgrowth of antigen escape variants. ReACT also provided optimal priming for endogenous T cells to differentiate into tissue-resident and circulating memory cells that provided long-term protection against local relapse and distant metastasis.

Antigen escape is reported in CD19 CAR T-cell trials in B-cell acute lymphoblastic leukemia (38). The spontaneous mutation and selective expansion of antigen-negative tumor cells in CD19 CAR T-cell therapy are well-documented, and the durability of clinical response is severely limited by the outgrowth of CD19-negative leukemia cells (3). Currently, clinical trials demonstrate that the loss of the target antigen CD19 accounts for 38% to 75% of total relapses (35–37). The outgrowth of tumor escape variant cells has also been observed in solid tumors and contributes to acquired resistance of melanoma to vemurafenib (39). Therefore, an innovative therapy is desperately needed to induce an antitumor immune response targeting a broad range of TAAs to overcome this challenge. To combat antigen escape, transferred T cells can be equipped to simultaneously target two TAAs (2, 40, 41). Despite the initial success of bispecific ACT, high
A more reasonable strategy to successfully tackle this problem is engaging endogenous escape variants that lose both targeted antigens. A more reasonable tumor mutation rate will inevitably result in the emergence of tumor escape variants that lose both targeted antigens. Indeed, the endogenous immune system is capable of antigen spreading, the generation of an immune response against antigens distinct from the initial immune-targeted epitope (4, 5). Such a phenomenon has also been observed in a study using a murine CAR model targeting EGFR (42). Mice that cleared EGFR\(^+\) tumors with the therapy later rejected EGFR\(^-\) tumors when rechallenged (42). This elegant model demonstrated that antigen spreading can be induced by ACT therapy and may be important in dealing with tumor heterogeneity and preventing tumor escape via antigen loss variants (43). However, it is unclear how to enhance the incidence and effectiveness of this phenomenon to counter immune escape.

As we previously reported (24), we developed ReACT, which mediates significant initial tumor destruction, providing a source of new TAAs, which can be cross-presented by DCs, priming endogenous CD8\(^+\) T cells toward an effector phenotype, thus potentially allowing enhanced antigen spreading. Indeed, infectious agents have been shown to induce antigen spreading from microbial to self-epitopes in several autoimmune diseases (5, 44, 45), which is associated with their ability to activate DCs. Stimulation of Toll-like receptor signaling on the cDC1 lineage induces prominent secretion of proinflammatory cytokines, IL12p70 (46), and chemokines such as CCL3, CCL4, and CCL5 (47). The combination of T cells causing tumor cell lysis, bacteria-induced recruitment of DCs, and the inflammatory milieu of immune cytokines creates an optimal environment for DC activation and cross-priming in the presence of tumor antigen. Using a model of mice engrafted with a mixture of B16-F10 and target-loss mutant (B16-gp100-KO) cells, we demonstrated that ReACT successfully elicited an endogenous CD8\(^+\) T-cell response with heightened effector function and reactivated to the surrogate TAAs not targeted by ReACT. Consequently, tumors containing escape mutants effectively regressed or were completely eradicated. Our approach also has potential to overcome baseline tumor heterogeneity. Due to the substantial diversity of tumor cells, single-target ACT often fails to achieve complete eradication of the tumor. ReACT-induced antigen spreading provides an opportunity to eliminate the tumor cells that do not express the originally targeted antigen.

Mechanistically, we also discovered that the expanded T-cell response critically relied on cDC1s, which are required to orchestrate an effective endogenous effector T-cell response (9). These migratory cDC1s depend on a transcription factor, BATF3, and very efficiently cross-present extracellular antigens, particularly cell-associated antigens, to CD8\(^+\) T cells (47). Therefore, many studies employ Batf3-knockout mice that are deficient in cDC1s to reveal its critical role in T-cell recruitment to the tumor site (9). cDC1s produce chemokines CXCL9 and CXCL10, the corresponding ligands for CXCR3 on effector T cells, which mediates their infiltration into the tumor (9). Our results are also supported by reports on Listeria monocytogenes, the bacteria used in the ReACT, showing that cDC1s are found to not only recruit effector memory T cells but also reactivate them at the site of infection (48, 49). Consistent with these studies using the same pathogen, we have clearly demonstrated that ReACT failed to induce effector CD8\(^+\) T-cell migration into tumors in the absence of cDC1s. Lack of Batf3 in these mice led to a selective loss of cDC1s in the tumor site, whereas all other CD11b\(^+\) DC subsets remained undisturbed. When these mice were challenged with B16-GP33 tumors and treated with ReACT, a significant reduction in the intratumoral endogenous CD8\(^+\) T cells was seen, which is crucial for overcoming antigen escape.

Optimal tumor immunotherapy should offer long-term protection against relapse by generating a potent memory CD8\(^+\) T-cell response, which requires specific subsets of DCs to provide optimal priming (11). It has been shown that Batf3-driven DCs provide unique priming signals including IL12, IL15, and CD24-mediating costimulation for differentiation of skin TRM cells (11). Compared with circulating memory T cells, TRM cells respond faster to stimulation and are equipped with a more potent effector response, thus providing instant protection against local rechallenge (12, 15, 17). In our study, we demonstrated that ReACT generated functional endogenous TRM cells in skin following tumor elimination, which contributes to its robust recall response, resulting in
tumor rejection. We also provided evidence on the formation of circulating memory T cells in mice with ReACT-eradicated tumors, which played a vital role in preventing distal metastasis.

Despite the translational potential of ReACT, several limitations and potential side effects must also be considered and addressed. First, therapy is based on the local injection of bacterial material; therefore, the targeted tumor has to be relatively accessible and over a certain threshold of size. Consistent with other intratumoral immunotherapies (50), our preliminary study also revealed that ReACT could induce an antitumor immune response against the distant tumor, which requires further characterization in future studies. Secondly, antigen spreading can be beneficial to the host by resulting in protection against antigen-negative tumor cells, but it carries the risk of autoimmunity (21). No obvious adverse effects and health issues were observed in treated mice. Occasionally, we did observe that few mice developed mild vitiligo that was confined in the local region of tumor inoculation, without affecting their health condition. Consistently, a study has elegantly shown that skin-resident memory T-cell responses to melanoma are generated naturally as a result of vitiligo (51). Therefore, future work must be done to eliminate the possibility of immune response specificity spread to self-antigens, causing severe inflammation and tissue damage. As to the choice of pathogens, because multiple genera of bacteria, such as Clostridia and Salmonella, have been clinically verified for safety (52), future studies are needed to determine the efficacy of ReACT using other pathogens with proper tropisms to various types of cancer for broader clinical applications.

Overall, although the therapeutic efficacy of ACT could be impeded by tumor-mediated immune escape via loss of targeted antigen expression, we presented a possible solution to this challenge. ReACT, an approach that promotes endogenous CD8+ T-cell priming with TAAVs via cDC1s, effectively prevented the outgrowth of antigen escape variants. We also demonstrated that ReACT optimized TRM development, forming long-term immunologic memory to prevent cancer relapse.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: G. Xin, W.A. See, W. Cui
Development of methodology: G. Xin, A. Khatun, W.A. See, W. Cui
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Xin, P. Topchyan, P.J. Volberding, Y. Chen, J. Shen, C. Fu, A. Jiang, W.A. See, W. Cui
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Xin, A. Khatun, C. Fu, A. Jiang, W. Cui
Writing, review, and/or revision of the manuscript: G. Xin, P. Topchyan, R. Zander, C. Fu, A. Jiang, W.A. See, W. Cui
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Cui
Study supervision: W. Cui

Acknowledgments
This work is supported by NIH grants AI125741 (W. Cui) and CA 198105 (A. Jiang) and by American Cancer Society Research Scholar (W. Cui). R. Zander is supported by the Cancer Research Institute Irvington Fellowship. G. Xin is supported by The Elizabeth Elser Doolittle Postdoctoral Fellowship. W. Cui is supported by the Medical College of Wisconsin Cancer Center and The Barlog Endowment Fund.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 4, 2019; revised September 6, 2019; accepted November 1, 2019; published first online December 12, 2019.

References
Pathogen-Boosted Adoptive Cell Transfer Therapy Induces Endogenous Antitumor Immunity through Antigen Spreading

Gang Xin, Achia Khatun, Paytsar Topchyan, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-19-0251

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2019/11/12/2326-6066.CIR-19-0251.DC1

Cited articles
This article cites 48 articles, 15 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/8/1/7.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/8/1/7.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/8/1/7.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.