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Activation of Tolerogenic Dendritic Cells in the Tumor Draining Lymph Nodes by CD8⁺ T Cells Engineered to Express CD40 Ligand

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Tolerogenic dendritic cells in the tumor microenvironment can inhibit the generation and maintenance of robust antitumor T cell responses. In this study, we investigated the effects of local delivery of CD40L by tumor-reactive CD8⁺ T cells on dendritic cell activation and antitumor T cell responses in the TRAMP model. To increase the immunostimulatory signal, CD40L was engineered, by deleting the majority of the cytoplasmic domain, to increase its levels of expression and duration on the surface of CD8⁺ T cells. Tumor-reactive CD8⁺ T cells expressing the truncated form of CD40L stimulated maturation of dendritic cells in vitro and in the prostate draining lymph nodes in vivo. Following dendritic cell maturation, a significantly higher fraction of adoptively transferred, tumor-reactive (reporter) CD8⁺ T cells was stimulated to express IFN-γ and infiltrate the prostate tissue. The antitumor CD8⁺ T cell response was further enhanced if TRAMP mice were also immunized with a tumor-specific Ag. These findings demonstrate that augmented T cell responses can be achieved by engineering tumor-reactive T cells to deliver stimulatory signals to dendritic cells in the tumor microenvironment.

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Abbreviations used in this paper: 2C/RAG, RAG1⁻/⁻ 2C TCR transgenic; ACT, adoptive cell transfer; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; PDLN, prostate-draining lymph node; PLN, peripheral lymph nodes; SIY, SYFV-RYYGL; TDLN, tumor-draining lymph node; WSN-SIY, WSN strain of influenza virus expressing SIY.

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can stimulate the maturation of dendritic cells in the prostate-draining lymph nodes (PDLNs) and augment antitumor responses of adoptively transferred, tumor-specific reporter CD8+ T cells. These findings demonstrate that augmented anti-tumor T cell responses can be induced by engineering T cells to deliver a CD40L-mediated stimulatory signal to dendritic cells in the tumor environment.

Materials and Methods

Mice, influenza virus, Abs, and flow cytometry

TRP-SIY mice were generated by expressing a nominal Ag SIYRYYGL (SIY) in the prostate tissue of TRAMP mice, as reported previously (39). Mice used for all experiments were at 3.5–4.5-mo-old heterogeneous males. The RAG1−/− 2C TCR transgenic (2C/RAG) mice were maintained on C57BL/6 or C57BL/6ByThy1.1 backgrounds. Recombinant WSN strain of influenza A expressing a SIY-neuraminidase fusion protein was described previously (40). For infection, TRP-SIY mice were infected intranasally with 100 pfu WSN-SIY virus in 50 μl PBS. Mice were maintained in a specific pathogen-free facility, and all animal experiments were performed in compliance with the institutional guidelines on animal care.

Agonist anti-CD40 Ab (PGK45.5) was a gift from Dr. A. Rolink of the Basel Institute for Immunology, Basel, Switzerland (41). Abs to CD40L (eBioscience, San Diego, CA), CD8 (BioLegend, San Diego, CA), Thy1.1 (BioLegend), CD11c (BioLegend), CD80 (BD Pharmingen, San Diego, CA), and CD86 (BD Pharmingen) were conjugated to FITC, PE, or allophycocyanin. The 2C TCR was identified using a biotin-conjugated 1B2 clonotypic Ab, detected with streptavidin-APC (BioLegend). Retrovirally-transduced 2C T cells were identified using 1B2 plus an Ab to either Thy1.1 or CD8. Ab-stained cells were analyzed using a FACScalibur (BD Biosciences) instrument, and the data were processed and evaluated using FlowJo software (Tree Star, Ashland, OR).

Intracellular IFN-γ staining was performed using a BD Cytofix/Cytoperm Kit (BD Biosciences). 2C T cells were stimulated in vitro with 1 μg/ml SIY peptide for 4 h at 37°C in the presence of BD GolgiPlug (BD Biosciences) containing brefeldin A. Cells were stained for 2C TCR, plus SIY peptide for 4 h at 37°C in the presence of BD GolgiPlug (BD Biosciences) containing brefeldin A. Cells were stained for 2C TCR, plus CD8 or Thy1.1, and dead cells were marked using a LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen, Carlsbad, CA). After fixation and permeabilization, cells were stained intracellularly with a PE-conjugated anti-IFN-γ Ab (BD Pharmingen).

Anti-CD40 treatment

TRP-SIY mice were injected i.p. with 500 μl of either PBS or 200 μg anti-CD40 Ab in PBS daily for 3 d. One day after the first anti-CD40 injection, the treated mice were injected retroorbitally with 100 μl PBS containing 1.0 or 1.5 x 10^6 naive 2C cells from the spleens and lymph nodes of 2C/RAG mice. Immediately following T cell transfer, mice were infected intranasally with WSN-SIY virus (39). Five and 10 d postinfection (dpi), 2C T cells from the PDLNs, spleens and peripheral lymph nodes (PLNs) were analyzed for IFN-γ expression, as above.

Construction of retroviral vectors expressing wild type and mutant CD40L

pMierCD40L plasmid, which contains full-length murine CD40L (GenBank accession no. X0545532.1 (42)), was obtained from Dr. Richard Kornbluth of the University of California at San Diego. A BglII/SalI generated full-length CD40L fragment (wild type) was then subcloned into a retroviral vector that expresses Thy1.1. Mutation of tyrosine at amino acid residue 5 to alanine (TAC/GCC) of CD40L (CD40L Y5A) was performed through site-directed mutagenesis, using a QuickChange II Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA). To delete the first threonine amino acid residues of the N-terminal cytoplasmic domain of CD40L (CD40L Δ1-13), amino acid residue 12 was first mutated from TCT to TTC through site-directed mutagenesis to generate a BglII site. The deletion was then completed by BglII digestion and subcloning into the retroviral vector above. Following ligation, a start codon was reintroduced at amino acid 13 (GTG→ATG). Retroviruses expressing wild type and mutant CD40L were produced in 293FT cells via cotransfection with pCL-Eco (Imgenex, San Diego, CA), which encodes the retroviral packing components, using TransIT-LT1 (Mirus, Madison, WI).

Retrovirus infection and generation of in vitro memory T cells

Naive 2C cells were pooled from the spleens and lymph nodes of 2C/RAG mice after lysis of RBCs. 2C cells were stimulated for 36 h with 1 μg/ml SIY peptide in RPMI plus 10% FCS, 50 μM 2-ME, 10 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 U/ml IL-2. Activated 2C cells were then transduced by spin infection with the indicated retroviral supernatant plus 4 μg/ml polybrene (American Bioanalytical, Natick, MA), and cultured for another 48 h at 5 x 10^6 cells/ml in the above medium. Cells were then rinsed and cultured in the same medium, except that IL-2 was substituted with recombinant murine IL-7 (Peprotech, Rocky Hill, NJ), for 6 d to generate in vitro memory T cells.

In vitro bone marrow-derived DC maturation assay

Retrovirally-transduced in vitro memory 2C cells were restimulated by mixing the cells 1:1 with C57BL/6 splenocytes in the presence of 1 μg/ml SIY peptide for 48 h. CD40L-expressing 2C cells were purified by sorting for Thy1.1 cells. The sorted T cells were then cocultured at a 10:1 ratio with day 6 immature bone marrow-derived dendritic cells (BMDCs). To generate immature BMDCs, bone marrow was harvested from the femurs of C57BL/6 mice. After lysis of RBCs, the cells were resuspended at 2 x 10^7 cells/ml and cultured for 6 d in RPMI plus 10% FCS, 50 μM 2-ME, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and I5 cell supernatant containing GM-CSF. As controls, BMDCs were cultured alone, in either the presence or absence of 1 μg/ml LPS. In the absence of LPS, control BMDCs were either left unmanipulated or were taken off the tissue culture plate and concentrated (10^8 cells/ml), to simulate the other coculture conditions. Following a 24 h coculture, maturation of BMDCs was evaluated by flow cytometry for CD11c, CD86, and CD80 expression.

In vivo DC activation and reporter T cell response

In vivo memory 2C cells either expressing or not expressing CD40L were transferred into TRP-SIY mice (1 to 5 x 10^6 cells/mouse). A subset of mice was also infected with WSN-SIY. Six days later, the maturation status of the DCs in the PDLNs, PLNs, mediasstemial lymph nodes, and/or spleens was evaluated by assaying for CD11c, CD11b, CD86, and CD80 expression. Alternatively, 6 d after the transfer of in vivo memory 2C cells, naive Thy1.1(high) reporter 2C cells were transferred into the TRP-SIY mice. Five and 10 d after transfer, reporter 2C cells from the PDLNs, PLNs, and spleens were evaluated for IFN-γ expression, as above.

Results

Systemic anti-CD40 treatment breaks tolerance transiently in TRAMP mice

We have previously developed transgenic mice that express a nominal Ag SIYRYYGL (SIY) in the prostate tissue that can be recognized by CD8+ T cells displaying the 2C TCR (39). Introduction of the transgene into TRAMP mice results in the generation of double transgenic TRP-SIY mice, which develop SIY-expressing prostate cancer. When TRP-SIY mice are infected intranasally with WSN-SIY, adoptively transferred 2C cells become fully activated, infiltrate the prostate tissue, but rapidly lose their function in the tumor tissue (39).

To determine the effects of anti-CD40 treatment on T cell activation and function, TRP-SIY mice were injected with either PBS or an agonist anti-CD40 Ab one day before, on the same day and one day after 2C cell transfer and WSN-SIY infection. Five days postinfection, ~67% of 2C cells from the prostate draining lymph nodes (PDLN) of anti-CD40 treated mice were stimulated to produce IFN-γ, whereas <40% of 2C cells from the PDLN of PBS-treated mice produced IFN-γ (Fig. 1A). However, by 10 d postinfection, only ~10% of 2C cells from the PDLNs of anti-CD40–treated mice still expressed IFN-γ, whereas 77% of 2C cells from the PDLNs of PBS-treated mice expressed IFN-γ (Fig. 1B). Although anti-CD40 treatment also resulted in an earlier appearance of 2C T cells in the spleens and peripheral lymph nodes (PLNs), most of these cells were incapable of producing IFN-γ. In contrast, in PBS-treated mice, almost all 2C cells from the spleens and PLNs were able to produce IFN-γ. These results suggest that whereas systemic anti-CD40 treatment can stimulate a tumor-specific CD8+ T cell response transiently in the PDLNs of TRP-SIY mice, it can lead to severe immune suppression in the long term.

Truncation of the cytoplasmic domain of CD40L leads to increased levels and duration of surface expression

One approach to deliver localized CD40 stimulation is to express CD40L on tumor-reactive CD8+ T cells, which naturally traffic to...
TDLNs and tumor tissues. Therefore, we constructed a retrovirus expressing CD40L and the surface marker Thy1.1. To test CD40L expression on CD8+ T cells, 2C T cells were activated with SIY peptide for 36 h, spin-infected with the retrovirus, and monitored for CD40L and Thy1.1 expression. Twenty-four hours after retroviral transduction, 12% of Thy1.1+ cells were also CD40L+ (Fig. 2). However, by 48 h after transduction, only 2% of Thy1.1+ cells were still CD40L+. Because both CD40L and Thy1.1 expression were driven by the same viral LTR, these results suggest that CD40L expression is tightly regulated post-translationally on the surface of CD8+ T cells, as observed with CD4+ T cells (11).

To achieve a more sustained and higher level of CD40L expression, we constructed CD40L mutants with potentially reduced susceptibility for receptor-mediated endocytosis (43). In one mutant, referred to as CD40LY5A, a tyrosine at position 5 was mutated to an alanine, because this residue was thought to be critical for the maintenance of a conserved endocytosis domain (43). However, the mutant displayed expression kinetics and levels comparable to the wild type CD40L (Fig. 2). However, by 48 h after transduction, only 2% of Thy1.1+ cells were still CD40L+. Because both CD40L and Thy1.1 expression were driven by the same viral LTR, these results suggest that CD40L expression is tightly regulated post-translationally on the surface of CD8+ T cells, as observed with CD4+ T cells (11).

Thy1.1+ 2C cells expressed surface CD40L by 24 h after retroviral transduction, and 10% of Thy1.1+ 2C cells still expressed CD40L by 48 h after transduction (Fig. 2). In addition, the level of CD40L Δ1-13 expression was substantially higher than wild type CD40L expression at 24 h after transduction. Therefore, deletion of the terminal 13 aa residues significantly increases the level and duration of CD40L expression on activated CD8+ T cells. CD40L Δ1-13 was used for all subsequent experiments.

CD40L-expressing 2C cells stimulate maturation of DCs in vitro and in vivo

Our strategy for localized CD40 ligation was to engineer tumor-reactive 2C T cells to express CD40L Δ1-13. Because even the truncated form of CD40L is expressed only transiently after T cell activation, an important requirement for the success of this approach is that the retrovirally-transduced CD8+ T cells express CD40L again when they encounter Ag a second time. Thus, retrovirally-transduced 2C cells were transitioned into a memory phenotype in vitro (Supplemental Fig. 1) to ensure that they could respond to a second Ag encounter in TRP-SIY mice. To demonstrate the ability of these retrovirally-transduced in vitro memory 2C cells to re-express CD40L, the cells were stimulated with SIY peptide 10 d after transduction, long after CD40L had ceased expression. The restimulated 2C cells

FIGURE 1. Effect of systemic anti-CD40 treatment on a tumor-reactive CD8+ T cell response in TRP-SY mice. TRP-SIY mice were injected with PBS or anti-CD40 Ab on days -1, 0, and +1. On day 0, mice were injected with naive 2C cells and infected with WSN-SIY virus. Five (A) and 10 d (B) postinfection, single-cell suspensions were prepared from the PDLNs, spleens, and PLNs. Cells were restimulated in vitro with SIY peptide for 4 h. Samples were stained for 2C TCR, CD8, and intracellular IFN-γ. Dot plots show 2C TCR versus CD8 staining profiles of live cells. Histograms show IFN-γ expression of 2C TCR+CD8+ cells. Numbers indicate percentage of positive cells. Representative data from one of two similar experiments are shown.

FIGURE 2. Surface expression of wild type and mutant CD40L. Naive 2C cells were activated with SIY peptide plus IL-2 for 36 h. Cells were then transduced with a retrovirus expressing Thy1.1 alone (control), Thy1.1 plus the wild type CD40L, CD40LY5A, or CD40L Δ1-13, and were analyzed for CD40L expression 24 and 48 h later. The time in parentheses corresponds to days poststimulation. Dot plots show CD40L versus Thy1.1 staining profiles of live cells. Numbers indicate the percentage of Thy1.1+ cells that are CD40L+. Transduction efficiencies were 20–25% in all cases, and representative data from one of at least two similar experiments are shown.
began to upregulate CD40L 2 d after restimulation, reached maximal levels of expression 3 d after restimulation, and began to down-regulate expression by 4 d after restimulation (Fig. 3). Therefore, the retrovirally transduced in vitro memory CD8+ T cells are capable of re-expressing CD40L when they encounter Ag again.

To demonstrate the functionality of CD40L expression on CD8+ T cells, we determined whether CD40L-expressing 2C cells could mature BMDCs in vitro. 2C T cells were transduced with the CD40L Δ1-13 retrovirus, transitioned in vitro into memory cells, restimulated with SIY peptide for 48 h, and cocultured with immature BMDCs. To assess maturation, BMDCs were stained for CD11c, CD80, and CD86. Dot plots show CD11c+, CD80+, and CD86+ expression of BMDCs under the indicated conditions. Numbers indicate the percentage of CD80+CD86+ BMDCs. Representative data from one of at least two similar experiments are shown.

FIGURE 3. Kinetics of CD40L Δ1-13 re-expression on retrovirally transduced in vitro memory 2C cells. Naive 2C cells were activated with SIY peptide plus IL-2 for 36 h and then transduced with a retrovirus expressing Thy1.1 and CD40L Δ1-13. Cells were cultured for 2 d in IL-2 and transitioned into a memory phenotype by culturing them for 6 d in IL-7. In vitro memory cells were restimulated with SIY peptide plus IL-2 in the presence of a 1:1 mix of C57BL/6 splenocytes and then stained for Thy1.1, 2C TCR, and CD40L on days 2, 3, and 4. Histograms show expression on 2C TCR+Thy1.1+ transduced cells or 2C TCR+Thy1.1− nontransduced cells. Representative data from one of at least two similar experiments are shown.

FIGURE 4. CD40L Δ1-13–expressing 2C cells stimulate maturation of dendritic cells in vitro and in vivo. Schematic diagram (A) and results (B) of in vitro BMDC maturation assay. Retrovirally transduced in vitro memory 2C cells were generated as in Fig. 3 and restimulated with SIY peptide for 48 h. Thy1.1+ transduced 2C cells were purified by cell sorting and cultured with immature BMDCs for 24 h. Cells were stained for CD11c, CD80, and CD86. Dot plots show CD80 and CD86 expression of CD11c+ BMDCs under the indicated conditions. Numbers in graphs indicate the percentage of CD80+CD86+ cells. Numbers below graphs indicate the average percentage of CD80+CD86+ cells ± SD. Representative data from one of at least two similar experiments are shown. p < 0.05 when percentages of CD80+CD86+ BMDCs were compared between samples stimulated with either control 2C cells or CD40L Δ1-13 2C cells. In vivo DC maturation. In vitro memory 2C cells either expressing or not expressing CD40L Δ1-13 retrovirus were transferred into TRP-SIY mice. Six days later, cells from the PDLN and spleens were pooled from five mice and stained for CD11c, CD11b, CD80, and CD86. Dot plots show CD80 and CD86 expression of CD11c+CD11b+ cells. Numbers indicate the percentage of positive cells. Representative data from one of at least two similar experiments are shown.
BMDCs (Fig. 4A). Twenty-four hours later, the maturation status of the BMDCs was evaluated by assaying for CD80 and CD86 expression. As controls, BMDCs were cultured alone, with control retrovirally-transduced in vitro memory 2C cells that do not express CD40L, or with LPS. Approximately 15% of BMDCs expressed both CD80 and CD86 when cultured alone, whereas ~55% of BMDCs expressed both markers following stimulation with LPS. Although ~34% of BMDCs expressed both CD80 and CD86 when cultured with 2C cells that did not express CD40L, a significantly higher percentage (~45%) of BMDCs expressed both markers when cultured with CD40L-expressing 2C T cells (Fig. 4B). Thus, CD40L-expressing 2C cells can stimulate BMDC maturation in vitro.

We also determined whether CD40L-expressing (therapeutic) 2C cells could stimulate maturation of DCs in the PDLNs of TRP-SIY mice. Retrovirally transduced in vitro memory 2C cells begin to re-express CD40L in the PDLN ~5 d after transfer (Supplemental Fig. 2). We therefore evaluated the maturation status of CD11c<sup>+</sup>CD11b<sup>+</sup> DCs in the PDLNs, spleens, and PLNs 6 d after transferring in vitro memory 2C cells either expressing or not expressing CD40L (Fig. 4C). A comparable fraction of DCs from the PDLNs of mice that received either no cells or control 2C cells expressed CD80 and/or CD86, whereas a higher fraction of DCs from the PDLNs of mice that received CD40L-expressing 2C cells expressed CD80 and/or CD86 (Fig. 4C). A minimal difference was seen in the maturation of DCs in the spleens of these mice (Fig. 4C), and no difference was seen in the PLNs (data not shown). These results suggest that CD40L-expressing 2C cells can stimulate DC maturation in the PDLNs of TRP-SIY mice, where they encounter tumor-derived Ag.

**FIGURE 5.** CD40L Δ1-13–expressing 2C cells stimulate a more functional antitumor T cell response in TRP-SIY mice. *A,* Schematic diagram of experimental protocol. In vitro memory 2C cells either expressing or not expressing CD40L Δ1-13 were transferred into TRP-SIY mice. As a control, some TRP-SIY mice were not injected with any in vitro memory 2C cells. The mice were divided into two groups. One group was infected intranasally with WSN-SIY, and the other group was not infected. Six days later, naive Thy1.1High reporter 2C cells were transferred into all mice. Five days after transfer, reporter 2C cells were analyzed for IFN-γ expression. *B,* Reporter 2C T cell responses. Cells from the PDLN and spleens of above treated mice were restimulated with SIY peptide for 4 h and then stained for Thy1.1, CD8, and intracellular IFN-γ. Dot plots show IFN-γ versus Thy1.1 staining profiles gating on Thy1.1High CD8<sup>+</sup> reporter 2C cells. Numbers in graphs indicate the average percentage of IFN-γ<sup>+</sup> cells ± SD of the analyzed tissues. Representative data from at least two similar experiments are shown.

CD40L-expressing 2C cells stimulate a more robust antitumor T cell response

The ability of CD40L-expressing 2C cells to stimulate local DC maturation is expected to stimulate a more robust T cell response in TRP-SIY mice. Thus, TRP-SIY mice were injected with in vitro memory 2C cells, either expressing or not expressing CD40L, or were given no cells. Some of the TRP-SIY mice were also infected with WSN-SIY at the time of 2C cell transfer (Fig. 5A). When DCs had matured locally in the PDLN, ~6 d after transfer (Fig. 4C), naive Thy1.1High reporter 2C cells were transferred into the mice. Because Thy1.1 expression on the reporter cells is at least 1 log higher than on the retrovirally transduced therapeutic 2C cells, the populations could be clearly differentiated (data not shown). Five days later, reporter 2C cells from the PDLNs, spleens, and PLNs were evaluated for their ability to express IFN-γ following in vitro restimulation (Fig. 5A). Without transfer of therapeutic 2C cells, ~10% of the reporter 2C cells from the PDLNs of TRP-SIY mice were able to express IFN-γ (Fig. 5B). This baseline level increased to ~20% if mice were transferred with control in vitro memory 2C cells, either with or without a WSN-SIY infection. In contrast, the fraction of reporter 2C cells that could express IFN-γ increased to ~40% if the mice were transferred with CD40L-expressing 2C cells. If the mice received a WSN-SIY infection at the time the CD40L-expressing 2C cells were transferred, up to 75% of the reporter 2C cells in the PDLNs could express IFN-γ (Fig. 5B). A smaller difference was observed in the percentages of reporter 2C cells that could express IFN-γ in the spleens at this timepoint, consistent with the minimal effects of CD40L-expressing 2C cells on the maturation of the dendritic cells in the spleens (Fig. 4C). No significant or consistent trends were seen in the PLNs (data not shown).
observed in the total reporter cell numbers in the PDLNs, spleens, or PLNs between the different treatment groups (data not shown).

By 10 d after reporter 2C cell transfer, the effector 2C cells had exited the PDLN, and the immune response had begun to contract. At this time point, more reporter 2C cells were able to produce IFN-γ in the periphery of mice that received CD40L-expressing 2C cells than in those that received control 2C cells (Supplemental Fig. 3). Furthermore, 4–6-fold as many reporter 2C cells infiltrated the prostate tissue of TRP-SIY mice that received CD40L-expressing 2C cells than did those that received control 2C cells (Supplemental Fig. 3). However, there was no difference in the ability of the reporter 2C cells from the prostate tissue to express IFN-γ.

Together, these results suggest that the transfer of CD40L-expressing therapeutic 2C cells conditions TRP-SIY mice for more robust antitumor T cell responses, which can be further enhanced by active immunization.

**Discussion**

One problem that limits the efficacy of adoptive T cell therapies is rapid tolerization or deletion of transferred tumor-reactive T cells in cancer patients. Although recent studies have shown that younger or central memory-like CD8+ T cells are more potent and persist longer than effector memory-like T cells in the setting of ACT (44, 45), their increased requirement for costimulatory support may heighten the influence of tolerogenic DCs on these transferred cells. Because of its critical and natural role in DC activation, CD40 ligation has been explored to activate tolerogenic DCs in the tumor environment. Although systemic administration of agonist anti-CD40 Abs has been shown to replace the need for CD4+ T cell help (12–14), boost CD8+ T cell responses to tumors, and break peripheral self-tolerance (15–17), there is also evidence that it can induce immune suppression (18–21). Similarly, we found that systemic administration of an agonist anti-CD40 Ab in TRP-SIY mice initially stimulated an antitumor CD8+ T cell response, but eventually led to severe immune suppression, in the context of an influenza infection (Fig. 1). In addition, systemic anti-CD40 administration is associated with significant side effects (data not shown). The complicated and variable outcomes of systemic CD40 ligation in immune responses highlight the need to induce CD40 ligation locally in the tumor tissue.

In this study, we report a novel strategy to activate tolerogenic DCs by using tumor-reactive CD8+ T cells to deliver an activating CD40L signal in the tumor environment. This strategy allows us to localize the immunostimulatory signal in both time and space. CD40L is normally expressed on activated CD4+ T cells for <24 h (11), and its expression on activated CD8+ T cells is similarly transient. Because CD40L transcription is driven by a retroviral LTR in our study, the tight regulation of CD40L expression on the surface of CD8+ T cells is likely regulated at the posttranslational level. Supporting this notion, deletion of the terminal 13 aa residues of the cytoplasmic domain led to a higher level and extended duration of CD40L expression on the surface of CD8+ T cells. This deletion mutant is designed to minimize receptor-mediated endocytosis, but does not likely affect other regulatory mechanisms that also control CD40L expression, such as downregulation of CD40L transcription, proteolytic cleavage, and release of soluble CD40 (11), and thus surface CD40L expression is still transient. We used in vitro memory 2C T cells to deliver CD40L to the correct location, because they recognize a tumor-derived epitope (SIY) and naturally traffic to the TDLNs and tumor tissue. After adoptive transfer, only those retrovirally transduced 2C cells that traffic to the PDLN have the opportunity to encounter SIY and re-express CD40L. Even with truncation of the majority of the cytoplasmic domain, CD40L is expressed only on the surface of 2C T cells for 48–72 h after activation (Fig. 2). Consequently, delivery of the immunostimulatory CD40L signal is limited spatially and temporally. This should minimize the potential autoimmune complications and immune suppression associated with systemic CD40 ligand.

Like CD40L expressed on activated CD4+ T cells, CD40L expressed on the surface of activated CD8+ T cells also stimulates maturation of DCs both in vitro and in vivo (Fig. 4). Coculture of BMDCs with CD40L-expressing 2C cells for 24 h is sufficient to stimulate upregulation of CD80 and CD86 on DCs, to a similar extent as observed with LPS stimulation (Fig. 4B). Similarly, transfer of CD40L-expressing 2C cells into TRP-SIY mice stimulated upregulation of CD80 and CD86 on some DCs in the PDLN. Despite the modest observable effect on DC maturation in vivo, the antitumor CD8+ T cell response was significantly enhanced, as indicated by the increased percentage of reporter 2C cells that could produce IFN-γ in the PDLN and infiltrate the prostate tissue. The enhanced antitumor response was more dramatic when the CD40L-expressing 2C cells were activated by an influenza infection, as opposed to by the tolerizing environment of the tumor (Fig. 5). Following influenza infection, a higher fraction of retrovirally transduced 2C T cells re-expressed CD40L, which stimulated maturation of more DCs in the PDLN (Supplemental Fig. 4). Thus, augmented antitumor T cell responses can be induced by engineering T cells to deliver a CD40L-mediated stimulatory signal to DCs in the TDLNs.

There are two critical environments in which tolerance needs to be prevented or broken for adoptive T cell transfer to be most effective: the TDLNs and the tumor itself. If tolerance is broken in the tumor, but not in the TDLNs, adoptively transferred T cells may be tolerized upon initial transfer. This is particularly true for central memory-like T cells that traffic through the secondary lymphoid tissues before entering the tumor. If tolerance is broken in the TDLNs, but not in the tumor, adoptively transferred T cells may be tolerized upon entering the tumor tissue. Our approach breaks tolerance in the lymphoid tissues, but not in the tumor itself. Because the retrovirus-mediated CD40L expression is transient, the engineered 2C cells no longer express CD40L when they infiltrate the tumor, ~7 d after transfer (data not shown, 39). As a result, neither DCs nor CD40-expressing tumor cells in the prostate tissue are affected. Therefore, after transfer of CD40L-expressing therapeutic 2C cells, adoptively transferred reporter 2C cells become productively primed in the PDLN, maintain their function in the periphery, and infiltrate the tumor tissue extensively, but become rapidly tolerized in the tumor (Supplemental Fig. 3). Because the tumor-infiltrating T cells become tolerized, we did not evaluate alterations in disease progression or overall survival of treated mice. To overcome tolerance in both the TDLNs and tumor tissue, our approach needs to be combined with other approaches that break tolerance in the tumor tissue. Alternatively, CD40L could be further engineered to achieve more prolonged surface expression.

Recent preclinical and clinical studies have made significant progress in advancing the success of adoptive cell therapy in the clinic. T cell engineering strategies may further augment the promise of such approaches. This study demonstrates that adoptively transferred T cells can be engineered to deliver functional signals to DCs in the tumor environment, and that those dendritic cells can then stimulate more robust T cell responses. As we better understand the critical role of DCs in the activation, maintenance, and tolerization of T cells in the tumor environment, similar approaches can be explored to engineer tumor-reactive CD8+ T cells to deliver important functional signals to DCs to overcome tumor tolerance.

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Disclosures
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Supplemental Figure 1. In vitro memory 2C cells have a CD44^+CD62L^{High} phenotype. Naïve 2C cells were activated with SIY peptide plus IL-2 for 36hrs, and were then retrovirally-transduced. Cells were cultured for 2 additional days in IL-2 and then transitioned into a memory phenotype by culturing them for 6 days in IL-7. Histograms show representative CD44 or CD62L expression on naïve 2C cells and in vitro memory 2C cells.
Supplemental Figure 2. Kinetics of CD40L Δ1-13 re-expression by transduced 2C cells in the PDLN of TRP-SIY mice. In vitro memory 2C cells transduced with a retrovirus expressing CD40L Δ 1-13 were transferred into TRP-SIY mice. Three, 4 and 5 days later, cells from the PDLN were stained for 2C TCR, CD8 and CD40L. Dot plots show CD40L versus CD8 staining profiles gating on 2C TCR+ cells.
**Supplemental Figure 3.** Transfer of CD40L Δ1-13-expressing 2C cells leads to increased infiltration of reporter 2C cells into the prostate tissue of TRP-SIY mice.

The experimental protocol is the same as outlined in Figure 5A, but the reporter 2C cells were analyzed 10 days after transfer. Dot plots show 2C TCR versus Thy1.1 staining profiles gated on live cells. IFN-γ versus Thy1.1 staining profiles are gated on Thy1.1\text{High}2C TCR\textsuperscript{+} reporter 2C cells. Numbers in graphs indicate percentage of positive cells in the gated region. Numbers below graphs indicate the average percentage of positive cells in the prostate tissue ± SD. Increased reporter cell infiltration in the prostate is indicated by percentages of Thy1.1\text{High}2C\textsuperscript{+} cells.
**Supplemental Figure 4.** CD40L Δ1-13-expressing 2C cells induce an increased number of mature dendritic cells in the PDLN of TRP-SIY mice with WSN-SIY virus. TRP-SIY mice were injected with in vitro memory 2C cells expressing or not expressing CD40L Δ1-13, followed immediately with an intranasal WSN-SIY infection. Six days later, cells from the spleens, MLN and PDLN were pooled from 5 mice and stained for CD11c, CD11b, CD80 and CD86. Dot plots show CD80 and CD86 expression of CD11c⁺CD11b⁺ cells, and numbers indicate percentage of positive cells in the gated region. Representative data from one of at least two similar experiments are shown. Compared to non-infected mice (Fig. 4C), the percentage of DCs that expressed CD86 in the PDLN was increased if TRP-SIY mice were infected with WSN-SIY virus.