Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells

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Current T-cell engineering approaches redirect patient T cells to tumors by transducing them with antigen-specific T-cell receptors (TCRs) or chimeric antigen receptors (CARs) that target a single antigen1–3. However, few truly tumor-specific antigens have been identified, and healthy tissues that express the targeted antigen may undergo T cell–mediated damage4–7. Here we present a strategy to render T cells specific for a tumor in the absence of a truly tumor-restricted antigen. T cells are transduced with both a CAR that provides suboptimal activation upon binding of one antigen and a chimeric costimulatory receptor (CCR) that recognizes a second antigen. Using the prostate tumor antigens PSMA and PSCA, we show that co-transduced T cells destroy tumors that express both antigens but do not affect tumors expressing either antigen alone. This ‘tumor-sensing’ strategy may help broaden the applicability and avoid some of the side effects of targeted T-cell therapies.

Adaptive cell therapies using genetically modified autologous T cells have shown efficacy for melanoma and indolent B-cell malignancies4–7. However, their broad applicability is limited by the paucity of truly tumor-specific target antigens. Extra-tumoral antigen expression may indeed result in “on-target, off-tumor” effects. These effects can be acceptable, as is the case with CD19, an antigen expressed in B-cell malignancies and normal B-lineage cells, resulting in B-cell aplasia5–7. In other instances, targeting, for example, carbonic anhydrase IX or human epidermal growth factor receptor 2 (HER2), the side effects may be intolerable and potentially life-threatening8,9.

Here we present an approach to render engineered T cells specific for a tumor even in the absence of a truly tumor-restricted antigen. T-cell activation requires TCR- or CAR-mediated recognition of one antigen, which in this work is either CD19 or prostate stem cell antigen (PSCA). T-cell costimulation must be independently mediated by a CCR10 specific for a second antigen, here being prostate-specific membrane antigen (PSMA). This dual-targeting approach facilitates augmented T-cell reactivity against tumors positive for two antigens compared to single-positive tumors, that is, those positive for only one antigen10–12. However, this approach alone fails to prevent T-cell reactivity to single-positive tumors, as we show here. To achieve tumor selectivity, we diminished the efficiency of T-cell activation to a level where it is ineffective in the absence of simultaneous CCR recognition of the second antigen. We hypothesized, and demonstrate below, that T cells expressing suboptimal activation receptors are functionally rescued at the tumor site by a CCR engaging a co-expressed tumor antigen.

To demonstrate that both T-cell activation and costimulation signals can be supplied in vivo using two distinct antigen-specific receptors, we initially evaluated the combination of a CAR that provides a CD3ζ-mediated activation signal upon recognition of the B-cell marker CD19 (19z1)13 and a CCR specific for PSMA10,14. Based on results showing synergy between CD28 and 4-1BB costimulation15,16, including through their cytoplasmic domains arranged in tandem17–20, we added the 4-1BB cytoplasmic domain to the PSMA-specific CCR P28 (ref. 14), as described20, to generate P28BB (Supplementary Fig. 1a). Primary human peripheral blood T cells were retrovirally transduced with 19z1 and/or P28BB, typically yielding expression of both receptors in 45–70% of T cells (Supplementary Fig. 1b). Four groups of T cells were analyzed in all subsequent studies, expressing 19z1, P28BB, 19z1+P28BB or neither (mock).

We first measured the in vitro cytotoxic and proliferative responses of transduced T cells exposed to mouse lymphoma cell line EL4 target cells expressing CD19 and/or PSMA. Cytotoxicity against CD19-expressing target cells was, as expected, imparted by 19z1 expression and was unaltered in the presence of PSMA in all T-cell groups (Fig. 1a). A quantitative comparison after normalizing to the fraction of 19z1-transduced T cells for the 19z1 and 19z1+P28BB groups and the P28BB-transduced fraction for the P28BB group showed that 19z1 and 19z1+P28BB T cells specifically lysed 40–47% CD19-expressing target cells at the 50:1 effector/target ratio, whereas the P28BB-transduced T cells did not lyse PSMA-expressing targets (Fig. 1a). We next examined the proliferation of T cells repeatedly exposed to artificial antigen-presenting cells (AAPCs) expressing CD19 and/or PSMA in the absence of exogenous cytokine. Over 4 weeks, only the 19z1+P28BB T cells underwent robust proliferation (58-fold expansion) when co-cultured on AAPCs expressing both antigens. In contrast, 19z1 or P28BB T cells underwent modest expansion over the first 14 d, as did the 19z1+P28BB T cells exposed to CD19+PSMA− AAPCs (Fig. 1b).

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activation in the presence of both antigens was provided by quantita-
tive assessment of cytokine production and the induction of the anti-
apoptotic molecule Bcl-2, which were maximal in 19z1+P28BB T cells
(Supplementary Fig. 2a,c).

We then tested the ability of these dual receptor (19z1+P28BB)
expressing T cells to eradicate established systemic human prostate
tumors in immunocompromised, nonobese diabetic severe/combined
immunodeficient (NOD.SCID) Il2rg−/− (NSG) mice. We intravenously
infused 2.0×10^6 green fluorescent protein/firefly-luciferase
(GFP/Luc) expressing PC3 tumor cells that expressed both CD19 and
PSMA (Supplementary Fig. 3) followed, 19 d thereafter, by a
single intravenous infusion of 1.0×10^6 19z1, P28BB or 19z1+P28BB
T cells intravenously. All three tumors progressed in mice that received
P28BB T cells, and these mice had to be euthanized within 35 d (Fig. 1d).
In mice treated with 19z1 T cells, the CD19+PSMA− and CD19+PSMA+ tumors initially underwent substan-
tial reduction in volume but eventually progressed. Mice treated with
19z1+P28BB T cells showed complete eradication of CD19+PSMA−
tumors. However, CD19+PSMA+ tumors were also rejected in these
mice more efficiently than in mice bearing only CD19+PSMA−
tumors (Fig. 1d). Thus, this dual-targeting combinatorial antigen approach did
not restrict T-cell reactivity to double-positive tumors.

To remedy this failure, we reasoned that T-cell activation would
have to be minimized, almost to the point of extinction, only to be

Figure 1 Dual chimeric receptor-mediated activation and costimulation of human T cells facilitates robust cytotoxicity, proliferation and tumor
eradication. (a,b) T cells were mock transduced or were transduced with retroviruses encoding a CD19-specific CAR (19z1) and/or a PSMA-specific CCR
(P28BB). (a) T cells were incubated at indicated effector/target ratios with 51Cr-loaded target cells expressing CD19 and/or PSMA, and target cell lysis
was measured. Plots represent at least four experiments with error bars representing s.d. of the mean of three replicates. (b) T cells were co-cultured with PC3 tumour cell lines expressing CD19 and/or PSMA (arrows indicate restimulation of T cells using freshly irradiated tumor cells). T-cell numbers were measured at indicated time intervals. Plots represent at least four experiments with error bars representing s.d. of the mean of three replicates. (c) T cells (1.0×10^6) described in a and b were injected intravenously into NSG mice bearing green fluorescent protein/firefly-luciferase
fusion protein (GFP/Luc) expressing CD19+PSMA+ PC3 human prostate tumors. Tumor burden was measured weekly by bioluminescent imaging.
Top, images of two representative mice from each group at each time point, with the pixel intensity represented in color. Bottom, average tumor burden
as quantified by luminescence of the tumors using units of photons per second per square centimeter per steradian (p/sec/cm²/sr); error bars represent
s.d. from the mean. Values were generated from six mice per group. (d) 1×10^6 CD19+PSMA−, 1×10^6 CD19+PSMA+ and 1×10^6 CD19+PSMA+ PC3
cells were injected subcutaneously into the left flank, right flank and back, respectively, of NSG mice. T cells expressing 19z1 and/or P28BB were
infused intravenously 7 d later. Top, representative images of two mice per time point per group as analyzed by bioluminescent imaging. Graphs, tumors
were quantitatively measured using calipers and tumor volumes were plotted versus time for each tumor. Error bars represent s.d. from the mean of
each six mice. Statistical significance was determined using two-tailed unpaired t-tests to compare values obtained from 19z1 T cells and 19z1 + P28BB
T cells; *P<0.05, **P<0.01.
rescued by simultaneous engagement of the CCR. We thus searched for CARs with diminished activity, and while doing so switched from CD19 and PSMA to a combination of prostate antigens, PSCA and PSMA. We evaluated three PSCA-specific scFvs with different binding affinities for PSCA. We incorporated each PSCA-specific scFv into bispecific PSCA/CD3 antibodies and incubated them with T cells and PSCA-expressing PC3 tumor cells to quantify tumor cell lysis (Fig. 2a). PSCA/CD3 bispecific antibody containing the CAR Hz1 lysed tumor cells 1,000- to 10,000 fold more efficiently than antibody containing the CAR Lz1, even in picogram quantities. We then used these scFvs to construct CARs by linking them to CD3cytoplasmic domains. As expected, T cells expressing the different CARs showed different activities in cytotoxicity assays (Fig. 2b). Hz1 and Mz1 CARs directed moderate lysis of PSCA++ targets (20% specific lysis at the 50:1 effector/target ratio), whereas the Lz1 CAR only reached 10% specific lysis, qualifying it as a suboptimal antigen receptor. This hierarchy was further confirmed in cytokine release assays using T cells expressing each PSCA CAR together with the P28BB CCR (Supplementary Fig. 2b–d). T cells transduced with 19z1 and Hz1 CARs produced relatively high amounts of T-helper type 1 (T H1) and TH2 cytokines, whereas T cells expressing less efficient CARs such as Mz1 and Lz1 produced lower amounts of cytokines. The enhancement of cytokine levels in Lz1+P28BB T cells compared to Lz1 T cells was minimal except for interleukin (IL)-2 and IL-13. IL-2 induces proliferation and can promote either a T H1 or T H2 response21, whereas IL-13 is associated with a T H2 response specific to 4-1BB signaling22.

We next tested the anti-tumor activity of these T cells in animals bearing tumors expressing PSCA and/or PSMA. We inoculated mice intravenously with 2 × 10^6 GFP/Luc PC3 cells expressing PSMA, PSCA or both antigens (Supplementary Fig. 3). Fourteen days later, we intravenously injected 1 × 10^6 Mz1+P28BB T or 1 × 10^6 Lz1+P28BB T cells into the mice. PSCA++PSMA++ tumors decreased in size in recipients of Mz1+P28BB T cells but not in recipients of Lz1+P28BB T cells, confirming the inefficiency of the Lz1 CAR (Fig. 2c,d). Similar to the CD19 CAR experiment (Fig. 1c), PSCA++PSMA++ tumors responding to Mz1 eventually relapsed and increased in size. In mice bearing PSCA++PSMA++ tumors, however, Mz1+P28BB T cells induced robust and long-term tumor eradication. Most importantly, PSCA++PSMA++ tumors were eradicated in mice treated with Lz1+P28BB T cells, resulting in complete long-term survival of all treated mice (Fig. 2d). Tumor eradication was not induced in control mice bearing PSCA++PSMA++ tumors (Fig. 2c,d).

To thoroughly evaluate the effect of Lz1+P28BB T cells on PSCA++PSMA++ tumors in animals where these T cells could be
costimulated, we analyzed the effect of Lz1+P28BB T-cell infusion on PSCA+PSMA+ tumors in animals also bearing PSCA−PSMA− and PSCA−PSMA+ tumors. Strikingly, Lz1+P28BB T cells eradicated PSCA−PSMA+ tumors but not PSCA−PSMA− tumors (Fig. 2e). These results demonstrate the feasibility of decreasing T-cell activation to the extent where dual CAR + CCR expressing T cells do not react against tissues expressing either the CAR-targeted antigen or the CCR-targeted antigen, and promoting T-cell activation only on encounter with the two coexpressed antigens. These data also provide proof-of-principle evidence for achieving two complementary outcomes that determine specificity and safety of T-cell tumor therapy: (i) the ability to harness combinatorial antigen recognition to design T cells specific for a tumor in the absence of a truly tumor-specific target antigen and (ii) the ability to protect cells that express only one of the targeted antigens by titrating activation and costimulatory signals so as to confine T-cell activation to sites of target antigen coexpression (Fig. 3).

This titration of CAR signals distinguishes our approach from studies that showed that two coexpressed antigen receptors boost T-cell responsiveness against double-positive cells beyond that induced by a single antigen receptor10,12 and addresses the central problem of abolishing or reducing T-cell reactivity against single-positive tissues. Our approach ultimately parallels physiological antigen presentation and T-cell priming23 wherein T cells are primed in lymph nodes where they receive TCR and costimulatory signals that are restricted to specialized antigen-presenting cells. T cells then migrate to peripheral sites where their activation does not depend on additional costimulation. Similarly, T cells stimulated through both a CAR and a CCR at one site may also recirculate to other peripheral sites and therein eliminate tumor cells without costimulatory dependence. However, we showed that if these sites express either the CAR target antigen or the CCR target antigen, “tumor-sensing” T cells, which exhibit carefully titrated CAR signaling, will not eradicate the single-positive tumor, which we used here as a surrogate for normal single-positive tissue. Studies in a murine syngeneic model will be conducted to validate the tumor-sensing concept in a fully immune competent model. However, these studies would have the caveats of using a mouse system to develop a human therapy that may not share the same biological and technical aspects. Notably, as CARs and CCRs recognize cell surface antigens rather than human leukocyte antigen (HLA)-peptide complexes, T cells engineered in this manner will recognize these antigens directly on the tumor but will not be costimulated by interacting with professional antigen-presenting cells that cross-present the targeted antigens in the context of HLA molecules. They will also not be activated by healthy tissues that may express a targeted antigen but do not express costimulatory ligands.

PSCA and PSMA are promising targets for the treatment of metastatic prostate cancer24, although neither antigen is absolutely specific to prostate tissue. PSCA is expressed in prostate tumors, and also in the renal pelvis, ureter, urinary bladder and urethra25. PSMA is highly expressed in metastatic prostate cancer, as well as in astrocytes type II, the kidney proximal tubule and the intestinal brush border26. Combinatorial PSCA/PSMA targeting is thus expected to increase prostate cancer targeting and reduce reactivity against healthy tissues expressing either antigen alone.

The “tumor-sensing” approach can be extended to other tumor types for which a combination of antigens may confer enhanced tumor specificity. For example, one may target breast cancer through combinatorial recognition of HER2, MUC1, CD44, CD49f and/or EpCAM, none of which is truly cancer-specific27,28. Likewise, one may selectively target ovarian cancer through a combination of mesothelin, folate receptor-α, CD44 and/or CD133 (refs. 29,30). When determining candidate sets of antigens for targeting a particular tumor type, an antigen should be selected based on high cell expression in cancerous tissue, but not normal tissue or lymphocytes. A second antigen should be selected so that expression of both antigens is restricted to cancerous tissue. The antigen whose expression in normal tissue would have more detrimental off-target effects should be targeted using a CCR to minimize lysis of single-positive cells. For example, HER2, MUC1 and EpCAM are not highly expressed by normal tissues and coexpression of a pair should be limited to cancerous tissue. The antigen whose expression in normal tissue would have more detrimental off-target effects should be targeted using a CCR to minimize lysis of single-positive cells. For example, HER2, MUC1 and EpCAM are not highly expressed by normal tissues and coexpression of a pair should be limited to cancerous tissue. HER2 is expressed by lung epithelial cells at low levels, target- ing HER2+MUC1 would be attractive by directing the CAR to MUC1 and the CCR to HER2. As many breast cancers are HER2 negative, MUC1+EpCAM may represent an attractive alternative. The targeting of tumor-initiating cells or cancer stem cells by tumor-sensing T cells represents another enticing application. Altogether, our work provides a path for restricting the selectivity and activity of engineered T cells in a manner that reconciles potency with safety.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.C.K. designed and performed experiments, analyzed data, and wrote the manuscript. M. Condomines contributed reagents, performed experiments.
interpreted results and reviewed the manuscript. M. Cartellieri contributed reagents and performed experiments. M.B. contributed reagents and designed experiments. M.S. designed experiments, analyzed data, interpreted results and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Gammaretroviral vector construction and viral production. The gammaretroviral vector SFG-19z1 has been extensively described.\textsuperscript{31} This backbone construct was used to exchange scFvs to generate SFG-Hz1, SFG-Mz1 and SFG-Lz1 by directional cloning using a NcoI site located 5′ of the scFv and a NotI site located 3′ of the scFv. To construct SFG-P28BB, we amplified by PCR the fused CD28 and 4-1BB domains from a third-generation CAR vector (that is, one that supplies two costimulatory signals and an activation signal upon scFv binding), SFG-P28BBz1 and ligated 3′ of the PSMA scFv using a 5′ NcoI site and a 3′ BamHI site to include a stop codon 3′ of the BB domain, while the CD3ζ′ domain was removed.\textsuperscript{30} We inserted an internal ribosomal entry site to facilitate bicistronic expression of CARs and CCRs with dsRED and hrGFP, respectively. Vectors were used to transiently transfect cell lines to generate stable virus-producing lines as previously described.\textsuperscript{31}

Generation of anti-PSCA scFvs. Three PSCA specific scFvs, termed Hz1, Mz1 and Lz1 were generated by amplifying the variable heavy (Vh) and variable light (Vl) domains from hybridomas, using degenerate primers as previously described.\textsuperscript{12-14} These Vh and Vl domains were fused together using a glycine-serine linker and were used to replace the CD19 scFv in the SFG-19z1 backbone using 5′ SphI to 3′ NotI sites.

Isolation, retroviral transduction and culture of primary human T cells. Peripheral blood leukocytes were isolated using Ficoll gradients and transduced as previously described.\textsuperscript{31} Briefly, after 48 h of activation with 2 µg/mL phytohemagglutinin, cells were transduced twice by spinoculation for 1 h on retronectin-coated plates over the next 48 h and 20 U/mL of IL-2 was added. After allowing 3 d for vector expression, transduction efficiencies were determined by flow cytometry, and bulk unsorted cells were used for various assays or adoptive transfers.

Generation of antigen-expressing tumor cell lines. The PC3 human prostate tumor line was obtained from ATCC and retrovirally transduced to express the green fluorescent protein/firefly luciferase fusion protein (GFP/Luc) for future fluorescent/luminescent quantification to generate PC3-GFP/Luc\textsuperscript{16}. This line was subsequently transduced to create PC3-CD19, PC3-PSMA, PC3-CD19-PSMA, PC3-PSCA and PC3-PSCA-PSMA by means of multiple retroviral transductions.

Cytotoxicity assays. Target cells expressing desired antigen were labeled with \textsuperscript{51}Cr and co-cultured with T cells at decreasing effector/target ratios. After 4 h of culture, supernatant was removed and radioactivity released from chromium was measured. Specific lysis was determined by subtracting background radioactivity of target cells not cultured with T cells and dividing by the radioactivity measured from target cells completely lysed by treatment with 0.2% Triton X-100.

T-cell proliferation assays. Tumor cells expressing desired antigen were irradiated with 30 Gy before co-culture with 1.0 × 10^6 T cells at a 5:1 effector/target ratio. T cells were counted weekly using an Invitrogen Countess cell counter and then restimulated with irradiated tumor cells. No exogenous cytokines were added to these co-cultures.

Mouse tumor models. NOD:SCID Il2rg^−/− (NSG) mice were obtained from either Jackson Laboratories or from in-house breeding under the protocol 04-10-024 approved by the MSKCC Institutional Animal Care and Use Committee. For systemic tumor experiments, 2.0 × 10^6 tumor cells were infused intravenously through the tail-vein into mice, and 1.0 × 10^6 T cells were infused 14 d later. For subcutaneous tumor experiments, 1.0 × 10^6 tumor cells were injected subcutaneously per tumor site, and 7 d later 1.0 × 10^6 T cells were infused intravenously.

Quantification of tumor burden. For systemic tumor experiments, bioluminescent imaging was used to quantitatively measure tumor burden by correlating the amount of tumor burden to luminescence using an IVIS 100 system (Caliper Life Sciences) as previously described.\textsuperscript{31} For subcutaneous tumors, calipers were used to measure tumor size. Tumor volume was calculated by multiplying the length, width and height of each tumor.

Bispecific antibody-mediated tumor lysis. The Vh and Vl domains of an anti-PSCA antibody were PCR amplified and cloned in tandem with the Vh and Vl domains of an anti-CD3 antibody to create single-chain bispecific tandem fragment variables binding to PSCA and CD3. Bispecific antibodies were added at various amounts to untransduced T cells co-cultured with \textsuperscript{51}Cr-labeled PSCA+ PC3 cells at a 20:1 ratio. Four hours later, chromium release was measured as described above.

Flow cytometry. Cells were analyzed using an LSR II flow cytometer or sorted using a FACS Aria cell sorter (BD Biosciences) as previously described.\textsuperscript{16} Detection of chimeric receptor at the cell surface could be achieved directly by using AF647-conjugated goat-anti-mouse antibody (Invitrogen), CD4-PE-Cy7, CD8-Pacific Blue and CD19-APC antibodies were obtained from Invitrogen while PSCA antibodies were purified from hybridoma supernatants and PSMA antibodies were obtained from MBL International.

Cytokine analysis. Supernatants were harvested 48 h after the second tumor stimulation (in T-cell proliferation experiments). Cytokines were measured using a custom multiplex system HCYTMMAG-60K (Millipore) and analyzed using a Luminex 100 instrument (Luminex) as previously described.\textsuperscript{30}

Western blot analysis. Cells were harvested 24 h after initial tumor stimulation (in T-cell proliferation experiments). Western blots were done as previously described using Bcl-xL (Clone 5H6, #27645) and Akt (Clone C67E7, #4691S) primary antibodies from Cell Signaling Technology with the PhosphoPlus(R) Akt (S473) Ab Kit (#9270S) at 1:1,000 dilution.

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