Rational Combination of Immunotherapies with Clinical Efficacy in Mice with Advanced Cancer

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Abstract

In the context of cancer, naïve T cells are insufficiently primed and become progressively dysfunctional. Boosting antitumor responses by blocking PD-1 or CTLA-4 results in durable clinical responses only in a limited proportion of cancer patients, suggesting that other pathways must be targeted to improve clinical efficacy. Our preclinical study in TRAMP mice comparing 14 different immune interventions identified anti-CD40 + IL2/anti-IL2 complexes + IL12Fc as a uniquely efficacious treatment that prevents tolerance induction, promotes priming of sustained, protective tumor-specific CD8+ T cells, and cures late-stage cancer when given together with adoptively transferred tumor-specific T cells. We propose that improving signals 2 (costimulation) and 3 (cytokines) together with fresh tumor-specific rather than boosting of dysfunctional preexisting memory T cells represents a potent therapy for advanced cancer. Cancer Immunol Res; 1–10. ©2015 AACR.

Introduction

The immune system plays a tumor-promoting and -suppressing role (1). Tumor-infiltrating Th1 and cytotoxic T cells are positive prognostic factors in human cancers (2); however, advanced tumors can evade immune attack and suppress tumor-specific immunity, which also limits the efficacy of standard therapies (1).

Immunotherapy aims to improve the protective effector function of tumor-specific T cells, which relies on 3 signals: stimulation of the T-cell receptor (TCR) receptor (signal 1), costimulation (signal 2), and cytokines (signal 3; ref. 3). Immature dendritic cells (DC) do not sufficiently provide signals 2 and 3 and induce robust, antigen-specific T-cell tolerance (4–6). Immunotherapies boosting tumor-specific immunity in mice and humans include vaccines (7), adoptive cell transfers (8), induction of antigen-presenting cell (APC) maturation (9, 10), checkpoint blockade (11, 12), and cytokines (13, 14), but the frequency of responders remains low.

We therefore aimed to prime tumor-specific CD8+ T cells, prevent induction of tolerance, and achieve control of large, established tumors. We used TRAMP mice that develop autochthonous prostate cancer (15) combined with adoptive transfer of tumor-specific TCR transgenic CD8+ T cells (TCR-I; ref. 16) as well as advanced B16 melanoma. We found that the combination of agonistic anti-CD40 + IL2/anti-IL2 complexes (IL2cx) + IL12Fc was a distinctively effective treatment with respect to priming protective, tumor-specific immunity and eradicating tumors at advanced disease stage.

Materials and Methods

Mice

C57BL/6-Tg(TRAMP)8247Ng/J (TRAMP; ref. 15) and B6.Cg-Tg(TcraY1,TcrbY1)416Tev/J (TCR-I) mice (16) were purchased from The Jackson Laboratory. B6.D2-Tg(TcrLCMV)327Sdz (P14) and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were obtained from the Laboratory Animal Service Center (University of Zurich, Zurich, Switzerland) and maintained on C57BL/6 background (17, 18). C57BL/6/OlaHsd male mice were obtained from Harlan Laboratories. Homozygous TRAMP female mice were bred with C57BL/6/OlaHsd males, generating heterozygous TRAMP males. Age- and sex-matched nontransgenic littersmates or C57BL/6/OlaHsd were used as wild-type (WT) controls. CD45.1+ TCR transgenic mice were maintained heterozygously. All mice were kept under specific pathogen-free conditions at the Institute of Laboratory Animal Science (University Hospital Zurich) and received standard chow (Provimi Kliba Cat.No. 3436) and water ad lib. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by the Swiss cantonal veterinary office (Zurich).

Tumor models

TRAMP mice carry the oncogenic SV40 large T antigen (SV40LT) as a transgene under control of the prostate-specific rat probasin promoter and show prostatic intraepithelial neoplasia at 8 weeks and adenocarcinomas at 12 weeks of age. Adenocarcinomas progress to large poorly differentiated tumors. Metastases occur in half of TRAMP mice by 24 weeks of age (19). For survival experiments, body weight measurements and pelvic palpations were performed on the mice every 1 to 2 weeks from the age of 20 weeks. Mice with palpable tumors at 20 weeks were excluded from the study. The death
event in tumor-free survival was defined as a tumor > 1 cm³ by palpation. The death event for overall survival was defined as at least one of five of the following termination criteria: lack of flight, poor general condition, hunched back, ruffled fur, or abdominal distension. B16F10 melanoma cells (ATCC CRL-6475) and B16F10-OVA (B16F10 stably transfected to express chicken ovalbumin, kindly provided by Dr. Melody Swartz, EPFL Lausanne, Switzerland) were cultured in DMEM (GIBCO Invitrogen) supplemented with 10% FBS (Gibco), 0.1 mmol/L 2-mercaptoethanol, 2 mmol/L l-glutamine, and antibiotics. 

Cells (2 × 10⁵) were injected subcutaneously (s.c.) in 100 μL PBS, and tumors were measured with a caliper every 2 to 3 days in two dimensions. The death event was defined as tumor size reaching 150 mm². Mice were randomized based on tumor size on the day before the scheduled start of therapy.

Adoptive transfer of CD8⁺ T cells

CD8⁺ cells were isolated from spleens using CD8α MicroBeads according to the manufacturer's protocol (Miltenyi Biotec). To track cell divisions, CD8⁺ T cells were incubated for 10 minutes at 37°C with 1 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich) in PBS, washed with media containing 10% FBS, and resuspended in PBS. To generate effector CD8⁺ T cells, CD8α⁺ cells were stimulated for 3 days in RPMI-1640 supplemented with 1 × MEM nonessential amino acids, antibiotics, 10 mmol/L Hepes, 2 mmol/L l-glutamine, 10% FBS, and 0.1 mmol/L 2-mercaptoethanol (Gibco) with Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) with a bead-to-cell ratio of 1:1 and recombinant murine cytokines (60 IU/μL IL2, 1 ng/mL IL7, 10 ng/mL IL15, and 10 ng/mL IL21; PeproTech). Unless otherwise stated, 10⁶ CD8⁺ T cells were i.v. injected.

Immunotherapies

Anti-CD40 (FGK45), anti–CTLA-4 (UC10-4F10-11), and anti–PD-1 (RMP1-14) were purified from culture supernatant using protein G sepharose 4 Fast Flow (GE Healthcare) columns according to the manufacturer’s protocol. Antibodies were administered at 50 μg (anti-CD40) or 250 μg (all others) in 200 μL PBS. IL2/anti-IL2 complexes were prepared with the 5486 Mab (20), and an equivalent of 15,000 units of recombinant mouse IL2 (eBioscience) was used per injection. Recombinant mouse IL2Fc was produced (21), and bioactivity was quantified by an IFNγ secretion assay with ConA-stimulated splenocytes (22) using the following formula: specific activity (units/mg) = 10⁶/ED₅₀ (ng/mL). IL2Fc was administered in PBS at 10,000 units/kg per i.p. injection for TRAMP mice or 100 units/kg per intratumoral (i.t.) injection for subcutaneous models. All other substances were injected intraperitoneally (i.p.).

Generation of recombinant vaccinia virus and infection

Vaccinia virus (VV) WR was originally obtained from Dr. B. Moss (NIH, Bethesda, MD). Recombinant VV expressing LCMV glycoprotein (rVV-G2) was originally obtained from Dr. D. Bishop (Institute of Virology, Oxford, UK). A recombinant VV expressing the SV40LT epitope I (aa 206-215) was generated (23) using Ncol/BglII-digested pSC11.3OR.2 (24) as vector and the following annealed oligonucleotides (fwd: 5'-CATGATCCGGCATAACAATTCGCCTCCAGAACGCTGTA-3'; rev: 5'-GATTCAGCTCCTCCTG GCCGAATTGTGTAGGCGCGA-3') as insert. Recombinant VV were generated as previously described (25). Viral titers were determined using BSC40 cells (ATCC CRL-2761). All rVV were propagated on BSC40 cells at a multiplicity of infection of 0.1. Mice received 2 × 10⁶ plaque-forming units i.p.

Peptides

SV40LT206–215 (SAINNYAQKL), SV40LT223–231 (CKGVNKEYL), SV40LT240–241 (VYDFLKIC), and LCMV gp33–41 (KAVYNFATC, PolyPeptide Laboratories, immunograde) were dissolved in DMSO to 10 mmol/L and stored at −20°C.

Flow cytometry

Spleens and lymph nodes were mechanically homogenized. Prostates were cut into small pieces and digested for 1 hour at 37°C with agitation in RPMI containing 1 mg/mL collagenase IV and 2.4 μg/mL (6 U/mL) DNase I (Sigma-Aldrich), washed once with RPMI followed by filtration (40 μm). Cells were resuspended in PBS and stained for 20 minutes at room temperature with fluorochrome-labeled antibodies. To detect intracellular IFNγ, cells were restimulated for 5 hours at 37°C with 10 μg/mL peptide and 10 μg/mL brefeldin A (Sigma-Aldrich). In some cases, 2.5 μg/mL of FITC anti-mouse CD107a (clone 1D4B; BioLegend) was added during restimulation. Subsequently, cells were washed in PBS followed by surface staining for 20 minutes at room temperature, washed again, and fixed with 4% paraformaldehyde in PBS. Cells were washed with permeabilization buffer (20 mmol/L EDTA + 2% FBS + 0.03% NaN₃ + 0.1% saponin in PBS). Samples were incubated overnight at 4°C with APC anti-mouse IFNγ (clone XM1G1.2; BioLegend). For staining of regulatory T cells (Treg), APC anti-mouse FoxP3 (clone FJK-16s; eBioscience) was used according to the manufacturer’s protocol. Unless otherwise stated, the following antibodies were purchased from BioLegend: anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD8α (53-6.7), anti–CD8β (53-5.8), anti–TCR β7 (TR310), anti–TCR α/β (B20.1; Becton Dickinson), anti–CD4 (RM4-5), anti–CD25 (PC61), and anti–PD-1 (RMP1-30 and 29F.1A12). Cells were gated on live singlets (live/dead Fixable Violet Dead Cell Stain Kit; Life technologies). Absolute counts were determined with CountBright Absolute Counting Beads (Life Technologies).

Histology

Sections (4 μm) were prepared from formalin-fixed, paraffin-embedded prostate samples. Hematoxylin and eosin staining was performed according to standard protocol. Immunohistochemistry was performed by Sophistolab AG using antibodies against CD3β (Santa Cruz Biotechnology: sc-1144, clone M-20), cleaved caspase 3 (Cell Signaling Technology; #9661, polyclonal), and hematdylin counter-staining. Whole slides were scanned with a Zeiss Mirax Midi slide scanner (40 objective, NA0.8) equipped with a 3-CCD color camera (Hitachi HV-F22) and analyzed using Pannoramic viewer 1.15.4 (3DHISTECH).

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.03 (GraphPad Software, Inc.). For comparisons, unless otherwise stated, unpaired, two-tailed Student t tests with systematic Welch correction were done with an alpha of 0.05. For
comparison of more than two experimental groups, one-way ANOVA either Tukey-Kramer post-test was used to compare all pairs or Dunnett post-test to compare all groups to the control, as mentioned in figure legends. A log-rank test (Mantel-Cox) was used for Kaplan-Meier survival curves comparison between selected pairs. A value of \( P < 0.05 \) was considered statistically significant and marked with asterisks (\(^*/_C_3^C\), \( P < 0.05 \); \(^*/_C_3/C_3\), \( P < 0.01 \); \(^*/_C_3/C_3/C_3\), \( P < 0.001 \)). In Fig. 3, two outliers identified with a significant Grubb test (alpha 0.05) with the online tool (http://graphpad.com/quickcalcs/Grubbs1.cfm) are shown in the bar graph, but were excluded from the statistical analysis.

Results

Tumor-specific CD8\(^{+}\) T cells are insufficiently primed in tumor-bearing mice

To study whether tumor-specific CD8\(^{+}\) T cells are primed to full effectors in the context of established tumors, we transferred CFSE-labeled tumor-specific CD8\(^{+}\) T cells (TCR-I) into 13- to 14-week-old male TRAMP or WT mice. The percentage of TCR-I cells of CD8\(^{+}\) T cells in spleen, PDLNs, and prostate 7 days after transfer (B), absolute number of CD8\(^{+}\) CD45.1\(^{+}\) TCR-I cells of live singlets per mg prostate (C), proliferation of TCR-I cells in spleen, PDLNs, and prostate 5 days after transfer (D), IFN\(\gamma\) production by TCR-I cells in spleen, PDLNs, and prostate (E), degranulation of TCR-I cells in spleen and PDLNs (F), PD-1 surface expression on TCR-I cells in the prostate (G). Two representative mice of 9 are shown (\( n = 4-5/group \)). WT, (\(*\)); TRAMP, (\(\bullet\)). Data from one of two independent experiments are shown. Horizontal lines represent mean values \(^*/_C_3\), \( P < 0.05 \); \(^*/_C_3/C_3\), \( P < 0.01 \); \(^*/_C_3/C_3/C_3\), \( P < 0.001 \); ns, not statistically significant. PDLN, prostate-draining lymph nodes.

Naive and effector tumor-specific CD8\(^{+}\) T cells lose function in tumor-bearing mice

To investigate whether tolerance induction is antigen dependent, we cotransferred TCR-I and P14 cells (as control) into WT and TRAMP mice followed by infection with two recombinant VVs, one encoding SV40LT206-215 (rVV-I), the other LCMV gp (rVV-G2), to trigger responses in both transferred populations (Fig. 2A). Whereas a high proportion of TCR-I and P14 cells in the spleen and prostate-draining lymph nodes (PDLN) of WT
mice produced IFNγ, responses of TCR-I, but not P14 cells, were significantly lower in TRAMP mice (Fig. 2B and Supplementary Fig. S2A–S2L). PD-1 surface expression was elevated only on TCR-I cells in TRAMP mice (Fig. 2C). Thus, antigen encounter by CD8+ T cells in the context of established cancer induces robust T-cell tolerance, which cannot be overcome by subsequent viral challenge.

Because adoptive T-cell therapy is a promising approach to treat cancer (8), we transferred tumor-specific CD8+ effector T cells, challenged mice 7 days later with rVV-I, and assessed TCR-I function on day 12 (Fig. 2D). Both in spleen and PDLNs of TRAMP mice, effector TCR-I cells displayed a 2-fold reduction of the frequency of IFNγ+ cells compared with WT mice and produced significantly less IFNγ on a per-cell basis (Fig. 2E), although...
prior activation of TCR-I cells prevented PD-1 upregulation in vivo on effector (Fig. 2F) but not naive cells (Fig. 2C). Thus, naive and, to a lesser extent, effector tumor-specific CD8+ T cells rapidly lose function in tumor-bearing mice.

Preventing tolerance induction by immune intervention

We used challenge with rVV-I to identify interventions that prevent tolerance induction (Supplementary Fig. S3A) and tested different treatments, including anti-CD40, IL2cx, IL12Fc, and blockade of CTLA-4 and PD-1. Data from three independent experiments were pooled, and for the purpose of comparison, we normalized the results to responses without intervention in WT (100%) and TRAMP mice (0%) for each of the six read-outs (Fig. 3A–C). Depending on the read-out, the individual treatments had different rankings (Fig. 3C and Supplementary Table S1), thus providing a rationale for combined use. Anti-CD40 was present in the five best treatments and was also the most potent single-agent therapy, underscoring the importance of DC activation. Anti-CD40+IL2cx+IL12Fc was the most potent regimen tested overall with respect to TCR-I numbers, IFNγ production, degranulation, and concomitant reduction in PD-1 expression (Fig. 3C; Supplementary Fig. S3C–S3E; and Supplementary Table S1). Thus, simultaneous targeting of multiple pathways prevents the induction of tumor-specific CD8+ T-cell tolerance in tumor-bearing mice.

Treatment with anti-CD40+IL2cx+IL12Fc turns tolerance of tumor-specific immunity into priming

To investigate whether anti-CD40+IL2cx+IL12Fc promotes priming of tumor-specific CD8+ T cells, we transferred naive TCR-I cells into TRAMP mice and concomitantly started therapy (Fig. 4A). One week after transfer, we found a statistically significant increase in absolute numbers of TCR-I cells and in the frequency and quality of IFNγ-producing TCR-I cells in the periphery and tumor of treated mice (Fig. 4B and Supplementary Fig. S4A), and high surface PD-1 expression was prevented upon treatment (Supplementary Fig. S4B). In addition, immunohistochemistry confirmed the flow cytometry data shown in Supplementary Fig. S4A and S4B: Treatment with anti-CD40+IL2cx+IL12Fc resulted in higher infiltration of the prostate by CD8β+ cells at day 7 after adoptive transfer of TCR-I cells (Supplementary Fig. S4G). On day 21 after transfer, hardly any CD8β+ cells were found in the control group, whereas CD8β+ cells infiltrated the tumor in mice treated with anti-CD40+IL2cx+IL12Fc (Supplementary Fig. S4H). Also, anti-CD40+IL2cx+IL12Fc outperformed all other combinations tested with this experimental setup (Supplementary Fig. S5A–S5F).

To investigate whether anti-CD40+IL2cx+IL12Fc can rescue tolerized TCR-I cells, we started treatment 2 weeks after...
adoptive transfer and performed analysis a week later (Fig. 4C). Anti-CD40+IL2cx+IL12Fc rescued tolerated TCR-I cells with respect to their numbers (Supplementary Fig. S4C), the frequency of IFNγ-producing TCR-I cells in the spleen, PDLNs, and prostate. C and D, rescue of tolerated TCR-I cells. E–H, sustained responses by TCR-I cells. G, weight of the genitourinary tract (seminal vesicles + prostate gland) on day 21. H, weight of prostate (day 21). I, logarithmic transformation of PD-1 geometric mean fluorescence intensity (GMFI) and IFNγ GMFI gated on IFNγ+ TCR-I cells was plotted with prostate samples from A, C, and E. Samples with less than 20 IFNγ+ TCR-I cells were excluded from the correlation. Symbols represent individual mice (n = 6–8/group). Data from three of seven independent experiments are shown; **, P < 0.01; ***. P < 0.001. Horizontal lines represent mean values.

To investigate whether anti-CD40+IL2cx+IL12Fc induces sustained tumor-specific immunity, we treated adoptively transferred mice for a week and analyzed the data 2 weeks later (Fig. 4E). Again, the treated group showed superior responses in terms of absolute numbers (Supplementary Fig. S4E), frequency and intensity of IFNγ production (Fig. 4F), and PD-1 expression (Supplementary Fig. S4F) by TCR-I cells. On day 21 after the start...
of the therapy, the treated mice had significantly reduced tumor burden (Fig. 4G and H). This reduction was not observed 1 week after treatment (Fig. 4A) or when treatment was started 2 weeks after adoptive transfer (Fig. 4C). When combining the data from these three different setups (Fig. 4A, C, and E), we found an inverse correlation between IFNγ and PD-1 geometric mean fluorescence intensity (GMFI) restricted to IFNγ+ TCR-I cells, with the strongest association ($R^2 = 0.9315$) in the prostate (Fig. 4I). Thus, treatment with anti-CD40+II.2cx+IL12Fc leads to clinical responses with durable local and systemic immunity. Moreover, tolerized tumor-specific CD8+ T cells were rescued to a limited extent, albeit without impact on tumor burden at the time point investigated.

To investigate the impact of the treatment on endogenous tumor-specific CD8+ T cells, we used 3 H-2k-restricted CD8 epitopes derived from SV40LT (26). Mice were treated as described (Fig. 4A and C), and single-cell suspensions from spleen, PDLNs, and prostate were stimulated in vitro with the three pooled SV40LT peptides, followed by intracellular staining for IFNγ. Seven days after adoptive transfer, the frequency of IFNγ+ endogenous CD8+ cells was significantly higher in treated mice (Fig. 5A), as were absolute counts of endogenous CD8+ T cells in the prostate (Fig. 5B). However, this effect was no longer detected 2 weeks after cessation of therapy. Higher numbers of IFNγ-producing endogenous CD8+ T cells correlated with higher surface PD-1 levels as compared with the control group (Fig. 5C), in contrast with the observations with TCR-I cells (Fig. 4C and Supplementary Fig. S5). The highest endogenous CD8+ T-cell response was observed on day 21 (Fig. 5D), but this was due in part to the low number of CD8+ T cells in the prostate (Fig. 5E). Because we only analyzed the response against three SV40LT-derived epitopes, we most likely underestimated the endogenous CD8+ T-cell response to the tumor. In addition, the absolute number of endogenous CD8+ T cells and the endogenous CD8/Treg ratio in the prostate increased upon treatment with anti-CD40 (Supplementary Fig. S1B and S1E). Thus, anti-CD40+II.2cx+IL12Fc enhances endogenous tumor-specific immunity.

Clinical efficacy of anti-CD40+II.2cx+IL12Fc in advanced cancer

We investigated the clinical efficacy of the therapy for advanced tumors using TRAMP mice and subcutaneously injected B16F10 syngeneic melanoma that does or does not express ovalbumin (OVA; Fig. 6A and E). TRAMP mice were treated at 20 weeks when all mice have advanced cancer (19). Although the control group had a median tumor-free survival of 32.3 weeks and median overall survival of 37.6 weeks, mice that received either TCR-I cells alone or TCR-I tumor-free survival of 32.3 weeks and median overall survival of (OVA; Fig. 6A and E). Clinical efficacy of anti-CD40+II.2cx+IL12Fc improves endogenous tumor-specific immunity. Endogenous CD8+ (CD45.1+) cells were stimulated for 5 hours with a combination of 3 SV40LT-derived peptides. A, frequency of IFNγ production by CD8+ T cells. B, absolute number and C, surface PD-1 expression on CD8+ T cells. D, ratio of absolute number of CD8+ cells to FoxP3+ CD4+ Treg cells. E, absolute number of FoxP3+ CD4+ Treg cells in the prostate. Symbols represent individual mice ($n = 7–8/group$). Data from one of two independent experiments are shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Statistical analysis was done by one-way ANOVA and Tukey-Kramer post-test to compare all pairs per organ. Horizontal lines represent mean values.
Figure 6.
Anti-CD40+IL2cx+IL12Fc controls late-stage cancer. A, treatment schedule for TRAMP mice. B, tumor-free survival of TRAMP mice. C, overall survival of TRAMP mice. D, frequency of IFNγ+ and CD107a+ TCR-I cells 6 months after immune intervention. E, experimental design for late-stage treatment of subcutaneous B16 melanoma. F, Kaplan-Meier survival analysis of mice with B16F10 tumors. G, Kaplan-Meier survival analysis of mice bearing B16F10-OVA tumors, some of which received 10⁶ naive OT-I CD8+ cells. H, two of three cured mice from G show vitiligo at the site of the rejected tumor (day 100). TRAMP survival analysis was performed once (n = 11-14/group). Data from two of four experiments are shown for the subcutaneous models (n = 6-7/group). **P < 0.01; ***P < 0.001. Log-rank (Mantel-Cox) tests were performed between pairs as described.
Effective Immunotherapy of Advanced Cancer in Mice

Treatment of B16F10 or B16F10-OVA tumors started on day 13 after tumor injection (tumor size ~40–50 mm³), a time at which single reagents cannot control tumor growth (27 and unpublished data). The median survival of B16F10-bearing mice treated with anti-CD40+IL2cx+IL12Fc nearly doubled (34 days) compared with the controls (18 days; Fig. 6F and Supplementary Fig. S6A).

Some of B16F10-OVA–bearing mice received adoptively transferred OT-I cells intravenously on day 13. Without anti-CD40+IL2cx+IL12Fc, mice with B16F10-OVA tumors had a median survival time of 21 days irrespective of OT-I transfer. Treatment with anti-CD40+IL2cx+IL12Fc + OT-I cells prolonged the median survival to 52 days and cured 43% (3/7) of the mice (Fig. 6G and Supplementary Fig. S6B). Tumor rejection was accompanied by local vitiligo, indicating an endogenous immune response against melanocytes (Fig. 6H). Thus, supporting tumor-specific CD8⁺ T cells on three levels (anti-CD40, IL2cx and IL12Fc) generates protective immunity that can eradicate advanced tumors.

Discussion

The clinical response to immune-modulating drugs, such as anti-CTLA-4 and -PD-1, illustrates that the immune system can be exploited to manage cancer (12, 28, 29). However, only a fraction of the patients respond to such therapies, suggesting that additional pathways must be targeted to improve clinical efficacy.

We identified the combination of anti-CD40+IL2cx+IL12Fc as uniquely efficacious in inducing sustained tumor-specific immunity that controls or cures advanced cancer when combined with adoptive T-cell transfer. Without intervention, naïve TCR-I cells proliferate upon transfer into TRAMP mice, but are subsequently tolerated, suggesting that signals 2 (costimulation) and 3 (cytokines) rather than signal 1 (TCR) are limiting.

A major result of concomitant TCR and CD28 signaling is the production of IL2 by T cells. However, IL2 production is transient and Tregs form a sink for IL2 (30, 31), which may be especially relevant in the Treg-rich tumor microenvironment. Specific support to effector T cells is possible by using IL2cx, which targets IL2 to antigen-experienced CD122high (IL2Rβ⁺) T cells (20).

While anti-CD40+IL2cx supports priming and expansion of advanced tumor-specific CD8⁺ T cells in the context of advanced cancer, their differentiation into protective effectors requires additional signals, such as IL12 (5). Accordingly, combining anti-CD40+IL2cx with IL12Fc significantly improved the quality of tumor-specific T cells with respect to many parameters. Together, our data are in line with recent work showing that T-cell division destiny depends on the integrated quality of signals 1, 2, and 3 (32).

Although APC maturation is essential and usually sufficient to prevent the induction of peripheral T-cell tolerance, the immunosuppressive tumor microenvironment poses an additional challenge. PD-1 was originally described as an activation marker on T cells (33), but later data showed that PD-1 acts as a coinhibitory molecule (34) involved in peripheral T-cell tolerance (35). TCR-I cells progressively upregulated the expression of PD-1 upon adoptive transfer into TRAMP mice, which negatively correlated with IFNγ production, in agreement with PD-1 marking T-cell exhaustion/dysfunction (36). However, endogenous CD8⁺ T cells behaved differently with respect to PD-1 expression: Treatment with anti-CD40+IL2cx+IL12Fc resulted in increased IFNγ production and PD-1 expression. Because the expression of PD-1 on endogenous CD8⁺ T cells in TRAMP mice treated with anti-CD40+IL2cx+IL12Fc is substantially lower than that on TCR-I cells in untreated TRAMP mice, we think that intermediate expression of PD-1 marks T-cell activation, whereas high expression indicates exhaustion/dysfunction (37). The greater tendency of TCR-I cells to express high levels of PD-1 in the context of advanced cancer when compared with endogenous CD8⁺ T cells may be related to TCR affinity (38). All interventions tested here had an impact on PD-1 expression by TCR-I cells, pointing toward PD-1 as a central player in the negative feedback mechanism following activation. However, blocking PD-1 or reducing its surface expression seems insufficient to induce protective immunity.

Recent studies have suggested that the efficacy of checkpoint blockade depends on de novo priming toward tumor-specific mutant antigens (39, 40). Our study supports the importance of de novo priming by showing that rescuing preexisting tumor-infiltrating CD8⁺ T cells is inefficient. Preexisting immunity is presumably compromised and cannot be sufficiently boosted, but, instead, efficacy may rely on new thymic emigrants (or transferred T cells) that encounter tumor-specific antigens in an immunogenic context. Indeed, our data show that treatment with anti-CD40+IL2cx+IL12Fc during the first week of adoptive transfer results in clinical efficacy and maintenance of protective effector TCR-I cells over a period of at least 6 months in TRAMP mice with advanced cancer. Therefore, combining anti-CD40+IL2cx+IL12Fc with adoptively transferred, tumor-specific T cells may be a promising approach to translate into clinical practice, for example, in the context of transferred autologous T cells, which express engineered TCRs or chimeric antigen receptors (CAR).

In conclusion, we have identified a novel therapeutic intervention for advanced tumors that durably supports tumor-specific immunity and shows clinical responses in multiple cancer models. The efficacy of anti-CD40+IL2cx+IL12Fc outperformed all other treatments we tested here, and we think that combined improvement of signals 2 and 3 explains this result.

We propose that an optimal cancer immunotherapy based on T cells should tackle three major obstacles. First, the frequency of fresh tumor-specific T cells needs to be increased. Second, appropriate costimulation must be provided. Third, survival and differentiation of tumor-infiltrating T cells must be supported by (local) cytokines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Bransi, O.C. Salgado, A. Knuth, M. van den Broek
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