

## Interleukin-7 Mediates Selective Expansion of Tumor-redirected Cytotoxic T Lymphocytes (CTLs) without Enhancement of Regulatory T-cell Inhibition

Serena K. Perna<sup>1</sup>, Daria Pagliara<sup>1,5</sup>, Aruna Mahendravada<sup>1</sup>, Hao Liu<sup>1</sup>, Malcolm K. Brenner<sup>1,2,4</sup>, Barbara Savoldo<sup>1,2</sup>, and Gianpietro Dotti<sup>1,3,4</sup>

### Abstract

**Purpose:** The antitumor activity of chimeric antigen receptor (CAR)-redirected CTLs should be enhanced if it were possible to increase their proliferation and function after adoptive transfer without concomitantly increasing the proliferation and function of regulatory T cells (Treg). Here, we explored whether the lack of IL-7R $\alpha$  in Treg can be exploited by the targeted manipulation of the interleukin-7 (IL-7) cytokine-cytokine receptor axis in CAR-engrafted Epstein-Barr Virus-specific CTLs (EBV-CTLs) to selectively augment their growth and antitumor activity even in the presence of Treg.

**Experimental Design:** We generated a bicistronic retroviral vector encoding a GD2-specific CAR and the IL-7R $\alpha$  subunit, expressed the genes in EBV-CTLs, and assessed their capacity to control tumor growth in the presence of Treg *in vitro* and *in vivo* when exposed to either interleukin-2 (IL-2) or IL-7 in a neuroblastoma xenograft.

**Results:** We found that IL-7, in sharp contrast with IL-2, supports the proliferation and antitumor activity of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs both *in vitro* and *in vivo* even in the presence of fully functional Treg.

**Conclusions:** IL-7 selectively favors the survival, proliferation, and effector function of IL-7R $\alpha$ -transgenic/CAR-redirected EBV-CTLs in the presence of Treg both *in vitro* and *in vivo*. Thus, IL-7 can have a significant impact in sustaining expansion and persistence of adoptively CAR-redirected CTLs. *Clin Cancer Res*; 20(1); 131–9. ©2013 AACR.

### Introduction

The expression of chimeric antigen receptors (CAR) in T lymphocytes to redirect their antigen specificity has significantly expanded the clinical application of adoptive T-cell immunotherapies against a variety of human malignancies (1, 2). CAR molecules are chimeric proteins, in which a single chain antibody-binding site is fused with the signaling domain CD3 $\zeta$  that activates T lymphocytes upon binding to the tumor antigen (3). However, in this form, CAR molecules do not provide adequate costimulation to T cells (1, 4, 5). To overcome this limitation, CARs can be expressed by CTLs whose native receptors are specific for virus latency proteins such as those derived from the Epstein-Barr Virus-specific CTLs (EBV-CTLs; refs. 6, 7).

These virus-specific CTLs can receive physiologic costimulation from professional antigen presenting cells processing latent viral antigens and kill tumor cells through their CAR (6, 7). Although this approach can produce complete and sustained antitumor responses, for example in some patients with neuroblastoma, in most recipients, CAR-engrafted EBV-CTLs have limited *in vivo* survival and fail to consistently eradicate disease (8, 9). It is likely that the combination of host/tumor associated inhibitory factors and insufficient *in vivo* immunostimulation limit the expansion and persistence of these cells (10).

Regulatory T cells (Treg) play a significant role in impairing the antitumor effects of tumor-specific CTLs (11). Treg are frequently increased in the peripheral blood and in tumor biopsies of patients with cancer (12–17) and their presence often correlates with poor clinical outcome (15). Thus, the development of strategies aimed at eliminating Treg or at selectively favoring the expansion of antitumor CTLs may significantly contribute in enhancing the engraftment and antitumor effects of adoptively transferred CTLs. To date, most efforts to increase *in vivo* immunostimulation of adoptively transferred T cells have focused on administration of interleukin (IL)-2 (18). Although this cytokine is a potent T-cell growth factor, it is not selective for effector T-cell subsets and can also enhance the growth and inhibitory activity of Treg (19).

**Authors' Affiliations:** <sup>1</sup>Center for Cell and Gene Therapy, and Departments of <sup>2</sup>Pediatrics, <sup>3</sup>Immunology, and <sup>4</sup>Medicine, Baylor College of Medicine, Methodist Hospital and Texas Children's Hospital, Houston, Texas; <sup>5</sup>Dipartimento di Ematologia ed Oncologia Pediatrica, IRCCS Ospedale Pediatrico Bambino Gesù, Roma, Italy

**Corresponding Author:** Gianpietro Dotti, Center for Cell and Gene Therapy, Baylor College of Medicine, 6621 Fannin St. MC 3-3320, Houston, TX 77030, Phone: 832-824-6891; Fax: 832-825-4732; E-mail: gdotti@bcm.edu

doi: 10.1158/1078-0432.CCR-13-1016

©2013 American Association for Cancer Research.

### Translational Relevance

Adoptive transfer of virus-specific CTLs expressing a chimeric antigen receptor (CAR) represents a promising therapy for patients with cancer. However, the *in vivo* expansion of these cells remains suboptimal so that new strategies are required to selectively expand them without favoring the concomitant proliferation and function of regulatory T cells (Treg) that are often abundant in patients with cancer. Our study provides preclinical data, indicating that the manipulation of the interleukin (IL)-7 cytokine–cytokine receptor axis in CAR-engrafted Epstein–Barr Virus–specific CTLs (EBV-CTLs) can be used to selectively expand the CTLs while avoiding the inhibitory effects of Treg, which would otherwise be enhanced by use of the more broadly acting T-cell growth factor IL-2.

One means by which T lymphocytes can be selectively expanded is by using IL-7, a  $\gamma$ -chain cytokine that promotes homeostatic expansion of naïve and memory T cells but has no activity on Treg, which lack the IL-7R $\alpha$  (the private chain of the IL-7 receptor; refs. 20–23). Administration of recombinant IL-7 was well tolerated in early-phase clinical trials, and expanded naïve and central-memory T-cell subsets but not Treg (20, 21). Unfortunately, under physiologic conditions, IL-7 cannot support the *in vivo* expansion of adoptively transferred CAR-redirection CTLs as this is an effector-memory T-cell subset that, like Treg, also lacks IL-7R $\alpha$  (24).

Here, we developed models *in vitro* and *in vivo* to demonstrate that human Treg clearly inhibit the antitumor effects of CAR-redirection EBV-CTLs. We also show that selective modulation of the IL-7 cytokine–cytokine receptor axis in CAR-engrafted EBV-CTLs augments their antitumor effects *in vivo* in the presence of Treg. This strategy should safely enhance the persistence and survival of adoptively transferred CAR-redirection virus-specific CTLs in patients with cancer.

### Materials and Methods

#### Plasmid construction, retrovirus production, and tumor cell lines

The full-length human IL-7R $\alpha$  linked through the 2A (TAV) sequence to the CAR-GD2 encoding the CD28 endodomain (25) was cloned into the SFG retroviral vector to generate the bicistronic vector SFG.IL-7R $\alpha$ .CAR-GD2. The retroviral vectors encoding eGFP and Firefly Luciferase (FFLuc) were previously described (26). Retroviral supernatant was prepared using transient transfection of 293T cells (26). The neuroblastoma cell line CHLA-255 (ref. 27; kindly provided by Dr. Leonid Metelitsa, Texas Children's Hospital, Baylor College of Medicine, Houston, TX) was derived from a patient, and we verified that this line retains the surface expression of the target antigen GD2.

#### Generation and transduction of EBV-CTLs

EBV-transformed lymphoblastoid cells (LCL) and EBV-CTLs were prepared using peripheral blood mononuclear

cells (PBMC), obtained from healthy donors as previously described (28). EBV-CTLs were transduced with retroviral supernatant after three stimulations with autologous LCLs, as previously described (8), and then maintained in culture by weekly stimulation with LCLs and recombinant IL-2 (50 IU/mL) or IL-7 (2.5 ng/mL; PeproTech).

#### Expansion of Treg

To obtain significant numbers of cells for the *in vitro* and *in vivo* experiments, Treg were isolated and expanded as previously described (29). Briefly, CD25<sup>bright</sup> T cells were purified from PBMCs by positive selection using immunomagnetic selection in the presence of nonsaturating concentrations (2  $\mu$ L/ $1 \times 10^7$  PBMCs) of anti-human CD25 magnetic beads (Miltenyi Biotec). On day 0, the purified CD25<sup>+</sup> T cells were activated in 24-well plates coated with OKT3 (1  $\mu$ g/mL) and anti-CD28 antibody (BD Pharmingen; 1  $\mu$ g/mL) in RPMI 1640 in the presence of rapamycin (Sigma) at a final concentration of 100 nmol/L. On days 7 and 14, cells were restimulated with OKT3/CD28 antibodies, irradiated feeder cells, rapamycin, and IL-2 (50 IU/mL) in small bioreactors (G-REX; ref. 29). At the end of the 3-week culture (day 21), cells were used for *in vitro* and *in vivo* experiments. The cell fraction obtained from buffy coats after the selection of CD25<sup>bright</sup> T cells was further enriched for CD4<sup>+</sup> cells which were then used as negative control in parallel culture experiments, in which we evaluated the immunosuppressive activity of Treg (29, 30).

#### Immunophenotyping

Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin-chlorophyll-protein complex (PerCP)-, or allophycocyanin (APC)-conjugated monoclonal antibodies (mAb). We used CD3, CD4, CD8, CD25, and CD127 (IL-7R $\alpha$  specific) from Becton Dickinson (BD Bioscience) and FoxP3 from eBioscience Inc. CAR-GD2 expression by transduced EBV-CTLs was detected using the specific anti-idiotypic antibody 1A7, followed by staining with the secondary antibody RAM-IgG1-PE (Becton Dickinson; ref. 8). STAT5 phosphorylation in Treg and EBV-CTLs was assessed after cytokine stimulation for 15 minutes using the anti-phospho-STAT5 (Y694) mAb-Alexa Fluor 647 Conjugate (BD Phosflow Reagents). Cells were analyzed using a BD FACSCalibur system equipped with the filter set for quadruple fluorescence signals and the CellQuest software (BD Biosciences). For each sample, we analyzed a minimum of 10,000 events.

#### Carboxyfluorescein diacetate succinimidyl ester–based assays

Proliferation of Treg or EBV-CTLs or activated PBMC was assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. Briefly, EBV-CTLs were labeled with 1.5  $\mu$ mol/L CFSE (Invitrogen) and activated with LCLs (ratio 4:1) with or without IL-2 (12.5 IU/mL) or IL-7 (10 ng/mL). CFSE dilution was measured by flow cytometry after 7 days of culture. A similar protocol was used to evaluate the proliferation of CFSE-labeled Treg post activation with

OKT3, irradiated feeders, and IL-2 or IL-7. To evaluate the suppressive activity of Treg, CFSE-labeled EBV-CTLs were stimulated with LCLs (ratio 4:1) in the presence of Treg or control CD4<sup>+</sup>CD25<sup>-</sup> cells (ratio, 1:1; ref. 30), and of IL-2 (12.5 IU/mL) or IL-7 (10 ng/mL). Similarly, PBMC depleted of CD25<sup>bright</sup> cells were stained with CFSE and activated in the presence of irradiated allogeneic feeders (ratio 2:1) and OKT3 (500 ng/mL; refs. 29, 30). After 7 days, cells were stained with CD8-APC and CD4-PerCP, analyzed by fluorescence-activated cell sorting (FACS) and cell division assessed by CFSE dilution.

### Evaluation of antitumor activity

EBV-CTLs were cultured in the presence of the neuroblastoma cell line (CHLA-255) genetically modified to stably express GFP in the presence or in the absence of Treg (at the EBV-CTLs:CHLA-255:Treg ratio of 1:2:1) and of IL-2 (12.5 IU/mL) or IL-7 (5 ng/mL). After 7 days, cells were collected, stained with CD3 to identify T cells, and analyzed by FACS. GFP was used to quantify residual tumor cells in culture.

### Xenogenic mouse model

To assess the antitumor effect of EBV-CTLs *in vivo* in the presence of Treg, we used the xenograft mouse model and an *in vivo* imaging system as previously described (7, 24). Mouse experiments were performed in accordance with Baylor College of Medicine's Animal Husbandry guidelines. Briefly, 8- to 10-week-old NOD.Cg-Prkdcscid IL2rgtmWjl/Sz (NSG) mice (Jackson Laboratory, Bar Harbor, ME) were engrafted intraperitoneally with the CHLA-255 cells ( $1 \times 10^6$  cells per mouse) genetically modified with FFluc to monitor tumor growth using the IVIS bioluminescence system (Xenogen IVIS 200 Biophotonic Imaging System). The intraperitoneal model was selected to minimize confounding issues due to suboptimal cell biodistribution and simultaneous colocalization at the tumor site of CAR-modified EBV-CTLs and Treg. When the signal (measured as p/sec/cm<sup>2</sup>/sr) was consistently increasing, usually by day 7 to 10, mice received intraperitoneal EBV-CTLs ( $10 \times 10^6$  T cells per mouse) with or without Treg ( $10 \times 10^6$  T cells per mouse; two infusions 1-week apart). IL-2 (500 IU/mouse) or IL-7 (200 ng/mouse) were administered intraperitoneally three times a week.

### Statistical analysis

All *in vitro* data were summarized by means and SEM. For the bioluminescent experiments, intensity signals were log-transformed and summarized using mean  $\pm$  SD at baseline and multiple subsequent time points for each group of mice. Changes in intensity of signal from baseline at each time point were calculated and compared using paired *t* tests or Wilcoxon signed-ranks test. When the *P* value was less than 0.05, a mean difference was accepted as statistically significant. For the bioluminescence experiments, intensity signals were log-transformed and summarized using mean and SDs at baseline and multiple subsequent time points for each group of mice. The response profiles over time were analyzed by the generalized estimating equations method for repeated measurements.

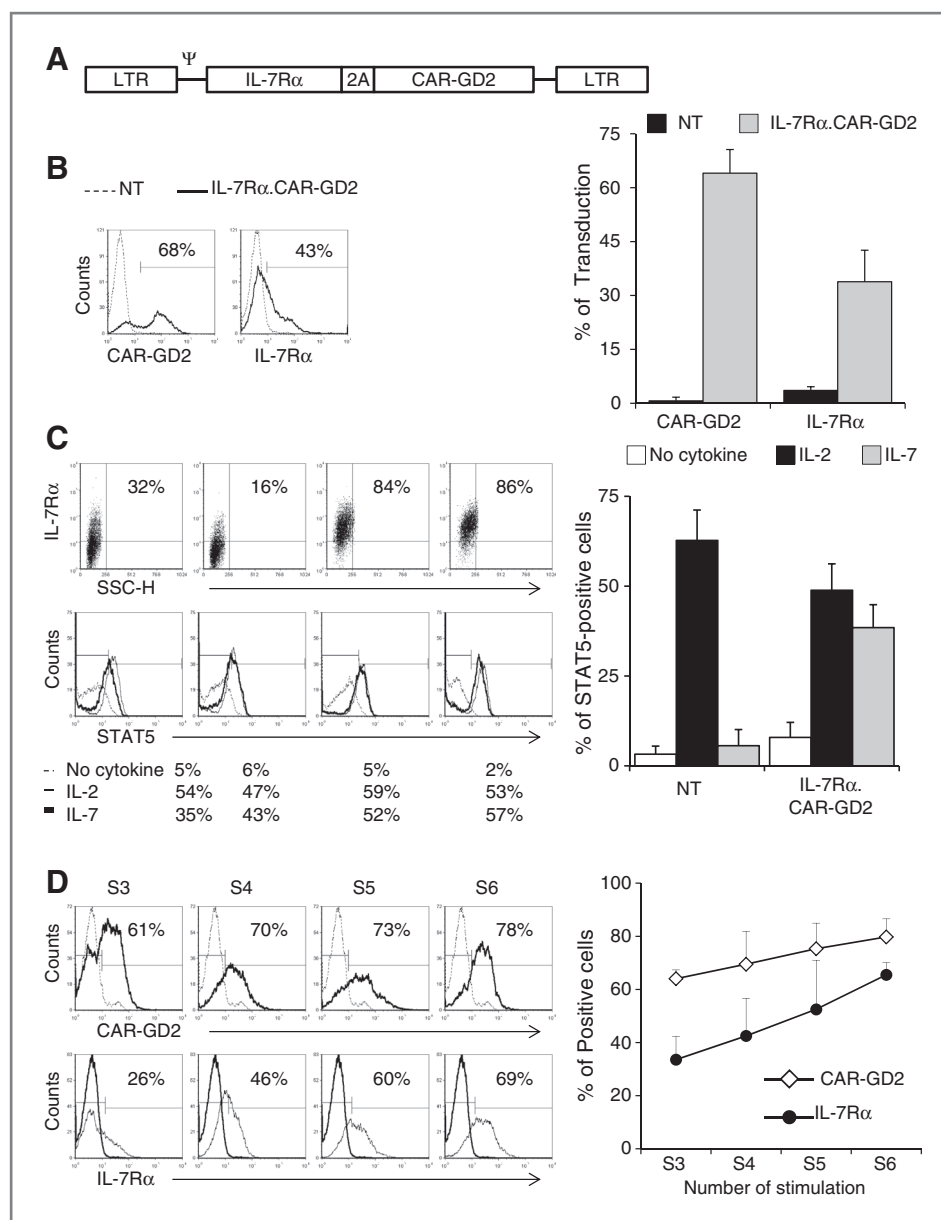
## Results

### Functional IL-7R $\alpha$ and CAR-GD2 can be coexpressed in EBV-CTLs

To restore the responsiveness to IL-7 and to redirect the antigen specificity of EBV-CTLs against neuroblastoma, we generated a bicistronic  $\gamma$ -retroviral vector encoding the IL-7R $\alpha$  and a GD2-specific CAR linked through a 2A (TAV) sequence (SFG.IL-7R $\alpha$ .2A.CAR-GD2; Fig. 1A). EBV-CTLs established from 5 healthy EBV-seropositive donors were transduced with the vector, and the expression of both IL-7R $\alpha$  and CAR-GD2 was measured by FACS analysis. As shown in Figure 1B, both CAR-GD2 and IL-7R $\alpha$  were stably expressed ( $64\% \pm 3\%$  and  $34\% \pm 9\%$ , respectively) in transduced EBV-CTLs, whereas the expression of the native IL-7R $\alpha$  on control cells remained negligible ( $4\% \pm 1\%$ ).

To evaluate the functionality of the transgenic IL-7R $\alpha$ , we measured the phosphorylation of STAT5 in response to either IL-2 or IL-7. In the absence of cytokines, control and IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs showed negligible phosphorylation of STAT5 ( $3\% \pm 2\%$  and  $8\% \pm 4\%$ , respectively). In IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs, near equal STAT5 phosphorylation of Tyr-694 was detected in response to IL-2 ( $49\% \pm 7\%$ ) or IL-7 ( $38\% \pm 6\%$ , respectively; *P* = NS). In contrast, in control cells, STAT5 was phosphorylated in response to IL-2 ( $63\% \pm 8\%$ ) but not to IL-7 ( $6\% \pm 5\%$ ; *P* < 0.05; Fig. 1C). The levels of IL-7R $\alpha$ -dependent STAT5 phosphorylation in IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs exposed to IL-7 were very similar to the amount observed in T lymphocytes physiologically expressing the IL-7R $\alpha$  and exposed to IL-7 (Supplementary Fig. S1A). The functionality of the transgenic IL-7R $\alpha$  was further supported by progressive selection of transgenic cells if cultures were supplemented with IL-7. As illustrated in Figure 1D (and Supplementary Fig. S1B), when IL-7R $\alpha$ .CAR-GD2<sup>+</sup> CTLs were stimulated weekly with autologous LCLs and IL-7, the expression of both IL-7R $\alpha$  and CAR-GD2 progressively increased between the third and sixth antigen-specific stimulation (from  $34\% \pm 9\%$  to  $66\% \pm 5\%$  for IL-7R $\alpha$ , and from  $64\% \pm 3\%$  to  $80\% \pm 7\%$  for CAR-GD2). In contrast, when CTLs were expanded in the presence of IL-2, no enrichment of either transgenes was observed, as this cytokine equally supports the *ex vivo* growth of transduced and non transduced CTLs (data not shown).

The enrichment of transgenic T cells following exposure to IL-7 was a consequence of the proliferation of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs. As illustrated in Figure 2A, CFSE labeled-control and IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs divided equally well when stimulated with LCLs (ratio 4:1) in the presence of IL-2 (proliferation,  $68\% \pm 6\%$  and  $68\% \pm 4\%$ , respectively). In contrast, in the presence of IL-7, IL-7R $\alpha$ .CAR-GD2<sup>+</sup> but not control EBV-CTLs had significantly greater proliferation,  $63\% \pm 3\%$  versus  $14\% \pm 1\%$ , respectively (*P* < 0.001). The number of EBV-CTLs proliferating in response to EBV-LCLs and IL-7 was generally higher than expected based on the ectopic expression of IL-7R $\alpha$ . This higher level is likely a consequence of the physiologic production of IL-2 by EBV-CTLs in response to their cognate EBV antigens (EBV-LCLs; Supplementary Fig. S2). Finally,



**Figure 1.** EBV-CTLs are effectively transduced with the bicistronic vector encoding both the IL-7R $\alpha$  and the CAR-GD2. A, schema of the bicistronic  $\gamma$ -retroviral vector encoding the IL-7R $\alpha$  and GD2-specific CAR linked through a 2A (TAV) sequence. B, expression of CAR-GD2 (top) and IL-7R $\alpha$  (bottom) evaluated by FACS analysis day 7 after transduction. The dotted line indicates control EBV-CTLs and the bold line indicates the transduced EBV-CTLs. The graph represents mean  $\pm$  SD of 5 donors. C, IL-7R $\alpha$  expression in four IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs generated (top) and STAT5 phosphorylation (bottom) in the absence of cytokines (thin black line), in response to IL-2 (dotted line), or IL-7 (black bold line). D, progressive enrichment in cells expressing the two transgenes IL-7R $\alpha$  and CAR-GD2 when IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs were expanded in the presence of IL-7. S3, S4, S5, and S6 indicate the transgene expression detected week 3 (S3), week 4 (S4), week 5 (S5), and week 6 (S6), respectively after transduction. Graph represents mean  $\pm$  SEM of four different EBV-CTL lines.

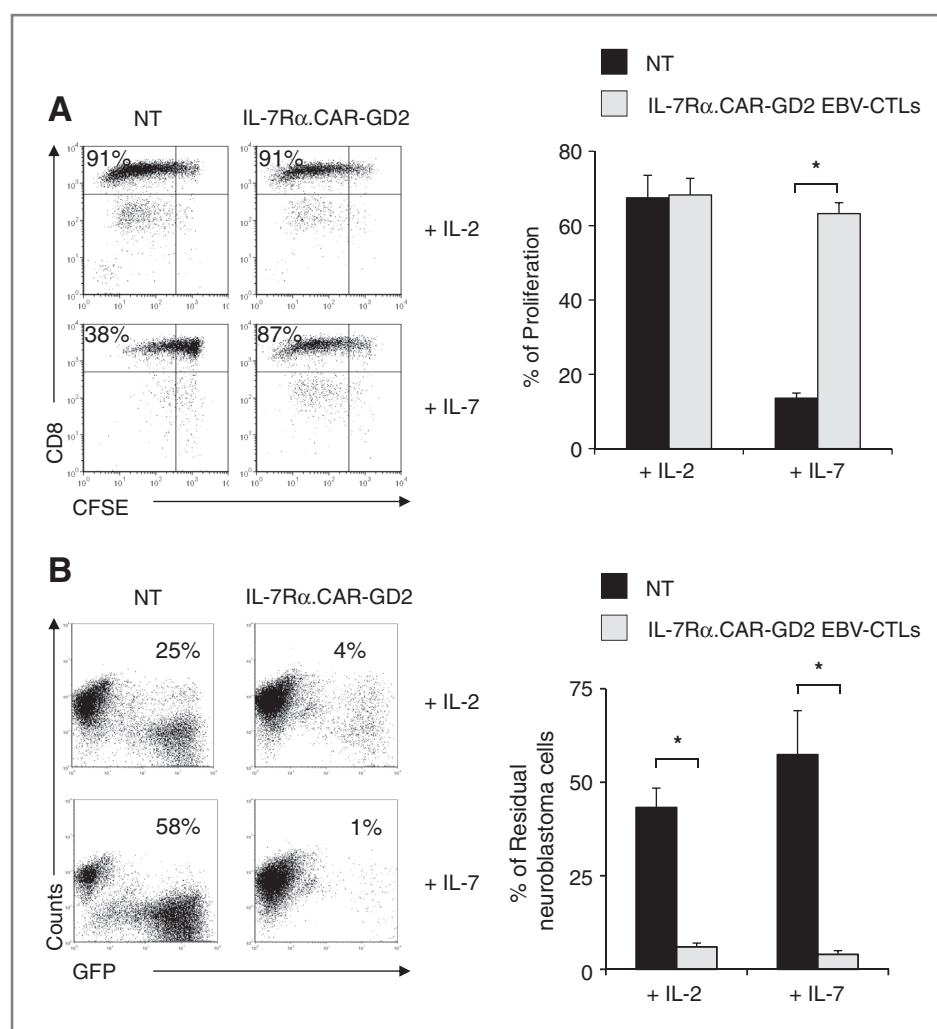
exposure of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs to IL-7 did not affect their antitumor properties. As shown in Figure 2B, when EBV-CTLs were cultured with CHLA-255 cells, only IL-7R $\alpha$ .CAR-GD2<sup>+</sup> cells controlled tumor growth in the presence of either IL-2 or IL-7 (6%  $\pm$  1% and 4%  $\pm$  1%, respectively), whereas tumor cells outgrew in cultures containing control EBV-CTLs irrespective of the cytokine added (43%  $\pm$  5% and 57%  $\pm$  12%, respectively;  $P < 0.001$ ).

#### Ex vivo expanded Treg do not respond to IL-7

We used *ex vivo* expanded CD4<sup>+</sup>CD25<sup>+</sup> Treg isolated from healthy donors rather than freshly isolated Treg for the following reasons. First, the experiments required a significant number of Treg that could not be obtained upon fresh isolation even from buffy coat preparations.

Second, circulating Treg obtained after immunomagnetic selection on the basis of CD4 and CD25 expression are frequently contaminated by CD4<sup>+</sup>CD25<sup>+</sup>IL7R $\alpha$ <sup>+</sup> cells that lack regulatory activity, but respond to IL-7 (data not shown; ref. 31). We first confirmed that the nominal Treg population retained their inhibitory properties. As shown in Figure 3A, the proliferation of activated PBMCs (80%  $\pm$  3% in the presence of control CD4<sup>+</sup>CD25<sup>-</sup> cells) was significantly inhibited in the presence of the expanded Treg population (27%  $\pm$  6%;  $P < 0.001$ ). We then confirmed that these Treg, like freshly isolated Treg (22), lacked expression of IL-7R $\alpha$  (3%  $\pm$  0.4% positive; Fig. 3B). As a consequence, STAT5 was only phosphorylated in these Treg in response to IL-2 (MFI = 75  $\pm$  9;  $P < 0.001$ ) and not in response to IL-7 (MFI = 23  $\pm$  3; Fig. 3C).

**Figure 2.** IL-7 supports the proliferation and effector function of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs. A, representative CFSE-based proliferation assay of control and IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs. Control and IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs were activated in the presence of autologous irradiated LCLs and either IL-2 or IL-7. CFSE dilution was evaluated on day 7 using FACS analysis. The graph represents mean  $\pm$  SD of five independent experiments. B, representative coculture experiment in which control and IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs were cocultured with CHLA-255 GFP-tagged tumor cells (at ratio 1:2) in the presence of IL-2 or IL-7. Residual tumor cells were enumerated by flow cytometry on day 7 of culture. The graph shows mean  $\pm$  SD of five independent experiments. \*,  $P < 0.001$ .



Finally, a CFSE-based dilution assay showed that Treg only proliferated after polyclonal activation in the presence of IL-2 and not on exposure to IL-7 (MFI 1439  $\pm$  207 vs. 445  $\pm$  68, respectively;  $P < 0.001$ ; Fig. 3D).

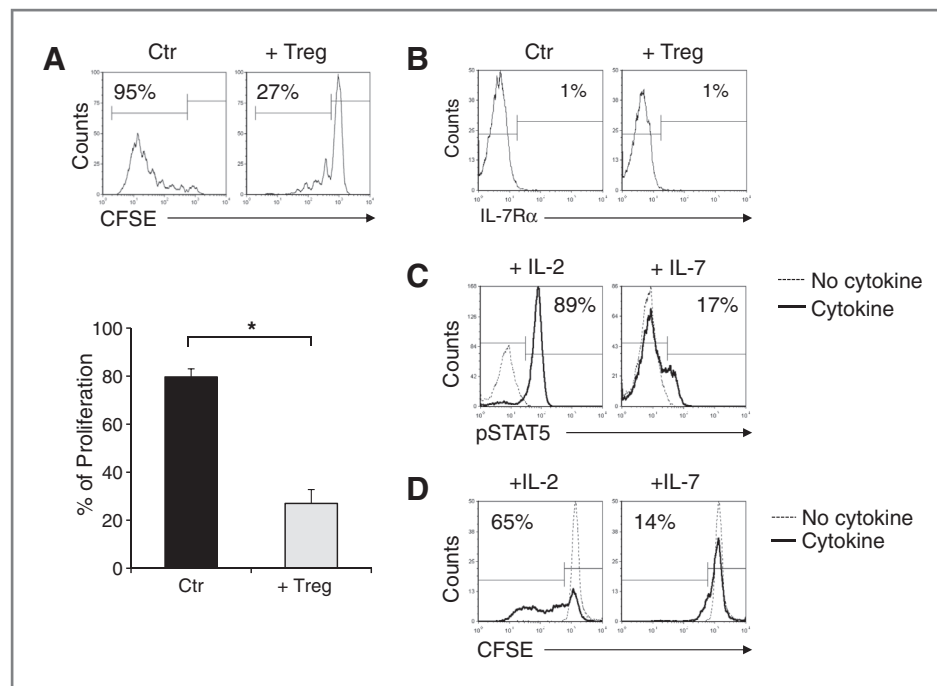
### IL-7 supports the proliferation and effector function of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs in the presence of Treg

Having demonstrated that IL-7 supports the proliferation and function of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs, we then investigated whether the beneficial effects of IL-7 were maintained in the presence of functional Treg. As illustrated in Figure 4A, when IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs were cultured with CHLA-255 cells (effector:target ratio of 1:2) they significantly controlled the growth of these tumor cells by day 7 of culture in the presence of either IL-2 or IL-7 (residual cells were 6%  $\pm$  1% and 4%  $\pm$  1%, respectively). In contrast, when expanded Treg were added to the coculture (ratio CTLs:Treg 1:1), the antitumor activity of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs was significantly inhibited in the presence of IL-2 but not of IL-7 (residual cells in culture, 14%  $\pm$  3% vs. 7%  $\pm$  2%, respectively;  $P < 0.05$ ). In addition, IL-7 also

supported the proliferation of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs in the presence of Treg upon physiologic costimulation with autologous LCLs. As the CFSE dilution assay shows in Figure 4B, the proliferation of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs in response to IL-2 (68%  $\pm$  4%) was significantly compromised in the presence of Treg (to 34%  $\pm$  6%;  $P < 0.01$ ). In contrast, when IL-7 was added to the culture, IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs divided well even in the presence of Treg (proliferation was 63%  $\pm$  3% without Treg and 56%  $\pm$  2% in the presence of Treg). The CFSE dilution of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs cocultured with Treg was significantly increased in the presence of IL-7 as compared with IL-2 ( $P = 0.005$ ).

### IL-7 supports the *in vivo* antitumor activity of IL-7R $\alpha$ .CAR-GD2 EBV-CTLs even in the presence of Treg

To assess the *in vivo* capacity of IL-7 to support the antitumor activity of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs, we used NSG mice engrafted intraperitoneally with the FFLuc<sup>+</sup> cell line CHLA-255. As shown in Figure 5, control mice that received only tumor cells or control CTLs showed a rapid



**Figure 3.** *Ex vivo* expanded Treg do not respond to IL-7. **A**, CFSE-based assay to illustrate the inhibitory activity of *ex vivo* expanded Treg. PBMCs labeled with CFSE were activated in the absence (left) or in the presence of Treg (right) at a ratio of 1:1. CFSE dilution was measured on day 7 of culture by flow cytometry. The graph represents mean  $\pm$  SEM of six independent experiments. **B**, expression of IL-7R $\alpha$  in *ex vivo* expanded Treg in a representative experiment. The plot on the left shows the isotype control, whereas the plot on the right shows the IL-7R $\alpha$  profile. \*,  $P < 0.001$ . **C**, phosphorylation of STAT5 in Treg not stimulated (dotted lines) or stimulated with IL-2 (left) or IL-7 (right). **D**, proliferative response of Treg exposed to IL-2 or IL-7. Treg were labeled with CFSE and stimulated in the presence of IL-2 (left) or IL-7 (right). CFSE dilution was evaluated on day 7 by flow cytometry. The solid and dotted lines represent the CFSE dilution of Treg stimulated with or without cytokines, respectively.

increase of the bioluminescence signal ( $2.3 \times 10^8 \pm 3 \times 10^7$  photons) and were sacrificed by day 18. Mice infused with IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs and IL-2 had superior tumor control ( $1.6 \times 10^8 \pm 2 \times 10^7$  photons at day 34), but this effect was abrogated when Treg were coinfused ( $2.4 \times 10^8 \pm 4 \times 10^7$  photons at day 34;  $P < 0.05$ ). In contrast, mice infused with IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs and IL-7 controlled tumor growth equally well in the absence ( $1.2 \times 10^8 \pm 3 \times 10^7$  photons) or in presence of Treg ( $1.3 \times 10^8 \pm 6 \times 10^6$  photons) at day 34.

## Discussion

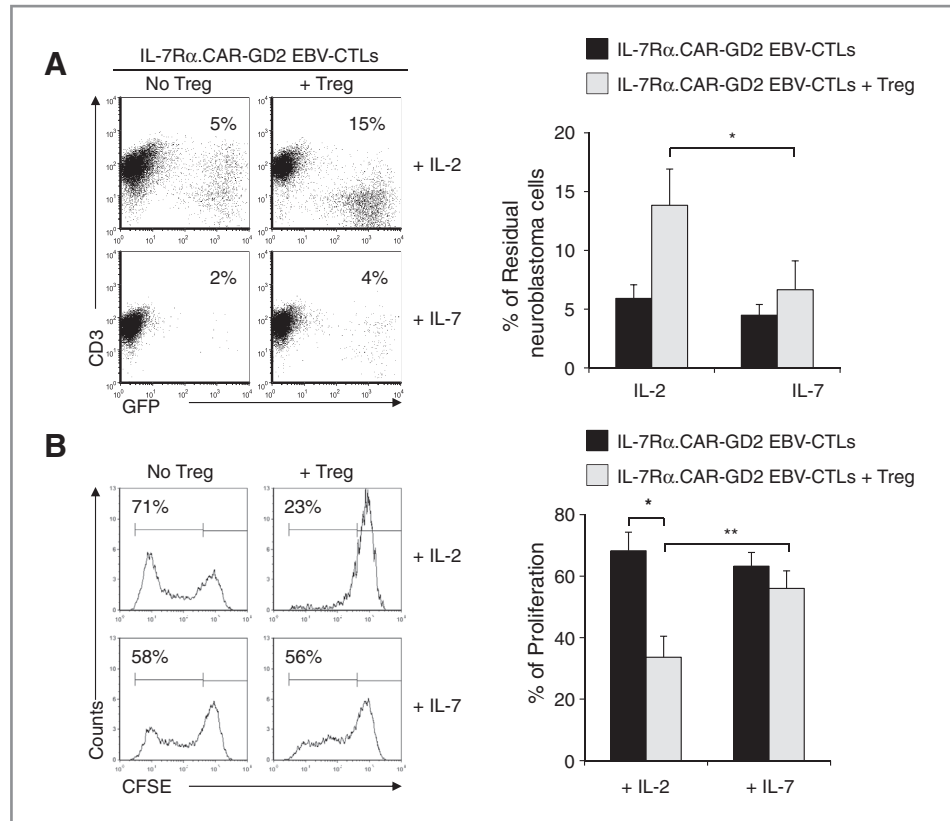
The adoptive transfer of CAR-redirection EBV-CTLs safely induces tumor regression in patients with neuroblastoma and the approach is potentially applicable to other human malignancies (8, 9). To further improve the clinical benefits of this approach, we developed a strategy that selectively promotes the *in vivo* expansion of CAR-redirection CTLs without favoring the proliferation and function of Treg that may limit long-term persistence and activity of the infused effector cells and thereby compromise antitumor efficacy. Here, we demonstrate that CAR-redirection EBV-CTLs engineered to regain responsiveness to IL-7 by restoring their expression of IL-7R $\alpha$  proliferate in response to a combination of native T-cell receptor (TCR) and IL-7 stimulation without favoring the expansion and function of Treg. As a

consequence, we observed an increase in their CAR-mediated antineuroblastoma activity, even in the presence of Treg.

Successful clinical outcome following adoptive transfer of tumor-specific T cells strongly correlates with the *in vivo* survival and proliferation of these cells (18, 32, 33). In addition to the intrinsic properties of T lymphocytes, such as central-memory versus effector-memory versus naïve phenotype that directly dictate the self-maintenance capacity of tumor-specific T cells (34), several tumor-associated mechanisms are also pivotal in determining the consequences of administering tumor-specific T cells (10, 35). Treg in particular are abundant in the tumor microenvironment and are a major factor in impairing T-cell function. Hence, strategies that selectively increase persistence and expansion of adoptively transferred T cells or that eliminate the influence of this cell subset should be as relevant for T-cell therapies as they have proved to be for cancer-vaccine trials (36).

The administration of recombinant cytokines or the use of cytokine-engineered T cells (30, 37, 38) that selectively support T-cell growth without providing functional or proliferative advantages to Treg represent appealing approaches to overcome the inhibitory function of Treg within the tumor microenvironment. However, IL-2 that is frequently used to sustain the *in vivo* proliferation and persistence of adoptively transferred CTLs is nonselective, stimulating both tumor-specific effector T cells and Treg, as both these cell

**Figure 4.** IL-7, unlike IL-2, supports *in vitro* the proliferation and function of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs in the presence of Treg. A, IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs were cocultured with CHLA-255 GFP-tagged cells (ratio 1:2) in the presence of IL-2 or IL-7, with or without Treg. The percentage of residual tumor cells was measured by flow cytometry on day 7 of culture. The plots on the left show a representative experiment, whereas the graph on the right summarizes mean  $\pm$  SD of five independent experiments. B, IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs were labeled with CFSE and activated with autologous LCLs in the presence of IL-2 (top) or IL-7 (bottom) with or without Treg. CFSE dilution was measured at day 7 of culture by flow cytometry. The plots on the left show a representative experiment, whereas the graph represents mean  $\pm$  SD of five independent experiments. \*,  $P < 0.01$ ; \*\*,  $P = 0.005$ .

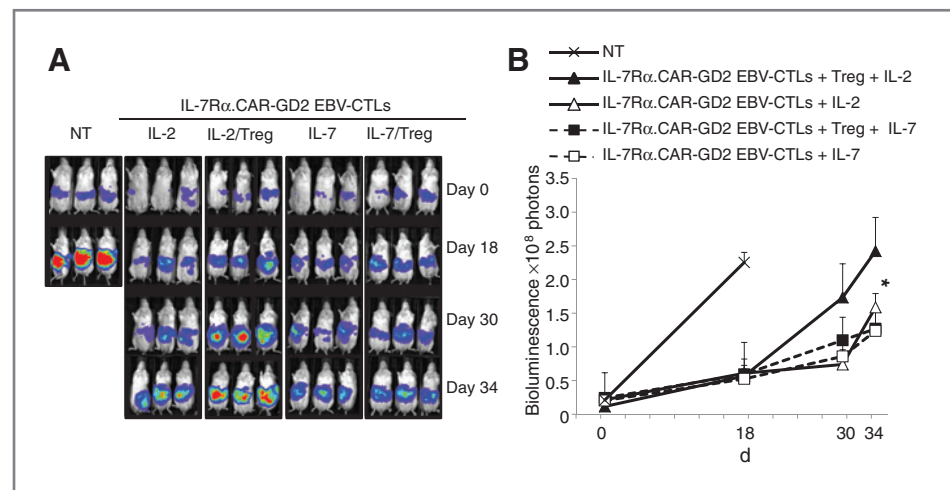


subsets express the IL-2 high affinity receptor (CD25; refs. 19, 39). Thus, as illustrated in the current and prior studies, the net effect of IL-2 administration is to block the proliferation and antitumor effects of CAR-redirection CTLs both *in vitro* and *in vivo*.

Although IL-7 shares several functions with IL-2, it also has effects on specific T-cell subsets that depend on their expression of the private IL-7R $\alpha$  subunit (23). Our experiments demonstrate both *in vitro* and *in vivo* that IL-7 can nonetheless support the survival, expansion, and effector

function of CAR-redirection EBV-CTLs if these cells are engineered to reexpress the IL-7R $\alpha$  and that it can thereby overcome the inhibitory effects of Treg. Our approach has significant advantages over the use of IL-2 or cytotoxic drugs to eliminate Treg in a nonselective manner (40) as it may promote the long-term persistence of CAR-redirection EBV-CTLs both in steady-state conditions and in a lymphopenic environment (23). In addition, the administration of recombinant IL-7 unlike recombinant IL-2 seems to be well tolerated even at high doses (20, 21, 41). Finally, as

**Figure 5.** IL-7, but not IL-2, supports *in vivo* antitumor activity of IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs in the presence of Treg. NSG mice engrafted intraperitoneally with CHLA-255 cells tagged with FFLuc were infused with IL-7R $\alpha$ .CAR-GD2<sup>+</sup> EBV-CTLs and received IL-2  $\pm$  Treg or IL-7  $\pm$  Treg. Tumor growth was monitored using an *in vivo* imaging system (Xenogen IVIS imaging system). A group of mice received control EBV-CTLs or tumor cells only (Control). A, images of different groups of mice. B, mean  $\pm$  SD of photons for 8 mice/group in two independent experiments. \*,  $P < 0.05$ .



the infusion of virus-specific CTLs after allogeneic stem-cell transplant does not induce the occurrence of graft versus host disease (28), our proposed approach of infusing CAR-redirectioned CTLs with restored responsiveness to the homeostatic cytokine IL-7 may significantly increase the application of CAR technology in the allogeneic setting (42).

One potential concern associated with any genetic manipulation of T cells is that the cells will undergo malignant transformation, or grow in an antigen independent manner. This concern is particularly prominent when the genetic manipulation modifies a growth factor receptor or other portions of a signaling pathway. However, the experience of our own and other groups has been that the genetic manipulation of differentiated T cells to express cytokines or cytokine receptors does not affect the antigen specificity of these cells and does not elicit uncontrolled proliferation (24, 37, 43). These results were confirmed in the current study even if we cannot completely exclude the possibility of secondary paracrine effects due to the ectopic expression of IL-7R $\alpha$ . If such a concern remains, however, incorporation of suicide or safety switches within the cells may provide a further level of reassurance (37, 44).

Our study suggests that restoring the responsiveness to IL-7 of virus-specific CTLs redirectioned with a CAR is a strategy that may allow enhanced T-effector cells without concomitant inhibition by Treg and may thereby further

improve the clinical outcome of a promising therapeutic approach.

#### Disclosure of Potential Conflicts of Interest

M.K. Brenner is a consultant/advisory board member of Bluebird Bio. The Center for Cell and Gene Therapy has a research collaboration with Celgene and Bluebird Bio. GD, BS and MKB have patent applications in the field of T cell and gene-modified T-cell therapy for cancer. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

**Conception and design:** S.K. Perna, M.K. Brenner, B. Savoldo, G. Dotti  
**Development of methodology:** S.K. Perna, B. Savoldo, G. Dotti  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S.K. Perna, D. Pagliara, B. Savoldo, G. Dotti  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S.K. Perna, D. Pagliara, H. Liu, B. Savoldo, G. Dotti  
**Writing, review, and/or revision of the manuscript:** S.K. Perna, M.K. Brenner, B. Savoldo, G. Dotti  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S.K. Perna, M.K. Brenner  
**Study supervision:** B. Savoldo, G. Dotti  
**Provided technical assistance for some of the *in vitro* and *in vivo* experiments:** A. Mahendravada

#### Grant Support

This work was supported in part by R01 CA142636 NIH-NCI, W81XWH-10-10425 Department of Defense, Technology/Therapeutic Development Award.

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 12, 2013; revised September 20, 2013; accepted September 26, 2013; published OnlineFirst October 4, 2013.

#### References

- Sadelain M, Brentjens R, Riviere I. The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 2009;21:215–23.
- Jena B, Dotti G, Cooper LJ. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. *Blood* 2010;116:1035–44.
- Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A* 1993;90:720–4.
- Imai C, Mihara K, Andreansky M, Nicholson IC, Pui CH, Geiger TL, et al. Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia* 2004;18:676–84.
- Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, et al. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J Clin Invest* 2011;121:1822–6.
- Rossig C, Bollard CM, Nuchtern JG, Rooney CM, Brenner MK. Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy. *Blood* 2002;99:2009–16.
- Savoldo B, Rooney CM, Di Stasi A, Abken H, Hombach A, Foster AE, et al. Epstein Barr virus specific cytotoxic T lymphocytes expressing the anti-CD30(zeta) artificial chimeric T-cell receptor for immunotherapy of Hodgkin disease. *Blood* 2007;110:2620–30.
- Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 2008;14:1264–70.
- Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood* 2011;118:6050–6.
- Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 2005;5:263–74.
- Baecher-Allan C, Hafler DA. Suppressor T cells in human diseases. *J Exp Med* 2004;200:273–6.
- Johnson BD, Jing W, Orentas RJ. CD25+ regulatory T cell inhibition enhances vaccine-induced immunity to neuroblastoma. *J Immunother* 2007;30:203–14.
- Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 2003;9:606–12.
- Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001;61:4766–72.
- Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942–9.
- Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, et al. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 2004;103:1755–62.
- Jing W, Yan X, Hallett WH, Gershon JA, Johnson BD. Depletion of CD25(+) T cells from hematopoietic stem cell grafts increases post-transplantation vaccine-induced immunity to neuroblastoma. *Blood* 2011;117:6952–62.
- Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* 2008;8:299–308.
- Ahmadzadeh M, Rosenberg SA. IL-2 administration increases CD4+CD25(hi) Foxp3 +regulatory T cells in cancer patients. *Blood* 2006;107:2409–14.
- Sportes C, Hakim FT, Memon SA, Zhang H, Chua KS, Brown MR, et al. Administration of rhIL-7 in humans increases *in vivo* TCR repertoire



- diversity by preferential expansion of naive T cell subsets. *J Exp Med* 2008;205:1701–14.
21. Rosenberg SA, Sportes C, Ahmadzadeh M, Fry TJ, Ngo LT, Schwarz SL, et al. IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. *J Immunother* 2006;29:313–9.
  22. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006;203:1701–11.
  23. Ma A, Koka R, Burkett P. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu Rev Immunol* 2006;24:657–79.
  24. Vera JF, Hoyos V, Savoldo B, Quintarelli C, Giordano Attianese GM, Leen AM, et al. Genetic manipulation of tumor-specific cytotoxic T lymphocytes to restore responsiveness to IL-7. *Mol Ther* 2009;17:880–8.
  25. Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Mol Ther* 2005;12:933–41.
  26. Vera J, Savoldo B, Vigouroux S, Biagi E, Pule M, Rossig C, et al. T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells. *Blood* 2006;108:3890–7.
  27. Song L, Ara T, Wu HW, Woo CW, Reynolds CP, Seeger RC, et al. Oncogene MYCN regulates localization of NKT cells to the site of disease in neuroblastoma. *J Clin Invest* 2007;117:2702–12.
  28. Rooney CM, Smith CA, Ng CY, Loftin SK, Sixbey JW, Gan Y, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 1998;92:1549–55.
  29. Chakraborty R, Mahendravada A, Perna SK, Rooney CM, Heslop HE, Vera JF, et al. Robust and cost effective expansion of human regulatory T cells highly functional in a xenograft model of graft-versus-host disease. *Haematologica* 2013;98:533–7.
  30. Perna SK, De AB, Pagliara D, Hasan ST, Zhang L, Mahendravada A, et al. Interleukin 15 Provides Relief to CTLs from Regulatory T Cell-Mediated Inhibition: Implications for Adoptive T Cell-Based Therapies for Lymphoma. *Clin Cancer Res* 2013;19:106–17.
  31. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 2006;177:8338–47.
  32. Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* 2010;115:925–35.
  33. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 2011;3:95ra73.
  34. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, et al. Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest* 2005;115:1616–26.
  35. Hoyos V, Savoldo B, Dotti G. Genetic modification of human T lymphocytes for the treatment of hematologic malignancies. *Haematologica* 2012;97:1622–31.
  36. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 2005;115:3623–33.
  37. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia* 2010;24:1160–70.
  38. Liu K, Rosenberg SA. Transduction of an IL-2 gene into human melanoma-reactive lymphocytes results in their continued growth in the absence of exogenous IL-2 and maintenance of specific antitumor activity. *J Immunol* 2001;167:6356–65.
  39. Terabe M, Berzofsky JA. Immunoregulatory T cells in tumor immunity. *Curr Opin Immunol* 2004;16:157–62.
  40. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 2008;26:5233–9.
  41. Rosenberg SA, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, et al. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 1994;86:1159–66.
  42. Micklethwaite KP, Savoldo B, Hanley PJ, Leen AM, Miller-Harrison GJ, Cooper LJ, et al. Derivation of human T lymphocytes from cord blood and peripheral blood with antiviral and antileukemic specificity from a single culture as protection against infection and relapse after stem cell transplantation. *Blood* 2010;115:2695–703.
  43. Quintarelli C, Vera JF, Savoldo B, Giordano Attianese GM, Pule M, Foster AE, et al. Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor-specific cytotoxic T lymphocytes. *Blood* 2007;110:2793–802.
  44. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* 2011;365:1673–83.