In vivo Inhibition of Human CD19-Targeted Effector T Cells by Natural T Regulatory Cells in a Xenotransplant Murine Model of B Cell Malignancy

James C. Lee¹,6, Erik Hayman¹,2, Hollie J. Pegram¹,2, Elmer Santos³, Glenn Heller⁴, Michel Sadelain¹,2,5, and Renier Brentjens¹,2,5

Abstract

Human T cells genetically modified to express chimeric antigen receptors (CAR) specific to the B cell tumor antigen CD19 can successfully eradicate systemic human CD19⁺ tumors in immunocompromised SCID (severe combined immunodeficient)-Beige mice. However, in the clinical setting, CD4⁺ CD25⁺ T regulatory cells (Treg) present within the tumor microenvironment may be potent suppressors of tumor-targeted effector T cells. In order to assess the impact of Tregs on CAR-modified T cells in the SCID-Beige xenotransplant model, we isolated, genetically targeted and expanded natural T regulatory cells (nTreg). In vitro nTregs modified to express CD19-targeted CARs efficiently inhibited the proliferation of activated human T cells, as well as the capacity of CD19-targeted 19-28z⁺ effector T cells to lyse CD19⁺ Raji tumor cells. Intravenous infusion of CD19-targeted nTregs into SCID-Beige mice with systemic Raji tumors traffic to sites of tumor and recapitulate a clinically relevant hostile tumor microenvironment. Antitumor efficacy of subsequently infused 19-28z⁺ effector T cells was fully abrogated as assessed by long-term survival of treated mice. Optimal suppression by genetically targeted nTregs was dependent on nTreg to effector T-cell ratios and in vivo nTreg activation. Prior infusion of cyclophosphamide in the setting of this nTreg-mediated hostile microenvironment was able to restore the antitumor activity of subsequently infused 19-28z⁺ effector T cells through the eradication of tumor-targeted nTregs. These findings have significant implications for the design of future clinical trials utilizing CAR-based adoptive T-cell therapies of cancer. Cancer Res; 71(8); 2871–81. ©2011 AACR.

Introduction

T cells may be genetically targeted to tumor antigens through the expression of chimeric antigen receptors (CAR) transduced using retroviral vectors (1). We have previously demonstrated that human T cells genetically modified to express a CD19-targeted CAR successfully eradicate established systemic human CD19⁺ B cell tumors in an immune suppressed SCID (severe combined immunodeficient)-Beige mice (2). However, despite promising preclinical in vivo studies (2–5), results from initial clinical trials utilizing CAR-modified T cells have to date been disappointing (6–8).

A potential etiology of treatment failure in the clinical setting may be the suppression of targeted T cells by a hostile tumor microenvironment infiltrated with CD4⁺ CD25⁺ regulatory T cells (Treg) and myeloid derived suppressor cells, as well as tumor expression of inhibitory ligands (PD-L1) and cytokines (TGF-β and IL-10; refs. 9–11). This hostile tumor microenvironment is largely unaddressed in pre-clinical models utilizing immune compromised mice. To address this limitation, we sought to investigate the impact of Tregs, a potent endogenous suppressive element of the immune system, on the antitumor activity of adoptively transferred CAR-modified T cells in a previously established SCID-Beige mouse tumor model.

Natural Tregs (nTreg) are CD4⁺ T cells derived from the thymus and defined by a CD4⁺ CD25⁺ CD127⁻ Foxp3⁺ phenotype. nTregs have been found to facilitate suppression of autoimmune T-cell responses and maintenance of peripheral tolerance (12–14), represent approximately 5% to 10% of peripheral CD4⁺ T cells in both mice and humans (13, 15), and express high levels of cytotoxic T lymphocyte associated antigen 4 (CTLA-4), glucocorticoid-induced TNFR-related protein, CD39, and CD73 (16–18). Patients with cancer, including B cell malignancies, have elevated numbers of Tregs in the peripheral blood and within the tumor microenvironment (19–21). Furthermore, in a variety of cancers, increased numbers of Tregs portend a poor prognosis (19, 22). Although the mechanism of suppression by Tregs appears to be multifactorial (23), it is clear that the presence of Tregs within the tumor microenvironment could markedly hinder the antitumor efficacy of adoptively transferred tumor-targeted effector T cells (24).

Authors’ Affiliations: ¹Center for Cell Engineering, ²Department of Medicine, ³Department of Radiology, ⁴Department of Epidemiology and Statistics, and ⁵Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York; and ⁶Yale University School of Medicine, New Haven, Connecticut

Corresponding Author: Renier J. Brentjens, 1275 York Avenue, Box 242, New York, NY 10065. Phone: 212-639-7053; Fax: 212-772-8441; E-mail: brentjens@mskcc.org

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Many studies have been published implicating Tregs as the cause of failed antitumor immune responses using clinical correlates. Treg depleting strategies (22, 25), and systemic lymphodepletion (26, 27). Recently, investigators have developed protocols to readily isolate (28), stimulate, and expand enriched Treg populations for pre-clinical experimental purposes (29, 30).

In this report, we investigate the in vivo impact of nTregs on CD19-targeted CAR⁺ T-cell therapy in a previously established xenotransplant SCID-Beige tumor model of Burkitt lymphoma (2, 3) by recapitulating a clinically relevant tumor microenvironment hostile to effector T-cell function through the infusion of CD19-targeted nTregs. Systemic injection of targeted nTregs into SCID-Beige mice bearing established systemic Raji tumors prior to infusion of CD19-targeted CAR⁺ effector T cells wholly abolished effector T-cell antitumor benefit while prior treatment with cyclophosphamide effectively reversed in vivo nTreg-mediated suppression of CD19-targeted CAR⁺ effector T cells. Taken together, our data support the hypothesis that tumor specific nTregs may significantly compromise the antitumor efficacy of CAR-modified tumor-targeted effector T cells in the clinical setting and may, in part, explain the modest clinical outcomes reported in previously published clinical trials utilizing adoptively transferred CAR-modified T cells (6–8).

Materials and Methods

Cell lines and T cells

The Raji tumor cell line was cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS (fetal calf serum), nonessential amino acids, HEPES buffer, pyruvate, and BME (Beta-mercaptoethanol; Life Technologies). T cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS supplemented with 20IU IL-2/mL (R&D Systems). PG-13 and gpg29 retroviral producer cell lines were cultured in DMEM supplemented with 10% FCS, and NIH-3T3 artificial antigen-presenting cells (AAPC), were cultured in DMEM supplemented with 10% heat-inactivated donor calf serum. All media were supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies).

Isolation of CD4⁺ CD25⁺ effector T cells and CD4⁺ CD25⁻ nTregs

Peripheral blood from healthy donors, obtained under institutional review board–approved protocol 95-054, was fractionated in BD Vacutainer CPT tubes (BD Medical), to isolate peripheral blood mononuclear cells (PBMC). CD4⁺ CD25⁺ responder T cells and CD4⁺ CD25⁻ nTregs were isolated from PBMCs using the CD4⁺ CD25⁺ Regulatory T-Cell Isolation Kit (Dynal brand; Invitrogen).

Retroviral genetic modification of T cells

Generation of retroviral producer PG-13 cell lines and gene transfer into effector T cells have been previously described (3, 31). For nTreg retroviral gene transfer, isolated nTregs were activated with Dynal CD3/CD28 Human Treg Expander magnetic beads (Invitrogen), cultured in RPMI media supplemented with IL-2 500IU and Rapamycin 100ng/mL (Sigma) for 48 hours (30, 32), and similarly transduced.

Expansion of CAR⁺ T cells

CAR⁺ effector T cells were expanded ex vivo on NIH-3T3 derived AAPCs as described previously (3). CAR⁻ nTregs were expanded with either Dynal CD3/CD28 Human Treg Expander beads (Invitrogen), or with AAPCs in RPMI medium supplemented with IL-2 and Rapamycin.

In vitro nTreg proliferation and suppression assay

5 × 10⁵ T effector cells were labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and cultured simultaneously with titrated numbers of purified autologous CAR⁺ CD4⁺ CD25⁻ Foxp3⁺ nTregs in 24-well tissue culture plates (Costar; ref.33). T-cell co-cultures were stimulated with Dynabeads CD3/CD28 T-Cell Expander beads (Invitrogen) at a bead-to-responder T-cell ratio of 1:1 in the absence of exogenous IL-2 and proliferation was assessed by flow cytometry (FACS) at 72 hours.

Cytokine detection assays

Cytokine levels in tissue culture supernatant as well as serum were assessed using the multiplex Human Cytokine Detection System (Millipore Corp.) in conjunction with the Luminex IS100 system and IS 2.2 software (Luminex Corp.).

In vitro cytotoxicity assay

19-28z⁻ effector T cells were cocultured with Raji cells in RPMI media at 1:1 ratio with or without equal numbers of 19z1⁺ nTregs for 24 hours. Tumor lysis was subsequently assessed by FACS to detect residual CD19⁺ tumor cells. The GranToxiLux (OncoImmunin, Inc.) cytotoxicity assay was performed per manufacturer’s instructions.

In vivo analyses of Treg function

We inoculated 8- to 12-week-old FOX CHASE C.B-17 (SCID-Beige) mice (Taconic) with Raji tumor cells by tail vein injection or subcutaneously as indicated. In the systemic tumor model, mice were injected by tail vein (i.v.) with 5 × 10⁵ Raji tumor cells on day 1, and on day 5 were treated with a single i.v. infusion of 1 × 10⁵ CAR⁻ nTregs, followed by a single i.v. infusion of 1 × 10⁵ CAR⁺ effector T cells on day 6. For cyclophosphamide experiments, mice were injected by tail vein with 5 × 10⁵ Raji tumor cells on day 1, and on day 5 were treated with a single i.v. infusion of 1 × 10⁵ CAR⁻ Tregs, followed by intraperitoneal (i.p.) injection of 100 mg/kg cyclophosphamide on day 6, and on day 7 injected with an i.v. dose of 1 × 10⁵ CAR⁺ effector T cells. Mice were sacrificed when disease became clinically evident. All in vivo studies were done in the context of an Institutional Animal Care and Use Committee approved protocol (#00-05-065).

Bioluminescent imaging

For in vivo imaging of Raji tumor cells we utilized Raji tumor cells modified to express GFP-FFLuc (Clontech Laboratories). Imaging of nTregs was performed using nTregs modified...
with the previously described 19z1 IRES extGLuc bicistronic retroviral vector (34). Tumor and T cells were imaged using the Xenogen IVIS Imaging System (Xenogen; ref. 34).

**Immunohistochemistry staining**

Mouse bone marrow samples were fixed in 10% buffered formalin phosphate (Fisher Scientific). All tissues were processed by routine methods and embedded in paraffin wax. Five-micrometer sections were stained with H&E (Prol Scientific). Human T cells in the paraffin-embedded mouse tissues were detected using rabbit polyclonal sera specific to human CD3 (DakoCytomation).

**Flow cytometry**

We performed FACS with a FACScan cytometer with FlowJo software (Tree Star), using PE-labeled CAR-specific monoclonal antibody (12D11, MSKCC monoclonal antibody core facility), FITC-labeled human CD4 specific antibody (S3.5, Caltag), CD8 specific antibody (3B5, Caltag), CD62L specific antibody (DREG 56, BD Pharmingen), PE-labeled human CD25 specific antibody (CD25.3G10, Caltag), and APC-labeled human CD19 specific antibody (SJ25-C, Caltag). Foxp3 expression was assessed using the Human Regulatory T cell Staining Kit (eBioscience).

**Statistical analysis**

Statistical analysis utilizing the GraphPad Prism software (GraphPad Software) was done using log-rank analyses for survival and the Student’s t-test with Wilcoxon rank sum test for line and bar graph comparisons.

**Results**

**nTregs are efficiently modified to express CARs by retroviral transduction**

To assess whether human nTregs can be genetically manipulated despite their anergic nature and difficulty to maintain in culture (30, 35), we isolated nTregs using immunomagnetic sorting for the CD4+ CD25hi population from PBMCs consistently achieving CD4+ T-cell populations that were more than 95% CD25hi, 70% to 90% Foxp3+ (Fig. 1A and B), CD62L2hi and CD127lo (2) enriched the CAR adapted nTregs. 19z1-nTregs were subsequently transduced with CD19+ CARs using retroviral supernatants, routinely resulting in more than 60% gene transfer (Fig 1C).

**Expanded genetically modified nTregs inhibit naïve T-cell proliferation and CAR+ effector T-cell cytotoxicity in vitro**

We next cocultured activated CFSE-labeled naïve T cells with varying numbers of 19z1+ nTregs, and as controls, nTregs transduced with the irrelevant Pz1 CAR specific to the prostate specific membrane antigen (31), and control 19z1+ non-Treg CD4+ T cells. 19z1+ and Pz1+ nTregs, but not 19z1+ non-Treg control T cells, induced a potent inhibition of nonspecific T-cell expansion even at low nTreg to effector T-cell ratios (Fig. 2A). Consistent with our CFSE studies, IL-2 levels in nTreg cocultures decreased in a dose dependent manner (Fig 2B), a finding similar to results published elsewhere (35, 36). Coculture of CFSE-labeled 19z1+ T cells with titrated numbers of 19z1+ nTregs or control 19z1+ non-Tregs demonstrated that only 19z1+ nTregs suppressed 19z1+ effector T-cell expansion (Fig. 2C).

To assess the role of 19z1+ nTregs on effector T-cell cytotoxicity, 19z1+ effector T cells were cocultured with 19z1+ nTregs at a 1:1 ratio for 24 hours followed by the addition of target CD19+ Raji tumor cells at a 1:1 ratio with effector 19z1+ T cells. We found that 19z1+ nTregs inhibited killing of Raji tumor cells by 19z1+ effector T cells (Fig. 2D). As expected, 19z1+ nTregs cocultured with CD19+ Raji tumors alone failed to eradicate Raji tumor cells consistent with the notion that CD19-targeted nTregs lack cytotoxic potential (data not shown). Similar to the above findings, nTregs markedly abrogated the lysis of Raji tumor cells by 19z1+ effector T cells in a standard cytotoxicity assay (Fig. 2E).

**19z1+ nTregs traffic to CD19+ Raji tumors**

In order to assess whether CAR-modified nTregs efficiently traffic to Raji tumors in SCID-Beige mice, we employed dual bioluminescent imaging (BLI) enabling simultaneous imaging of both tumor cells and T cells within the same animal (34). SCID-Beige mice previously injected subcutaneously with Raji (GFP-FLLuc) tumor cells underwent BLI to verify detectable tumor (Fig. 3A). Subsequently, mice were infused i.v. with either 19z1+ or Pz1+ nTregs further modified to express extGLuc bioluminescent enzyme (extGLuc+) nTregs. BLI at 24 hours following nTreg infusion demonstrated 19z1+ extGLuc+ nTreg but not Pz1+ extGLuc+ nTreg signal localized to the Raji tumor (Fig. 3A). Immunohistochemistry studies confirmed the presence of 19z1+ extGLuc+, but not Pz1+ extGLuc+ nTregs within the Raji tumors (Fig. 3B). Similar results were obtained in mice bearing systemic Raji tumors, which primarily infiltrate the bone marrow and lymphnodes, demonstrating specific localization of 19z1+ extGLuc+ nTregs but not Pz1+ extGLuc+ nTregs to these sites at 24 hours (Fig. 3C). These findings indicate that by 24 hours, 19z1+ nTregs successfully traffic to CD19+ tumors, recapitulating a hostile tumor microenvironment which may be seen in the clinical setting.

**Tumor infiltrating 19z1− nTregs inhibit eradication of systemic CD19+ Raji tumors by 19-28z+ effector T cells**

We have previously demonstrated that CD19-targeted effector T cells successfully eradicating systemic Raji tumors...
in SCID-Beige mice as assessed by long-term survival (2, 3). In SCID-Beige mice, Raji tumors have a primary tropism for the bone marrow and untreated mice reliably develop hind-limb paralysis at 3 to 5 weeks as a consequence of spinal cord compression by tumor expanding from vertebral bodies (2). To determine whether CD19-targeted nTregs within the tumor microenvironment could inhibit successful tumor eradication in this model, mice were injected systemically with Raji tumor cells on day 1, with 19z1 nTregs on day 5, and with 19-28z effector T cells at a nTreg to T effector ratio of 1:1 on day 6. In all in vivo experiments, more than 80% of infused CARþ effector T-cell populations retained a central memory phenotype (CD62Lhi CCR7þ) and consisted of 50% to 65% CD8þ and 35% to 50% CD4þ T cells as assessed by FACS prior to infusion (data not shown). Prior infusion of 19z1 nTregs, in contrast to Pz1 nTregs, wholly abolished any antitumor effect by subsequently infused 19-28z effector T cells as assessed by overall survival (Fig 4A). To confirm the in vivo presence of Tregs, we further assessed the nTreg to effector T-cell ratio in the bone marrow of mice infused with 19z1 nTregs followed by 19-28z effector T cells by FACS analysis at 24 hours following effector T-cell infusion, demonstrating a 1:1 nTreg to effector T-cell ratio (Fig. 6B).

To assess in vivo potency of nTreg effector suppression, we titrated the 19z1 nTreg to effector 19-28z T-cell ratio in Raji tumor bearing SCID-Beige mice. We observed that the 19z1 nTregs were able to fully suppress the in vivo antitumor efficacy of effector 19-28z T cells, as assessed by survival,
at a nTreg to effector T-cell ratio as low as 1:8, but found recovery of effector T-cell antitumor efficacy at a 1:16 nTreg to effector T-cell ratio (Fig. 4B). Similar results were obtained when this experiment was conducted using 19-28z⁺ nTregs (data not shown).

Optimal suppression of 19-28z⁺ effector T cells requires nTreg activation within the tumor microenvironment

We next generated a CD19-targeted CAR lacking the ζ chain signaling domain termed 19(del) (Fig 5A). 19(del)⁺ nTregs failed to expand on 3T3(CD19/CD80) AAPCs verifying the lack of T-cell activating signaling by the ζ chain-deleted 19(del) CAR (Fig 5B). However, despite loss of CAR signaling, 19(del)⁺ nTregs retained the capacity to traffic to CD19⁺ Raji tumors in vivo (Fig 5C) and the ability to potentely suppress both naïve T-cell proliferation (Fig. 5D) and 19-28z effector T-cell cytotoxicity in vitro (data not shown).

In order to assess the in vivo inhibitory capacity of 19(del)⁺ nTregs, SCID-Beige mice were injected i.v. with Raji tumor cells on day 1, injected with either 19z1⁺ or 19(del)⁺ nTregs on day 2 and mice were sacrificed on day 3. As shown in Figure 5E, nTregs were found to reduce the number of Raji tumor cells by 90% in mice receiving 19z1⁺ nTreg, but not in any of the other groups tested (Fig. 5E). These results suggest that nTregs are able to suppress both naïve T-cell proliferation and 19-28z effector T-cell cytotoxicity in vivo.
day 5, followed by injection of 19-28z+ effector T cells, at a 1:1 nTreg to effector T-cell ratio, on day 6. As expected, 19z1+ nTregs conferred full suppression of 19-28z+ effector T cells, while 19(del)+ Tregs conferred only partial suppression (Fig. 5E). These findings suggest that optimal suppression requires both nTreg localization to and activation within the tumor microenvironment.

To further define the mechanism whereby 19(del)+ nTregs-mediated partial inhibition of 19-28z+ T-cell effector function, we next measured serum levels of human IL-2 and IFNγ as well as the inhibitory human cytokines TGF-β and IL-10 in treated mice. Human TGF-β and IL-10 levels were consistently low to undetectable in all treated cohorts (data not shown), while human IL-2 and IFNγ levels were significantly and equally decreased in both 19z1+ and 19(del)+ nTreg infused cohorts (Fig. 5F and G). This finding is consistent with sequestration of IL-2 by nTregs, a previously described mechanism of nTreg-mediated T-cell suppression (35, 36), and inhibition of effector T-cell activation as assessed by IFNγ levels. These findings were independent of nTreg activation status, and may explain, in part, the observed partial inhibition mediated by 19(del)+ nTregs.

**Cyclophosphamide lymphodepletion eradicates 19z1+ nTregs and restores antitumor efficacy of 19-28z+ effector T cells**

Lymphodepleting preconditioning regimens can enhance the antitumor efficacy of adoptively transferred cytotoxic T cells which may be mediated in part through the eradication of Tregs in the host (26, 27). In particular, cyclophosphamide chemotherapy has been shown to effectively eliminate Tregs (37–39). To this end, we next investigated whether cyclophosphamide therapy (100 mg/kg) following infusion of 19z1+ nTregs can abrogate effector T-cell suppression by nTregs. SCID-Beige mice bearing systemic Raji tumors were injected subcutaneously with Raji (GFP-FFLuc) cells and 10 days later, with 19z1+ extGLuc+ or control Pz1+ extGLuc+ nTregs. Tumor cells were imaged using the FFLuc specific luciferin substrate, while T cells were imaged using the GLuc specific coelenterazine substrate. B, immunohistochemistry staining with an anti-human CD3 antibody confirms the presence of 19z1+ nTregs, but not Pz1+ nTregs, within Raji tumor microenvironment. C, BLI CAR+ nTregs show trafficking of 19z1+ nTregs, but not Pz1+ nTregs, to systemic Raji tumors at 24 hours, with predicted signal in the bone marrow of the femurs, tibia, and humeri (green arrows) of 19z1+ extGLuc+ nTreg infused mice, as well as infiltration of these nTregs into the submandibular lymphnodes (orange arrow).
T-cell ratios in the bone marrow of cyclophosphamide treated (Fig. 6B). These data verify cyclophosphamide depletion of nTreg to effector T-cell ratio seen in nonlymphodepleted mice abrogated eradication of systemic Raji tumors by 19-28z alone cohort; (data not shown). d, days since Raji tumor cell injection.

**Discussion**

The generation of tumor-targeted T cells for adoptive therapy may be insufficient to achieve significant antitumor responses in the clinical setting. Specifically, the tumor itself may foster an environment capable of impairing targeted effector T-cell function. Specifically, Tregs, which are relevant in both the clinic and in immune-competent animal models of disease (40, 41), are absent in the xenograft tumor models previously used to study modified human T cells in vivo. Herein we report that isolated human nTregs from healthy donors may be efficiently transduced to express CARs and subsequently expanded in vitro. While other groups have examined the effects of induced Tregs (iTreg) toward similar ends (42), we opted to isolate and expand nTregs due to concerns regarding the stability of FOXP3 expression in iTregs (43). The resulting tumor-targeted nTregs successfully traffic to tumor in SCID-Beige mice recapitulating a hostile tumor microenvironment seen in the clinical setting. nTreg infiltrated tumors were markedly resistant to eradication by CAR-modified effector T cells even at low (1:8) nTreg to effector T-cell ratios, while tumor resistance to effector T-cell eradication was no longer apparent at a ratio of 1:16, consistent with a dose dependent nTreg-mediated suppression. This dose dependent nature of inhibition, evidence of persistence of tumor-targeted nTregs within the tumors, and the inability of nontumor-targeted control Pz1 nTregs to inhibit 19-28z+ effector T cells even at a 1:1 ratio, all support the notion that the suppression of tumor-targeted effector T cells is specifically dependent upon the presence of these nTregs within the tumor microenvironment.

While our in vitro data suggest that 19z1+ Tregs are capable of inhibiting both CAR-mediated cytotoxicity and proliferation, which of these factors predominates in vivo in this tumor model is currently unclear. However, our prior studies suggest that proliferation or persistence does not play a dominant role in this tumor model, as multiple infusions of 19-28z- T cells are needed to achieve optimal antitumor efficacy in a pre B cell ALL tumor model (3). These data are consistent with limited in vivo persistence and proliferation of the CAR-modified cells in SCID-Beige mice favoring inhibited effector T-cell cytotoxicity by colocalized nTregs as the primary mechanism of suppression observed in our studies.

The mechanism of in vivo nTreg suppression in our model further appears to be dependent, in part, on the activation status of nTregs at the tumor site since nTregs modified to express the 19(del) CAR retained a statistically significant ability to suppress in vivo effector T-cell function when
compared to mice treated with 19-28z⁺ effector T cells alone, but demonstrated statistically inferior suppression when compared to mice pretreated with 19z1⁺ nTregs (Fig. 5E). While both sets of CAR-modified nTregs were previously activated using CD3/28 beads and IL-2, only the 19z1⁺ nTregs receive additional intratumoral activation via a functional CAR. This phenomenon is consistent with prior literature reporting the requirement for activation to induce Treg-mediated suppression (36) although activation and suppression can be separated temporally (44). The mechanism of this retained but attenuated suppression may be partially mediated by direct contact of the nTreg with effector T cells since the 19(del)⁺ nTregs successfully traffic to the tumor (35). Additionally, we found that both 19z1⁺ and 19(del)⁺ nTregs infused mice demonstrated equal levels of IL-2 reduction in serum suggesting that 19(del)⁺ nTregs may further inhibit effector T-cell function through a retained ability to sequester of IL-2, a known mechanism of Treg-mediated suppression (23).
modified effector T-cell antitumor efficacy in the clinical tumor microenvironment may markedly compromise CAR-nTreg-mediated suppression of effector T cells (Fig. 4B).

Our data are consistent with the notion that tumor infiltrating Tregs may represent a significant obstacle to the successful application of this adoptive cell therapy. One relatively direct method of overcoming this obstacle would be to eliminate Tregs prior to CAR-modified T-cell infusion. While several therapeutic options exist for this purpose, including anti-CD25 antibodies and immunotoxins (45), we chose cyclophosphamide due to its established ability to eliminate Tregs in the context of an antitumor response (46, 47) as well as the applicability of this approach to ongoing Phase I clinical trials at our center using autologous 19-28z modified T cells to treat patients with relapsed or refractory B cell malignancies. In our studies, prior lymphodepletion with cyclophosphamide before adoptive cell therapy of SCID-Beige cell malignances. In our studies, prior lymphodepletion with cyclophosphamide before adoptive cell therapy SCID-Beige mice bearing systemic established Raji tumors infiltrated with 19z1 nTregs abrogated the suppression of subsequently infused 19-28z effector T cells by significantly lowering the nTreg to effector T-cell ratios from 0.90 to 0.14 within the tumor microenvironment (Fig. 6B). These data are furthermore consistent with our observed in vivo dose dependent nTreg-mediated suppression of effector T cells (Fig. 4B).

In conclusion, these data validate concerns that a hostile tumor microenvironment may markedly compromise CAR-modified effector T-cell antitumor efficacy in the clinical setting. Further our data support the incorporation of lymphodepleting chemotherapy prior to infusion of CAR-modified tumor-targeted T cells in the modification of ongoing clinical trials and the design of future clinical trials. Finally, our data support the potential of CAR-modified nTregs as a novel approach for the treatment of autoimmune diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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