**Microenvironment and Immunology**

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

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**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 tumor cell lines in 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).
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 \textit{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 \textit{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).

\section*{Materials and Methods}

\subsection*{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 20U IL-2/mL (R&D Systems). PG-13 and gp92 retroviral producer cell lines were cultured in DMEM (Life Technologies) 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 $\mu$g/mL streptomycin (Life Technologies).

\subsection*{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).

\subsection*{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.

\subsection*{Expansion of CAR$^+$ T cells}

CAR$^+$ effector T cells were expanded \textit{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.

\subsection*{In vitro nTreg proliferation and suppression assay}

$5 \times 10^7$ T effector cells were labeled with 5 $\mu$mol/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 cocultures 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.

\subsection*{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.).

\subsection*{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 19z$^+$ 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.

\subsection*{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 $5 \times 10^5$ Raji tumor cells on day 1, and on day 5 were treated with a single i.v. infusion of $1 \times 10^7$ CAR$^+$ nTregs, followed by a single i.v. infusion of $1 \times 10^7$ CAR$^+$ effector T cells on day 6. For cyclophosphamide experiments, mice were injected by tail vein with $5 \times 10^5$ Raji tumor cells on day 1, on day 5 were treated with a single i.v. infusion of $1 \times 10^7$ 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 $1 \times 10^7$ CAR$^+$ effector T cells. Mice were sacrificed when disease became clinically evident. All \textit{in vivo} studies were done in the context of an Institutional Animal Care and Use Committee approved protocol (#00-05-065).

\subsection*{Bioluminescent imaging}

For \textit{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 (Poli 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 and 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), CD62Lhi and CD127− (data not shown). CD3/CD28 bead activated nTregs were subsequently transduced with CD19-targeted CARs using retroviral supernatants, routinely resulting in more than 60% gene transfer (Fig 1C).

We next compared CAR+ nTreg expansion in the context of high dose IL-2 and rapamycin, either by coculture on NIH-3T3 AAPCs [3T3(CD19/CD80); ref. 2] or through the addition of Dynal CD3/CD28 Human Treg Expander beads. CAR+ nTregs proliferated equally well under either condition with largely retained Foxp3 expression (Fig. 1D and E) while only expansion of CAR+ nTregs by coculture on 3T3(CD19/CD80) AAPCs (2) enriched the CAR+ nTreg fraction (Fig. 1E and F) resulting in a significantly increased absolute number of CAR+ nTregs (Fig. 1G). However, due to contamination by persistent 3T3 fibroblasts in AAPC expanded nTreg populations, we utilized nTregs generated by CD3/CD28 Human Treg Expander beads for further studies.

**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− 28z+ T cells with titrated numbers of 19z1+ nTregs or control 19z1− non-Tregs demonstrated that only 19z1+ nTregs suppressed 19z1− 28z+ effector T-cell expansion (Fig. 2C).

To assess the role of 19z1+ nTregs on effector T-cell cytotoxicity, 19z1− 28z+ 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− 28z+ T cells. We found that 19z1+ nTregs inhibited killing of Raji tumor cells by 19z1− 28z+ 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− 28z+ 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-FLuc) 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 19z1− 28z+ effector T cells**

We have previously demonstrated that CD19-targeted effector T cells successfully eradicate 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 CAR+ effector T-cell populations retained a central memory phenotype (CD62Lhi CCR7+), and consisted of 50% to 65% CD8+ and 35% to 50% CD4+ 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 potently 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 tumor sizes were measured over the following 7 days (Fig. 5E). 19z1⁺ nTregs were able to inhibit tumor growth in a dose-dependent manner, whereas 19(del)⁺ nTregs had no effect on tumor growth (Fig. 5F). These results demonstrate that nTreg activation within the tumor microenvironment is required for optimal suppression of 19-28z⁺ effector T cells.
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 with 19z1+ nTregs on day 5 following tumor cell infusion. On day 6, 24 hours prior to treatment with 19-28z+ T cells, mice were injected i.p. with cyclophosphamide. In contrast to mice infused with 19z1+ nTregs followed by 19-28z+ effector T cells with no long-term survival, mice infused sequentially with 19z1+ nTregs, i.p. cyclophosphamide, and 19-28z+ effector T cells demonstrated a prolonged long-term survival (67%) which compares favorably to mice treated with 19-28z+ effector T cells alone (78%; Fig. 6A). Furthermore, these studies demonstrated Raji tumors to be largely refractory to cyclophosphamide treatment at this dose level as evidenced by the poor
Inhibition of Genetically Targeted T Cells by nTregs

Figure 4. CD19-targeted nTregs within the Raji tumor microenvironment suppress 19-28z+ effector T cell function in vivo. A, SCID-Beige mice were injected i.v. with Raji tumor cells on day 0, followed by CAR⁻ nTregs on day 5 (filled arrow) and CAR⁺ effector T cells on day 6 (open arrow). 19z1⁺ nTregs fully abrogated eradication of systemic Raji tumors by 19-28z⁺ effector T cells as assessed by survival over time when compared to mice treated with 19-28z⁺ effector T cells alone (P < 0.001). Pz1⁺ nTregs did not demonstrate significant suppression (P = 0.09 when compared to the 19-28z⁺ effector T cells alone cohort; P < 0.001 compared to the 19z1⁺ nTreg plus 19-28z⁺ effector T cells cohort). Data represent combined results from 2 independent experiments. B, 19z1⁺ nTregs inhibited 19-28z⁺ T cells in a dose-dependent manner with infused nTreg to effector T-cell ratios of 1:1, 1:4, and 1:8 resulting in no long-term surviving mice (all with P < 0.001, compared to 19-28z Teff alone cohort) while a 1:16 nTreg to effector T-cell ratio allowed for a 50% long-term survival of treated mice (P = 0.02, compared to Pz1⁺ Teff treated control cohort). Survival of the 1:16 nTreg to effector T-cell-treated cohort was statistically similar to the 19-28z Teff alone control cohort (P = 0.3). Similar results were obtained in tumor-bearing mice following prior infusion with 19-28z⁺ nTregs (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).
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.

**Grant Support**

This work was supported by CA138738, CA95152, CA059350, CA08748, CA69438, CA096945, CA094060. The Alliance for Cancer Gene Therapy, Damon Runyon Clinical Investigator Award (R.J. Brentjens), The Annual Terry Fox Run for Cancer Research (New York, NY) organized by the Canada Club of New York, Kate’s Team, Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Cancer Foundation for Research and the Experimental Therapeutics Center of MSKCC, and the Geoffrey Beece Cancer Foundation. E. Hayman is a Howard Hughes Medical Institute award recipient.

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Received February 16, 2010; revised January 18, 2011; accepted January 28, 2011; published online April 12, 2011.

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