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Inventing New CARs: Analysis of Chimeric Antigen Receptor Gene-Targeted T cells Modified to Overcome Regulatory T cell Suppression in the Tumor Microenvironment

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Abstract

Human T cells may be genetically modified to express targeted chimeric antigen receptors (CARs). We have previously demonstrated that T cells modified to express a CAR specific to the B cell tumor antigen CD19, termed 19-28z, successfully eradicate systemic human $CD19^+$ tumors in SCID-Beige mice. While these results are encouraging, this xenogeneic tumor model fails to address potential limitations of this therapeutic approach in the clinical setting wherein these modified T cells encounter a hostile tumor microenvironment. Specifically, these models fail to address potential effector T cell inhibition mediated by endogenous regulatory T cells (Tregs). To investigate the role of inhibitory Tregs, we initially assessed the *in vitro* function of CAR-modified T cells in the context of Tregs. We found that CD19-targeted T cell proliferation and cytotoxicity were inhibited by purified natural Tregs. To further assess the role of these Tregs *in vivo*, we isolated and genetically modified Tregs to express the CD19-targeted 19z1 CAR. We verified specific trafficking of targeted Tregs to $CD19^+$ tumors *in vivo*, and demonstrate that 19z1 Tregs wholly inhibit anti-tumor function of subsequently injected 19-28z effector T cells even at low Treg to effector T cell ratios (1:8). In order to overcome this limitation, we assessed whether the addition of a pro-inflammatory cytokine *in vitro* could overcome Treg inhibition. Indeed, the addition of exogenous IL-12 mediated resistance of 19-28z T cells to Treg inhibition. In light of this data we generated a bicistronic retroviral vector containing both the 19-28z CAR as well as the murine IL-12 fusion gene (19-28z IRES IL-12). Significantly, we found that 19-28z/IL-12^+ T cells when compared to 19-$28z^+$ T cells exhibited enhanced proliferation *in vitro* as well as resistance to Treg mediated inhibition. Finally, we demonstrate that 19-28z/IL-12^+ T cells overcome Treg inhibition *in vivo* in our SCID-Beige Treg tumor model. In conclusion, tumor targeted T cells modified to express IL-12 demonstrate significantly enhanced *in vivo* anti-tumor efficacy in the presence of Tregs that are similarly targeted to the site of tumor. These results validate utilization of IL-12 secreting tumor targeted T cells in future clinical trials.

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Introduction

Adoptive T cell Therapy For Cancer

Adoptive T-cell therapy refers to the infusion of tumor-targeting T-cells into the tumor-bearing host. The principle that T cells may serve as an effective and specific reagent for the eradication of tumor cells is demonstrated by the following observations from the allogeneic setting- 1. Patients undergoing allogeneic bone marrow transplantation (allo-BMT) for hematologic malignancies have lower relapse rates when compared to patients receiving a syngeneic bone marrow graft from an identical twin sibling; 2. Patients who have received a T-depleted allo-BMT graft, while experiencing fewer undesirable graft versus host T cell-mediated complications (GvHD), also experience a higher degree of tumor relapse; and 3. Patients who experience chronic GvHD are overall less likely to have disease relapse when compared to those patients who do not develop GvHD. These clinical findings strongly suggest that T cells mediate both a GvHD effect as well as a graft versus leukemia (GvL) effect. Finally, the role of T cell-mediated targeting and eradication of tumor is most powerfully demonstrated by the high rates of remissions seen in patients with chronic myelogenous leukemia (CML) who relapse after allo-BMT but are subsequently treated with T cells harvested from their original stem cell donor, a procedure known as donor leukocyte infusion (DLI)[1-3]. While these data support the contention that T cells may eradicate systemic disease, enthusiasm for the use of DLI in the allo-BMT setting is tempered by the lack of suitable HLA-identical bone marrow donors for many patients, associated GvHD complications, and the fact that efficacy of this approach appears to be largely limited to patients with CML.

In the autologous setting, adoptive T cell therapy has achieved very limited success. In this setting of melanoma, tumor infiltrating lymphocytes (TILs), specific to antigens on melanoma tumor cells, may be isolated from resected tumor, expanded *ex vivo*, and subsequently re-infused back into patients with metastatic disease [4-7]. While this approach has resulted in significant tumor regressions in some patients, over 80% of patients eventually relapse or fail to respond at all. Furthermore, application of this approach in most other cancers has not been studied, and would likely be limited by a paucity of TILs present in tumors less immunogenic to the host than melanoma.

Chimeric Antigen Receptors

To overcome lack of suitable donors, tumor specificity and complications from GvHD , autologous T cells may be genetically targeted to tumor antigens through chimeric antigen receptors (CARs) using gammaretroviral vectors[8]. These genetically engineered CARs fuse a tumor antigen specific single chain fragment (scFv) derived from mouse antibody to the TCR ζ-signaling domain, and will serve as artificial T-cell receptors (TCRs) that allow us to mimic a functional TCR signal transduction response with effective activation. The specificity to the tumor antigen and autologous nature of the modified T-cells to the patient may provide effective therapy while minimizing the risk for the development of GVHD or autoimmune reactivity against normal tissues. Furthermore, targeting tumor cells with CAR-modified T cells has the advantage of overcoming several tumor immune escape mechanisms including the failure of tumors to express immunogenic proteins, the failure of tumors to express co-stimulatory ligands, and tumor downregulation of MHC-mediated antigen presentation[8-10]. However,

although this form of therapy appears promising, recent clinical trials using genetically modified T cells have largely failed to eradicate patient disease[11,12] It is increasingly apparent that the factors determining the success of therapy are not only the effective targeting and trafficking of modified T cells to the site of tumor, but also their ability to persist in a hostile tumor microenvironment. In order to better understand treatment failures using this approach, models that study anti-tumor response of T cells as they encounter an inhibitory environment are needed. To this end, we sought to investigate the impact of regulatory T cells (Tregs), a potent endogenous suppressive element of the immune system, on the anti-tumor activity of adoptively transferred modified T cells at the site of tumor.

Regulatory T cells

Tregs are a subset of $CD4^+$ $CD25^+$ T cells that are either derived from the thymus, referred to as natural Tregs (nTregs), or from the periphery where non-Tregs may be induced to acquire a Treg phenotype[13]. Such "induced" Tregs have been categorized as phenotypically distinct Tr1 and Th3 subsets of Tregs[14]. Of all the subsets, the best characterized are nTregs. These cells, defined as $CD4^+$ $CD25^+$ $CD127^-$ $Foxp3^+$ T cells, have been extensively studied and widely accepted to facilitate suppression of autoimmune T cell responses and maintain peripheral tolerance[15-18]. These cells represent approximately 5-10% of the peripheral $CD4^+$ T cells in both mice and humans[16,19,20], and have high expressions of cytotoxic T lymphocyte associated antigen 4 (CTLA-4), glucocorticoid-induced TNFR-related protein (GITR), CD39, and CD73[21-23] in addition to the above markers. In many cancers, including the B cell malignancies

chronic lymphocytic leukemia (CLL) and non-Hodgkins lymphoma, it is reported that elevated numbers of Tregs are present in the periphery and within the tumor of the patient[24-26]. Furthermore, there appears to be a direct correlation between increased amount of Tregs and poorer prognosis amongst many malignancies[24,27,28]. Although the precise mechanism of suppression by Tregs remain controversial and appear to be environment dependent[29], the increased presence of this suppressive cellular entity raises the concern that the hostile microenvironment it creates could hinder the success of cancer adoptive cell therapies[30].

The difficulty with studying the tumor microenvironment and how it causes tumor immune escape is the laborious dissection through the many players in the immune system. While there have been many studies implicating Tregs as the cause of failed anti-tumor immune response using either specific Treg depleting strategies[28,31,32] or even systemic lymphodepletion[33,34]. Because there are so few Tregs naturally, the creation of models that study the effect of Tregs on anti-tumor immunity has been technically challenging. Until recently, Tregs were thought to be anergic and infeasible to expand in culture. But new discoveries in the biology of Tregs has enabled investigators to effectively and readily isolate[35], stimulate, and expand pure Tregs for experimental purposes[36-38]. Despite this advancement in *ex vivo* culture methods, progress has been slow in the study of Tregs *in vivo* due to the incomplete understanding in what causes increased levels of Tregs in cancer. As a result, investigators have been unable to study the impact of Tregs prospectively, in a manner that mimics the clinical setting.

Interleukin-12

IL-12 is a heterodimeric cytokine composed of an α chain (p35 subunit) and a β chain (p40 subunit) dimerized via a disulfide link. When first isolated, IL-12 was reported to enhance the cytotoxic potential of both NK cells as well as T cells, enhance activated T cell proliferation, and mediate Th1 differentiation[39,40]. Mescher and co-workers equated IL-12-mediated signaling to a "signal 3" for T cells wherein co-stimulation (signal 2) with associated IL-2 secretion augments T cell proliferation, while IL-12 signaling (signal 3) specifically enhances T cell cytotoxic effector function through upregulation of granzyme B, thereby effectively overcoming T cell tolerance[41-43].

More recently, investigators have found that IL-12 has a marked influence on the tumor microenvironment and T cell resistance to Tregs and apoptosis. Kilinc et al have demonstrated profound reversal of tumor immune suppression in Balb/c mice bearing established lung carcinoma tumors following intra-tumoral injection of IL-12[44]. Investigators found that following intra-tumoral IL-12 injection, there was a steady decrease in the Treg population within the tumor associated with an increased secretion of IFNγ and restored expression of granzyme B by infiltrating CD8+ effector/memory T cells. Furthermore, the authors noted an increased infiltration of exogenous tumor-targeted T cells into the tumor and this cascade of events resulted in tumor regression. Similarly, studies by Bankert et al found that IL-12 could reverse anergy of T cells derived from human lung tumor tissues[45-47]. Xenografted human lung tumor tissue into SCID mice followed by intra-tumoral injection with IL-12 microbeads resulted in complete eradication of tumor cells in the xenograft, a process of tumor regression primarily mediated by endogenous $CD4^+$ T cells through an IFNγ dependent mechanism.

These studies demonstrate that IL-12 may mediate reactivation of anergic $CD4^+$ T cells within the tumor leading to profound expansion of these reactivated T cells with associated secretion of IFNγ. Finally, work by King and Segal expanded on these observations by demonstrating in the murine setting that anergy of $CD4^+$ T cells induced upon co-culture with Tregs was reversed by the addition of exogenous IL-12[48]. The authors demonstrate that this effect was due solely on effects by IL-12 on the effector T cell population, with IL-12 having no effect on the Treg population. These observations make IL-12 an attractive agent to incorporate into gene-modified adoptive T cell therapy as a means to overcome the suppressive tumor microenvironment.

Here, we sought to investigate the *in vivo* impact of Tregs on targeted T cell therapy in a systemic tumor model and devise a way to overcome the suppression, if any, that would be present. We have previously generated CARs specific for the human CD19 (hCD19) antigen expressed on normal B cells and most B cell malignancies[49,50]. While human CD19-targeted T cells eradicate established human $CD19^+$ Raji Burkitt lymphoma tumors in a majority of treated SCID-Beige mice[49], this xenogeneic tumor model fails to address potential limitations of immune suppression by Tregs. We therefore utilized CAR-modified Tregs, similarly targeted to CD19, to generate a suppressive tumor microenvironment that mimics the clinical setting. In this model, the CD19 targeted human Tregs are infused into SCID-Beige mice bearing established $CD19^+$ human tumors prior to the adoptive transfer of targeted cytotoxic T cells. Our results demonstrate that CAR^+ Tregs traffic to the site of tumor and exert potent suppression on similarly targeted effector T cells. Full suppression by the Tregs require their localization to the site of tumor as well as *in vivo* activation through the CAR.

Utilizing a bicistronic retroviral vector containing both 19-28z CAR as while as the IL-12 fusion gene, we demonstrate that targeted delivery of a pro-immune cytokine can reverse such Treg-mediated suppression. Taken together, our data support the hypothesis that antigen specific Tregs may significantly compromise the efficacy of genetically modified T cell therapy. The inhibition, however, can be overcome by the incorporation of genes of proinflammatory cytokines in the CAR which facilitates its delivery specifically at the tumor microenvironment.

Statement of Purpose, Specific Aims, and Hypothesis

The purpose of this research project is to study the biology of CAR modified adoptive T cell therapy in the presence of a hostile tumor microenvironment. We decided to study this in the context B cell malignancies, because a majority of patients with B cell malignancies either die from their disease or are incurable despite a multitude of treatment options such as chemotherapy, bone marrow transplantation, radiation, and in recent years, monoclonal antibodies such as Rituximab and Alemtuzumab. This heterogeneous group of neoplasms comprise a vast majority of non-Hodgkin's lymphomas (NHL), acute lymphoblastic leukemias (ALL), and chronic lymphocytic leukemias (CLL). The targeting of CD19 using autologous anti-tumor T cells would be a promising novel treatment approach that could improve the outcome of these patients.

A better understanding of the tumor microenvironment provides clues as to why adoptive T cell therapies in their current form have only modest potential for full tumor eradication. While T cells may be successfully targeted to the tumor, they are likely to encounter a wide array of suppressive elements that may facility tumor immune escape. For this reason, future research in the field of adoptive T cell therapy of requires studies not only on the T cell specificity to tumor, but also a focus on how these T cells are capable of maintaining effector function within the tumor microenvironment. In our proposed studies, we sought to investigate first the impact Tregs at the site of tumor on the efficacy of adoptively transferred cytotoxic T cells, and, second, the impact of IL-12 secretion by tumor targeted T cells on the resistance of these T cells to inhibition by the suppressive tumor microenvironment. We anticipate that this clinically relevant model

will have broad implications to adoptive T cell therapies of cancer and the design of future clinical trials using this treatment modality. The specific aims of this project are:

Aim 1. To isolate and expand a viable population of CD4+CD25+*FOXP3*+ Tregs from human peripheral blood for CD19 CAR transduction.

Hypothesis: We hypothesize that a viable population of CD4+CD25+*FOXP3*+ Tregs can be isolated from human peripheral blood and successfully expanded for CD19 CAR transduction.

Aim 2. To investigate whether CAR modified Tregs retain functionality on effector T cell proliferation and cytotoxicity *in vitro.*

Hypothesis: We hypothesize that Tregs transduced with CARs will retain suppressive functionality when compared to non-transduced Tregs and potently inhibit effector T cell functions.

Aim 3. To assess whether adoptively transferred CAR modified Tregs traffic to site of tumor and hinder the successful treatment of SCID-beige mice with $CD19^+$ Burkitt's lymphoma with 19-28z$^+$ T cells.

Hypothesis: We hypothesize that CD19 specific Tregs will successfully traffic to the sites of tumor and suppress the antitumor effects of 19-28z^{+} T cells against CD19^{+} Burkitt's lymphoma in a dose dependant manner *in vivo*.

Aim 4. To demonstrate the ability of IL-12 modified tumor targeted T cells to overcome Treg inhibition within the tumor microenvironment.

Hypothesis: IL-12 protects CAR-modified T cells from Tregs, reversing the previously observed Treg suppression of effector T cell cytotoxicity at the site of tumor.

Methods:

Isolation of $CD4^+$ $CD25^-$ responder T cells and $CD4^+$ $CD25^+$ nTregs

Peripheral blood from healthy donors, obtained under institutional review board (IRB)-approved protocol 95-054, was collected in BD Vacutainer CPT tubes (BD Medical, Sandy, UT), and fractionated by subsequent centrifugation as per the manufacturer's instructions to yield peripheral blood mononuclear cells (PBMNCs). $CD4^+$ $CD25^-$ responder T cells and $CD4^+$ $CD25^+$ Tregs were then isolated from PBMNCs using the $CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kit (Dynal brand; Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Briefly, $CD4^+$ T cells were negatively isolated with magnetic microbeads, the population is then positively selected for $CD25^+$. FACS analysis is then used to confirm CD25 and Foxp3 double-positivity. Purity of the isolated cells was always above 90% as assessed by FACS analysis.

Genetic Modification of T cells

We performed retroviral transduction of healthy human donor T cells, obtained following informed consent under the MSKCC institutional review board approved protocol #90-095, by activating the cells using PHA or in the case of Tregs the Dynal CD3/CD28 human Treg expander and transducing using PG-13 retroviral supernatants with spinoculation in retronectin coated 6 well plates for 1 hour with retroviral supernatent at 3200 rpm, 37^{o}C, for 2 consecutive days as previously reported[51].

Culture and expansion of CAR$^+$ T cells

CAR$^+$ effector T cells were expanded in RPMI media supplemented with IL-2 (100IU/ml) with weekly stimulation on CD19/CD80 NIH-3T3 fibroblasts as artificial antigen presenting cells[51]. CAR$^+$ Tregs were expanded in RPMI media supplemented with IL-2 (500IU/ml) and rapamycin 1ug/ml with Dynal CD3/CD28 human Treg Expander beads or CD19/CD80 NIH-3T3 fibroblasts.

In vitro Cytotoxicity Assay

19-28z$^+$ effector T cells were co-cultured with Raji cells in RPMI media at 1:1 ratio with or without equal amounts of 19z1$^+$ Tregs for 24 hours. Flow cytometric analysis with anti-CD19 antibody (Caltag Laboratories, Burlingame, CA) was done at 24 hours after start of co-culture, CD19 positive cells were gated and regarded as viable Raji cells.

Suppression Assay

5 x10^5 T cells were labeled with 5 μM CFSE (Invitrogen, Carlsbad, CA) and cultured with titrated numbers of purified autologous CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs in RPMI (GIBCO brand; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT) in 24-well tissue culture plates (Costar, Corning, NY). Stimulation was carried out in the absence of IL-2 and with 5 x10^5 Dynabeads CD3/CD28 T Cell Expander (Dynal brand; Invitrogen, Carlsbad, CA) for a bead-to-responder T cell ratio of 1:1. After 72 hours of incubation at 37°C, responder T cell proliferation was determined by flow cytometric detection of CFSE fluorescence as well as Luminex assay detection of IL-2 in the media.

Bioluminescence imaging

Bioluminescence was detected using a Xenogen IVIS Imaging System (Xenogen). We performed imaging either 10-15 sec following bolus IV injection with coelenterazine (250 μg) (Nanolight Technology), substrate for extGLuc, or 10-15 min after intraperitoneal injection of D–luciferin (150 mg kg^{-1}) (Xenogen), substrate for FFLuc. We imaged mice individually whenever coelenterazine substrate was used whereas 2-5 mice were simultaneously imaged in luciferin-based acquisitions. Time of image acquisition was in the range of 0.5 to 3 min. Field of view of 15, 20, or 25 cm with low, medium, or high binning in an open filter was utilized to maximize signal intensity and sensitivity. We obtained acquisition of image data sets and measurement of signal intensity through region of interest (ROI) analysis using Living Image software (Xenogen), and normalized images displayed on each data set according to color intensity.

Statistical Analysis

Statistical analysis done using paired t test for line graph comparisons and log rank analysis for survival curve comparisons. P values of less than 0.05 were considered statistically significant.

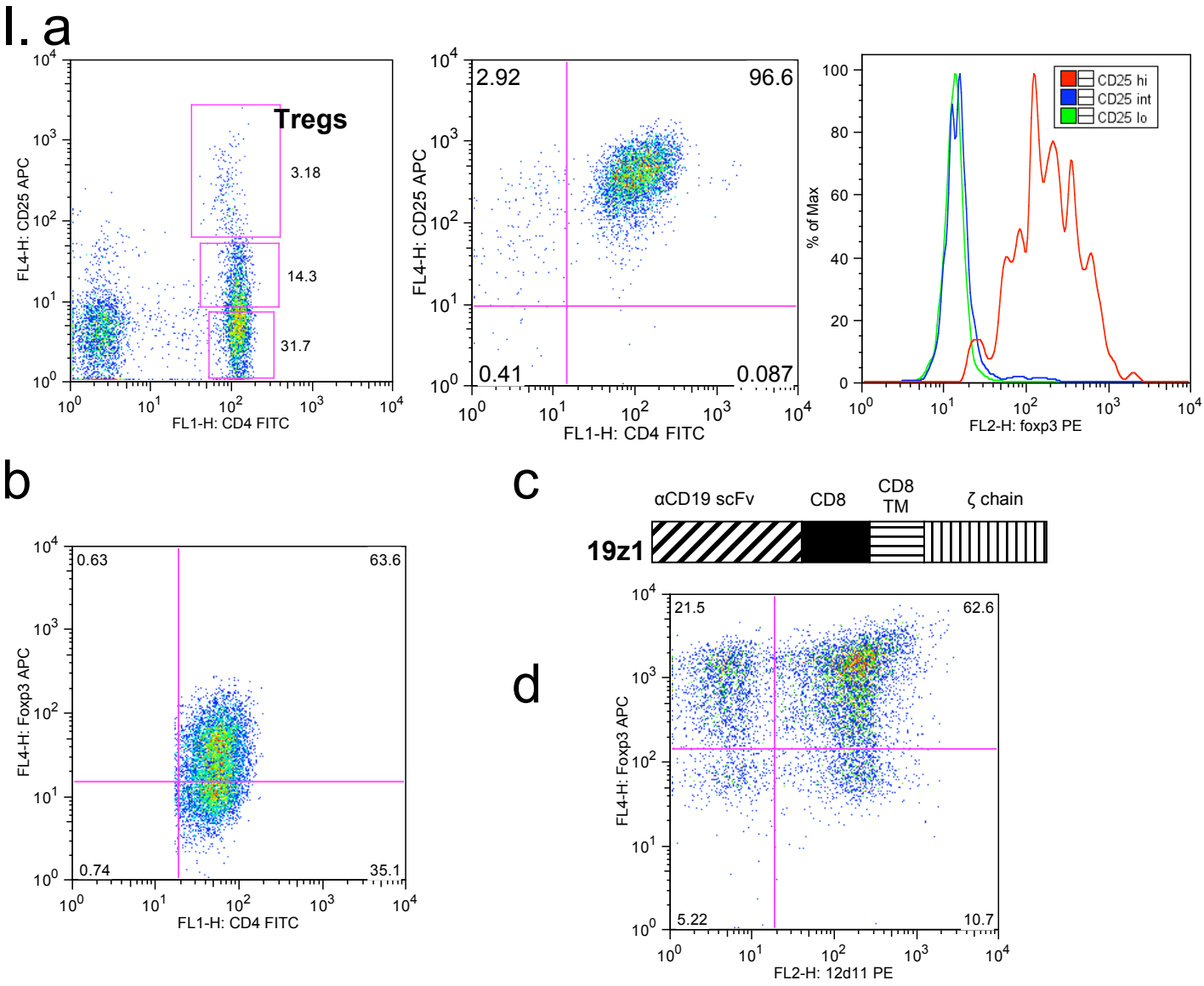
Results

I. Natural and induced Tregs can be modified with CARS and are readily expandable.

To establish a tumor microenvironment similar to the clinical setting, where Tregs are elevated at the tumor, we sought to genetically redirect them to the actual location of the cancer cells. We have previously shown that effector T cells may be modified to express a functional CD19-specific CAR, termed 19z1[49], that facilitates specific accumulation of T cells at the site of tumor and exert effector function. However, it is less certain whether human Tregs can be manipulated with equal efficiency due to their anergic nature and difficulty to maintain in culture[37,52]. To assess the feasibility of genetic modification in Tregs, we isolated natural Tregs (nTregs) and generated induced Tregs (iTregs) for our experiments. nTregs were isolated using immunomagnetically sorting for the $CD4^+$ $CD25^{hi}$ population from PBMNCs. The nTregs isolated were consistently >95% in purity with their Foxp3 expression routinely 70-90% (Fig. 1a). iTregs were generated using a published method by coculturing $CD4^+$ $CD25^-$ $Foxp3^-$ cells with TGF-β[53,54], with the induced Foxp3 expression routinely 60-80% (Fig. 1b). The cells were then retrovirally transduced with 19z1 using a previously described method (Fig. 1c)[49], and over 60% $Foxp3^+$ CAR^+ cells are readily obtainable for both nTreg (Fig. 1d) and iTreg (data not shown) groups.

We next assessed the proliferation potential of the CAR^+ Tregs, to see if sufficient cell numbers could be obtained for larger scale *in vivo* experiments. We have previously developed a method to expand CAR^+ T cells using NIH-3T3 murine fibroblasts

genetically engineered to express CD19 and CD80 [3T3(CD19/CD80)][49]. To compare the efficacy of commercial CD3/CD28 T cell expansion beads with 3T3 (CD19/CD80) AAPCs at expanding Tregs, equal numbers of CAR$^+$ nTregs and iTregs were cocultured with beads per manufacturer's recommended protocol or 3T3 (CD19/CD80) AAPCs after initial T-cell transduction. CAR$^+$ nTregs and iTregs proliferated equally well for both conditions, with greater than 120 fold total cell expansion over 28 days (Fig. 1e). However, in terms of expanding pure populations of transduced Tregs, we found in a follow-up experiment that the 3T3 (CD19/CD80) AAPCs provided specific stimulation for the CAR$^+$ cells and generated nearly twice the amount of absolute CAR$^+$ cells over beads at 28 days (Fig. 1f).



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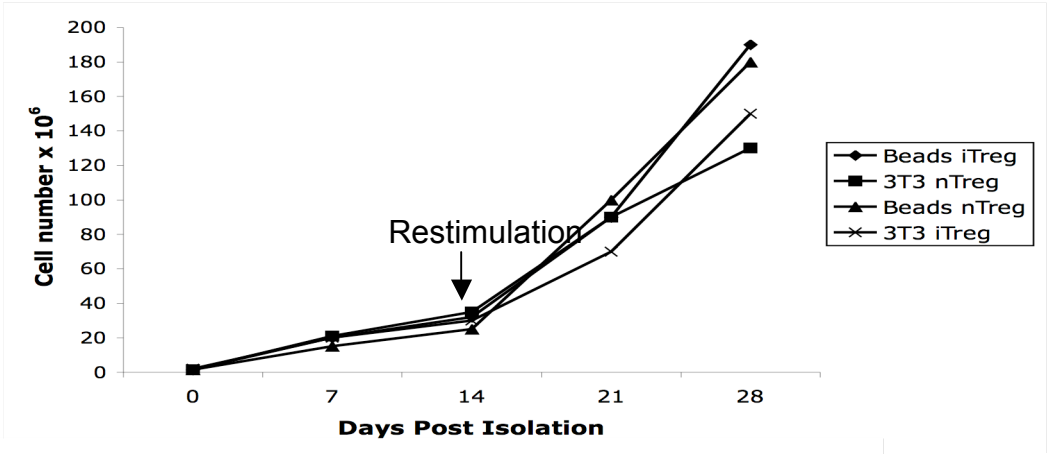

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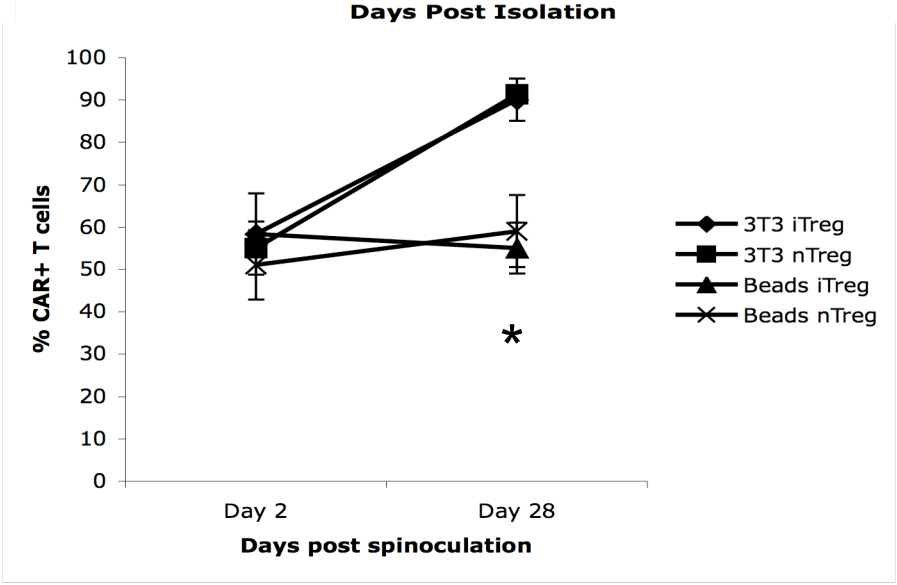

Figure 1. Isolation and transduction of Tregs. **(A)** Isolation of nTregs from peripheral blood utilizing the Dynal Treg isolation kit. Treg phenotype is verified by CD4 CD25 surface staining as well as Foxp3 intracellular staining. **(B)** Generation of iTregs using 10ng/mL TGF-b for 3 days during initial activation of $CD4^+$ $CD25^-$ naïve T cells. **(C)** Schematic of the 19z1 CAR. **(D)** Isolated nTregs are successfully transduced with the 19z1 CAR, FACS analysis is done using intracellular Foxp3 staining as well as an antibody specific for the CAR, 12d11, created at Sloan-Kettering Cancer Center. **(E)** $19z1^+$ Tregs exhibit capacity for expansion on 3T3(hCD19/CD80) AAPCs and CD3/CD28 microbeads. **(F)** The absolute percentage of $19z1^+$ Tregs in increased with 3T3(hCD19/CD80) AAPC expansion in comparison to CD3/CD28 microbeads (n = 3; *, $p < 0.01$).

II. Expanded natural but not induced Tregs confer potent suppressive activity.

To better mimic a hostile tumor microenvirnment, we sought to define the suppressive activity of expanded CAR^+ nTreg and iTregs and identify the more potent and consistent suppressor cell type of the two. Because both nTreg and iTreg share approximately the same number of $Foxp3^+ CAR^+$ cells, we predicted that their suppression capability would be comparable. We first conducted a standard CFSE-labeled suppression assay where 5 $x10^5$ effector cells were cocultured with different ratios of suppressor cells. Unexpectedly, while nTregs showed visible inhibition even at 1:16 suppressor to effector cell ratio, iTregs showed no inhibition at all for all ratios compared to control, non-suppressive $CD4^+$ $Foxp3^-$ cells (Fig. 2a).

To confirm the inhibitory activity of the Tregs, we used the Luminex assay to detect IL-2 as a surrogate marker for functional suppression in the cocultures. The IL-2 level for cocultures with nTregs decreased as we increased the suppressor to effector ratio, consistent with published data[52,55]. However, in contrary to the nTreg results, the IL-2 level in the cocultures with iTregs increased as we added more suppressors (Fig. 2b).

To further characterize the hostile tumor microenvironment mediated by Tregs, we next sought to compare the suppressive capability of nTreg and iTregs in terms of inhibition of effector T cell cytotoxicity. Effector T cells were first cocultured with nTreg or iTregs at 1:1 ratio for 24 hours to ensure the Tregs can exert their suppressive function upon the effector T cells. Next, Raji cells, also at 1:1 ratio with the effector T cells, were added to the cocultures. 24 hours later, effector T cell cytotoxicity was assessed by the amount of Raji cells that remain in culture. CAR^+ effector T cells were

cocultured with Raji cells alone as positive control, and nTreg as well as iTregs were cocultured with Raji cells alone for negative control. The data showed that nTregs completely inhibited killing of Raji cells in culture, as the Raji cell population remaining were nearly identical to negative controls. iTregs were unable to inhibit effect T cells, as CAR^{+} effector T cells were able to completely eradicate the Raji cells in the coculture in similar fashion to effector T cells alone (Fig. 2c).

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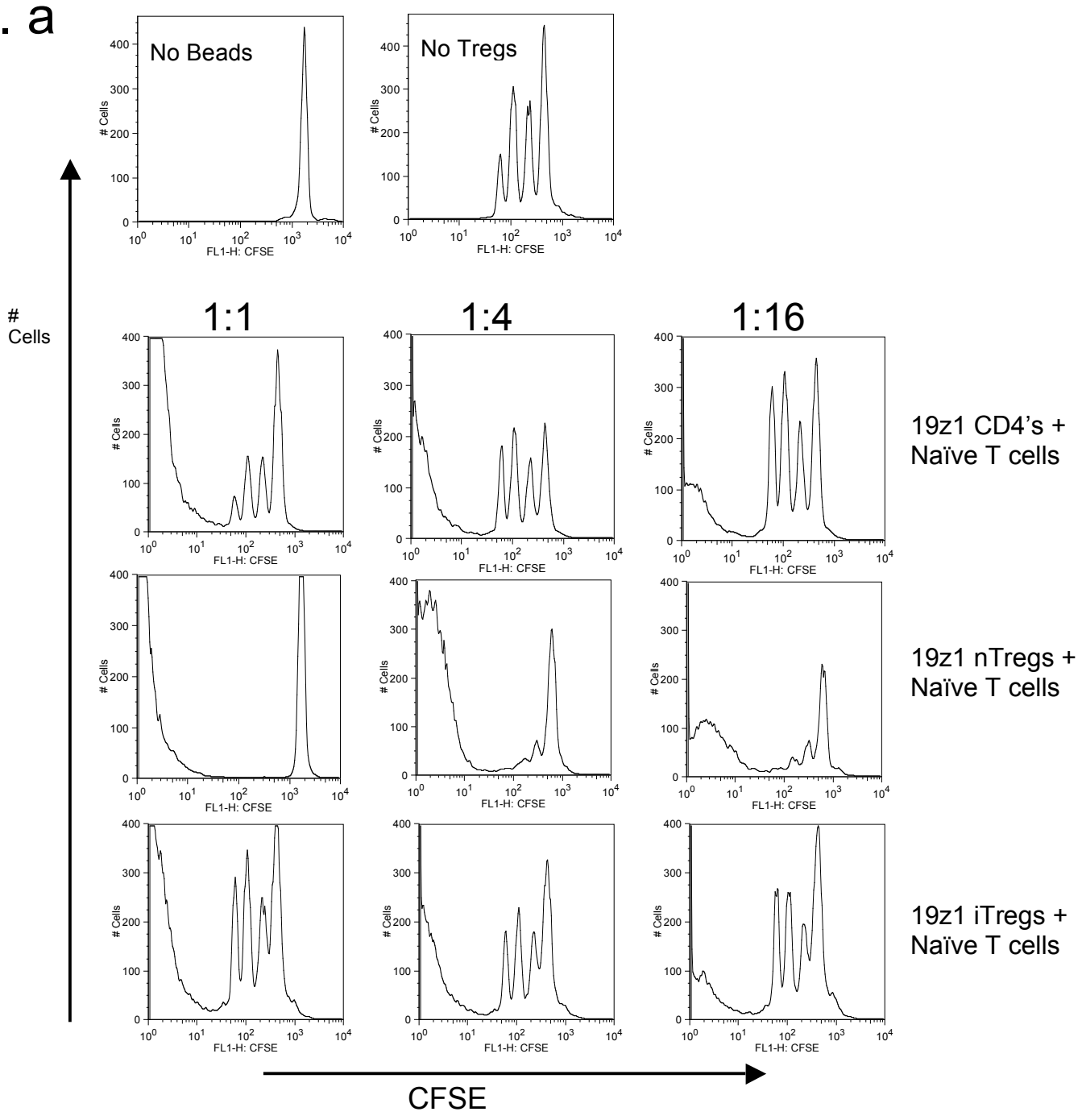


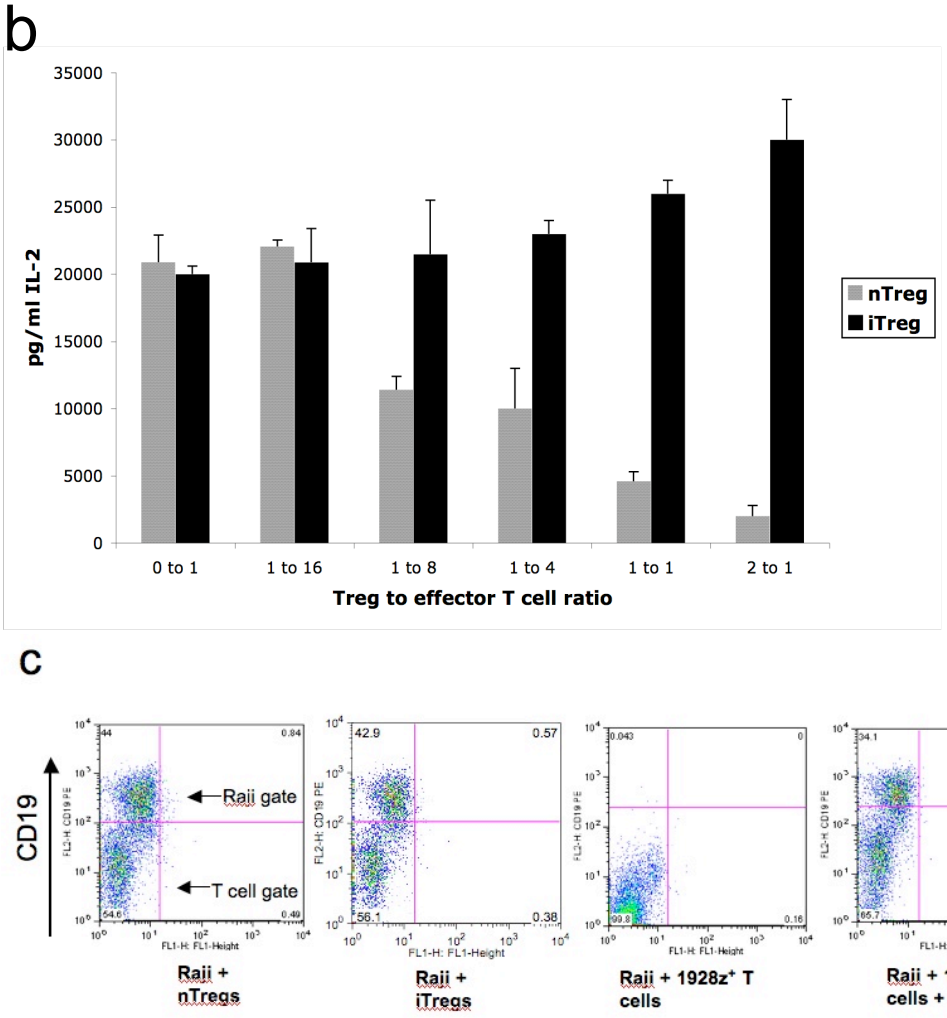
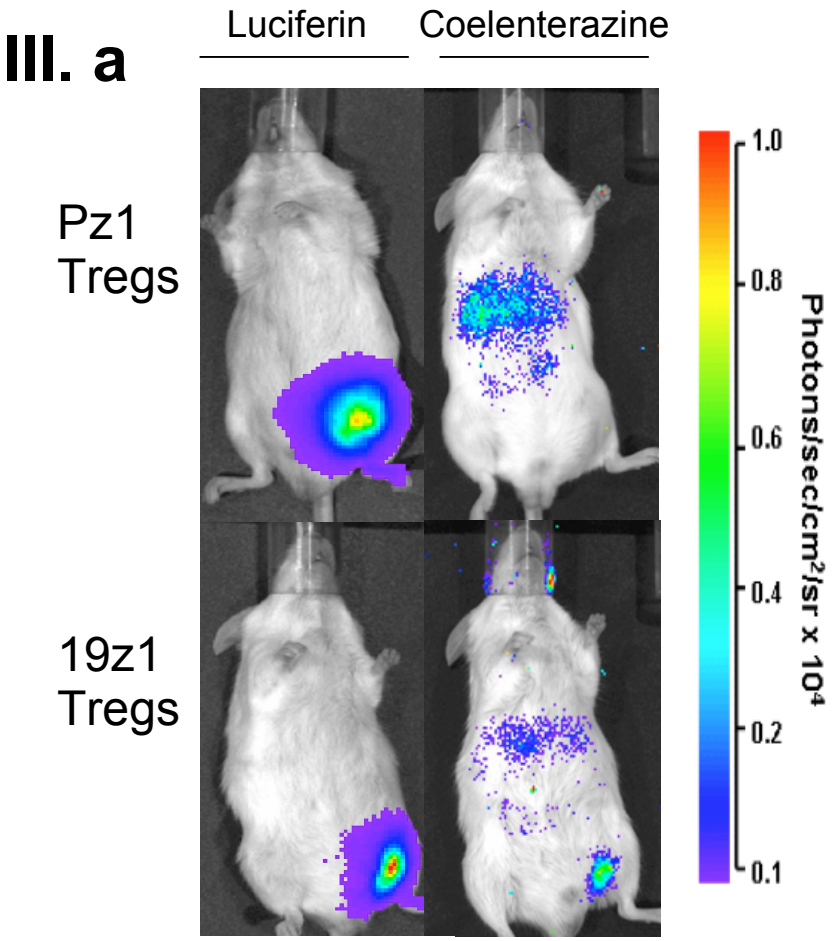


Figure 2. CAR$^+$ nTregs mediate potent suppression *in vitro*. **(A)** 19z1$^+$ nTregs inhibit proliferation of CD3/CD28 microbead stimulated T cells as assessed by FACS analysis of CFSE stained T cells at 3 days after co-culture. Inhibition is evident even at a nTreg to T cell ratio of 1:16. iTregs and control group with non-Treg CD4$^+$ T cells demonstrated no inhibition. **(B)** nTreg inhibits naïve T cell secretion of IL-2 in a dose dependent manner as assessed by Luminex cytokine assay of culture media 1 day after activation of T cells with CD3/CD28 microbeads. iTreg cultures resulted in more IL-2 secretion, in a dose dependant manner, when compared to no Treg controls (n = 3). **(C)** Isolated nTregs, as opposed to iTregs, inhibit 19-28z effector T cell lysis of Raji tumor cells at 24 hours following addition of targeted tumor cells.

III. CAR$^+$ nTregs specifically traffic to site of tumor.

Having determined that nTregs are the more potent suppressor cells, we decided to utilize them instead of iTregs for all subsequent experiments, and elected to characterize their trafficking pattern *in vivo* to see if they can be brought to the site of tumor. We employed a dual bioluminescent imaging model that will allow us to simultaneously image tumor cells and T cells within the same animal. We injected SCID-Beige mice subcutaneously with tumor cells that are modified to express the bioluminescent enzyme Firefly luciferase (Raji-FFLuc) in the left flank to establish a solid tumor as a target for the CAR$^+$ nTregs to see if they will successfully traffic to tumor. At 7 days, when the tumors were palpable, mice were injected intravenously with 1 x 10^7 targeted nTregs modified to express the membrane anchored bioluminescent enzyme external-Gaussia luciferase (19z1$^+$extGLuc$^+$ nTregs) to allow for dual imaging of the tumor as well as the nTregs. nTregs co-transduced with the irrelevant Pz1 CAR[56] (an irrelevant CAR targeted to the prostate tumor antigen PSMA) and extGLuc, were used as a control. Bioluminescent imaging at 24 hours demonstrated 19z1$^+$extGLuc$^+$ T cell signal localization at the Raji tumor on the left flank as well as the liver. In contrast, Pz1$^+$ extGLuc$^+$ nTregs did not traffic to the Raji tumor but only to the liver (Fig. 3a). At autopsy, immunohistochemistry staining for T cells using CD3 confirms the presence of Tregs in the Raji tumor for the mouse that received the19z1$^+$extGLuc$^+$ but not the Pz1$^+$ extGLuc$^+$ nTregs (Fig. 3b). These observations strongly suggest CAR$^+$ nTregs are capable of specifically trafficking to tumor.



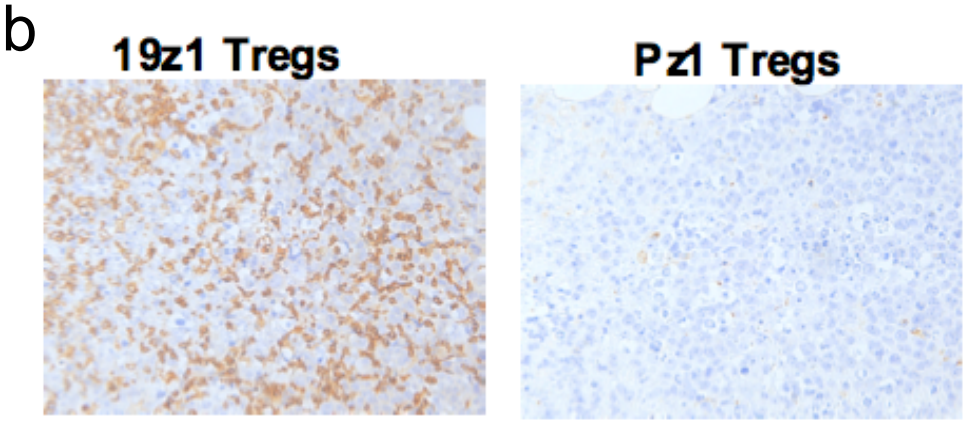


Figure 3. CAR$^+$ nTregs traffic specifically to tumor. **(A)** 19z1$^+$ nTregs labeled with exGLuc were imaged 24 hours following systemic infusion into SCID-Beige mice with subcutaneous Raji-FFLuc tumors. Differential bioluminescent imaging of tumor and T cells demonstrates trafficking of 19z1$^+$ nTregs to the tumor while control Pz1$^+$ nTregs fail to accumulate in the tumor. **(B)** Immunohistochemistry of subcutaneous Raji tumors

verify the presence of $CD3^+$ Tregs in the $19z1^+$ nTreg infused mice with little presence of Tregs in the $Pz1^+$ nTreg treated mice.

IV. CAR+ nTregs prevent the successful eradication of established systemic Raji tumor by adoptive transfer of similarly targeted CAR+ effector T cells.

To investigate the impact of nTregs at the site of tumor on the efficacy of adoptively transferred CAR^+ effector T cells *in vivo*, we next employed a previously described systemic Raji tumor model where a single injection of $19\text{-}28z^+$ effector T cells can eradicate established systemic Raji tumors 60%-80% of the time[49]. In this experiment, SCID-Beige mice were injected intravenously with 1×10^6 Raji tumor cells, 4 days later, 1×10^7 $19z1^+$ nTregs were systemically injected in the tumor bearing mice 24 hours prior to the$19\text{-}28z^+$ effector T cell injection (also 1×10^7 cells) to ensure that the Tregs arrive at the site of tumor first to exert suppression . We observed that, consistent with our *in vitro* data, $19z1^+$ nTregs were capable of completely inhibiting the eradication of tumor by the $19\text{-}28z^+$ effector T cells, with 80% survival in the $19\text{-}28z^+$ effector T cell alone group and 0% survival in the effector T cell plus Treg group as well as the Treg alone and $Pz1^+$ effector T cell control groups (Fig. 4a).

To assess for the minimum dose of nTregs required to exert suppression, we titrated the amount of nTregs to effector ratios from 1:16 to 1:1 in the same animal model. We observed that the CAR^+ nTregs were able to fully suppress the anti-tumor efficacy of effector T cells at a dose as low as 1:8 suppressor to effector cell ratio, suggesting that CAR^+ nTregs are potent inhibitors of adoptively transferred T cell therapy

that do not require high cell number to generate a hostile tumor microenvironment (Fig. 4b).

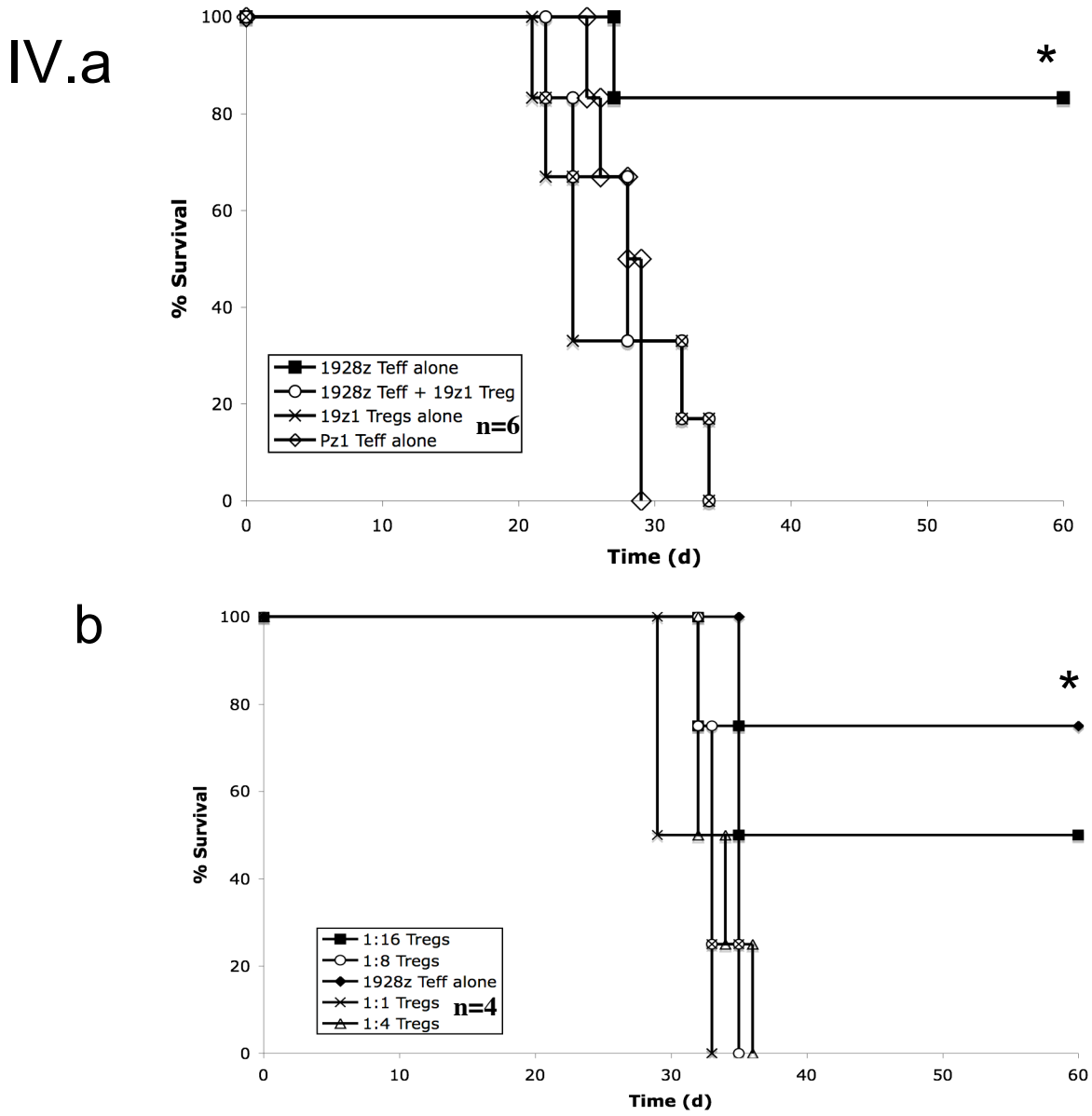
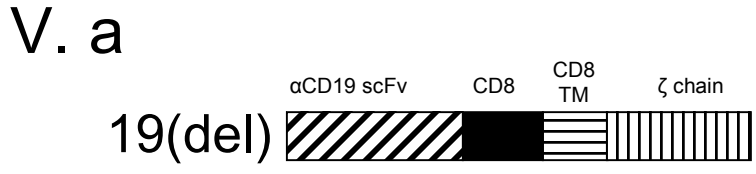


Figure 4. CAR$^+$ nTregs inhibit antitumor efficacy of adoptively transferred CAR$^+$ T cells *in vivo*. **(A)** 19z1$^+$ nTregs infused 24 hours prior to 19-28z$^+$ effector T cell treatment completely abrogated anti-tumor efficacy of these effector T cells when compared to mice treated with 19-28z$^+$ effector T cells alone (*, $p < 0.01$ compared to 1928z Teff + 19z1 Treg). **(B)** Four doses of nTregs were compared for ability to suppress T cell therapy; compete suppression of anti-tumor efficacy were noted at 1:1, 1:4, and 1:8 Treg-to-effector T cell ratios (*, $p < 0.02$ compared to 1:8 Tregs).

V. Full *in vivo* suppression by CAR+ nTregs requires their localization at the site of tumor and presence of zeta chain signaling domain.

To dissect the mechanism contributing to Treg suppression observed in our system, we employed the use of three different CARs, each with distinct properties. $19z1^+$ Tregs localizes to the site of tumor and exerts effector in the presence of CD19 antigen stimulation as stated above. Also as shown above, $Pz1^+$ Tregs do not traffic to the site of tumor nor do they respond to CD19 antigen stimulation (Fig. 5b). A third CAR, termed 19(del), which contains the anti-CD19 scFv but lacks the TCR ζ-signaling domain (Fig. 5a), generates Tregs that do not respond to CD19 antigen stimulation (Fig. 5b) but retains the ability to traffic specifically to Raji tumor (data not shown), possibly due to the ability of the anti-CD19 scFv to facilitate accumulation of cells upon contact with its cognate antigen at the tumor bed.

To test whether localization as well as TCR signaling were necessary for the observed suppression, mice were injected intravenously with Raji tumor on day -4, then injected with $19z1^+$, $19(del)^+$, and $Pz1^+$ nTregs on day 0, followed by injection of $19\text{-}28z^+$ effector T cells 1 day later as described previously. Our data demonstrates that while $19z1^+$ Tregs conferred full suppression, resulting in 0% long term survival for the 19-28z T cell treated mice, both $19(del)^+$ and $Pz1^+$ Tregs were able to confer partial suppression, resulting in 33% long term survival (Fig. 5c). This finding suggests that full suppressive activity requires both localization at the site of tumor and TCR signaling, but a partial suppression is possible without either property.



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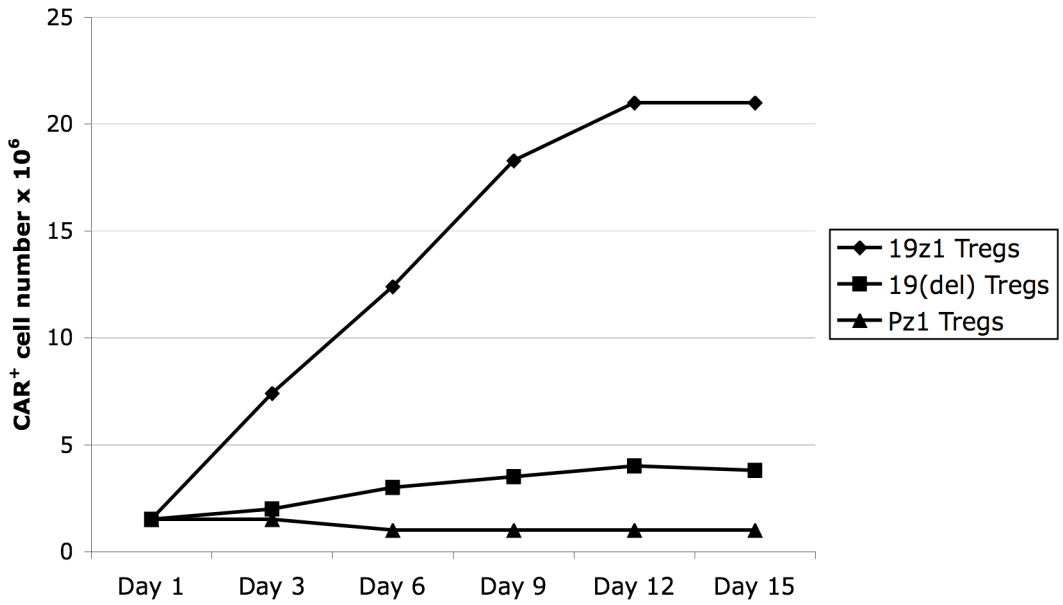

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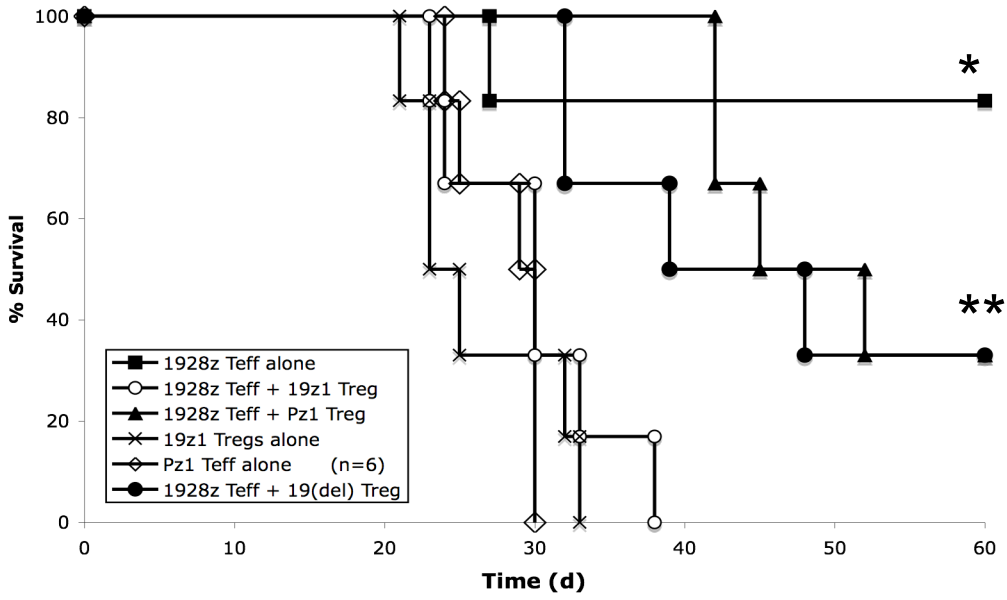

Figure 5. Full nTreg suppression *in vivo* is dependent on cell localization at site of tumor and TCR signaling. **(A)** Schematic of the 19(del) CAR. **(B)** 19z1, 19(del), and Pz1 transduced nTregs were expanded on 3T3 (hCD19/CD80) AAPCs to assess proliferation. Only 19z1$^+$ proceeded to expand significantly (20 fold) over 15 days. **(C)** 19z1$^+$ nTregs infused 24 hours prior to 19-28z$^+$ effector T cell treatment completely abrogated anti-tumor efficacy of these effector T cells when compared to 19(del)$^+$ and Pz1$^+$ nTreg infused mice which only conferred partial suppression. (*, $p < 0.01$ compared to 1928z Teff + 19z1 Treg; **, $p < 0.02$)

VI. Exogenous IL-12 protects 19-28z T cells from Treg inhibition of proliferation.

Having observed that Tregs localized at the site of tumor can act as inhibitors against adoptively transferred tumor-targeted effector T cells, we sought to discover ways to overcome this suppression. We decided to test the effect of the pro-inflammatory cytokine IL-12 on our T cells, because it has been well documented to promote T cell effector function (see introduction- interleukin 12). We used a CFSE-based proliferation assay to assess the suppression of effectors by Tregs in the presence or absence of IL-12. Resting 19-28z$^+$ T cells (effectors) were CFSE-labeled and co-cultured with Tregs at three different effector to suppressor ratios in a standard suppression assay. Dynal CD3/CD28 beads were added at 1:1 bead to effector cell ratio to activate the 19-28z$^+$ T cells. Flow cytometric analysis for CFSE$^+$ T cells were done on day 3 post activation, showing effects of Treg suppression with/without IL-12 1ng/ml in the culture media. **(Fig. 6a)** Proliferation of 19-28z$^+$ T cells in the presence of Tregs without IL-12 in the media. **(Fig. 6b)** Proliferation of 19-28z$^+$ T cells in the presence of Tregs with IL-12 supplementation. Data demonstrates increased proliferation of 19-28z$^+$ T cells (69.1, 67.3,and 54.5% vs 30.3, 26.6, 18.6%) with the addition of IL-12.

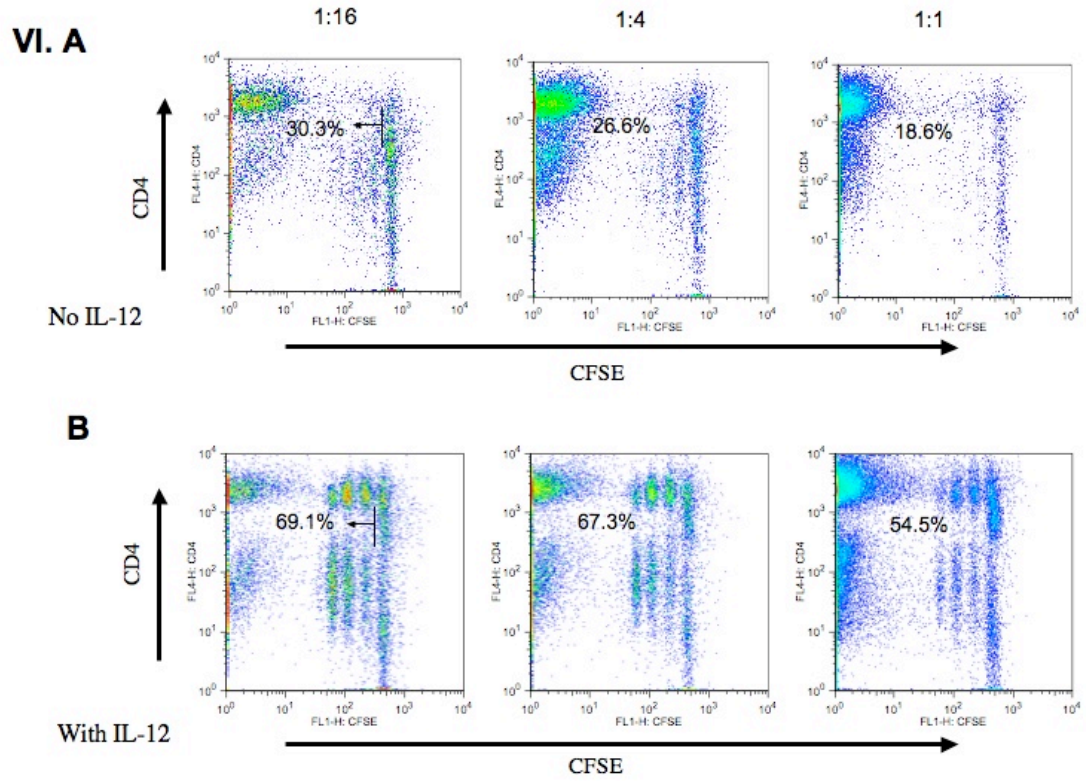

Figure 6. IL-12 protects T cells from Treg suppression. **(A)** nTregs co-cultured with 19-28z^{+} effector T cells followed by stimulation with CD3/CD28 microbeads results in the inhibition of 19-28z^{+} T cell expansion. **(B)** The addition of exogenous IL-12 (1ng/mL) to the T cell co-culture restores 19-28z^{+} effector T cell proliferation despite the presence of inhibitory nTregs.

VII. Anti-CD19 T cells genetically modified to express IL-12 overcome Treg inhibition *in vivo.*

In light of the above observation that IL-12 can mediate effector T cell resistance to Treg inhibition, we elected to generate CD19 targeted T cells capable of secreting IL-12, using a bicistronic retroviral vector containing the 19-28z CAR and murine IL-12 fusion gene (Fig. 7a). We next tested the utility of T cells modified using this construct using the previously established SCID-Beige tumor model. The expression of the 19-28z IRES IL-12 transduced T cells readily express the CAR at a level comparable to the 19-28z parent CAR transduced T cells. An animal tumor model with 1:1 Treg to T cell

inhibition were set up as described in the previous experiment, and data shows 60% long term survival of 1928z/IL-12^{+} T cell treated mice vs. 1928z^{+} T cell and irrelevant Pz1^{+} T cell treated mice which had 0% survival (Fig. 7b).

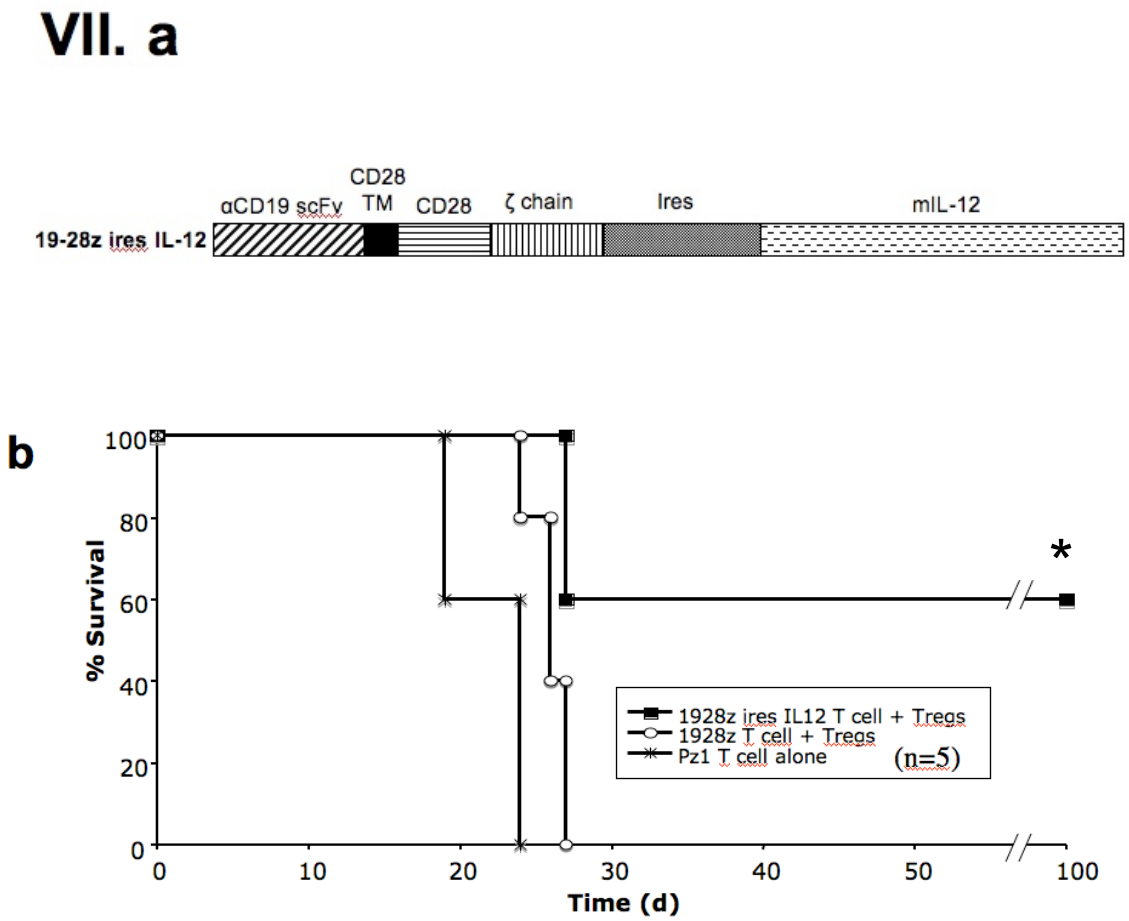

Figure 7. 19-28z IRES IL-12 T cells are resistant to Treg suppression *in vivo*. **(A)** Schematic of the 19-28z IRES IL-12 CAR. **(B)** Infusion of 1 x 10^{7} 19-28z IRES IL-12 in Raji tumor bearing SCID-Beige mice 24 hours following infusion of 19z1^{+} nTregs resulted in 60% complete tumor eradication in contrast to infusion with 19-28z^{+} effector T cells which failed to demonstrate survival benefit when compared to the Pz1^{+} T cell control mice (*, $p < 0.02$ compared to 1928z T cell + Tregs).

Discussion

To better understand the biology of tumor targeted genetically modified T cells in the setting of a suppressive environment, we have developed a novel tumor model where Tregs actively inhibit adoptively transferred cytotoxic T cells at the site of tumor. This model enables investigators for the first time to prospectively generate a suppressive tumor microenvironment using tumor targeted Tregs, allowing for the studying of their impact on adoptive cellular cancer therapy. In this report, we demonstrated that human Tregs are readily expandable, transduceable with CARs, and retain potent suppressive capabilities. When these tumor targeted Tregs are infused to the tumor bearing host, they specifically travel to sites of tumor and actively suppress the anti-tumor killing mediated by adoptively transferred cytotoxic T cells. We further utilize this model to demonstrate that, when the pro-inflammatory cytokine IL-12 is incorporated into the CAR, effector T cells can be made to resist the Treg suppression. When IL-12 secreting T cells are used for adoptive T cell therapy, they have the ability to overcome the hostile tumor microenvironment generated by Tregs.

Although adoptive T cell therapy in the setting of allo-BMT has demonstrated the potential of T cells as an effective anti-tumor reagent, in the autologous setting, most clinical trials have been met with only modest if any anti-tumor responses. A better understanding of the tumor microenvironment will provide clues as to why adoptive T cell therapies fail despite promising preclinical data. Tumor specificity of T cells can be generated *ex vivo* through gene transfer of tumor targeted CAR genes, however, this alone is likely not sufficient for the generation of truly effective anti-tumor activity. Adoptively transferred CAR targeted T cells will encounter a wide array of inhibitory

factors upon successful trafficking to the site of tumor following infusion into the patient, and these factors are likely to quickly negate meaningful anti-tumor effects mediated by these T cells. Therefore, future research must focus on generating means whereby these T cells are made capable of maintaining effector function within the tumor microenvironment. Furthermore, the concept of adoptively transferred tumor targeted T cells solely as agents of tumor cell destruction needs to be modified. In other words, since tumor-targeted T cells may specifically home to sites of tumor, these T cells could further serve as agents capable of targeted delivery of other anti-tumor, pro-immune reagents. The impact of IL-12 secretion by tumor targeted T cells may not only be the resistance of these T cells to inhibition by the tumor microenvironment, but also the ability of targeted IL-12 secretion at the tumor site to modify the tumor microenvironment, reactivate anergic endogenous tumor specific tumor infiltrating lymphocytes, and further recruit other anti-tumor immune effectors such as NK cells and additional T cells to the tumor thereby generating a broader and more potent anti-tumor immunity. Moreover, since the IL-12 cytokine is secreted locally with markedly lower levels of systemic IL-12, this approach is likely safer and has less risk for the toxicities seen previously in clinical trials utilizing systemic high dose IL-12 infusions.

Our laboratory studies T cells genetically targeted to the CD19 antigen expressed on B cell malignancies through the introduction of CD19 targeted CARs in xenogeneic SCID-Beige $CD19^{+}$ tumor models. Although these models are highly useful in studying the cytotoxicity of our T cells and their ability to traffic to the site of tumor, they do not provide any insight on how the adoptively transferred T cells would respond to a suppressive microenvironment as one that is likely encountered clinically, mediated by

Tregs. We therefore sought to utilize our genetic modification technique on Tregs and similarly target them to the site of tumor, mimicking the scenario where elevated levels of Tregs are present at the site of tumor.

Recent discoveries in Treg biology have enabled investigators to better understand what is needed to effectively stimulate and therefore expand Tregs to meaningful numbers for experiments. Moreover, in contrast to traditional beliefs where Tregs are described as anergic cells, they are now shown to undergo rapid cell division when they are activated in the appropriate culture environment. This has allowed for the effective gene transfer previously not thought possible into Tregs. To date, there are two published reports of genetically redirected Tregs. One of which is in the setting of treatment for experimental colitis in mice[57], and the other for the treatment of experimental autoimmune encephalomyelitis in mice[58]. We for the first time demonstrate that redirected Tregs can potently suppressive effector T cells in the setting of adoptive tumor immunotherapy for cancer. However, the utility of redirected Tregs utilizing CAR targeting technology may be extended to a myriad of uses to suppress autoimmunity whenever a target antigen is identifiable.

We conclude that the novel Treg model presented here represents a highly useful tool in the biological study of adoptive T cell therapy. Furthermore, the promising results demonstrated by combining IL-12 secretion along with CAR-targeting represents a potent and available advancement that may be readily applied to the design of clinical trials in the future.

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