Published in final edited form as:

Nat Med. 2017 February ; 23(2): 242–249. doi:10.1038/nm.4258.

Donor CD19-CAR T Cells Exert Potent Graft-versus-Lymphoma Activity With Diminished Graft-versus-Host Activity

Arnab Ghosh^{1,*}, Melody Smith^{1,*}, Scott E. James^{1,2,*}, Marco L. Davila^{2,*,\$}, Enrico Velardi¹, Kimon V. Argyropoulos¹, Gertrude Gunset², Fabiana Perna², Fabiana M. Kreines¹, Emily R. Levy¹, Sophie Lieberman¹, Hilary Jay¹, Andrea Z. Tuckett¹, Johannes L. Zakrzewski³, Lisa Tan¹, Lauren F. Young¹, Kate Takvorian¹, Jarrod A. Dudakov^{1,@}, Robert R. Jenq¹, Alan M. Hanash¹, Ana Carolina F. Motta^{4,#}, George F. Murphy⁴, Chen Liu⁵, Andrea Schietinger¹, Michel Sadelain^{1,2,*}, and Marcel R.M. van den Brink^{1,2,*}

¹Department of Medicine and Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY

²Center of Cell Engineering, Memorial Sloan Kettering Cancer Center, New York, NY

³Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, NY

⁴Program in Dermatopathology, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

⁵Department of Pathology and Laboratory Medicine, Rutgers-Robert Wood Johnson Medical School, NJ

Abstract

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for hematological malignancies. However, graft-versus-host disease (GVHD) and relapse after allo-HSCT remain major impediments. Chimeric antigen receptors (CARs) direct tumor cell recognition of adoptively transferred T cells.^{1–5} CD19 is an attractive CAR target, expressed in most B cell malignancies as well as normal B cells.^{6,7} Clinical trials using autologous CD19-targeted T cells have shown remarkable outcomes in various B cell malignancies^{8–15}. The use of allogeneic CAR T cells poses a concern of increased GVHD, which however has not been reported in selected patients infused with donor-derived CD19-CAR T cells after allo-HSCT.^{16,17} To understand the mechanism whereby allogeneic CD19-CAR T cells may mediate anti-lymphoma

Correspondence should be addressed to M.R.M.v.d.B (vandenbm@mskcc.org) or M.S. (sadelaim@mskcc.org).

^{\$}MLD: Department of Blood and Marrow Transplantation, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL;

[@]JAD: Program in Immunology, Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA;

[#]ACFM: University of Sao Paulo, Brazil.

*These authors contributed equally.

Author contributions:

AG and MLD conceived the project. AG, MeS, SEJ and MLD contributed to the design of the experiments, supervised and conducted the experiments, generated the manuscript figures and wrote the manuscript. EV and KVA conducted the experiments and generated the manuscript figures. FP contributed to the conduct and interpretation of microarray analyses. ACFM, GFM and CL contributed to the conduct and interpretation of histological analyses. GG, FMK, ERL, SL, HJ, AZT, LT, LFY and KT assisted in conducting experiments. JLZ, JAD, RRJ, AMH and AS contributed to the design of the experiments and their analyses. MiS and MRMvdB supervised the project, contributed to the design of the experiments and their analyses, and wrote the manuscript.

Competing Financial Interest

M. Sadelain is a scientific cofounder of Juno Therapeutics. All other authors declare no competing financial interests.

activity without significant GVHD, we studied donor-derived CD19-CAR T cells in allo-HSCT and lymphoma models in mice. We demonstrate that alloreactive T cells expressing CD28-costimulated CD19-CARs experienced enhanced T cell stimulation, resulting in progressive loss of effector function and proliferative potential, clonal deletion, and significantly decreased GVHD. Concurrently, other CAR T cells present in bulk donor T cell populations retained their anti-lymphoma activity consistent with the requirement for engaging both the TCR and the CAR to accelerate T cell exhaustion. In contrast, first generation and 4-1BB-costimulated CARs increased GVHD. These findings could explain reduced risk of GVHD with cumulative TCR and CAR signaling.

To evaluate the impact of CAR signaling on anti-lymphoma and GVHD activity of allogeneic T cells, we constructed a panel of retroviral vectors encoding CARs targeting mouse-CD19 (Figure 1A). The mouse-1928z (m1928z) CAR encodes murine CD28 linked to CD3-zeta endodomains and is specific for mouse-CD19¹⁸. m19delta lacks the CD3-zeta signaling domain, serving as a non-signaling control CAR. m19z lacks a costimulatory signal. m19BBz encodes murine 4-1BB and CD3-zeta endodomains. hum1928z contains a human-CD19-specific scFv and does not cross-react with mouse-CD19. m19delta.GFP and m1928z.GFP are GFP fusion proteins¹³. CAR expression was verified by flow cytometry (Suppl Figure 1) and m1928z, but not m19delta, T cells specifically lysed CD19-expressing syngeneic targets (Figure 1B). In an MHC-disparate model of allo-HSCT (B6→BALB/c) m1928z and m19delta T cells were compared in mice inoculated with A20-TGL B cell lymphoma to model lymphoma relapse. Recipients of allogeneic m19delta T cells developed lethal acute GVHD, while recipients of only T cell depleted BM allografts died of lymphoma. Strikingly, recipients of m1928z T cells demonstrated reduced tumor growth and mortality due to GVHD, resulting in significantly improved overall survival compared to those treated with m19delta T cells and untreated controls ($p<0.0001$, Figure 1C and 1D, Suppl Figure 2). We identified a dose-dependent increase in the survival of BALB/c recipients of B6 BM infused with A20 cells when treated with varying doses of m1928z T cells (Figure 1E), demonstrating increasing anti-lymphoma activity without increased GVHD in $0.125\text{--}0.5\times 10^6$ /mouse T cell dose range. Transfer of at least 0.5×10^6 m1928z T cells was required to promote anti-lymphoma activity beyond that conferred by the alloreactive GVL effect mediated by m19delta T cells (Suppl Figure 3).

The diminished GVHD activity of m1928z T cells in allo-HSCT recipients was confirmed by evaluating the survival and clinical GVHD scores in the absence of tumor (Figure 1F). We performed experiments in acute GVHD models comprising MHC-disparate (B6→BALB/c)¹⁹ and haploidentical (B6→CBF1)²⁰ combinations to determine if the inhibition of GVHD by the m1928z T cells was consistent in mice on different genetic backgrounds with heterogeneous alloreactive TCR antigen specificities. Histopathological analyses revealed significantly lower GVHD scores in major GVHD target organs (skin, liver, small and large intestines) of allo-HSCT recipients of m1928z vs. m19delta T in both transplant models (Figure 1G). However, at very high doses of T cells (10^7 cells), we noted increased mortality in both m1928z and m19delta T cell recipients (Suppl Figure 4), indicating that transfer of large numbers of non-transduced T cells was sufficient to promote comparable GVHD in recipients of m1928z and m19delta T cells. Our findings suggest that

GVHD in m1928z T cell recipients may occur as a result of either (1) a high number of non-transduced cells mediating GVHD or (2) excess alloreactive m1928z T cells that direct effector function towards GVHD target organs in the setting of limited numbers of B cells. Importantly, this argues against a mechanism in which alteration of the host environment, for example by B cell depletion by m1928z T cells, dominantly inhibits GVHD. These data are of significance because the current CAR T clinical protocols do not involve post-transduction sorting, and in addition to cytokine release syndrome seen with high doses of CAR T cells, there is an increased risk of GVHD when transferring greater numbers of transferred non-transduced cells²¹.

Despite decreased GVHD, the bulk m1928z T cell population maintained anti-CD19 activity *in vivo* as demonstrated by persistent B cell aplasia in allo-HSCT recipients of m1928z T cells (Figure 2A) and m1928z T cells were seen at least 60 days after HSCT (Figure 2B). Additionally, harvested m1928z T cells mediated *ex vivo* anti-CD19 lytic activity (Figure 2C). To study alloreactive CD19-CAR T cell activity over a more prolonged time-frame, we analyzed m1928z T cell activity against normal B cells in a chronic GVHD model (B6→B10.BR) that results in reduced intestinal pathology and attenuated lethality (Suppl Figure 5A) compared with acute GVHD models²². m1928z T cells again promoted reduced GVHD in this model, but mediated severe B cell aplasia compared with m19delta T cells that produced GVHD and concomitant mild alloreactivity-related B cell depletion in peripheral blood (Suppl Figure 5B). Histopathological analyses demonstrated loss of B cell follicles as well as loss of B220⁺ follicular B cells in recipients of m1928z T cells compared to m19delta T cells or BM only (Figure 2D).

We hypothesized that the decreased GVHD potential of m1928z T cells resulted from cumulative CAR and alloreactive TCR signaling leading to exhaustion and eventual deletion of alloreactive CAR⁺ T cells, while non-alloreactive CAR⁺ T cells retained activity against CD19⁺ targets. To test this hypothesis, we first assessed the *in vivo* expansion and distribution of m1928z T cells by infusing luciferase-expressing B6 m1928z or m19delta T cells into B6→BALB/c recipients. Bioluminescence imaging (BLI) revealed significantly reduced expansion of m1928z T cells compared to m19delta T cells (Figure 3A). Additionally, there were fewer splenic donor CD8⁺ T cells in recipients of m1928z T cells vs. m19delta T cells at day-7 post-HSCT (Suppl Figure 6A). GVHD target organs and lymph nodes also revealed fewer Thy1.1⁺ donor m1928z T cells compared to m19delta T cells (Figure 3B). Splenocytes isolated at day-14 displayed reduced frequency of Thy1.1⁺ donor T cells expressing the gut homing markers CCR5 and LPAM in recipients of m1928z T cells vs. m19delta T cells (Figure 3C). Further T cell immunophenotypic analyses revealed significantly fewer activated and effector-memory CD8⁺ T cells in spleens of mice receiving m1928z vs. m19delta T cells 7 days post-transplant (Suppl Figure 6B). No differences in T cell polarization were identified in the expression of transcription factors for Th1 (T-bet), Th2 (GATA-3) or T-regulatory (Foxp3) cells in splenocytes (Suppl Figure 6C). We also found significantly lower levels of IFN-gamma, TNF-alpha and IL-6 in sera from B6→BALB/c recipients of m1928z vs. m19delta T cells at day-7 (Figure 3D). We investigated if a high burden of malignant CD19⁺ cells might enhance m1928z T cell cytokine production and activation, which could phenocopy cytokine release syndrome and exacerbate GVHD. In a modification of the B6→BALB/c model that included pre-

transplant, established A20 lymphoma we identified lower serum levels of GM-CSF, IFN-gamma, IL-6, and TNF-alpha in recipients of m1928z vs. m19delta T cells (Suppl Figure 7). These data suggest that the reduced proliferation (Figure 3A) and *ex vivo* recovery (Figure 3B and Suppl Figure 6A) of m1928z T cells compared with m19delta T cells result in fewer functional T cells to elaborate cytokines in response to lymphoma cells *in vivo*. Importantly, our data are in disagreement with a model in which enhanced cytokine secretion by m1928z CAR T cells in response to lymphoma cells promotes GVHD or cytokine release-mediated lethality as was seen in the recent murine study²³. Possible reasons for this difference include intrinsic structural differences in the CAR constructs which can influence the survival and activity of CAR T cells in response to antigen challenge²⁴.

To assess the contribution of the alloreactive TCR in decreased GVHD, we took advantage of a monoclonal TCR transgenic GVHD model in which donor ABM-TCR-transgenic RAG1 knockout (KO) donor T cells target the allelic MHC class II molecule I-A^{bm12} expressed by B6.C-H2^{bm12} (BM12) recipients. Transfer of ABM T cells is sufficient to cause GVHD in BM12 recipients, as well as BM12 allo-HSC graft rejection in immunodeficient mice²⁵. We again observed significantly less GVHD in allo-HSCT BM12 recipients of ABM m1928z vs. m19delta T cells (Figure 3E). Combined with the results above demonstrating reduced proliferation and cytokine production by m1928z vs. m19delta T cells, this result further compounds a model where m1928z T cells develop functional exhaustion in allogeneic hosts.

To investigate the role of costimulatory signals in CAR-mediated protection from GVHD, we compared first generation m19z T cells lacking enforced costimulation with. CD28-costimulated m1928z T cells (Figure 1A) in the B6→BALB/c GVHD model (Figure 3F). m19z T cells produced significantly more GVHD mortality than m1928z T cells (even though less severe than m19delta T cells), suggesting that enforced costimulation affects the strength of protection from GVHD. As 4-1BB based CARs have been shown to attenuate development of exhaustion in response to repetitive CAR signaling^{26,27}, we compared m19BBz T cells with m1928z T cells (Figure 1A and Figure 3G). Donor m19BBz B6 T cells mediated significantly more lethal GVHD than m1928z T cells, similar to m19delta T cells. Finally, we assessed if the autonomous signaling from the CD28z cytoplasmic domain in the absence of antigen triggering was sufficient to protect against GVHD by comparing GVHD in recipients of hum1928z (Figure 1A) vs. m1928z T cells. Donor hum1928z T cells mediated significantly more lethal GVHD vs. m1928z T cells indicating that antigen-triggered signaling through the m1928z CAR was required for suppression of GVHD (Figure 3H).

To demonstrate the requirement of m1928z signaling in protection from GVHD, we utilized *Ighm^{tm1Cgn}* (mu-deficient) mice (which lack mature B cells) as bone marrow donors and RAG2KO BALB/c mice (which lack mature T and B cell population) as recipients (Suppl Figure 8). m1928z B6 T cells mediated significantly more GVHD mortality in RAG2KO BALB/c recipients of mu-deficient BM vs. RAG2KO BALB/c recipients of B6 wildtype BM, suggesting that reduction of B cell number in hosts prevented m1928z signaling-mediated protection from GVHD. We hypothesized that the mu-deficient reconstituted RAG2KO mice could still promote activation of m1928z signaling due to the presence of

CD19⁺ pre-B cells in these mice²⁸. We therefore used B6 CD19KO donors to reconstitute BALB/c CD19KO recipients, which lack the cognate antigen of the CD19-CAR²⁹. CD19-deficient allo-HSCT recipients of CD19-deficient BM infused with B6 m1928z T cells demonstrated similar lethal GVHD as those infused with B6 m19delta T cells (Figure 3I). These data indicate that m1928z signaling in response to normal B cells is necessary for inhibition of GVH responses.

GVHD is mediated by alloreactive TCR activation, and CAR-expressing alloreactive T cells likely experience concurrent stimulation via both the CAR and the TCR in response to allogeneic B cells. To study the effect of CAR stimulation on TCR-directed killing, we generated a novel tri-cistronic vector, m1928z-OT-1, which promotes coordinated expression of m1928z and the OT-1 (OVA-specific) TCR (Suppl Figure 9). Polyclonal B6 m1928z-OT-1 T cells were tested as effectors against EL4 targets pulsed with OVA peptide (EL4-OVA) in the presence or absence of excess EL4-mCD19 targets. Unlabeled and unpulsed EL4-CD19 (CAR-directed distractor) cells were added at 30x-excess relative to CFSE-labeled EL4-OVA primary targets (TCR target). Presence of EL4-CD19 target cells resulted in a significant reduction in cytolysis of EL4-OVA targets mediated through the OT-1 TCR (Figure 4A left panel). TCR-stimulation by excess ova-pulsed targets also inhibited killing of targets mediated by CAR signaling (Figure 4A right panel). This demonstrates that both a CAR and TCR can remain functional when simultaneously expressed, and activation of a T cell through one receptor can impair lytic activity mediated by the other.

Chronic, repetitive activation of T cells via the endogenous TCR has been shown to result in anergy, exhaustion, and AICD in chronic viral infection and tumor/self-antigen pre-clinical models³⁰. Mouse models of dual-TCR expressing T cells have demonstrated that T cells can be tolerized by chronic exposure to antigen leading to chronic exhaustion^{31,32}. More recently, an exhaustion signature has been demonstrated in autonomously signaling GD2-CAR T cells signaling through CD28 but not 4-1BB costimulatory domains²⁷. We studied the immunophenotypic markers of exhaustion in the m1928z T cells post-transplant and found a significantly higher expression of PD-1, LAG3 and Tim3 in splenic m1928z T cells of allo-HSCT recipients on day 14 (Figure 4B). While our findings suggest an exhaustion phenotype, these markers are also expressed in response to strong and chronic T cell activation. We therefore assessed the signal transduction pathways of B6 m1928z T cells obtained from BALB/c transplant recipients on days 7, 10 and 14 post-HSCT, by analyzing TCR signaling using Phosflow analysis (Figure 4C). We noted significantly higher levels of phosphorylated PKCa, pERK1/2, pS6, pSTAT1, pSTAT3, and pSTAT5 in donor m1928z T cells compared to m19delta T cells. These results suggest enhanced T cell stimulation in m1928z T cells as a consequence of stimulation via both the CAR and the alloreactive TCR.

We next performed experiments to determine if this enhanced T cell signal transduction produces a gene expression profile consistent with exhaustion or AICD. We adoptively transferred ABM T cells transduced with either the m19delta.GFP or m1928z.GFP (Figure 1A) into BM12 recipients of B6 BM and performed transcriptome profiling on day-14-sorted splenic GFP⁺ CAR T cells from allo-HSCT recipients, an approach that ensured that only cells capable of receiving signals through both the TCR and CAR were included in the analysis (Figure 4D). Microarray data were consistent with a population of T cells

undergoing exhaustion and deletion as evidenced by upregulation of Bim, Bid, FasL, Apaf1 and Eomes, as well as down-regulation of Tcf7, which is associated with progressive T cell differentiation and loss of self-renewal function^{33,34}. Pathway analysis showed that the greatest differences between m1928z vs. m19delta T cells were in apoptosis pathways, especially pathways related to programmed cell death, caspase activation, and FasL signaling. We validated the array data by flow cytometry of GFP⁺ CAR T cells, confirming increased surface expression of Fas, along with the previously observed increase in PD-1 expression (Figure 4E). These data suggest that alloreactive m1928z cells receive increased cumulative T cell signaling in response to CD19 and allo-antigens leading to chronic activation and eventual functional exhaustion and programmed cell death.

Recent clinical studies have reported minimal incidence of GVHD in recipients of donor CD19-CAR T cells for treatment of B cell malignancies, which had progressed after allo-HSCT^{17,16}. The donor 1928z T cells were infused in the post-transplant setting without lymphodepletion prior to T cell infusion. Consistent with our data, one study also reported increased expression of PD-1 on donor-derived allogeneic CAR T cells¹⁶. Our data demonstrate that alloreactive CAR T cells undergo hyperactivation, exhaustion, and subsequent deletion mediated by CD28 costimulation and CD3-zeta signaling in multiple GVHD models characterized by different MHC disparities and alloreactive T cells. We also show that concurrent activation of the CD19-specific CAR and TCR can promote acute lytic exhaustion of dual-specific T cells. Together, these results suggest that both acute and chronic stimulation of T cells via the m1928z can negatively affect the responsiveness of T cells to antigen presented to the TCR, independent of the TCR specificity.

These findings may explain results showing impaired activity of virus-specific CD19-CAR T cells, which would be expected to receive dual stimulation through their CAR and TCR *in vivo*³⁵. Phase 1 data suggest that while donor-derived CD19-CAR-modified virus-specific T cells maintained activity against CD19⁺ targets when administered in the post-transplant setting to patients with relapsed B cell malignancies, there was a suggestion of an inverse correlation between T cell persistence and CD19⁺ cell counts (both ALL/CLL and normal B cells)⁷. In this setting, T cell survival could be negatively affected by recurrent CAR activation by CD19 and allo-antigens that were not eliminated by pre-infusion chemotherapeutic lymphodepletion.

We show that the presence of CD19 antigen, either in mature B cells or tumor cells, is critical to protection from GVHD. When intended for management of minimal residual disease (MRD), adoptive transfer of 1928z T cells should thus be performed after a timeframe of B cell recovery, which is transplant dependent³⁶.

In summary, our data provide mechanistic insight into recent clinical trial results demonstrating that allogeneic donor CD19-specific CD28z CAR T cells promote anti-lymphoma activity, with minimal GVHD. Our results support a model in which alloreactive T cells expressing CD19-specific CD28z CARs undergo progressive activation in response to dual TCR and CAR signaling, resulting in loss of function and possible deletion. These findings caution about the complexity of relying on multiple antigen receptors in clonal T cells. Our findings further demonstrate a requirement for the presence of CD19⁺ targets to

mediate the inhibition of the GHVD potential of m1928z T cells and indicate that infusion of donor 1928z T cells in the post-transplant setting will produce the lowest incidence of GVHD when infused after B cell recovery.

Online Materials and Methodology

Mouse HSCT, GVHD and GVL models

We obtained C57BL/6 (B6), C57BL/7.Thy1.1 (Thy1.1⁺B6), CBF1, B10.BR, BALB/c and BM12 mice from the Jackson Laboratory (Bar Harbor, ME). ABM transgenic mice¹ were provided by M. Sayegh (Brigham and Women's Hospital and Children's Hospital Boston, Boston, MA) and had been backcrossed onto a B6 background for at least 20 generations. These mice were then crossed on a RAG1KO background derived from the Jackson Laboratory, which had been previously backcrossed to B6 background for ten generations. Mice used for experiments were 6–9 weeks old. They were co-housed in three to five mice/cage in all experiments. Mouse HSCT experiments with a MHC disparate model (B6 → BALB/c) and a haploidentical model (B6 → CBF1) have been used extensively by others as well as by us^{2,3}. The chronic GVHD model (B6 → B10.BR) has been described in detail previously⁴. The T cells were generated from splenocytes, as outlined, and injected via tail vein at the time of the allograft injection. The recipients were monitored daily for survival and scored weekly on a 10-point scale in a blinded fashion for signs of clinical GVHD as described previously⁵. Animals with scores greater than five were euthanized. Blinded histopathological assessment of GVHD in liver, small intestine, and large intestine was performed at Massachusetts General Hospital (Boston, Massachusetts, USA), and University of Florida (Gainesville, Florida, USA) as previously described².

In A20 lymphoma experiments, animals received 0.5×10^6 /mouse tumor cells on day 0 in a separate injection or 10×10^6 /mouse on day –7 intravenously, where indicated. All studies were approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee under protocol 99-07-025.

CAR constructs and CAR T cell production

The CAR constructs and T cell production have been described previously⁶. The constructs include a single-chain fragment variable (scFv), composed of a mouse CD8 signal peptide, IgH rearrangement, glycine-serine linker, and IgL rearrangement. The scFv was fused to the mouse CD8 hinge and transmembrane region and mouse stimulatory domains including mouse CD28 and/or mouse CD3 ζ . The CAR construct was cloned into the vector backbone SFG, which is a Moloney murine leukemia-based retroviral vector.⁷ The m1928z-OT-1 TCR tricistronic retroviral construct was generated using standard cloning techniques. pENTR1A no ccDB (w48-1) was a gift from Eric Campeau (Addgene plasmid # 17398). Murine TCR OTI-2A.pMIG II was a gift from Dario Vignali (Addgene plasmid # 52111). The m1928z construct was cloned into pENTR1A in frame followed by the E2A self-cleaving peptide. The OT-I TCR alpha and beta chains were separated by the T2A self cleaving peptide. The tricistronic construct was recombined into the LZRS-Rfa vector using Gateway LR Clonase II Enzyme mix (Invitrogen). LZRS-Rfa was a gift from Paul Khavari (Addgene plasmid # 31601). For retrovirus production, Phoenix-E packaging cells were transfected with the

retroviral vectors using Effectene transfection reagent (Qiagen) and viral supernatant used for transduction. For CAR T cell generation, splenic T cells were activated with CD3/CD28 Dynabeads (Invitrogen) and IL-2 (R & D Systems, Minneapolis, MN). Spinoculations were done twice with the viral supernatant.⁸

In vivo bioluminescence imaging

A20-TGL and T cells from luciferase-expressing mice (a gift from Robert Negrin, Stanford University, Palo Alto, California, USA) were visualized using in vivo bioluminescence imaging systems (Caliper Life Sciences)². The bioluminescent flux was analyzed using Living Image software, version 4.3 (Caliper Life Sciences).

In vitro cytotoxicity

In vitro cytotoxicity of targets were determined by measuring chromium-51 (⁵¹Cr) release from labeled target cells as described previously³. For cold target inhibition assay polyclonal B6 m1928z-OT-1 TCR T cells were added at varying effector:target ratios to targets with or without secondary “cold” targets. Primary targets – EL4-CD19 or EL4 pulsed with 10 μM SIINFEKL (OVA) peptide (Invivogen) – were labeled with 0.5 μM CFSE (Molecular Probes) and added to 96-well plates at 10⁴ targets/well. In cold target wells, a 30× excess of non-CFSE labeled secondary target was added. Target lysis was assessed by flow cytometry at 5 hours as the percentage of 7AAD+ targets (Molecular Probes).

Flow cytometry

Antibody staining of cultured T cells or tissues obtained from sacrificed mice was performed at 4°C with mouse Fc-block (Ebioscience) in 1% FBS in PBS. Stained cells were washed once with 1% FBS in PBS before being processed through a 5-laser BD LSR II (BD Biosciences). Flow cytometry of blood cells was performed with a lyse-no wash preparation. Briefly, 25 μL of retro-orbital or cheek blood was incubated with antibodies for 25 minutes at 4°C. Afterwards, FACS Lysing Solution was added (BD Biosciences) and the cells were evaluated. All flow cytometry data files were analyzed with FlowJo software (Tree Star, Ashland, OR). Protein L, an immunoglobulin-binding protein that binds to variable light chains of immunoglobulin without interfering with the antigen-binding site, was used to detect expression of the CAR⁹. To detect m1928z-OT1 TCR, T cells were stained with CD19-His (Sinobiological) followed by anti-His-PE staining (Miltenyi) to demonstrate CD19-CAR expression, and then stained with anti-Vα2-APC (clone B20.1, Pharmingen) and anti-Vβ5.1/5.2-FITC (clone MR9-4, Pharmingen) to detect OT-1 TCR chains.

For Phosflow cytometry, transplanted mice receiving m19delta or m1928z CAR T-cells were harvested at day 7, 10 and 14 post-transplantation. Immediately after euthanasia, spleens were harvested and homogenized over a 70 μm cell-strainer. Cells were rinsed through the strainer with pre-warmed RPMI-1640 containing 2% paraformaldehyde (PFA). After centrifugation, cells were resuspended in RPMI-1640 - 2% PFA and incubated at 37°C for 10 minutes. Subsequently cells were washed with PBS, resuspended in ice-cold methanol and kept at -20°C for 1 hour. After washing with PBS, permeabilized cells were finally stained with CD90.1 and the following phospho-specific antibodies: pPKCα-pT497, ppERK1/2-pT202/pY204, pS6-pS235/pS236, pSTAT1-pS727, pSTAT3-pY705, pSTAT5-

pY694 (BD). CAR T-cell phosphorylation status was evaluated in CD90.1/GFP double positive cells.

Microarray

Spleens were harvested on day 14 and FACS-sorted on GFP-expressing CAR T cells. The sorted T cells were placed in Trizol and RNA was isolated. Microarray was performed with Affymetrix MOE 430A 2.0. Microarray data was processed using the standard R/Bioconductor packages: gcRMA (based on Robust Multi-Array Average method) for quantitation and normalization and LIMMA empirical Bayes method for differential expression^{10,11}. Hierarchical clustering was performed using the R hclust function with the Euclidean distance measure. A heatmap was generated using the heatmap.2 function from the gplots R package. All generated microarray data are available at the Gene Expression Omnibus (GEO) with accession code GSE85397 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qnehygkqljmtjop&acc=GSE85397>).

Cytokine Detection

Retro-orbital blood was collected from mice and serum prepared by centrifugation after clotting of blood. Serum was incubated with a Milliplex multi-analyte panel for mouse cytokines (EMD Millipore, Billerica, Massachusetts) and analyzed on a Luminex 100 system.

Statistics

Survival curves were analyzed with a Mantel-Cox (Log rank) test, and grouped comparisons were made using a Mann-Whitney *U* test or 2-way ANOVA. Calculations were performed using Excel (Microsoft) and Prism software (GraphPad Software). Data represent the means $\pm$ SEM. In box whisker diagrams, median is shown with a horizontal line, the box extends from the 25th to 75th percentiles and the whiskers extend from the smallest value and up to the largest. *P* values less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by National Institutes of Health award numbers R01-HL069929 (M.R.M. van den Brink), R01-AI101406 (M.R.M. van den Brink), P01-CA023766 (M.R.M. van den Brink) and R01-AI100288 (M.R.M. van den Brink). The Lymphoma Foundation, The Susan and Peter Solomon Divisional Genomics Program, MSKCC Cycle for Survival, and P30 CA008748 MSK Cancer Center Support Grant/Core Grant. A. Ghosh is a fellow of the Lymphoma Research Foundation. M. Smith received funding from a NIH Diversity Supplement (PA-16-288) under R01 AI100288-08S1, the M. J. Lacher Research Fellowship (The Lymphoma Foundation), and the American Society for Blood and Marrow Transplantation New Investigator Award. M. Smith and S. James are supported under an NIH T32 grant (T32-CA009207). S. James is also a Young Investigator Awardee from the Conquer Cancer Foundation of ASCO. M.L. Davila is supported by an ASH-AMFDP career development award, Damon Runyon Fund and K08 CA148821 (NCI). J.L. Zakrzewski is supported by LLS TRP grant 6465-15 and K08 CA160659 (NCI). A.Z. Tuckett is supported by a grant from the Imaging and Radiation Sciences (IMRAS) Program of MSKCC. The content is solely the responsibility of the authors and does not represent the official views of the National Cancer Institute or the National Institutes of Health. We are thankful to R. Negrin, Stanford for the A20-TGL cell line, E Campeau, UMASS for pENTR1A, D Vignali, UPMC for murine TCR OTI-2A.pMIG II and P. Khavari, Stanford for LZRS-Rfa plasmid. We appreciate the help of J. White, LCP,

RARC, flow cytometry core facility, integrative genomics core and computational biology core, MSKCC. We also appreciate the help of H. Poeck for critical reading of the manuscript.

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Figure 1. m1928z T cells eliminate CD19-expressing lymphoma while exerting significantly less GVHD activity

(A) CD8L = mouse CD8 leader, CD8TM= mouse CD8 transmembrane region, Gly-Ser = glycine-serine linker. Representation of murine CD19-CAR constructs: m19delta (mouse-specific CAR lacking non-functional zeta-chain); m19z (mouse-specific functional CAR, no costimulation); m1928z (mouse-specific functional CAR, CD28-stimulation); m19BBz (mouse-specific functional CAR, 4-1BB costimulation); hum1928z (human-specific functional CAR, mouse CD28 costimulation); m19delta.GFP and m1928z.GFP (CARs with GFP reporter). (B) *In vitro* cytotoxicity assay using m19delta and m1928z CAR T cells as effectors and EL4-CD19 or EL4-OVA (control). (C, D) Lethally irradiated BALB/c recipients were reconstituted with B6 lin-depleted bone marrow cells and inoculated with

A20-TGL lymphoma cells. Designated groups were treated with 1×10^6 B6 m19delta or m1928z T cells per mouse. Tumor growth was monitored by *in vivo* bioluminescence and images from one of multiple independent experiments are depicted. The BLI images are depicted from one of two experiments (C). Survival was monitored for up to 100 days. Data are representative of two independent experiments (D). (E) Lethally irradiated BALB/c recipients were reconstituted with B6 lin-depleted bone marrow cells and inoculated with A20-TGL lymphoma cells. Designated groups were treated with 0.5×10^6 , 0.25×10^6 , or 0.125×10^6 B6 m19delta or m1928z T cells per mouse. Survival was monitored. The mice treated with B6 m19delta T cells are depicted in Suppl Figure 3 for simplicity. (F) Lethally irradiated BALB/c recipients (upper panel) and CBF1 recipients (lower panel) were reconstituted with B6 lin-depleted bone marrow cells. Designated groups were treated with 1×10^6 B6 m19delta or m1928z T cells. Survival and weekly clinical GVHD scores were monitored. Results are pooled from two independent experiments. (G) Skin, liver, small intestine and large intestine were harvested from the recipients on day 14 post-transplant. H&E sections were analyzed for GVHD in a blinded fashion. Results pooled from 2 independent experiments and representative micrographs are shown. Black bar on micrographs indicate a field length of 100 microns. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; NS, not significant.

Figure 2. Allogeneic m1928z T cells mediate persistent B cell hypoplasia

(A–C) Lethally irradiated BALB/c recipients were reconstituted with B6 lin-depleted bone marrow cells. Designated groups were treated with 1×10^6 B6 m19delta and m1928z Thy1.1⁺B6 T cells. (A) Peripheral blood was drawn at day 60 or day 90 and CD45-gated events analyzed for B220⁺B cells by flow cytometry. (B) CD45⁺Thy1.1⁺T cells were detected in peripheral blood. (C) Splenocytes were harvested at day 14 and used as effectors, and labeled EL4-CD19 were used as targets in Cr-51 release *in vitro* cytotoxicity assay. The proportions of donor T cells in spleens were determined by flow cytometry and the effective effector-to-target ratios are depicted. A representative of two independent experiments with

similar results is shown (n=4, with lysis set up in triplicates). **(D)** B10.BR recipients were conditioned with cyclophosphamide on days –3 and –2 and TBI (7.5 Gy) on day –1 and reconstituted with 10×10^6 lin-depleted BM cells. Designated groups were treated with 1×10^6 B6 m19delta or m1928z T cells. The size of B220⁺ splenic follicles were scored after IHC staining. Pooled data from two independent experiments are depicted in the right panel and representative micrographs are shown in the lower panel. Black bar on micrographs indicate a field length of 200 microns. ****P < 0.0001; NS, not significant.

Figure 3. Allogeneic m1928z T cells exhibit decreased alloreactive proliferation and GVHD target organ infiltration

(A) Lethally irradiated BALB/c recipients were reconstituted with B6 lin-depleted bone marrow cells. Designated groups were treated with luciferase⁺Thy1.1⁺B6 1×10^6 m19delta, or m1928z T cells. Bioluminescence imaging of the transplanted mice was performed weekly and flux was measured. Animals from one representative experiment and flux pooled from two independent experiments are shown. (B–D) Lethally irradiated BALB/c recipients were reconstituted with B6 lin-depleted bone marrow cells. Designated groups were treated with 1×10^6 B6 m19delta, or m1928z T cells. Data from two independent experiments are depicted. (B) Tissues were harvested at day 14, homogenized and counted, and single cell suspensions analyzed by flow cytometry. The calculated numbers of donor Thy1.1⁺T cells in pLN, LPL and liver are shown. Results are representative of one of two independent experiments. (C) Splenocytes were harvested and frequencies of CCR5 and L-PAM determined by flow cytometry. Data from two independent experiments are depicted. (D) Cytokine expression in the serum was assessed at day 7 with Luminex multiplex assay. Results are representative of one of two independent experiments. (E) Lethally irradiated BM12 recipients were reconstituted with B6 lin-depleted bone marrow cells. Designated groups were treated with 2×10^5 ABM m19delta or m1928z T cells and survival and GVHD

were monitored. **(F–H)** Lethally irradiated BALB/c recipients were reconstituted with B6 lin-depleted bone marrow cells and designated groups were treated with B6 1×10^6 m19delta, m1928z, m19z, m19BBz and hum1928z T cells. Survival and weekly clinical GVHD scores were monitored. Results are pooled from two independent experiments. **(I)** Lethally irradiated recipients (BALB/c or BALB/c CD19KO) were reconstituted with B6 or B6 CD19KO lin-depleted bone marrow cells. Designated groups were treated with 1×10^6 B6 m19delta or m1928z T cells. Survival and weekly clinical GVHD scores were monitored. Results are depicted from one of two independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; NS, not significant.

Figure 4. Alloreactive m1928z T cells are hyperactive, exhausted and undergo deletion
(A) m1928z-OT-1 TCR T cells or untransduced T cells were used as effectors. **Left panel:** CFSE labeled EL4-cells pulsed with OVA peptide (10 μ M SIINFEKL) were used as targets with or without non-labeled EL4-CD19 cells as distractor. **Right panel:** CFSE labeled EL4-CD19 cells were used as targets with or without non-labeled EL4 cells pulsed with OVA peptide (10 μ M SIINFEKL) as a distractor. Lysis at 5 hours was estimated as the percentage of 7AAD⁺ CFSE⁺ targets divided by total CFSE⁺ targets. Combined data from three experiments are shown (n = 9 replicates/data point). Each data point reflects the average + SEM. Two-tailed paired t-tests were performed for selected comparisons as depicted. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant. **(B, C)** Lethally irradiated BALB/c recipients were reconstituted with B6 lin-depleted bone marrow cells. Designated groups were treated with Thy1.1⁺B6 1 $\times$ 10⁶ m19-delta.GFP or m1928z.GFP T cells. **(B)** Splenocytes harvested at day 14 post-HSCT were counted and surface expression of exhaustion markers was measured on Thy1.1⁺GFP⁺ gated cells. Results are representative

of one of two independent experiments. **(C)** Splenocytes harvested on designated days post-HSCT were harvested and phosphorylation status evaluated on Thy1.1⁺GFP⁺ gated cells. **(D, E)** Lethally irradiated BM12 recipients were reconstituted with B6 lin-depleted bone marrow cells. Designated groups were treated with 1×10^5 ABM m19delta.GFP or m1928z.GFP T cells. Splenocytes harvested at day 14 post-HSCT. Results are representative of one of two independent experiments. **(D)** Global transcriptional profiles of FACS sorted GFP⁺CAR T cells, **(E)** Splenocytes were counted and surface expression of exhaustion markers measured on GFP⁺CAR T gated cells. Results are representative of one of two independent experiments.