PKCθ is required for alloreactivity and GVHD but not for immune responses toward leukemia and infection in mice

Javier O. Valenzuela,1 Cristina Iclozan,1 Mohammad S. Hossain,2 Martin Prlic,3 Emily Hopewell,1 Crystina C. Bronk,1 Junmei Wang,1 Esteban Celis,1,4 Robert W. Engelman,1,4 Bruce R. Blazar,5 Michael J. Bevan,6 Edmund K. Waller,7 Xue-Zhong Yu,1,4 and Amer A. Beg1,4

1H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA. 2Hematology and Oncology Department, Division of Stem Cell and Bone Marrow Transplantation, Emory University School of Medicine, Atlanta, Georgia, USA. 3Department of Immunology and Howard Hughes Medical Institute, University of Washington, Seattle, Washington, USA. 4Department of Oncologic Sciences, College of Medicine, University of South Florida, Tampa, Florida, USA. 5Pediatric Blood and Marrow Transplantation Program, University of Minnesota, Minneapolis, Minnesota, USA.

When used as therapy for hematopoietic malignancies, allogeneic BM transplantation (BMT) relies on the graft-versus-leukemia (GVL) effect to eradicate residual cells through immunologic mechanisms. However, graft-versus-host disease (GVHD), which is initiated by alloreactive donor T cells that recognize mismatched major and/or minor histocompatibility antigens and cause severe damage to hematopoietic and epithelial tissues, is a potentially lethal complication of allogeneic BMT. To enhance the therapeutic potential of BMT, we sought to find therapeutic targets that could inhibit GVHD while preserving GVL and immune responses to infectious agents. We show here that T cell responses triggered in mice by either Listeria monocytogenes or administration of antigen and adjutant were relatively well preserved in the absence of PKC isoform θ (PKCθ), a key regulator of TCR signaling. In contrast, PKCθ was required for alloreactivity and GVHD induction. Furthermore, absence of PKCθ raised the threshold for T cell activation, which selectively affected alloresponses. Most importantly, PKCθ-deficient T cells retained the ability to respond to virus infection and to induce GVL effect after BMT. These findings suggest PKCθ is a potentially unique therapeutic target required for GVHD induction but not for GVL or protective responses to infectious agents.

Introduction

The primary signal for T cell activation is delivered by engagement of the TCR with MHC/peptide complexes on APCs. In addition, a second signal is provided by costimulatory molecules belonging to B7 and TNF receptor (TNFR) superfamilies (1, 2), while inflammatory cytokines provide the third signal (3). TCR signaling requires key protein tyrosine kinases, including Lck and ZAP70, which mediate activation of multiple signaling pathways (4). PKC isoform θ (PKCθ) is thought to be a key modulator of TCR signaling (5, 6). PKCθ is positioned in the immunological synapse during T cell activation and, together with the CARMA and Bcl-10 adaptors, mediates TCR activation by inducing NF-κB, NF-AT, and AP-1 transcription factors (5, 6). However, the specific roles of these transcription factors in mediating different PKCθ-induced responses are unclear. Studies of PKCθ−/− mice have shown normal T cell development but greatly impaired in vitro proliferative responses (7–9).

In vivo studies have indicated important roles for PKCθ in T cell survival and in promoting activation versus tolerance (10, 11). Recent studies have also shown that PKCθ is important in the induction of experimentally induced autoimmune diseases in the mouse, including encephalomyelitis, arthritis, and myocarditis (12–14). Additionally, PKCθ is involved in generation of Th2 responses (15). However, PKCθ is not required for induction of Th1 responses against Leishmania major, and most important, PKCθ−/− mice mount normal CD8 T cell proliferative and cytotoxic responses against several different viruses (15, 16). The molecular basis for why PKCθ−/− T cell proliferation is impaired in vitro yet normal under certain conditions in vivo is not completely clear.

Although PKCθ has been shown to be important for induction of experimental autoimmune diseases in the mouse, the human counterparts of these ailments likely follow a different etiology. Thus, specific situations in which PKCθ inhibition may be therapeutically efficacious have yet to be defined. Graft-versus-host disease (GVHD) is a potentially lethal consequence of allogeneic BM transplantation (BMT) for which mouse models that recapitulate human GVHD have been established (17). GVHD is initiated by donor T cells that specifically recognize mismatched major (MHC) and/or minor (MiHA) histocompatibility antigens of the recipient (17–19). These alloreactive T cells undergo robust expansion and functional differentiation within recipients and cause severe damage to the gut, liver, and skin (17–19). Consequently, therapeutic immunosuppressive regimens that prevent T cell activation can limit the deleterious effects of GVHD as well as organ transplant rejection (17–19). However, because commonly used agents such as cyclosporine and FKS06 are broadly immunosuppressive, they also render recipients susceptible to life-threatening infections (20, 21).

The use of allogeneic BMT in patients with nonmalignant disorders, such as sickle-cell anemia, is limited by GVHD toxicity as well as increased risk of infection following immunosuppression (17). When used as immunotherapy for hematopoietic malignancies...
(e.g., leukemia), the therapeutic potential of allogeneic BMT relies on the graft-versus-leukemia (GVL) effect to eradicate residual tumor cells through immunologic mechanisms (22). A key goal of research in this area is to identify targets and modalities that can be used to prevent GVHD while preserving GVL and responses against infectious agents. The studies described here help define key aspects of PKCθ function and validate PKCθ as a potential therapeutic target for inhibition of GVHD while sparing donor T cell–mediated antileukemia and antiinfection responses.

Results

Distinct roles of PKCθ in regulating T cell proliferation in vitro and in vivo. To utilize a system whereby TCR stimulation is provided by the same agonist in vitro and in vivo, we crossed PKCθ−/− mice (7) to OVA257-264-specific OT-1 TCR Tg mice. CD8 T cells from WT and PKCθ−/− OT-1 mice were stimulated with microspheres coated with OVA257-264-pulsed dimeric H-2Kb Ig plus the CD28 ligand B7.1:Fc. WT cells proliferated vigorously to TCR plus CD28 stimulation. In contrast, PKCθ−/− cells proliferated weakly during the first 48 hours of culture and did not proliferate beyond that point (Figure 1A). Impaired proliferation of PKCθ−/− cells was evident over a wide range of peptide concentrations (Figure 1B). These results demonstrate the crucial role of PKCθ−/− in the proliferation of CD8 T cells responding to antigen in agreement with previous studies using agonistic antibodies to CD3 and CD28 (7). Furthermore, increased cell death in PKCθ−/− cells likely contributes to their low levels of proliferation (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI39692DS1).

To determine whether the requirement for PKCθ in T cell proliferation observed in vitro was also evident in vivo, CFSE-labeled WT versus PKCθ−/− OT-1 CD8 T cells were adoptively transferred by i.v. injection into C57BL/6/6 CD45.1 congenic recipients, and their levels of CFSE dilution (Figure 1C) and clonal expansion (Figure 1D) were determined after 3 days. s.c. immunizations with LPS (used as microbial adjuvant) alone did not induce T cell proliferation or clonal expansion in draining lymph nodes when compared with mock injections of PBS (data not shown). Immunization with OVA alone induced proliferation in a small subset of WT and PKCθ−/− T cells (Figure 1C), but this was not accompanied by clonal expansion (Figure 1D). In contrast, LPS plus OVA immunizations induced a strong proliferation response in both WT and PKCθ−/− T cells, and the total numbers of PKCθ−/− cells were only 2- to 2.5-fold lower than WT cells. Thus, the in vitro requirement for PKCθ in T cell proliferation is not observed in vivo, although it is still important for optimal clonal expansion. To determine whether PKCθ−/− CD8 T cells are also able to kill target cells, CFSE-labeled and OVA-pulsed syngeneic splenocytes from CD45.2 B6 mice were injected as target cells on day 3 after immunization and their elimination from the host followed 10–12 hours later. As shown in Figure 1E, PKCθ−/− CD8 T cells efficiently cleared injected target cells, indicating apparently normal development of cytolytic effector function in vivo. Thus, PKCθ is not essential for antigen-induced CD8 T cell proliferation or differentiation into effector cells.

In vivo activation of endogenous PKCθ−/− CD8 T cells by Listeria monocytogenes. We next determined the immune response of endogenous PKCθ−/− CD8 T cells to the bacterial agent L. monocytogenes. Importantly, eradication of this agent is known to be dependent on CD8 T cells (23). WT and PKCθ−/− mice were immunized i.v. with a recombinant Listeria strain expressing OVA (LM-OVA), and the levels of OVA-specific T cells and IFN-γ and IL-2 production were measured by K/ova tetramer and intracellular cytokine staining (Figure 1, F and G). Clonal expansion of endogenous OVA-specific PKCθ−/− CD8 T cells was only 2.1-fold lower than that of WT cells in response to LM-OVA infection. In addition, IFN-γ–producing cells were only 52% lower in PKCθ−/− mice than WT cells, although they were severely affected in their ability to produce IL-2. This apparent lack of IL-2 production, however, did not prevent the clonal expansion of tetramer+ PKCθ−/− CD8 T cells (Figure 1F). Most importantly, no detectable Listeria was found in PKCθ−/− mice or WT mice 7 days after infection (data not shown). Thus, PKCθ is not essential for mounting a T cell response against Listeria or for efficient clearance of this bacterial agent. A recent study showed impaired responsiveness in PKCθ−/− mice after Listeria infection (24). Although the reason for differences from our study are not clear, this study used a 25-fold higher LM-OVA amount, indicating potential PKCθ dependence in response to very-high-level infectious agent challenge.

Crucial role of NF-κB in mediating PKCθ-dependent and -independent responses in CD8 T cells. DCs play a crucial role in naive T cell activation in vivo. To better understand mechanisms responsible for PKCθ−/− T cell proliferation in vivo, we used DCs derived from mouse BM (BMDCs). In contrast to microspheres, PKCθ−/− T cell proliferation was only slightly reduced compared with WT T cells in the presence of BMDCs (Figure 2A). Thus, CD8 T cell activation can take place in the absence of PKCθ, provided that compensatory signals (e.g., costimulatory molecules) on DCs are available. Members of the B7 and TNF families of costimulatory molecules are known to activate NF-κB (1, 2), and therefore, PKCθ−/− CD8 T cell proliferation may be rescued through NF-κB activation by these molecules expressed on DCs. We first examined NF-κB activation when PKCθ−/− OT-1 CD8 T cells were stimulated with OVA peptide–pulsed microspheres. NF-κB activation in PKCθ−/− cells stimulated with OVA alone (i.e., without B7.1) was greatly reduced compared with WT T cells after 18 hours (Figure 2B). The presence of B7 costimulation increased the level of NF-κB activation after 18 hours in PKCθ−/− cells, but it remained lower than in WT cells (Figure 2B). We next determined NF-κB activation when WT and PKCθ−/− OT-1 CD8 T cells were stimulated with OVA peptide–pulsed BMDCs (Figure 2C). In contrast to microspheres, BMDCs induced roughly equivalent levels of nuclear NF-κB in WT and PKCθ−/− cells after 18 hours of stimulation (Figure 2C). These results suggest that the presence of additional NF-κB–activating molecules on DCs, such as costimulatory molecules, can allow activation of NF-κB in the absence of PKCθ.

We then determined whether NF-κB activation merely correlated with increased proliferation of PKCθ−/− cells by DCs or whether it was indeed required for DC-induced T cell proliferation. For these studies, we used T cells deficient in NF-κB subunits p50 and cRel, in which overall NF-κB activity in CD4 (25) and CD8 T cells (data not shown) is virtually undetectable after activation. CD8 T cells from WT, PKCθ−/−, and p50−/−cRel−/− mice were stimulated with anti-CD3 plus anti-CD28 antibodies in the presence or absence of BMDCs, and the levels of [3H]thymidine incorporation were measured after 72 hours (Figure 2D). As expected, proliferation of PKCθ−/− and p50−/−cRel−/− cells was greatly reduced compared with that of WT cells in the absence of BMDCs. In the presence of BMDCs, PKCθ−/− cells were able to recover and proliferate. In contrast, NF-κB p50−/−cRel−/− cells were unable to proliferate in the presence of BMDCs (93% reduction from WT levels; Figure 2D). Taken together, these results suggest that NF-κB activation
in T cells, presumably via cell-surface costimulatory molecules or secreted products such as cytokines expressed by BMDCs, is required for rescue of PKC\(\theta\)–/– CD8 T cell proliferation.

To determine whether NF-κB activation is also sufficient for rescue of PKC\(\theta\)–/– cell proliferation by microspheres, WT and PKC\(\theta\)–/– OT-1 T cells were first stimulated with microspheres coated with H2K\(^b\)/OVA/B7. After 24 and 48 hours of activation, the cells were infected with a GFP-expressing retroviral construct bearing a constitutively-active mutant of NF-κB–activating IκB kinase, β subunit (IKKβ) kinase, which has been shown previously to be sufficient for NF-κB activation in T cells (25). Infected cells (GFP\(^+\)) were restimulated with H2K\(^b\)/OVA/B7 microspheres for an additional 2 days. While PKC\(\theta\)–/– cells infected with the control (murine stem cell virus internal ribosome entry site GFP [MIG]) virus were unable to proliferate like WT cells (Figure 2E), PKC\(\theta\)–/– cell proliferation was largely rescued by IKKβ. These results demonstrate that activation of NF-κB is not only required but also largely sufficient for rescuing PKC\(\theta\)–/– CD8 T cell proliferation. Together, our
results suggest that NF-κB activation by PKCθ-independent pathways is likely responsible for rescue of PKCθ−/− T cell proliferation in vivo. Furthermore, such PKCθ-independent pathways may be enhanced by microbial signals and thus explain a previously documented requirement for innate immunity (via microbial stimulation) in rescue of PKCθ−/− T cell proliferation (16, 26).

PKCθ plays a critical role in CD8 T cell–induced GVHD. In light of the above findings, we evaluated the role of PKCθ in the generation of alloreactivity and GVHD, which typically does not require microbial stimulation. To this end, CD8 T cells from WT or PKCθ−/− B6 donors were transferred into sublethally irradiated (500 cGy) MHC class I mismatched bm1 recipients. In this model, donor T cells severely damage the hematopoietic system, resulting in marrow failure and GVHD lethality (27). As expected, WT CD8 T cells induced death of all the recipients within 20 days after cell transfer (Figure 3A). In contrast, the ability of PKCθ−/− CD8 T cells to cause GVHD lethality was severely ameliorated (P = 0.0008; Figure 3, A and B). Additionally, in contrast to OVA/LPS-induced proliferation in vivo (Figure 1C), PKCθ−/− CD8 T cell proliferation in bm1 mice was significantly impaired compared with WT CD8 T cells (Figure 3C). However, homeostatic proliferation of PKCθ−/− CD8 T cells in irradiated syngeneic B6 mice was comparable to WT T cells (Supplemental Figure 2). These results therefore indicate that PKCθ plays a critical role in alloantigen-induced CD8 T cell proliferation and GVHD induction.

PKCθ is required for GVHD development in B6→BALB/c BMT model. In the clinical hematopoietic cell transplantation setting, GVHD typically refers to the epithelial damage induced by donor CD4 and CD8 T cells in MHC and/or MhA recipient cells that are lethally irradiated (i.e., myeloablated) and reconstituted with hematopoietic stem cells containing peripheral T cells from the donor. Therefore, we next evaluated the role of PKCθ in myeloablated recipients in which GVHD lethality is induced through epithelial damage (28). CD4 and CD8 T cells were purified from WT or PKCθ−/− B6 mice and transferred together with WT T cell–depleted (TCD) BM cells into lethally irradiated MHC- and MhA-mismatched BALB/c recipients. To independently evaluate the role of NF-κB in GVHD induction, we utilized p50−/− or p50−/−cRel−/− T cells, which have greatly reduced TCR-induced NF-κB activity (25). As expected, recipients of 2 × 10⁶ WT T cells showed typical clinical features of GVHD induction, including hunched back, ruffled fur, hair loss, diarrhea, and body weight loss. More than 70% of WT mice died within 60 days after BMT whereas, in sharp contrast, the recipients of PKCθ−/− or p50−/−cRel−/− T cells did not have GVHD manifestations and survived through
the entire observation period (Figure 4, A and B). Together, these results indicate that PKCθ−/− and p50−/−cRel−/− T cells have severely reduced ability to induce GVHD. Histopathological appearance of the small intestine of BALB/c mice injected with WT CD8 T cells showed clear evidence of GVHD including glandular destruction, lymphocytic infiltrate, and loss of mucosa compared with the unaffected intestines of mice injected with PKCθ−/− T cells (Figure 4C). Donor reconstitution in the recipient was measured at 100 days after BMT, at which point recipients of PKCθ−/− or p50−/−cRel−/− T cells showed normal T and B cell reconstitution compared with mice of the BM alone group (Supplemental Figure 3).

We next determined whether the impaired ability of alloreactive PKCθ−/− T cells to cause GVHD could be attributed to a defect in T cell expansion. To test this, CFSE-labeled T cells were transferred into lethally irradiated BALB/c recipients, and the kinetics of cell proliferation in the spleen were compared between WT and PKCθ−/− T cells. Our results indicate that PKCθ−/− CD8 T cells have impaired proliferation compared with WT CD8 T cells (Figure 4D). In addition, annexin V staining revealed greater cell death in PKCθ−/− CD8 T cells than in WT cells (P = 0.04; Figure 4D). Similar observations were made with PKCθ−/−CD4 T cells (data not shown) and were further confirmed by the significant decrease in absolute numbers of both PKCθ−/−CD4 and CD8 T cells (Figure 4E). Th1 responses primarily account for GVHD development (29). We next determined whether PKCθ affects T cell differentiation following allogeneic BMT. By measuring intracellular cytokine expression (Supplemental Figure 4), we found that a smaller percentage of PKCθ−/− CD4 and CD8 produced IFN-γ compared with WT T cells but that these differences were not statistically significant (P > 0.05). Very few WT or PKCθ−/− T cells produced IL-4 (<2%; data not shown), indicating there was no significant Th2 response after allogeneic BMT. These results suggest that impaired ability of PKCθ−/− to induce GVHD more likely results from impaired activation and/or survival (Figure 4, D and E) rather than from differentiation defects.

A recent study suggested that PKCθ−/− accessory cells (i.e., non-T cells) could promote cardiac graft rejection by PKCθ−/− T cells (30). We further asked whether whole splenocytes (i.e., containing accessory cells, including B cells and DCs) used as donor cells could induce GVHD in BALB/c recipients. As seen with T cells, splenocytes from WT but not PKCθ−/− donors could induce GVHD (Supplemental Figure S). Thus, PKCθ is essential for alloantigen-induced T cell proliferation and GVHD induction in this clinically relevant mouse BMT model.

**Figure 3**

PKCθ plays a critical role in CD8 T cell--induced GVHD. 1 × 10⁶ MACS-purified WT and PKCθ−/− CD8 T cells were transferred into sublethally irradiated (500 cGy) bm1 recipients, and the recipients were monitored for survival (A) and weight loss (B). (C) 5–8 × 10⁶ MACS-purified and CFSE-labeled B6 WT or PKCθ−/− CD8 donor T cells were injected i.v. into sublethally irradiated bm1 recipients, and their proliferation was monitored in the spleen 4 days after transfer. Representative results from 2 separate experiments are shown.

Strength of TCR stimulation determines the requirement for PKCθ in T cell activation. Based on our above findings, we hypothesized that provision of microbial stimulation may rescue proliferation of alloreactive PKCθ−/− T cells and GVHD induction. However, neither LPS nor CpG administration increased PKCθ−/− T cell--induced GVHD development or enhanced lethality (data not shown). While these results do not discount the significance of microbial agents in stimulating PKCθ−/− T cell responses, they suggest that additional mechanisms may account for lack of GVHD induction by PKCθ−/− T cells, including a fundamental difference in requirement for PKCθ in TCR-induced responses by self-MHC/antigen versus allo-MHC/antigen. To address this possibility, we compared the in vitro activation of WT and PKCθ−/− CD8 T cells in response to spleen APC isolated from H-2B syngeneic (C57BL/6) versus H-2d alloageneric (BALB/c) mice (Figure 5, A and B). Importantly, allogeneic activation of PKCθ−/− cells was severely reduced (~10-fold reduction from WT levels; Figure 5B) when compared with antigen-induced activation (~2-fold or less reduction from WT levels; Figure 5A).

Since the activation/maturaion state of syngeneic and alloageneric APC used here is the same, the underlying difference in PKCθ requirement likely lies in TCR-induced signals. A key difference between these 2 systems is that the response of OT-1 T cells involves high-affinity interactions between monoclonal TCR and self-MHC/peptide. In contrast, allogeneic proliferation involves activation of T cells bearing TCRs with a wide range of affinities for different allo-MHC/antigens. Furthermore, lower affinity interactions between TCR and allo-MHC/antigen play a crucial role in GVHD induction (31, 32). Thus, a possible explanation for impaired allogeneic proliferation of PKCθ−/− T cells may be that they respond poorly to lower affinity TCR agonists.

We next examined the role of dose and affinity on alloreactive PKCθ−/− T cell proliferation using 2C Tg mice. The CD8 2C TCR is positively selected in H-2Kb mice and negatively selected in H-2Ld mice (e.g., BALB/c mice) (33, 34). 2C Tg T cells recognize the naturally occurring p2Ca peptide presented by H-2Ld with high affinity (35). A variant of p2Ca (QL9 peptide) binds 2C TCR with 10-fold greater affinity when complexed with H-2Ld (36). PKCθ−/− 2C Tg T cells can therefore allow us to study the role of TCR-MHC/peptide interaction affinity on T cell activation. Using BMDCs from BALB/c × B6 F1 mice (CB6), we determined the role of both affinity and antigen dose on PKCθ−/− 2C Tg cell activation. WT 2C T cells proliferated strongly when cocultured with CB6 BMDCs through endogenously expressed p2Ca by these BMDCs (Figure 5C). WT 2C T cell proliferation was enhanced in the presence of 1 µM or
10 μM exogenously added p2Ca. In notable contrast, PKCθ−/− 2C T cells showed greatly reduced proliferation in the presence of endogenous p2Ca presented by BMDCs (Figure 5C). However, this was partially rescued by high levels of exogenous peptide (e.g., 10 μM p2Ca). Thus, while PKCθ−/− 2C T cells can respond well to higher concentrations of p2Ca peptide, the amount of naturally expressed p2Ca by APC is apparently insufficient to support proliferation of PKCθ−/− 2C T cells. In the presence of the higher affinity QL9 peptide, PKCθ−/− 2C T cells proliferated strongly at both 1-μM and 10-μM peptide concentrations (Figure 5D). Similar to that of PKCθ−/− 2C T cells, PKCθ−/− OT-1 T cell proliferation was more severely impaired in response to low-affinity peptide agonists SIIGFEKL (G4) and EIINFEKL (E1) (37, 38) than to the high-affinity SIINFEKL peptide, relative to WT OT-1 T cells (Supplemental Figure 6). Collectively, these observations suggest that insufficient expression and/or affinity of allo-MHC/peptide complexes may be responsible for the inability of PKCθ−/− T cells to undergo robust expansion in allogeneic settings.

Essential requirement for PKCθ in 2C T cell expansion and cytotoxicity in vivo. Our in vitro studies demonstrate the inability of PKCθ−/− T cells expressing the 2C Tg to undergo robust proliferation in response to naturally expressed p2Ca antigen. We next investigated whether PKCθ−/− 2C T cells are similarly unable to expand in vivo. In this model, sublethally irradiated CB6 mice are injected with donor 2C T cells, which severely damage the recipient hematopoietic system, especially B cells (39). Peripheral blood from WT 2C T cell–injected CB6 mice showed that numbers of these T cells peaked on day 14 (Figure 6A). In contrast, PKCθ−/− 2C T cell numbers were significantly reduced on days 14 and 22 (P < 0.05; Figure 6A). As expected from low T cell numbers, substantial recipient B cell reconstitution was observed in PKCθ−/− 2C T cell recipients but not in WT 2C T cell recipients (P < 0.01; Figure 6B). Thus, impaired expansion of PKCθ−/− 2C T cells allows robust recipient B cell reconstitution in this sublethal irradiation model. Next, we determined the effect of microbial/inflammatory agents on PKCθ−/− 2C T cell expansion. Recent studies indicate that a combination of TLR ligands and agonistic anti-CD40 Abs (FGK45) provide an exceptionally strong signal for T cell proliferation (40–42). Furthermore, studies have shown that TLR ligands can exacerbate GVHD (43). We employed the TLR3 ligand poly(I:C) (a synthetic dsRNA mimic) and anti-CD40 to stimulate proliferation of PKCθ−/− T cells. A single treatment with anti-CD40 plus polyI:C enhanced expansion of WT 2C (P < 0.02 at all 3 time points) and elimination of host B cells (P = 0.01 on day 6; Figure 6B). PKCθ−/− 2C T cells showed enhanced expansion at days 14 and 22 in the presence of anti-CD40 plus polyI:C, although the differences were not significant (P > 0.05; Figure 6A). Furthermore, B cell reconstitution was significantly decreased in the recipients of PKCθ−/− 2C T cells when anti-CD40 plus polyI:C was administered (P < 0.01 at all 3 time points). Overall, these results indicate that the presence of strong microbial/inflammatory agents can enhance but is insufficient to fully rescue PKCθ−/− 2C T cell expansion.

PKCθ−/− T cells can mount an effective anti–murine CMV response after BMT. Allogeneic BMT results in increased risk of life-threatening infections. The above findings (Figure 1) and previous studies show that responses to bacterial and viral agents remain intact in...
the absence of PKCθ (26). Similar to these studies, PKCθ−/− mice mounted comparable murine CMV–specific (MCMV-specific) T cell responses to WT mice, and viral clearance was only slightly reduced in PKCθ−/− mice (data not shown). How absence of PKCθ has an impact on responses against infectious agents following BMT is not clear. To this end, we used an MCMV infection model (44, 45). CMV is one of the most common infections in BMT patients (46) and thus a highly relevant infectious agent for these studies. 60 days after WT and PKCθ−/− BM transfer into lethally irradiated CB6 recipients, comparable numbers of MCMV tetramer–positive cells were generated 10 days after infection (Figure 7A). Furthermore, clearance of MCMV was comparable in WT and PKCθ−/− BM–transplanted mice (Figure 7B). For both tetramer analysis and virus titer, BM-transplanted mice were also sacrificed on day 3, at which point there is minimal T cell activation or virus clearance (Figure 7, A and B). Inclusion of 5 × 10⁵ WT splenocytes results in mild GVHD in this mouse model, which typically reduces responses to infectious agents. Consistent with this and our previous results showing lack of GVHD by PKCθ−/− splenocytes (Supplemental Figure 5), recipients of the WT splenocyte group showed significantly fewer tetramer–positive cells than recipients of PKCθ−/− splenocytes (P = 0.03; Figure 7A). Although not statistically significant, recipients of WT splenocytes showed higher MCMV load than recipients of PKCθ−/− splenocytes (Figure 7B). Importantly, the viral load in each group on day 10 was significantly lower than that on day 3 after infection (P < 0.05), except in the group transplanted with BM plus WT splenocytes (P = 0.19). Thus, absence of PKCθ does not appear to have a substantial impact on responses to MCMV infection after BMT.

Preservation of GVL responses in the absence of PKCθ in T cells. When BMT is used as therapy for hematologic malignances, an important role for donor T cells is to prevent relapse of original disease through GVL effects. Therefore, it is critically important to determine whether T cells lacking PKCθ retain the beneficial GVL effect. To this end, we performed studies using the B6 → BALB/c BMT model and the A20 B cell lymphoma line that was retrovirally transduced with a luc/neom plasmid (28). The ability of WT and PKCθ−/− T cells was compared in the induction of GVHD and in mediating GVL. To quantitatively compare WT or PKCθ−/− T cells, we used 3 different cell doses: 0.5, 2.0, or 5 × 10⁶ per recipient. Mortality due to GVHD or tumor relapse was distinguished as described in Methods. As expected, all recipients of TCD BM survived without A20 cell infusion. However, when A20 cells were infused, all recipients of TCD BM died within 35 days (Figure 8, A and B) without weight loss (Figure 8, C and D) but with very strong bioluminescent imaging (BLI) signals prior to death (Figure 8, E–H), indicating that tumor growth was the cause of mortality. WT T cells induced severe GVHD in a dose-dependent manner, reflected by high lethality (Figure 8, A and B) but little or no BLI signals prior to death (Figure 8C) but significant weight loss (Figure 8C) but little or no BLI signals prior to death (Figure 8A).
death (Figure 8, E and G). In contrast, all recipients of PKC\(\theta^{−/−}\)-T cells survived through the 50-day observation period (Figure 8B) with mild to moderate body-weight loss (Figure 8D). Furthermore, all the recipients of 5 × 10⁶ KO cells and most recipients of 0.5 (80%) or 2.0 × 10⁶ (60%) KO cells had little or no BLI signal (Figure 8, F and H), indicating these recipients were largely free from tumor. The average signal intensity was 50- to 100-fold lower in recipients of TCD BM plus PKC\(\theta^{−/−}\)-T cells than in TCD BM alone (Figure 8, F and H). Most importantly, none of the recipients that were transplanted with PKC\(\theta^{−/−}\)-T cells died within the observation period (Figure 8B). Because 5 × 10⁶ PKC\(\theta^{−/−}\)-T cells did not induce GVHD lethality whereas 0.5 × 10⁶ WT cells induced 50% GVHD lethality (Figure 8, A and B), the ability of T cells to induce GVHD is likely more than 10-fold lower in the absence of PKC\(\theta\). Additionally, because 5 × 10⁶ PKC\(\theta^{−/−}\)-T cells were as capable as 2.0 × 10⁶ WT cells in eradicating tumor cells (Figure 8, E and F), the ability of T cells to induce GVL is likely approximately 2.5-fold lower in the absence of PKC\(\theta\). These results indicate that absence of PKC\(\theta\) impacts GVHD more severely than GVL responses.

**Discussion**

We show here that PKC\(\theta\) is not required for T cell responses triggered by *Listeria* infection or following administration of antigen with a microbial adjuvant. In contrast, we demonstrate an essential requirement for PKC\(\theta\) in alloreactivity and GVHD. Using 3 distinct mouse models of alloreactivity, we show that T cells lacking PKC\(\theta\) are unable to undergo robust expansion and cause damage to recipient hematopoietic or epithelial tissues. Importantly, PKC\(\theta^{−/−}\)-T cells retain both GVL and antinfection functions after BMT. These findings validate PKC\(\theta\) as a potentially unique thera peutic target that is required for detrimental but not beneficial functions of donor T cells after BMT.

Our results indicate that NF-\(\kappa\)B must be activated by PKC\(\theta\) following TCR engagement or, in the absence of PKC\(\theta\), through compensatory signals (e.g., costimulatory molecules) expressed on APC. These results suggest that activation of NF-\(\kappa\)B through PKC\(\theta\)-independent pathways likely rescues activation of PKC\(\theta^{−/−}\)-T cells in vivo. Previous studies indicated a role for innate/microbial signals in compensating for the absence of PKC\(\theta\) (16, 26). Since these signals enhance costimulatory molecule expression, it is likely that innate immunity functions by enhancing NF-\(\kappa\)B activation in PKC\(\theta^{−/−}\)-T cells. Based on this reasoning, we determined whether impaired alloreactivity of PKC\(\theta^{−/−}\)-T cells results from insufficient stimulation of innate immunity. However, our results suggest that the presence of microbial signals (e.g., LPS, CpG, or polyI:C plus anti-CD40) cannot fully rescue alloreactivity of PKC\(\theta^{−/−}\)-T cells. Instead, our results indicate that a main reason for lack of GVHD is the intrinsic inability of PKC\(\theta^{−/−}\)-T cells to undergo a strong activation response after stimulation with allogeneic APC. In studies with 2C Tg mice, we demonstrated that endogenous levels of alloantigens are insufficient to induce robust PKC\(\theta^{−/−}\)-T cell proliferation. Collectively, our results indicate that while PKC\(\theta\) is not absolutely required for T cell activation and proliferation, it is critical when T cells are activated by low antigen levels and/or low-affinity antigens (also see below). Thus, PKC\(\theta\) functions in lowering the overall signaling threshold required for T cell activation.

Alloreactivity is generated following interaction between TCRs with varying affinities for different allo-MHC/antigens. Indeed, lower affinity interactions between TCR and allo-MHC/antigen are more important for GVHD induction than the highest affinity interactions (31, 32). This is in contrast to T cell responses against infectious agents, which are typically dominated by high-affinity interactions with immunodominant antigen epitopes. While our results indicate that impaired alloreactivity of PKC\(\theta^{−/−}\)-T cells is likely due to inefficient TCR-induced stimulation, TCR-independent pathways (e.g., microbial/inflammatory agent–induced enhancement of costimulation) may nonetheless also play a role. However, enhancement of APC function by these agents was not sufficient to rescue proliferation of PKC\(\theta^{−/−}\)-T cells stimulated by low antigen levels or low-affinity antigens. A possible reason for this may be because non-TCR signals fail to induce sufficient NF-\(\kappa\)B activation in PKC\(\theta^{−/−}\)-T cells stimulated by low-level/affinity antigens. Thus, inadequate overall levels of NF-\(\kappa\)B may be responsible for impaired alloreactivity of PKC\(\theta^{−/−}\)-T cells. Consistent with this, we have shown that NF-\(\kappa\)B (p50 plus cRel) is independently required for GVHD induction. Tregs can suppress GVHD induction (47). In contrast to conventional T cells, PKC\(\theta\) is required for the development of Tregs in the thymus but dispensable for their suppressive function (48). To exclude the contribution of Tregs, e.g., because of differences in Treg numbers between WT and PKC\(\theta^{−/−}\)-mice, we removed Tregs (CD4+CD25+) from donor T cell populations in our studies. Thus, the precise role of PKC\(\theta\) in regulating Treg function after BMT remains to be determined.

After allogeneic BMT, the recipient relies on donor immune reconstitution to fight against infection. PKC\(\theta^{−/−}\)-T cells are impaired in the induction of GVHD but allow donor engraftment and immune reconstitution of the host (Figure 4 and Supplemen tal Figure 3). Using MCMV infection, we show that
PKC\(\theta\)–/– T cells derived from PKC\(\theta\)–/– BM can expand in response to MCMV infection, and this agent was cleared in these mice comparably to the way it was cleared in mice reconstituted with WT BM (Figure 7). These findings indicate that targeting PKC\(\theta\) can prevent GVHD without compromising the ability of BMT recipients to respond to infectious agents.

When BMT is used as immunotherapy for hematopoietic malignancies (e.g., leukemia), it is critically important to prevent GVHD development and tumor relapse. Despite the widely appreciated magnitude of this problem, no clinical strategy has been established to selectively prevent GVHD while preserving GVL effects. We found that the PKC\(\theta\)–/– T cells ranging from 0.5 to 5.0 \(\times 10^6\) mouse did not induce GVHD lethality and protected the majority of the recipients from tumor relapse (Figure 8). We believe that targeting PKC\(\theta\) represents a better strategy for reduction of GVHD and preservation of GVL than other previously described strategies, including targeting adhesion molecules and chemokine pathways. Targeting adhesion molecules including \(\beta_2\) or \(\beta_7\) integrin have been used to separate GVHD and GVL effects by limiting T cell migration. However, inhibition of GVHD was less profound than targeting PKC\(\theta\) in the current study (28, 49, 50). Chemokine signals also contribute to the development of both GVHD and GVL, but targeting a single chemokine receptor (e.g., CCR1, CCR2, or CCR6) typically only partially prevents GVHD, presumably because of functional redundancy (51–53). Antifibrosis activity has rarely been studied together with GVHD and GVL following BMT. Importantly, our data indicate that targeting PKC\(\theta\) will have the substantial additional advantage of preserving antifibrosis activity, which can further benefit patients after allogeneic BMT.

Clinical BMT recipients include individuals mismatched in either MiHA or MHC from the donor. It is well known that MHC is a stronger stimulus for T cell activation than MiHA. Although we have not specifically examined the role of PKC\(\theta\) in an MiHA setting, the dependence of PKC\(\theta\)–/– T cells on strong TCR stimulation suggests that their response to MiHA will be similarly or even more significantly diminished than the response to MHC. Importantly, MHC-mismatched transplantation, especially using cord blood, is being increasingly performed in the clinic (54–56). Thus, the ability to prevent GVHD, e.g., by inhibiting PKC\(\theta\) (see below), in an MHC/HLA mismatched setting can greatly increase eligible donor availability for BMT.

Since GVHD induction typically correlates with GVL responses, the mechanism or mechanisms by which PKC\(\theta\)–/– T cells specifically retain GVL activity are presently unclear. Nonetheless, nonmutually exclusive mechanisms that may contribute to this outcome include the following: (a) tumor cells are better targets for T cells than epithelial cells (which are targeted in GVHD). In this context, we examined levels of MHC I and ICAM-1, a key adhesion/costimulatory molecule, on A20 versus epithelial cells (TUBO cell line) and fibroblasts from BALB/c mice. Interestingly, approximately 50-fold greater expression of these molecules on A20 versus epithelial cells and fibroblasts was noticed (Supplemental Figure 7). Thus, lymphoid (e.g., A20) and potentially myeloid tumor cells may be better targets for alloreactive T cells than epithelial cells because of greater avidity T cell/target cell interaction. Furthermore, our results suggest that PKC\(\theta\)–/– T cells respond better when challenged with higher affinity and/or higher avidity (i.e., higher alloantigen levels; Figure 5, C and D). (b) It has previously been shown that PKC\(\theta\) is required for FasL expression but not for exocytosis of perforin granules (57, 58), and the Fas/FasL pathway is more important for GVHD while the perforin pathway is more important for GVL responses by CTL (59, 60). Thus, PKC\(\theta\)–/– T cells may be able to differentially induce GVL responses through selective retention of the perforin pathway. (c) PKC\(\theta\) has been shown to be crucial in the TCR-mediated activation of \(\beta_2\) integrin adhesion (61), and we recently provided evidence that \(\beta_2\) integrin adhesion can separate GVHD and GVL by regulating T cell migration (28). Thus, PKC\(\theta\)–/– T cells may fail to infiltrate into GVHD target organs, yet adequate numbers of activated PKC\(\theta\)–/– T cells may be generated to eliminate tumor cells. Additional studies will be required to determine which specific mechanism or mechanisms are responsible for differences in GVHD versus GVL responses in the absence of PKC\(\theta\).

In summary, our studies have provided what we believe are novel and fundamentally important insights into the biological function of PKC\(\theta\). First, we show that PKC\(\theta\) plays a key role in lowering the overall signaling threshold required for T cell activation. Thus, absence of PKC\(\theta\) selectively impairs T cell activation by low-level and low-affinity TCR agonists. In the context of infection, the natural function of PKC\(\theta\) may therefore be to allow T cell activation by lower-affinity antigens and/or allow T cell activation under conditions of limited APC activation. Second, our findings identify PKC\(\theta\) as a crucial regulator of T cell activation in allogeneic settings. Currently used immunosuppressive drugs broadly inhibit T cell activation by alloantigens as well as infectious agents, rendering recipients susceptible to life-threatening infections. In contrast, inhibition of PKC\(\theta\) is expected to be uniquely efficacious in preventing GVHD while not having a significant impact on protective responses to infectious agents. In addition, deficiency of PKC\(\theta\) in mice primarily has an impact on T cell responses (7), suggesting that targeting PKC\(\theta\) will not result in widespread toxicity in nonlymphoid tissues. However, whether PKC\(\theta\) targeting will also inhibit alloreactivity in the context of solid organ transplantation remains to be determined. Finally, we show that PKC\(\theta\) is dispensable for GVL responses. Together, our findings validate PKC\(\theta\) as a promising therapeutic target for preventing GVHD while preserving antifibrosis and antitumor responses. PKC\(\theta\) inhibitors may therefore broaden the use of allogeneic BMT in treatment of both malignant and nonmalignant disorders in the clinic.
Methods

Mice and reagents. PKCθ−/− mice were provided by D.R. Littman (New York University, New York, New York, USA). Founders of 2C TCR Tg mice were provided by D. Loh (Nippon Roche Research Center, Kamakura, Japan). OT-1 and 2C mice were crossed to PKCθ−/− mice and bred to homozygosity. All colonies were maintained under specific pathogen–free conditions. All animal studies were approved by the Institutional Animal Care and Use Committees of the H. Lee Moffitt Cancer Center and the University of South Florida. CD8–FITC, CD45.2-APC, Vα2-biotin, streptavidin-APC, CD44-FITC, IFN-γ–APC, IL-2–PE, and annexin V–PE fluorescent antibodies were purchased from BD Biosciences — Pharmingen or eBioscience. Kβ/OVA tetramer–PE was obtained from Beckman Immunometrics. Agonistic anti-CD3 and anti-CD28 Abs were obtained from eBioscience. The FGK45 agonistic anti-CD40 Ab has been previously described (62). OVA325–334 peptide (SIINFEKL; OVA3p) and its low-affinity variant E1 (EINFEKL) were purchased from AnaSpec. The G4 peptide (SIIIGFEKL) was provided by N. Gascoigne (Scripps Research Institute, La Jolla, California, USA). p2Ca (LSPPPFDL) and GL9 (QSLPPFDL) peptides were purchased from United Biochemical Research. The TLR ligand LPS was purchased from Sigma-Aldrich. Cell cultures were done using RPMI 1640 medium supplemented with 10% FBS, 4 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and streptomycin, 10 mM HEPES, and 5 μM 2-ME (RP-10).

T cell purification. CD8 T cell purification from TCR Tg OT-1 and 2C mice was done using MACS magnetic cell sorting (Miltenyi Biotec). In brief, cells isolated from lymph nodes of naive animals were resuspended in 2% FBS medium and incubated for 20 minutes with biotin-labeled Abs specific for CD4, B220, I-Aβ, and CD44. Streptavidin-conjugated microbeads were added to the cells for an additional 30 minutes before passing them over LS separation columns attached to the MACS magnet. Tg cells were typically more than 93% CD8+ and more than 95% CD44+. For experiments using non-Tg CD8 T cells, the Ab cocktail was supplemented with 0.5 μg of biotinylated anti-CD44. Non-Tg cells were typically more than 98% CD8+ and more than 99% CD44+. Total (CD4+ and CD8+) T cells were purified using the same protocol except for addition of biotin-labeled anti-CD4 mAbs.

Microsphere, BM-derived DCs, and whole-spleenocyte preparation. For preparation of microspheres, 5-μm sulfate latex microspheres (Invitrogen) were incubated with DimerX H-2Kb-Ig (BD Biosciences — Pharmingen) at 2.5 μg/107 microspheres for 15 minutes at 4°C and pulsed with 0.1 μM OVA325–334 peptide (or as indicated) for 2 hours at 37°C. When used, B7.1/Fc chimeric protein (R&D Systems) was coinublized at 0.6 μg/107 microspheres (or as indicated) for an additional 30 minutes at 4°C. Coated microspheres were washed extensively to remove free peptide. BMDCs were cultured from BM precursors. Cells were harvested, washed, and replated onto a 6-cm dish at 1 × 105 cells/ml for an additional day to induce maturation. DCs generated were more than 95% CD11c+. To obtain spleen APCs, spleens from syngeneic C57BL/6 or allogeneic Balb/c mice were perfused with 1 ml collagenase D at 2 mg/ml in buffered solution (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2). Perfused spleens were incubated in 5 ml of collagenase buffer at 37°C for 30 minutes, homogenized to suspension, and washed, then resuspended in rbc lysis buffer for an additional 5 minutes. After 2 to 3 washing steps, splenocytes were resuspended in supplemented RPMI 1640 medium and irradiated with 20 Gy before mixing with T cells.

In vitro proliferation. For in vitro proliferation experiments, 1 × 104 purified naive CD8 T cells were mixed with 2 × 105 microspheres in flat-bottom plates or with 1 × 105 BMDCs or 1 to 4 × 105 splenocytes in round-bottom plates in a total of 200 μl supplemented RPMI 1640 media. [3H]-Thymidine was added in the final 10 hours of culture. All conditions were tested in triplicate, and the average and SD of incorporated radioactivity are shown.

Experiments that required the activation of non-Tg CD8 T cells by BMDCs were done by mixing 1 × 105 purified naive CD8 T cells with 1 × 105 BMDCs in round-bottom plates that were precoated with anti-CD3 ± anti-CD28 agonistic antibodies at 1 μg/well each.

Adaptive transfer and immunizations. To measure the in vivo response of OT-1 CD8 T cells to OVA immunizations, 2 × 106 purified naive CD8 T cells from CD45.2+ OT-1 or OT-1/PKCθ−/− mice were transferred into age- and sex-matched congenic CD45.1+ C57BL/6 recipients by i.v. (tail vein) injections. One day after cell transfer, recipient mice were immunized with whole OVA protein (10 or 100 μg/mouse) with or without LPS (25 μg/mouse) in a total volume of 100 μl s.c. injection at the base of the tail. To measure the response of endogenous CD8 T cells to LM-OVA immunizations, the mice were immunized i.v. (tail vein) with 2 × 105 CFUs of the bacterium.

In vivo proliferation and effecter function. For in vivo proliferation of TCR Tg OVA-specific CD8 T cells, purified naive OT-1 or OT-1/PKCθ−/− CD45.2+ CD8 T cells were labeled with 5 μCFDA-SE (CFSE; Invitrogen) and adaptively transferred into congenic CD45.1+ recipient mice. After immunization, draining inguinal lymph nodes were harvested and CFSE dilution was analyzed by FACS using antibodies to CD8 and CD45.2. In some experiments, the combined proliferation and survival (i.e., clonal expansion) of nonlabeled donor cells was counted as the total number of CD8+ CD45.2+ donor T cells found in the draining lymph nodes of recipient mice. Clonal expansion of endogenous OVA-specific CD8 T cells after immunization with LM-OVA was detected by FACS using antibodies to CD8, CD44, and Kβ/OVA tetramer. To measure the cytotoxic effecter function of adoptively transferred OT-1 CD8 T cells after immunization, we used a previously described procedure (63). In brief, a suspension of syngeneic splenocytes obtained from C57BL/6 mice was divided into 2 parts. One sample was pulsed with 0.1 μM OVA325–334 for 45 minutes at 37°C, washed extensively, and incubated with a low concentration of CFSE (0.5 μM). The other population was incubated without peptide, washed, and labeled with a high concentration of CFSE (5 μM). Equal numbers of peptide-pulsed CFSEhi cells and unpulsed CFSElo cells were mixed, and a total of 2 × 105 CFSE-labeled splenocytes were transferred i.v. into recipient mice that had been previously injected with CD8 T cells and immunized as indicated. The fate of OVA peptide–pulsed versus unpulsed target cells was monitored 10–12 hours later in the spleen by FACS. The production of IFN-γ by activated endogenous CD8 T cells after LM-OVA immunizations was measured by intracellular staining.

Electrophoretic mobility shift assay and retroviral infection of CD8 T cells. Electrophoretic mobility shift assays (EMSAs) to detect nuclear localization of NF-κB were done as previously described (64). Retroviral infection of CD8 T cells with the constitutively active mutant IKKβ (EM), which contains both IκB and M10 mutations, was done as previously described (25). In brief, purified OT-1 and OT-1/PKCθ−/− CD8 T cells were mixed with Kβ/OVA-coated microspheres as described above. After 1 and 2 days of activation, cells were spin infected with retroviral supernatants at 1500 g for 1 hour at 30°C in the presence of 4 μg/ml polybrene (Sigma-Aldrich). Original culture supernatants were returned to the wells each time after infection, and the cells were allowed to remain in culture for an additional day. Because all retroviral constructs based on the MIG vector containing an internal ribosome entry site GFP cassette were used, infected cells were distinguished and sorted from uninfected cells by FACS at day 3. Sorted GFP+ cells were mixed with newly made Kβ/OVA-coated microspheres and cultured for an additional 2 days. Proliferation levels from this secondary culture were measured by means of [3H]-thymidine incorporation as described above.

MCMV infection. Mice were infected with 2 × 104 PFU MCMV by i.p. injection. 3 or 10 days later, mice were sacrificed and livers and spleens obtained to determine virus titer and MCMV-specific tetramer-positive CD8 T cells, respectively, as described previously (44, 45).
GVHD and GVL. In nonmyeloablative models, bm1 mice at 7–8 weeks were exposed to 500 cGy of total body irradiation from 137Cs source at 120 cGy/min. Freshly isolated CD8+ cells at indicated numbers were injected via the tail vein into bm1 recipients within 24 hours after irradiation. In myeloablative BMT models, BALB/c mice at 8–10 weeks old were exposed to 800–900 cGy of total body irradiation from 137Cs source at 120 cGy/min. Sulfamethoxazole trimethoprim (Hi-Tech Pharmacal Inc.) was added to the drinking water of irradiated mice starting the day before irradiation and continuing throughout the entire experiment. TCD BM cells alone or in combination with purified T cells from indicated donors were injected via the tail vein to recipients within 24 hours after irradiation. Recipient mice were monitored every other day for clinical signs of GVHD, such as ruffled fur, hunched back, inactivity or diarrhea, and mortality. Animals judged to be moribund were sacrificed and counted as GVHD lethality. To establish a leukemia/lymphoma in the BMT recipients, 2000/mouse luciferase-expressing A20 cells (B cell lymphoma line derived from BALB/c mice) were given on the day of BMT. To monitor for tumor growth weekly by BLI. If recipient death was associated with weight loss and clinical signs of GVHD 2–3 times a week, and monitored for tumor growth weekly by BLI. If recipient death was associated with weight loss and other signs of GVHD, with little or no BLI signal, we scored the death due to GVHD. If recipient death was associated with strong BLI signal, and other signs of GVHD, we scored the death due to tumor relapse.

Statistics. The log-rank test was used to detect statistical differences in recipient survival in GVHD experiments. P < 0.05 was defined as significant. Two-tailed Student’s t test was used to compare percentages or numbers of donor T cells, percentages of T cell apoptosis, tetramer+ cells, and viral load.

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Address correspondence to: Amer A. Beg or Xue-Zhong Yu, H. Lee Moffitt Cancer and Research Institute, 12902 Magnolia Drive, Tampa, Florida 33612, USA. Phone: (813) 745-5714; Fax: (813) 979-7265; E-mail: amer.beg@moffitt.org (A.A. Beg). Phone: (813) 745-3562; Fax: (813) 979-7265; E-mail: xue.yu@moffitt.org (X.-Z. Yu).

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