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Graft-versus-Host Disease Impairs Vaccine Responses through Decreased CD4\(^+\) and CD8\(^+\) T Cell Proliferation and Increased Perforin-Mediated CD8\(^+\) T Cell Apoptosis

Christian M. Capitini,*  Nicole M. Nasholm,*  Brynn B. Duncan,*  Martin Guimond,‡ and Terry J. Fry*

Tumor-targeted vaccines represent a strategy to enhance the graft-versus-leukemia effect after allogeneic blood and marrow transplantation (BMT). We have previously shown that graft-versus-host disease (GVHD) can negatively impact quantitative responses to vaccines. Using a minor histocompatibility Ag-mismatched BMT (B6→B6 × C3H.SW) followed by adoptive transfer of HY-specific T cells and HY-expressing dendritic cells, we assessed whether GVHD induced by donor lymphocyte infusion (DLI) affects the persistence, proliferation, and survival of vaccine-responding, nonalloantigen reactive T cells. Both CD8\(^+\) and CD4\(^+\) HY-specific T cells undergo less vaccine-driven proliferation in allogeneic recipients with GVHD, but there was no difference in CD4\(^+\) T cell apoptosis. Using Fas ligand–deficient or TRAIL-deficient DLI had no impact on mediated CD8\(^+\) T cell apoptosis. These results provide important insights toward optimizing vaccine responses after allogeneic BMT. The Journal of Immunology, 2013, 190: 000–000.

A llogeneic blood and marrow transplantation (alloBMT) is associated with prolonged lymphopenia that predisposes to infection and relapse. Because thymic function is limited early after alloBMT, mature T cells in the graft or provided as a donor lymphocyte infusion (DLI) contribute substantially to immune recovery but also induce graft-versus-host-disease (GVHD), which further impairs thymic function (1). Prior studies have documented that GVHD has direct deleterious effects on mature donor T cells transferred with the graft (2), because of shortened survival in the periphery (3, 4) and activation-induced cell death

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Abbreviations used in this article: alloBMT, allogeneic blood and marrow transplantation; BMT, blood and marrow transplantation; DC, dendritic cell; DLI, donor lymphocyte infusion; Fas-, Fas ligand; GVHD, graft-versus-host disease; GV, graft-versus-leukemia; LIP, lymphopenia-induced proliferation; mHA, minor histocompatibility Ag; PD-1, programmed death 1; TAA, tumor-associated Ag; TCD, T cell-depleted; TCRtg, TCR transgenic; TIM3, T cell Ig mucin-like domain 3.
responding T cells by examining the contribution of three effecter pathways: Fas ligand (Fasl), TRAIL, and perforin.

Materials and Methods

Mice

Congenic CD45.1* C57BL/6 (H-2b) (B6) and CD45.2* B6 mice were purchased from the National Cancer Institute animal production colony (Frederick, MD), and B6 Rag2−/−, B6 Fasl−/− (gld), B6 Ptf1−/−, C3H. SW, and B6 x C3H.SW (all H-2b) (F1) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). F1 mice underwent spontaneous removal of the thymus according to standard protocol. B6 TRAIL−/− mice were provided by Dr. Robert Willing from the National Cancer Institute-Frederick. CD45.2* B6 Rag2−/− Marilyn and Matahari mice obtained from the Taconic National Institute of Allergy and Infectious Diseases colony (Taunton, NY) with permission from Dr. Polly Matzinger (20, 21). Mice were age matched and used between 6 and 12 wk of age, and housed in a specific pathogen-free facility. The Animal Care and Use Committee at the National Institutes of Health approved all protocols.

T cell–depleted bone marrow transplantation and DLI

After lethal irradiation (10 Gy), thymectomized F1 mice were injected i.v. with 4 × 10^6 T cell–depleted (TCD) bone marrow cells in serum-free media from CD45.1* B6 (allogenic) or CD45.1*CD45.2* F1 (syngeneic) donors as previously described (12). On day +14, recipients received a 25% DLI of 30 × 10^6 cells in serum-free media from pooled single-cell suspensions of splenic and lymph node cells. Recipients were weighed and monitored for GVHD as previously described (22). Engraftment was confirmed by flow cytometric analysis of spleens of F1 recipients. Examination of moribund mice was performed by a veterinarian and veterinary technicians blinded to experimental design and groups, and they assessed mice daily according to approved institutional protocols.

Dendritic cell vaccination and adoptive transfer of CFSE-labeled HY-reactive T cells

On day +28, 10 x 10^6 CFSE (Molecular Probes, Carlsbad, CA)-labeled splenocytes from CD45.2* Marilyn or Matahari donors were injected i.v. with or without a male B6 dendritic cell (DC) vaccine. CFSE labeling was performed as previously described (23). DC vaccines were prepared from male B6 bone marrow and activated as previously described (12). DCs were injected i.p. in serum-free media at 1 x 10^6 cells/reipient on day +28 at the time of Marilyn or Matahari transfer. Spleens and lymph nodes were harvested from blood and marrow transplantation (BMT) recipients 3, 5, and 7 d after adoptive transfer and analyzed by flow cytometry for enumeration, proliferation, or apoptosis.

Flow cytometry

Spleens and lymph nodes were harvested on days +31, 33, or 35 after BMT, and flow cytometric–based enumeration of lymphocytes was accomplished using a FACSCalibur equipped with CellQuest software version 5.2.1 (Becton Dickinson, San Jose, CA). In brief, 1 x 10^6 freshly isolated, erythrocyte-depleted splenocytes or lymph node cells were treated with anti-FcγRI/II/III mAb (clone 24G2) and then stained at 4°C for 20 min with an mAb mixture containing Vβ6 (for Marilyn) or Vβ8.3 (for Matahari)-PE, CD45.2-PerCP Cy5.5, and CD45.1-allophycocyanin (BD Pharmingen, San Jose, CA), then washed in FACS buffer (phosphate-buffered salt solution with 0.2% FCS and 0.1% sodium azide). To examine for apoptosis, we performed Annexin V–PE staining according to the manufacturer’s instructions (Becton Dickinson, San Jose, CA). For phenotypic analysis of Marilyn and Matahari T cells in vivo, the following Abs were used: anti–CD127a-PE (clone I1D4B; BD Pharmingen), anti–programmed death 1 (PD-1)-allophycocyanin (clone J43; ebiosciences), and anti-T cell Ig mucin-like domain 3 (TIM3) (clone RMT3-23; ebiosciences). For intracellular staining of IFN-γ, cells were fixed and permeabilized according to the manufacturer’s instructions using Fix and Perm buffer (BD Pharmingen) and stained with anti–IFN-γ–PE (clone XMGL12; ebiosciences).

Tumor challenge

The MB49 tumor cell line is derived from a chemically induced urothelial carcinoma in a male B6 mouse (H-2b) and expresses the male-specific mHA H-Y (24). MB49 cells were maintained in culture at 37°C in 5% CO2 in complete mouse media. Exponentially growing tumor cells were prepared as a single-cell suspension in serum-free media and injected into the s.c. fat of the flank at a dose of 1 x 10^6 tumor cells on day +35 after BMT.

Results

GVHD decreases recovery of nonalloreactive, vaccine-responding T cells

Irradiated female mice were transplanted with TCD mHA-mismatched bone marrow (CD45.1* B6 → CD45.1*/CD45.2* B6 x C3H.SW), followed by infusion of congenic CD45.1* polyclonal donor T cells as a delayed DLI to induce GVHD. This platform was used to study vaccine-induced proliferation and apoptosis of CD8* and CD4* T cells with known specificity for the male HYAg (Fig. 1A). Thymectomized recipients were chosen to mimic poor thymic function observed in humans early after alloBMT and to restrict T cell recovery to those contained in the adoptively transferred inocula. Because female donors and recipients were used, Ags causing GVHD could not contribute to the proliferation of vaccine-responding HY-specific T cells. In this model, donor chimerism is nearly 100% (Fig. 1B), and a delayed DLI given on day +14 induces weight loss in allogeneic recipients (Fig. 1C) without lethality. Also, as expected, the donor-derived DC vaccine does not exacerbate GVHD because HY is not expressed by the female recipients. Furthermore, HY-specific T cells do not undergo lymphopenia-induced proliferation (LIP) (25) (Fig. 1D). Thus, proliferation could be interpreted as being either vaccine driven or a nonspecific effect of the allogeneic environment.

On day +28, CD8* TCR transgenic (TCRTg) T cells (Matahari, CD45.2*, Vβ8.3*) specific for the immunodominant class I HYAg (UTY) or CD4* TCRt T cells (Marilyn, CD45.2*, Vβ6*) specific for the immunodominant class II HYAg (DBY) were given in conjunction with a male DC vaccine. To examine the impact of the allogeneic environment on recovery of vaccine-responding T cells, we compared the quantity of Marilyn and Matahari cells in the spleens of unvaccinated and vaccinated allogeneic recipients with GVHD compared with syngeneic recipients. By gating on congenic markers and the associated TCR V-β molecule, we were able to identify the vaccine-responding T cells (Fig. 2A). There was no difference in recovery of CD4* Marilyn T cells between syngeneic and allogeneic transplant recipients in the absence of vaccine (Fig. 2B). However, there was decreased recovery of CD4* Marilyn T cells in the spleens of mice with GVHD after vaccination (Fig. 2C). There was an identical pattern with respect to CD8* Matahari T cells (Fig. 2D, 2E). These findings are also consistent with our prior observation of decreased HY-specific responses expanded from polyclonal T cells by HY vaccination (12). To exclude that the allogeneic environment did not change the trafficking pattern of HY-specific T cells...
to lymphoid organs, we also analyzed pooled lymph nodes and found similar results (Supplemental Fig. 1A–D).

**GVHD prevents proliferation of Ag-specific CD4+ and CD8+ T cells from DC vaccination to a nonalloantigen**

To determine the mechanism of diminished recovery of vaccine-responding T cells observed during GVHD, we measured proliferation of CFSE-labeled Marilyn and Matahari T cells in separate experiments. Neither Marilyn nor Matahari T cells proliferated in the absence of a vaccine (Fig. 3A–D), confirming earlier reports that LIP is insufficient to drive T cell division and demonstrating that the allogeneic environment does not induce proliferation. In syngeneic BMT recipients, Marilyn T cells (Fig. 3A, 3C) and Matahari T cells showed robust proliferation after vaccination (Fig. 3E). As expected with a single vaccine, proliferation was synchronized, resulting in two distinct peaks, representing Ag-responding and nonresponding cells, rather than the typical multiple divisions noted in polyclonal T cells during LIP (Fig. 1D) (26). However, GVHD significantly impaired proliferation of both Marilyn and Matahari vaccine-responding cells (Fig. 3A, 3C, 3E).

To assess additional functional characteristics of vaccine-responding T cells, we analyzed cytokine production and lytic potential of Matahari T cells after vaccination of allogeneic and syngeneic recipients. As shown in Fig. 3F, the absolute number of Matahari cells producing IFN-γ in response to HY DC vaccination was significantly reduced in the setting of GVHD, as was the absolute number CD107a+ Matahari T cells (Fig. 3G). Thus, in addition to reduced proliferation and total HY-specific T cell recovery, GVHD also affected the number of functional vaccine-responding CD8+ T cells.

To begin to address the mechanism for the reduced proliferation of Marilyn T cells in response to vaccination, we assessed the expression of markers of T cell dysfunction in both allogeneic and syngeneic recipients. As shown in Fig. 3H, both PD-1 receptor and TIM3 was more highly expressed on Marilyn T cells in allogeneic recipients as compared with syngeneic recipients. Interestingly,
although the expression of these markers was increased by DC vaccination, there was higher PD-1 and TIM3 on Marilyn T cells in allogeneic recipients in the absence of HY vaccination. Because HY is not an allogeneic Ag in this system, these results suggest a nonspecific “bystander effect” induced by GVHD that does not require the presence of cognate Ag.

GVHD increases apoptosis of CD8+, but not CD4+, T cells responding to vaccination

We next tested whether increased apoptosis also contributed to diminished recovery of vaccine-responding T cells. Because there was a large proportion of undivided (CFSE+) T cells present in mice with GVHD, we were particularly interested in assessing whether these cells were undergoing apoptosis and thus unable to proliferate to the vaccine. Whereas there were equivalent numbers of undivided, CFSE+ Annexin V+ Marilyn T cells after both syngeneic and alloBMT (Fig. 4A, 4B) with or without a vaccine, there was a significant increase in apoptosis of vaccine-responding Matahari T cells in the lymph nodes of alloBMT recipients (Fig. 4D). Interestingly, no differences in the percentage CFSE+ Annexin V+ T cells were observed in the spleen (data not shown). Although a reduction in CD4 and CD8 vaccine-induced proliferation was seen in both spleen and lymph nodes of mice with GVHD, there was no increase in CD4 apoptosis in splenic T cells. We hypothesized that, in this model, simultaneous presentation of alloantigens to alloreactive T cells and HY Ags to HY-specific T cells may partially explain the increased bystander apoptosis observed in HY-specific T cells. To test this, we performed in vitro coculture experiments with Matahari T cells and male DCs in the presence or absence of alloantigen presentation. Indeed, as shown in Fig. 4E, the recovery of Matahari T cells was reduced when cultured with allogeneic T cells plus DCs presenting both HY and alloantigens, with an increased fraction of Annexin V+ Matahari T cells noted.

Vaccine-induced apoptosis in the setting of GVHD can be reversed through disruption of the perforin pathway resulting in restoration of vaccine-mediated tumor protection

Because the TCRTg T cells in our model do not recognize an alloantigen and do not undergo expansion in either syngeneic or...
allogeneic recipients without a vaccine, alloantigen could not directly mediate apoptosis. Rather, we hypothesized that alloreactive T cells present in the DLI were mediating bystander apoptosis of vaccine-responding CD8+ T cells, as has been reported for polyclonal T cells of unknown specificity in the absence of a vaccine (5). To determine which molecules were regulating bystander apoptosis, we infused DLIs deficient in specific cytolytic pathways that could be potentially used by the DLI to induce apoptosis in the vaccine-responding CD8+ T cells (Fig. 5A). Prior work has implicated both Fas-FasL (3, 5, 27–37) and perforin (29, 33–35, 37–39) in alloantigen-reactive T cell apoptosis during GVHD. In our model, DLI deficient in FasL or TRAIL did not result in reduced apoptosis of vaccine-responding CD8+ T cells. However, infusing DLI deficient in perforin resulted in a modest, but significantly reduced, percentage of undivided, apoptotic Matahari in spleen (Fig. 5B) and 50% decrease in the lymph nodes (Fig. 5C) of alloBMT recipients. To determine whether this had functional implications, we challenged alloBMT recipients that were treated with perforin-deficient DLI followed by Matahari and DC vaccine with MB49 tumor, and observed mildly attenuated weight loss (Fig. 6A) and enhanced tumor protection by Matahari T cells when perforin was absent from the DLI (Fig. 6B).

**Discussion**

Tumor-targeted vaccines are a promising approach to direct immune responses after alloBMT. Indeed, a number of preclinical studies have demonstrated the efficacy of such a strategy (11–18), and clinical trials using vaccines after alloBMT are under way. However, preventative and therapeutic interventions for GVHD, as well as GVHD itself, are globally immunosuppressive. Furthermore, the impact of the allogeneic environment on T cells expanding in response to a vaccine has not been well characterized. We have previously demonstrated that sublethal GVHD is immunosuppressive, leading to decreased vaccine responses to TAAs (12). We confirm the reduction in vaccine responses after alloBMT in this study using CD4+ and CD8+ T cells with known specificity for a nonalloantigen. The ability to carefully track the behavior of these T cells in the allogeneic recipients allowed us to establish that this reduction in vaccine responses is due to diminished proliferation and, for CD8+ T cells, increased bystander apoptosis mediated, at least in part, by perforin.

Previous studies have used proliferative rate to distinguish between alloreactive and nonalloreactive T cells (8) but did not definitively identify nonalloreactive T cells based on known TCR specificity. Using this approach, cytokines have been shown to enhance global T cell reconstitution, with enhanced graft versus
Although tumor responses demonstrated in some of these studies (40–43), however, the impact on Ag-specific T reconstitution was not analyzed. A few studies have specifically assessed the impact of alloreactive T cells on cognate Ag-driven expansion of T cells with known specificity for a nonalloantigen (18, 19). Jenq et al. (19) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) used both an HY-alloreactivity model and mHA-disparate transplant model to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) (19) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) used both an HY-alloreactivity model and mHA-disparate transplant model to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demonstrate that recombinant keratinocyte growth factor could enhance thymic output, improve DNA vaccine responses, and enhance tumor protection, but did not specifically address the question whether GVHD affects vaccine responses. Manzo et al. (18) performed TCD alloBMT to demon
in humans, posttransplant interventions would likely be most effective after these medications are discontinued. Furthermore, because of graft manipulation, alloBMTs without or with limited use of immunosuppressive medications are being used with increasing frequency. Thus, we believe these results present important, and potentially clinically relevant, insights into the use of tumor-directed vaccine approaches after alloBMT.

Clinical studies have shown increased apoptosis of T cells after alloBMT (3, 4, 6, 10), but as with proliferation, these studies are not able to distinguish between T cells that recognized alloantigen and T cells with no reactivity against alloantigen. In a murine study by Alpdogan et al. (8), rapidly proliferating T cells that acquire CD44hi expression with presumed specificity for alloantigen demonstrated increased apoptosis. In our study, we show that known nonalloreactive CD8+ T cells, in particular, nondividing T cells with vaccine specificity, undergo increased apoptosis in the allogeneic environment. Interestingly, we did not see increased apoptosis of these T cells in absence of cognate Ag stimulation. However, the combination of cognate Ag plus the allogeneic environment resulted in marked apoptosis of CD8+ T cells. In contrast, our data indicate that vaccine-specific CD4+ T cell apoptosis does not contribute to impaired responses during GVHD. Although our results are consistent with a report that polyclonal CD8+ T cells from recipients of HLA-matched sibling alloBMT appear more sensitive to apoptosis than CD4+ T cells (3), they contrast with another report concluding the opposite in recipients of HLA-matched unrelated donors (4). In neither of these clinical studies and in prior murine studies was the specificity of the T cells analyzed for apoptosis known, and could have contained alloreactive T cells, nonalloreactive T cells, or both. Nonetheless, the studies presented in this article, where T cells with known specificity for nonalloantigen were evaluated, demonstrate that the impact of GVHD on CD8+ T cell apoptosis does not require recognition of alloantigen by the vaccine-responding cells.

**FIGURE 5.** Administration of perforin-deficient DLI attenuates GVHD-induced apoptosis of TCRTg CD8+ T cells in spleens and lymph nodes of alloBMT recipients. (A) C3H.SW recipients received TCD B6 bone marrow on day +0 followed by 30 × 10^6 DLI from B6 (wild type, WT), B6 FasL−/− (GLD), B6 TRAIL−/−, or B6 perforin−/− (perforin) donors on day +14. On day +28, 10 × 10^6 CFSE-labeled Matahari T cells were adoptively transferred and all recipients concurrently received B6 male DC vaccine. After 7 d (day +35), nonproliferated CFSE+ Annexin V+ cells were enumerated in (B) spleens and (C) pooled axillary, inguinal, and cervical lymph nodes from alloBMT recipients; 5–10 mice/group, *p < 0.05, data pooled from two independent experiments.

**FIGURE 6.** Infusion of perforin-deficient DLI enhances tumor protection by TCRTg CD8+ T cells expanded in vivo with a tumor-specific vaccine. C3H. SW recipients received TCD B6 bone marrow on day +0 followed by 30 × 10^6 DLI from B6 (wild type [WT]) or B6 perforin−/− (perforin) donors on day +14. On day +28, 10 × 10^6 Matahari T cells were adoptively transferred and all recipients concurrently received B6 male DC vaccine. (A) AlloBMT recipients were weighed twice weekly, and on day +35, (B) all recipients were challenged with 1 × 10^6 MB49 tumor cells in the flank and measured twice weekly; 5 mice/group, p < 0.01 on day 35, data representative from two independent experiments.
results also suggest that administering a vaccine with prosurvival cytokine such as IL-7 or IL-15 may decrease CD8+ T cell apoptosis further (40, 41), particularly of nonalloreactive T cells, such as those used in our alloBMT model.

A multitude of studies have demonstrated that FasL, perforin, and TRAIL can all contribute to the pathophysiology of GVHD (3, 5, 27–39). In the majority of these studies, these pathways were implicated in the effector mechanisms by which alloreactive T cells mediate tissue injury. In our model, we were specifically interested in the role these molecules play in the deletion of nonalloreactive T cells responding to a vaccine. Alpdogan et al. (8) demonstrated that apoptosis of rapidly proliferating cells resulted in impaired lymphocyte recovery via a mechanism that does not require Fas or TRAIL on donor bone marrow. Notably, these studies examined effects on polyclonal T cell populations. Brochu et al. (5) demonstrated that alloreactive T cells can cause bystander apoptosis of infused polyclonal nonalloreactive T cells via Fas-FasL. In this study, we used T cells with known Ag specificity to assess the mechanism of apoptosis in vaccine-responding T cells induced by the allogeneic environment. Surprisingly, this appears to be mediated, at least in part, by perforin rather than FasL. The difference in the pathway involved may reflect either vaccine-induced changes in T cell susceptibility to Fas or to trafficking. Indeed, the most marked decline in apoptosis with perforin-deficient DLI was noted in the lymph nodes rather than FasL. The difference in the pathway involved may require minimization of GVHD or specific targeting of relapse disease is constrained by the host environment. Blood 109: 5502–5510.


