PLZF Confers Effector Functions to Donor T Cells That Preserve Graft-versus-Tumor Effects while Attenuating GVHD

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University of Florida College of Medicine, Gainesville, Florida; and Child reable abrogation of GVHD (3–6).

Abstract

Efforts to limit GVHD mediated by alloreactive donor T cells after allogeneic bone marrow transplantation are limited by a concomitant decrease in graft-versus-tumor (GVT) activity and increased possibilities of tumor relapse. Using a novel approach, we adoptively transferred conventional T cells expressing the transcription factor promyelocytic leukemia zinc finger (PLZF), which confers effector properties resembling invariant natural killer T cells, such as copious production of cytokines under suboptimal stimulation. PLZF expression in T-cell allolografts attenuates expansion of alloreactive T cells, leading to lower GVHD. Intact alloreactivity-driven antitumor cytokine responses result in preserved GVT effects, leading to improved survival. Our findings suggest that therapy with PLZF-overexpressing T cells would result in overall improved outcomes due to less GVHD and intact GVT effects. Cancer Res; 73(15): 4687–96. © 2013 AACR.

Introduction

Allogeneic bone marrow transplantation (allo-BMT) is a curative therapy for hematopoietic malignancies. However, mortality and morbidity remain high due to malignant relapse, GVHD, and prolonged immunosuppression, necessitating additional strategies to improve BMT outcomes (1). Hematopoietic transplantation with allogeneic donor T cells reconstitutes the lymphoid compartment and enables antitumor immunity, but simultaneously increases the risk of GVHD (2). A growing body of literature suggests a crucial role of T helper (Th) responses in the pathogenesis of GVHD, although selective targeting of individual Th cell subsets does not lead to reliable abrogation of GVHD (3–6).

Materials and Methods

BALB/c (H2b), LP (H2d), C57BL6 (B6-H2b), and B6.SJL-Ptprc-a Pep3b/BoyJ (B6-Ly5.1-H2b) were obtained from the Jackson Laboratory. Lck-PLZF transgenic (PLZF-TG, B6-H2b) and their wild-type (B6-WT) littermates were bred in-house (7). All animal protocols were approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center (MSKCC). P815 (H2d), a mastocytoma cell line of DBA/2 origin, was obtained from the American Type Cultural Collection. A20 (H-2d), a BALB/c B-cell lymphoma, was kindly provided by A. Houghton (MSKCC) and A20-TGL generated as described previously (11).
were magnetically sorted (>90% purity confirmed) from donor mice (Miltenyi). Lin-BM was administered intravenously at a dose of 1 × 10^6 per mouse along with T cells in a single injection. In our A20 and P815 GVT models, tumor cells were inoculated via separate intravenous injection on the day of allo-BMT (4). To visualize and quantify tumor burden, A20-TGL inoculated mice were administered t-luciferin (Goldbio), anesthetized, and imaged using in vivo bioluminescence imaging systems (Caliper Life Sciences; ref. 11). All allo-BMT recipients were monitored daily for survival and weekly for weight loss (12). Liver, small intestine, large intestine, and skin were harvested for histopathologic assessment of GVHD 14 days post-BMT and formalin-preserved, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Blinded scoring was conducted as previously described (12).

Flow cytometry

Spleen, mesenteric lymph nodes (MLN), peripheral lymph node (PLN), liver, and lamina propria lymphocytes (LPL) from small intestine were harvested as described in previous publications (4, 5). Briefly, lymphoid organs were mashed into single-cell suspensions; liver and LPL were isolated after dissociation of the epithelium and digestion with DNaseI and Collagenase D (Roche). Surface and intracellular staining for single-cell suspensions; liver and LPL were isolated after dissociation of the epithelium and digestion with DNaseI and Collagenase D (Roche). Surface and intracellular staining for Flow cytometry was conducted as described previously and analyzed with FlowJo software (Tree Star; refs. 4 and 5). Donor cells were magnetically sorted (Miltenyi). Lin-BM T cells obtained from A20-TGL mice display a predominantly effector-memory (CD44^hiCD62L^lo) phenotype, whereas CD8^+ T cells display both effector and central (CD44^hiCD62L^hi) memory phenotypes (Fig. 1A and B). A significantly higher frequency of Foxp3^+ Tregs were seen in the CD4 compartment of PLZF-TG T cells in PLZF-TG splenocytes was similar to B6-WT splenocytes, whereas a lower but still high frequency of CCR7^+ CD8^+ T cells were seen in splenic PLZF-TG T cells (Fig. 1D). A high expression of CCR7 in steady-state PLZF-TG T cells indicated lymph node trafficking potential similar to naive B6-WT T cells. In addition to spontaneous acquisition of memory phenotype, PLZF-TG T cells display innate-like properties. With a brief stimulation with phorbol-12-myristate-13-acetate (PMA) and ionomycin, PLZF-TG T cells (Fig. 1C). We examined the homing potential of naive PLZF-TG T cells to secondary lymph nodes by examining the expression of CCR7. The frequency of CCR7^+ CD4^+ T cells in PLZF-TG splenocytes was similar to B6-WT splenocytes, whereas a lower but still high frequency of CCR7^+ CD8^+ T cells were seen in splenic PLZF-TG T cells (Fig. 1D). A high expression of CCR7 in steady-state PLZF-TG T cells indicated lymph node trafficking potential similar to naive B6-WT T cells. In addition to spontaneous acquisition of memory phenotype, PLZF-TG T cells display innate-like properties. With a brief stimulation with phorbol-12-myristate-13-acetate (PMA) and ionomycin, PLZF-TG T cells express significantly more IFN-γ, and specifically the CD8^+ subset more TNF-α, but less IL-2 compared with the B6 wild-type (B6-WT) T cells (Fig. 1E). We hypothesized that acquisition of innate-like properties would decrease GVHD whereas alloreactive stimulation would maintain GVT mediated by PLZF-TG T cells.

Recipients of PLZF-TG T cells have less GVHD than recipients of B6-WT T cells

Given the advanced activation status of PLZF-TG T cells, we sought to determine the GVHD potential of PLZF-TG T cells versus B6-WT T cells in 2 allo-BMT models: B6 → BALB/c (MHC-disparate) and B6 → LP (minor histocompatibility antigen-disparate). In both models, BMT recipients of PLZF-TG T cells had significantly less GVHD mortality and morbidity (as assessed by weight loss; Fig. 2A and B). To specifically assess whether PLZF expression affects CD4^+ driven alloreactivity, we conducted experiments with CD4-selected donor T cells
and noted significantly less GVHD mortality in recipients of PLZF-TG T cells compared with B6-WT T cells (Supplementary Fig. S1A). We further separated the role of CD4+ and CD8+ T cells in this model by mixing CD4+ and CD8+ T cells from B6-WT and PLZF-TG mice at a physiological T-cell ratio (0.66 × 10^6 CD4+ / 0.33 × 10^6 CD8+ ) in our B6 → BALB/c GVHD model (Supplementary Fig. S1B). PLZF-TG CD4+ T cells mixed with either PLZF-TG or B6-WT CD8+ T cells mediated significantly less lethal GVHD than B6-WT CD4+ T cells mixed with either PLZF-TG or B6-WT CD8+ T cells, showing a critical role of CD4+ T cells in the PLZF-mediated decrease in GVHD in this model.

We then assessed specific effects of PLZF-expressing T cells in individual GVHD target organs. Thymuses of the recipients of PLZF-TG T cells had significantly greater cellularity and higher numbers of CD4+ CD8+ double positive thymocytes, indicating significantly less thymic GVHD (Fig. 2C). Histopathologic analyses of GVHD target organs showed significantly less GVHD in BMT recipients of PLZF-TG T cells compared with B6-WT T cells in small and large intestine and skin, but not liver (Fig. 2D and E). Taken together, these studies show that PLZF expression, particularly in CD4+ T cells, significantly decreases GVHD in multiple target organs, leading to decreased mortality and morbidity.

PLZF-TG T cells can be allo-stimulated but undergo apoptosis under alloreactive conditions in vivo

We next explored the responses of PLZF-TG T cells under syngeneic conditions in B6 → B6 model and alloreactive conditions in the B6 → BALB/c model. The allo-BMT recipients of PLZF-TG T cells had markedly fewer T cells in the spleen on days 4 and 14 (Fig. 3A). We also found fewer PLZF-TG T cells in mesenteric and peripheral lymph nodes (MLN and PLN) on day 14 (Fig. 3B). The numbers of T cells in spleen and lymph nodes of syn-BMT recipients of PLZF-TG T cells were similar to those in recipients of B6-WT T cells. In the allo-BMT
Figure 2. PLZF-TG T cells cause limited GVHD. Lethally irradiated recipients (A, C, and D, BALB/c; B, LP) were reconstituted with $2 \times 10^5$ cells/recipient B6-WT BM. Designated groups were treated with B6-WT or PLZF-TG T cells. Survival was monitored (A and B, left) and weekly weight tabulated (A and B, right). A, T-cell dose of $0.5 \times 10^6$ cells/recipient. Results pooled from 2 independent experiments ($n = 20$ in treated groups). B, T-cell dose of $2 \times 10^6$ cells/recipient ($n = 10/gp$). C, thymocytes from the recipients were harvested at day 14 post-allo-BMT, counted (left), and double positive subset (DP) determined by flow cytometry of live gated CD45$^+$CD4$^+$CD8$^+$ cells (right). Results pooled from 2 independent experiments ($n = 18/gp$). D and E, small intestine, large intestine, skin, and liver were harvested from the recipients on day 14 post-allo-BMT. H&E sections were analyzed for GVHD in a blinded fashion. Results pooled from 2 independent experiments ($n = 18/gp$; D) and representative micrographs (E) are shown. $^*, P < 0.05; ^{**}, P < 0.01; ^{***}, P < 0.001; \text{NS}, \text{not significant.}$
recipients of PLZF-TG T cells, we observed significantly fewer mononuclear cells in the lamina propria of small intestines but not in the liver (Fig. 3C), reflecting the difference in the histopathologic GVHD scores of gut and liver.

PLZF-TG T cells have a lower threshold for activation and can proliferate with suboptimal costimulation (10). To study how the expression of PLZF modulates the T-cell responses under alloreactive conditions, we conducted an in vivo analysis...
by adoptively transferring CFSE-labeled B6-WT or PLZF-TG T cells into irradiated BALB/c recipients. We found that significantly more PLZF-TG T cells underwent apoptosis before dividing, as indicated by significantly higher Annexin V frequencies in CFSEhi populations of PLZF-TG T cells (Fig. 3D). To further confirm enhanced apoptosis in the allo-activated PLZF-TG T cells, we analyzed activated polycaspase activity in the donor B6-WT versus PLZF-TG T cells after adoptive transfer into irradiated BALB/c recipients. Significantly more PLZF-TG T cells showed activated polycaspase activity (Fig. 3E), supporting our hypothesis that PLZF expression led to increased apoptosis in PLZF-TG T cells under alloreactive conditions.

We next wondered whether impaired upregulation of activation markers on PLZF-TG T cells accounted for their decreased GVHD activity. In contrast to the differences observed in steady-state T cells (Fig. 1), we observed only minor differences between B6-WT and PLZF-TG T-cell subsets in spleen and the LPL from small intestine in alloreactive settings (Supplementary Fig. S2A and S2B). Phosphorylation of ERK1/2, STAT1, and STAT3 are key events leading to the development of alloreactivity (13). We found similar phosphorylation status of ERK1/2, STAT1, and STAT3 in alloreactive PLZF-TG and B6-WT T cells (Supplementary Fig. S2C–S2G). Therefore, PLZF-TG T cells have similar allo-activation compared to donor B6-WT T cells. Overall, these data indicate that in spite of intact activation signaling PLZF-TG T cells are more susceptible to apoptosis leading to impaired expansion following alloactivation.

We next investigated if PLZF expression renders T cells more susceptible to cell death via T-cell–mediated cytolytic mechanisms (Fig. 3F). We found significantly more PLZF-TG T cells expressed FasL. In addition, expression of its receptor Fas was also significantly higher on PLZF-TG T cells compared with B6-WT T cells, suggesting an increased susceptibility to apoptosis and fratricidal regulation (Fig. 3G).

Alloreactive PLZF-TG T cells display Th1 and Th2 polarization

We next interrogated whether PLZF-TG T cells could influence polarization of Th cells and the resultant cytokine production in alloreactive settings. We first assessed if the cytokine profile of transferred alloreactive donor T cells is affected by PLZF expression (Fig. 4A). We noted similar production of
IFN-γ and TNF-α from PLZF-TG and WT T cells. In contrast, both CD4+ and CD8+ PLZF-TG T cells showed increased production of IL-4 as compared with WT T cells, and CD8+ PLZF-TG T cells also showed significantly higher IL-2 expression.

The coexpression of IFN-γ (T₈₁) and IL-4 (T₈₂), which are typically antagonistic prompted us to analyze the polarization of alloreactive T cells (Fig. 4B). The total frequencies of T-bet and RORγt in PLZF-TG CD4+ T cells were similar to B6-WT controls, indicating a comparable T₈₁ and T₈₂ polarization. However, a significant increase in T₈₂ frequency was seen in PLZF-TG T cells, as evidenced by increased GATA-3+ CD4+ T cells. We also observed an increased frequency of CD4+ T cells coexpressing T-bet and GATA-3. In addition, we found an increased frequency of Foxp3+ Tregs in the recipients of PLZF-TG T cells (Fig. 4C, left). Certain reports have suggested that expression of Helios reflects thymic-derived Tregs (17). We also determined the frequency of Helios-expressing Foxp3+ Tregs in recipients of allo-BMT. The frequency of Helios-expressing Tregs was comparable in recipients of PLZF-TG T cells to that in the recipients of B6-WT T cells (Fig. 4C, right), indicating the presence of both thymic-derived and induced Tregs. Taken together, these data indicate a shift toward T₈₂/Treg polarization and wider GATA-3 and Foxp3 expressions among alloreactive PLZF-TG T cells.

Our results suggest that under alloreactive conditions PLZF-TG T cells exhibit increased susceptibility to apoptosis induced by Fas and enhanced IL-4 production leading to decreased alloreactive expansion and attenuated GVHD. To study the relative contributions of these cell intrinsic mechanisms, we tested proliferation in in vitro MLRs using BALB/c-derived dendritic cells as stimulators (Fig. 4D). PLZF-TG T cells proliferated significantly less than B6-WT T cells after allogeneic stimulation. Individually blocking the IL-4 pathway or Fas–FasL interactions, or supplementing with IL-2 did not rescue the decreased proliferation. However, simultaneous blocking of IL-4 and Fas, and supplementation of IL-2 to the reaction significantly rescued proliferation to the levels of B6-WT T cells. This indicates that attenuated GVHD elicited by donor PLZF-TG T cells is multifactorial including Fas–FasL interactions, increased IL-4 expression and decreased IL-2.

**Tumor-bearing recipients of PLZF-TG T cells have improved survival compared with tumor-bearing recipients of B6-WT T cells**

We next determined the GVHD and GVT activity of PLZF-TG T cells in our B6 → BALB/c model, in which recipients also received A20 lymphoma cells. Mice receiving PLZF-TG T cells had significantly improved overall survival compared with the recipients of B6-WT T cells (Fig. 5A). We ascertained by necropsy that fewer BMT recipients of PLZF-TG T cells died from GVHD compared with the BMT recipients of B6-WT T cell group (Supplementary Fig. S3A). Through a range of T-cell doses (0.25 × 10⁶/mouse to 1 × 10⁶/mouse) PLZF-TG T cells mediated significantly improved survival compared with the B6-WT T cells, indicating preserved GVT activity in the absence of GVHD-related mortality (Fig. 5B and C, Supplementary Fig. S3B and S3C). To visualize and validate the GVT effects, we inoculated luciferase-expressing A20-TGL in B6 → BALB/c allo-BMT recipients treated with PLZF-TG or B6-WT T cells and monitored tumor progression by bioluminescent imaging. Recipients of PLZF-TG T cells showed significant clearance of tumors compared with recipients of allografts without T cells, and less GVHD-related mortality (compared with recipients of allografts with B6-WT T cells), showing an intact GVT activity but less GVHD (Fig. 5D). To test the reactivity of PLZF-TG T cells in a distinct GVT model, we inoculated P815, an MHC-II negative mastocytoma cell line, in B6 → DBF1 allo-BMT recipients treated with B6-WT or PLZF-TG T cells. In this model too, we found significantly superior survival in mice treated with PLZF-TG T cells compared with recipients of B6-WT T cells and those without T-cell therapy (Fig. 5E).

**Discussion**

We show here that PLZF-TG T cells can mediate significantly less GVHD while sustaining GVT effects. Our data suggest that PLZF expression results in increased fratricidal regulation of effector cells, T₈₂ polarization and enhanced Foxp3+ Tregs leading to attenuated GVHD. Furthermore, enhanced T₈₂ polarization coupled with intact T₈₁ and T₈₁ 17 polarization, with typical expression of IFN-γ and TNF-α, leads to maintained GVT effects and superior survival in recipients.

PLZF is critical in the development of innate T cells in mouse (7) and man (18) and controls key effector functions. Developmentally, forced PLZF expression partially protects Vb5 TCR transgenic thymocytes from negative selection (19). This results in a subtle change in the TCR repertoire due to the partial protection of PLZF-expressing thymocytes from super-antigen-mediated negative selection. However, the overall repertoire is nearly identical, as shown by measuring the frequency of TCR Vb gene usage and by spectral typing of CDR3 lengths (10). The mature T cells from PLZF-TG mice spontaneously acquire effector and central memory phenotypes. Recent studies have suggested that effector memory T cells produce less IFN-γ, leading to attenuated GVHD (20). However, unlike B6-WT memory T cells, PLZF-TG can produce copious amounts of cytokines including IFN-γ and TNF-α under suboptimal conditions. In these respects, PLZF-TG T cells are similar to innate T cells such as NKT cells. Moreover, recent data suggest that donor NKT cells can suppress GVHD through IL-4–dependent mechanisms (21) and high doses of NKT cells in the donor graft are associated with decreased GVHD (22). There are no data to directly show that ectopic expression of PLZF in mature human T cells will induce these types of effector functions. However, it is clear that, similar to the mouse, PLZF controls effector functions in human innate T cells (18). Although our study uses Lck-driven PLZF, which comes on during thymic development, it is clear that modern tools of genetic engineering and novel adoptive T lineage-based therapies can adapt this strategy for human applications. Genetic engineering with PLZF may therefore be used to confer effector functions on conventional T lineage cells for therapeutic use.
Following adoptive transfer, PLZF-TG T cells led to significantly decreased GVHD. This was evidenced by significantly less GVHD-related deaths and morbidity in MHC-disparate and minor histo-incompatibility models. Histologically, significantly less GVHD was seen in skin, small and large intestines. This was accompanied by significantly reduced numbers of T cells in secondary lymphoid organs. We examined if decreased numbers of PLZF-TG T cells in the tissues were reflective of decreased homing of T cells to these organs. PLZF-induced LFA-1 expression on T cells has been shown to cause their accumulation in the liver and retention in the intravascular compartment (23). In our studies, steady-state PLZF-TG T cells expressed similar levels of CCR7 as compared with B6-WT T cells, suggesting similar homing preferences to the lymphoid organs. Furthermore, the numbers of PLZF-TG T cells and B6-WT T cells were not significantly different in the spleens and lymph nodes of syngeneic recipients. These data indicate that the decreased GVHD mediated by PLZF-TG T cells was attributable to impaired alloreactive expansion rather than potential differences in effects of forced PLZF expression on homing.

Decreased expansion of PLZF-TG T cells in alloreactive conditions is multifactorial. PLZF-TG T cells have increased susceptibility to apoptosis as evidenced by increased caspase activity and increased levels of Fas receptor on the T cells. Furthermore, under alloreactive conditions, PLZF-TG T cells produce enhanced levels of IL-4. The decreased allo-expansion of PLZF-TG T cells could be rescued to levels comparable to allo-activated B6-WT T cells only by blocking Fas and IL-4 in combination with IL-2 supplementation, and blocking any single pathway was not adequate to rescue allo-expansion. TH1, TH2, and TH17 subsets have been shown to have critical roles in pathogenesis of GVHD, especially in infiltration of GVHD target organs (3). It has also been suggested that selective deficiency of T11 subset can lead to dominant expression of the remaining T11-cell subsets and alter GVHD. Elegant studies in mice have further shown that elimination of T11 and T17 subsets using T-bet and RORγt-deficient mice.
prevented development of GVHD through preponderance of T\textsubscript{H2} subsets (24). Alloreactive PLZF-TG T cells maintain expression of T\textsubscript{H1} and T\textsubscript{H17} transcription factors. However, a significant proportion of PLZF-TG T cells express the T\textsubscript{H2} transcription factor GATA-3 and produce IL-4 in addition to IFN-\textgreek{g}, thus showing a unique biphenotypic T-cell subset. Moreover, increased proportions of Foxp3\textsuperscript{+} T cells were seen in PLZF-TG treated groups, possibly driven by enhanced IL-2 production. Alternatively, this could also reflect the reduced GVHD and a relative shift towards T\textsubscript{H2} immunity. Surprisingly, the activation and signaling of alloreactive PLZF-TG cells remained intact and similar to that of B6-WT T cells. Our data therefore suggest that rather than complete elimination of T\textsubscript{H1}/T\textsubscript{H17} subsets, an upregulation of T\textsubscript{H2} cytokines mediated by PLZF expression can attenuate GVHD. In spite of significant differences in GVHD-related morbidity and mortality, sections of livers from PLZF-TG treated mice scored similar to B6-WT T-cell–treated groups. This can in part be attributed to higher FasL expression on PLZF-TG T cells, which is a key driver of liver GVHD (25, 26) and indicates a differential organ-specific GVHD pathogenesis in target organs.

In spite of a significantly decreased GVHD mediated by PLZF-TG T cells, we found an intact GVT effect against cell lines of hematopoietic origin. An intact GVT mediated by alloreactive PLZF-TG T cells can be attributed to intact T-cell signaling, antitumor IFN-\textgreek{g} and TNF-\alpha expression, and cytolytic molecules expressed on the T cells. In spite of the decreased numbers of total PLZF-TG T cells in the recipients, we observed significant GVT activity. Furthermore, PLZF-TG T cells mediated significant GVT effects over a range of T-cell doses. These results are in agreement with previous studies that suggest the early suppression of allo-antigen–driven proliferation, while leaving alloactivation intact, can lead to preserved GVT effects with attenuated GVHD (27, 28).

In summary, our data suggest that PLZF-TG T cells mediate less GVHD due to Fas-mediated fratricidal regulation and biphenotypic T\textsubscript{H1}/T\textsubscript{H2} response leading to limited alloreactive expansion, and an intact GVT activity leading to improved survival outcomes. Our results suggest that genetic engineering strategies to express PLZF in donor T cells could reduce GVHD deaths while preserving GVT activity.

Disclosure of Potential Conflicts of Interest
G.F. Murphy has a commercial research grant from Bristol Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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