Graft-versus-Leukemia (GVL) against Mouse Blast-Crisis Chronic Myelogenous Leukemia (BC-CML) and Chronic-Phase Chronic Myelogenous Leukemia (CP-CML): Shared Mechanisms of T Cell Killing, but Programmed Death Ligands Render CP-CML and Not BC-CML GVL Resistant

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Graft-versus-Leukemia (GVL) against Mouse Blast-Crisis Chronic Myelogenous Leukemia (BC-CML) and Chronic-Phase Chronic Myelogenous Leukemia (CP-CML): Shared Mechanisms of T Cell Killing, but Programmed Death Ligands Render CP-CML and Not BC-CML GVL Resistant

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Graft-versus-leukemia (GVL) against chronic-phase chronic myelogenous leukemia (CP-CML) is potent, but it is less efficacious against acute leukemias and blast-crisis chronic myelogenous leukemia (BC-CML). The mechanisms underlying GVL resistance are unknown. Previously, we found that alloreactive T cell targeting of GVL-sensitive bcr-abl-induced mouse CP-CML (mCP-CML) required TCR–MHC interactions and that multiple and redundant killing mechanisms were in play. To better understand why BC-CML is resistant to GVL, we performed a comprehensive analysis of GVL against mouse BC-CML (mBC-CML) induced by the retrovirally transfer of the bcr-abl and NUP98/HOXA9 fusion cDNAs. Like human BC-CML, mBC-CML was GVL resistant, and this was not due to accelerated kinetics or a greater leukemia burden. To study T cell recognition and killing mechanisms, we generated a panel of gene-deficient leukemias by transducing bone marrow from gene-deficient mice. T cell target recognition and killing mechanisms absolutely required that mBC-CML cells express MHC molecules. GVL against both mCP-CML and mBC-CML required leukemia expression of ICAM-1. We hypothesized that mBC-CML would be resistant to some of the killing mechanisms sufficient to eliminate mCP-CML, but we found instead that the same mechanisms were effective against both types of leukemia, because GVL was similar against wild-type or mBC-CML genetically lacking Fas, TRAIL-R, Fas/TRAIL-R, or TNFR1/R2 or when donor T cells were perforin<sup>−/−</sup>. However, mCP-CML, but not mBC-CML, relied on expression of programmed death-1 ligands 1 and 2 (PD-L1/L2) to resist T cell killing, because only GVL against mCP-CML was augmented when leukemias lacked PD-L1/L2. Thus, mBC-CML cells have cell-intrinsic mechanisms, distinct from mCP-CML cells, which protect them from T cell killing. The Journal of Immunology, 2011, 187: 000–000.

A llogeneic hematopoietic stem cell transplantation can be a life-saving therapy for patients with hematologic malignancies. Much of the efficacy of allogeneic hematopoietic stem cell transplantation is due to an antitumor effect mediated by alloreactive donor T cells, called graft-versus-leukemia (GVL) (1, 2). GVL is extremely potent against chronic-phase chronic myelogenous leukemia (CP-CML) wherein allograft T cell depletion increases the risk for relapse by 5- to 6-fold (3), and donor leukocyte infusions can induce remissions in ~80% of patients who have relapsed posttransplant (4). However, a variety of data support the idea that GVL is less efficacious against many other hematopoietic malignancies, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myelogenous leukemia that has progressed to blast crisis (BC-CML) (3–8). Patients with AML and ALL are typically transplanted in complete remission, whereas CP-CML patients are transplanted with overt disease. Thus, differences in overall disease burden alone are unlikely to fully explain GVL resistance. Nonetheless, there clearly is a GVL effect against AML, ALL, and BC-CML, because the risks for relapse are decreased in patients with graft-versus-host disease (GVHD) compared with patients with none (3, 9, 10). Understanding why many neoplasms, as a group, are GVL resistant and why GVL is effective in a subset of patients with these neoplasms is important first steps in developing approaches to render GVL-resistant cancers more GVL sensitive.

A barrier in studying GVL resistance has been the paucity of mouse leukemia models that share genetic etiology and phenotype with human leukemias and are inducible on different genetic
backgrounds that will yield leukemias lacking critical molecules. In an effort to overcome these limitations, we began by establishing a clinically relevant model of a GVL-sensitive leukemia. We chose a murine model of CP-CML (mCP-CML) induced by the retroviral transduction of mouse bone marrow (BM) cells with a cDNA derived from the bcr-abl translocation, responsible for human CP-CML (11). mCP-CML is an oligocolonial myeloproliferative syndrome characterized by splenomegaly and a high WBC count, with hematopoiesis dominated by maturing myeloid cells (12). An advantage of this model is that we are able to create leukemias lacking molecules that could be important for immunogenicity by infecting BM from gene-deficient mice (13–15). Key findings from our prior work on GVL against mCP-CML were that both CD4 and CD8 T cells must make direct TCR–MHC contacts with mCP-CML cells (13–15) and that T cells use redundant effector mechanisms. Specifically, GVL was preserved when leukemias were Faβq, TNFR1/R2, or TRAIL-R2/3; when T cells were perforin−/−; and when mCP-CML was Faβq (13–15). We hypothesized that this redundancy, at least in part, accounted for GVL sensitivity and that GVL-resistant diseases would be more reliant on single pathways.

To establish a GVL system against a GVL-resistant leukemia, we adopted a mouse model of BC-CML (mBC-CML) induced by the cointroduction of the bcr-abl and the NUP98/HOXA9 (NH) fusion cDNAs (16). NUP98 is a nuclear pore protein that, in AML, is a fusion partner with ≥15 other genes, 8 of which encode class 1 homeodomain proteins, such as HOXA9. Importantly, NH fusions were reported in both AML and BC-CML (17–19). When NH alone is introduced by retrovirus into mouse BM cells, a clonal myeloblastic leukemia evolves with a long latency (20). However, when NH and p210 are cointroduced, a short-latency blast crisis-like disease develops, with hematopoiesis dominated by maturing myeloid cells (12). An mCP-CML or B6 BM→T cell-depleted C3H.SW BM, and CD4+ or CD8+ T cells. For the B6bm12 mice, mBC-CML→T cell-depleted C3H.SW BM, either B6 background mBC-CML cells, mBC-CML cells, or a combination of both, with no T cells or GVL-inducing C3H.SW CD4+ or CD8+ T cells. Unless otherwise indicated, 1 × 10^6 and 1.5 × 10^6 mBC-CML cells were infused for CD4- and CD8-mediated GVL experiments, respectively. A total of 7.5 × 10^6 BM cells that underwent spin infection with 600 C.G sublethally irradiated B6 hosts (primary mice; Supplemental Fig. 1). Premorbid mice were sacrificed, and splenocytes were frozen. These cells were passed in sublethally irradiated mice, from which splenocytes were harvested and frozen (secondary mice). We then cloned mBC-CML cells by injecting 1000 live EGFP+ cells into sublethally irradiated hosts, which results in end-stage leukemia in 40–60 d. Individual spleens were frozen in experiment-sized aliquots. This procedure was repeated to create mBC-CML on each gene-deficient background.

Cell purification

CD8 cells were purified from lymph nodes via negative selection, as previously described (26), and were >90% pure, with CD4+ T cell contamination <0.2% (data not shown). CD4 cells were similarly purified by negative selection, except that anti-CD4 (GR1.5) was omitted, and biontin-conjugated anti-CD8 (TIB105; laboratory-prepared) was added to the depletion mixture. BM T cells were depleted with anti-Thy1.2 magnetic microbeads (Miltenyi Biotech, Auburn Hills, CA) or anti-Thy1.2 biontin and streptavidin microbeads and the AutoMACS (Miltenyi Biotec) (26). BM in all experiments was T cell depleted.

BM transplant and follow-up

All transplants were performed according to protocols approved by the Yale University Institutional Animal Care and Use Committee. B6 mice received 900 cGy and were reconstituted with 5 × 10^6 T cell-depleted C3H.SW BM, either B6 background mBC-CML cells, mBC-CML cells, or a combination of both, with no T cells or GVL-inducing C3H.SW CD4+ or CD8+ T cells. Unless otherwise indicated, 1 × 10^6 and 1.5 × 10^6 mBC-CML cells were infused for CD4- and CD8-mediated GVL experiments, respectively. A total of 7.5 × 10^6 BM cells that underwent spin infection with p210-expressing retrovirus was infused in mCP-CML GVL experiments, except as noted. BALB/c mice received 800 C.G y and were reconstituted with 10^7 T cell-depleted B6 BM, BALB/c mBC-CML, or with or without purified B6 wild-type (wt) or perforin−/− CD8+ T cells. For the B6m12→B6 strain pairing, B6 mice received 900 C.G y, 10^7 B6m12 BM, B6 mBC-CML, with or without B6m12 wt or perforin−/− CD4 cells. In experiments with β2M−/− mBC-CML or β2M−/− donor BM, all recipients were treated with 250 μg anti-NK1.1 (PK13-16; laboratory-prepared) on days −2, −1, and 7 to prevent NK cell-mediated rejection of MHC class I (MHC)-deficient cells. Mice were bled weekly to quantitate leukemic cells by flow cytometry, beginning on day −9. Mice were scored as having died of leukemia if they had a dominant population of leukemia cells in peripheral blood prior to death and had splenomegaly at necropy. All deaths in mBC-CML experiments were due to leukemia. A small minority of mice in the mCP-CML experiments died of GVHD, and these events are represented by a tick on the survival plots at each occurrence.

Abs and flow cytometry

The following Abs were prepared in the laboratory: anti-CD4 (GK1.5), anti-CD8 (TIB105), and anti-NFGR (HB8737). The following Abs were used from the Jackson Laboratories: anti-CD3, anti-CD4 (GK1.5), anti-CD8 (GK1.5), anti-CD44 (eBio13-259), anti-CD25 (PC61.5), anti-CD80 (CD1L), and anti-CD86 (CD106). The following Abs were purchased from Taconic (Germantown, NY): C3H.SW, B6 β2M−/− mice were provided by Lieping Chen (Yale University School of Medicine) (23). Faslpr mice were purchased from the Jackson Laboratories (Bar Harbor, CA) (22). B6 perforin−/− mice were provided by the National Cancer Institute (Frederick, MD). IAb MAb was obtained from the Jackson Laboratories (Jacksonville, CA) (21). All other Abs used were prepared in the laboratory: anti-CD4 (GK1.5), anti-CD8 (GK1.5), anti-CD44 (eBio13-259), anti-CD25 (PC61.5), anti-CD80 (CD1L), and anti-CD86 (CD106). The following Abs were purchased from Taconic (Germantown, NY): C3H.SW, B6 β2M−/− mice were provided by Lieping Chen (Yale University School of Medicine) (23). Faslpr mice were purchased from the Jackson Laboratories (Bar Harbor, CA) (22). B6 perforin−/− mice were provided by the National Cancer Institute (Frederick, MD). IAb MAb was obtained from the Jackson Laboratories (Jacksonville, CA) (21). All other Abs used were prepared in the laboratory: anti-CD4 (GK1.5), anti-CD8 (GK1.5), anti-CD44 (eBio13-259), anti-CD25 (PC61.5), anti-CD80 (CD1L), and anti-CD86 (CD106). The following Abs were purchased from Taconic (Germantown, NY): C3H.SW, B6 β2M−/− mice were provided by Lieping Chen (Yale University School of Medicine) (23). Faslpr mice were purchased from the Jackson Laboratories (Bar Harbor, CA) (22). B6 perforin−/− mice were provided by the National Cancer Institute (Frederick, MD). IAb MAb was obtained from the Jackson Laboratories (Jacksonville, CA) (21). All other Abs used were prepared in the laboratory: anti-CD4 (GK1.5), anti-CD8 (GK1.5), anti-CD44 (eBio13-259), anti-CD25 (PC61.5), anti-CD80 (CD1L), and anti-CD86 (CD106).
purchased from BD PharMingen (San Diego, CA); anti-CD3 (17A2), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-TER-119 (TER-119), anti-H-2Kb (AF6-88.5), anti-I-AI-E (M5/14.15.2), anti-Fas (Jo2), anti-PD-L1 (MH5), anti-PD-L2 (TY25), anti-CD19 (ID3), anti-CD117 (2B8), and anti-sca-1 (D7). mCp-CML stem cells were identified as not staining with a lineage mixture of Abs (CD3, CD11b, CD19, GR-1, and TER-119) and expressing c-Kit, sca-1, and NGFR. mBC-CML stem cells were EGFP

Statistical methods

The p values for survival curves were calculated by the log-rank test. The p values for comparisons of the numbers of leukemia cells were calculated by the Mann–Whitney test.

Results

Generation of mBC-CML

mBC-CML cells were generated as described in Materials and Methods and in Supplemental Fig. 1. The majority of mBC-CML cells was lin–EGFP+NGFR+ (Fig. 1A) and were clonal, as measured by Southern blot analysis of retrovirus-integration sites (data not shown). The same protocol was used to establish mBC-CML lines using BM from mice deficient in β2M, IAβ, TRAIL-R, TNFR/R2, Fas (FasLpr), TRAIL-R/Fas, PD-L1, and PD-L2. Open graphs represent wt mBC-CML cells; shaded graphs are from mBC-CML cells genetically deficient in the surface protein being assessed. Data are representative of at least three independent experiments with at least three mice from each group analyzed.

mBC-CML is relatively GVL resistant

To test the GVL sensitivity of mBC-CML, we used the C3H.SW →B6 B6 (H-2b) MHC-matched, minor histocompatibility Ag (miHA)-mismatched strain pairing. This is the same strain pairing we used in GVL experiments against mCP-CML (13, 26), (miHA)-mismatched strain pairing. This is the same strain pair-ing we used in GVL experiments against mCP-CML (13, 26), (miHA)-mismatched strain pairing. This is the same strain pair-

FIGURE 1. Phenotype of mBC-CML cells. To assess expression of key molecules on wt mBC-CML cells, wt or gene-deficient mBC-CML cells were injected into sublethally irradiated B6 recipients. Shown are representatives of at least three independent experiments with at least three mice from each group analyzed.

Sw BM, 107 (CD4 experiments) or 1.5 × 107 (CD8 experiments) mBC-CML cells with no T cells, or graded doses of purified C3H. SW CD4+ or CD8 T cells. Mice that did not receive GVL-inducing T cells died of mBC-CML between days 18 and 23 (Fig. 2A, 2B). CD8 and CD4 doses beyond 106 prolonged survival, but the majority of mice that received T cell doses below 4 × 106 succumbed to mBC-CML, as determined by the presence of EGFP+NGFR+ cells in the peripheral blood of mice prior to death and by spleen weight at necropsy (data not shown). Even with 4 × 106 T cells, between 30 and 50% of mice died of mBC-CML.

This contrasts with GVL against mCP-CML. In an analysis of mice transplanted with mBC-CML or mCP-CML in the C3H. SW→B6 strain pairing using standard experimental conditions, 169 of 179 (94%) evaluable recipients of mCP-CML and 1.2 × 106 donor CD8 cells were leukemia-free survivors, whereas only 129 of 197 (65%) recipients of mBC-CML and 4 × 106 donor CD8 cells survived (Fig. 2C). For CD4-mediated GVL, both mBC-CML and mCP-CML mice received 4 × 105 CD4 cells. Of mCP-CML CD4 recipients, 65/66 mice were leukemia-free compared with 40/70 mBC-CML recipients (Fig. 2C). Survival in mice that received mBC-CML cells, donor BM, and no T cells was significantly longer than in the comparable mCP-CML groups (p < 0.0001), with high WBC counts and extensive BM and spleen involvement occurring in both. Thus, if anything, there was a longer window for allo-reactive T cell generation in mBC-CML experiments.

It was possible that mBC-CML was GVL-resistant because of a greater leukemia burden. To test this hypothesis, we performed a time-course analysis of mCP-CML and mBC-CML development in mice transplanted with two doses of M-p210-NGFR spininfected B6 BM or mBC-CML cells (Fig. 2D). We sacrificed mice on days +7 and +14 and enumerated mCP-CML and mBC-CML cells. At day +7, mCP-CML cells were more numerous than were mBC-CML cells in spleen and BM but not in blood. At day +14, mBC-CML and mCP-CML cells were present in similar numbers in BM and spleen, but mCP-CML cells were more numerous than were mBC-CML cells in blood. Importantly, mice that were not sacrificed for analysis died with kinetics similar to that in the majority of our experiments (Supplemental Fig. 2).

GVL against mBC-CML is directed against miHAS

mBC-CML cells express human NGFR, p210, NH, and EGFP, which could be immunogenic (27). To assess the contribution of these leukemia-specific Ags to GVL against mBC-CML, we performed syngeneic B6→B6 transplants with or without B6 lymph node cells containing 4.6 × 106 and 4 × 106 CD4 and CD8 cells, respectively, or 4 × 106 purified CD8 cells. The addition of syngeneic T cells had no impact on survival, because all T cell recipients died with the same kinetics as did mice that received no T cells (Supplemental Fig. 3). Thus, NGFR, p210, NH, and EGFP were insufficient as target Ags and GVL required an alloreponse against miHAS, which parallels our data on GVL against mCP-CML (13).

mCP-CML does not promote GVL against mBC-CML

In sum, these data suggested that mBC-CML is intrinsically GVL resistant. However, an alternative explanation is that mCP-CML cells promote alloreactivity because they are capable of differentiating into CD11cMHChigh cells (data not shown) and could have APC function. To address this possibility, we determined whether the presence of mCP-CML increases GVL against mBC-CML. To do so, we reconstituted irradiated B6 mice with C3H.SW BM and B6 mCP-CML, mBC-CML, or a mix of both leukemias. Some mice in each group also received 1.2 × 106 C3H.SW CD8 cells. As expected, survival was greater in recipients of CD8 cells and only mCP-CML compared with recipients of CD8 cells and...
Importantly, recipients of a mix of mCP-CML, mBC-CML, and CD8 cells died of mBC-CML with the same kinetics as did mice that received mBC-CML cells and no mCP-CML cells. Thus, the presence of mCP-CML did not augment GVL against mBC-CML. Similar data were obtained in a parallel experiment in which GVL was induced by CD4 cells (data not shown).

**GVL requires cognate interactions between donor T cells and MHC on mBC-CML cells**

To determine whether donor T cells must make cognate TCR–MHC interactions with mBC-CML cells, we created mBC-CML using BM from $b_2M^{-/-}$ (and therefore MHC-I-deficient) and $Ia^b^{-/-}$ (and therefore MHC class II [MHCII]-deficient) mice and used these leukemias in CD8- and CD4-mediated GVL experiments, respectively. $b_2M^{-/-}$ and $Ia^b^{-/-}$ mBC-CML cells were completely resistant to CD8-mediated (Fig. 3A) and CD4-mediated (Fig. 4A) GVL, respectively. Thus, both CD8 and CD4 cells must make cognate interactions with mBC-CML cells to mediate GVL.

**Killing mechanisms against mBC-CML are redundant**

Having established that direct cytotoxicity is required for both CD4- and CD8-mediated GVL, we investigated mechanisms of T cell killing by creating mBC-CML deficient in Fas (Faslpr), TRAIL-R, TNFR1/R2, and TRAIL-R and Fas (TRAIL-R/Faslpr). CD8-mediated GVL was also unimpaired against TRAIL-R$^{-/-}$/Faslpr mBC-CML (Fig. 3E). Importantly, in all experiments, recipients of wt or gene-deficient mBC-CML without donor T cells died with similar kinetics ($p > 0.288$), indicating that the absence of these death receptors did not have a major effect on leukemia pathogenicity.

Because no gene deletion renders cells specifically resistant to perforin/granzyme-mediated killing, we used donor perforin$^{+/+}$ T cells to examine the importance of this pathway. For these experiments, we used the B6→BALB/c (CD8-mediated GVL) and B6$^{env1257}$→B6 (CD4-mediated GVL) strain pairings, because perforin$^{+/+}$ C3H.SW mice were not available. BALB/c mice were irradiated and reconstituted with B6 BM and BALB/c mBC-CML.
with no CD8 cells or with purified B6 wt or perforin^{−/−} CD8^{+} T cells (Fig. 3F). For CD4-mediated GVL, B6 mice were irradiated and reconstituted with C3H.SW BM, with or without 4 × 10^{6} C3H.SW CD8 cells with wt mBC-CML or an equivalent number of β2M^{−/−} mBC-CML (A), Faslpr (B), TRAIL-R^{−/−} (C), or TNFR1/2^{−/−} (D) mBC-CML cells. Mice received either TRAIL-R^{−/−} or TRAIL-R^{−/−}/Faslpr mBC-CML cells in A, all mice were treated with anti-NK1.1, as in Materials and Methods. F, To determine whether perforin was required, BALB/c mice were irradiated and reconstituted with B6 BM, 5000 BALB/c mBC-CML cells, and 10^{6} CD8 cells from wt or B6 perforin^{−/−} donors. For A–E, data are from one of two experiments with similar results. Data in F are from one experiment.

FIGURE 3. CD8-mediated GVL against mBC-CML requires target MHCI expression, but killing mechanisms are redundant. B6 mice were irradiated and reconstituted with C3H.SW BM, with or without 4 × 10^{6} C3H.SW CD8 cells with wt mBC-CML or an equivalent number of β2M^{−/−} mBC-CML (A), Faslpr (B), TRAIL-R^{−/−} (C), or TNFR1/2^{−/−} (D) mBC-CML cells. E, Mice received either TRAIL-R^{−/−} or TRAIL-R^{−/−}/Faslpr mBC-CML cells. In A, all mice were treated with anti-NK1.1, as in Materials and Methods. F, To determine whether perforin was required, BALB/c mice were irradiated and reconstituted with B6 BM, 5000 BALB/c mBC-CML cells, and 10^{6} CD8 cells from wt or B6 perforin^{−/−} donors. For A–E, data are from one of two experiments with similar results. Data in F are from one experiment.

with no CD8 cells or with purified B6 wt or perforin^{−/−} CD8^{+} T cells (Fig. 3F). For CD4-mediated GVL, B6 mice were irradiated and reconstituted with B6^{b2m12} BM with no T cells or with B6^{b2m12} wt or perforin^{−/−} (14) spleen cells containing 1 × 10^{6} or 2 × 10^{6} CD4^{+} T cells (Fig. 4D). In both models, GVL induced by wt or perforin^{−/−} T cells was equivalent.

ICAM-1 on mBC-CML and mCP-CML cells is required for CD8-mediated GVL

ICAM-1 on mBC-CML and mCP-CML cells is required for CD8-mediated GVL. However, contrary to that expectation, we observed similar CD8-mediated GVL in wt B6 and B6 β2M^{−/−} hosts and CD4-mediated GVL in wt B6 and IA^{b} β^{−/−} hosts (data not shown). We were surprised by these results and considered the possibility that GVL was initiated by APCs contaminating the mBC-CML cells, because we used splenocytes from wt mice with mBC-CML as the source of leukemia cells. Therefore, we repeated these GVL experiments with sort-purified mBC-CML cells. With sort-purified mBC-CML cells, we observed no GVL in B6^{b2M^{−/−}} hosts, whereas GVL was intact in recipients of unsorted mBC-CML cells (Fig. 6A). GVL against sorted and unsorted mBC-CML cells was similar in control wt B6 hosts transplanted in the same experiment (data not shown). The presort frequency of EGFP^{+} cells was 65%; therefore, at most, 5250 nonleukemic splenocytes were sufficient to initiate GVL.

Donor-derived APCs are not required for CD8-mediated GVL

To determine whether donor-derived APCs are required for CD8-mediated GVL, we used C3H.SW β2M^{−/−} mice as BM donors.
B6 mice were irradiated and reconstituted with wt or b2M2/2 C3H.SW BM, with or without donor CD8+ T cells. All mice (including recipients of wt donor BM) were treated with anti-NK1.1 on days -2, -1, and +7 to prevent NK cell-mediated rejection of b2M2/2 donor BM. GVL was equivalent in recipients of wt and b2M2/2 BM (Fig. 6D). Thus, donor-derived T cells exclusively primed on host APCs were sufficient to mediate GVL.

PD-L1/L2 on mCP-CML cells, but not mBC-CML cells, inhibits GVL

The B7 family members PD-L1 (B7-H1) and PD-L2 (B7-DC) deliver inhibitory signals to activated PD-1+ T cells and have been implicated in resistance to cancer immunotherapy (30, 31). PD-L1 is expressed both by mCP-CML and mBC-CML cells, including their stem cells (Fig. 1, Supplemental Fig. 4). Therefore,

B6 mice were irradiated and reconstituted with wt or b2M−/− C3H.SW BM. B6 mBC-CML cells, with or without donor CD8+ T cells. All mice (including recipients of wt donor BM) were treated with anti-NK1.1 on days −2, −1, and +7 to prevent NK cell-mediated rejection of b2M−/− donor BM. GVL was equivalent in recipients of wt and b2M−/− BM (Fig. 6D). Thus, donor-derived T cells exclusively primed on host APCs were sufficient to mediate GVL.

ICAM-1 on mCP-CML and mBC-CML cells is required for GVL. A, B6 mice were irradiated and reconstituted with C3H.SW BM, 5000 wt or ICAM-1−/− mBC-CML cells, with or without 4 × 106 C3H.SW CD8+ T cells. Data combined from two experiments with similar results. p < 0.0001 comparing either CD8-recipient group with its BM-alone control. B, B6 mice were irradiated and reconstituted with C3H.SW BM, wt or ICAM-1−/− mCP-CML, with or without 1.2 × 106 C3H.SW CD8 cells. Data combined from two experiments with similar results. p < 0.0001 comparing either CD8-recipient group with its BM-alone control. C, Expression of ICAM-1 on mCP-CML stem cells. Stem cells were lin− (Gr-1, CD11b, TERR-119, CD19, and CD3) and sca-1−/− c-Kit+NordFR+. Open graph represents ICAM-1; shaded graph represents isotype control. Data are representative of at least three independent experiments.
we created PD-L1/PD-L2 hematopoietic in origin, they could also express PD-L2. Therefore, PD-1+ T cells in the effector phase. To distinguish these possibilities, we performed GVL experiments wherein hosts received PD-L1/PD-L2-deficient mCP-CML and mBC-CML and tested their immunogenicity in CD8-mediated GVL experiments in the C3H.SW→B6 strain pairing. Despite clear PD-L1 expression on wt leukemias, neither PD-L1-deficient mCP-CML nor mBC-CML was more sensitive to GVL, even when GVL was induced by lower numbers of CD8 cells that resulted in reduced survival (Fig. 7A, 7B).

We next considered that because mCP-CML and mBC-CML are hematopoietic in origin, they could also express PD-L2. Therefore, we created PD-L1/PD-L2−/− mCP-CML and mBC-CML cells and used these in GVL experiments in the C3H.SW→B6 strain pairing. We again included groups that received lower doses of donor CD8 cells to increase our ability to detect differences in GVL sensitivity. The absence of PD-L1/L2 did not render mBC-CML cells more GVL sensitive (Fig. 7C). In contrast, PD-L1/PD-L2−/− mCP-CML was far more GVL sensitive than was wt mCP-CML (Fig. 7D). Therefore, either PD-L1 or PD-L2 on mCP-CML cells can inhibit GVL, and both must be ablated for GVL to be enhanced.

Because mCP-CML cells can differentiate into APCs and not all cells that undergo spin infection are bcr–abl-transduced, it was possible that the absence of PD-L1/PD-L2 on infected cells augmented alloreactive T cell priming, rather than failing to inhibit PD-1+ T cells in the effector phase. To distinguish these possibilities, we performed GVL experiments wherein hosts received wt, PD-L1/PD-L2−/−, or a mix of wt and PD-L1/PD-L2−/− mCP-CML cells, with or without 3×10⁵ GVL-inducing CD8+ T cells. We reasoned that if the absence of PD-L1/PD-L2 primarily augments T cell priming, GVL against wt mCP-CML should be potentiated by PD-L1/PD-L2−/− mCP-CML cells, whereas if the action of PD-L1/PD-L2 is in the effector phase, PD-L1/PD-L2−/− mCP-CML cells would not increase GVL against wt mCP-CML. To distinguish the two leukemias, we used M-p210/NGFR and M-p210/EGFP to infect PD-L1/PD-L2−/− and wt BM, respectively. As expected, PD-L1/PD-L2−/− mCP-CML was more sensitive to GVL (Fig. 7E). However, survival in recipients of a mix of wt and PD-L1/PD-L2−/− mCP-CML cells and CD8 cells was identical to recipients of only wt mCP-CML cells and CD8 cells and worse than that in recipients of PD-L1/PD-L2−/− mCP-CML and CD8 cells. Mice were serially bled to enumerate NGFR+ and EGFP+ cells (Fig. 7F). At days +7 to 13, there were more NGFR+ PD-L1/PD-L2−/− cells than EGFP+ wt mCP-CML cells in recipients of a mix of the two leukemias, whether they did or did not receive CD8 cells. However, on days +18 and +21, there were significantly more EGFP+ wt cells in CD8 recipients, demonstrating more efficient clearance of PD-L1/PD-L2−/− mCP-CML cells.

**Discussion**

In clinical transplantation, leukemia relapse remains the greatest single cause of mortality (32). The risk for relapse is not uniform across different hematopoietic malignancies. Rather, certain leukemias are, as a group, GVL sensitive, whereas others are relatively resistant. Improved clinical outcomes will depend on a mechanistic understanding of both GVL sensitivity and resistance. A major obstacle in achieving this has been the absence of clinically relevant and genetically manipulatable GVL-sensitive and GVL-resistant mouse leukemias. This was the motivation in adapting mCP-CML and mBC-CML models for GVL experiments.

The central findings of our prior work on GVL against mCP-CML were that cognate interactions between leukemia cells and CD4+ or CD8+ T cells are required and that GVL-inducing T cells...
FIGURE 7. PD-L1 and PD-L2 on mCP-CML, but not mBC-CML, cells, inhibit GVL. A–E are survival curves. The $p$ values shown on the figures compare recipients of wt and gene-deficient leukemias given the same T cell dose. B6 mice were irradiated and reconstituted with C3H.SW BM, wt or PD-L1$^{−/−}$ (A) or PD-L1/L2$^{−/−}$ (C) mBC-CML, with or without C3H.SW CD8$^+$ T cells. For A, $p = 0.09$ and $p = 0.0023$ comparing recipients of wt or PD-L1$^{−/−}$ mBC-CML and $2 \times 10^6$ or $4 \times 10^6$ CD8 cells, respectively, with their BM-alone controls. For C, $p = 0.0046$ comparing recipients of wt or PD-L1/L2$^{−/−}$ mBC-CML and $4 \times 10^6$ CD8 cells with their BM-alone controls. B, D, and E, B6 mice were irradiated and reconstituted with C3H.SW BM, $7.5 \times 10^5$ wt, PD-L1/L2$^{−/−}$, or a mix of PD-L1/L2$^{−/−}$ and wt mCP-CML cells ($3.75 \times 10^5$ of each), with no T cells or with $1.2 \times 10^6$ or $3 \times 10^5$ C3H.SW CD8 cells. $B, p = 0.0007$ comparing any T cell-recipient group with its BM-alone control. $D, p = 0.0001$ comparing any T cell-recipient group with its BM-alone control. $E, p = 0.0001$ comparing recipients of $3 \times 10^5$ CD8 cells and PD-L1/L2$^{−/−}$ mCP-CML versus a mix of wt and PD-L1/L2$^{−/−}$ mCP-CML cells. $p = 0.0033$ comparing recipients of CD8 cells and wt or PD-L1/L2$^{−/−}$ mCP-CML with their BM-alone controls, $p = 0.069$ comparing recipients of wt plus PD-L1/L2$^{−/−}$ mCP-CML with or without CD8 cells. $F$, Mice from the experiment depicted in $E$ were bled at the indicated days, and EGFP$^+$ wt and NGFR$^+$ PD-L1/L2$^{−/−}$ mCP-CML cells were enumerated. Note the reduction in PD-L1/L2$^{−/−}$ mCP-CML cells versus wt mCP-CML cells in CD8 recipients of either PD-L1/L2$^{−/−}$ mCP-CML or a mix of wt and PD-L1/L2$^{−/−}$ mCP-CML (MIX). $p \leq 0.01$ comparing numbers of leukemia cells in recipients of CD8 cells and wt versus PD-L1/L2$^{−/−}$ mCP-CML on days 18 and 21. $p = 0.016$ comparing numbers of wt and PD-L1/L2$^{−/−}$ mCP-CML cells in MIX CD8 recipients on days 18 and 21. Data in $A$ and $B$ are from one experiment each. Data in $C$, $E$, and $F$ are representative of two experiments, each with similar results. Data in $D$ are representative of three experiments with similar results.
used redundant killing mechanisms (13–15). We speculated that this redundancy, at least in part, accounted for GVL sensitivity and predicted that more GVL-resistant leukemias would be more reliant on single death-inducing pathways (1). In the present work, we analyzed GVL against mBC-CML mostly using the same strain pairing (C3H.SW→B6) as in our mCP-CML studies, which allows for a direct comparison of GVL against both leukemias.

Because we aimed to understand GVL resistance by comparing GVL against mCP-CML with GVL against mBC-CML, it was important that we established that mBC-CML is relatively GVL-resistant, and we did so in several complementary ways. GVL against mBC-CML required much higher doses of donor CD8 cells. Even with these higher doses, survival, assessed in a large cohort of similarly transplanted mice from multiple independent experiments, was less in mBC-CML recipients than in mCP-CML recipients of >3-fold fewer CD8 cells. mCP-CML was not relatively GVL-sensitive due to mCP-CML cells with APC function promoting a more effective alloreactive T cell response because the confusion of mCP-CML cells did not render mBC-CML more GVL sensitive. Nor was GVL resistance due to a larger mBC-CML leukemia burden, because mCP-CML cells were at least as numerous in BM, spleen, and blood at multiple early time points. In most experiments, without donor T cells, recipients of mBC-CML died later than did recipients of mCP-CML; therefore, if anything, there was more time to develop an alloimmune response.

Cognate interactions between donor T cell Ag receptors and MHC on mBC-CML cells were absolutely required for both CD4- and CD8-mediated GVL. That MHCII− mBC-CML was completely resistant to CD4-mediated GVL is congruent with the same finding for CD4-mediated GVL against mCP-CML (13, 15). These data suggested that direct cytolysis is the general mode by which CD4+ T cells mediate GVL, rather than indirect killing through accessory cells, such as macrophages. In contrast, CD4 cells can mediate GVHD indirectly, without making TCR-mediated contact with MHCII* host nonhematopoietic cells (15, 33); thus, interfering with these indirect mechanisms may decrease GVHD while preserving GVL. Direct target killing by CD4 cells was also demonstrated in vivo by other investigators (34, 35). However, these experiments were performed with ex vivo activated TCR transgenic T cells or T cell lines, whereas our data were from experiments with polyclonal CD4 cells activated exclusively in vivo, which is more clinically relevant.

Indirect killing by CD4 cells has long been advocated as an important mechanism for antitumor T cell responses (36–40). However, this conclusion has been based on data with tumor cell lines in which MHCII was undetectable by flow cytometry, whereas we used primary leukemias that were genetically MHCII deficient. This is especially relevant given that MHCII expression in wt mCP-CML (data not shown) and mBC-CML stem cells is indistinguishable from that in IAb2−/− leukemias (Fig. 1), and had IAb2−/− leukemias not been available, we could have mistakenly concluded that CD4 cells acted indirectly. It is possible that very few MHCII molecules are sufficient (below what we can detect by flow cytometry), that MHCII is induced in vivo by the alloimmune response, or that a small subset of critical cells express MHCII.

Having established that TCR–MHC contact was required, we focused on T cell cytolytic mechanisms. We had anticipated that killing mechanisms would lack the redundancy observed for GVL against mCP-CML. However, impairment of any single effector mechanism, or Fas and TRAIL-R together, had no impact on GVL against mBC-CML. These data argue against resistance to single killing mechanisms as a cause for GVL resistance. Numerous prior GVL studies suggested reliance on a single killing mechanism (reviewed in Ref. 1). Perforin was most commonly implicated, although FasL and TRAIL had essential roles in some models. These prior studies used leukemia cell lines, commonly induced by mutagenesis and passaged over many years, as targets for alloreactive T cells. These lines may have initially lacked one or more death receptor types or lost their expression with extended passaging, leading to reliance on a smaller subset of potential killing mechanisms. Previous work on killing mechanisms also mostly used gene-deficient T cells and reagent-based blockade of death receptor ligands, which could alter effector T cell generation (41, 42), thereby clouding the importance of these pathways to end effector killing. In contrast, in our experiments (except for those with perforin−/− T cells), only end effector killing was blocked. Another advantage of our gene-deficient leukemias is that the targeted death receptors were unequivocally absent, whereas leukemia cell lines may still express a given death receptor at a low level or be induced to express it in vivo, even if this is not apparent by flow cytometry or RT-PCR.

To our knowledge, our data were the first to definitively identify ICAM-1 on leukemia cells as an absolute requirement for CD8-mediated GVL. Because ICAM-1–LFA-1 interactions both promote T cell activation by APCs and T cell target killing (43), reduced GVL with reagent-based ICAM-1 or LFA-1 blockade could be due to reduced T cell activation rather than target killing. The use of ICAM-1−/− leukemias allowed us to isolate the role for ICAM-1 in the effector phase.

Because we and other investigators proposed inhibiting APCs as a strategy for decreasing GVHD, it was important to evaluate APC requirements for GVL against mBC-CML. Recipient APCs were required for both CD8- and CD4-mediated GVL against mBC-CML, and this confirms results from Reddy et al. (44) for GVL against a different model leukemia. That host APCs were required for optimal CD8-mediated GVL was anticipated given their essential role in CD8-mediated GVHD in this strain pairing (29). The reliance on host APCs also supports the idea that leukemias themselves are not an important source of miHAs for alloreactive T cell priming. However, it was surprising that CD4-mediated GVL also required intact recipient APCs. Exogenously acquired Ags are more efficiently presented on MHCII+ than on MHCII. Consistent with this, recipient APCs are not required for CD4-mediated GVHD across only miHAs (45, 46). The divergence in the roles of host APCs in CD4-mediated GVH and GVHD could be due to how the kinetics of donor T cell activation affects outcomes.Recipient APCs are available immediately to prime donor CD4 cells, whereas donor-derived APCs must differentiate from BM precursors and traffic to secondary lymphoid tissues. Therefore, without functional host APCs, alloreactive T cell generation is likely delayed, which would compromise the early GVL response. No more than 5250 infused nonleukemic spleen cells restored GVL in MHC-deficient hosts. Only a fraction of these would be functional APCs, and even fewer would make it to secondary lymphoid tissues. This highlights how conducive the early posttransplant period is to alloreactive T cell priming.

Another potential mechanism for GVL resistance that we considered was the interaction between PD-L1/PD-L2 on leukemia cells with PD-1 on T cells. PD-L1 expression by cancer cell lines is mostly used gene-deficient T cells and reagent-based blockade of death receptor ligands, which could alter effector T cell generation (41, 42), thereby clouding the importance of these pathways to end effector killing. In contrast, in our experiments (except for those with perforin−/− T cells), only end effector killing was blocked. Another advantage of our gene-deficient leukemias is that the targeted death receptors were unequivocally absent, whereas leukemia cell lines may still express a given death receptor at a low level or be induced to express it in vivo, even if this is not apparent by flow cytometry or RT-PCR.

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Another potential mechanism for GVL resistance that we considered was the interaction between PD-L1/PD-L2 on leukemia cells with PD-1 on T cells. PD-L1 expression by cancer cell lines can suppress antitumor T cell immunity via engagement of PD-1 on T cells (31). mCP-CML, mBC-CML, and their stem cells clearly expressed PD-L1. Yet, the absence of PD-L1/PD-L2 only promoted GVL against mCP-CML. By analyzing GVL responses in recipients of a mix of wt and PD-L1/L2−/− mCP-CML, we demonstrated that PD-L1/L2 acts in the effector phase. Because GVL was not augmented when mCP-CML was only PD-L1−/−, we can conclude that PD-L1 and PD-L2 are redundant. There has been little prior evidence supporting a role for PD-L2 in inhibiting
antitumor effects, likely due to its relatively limited expression, mostly on dendritic cells, macrophages, and B1 cells (31). In one study, its overexpression on a cell line augmented tumor rejection in a PD-1–independent fashion (47). Of note, PD-L2 expression was minimal on mCp-CML leukemia stem cells (Supplemental Fig. 4). Either this low level expression is sufficient, or it is up-regulated in vivo by alloimmune-induced inflammation (48, 49).

These data highlighted that the mere expression of PD-L1 or PD-L2 on a cancer cell does not assure that PD ligand blockade will enhance T cell killing. We do not know why the absence of PD-L1/PD-L2 on mBC-CML did not augment GVL. Allorative CD8 cells in these experiments should have been suppressible by PD ligands, given that PD-L1/PD-L2 on mCp-CML cells inhibited allorative CD8 cells in the same C3H.SW→B6 strain pairing. This suggests that the absence of this suppression was insufficient to overcome the intrinsic GVL resistance of mBC-CML cells. PD-L1/PD-L2 may also act in part through outside-in signaling (30), and these pathways may differ in the two leukemias. This remains to be further explored.

The role of PD-1 in immunity against mBC-CML has also been studied by Mumprecht et al. (50), although in a syngeneic-rejection model. They found that survival in sublethally irradiated B6 PD-1−/− recipients of p210/NH-transduced wt B6 BM was improved compared with that in control sublethally irradiated wt B6 mice, which contrasts with our results with PD-L1 and PD-L1/L2–deficient recipients of p210/NH-transduced wt B6 BM and peripheral blood stem cell transplantation for multiple myeloma: a comparison between transplants performed 1983–94 and 1994–8 at European Group for Blood and Marrow Transplantation. 2001. Progress in allogeneic bone marrow and peripheral blood stem cell transplantation for multiple myeloma: an overview of determinants of survival. Semin. Hematol. 37(3, Suppl. 42):323–30.


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