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 retroviral 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 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−/−. However, mCP-CML, but not mBC-CML, relied on expression of programmed death-1 ligands 1 and 2 (PD-L1/2) to resist T cell killing, because only GVL against mCP-CML was augmented when leukemias lacked PD-L1/2. 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: 1653–1663.

Abbreviations used in this article: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BC-CML, blast-crisis chronic myelogenous leukemia; BM, bone marrow; CP-CML, chronic-phase chronic myelogenous leukemia; EGFP, enhanced GFP; GVHD, graft-versus-host disease; GVL, graft-versus-leukemia; mBC-CML, murine model of blast-crisis chronic myelogenous leukemia; mCP-CML, murine model of chronic-phase chronic myelogenous leukemia; MHCII, MHC class II; miHA, minor histocompatibility Ag; M-p210/EGFP, MSCV2.2 expressing the human bcr-abl p210 cDNA and enhanced GFP; M-p210/NFγR, MSCV2.2 expressing the human bcr-abl p210 cDNA and a nonsignaling truncated form of the human low-affinity nerve growth factor receptor driven by an internal ribosome entry site; NGFR, nerve growth factor receptor; NH, NUP98/ HOXA9; PD, programmed death; PD-L1, programmed death-1 ligand 1; PD-L2, programmed death-1 ligand 2; wt, wild-type.

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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 oligoclonal 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 Fas(b/-), TNFR1/R2(-/-), or TRAIL-R(-/-); when T cells were perforin(-/-); and when mCP-CML was Fas(b/-) (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 cotransduction 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 I 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 coinjected, a short-latency blast crisis-like disease develops, with hematopoiesis dominated by lin- myeloblasts (16, 21). Significant for our studies, gene-deficient mBC-CML can be induced by infecting BM from knockout mice.

In this article, we describe a comprehensive analysis of GVL against mBC-CML, including mechanisms of T cell recognition and killing and the roles of donor and recipient APCs. We also tested the roles of the inhibitory ligands programmed death (PD)-1 ligand 1 (PD-L1) and 2 (PD-L2) and the adhesion molecule ICAM-1 on mBC-CML and mBC-CML cells. As we found for GVL against mCP-CML, direct cognate interactions between T cells and mBC-CML cells were required. Contrary to our hypothesis and in disagreement with many studies evaluating GVL against leukemia cell lines (1), T cell-killing mechanisms used against mBC-CML were highly redundant. However, the absence of ICAM-1 on either mCP-CML or mBC-CML cells greatly reduced GVL. Surprisingly, although PD-L1/L2 on mCP-CML cells protected them from T cell killing, PD-L1/L2 on mBC-CML cells did not inhibit GVL, although PD-L1 was well expressed.

Materials and Methods

Mice

Mice were between 7 and 10 wk of age. B6 and BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). IA(b/-) mice were purchased from Tacconic (Germantown, NY). C3H.SW, B6 b2M(b/-), and Fas(b/-) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). TNFR1/R2(-/-) mice were created as described (13). TRAIL-R(-/-) and TRAIL-R(-/-) littermates were a gift from Asat Winoto (University of California, San Francisco, San Francisco, CA) (22). B6 perforin(-/-) mice were obtained from the Jackson Laboratories and crossed to B6b2M(b/-) mice (Jackson Laboratories), as described (14). PD-L1(-/-) and PD-L2(-/-) mice were purchased from Dr. Lieping Chen (Yale University School of Medicine, New Haven, CT). PD-L1/L2 double-deficient mice were kindly provided by Arlene Sharpe (Harvard Medical School, Boston, MA). ICAM-1(-/-) mice (24) were purchased from Jackson Laboratories.

Retrovirus production

MSCV2.2 expressing the human bcr-abl p210 cDNA and enhanced GFP (EGFP) (M-p210/EGFP) or a nonsignaling truncated form of the human low-affinity nerve growth factor receptor (NGFR) driven by an internal ribosome entry site (M-p210/NGFR) were expressed as described (13). MSCV2.2 expressing NH and EGFP downstream of the internal ribosome entry site was a gift from D.G. Gilliland (Merck Research Labs, Whitehouse Station, NJ). Retroviral supernatants were generated as described (13, 25).

mBC-CML generation

p210-infected progenitors were generated as previously described (13). Briefly, on days −1 and 0, cells underwent “spinning infection” with M-p210/NGFR or M-p210/EGFP retrovirus.

mBC-CML generation

To generate mBC-CML (Supplemental Fig. 1), mice were injected on day −6 with 5 mg 5-fluorouracil (Pharmacia & Upjohn, Kalamazoo, MI). On day −2, BM cells were harvested and cultured in prestimulation media (DMEM, 15% FBS, IL-3 [5 ng/ml], IL-6 [10 ng/ml], and stem cell factor [10 ng/ml]; all cytokines from PeproTech, Rocky Hill, NJ). On days −1 and 0, cells were resuspended at 2 × 10⁶/ml in prestimulation media with the addition of retroviral supernatants, polybrene (4 μg/ml; Sigma, St. Louis, MO), and HEPES (100 mm) (13). The final titer of MSCV2.2 expressing NH and EGFP downstream of the internal ribosome entry site was that which infects 50% of 10⁶ cells after 3 d in culture. The absence of these cells was determined by flow cytometry.

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 × 10⁵ T cell-depleted C3H.SW BM, either B6 background mBC-CML cells, mCP-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 × 10⁶ and 1.5 × 10⁶ mBC-CML cells were infused for CD4- and CD8-mediated GVL experiments, respectively. A total of 7.5 × 10⁵ 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 cGy and were reconstituted with 10⁵ 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 cGy, 10⁵ B6m12 BM, B6 mBC-CML, with or without B6m12 wt or perforin(-/-) CD4 cells. In experiments with β2M(b/-) mBC-CML or β2M(b/-) donor BM, all recipients were treated with 250 μg anti-NGFR (1D-136; laboratory prepared) on days −2, −1, and +7 to prevent NK cell-mediated rejection of MCH1 class I (MCH1c)-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 necropsy. All deaths in mBC-CML experiments were due to leukemia. A subpopulation 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-NGFR (HB8737). The following Abs were

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To test the GVL sensitivity of mBC-CML, we used the C3H.SW mBC-CML. mBC-CML is relatively GVL resistant. In experiments with at least three mice from each group analyzed.

Protein being assessed. Data are representative of at least three independent experiments.

**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 were 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, Iαβ, TRAIL-R, TNFR/R2, Fas (Fas−/−), TRAIL-R/Fas, PD-L1, PD-L1/L2, and ICAM-1 (Fig. 1B).

**mBC-CML is relatively GVL resistant**

To test the GVL sensitivity of mBC-CML, we used the C3H.SW (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), allowing a direct comparison of GVL mechanisms against both leukemias. B6 mice were irradiated and reconstituted with C3H.SW CD4+ or CD8+ T cells. Mice that did not receive GVL-mediating T cells 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 × 10⁸ 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 × 10⁸ donor CD8 cells were leukemia-free survivors, whereas only 129 of 179 (65%) recipients of mBC-CML and 4 × 10⁸ donor CD8 cells survived (Fig. 2C). For CD4-mediated GVL, both mBC-CML and mCP-CML mice received 4 × 10⁶ 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 lower 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 alloreactive 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 spin-infected 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 × 10⁸ 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 alloresponse 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 CD11c⁺MHCI⁺bright 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 × 10⁸ 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 SW BM, 10⁴ (CD4 experiments) or 1.5 × 10⁴ (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 10⁴ prolonged survival, but the majority of mice that received T cells doses below 4 × 10⁴ 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).

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 representative flow cytometry data from prenominal recipients, analyzed in the same experiment using identical flow cytometry settings. A, The majority of cells had a high forward light scatter/side scatter (FSC/SSC) profile and did not stain with Abs against lineage-specific markers Gr-1, CD11b, TERR-119, CD19, and CD3. B, Staining for MHCI, IAβ, Fas, ICAM-1, 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.
only mBC-CML (Fig. 2E). 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 b2M−/− (and therefore MHCI-deficient) and IAb−/− (and therefore MHC class II [MHCII]-deficient) mice and used these leukemias in CD8- and CD4-mediated GVL experiments, respectively. b2M−/− and IAb−/− 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 (Fig. 3) and CD4-mediated (Fig. 4) GVL were equivalent against Faslpr, TRAIL-R and TNFR1/R2 mBC-CML. 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→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 $\beta 2M^{−/−}$ mBC-CML (A), Faslpr (B), TRAIL-R$^{−/−}$ (C), or TNFR1/R2$^{−/−}$ (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 $\beta 2M^{−/−}$ mBC-CML (A), Faslpr (B), TRAIL-R$^{−/−}$ (C), or TNFR1/R2$^{−/−}$ (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.

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

ICAM-1 interactions with LFA-1 on T cells promote adhesion and formation of a stable immunologic synapse (28). ICAM-1 is also highly and uniformly expressed on mBC-CML cells (Fig. 1). Therefore, we created ICAM-1$^{−/−}$ mBC-CML and mCP-CML and tested them in CD8-mediated GVL experiments in the C3H.

**HOST APCs are required for both CD4- and CD8-mediated GVL**

To determine whether host APCs are required for GVL against mBC-CML, we used $\beta 2M^{−/−}$ or IA$^b\beta^{−/−}$ recipients in CD8 and CD4 GVL experiments, respectively. Based on our previous data that host APCs are essential for CD8-mediated GVHD in the C3H.

**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 $\beta 2M^{−/−}$ mice as BM donors.
B6 mice were irradiated and reconstituted with wt or \(b^2M^{+/2}\) 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 \(b^2M^{+/2}\) donor BM. GVL was equivalent in recipients of wt and \(b^2M^{+/2}\) BM (Fig. 6D). Thus, donor-derived T cells exclusively primed on host APCs were sufficient to mediate GVL.

\(\text{PD-L1/L2 on mCP-CML cells, but not mBC-CML cells, inhibits GVL}\)

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, donor-derived T cells exclusively primed on host APCs were sufficient to mediate GVL.

FIGURE 4. CD4-mediated GVL requires cognate interactions with MHCII on mBC-CML cells, but mechanisms of killing are redundant. B6 mice were irradiated and reconstituted with C3H.SW BM, with or without \(4 \times 10^6\) CD4 cells, with wt mBC-CML or an equivalent number of IAb\(^b^2\) (A), Fas\(^bw\) (B), or TNFR1/R2\(^bw\) (C) mBC-CML. To test the role of perforin-mediated killing, B6 mice were irradiated and reconstituted with wt B6 mBC-CML, B6bm12 BM, with no CD4 cells or \(1 \times 10^6\) or \(2 \times 10^6\) CD4 cells from B6bm12 perforin\(^bw\) or wt B6 mice. Data from one of two experiments with similar results for TNFR1/R2\(^bw\) and Fas\(^bw\) mBC-CML. Data from one experiment for IAb\(^b^2\) mBC-CML and perforin\(^bw\) CD4 cells.

FIGURE 5. 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\(^bw\) mBC-CML cells, with or without \(4 \times 10^6\) 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\(^bw\) mCP-CML, with or without \(1.2 \times 10^6\) 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\(^bw\) (Gr-1, CD11b, TERR-119, CD19, and CD3) and sca-1+c-Kit\(^NGFR^-\). Open graph represents ICAM-1; shaded graph represents isotype control. Data are representative of at least three independent experiments.
we created PD-L1–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-L2–deficient 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 than was wt mCP-CML cells (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 × 10^8 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 +7–13, 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<sup>−/−</sup> (A) or PD-L1/L2<sup>−/−</sup> (C) mBC-CML, with or without C3H.SW CD8<sup>+</sup> T cells. For A, p = 0.09 and p = 0.0023 comparing recipients of wt or PD-L1<sup>−/−</sup> mBC-CML and 2 × 10<sup>6</sup> or 4 × 10<sup>6</sup> CD8 cells, respectively, with their BM-alone controls. For C, p ≤ 0.0046 comparing recipients of wt or PD-L1/L2<sup>−/−</sup> mBC-CML and 4 × 10<sup>6</sup> CD8 cells with their BM-alone controls. B, D, and E. B6 mice were irradiated and reconstituted with C3H.SW BM, 7.5 × 10<sup>5</sup> wt, PD-L1/L2<sup>−/−</sup>, or a mix of PD-L1/L2<sup>−/−</sup> and wt mCP-CML cells (3.75 × 10<sup>5</sup> of each), with no T cells or with 1.2 × 10<sup>6</sup> or 3 × 10<sup>5</sup> 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 × 10<sup>5</sup> CD8 cells and PD-L1/L2<sup>−/−</sup> mCP-CML versus a mix of wt and PD-L1/L2<sup>−/−</sup> mCP-CML cells, p ≤ 0.0033 comparing recipients of CD8 cells and wt or PD-L1/L2<sup>−/−</sup> mCP-CML with their BM-alone controls. p = 0.069 comparing recipients of wt plus PD-L1/L2<sup>−/−</sup> mCP-CML with or without CD8 cells. F, Mice from the experiment depicted in E were bled at the indicated days, and EGFP<sup>+</sup> wt and NGFR<sup>+</sup> PD-L1/L2<sup>−/−</sup> mCP-CML cells were enumerated. Note the reduction in PD-L1/L2<sup>−/−</sup> mCP-CML cells versus wt mCP-CML cells in CD8 recipients of either PD-L1/L2<sup>−/−</sup> mCP-CML or a mix of wt and PD-L1/L2<sup>−/−</sup> mCP-CML (MIX). p ≤ 0.01 comparing numbers of leukemia cells in recipients of CD8 cells and wt versus PD-L1/L2<sup>−/−</sup> mCP-CML on days 18 and 21. p = 0.016 comparing numbers of wt and PD-L1/L2<sup>−/−</sup> 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 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 IAββ−/− leukemias (Fig. 1), and had IAββ−/− 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 MHCI. 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 GVL 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 leukemic 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-1. 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 upregulated 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. Alloreactive CD8 cells in these experiments should have been suppressible by PD ligands, given that PD-L1/L2 on mCP-CML cells inhibited alloreactive 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/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/ND-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 mBC-CML. Our studies differ substantially from these. mBC-CML cells in our study and others (16, 21) were mostly Lin−, whereas the majority of mBC-CML cells in the study by Mumprecht et al. (50) expressed CD11b and Gr-1. Their experimental design, which used PD-1–deficient T cells rather than PD ligand-deficient mBC-CML, could also have provided more efficient T cell priming, whereas in our experiments only the effector phase was affected.

In sum, our studies indicated that GVL against both mCP-CML and mBC-CML is directed at MHc and that at least the early GVL response requires competent host APCs. This reliance on host APCs and the failure of mCP-CML to rescue GVL against mBC-CML suggested that similar miHA-reactive T cells are generated, regardless of the type of leukemia, which supports the hypothesis that GVL resistance is cell intrinsic to mBC-CML cells. Despite this cell-intrinsic resistance to T cell killing, we found that GVL against both leukemias share the same pathways of recognition and killing and, individually, only MHc and ICAM-1 expression were essential. Our experiments looked at the extreme example: complete absence of MHc or ICAM-1. Nonetheless, these results indicated that decreased expression of either MHc or ICAM-1 would reduce the avidity of alloreactive T cells for their targets, thereby diminishing GVL. That T cells use redundant killing mechanisms against both mCP-CML and mBC-CML, but mBC-CML is nonetheless resistant to GVL, supports the hypothesis that even with equivalent T cell recognition, a class of leukemias could be resistant as the result of differences downstream from death receptor ligation or the introduction of granzymes. If identified, these resistance pathways would be targets for rendering GVL-resistant cancers more susceptible to T cell killing.

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Disclosures

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References


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