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Cross-Protective Murine Graft-Versus-Leukemia Responses to Phenotypically Distinct Myeloid Leukemia Lines

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ABSTRACT

A c-*myc* retrovirus–transformed myeloid leukemia line, MMB3.19, of C57BL/6 (B6) origin, was developed to investigate graft-versus-leukemia (GVL) activity in murine bone marrow transplantation (BMT) models. It was previously determined that both naive and leukemia-presensitized CD4$^+$-enriched T cells are capable of mediating GVL activity to MMB3.19 challenge in both syngeneic (B6) and allogeneic (C3H.SW→B6) strain combinations, with the latter coinciding with minimal graft-versus-host disease. In the present study, MMB3.19 and 2 other similarly derived, yet phenotypically diverse, B6 myeloid leukemia lines (MMB1.10 and MMB2.18) were investigated for potential shared tumor antigens in the syngeneic GVL model. Morphologically, all 3 tumor lines are blastic with high cytoplasmic:nuclear ratios, but MMB2.18 displays dendritic processes, whereas MMB1.10 and MMB3.19 have a more rounded appearance. Flow cytometric analysis of the 3 lines revealed constitutive surface molecule expression of Mac-1, Mac-2, F4/80, LFA-1, B7-1, B7-2, H2K^b, H2D^b, and macrophage scavenger receptor, consistent with macrophage/monocyte lineages. Furthermore, each of the lines expresses H2I-A^b, but to varying degrees, with MMB2.18 cells having the lowest percentage (31.6%). In vitro ^{51}Cr release assays using MMB3.19-primed T-cell effectors demonstrated equivalent specific lysis of all 3 leukemia-line target cells. In addition, enzyme-linked immunospot analysis of MMB3.19-primed CD4$^+$ T cells revealed significantly increased frequencies of tumor-stimulated interleukin (IL)-2–, IL-4–, and interferon-γ–secreting cells when restimulated with each of the 3 leukemia lines. Furthermore, when MMB3.19-primed CD4$^+$ T cells were administered in a BMT setting, a protective GVL effect was seen in those mice challenged with MMB1.10, MMB2.18, or MMB3.19. Therefore, in vitro and in vivo experiments indicate that the 3 distinct myeloid leukemia lines share 1 or more common major histocompatibility complex class II–restricted tumor antigens that can elicit a cross-protective in vivo T-cell GVL response.

KEY WORDS

Graft-versus-leukemia • Graft-versus-host disease • Myeloid leukemia • Bone marrow transplantation • Tumor antigen • Cross-protection

INTRODUCTION

Bone marrow transplantation (BMT) represents an effective treatment for many blood disorders, including acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) [1,2]. For autologous transplantation, the primary risk factor is leukemic relapse due to the inefficiency of the patient's immune system to mount a meaningful response against residual tumor cells. A better understanding of potential myeloid leukemia antigens in AML and CML could enhance the ability to stimulate autologous T-cell responses, either ex vivo or in vivo. For allogeneic BMT, the issue of leukemic relapse is still present and further complicated by an inverse relationship between the incidence and severity of graft-versus-host disease (GVHD) [3-7]. Donor cell populations responsible for mediating GVHD are also thought to mediate the graft-versus-leukemia (GVL) response, thereby reducing the incidence of leukemic relapse. An important step toward separating GVHD and GVL responses hinges on finding either tissue-specific or leukemia-specific antigens that can be selectively targeted by donor T-cell populations.

Many tumor-specific shared antigens have been identified to date, including those encoded by the MAGE, BAGE, and GAGE families of genes [8-11]. These antigens are

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expressed by many melanomas, lung carcinomas, sarcomas, and bladder carcinomas, but rarely, if ever, by leukemias [12-14]. In addition, although a tumor-specific antigen has been found in a radiation-induced leukemia line [15], and tumor-specific cytotoxic T lymphocytes (CTLs) have been shown to target synthetic *bcr-abl* leukemia antigens in vitro [16-18], shared leukemia antigens capable of stimulating GVL responses in vivo have yet to be described. To date, the closest things to leukemia-restricted antigens that may be of any value have been minor histocompatibility antigens (mHAs) (eg, the HA-5 peptide) that are restricted to expression in hematopoietic tissue, including host leukemia cells [19].

Previously, an allogeneic murine myeloid leukemia BMT model using a c-*myc*–transformed myeloid leukemia line, MMB3.19, was used to study GVL activity in an mHA-mismatch system [20]. Transformation was achieved in vivo by injection of a nonreplicating c-*myc* retrovirus construct, which randomly integrates the c-*myc* encoding exons 1 and 2 preferentially into the myeloid lineage and, as such, can induce tumorigenesis of myeloid leukemias in varying differentiation stages [21,22]. These tumor cells do not produce any detectable retrovirus product. Therefore, any antigens that they might express are likely to be a consequence of the oncogenic process itself, initiated by the overexpression of c-*myc*. The unique availability of several myeloid leukemia lines from the same strain with varying phenotypic origins allows us to investigate whether common tumor antigens may be targeted in a GVL response. To address this question, the syngeneic B6 BMT model was used with the different myeloid leukemia lines to focus on the specific antileukemia response without the added complications of histocompatibility differences.

In this report, we analyzed 3 leukemia lines, MMB1.10, 2.18, and 3.19, for morphological differences, surface molecule expression, cross-stimulatory potential in enzyme-linked immunospot (ELISPOT) and CTL assays, and cross-protective ability in GVL survival assays. All 3 tumor lines were found to be blastic, but with distinct morphology, and to express typical myeloid markers. ^{51}Cr release assays using MMB3.19-primed T cells demonstrated equal specific lysis of all 3 myeloid tumor lines. Similarly, ELISPOT assays and in vivo GVL survival experiments demonstrated that MMB3.19-presensitized $CD4^+$ T cells responded to restimulation with and protected against challenge by MMB1.10, MMB2.18, or MMB3.19, indicating that the 3 tumor lines share 1 or more common major histocompatibility complex (MHC) class II–restricted determinants capable of eliciting a cross-protective T-cell GVL response.

MATERIALS AND METHODS

Mice

Male $H2^b$ C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Donors were between the ages of 8 and 12 weeks; recipients were between the ages of 8 and 16 weeks. Mice were housed in a sterile environment in microisolators and given autoclaved food and acidified water ad libitum.

Cell Lines

The φ2myc8 cell line produces retroviral particles containing the pEVX-mycXH RNA construct, which, upon infection, causes stable integration and expression of the c-*myc* gene under the control of the Moloney LTR promoter [23]. Using an approach described previously [24], φ2myc8 supernatant was injected intraperitoneally (IP) into pristane-primed B6 mice, several of which after 3 to 6 months developed ascites from which tumor cells were collected. Tumor clones were then established by limiting dilution in the presence of irradiated thymocyte feeder cells. The MMB1.10, MMB2.18, and MMB3.19 cloned lines were originally derived from separate mice and were selected for use in the current investigation on the basis of their different morphology. Wright-Giemsa staining was used for microscopic examination of the MMB tumor lines. The B6 methylcholanthrene-induced fibrosarcoma MC57G (ATCC #CRL-2295) was used as a control transformed cell line in assays for immune responsiveness and was MHC class I–positive and class II–negative.

Media

The MMB tumor lines and splenocytes were grown in RPMI 1640 (Mediatech, Herndon, VA) plus 10% fetal calf serum (FCS) (Sigma, St. Louis, MO), supplemented with L-glutamine, penicillin/streptomycin (Mediatech), and 2-mercaptoethanol (Life Technologies, Grand Island, NY), at 37°C in 7% CO_2. The MC57G line was cultured in Iscove's (Mediatech) plus 10% FCS, supplemented with L-glutamine, penicillin/streptomycin, and 2-mercaptoethanol, at 37°C in 7% CO_2. Phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) (Sigma) was used for in vitro manipulation of donor bone marrow cells and lymphocytes. Cells were resuspended in PBS alone for all injections into recipients.

Monoclonal Antibodies

Anti-Thy-1.2 (J1j; rat immunoglobulin [Ig] M) [25], anti-CD8 (3.168; rat IgM) [26], and anti-NK1.1 (pk136; IgG2a) [27] monoclonal antibodies (MoAbs) were obtained from ascites fluid and used with guinea pig C′ (Rockland, Boyertown, PA) for cell depletions. Affinity-purified goat anti-mouse IgG antibody (Cappel-Organon Teknika, West Chester, PA) was used for B-cell panning. For phenotypic analysis of the leukemia lines and donor lymphocyte preparations, fluorescein isothiocyanate (FITC)-labeled anti-B7-1 (CD80), anti-B7-2 (CD86), anti-$H2K^b$, anti-$H2D^b$, anti-IA^b, anti-CD3, anti-CD4, anti-CD8, and anti-B220 MoAb (all from PharMingen, San Diego, CA) and rat anti-mouse macrophage scavenger receptor (MCA1322) MoAb (Serotec, Oxford, UK) were used. In addition, anti-Mac-1 (CD11b), anti-Mac-2, anti-F4/80, and anti-LFA-1 (CD11a) MoAb were used for analysis, as previously described [24]. For ELISPOT analysis, purified rat anti-mouse interleukin (IL)-2 MoAb (clone JES6-1A12; IgG2a) [28]; IL-4 MoAb (clone BVD4-1D11; IgG2b) [28]; and interferon (IFN)-γ MoAb (clone R4-6A2; IgG1) [29] were used for primary cytokine capture. Biotinylated rat anti-mouse IL-2 MoAb (clone JES6-5H4; IgG2b) [30]; IL-4 MoAb (clone BVD6-24G2; IgG1) [28]; and IFN-γ MoAb (clone XMG1.2; IgG1) [29] were used for detection of cytokines. All MoAbs for the ELISPOT assay were purchased from PharMingen.

Preparation of Cells

Anti-Thy-1–treated (T cell–depleted) bone marrow (ATBM) was prepared by flushing femurs and tibias of donor

mice and incubating bone marrow cells with J1j MoAb (1:200 dilution) and C′ (1:12 dilution) for 45 minutes at 37°C. To obtain T cell–enriched donor populations, spleen and lymph node cells were treated with red blood cell lysing solution containing 0.8% NH_4Cl and allowed to adhere to plastic petri dishes coated with 1:200 dilution of goat anti-mouse IgG for 1 hour at room temperature. The collected nonadherent T cells were further enriched for the $CD4^+$ subset by treatment with anti-CD8 MoAb (3.168; 1:100 dilution) and C′ (1:12 dilution) for 45 minutes at 37°C. $CD4^+$-enriched T-cell populations contained FITC background levels of $CD8^+$ T cells (<1%).

Flow Cytometry

For phenotypic analysis of leukemia lines and T-cell–enriched populations, appropriate FITC-labeled MoAbs in 25 mL were incubated with 2×10^5 cells in 96-well round-bottom plates for 30 minutes at 4°C and washed 3 times with 100 mL PBS, 1% BSA, and 0.02% sodium azide. Samples were then fixed with 1% paraformaldehyde and analyzed for fluorescence on an EPICS Profile II analyzer (Coulter Electronics, Hialeah, FL).

CTL Assays

Cross-cytolytic T-cell activity was determined by a standard ^{51}Cr release assay. T cells were isolated from MMB3.19-primed mice and restimulated with 1:1 irradiated (20 Gy) syngeneic splenocytes and 1:100 irradiated (30 Gy) MMB3.19 tumor cells in RPMI 1640, 10% FCS, and 1:10 T-Stim culture supplement (Collaborative Biomedical Products, Bedford, MA) for 5 days at 37°C, 7% CO_2. Directly before the CTL assay, effectors were depleted of natural killer (NK) cells using anti-NK1.1 MoAb and C′. MMB1.10, MMB2.18, MMB3.19, and MC57G targets were radiolabeled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 1 hour at 37°C, washed, resuspended, and then added (1×10^4/well) to the effectors (in triplicate) at various effector:target (E:T) cell ratios in 96-well U-bottom plates (Nalge Nunc, Naperville, IL) for 4 hours at 37°C, 7% CO_2. Culture supernatants were harvested and counted in a LKB-Wallac CliniGamma 1272 automatic γ counter (Wallac, Turku, Finland). Spontaneous release of ^{51}Cr was determined by incubation of targets in the absence of effectors. Maximal release was determined by incubation of targets in detergent. Results are expressed as percent specific lysis, which was calculated using the following equation: % specific lysis = [(experimental – spontaneous)/(maximal – spontaneous)] × 100.

ELISPOT Assay to Detect Single Cytokine–Secreting $CD4^+$ T Cells

The frequencies of IL-2–, IL-4–, and IFN-γ–secreting $CD4^+$ T cells in MMB3.19-primed mice were determined by the ELISPOT assay, as described previously [30,31]. Briefly, $CD4^+$ T cells (1×10^7 cells/15 mL) were isolated as described above from mice that were presensitized 2 to 3 weeks before with irradiated (30 Gy) MMB3.19 cells (5×10^6, IP). Then they were incubated with syngeneic irradiated (20 Gy) splenocytes (1×10^7) in the presence or absence of irradiated (30 Gy) MMB1.10, MMB2.18, MMB3.19, or control MC57G cells (1×10^5) for 48 hours at 37°C, 7% CO_2. The splenocytes were included as a source of MHC class II–positive antigen-presenting cells for optimum stimulation of responses to tumor-derived antigens. Nitrocellulose-backed microtiter plates (Millipore, Bedford, MA) were coated overnight at 4°C with cytokine-specific primary capture MoAbs, including purified rat anti-mouse IL-2 (50 μg/mL), IL-4 (25 μg/mL), or IFN-γ (50 μg/mL) MoAb in a final volume of 50 mL coating buffer (borate buffer, pH 8.5, 0.1 mol/L). Plates were then washed and blocked, and 1 to 2×10^6 cultured cells were added in triplicate and incubated overnight at 37°C, 7% CO_2. After extensive washing, spot-forming IL-2–, IL-4–, or IFN-γ–secreting cells were detected by addition of 50 μL/well biotin-labeled cytokine-specific secondary detecting MoAbs, including biotin rat anti-mouse IL-2 (2 μg/mL), IL-4 (0.33 μg/mL), and IFN-γ (4 μg/mL) MoAb. The plates were then washed and developed using alkaline phosphatase–labeled streptavidin (Southern Biotechnologies, Birmingham, AL) and 50 mL of Sigma FAST BCIB/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). Development was stopped by washing with deionized water.

Survival Assay for GVL Activity

B6 mice were lethally irradiated with 9.5 Gy (from a Shepherd Mark-I-68A ^{137}Cs source; 1.43 Gy/min) and 4 to 6 hours later were intravenously injected with a syngeneic donor inoculum of 2×10^6 ATBM cells, alone or including T cells (2×10^6). Donor T cells were either unfractionated or enriched for the $CD4^+$ subset. They were from mice that were either naive or presensitized 2 to 3 weeks previously with irradiated (30 Gy) MMB3.19 cells (5×10^6, IP). Recipients were challenged 1 day later with MMB1.10, MMB2.18, or MMB3.19 cells (0.5 to 2×10^5 cells, IP in 0.5 mL PBS). Mice were checked daily for morbidity and mortality until the termination of experiments. The nonparametric Wilcoxon signed rank test was used for statistical comparisons of survival curves.

RESULTS

Morphological and Phenotypic Comparison of MMB Cell Lines

Morphologic analysis of the MMB1.10, MMB2.18, and MMB3.19 cell lines revealed that all 3 tumors were blastic, with a high cytoplasmic:nuclear ratio (Figure 1). A high proportion of MMB2.18 cells exhibited extensive dendritic-like cytoplasmic processes (Figure 1B), whereas MMB1.10 (Figure 1A) and MMB3.19 (Figure 1C) cells were more rounded in appearance. In culture, MMB1.10 and MMB3.19 grew more rapidly (doubling time approximately 15.7 hours) than MMB2.18 cells (doubling time approximately 19.4 hours). In regard to adherence characteristics, MMB3.19 cultures contained the highest percentage of nonadherent cells (approximately 72%), compared with 12% to 14% in the other 2 lines (data not shown). Next, the MMB tumor lines were analyzed by flow cytometry for phenotypic expression of various myeloid-related determinants. All 3 lines contained a high percentage (>75%) of cells expressing Mac-1, Mac-2, F4/80, B7-1, B7-2, $H2K^b$, $H2D^b$, and macrophage scavenger receptor (Table). In addition, although all 3 lines also expressed $H2I\text{-}A^b$ and LFA-1, MMB1.10 and MMB3.19 cells

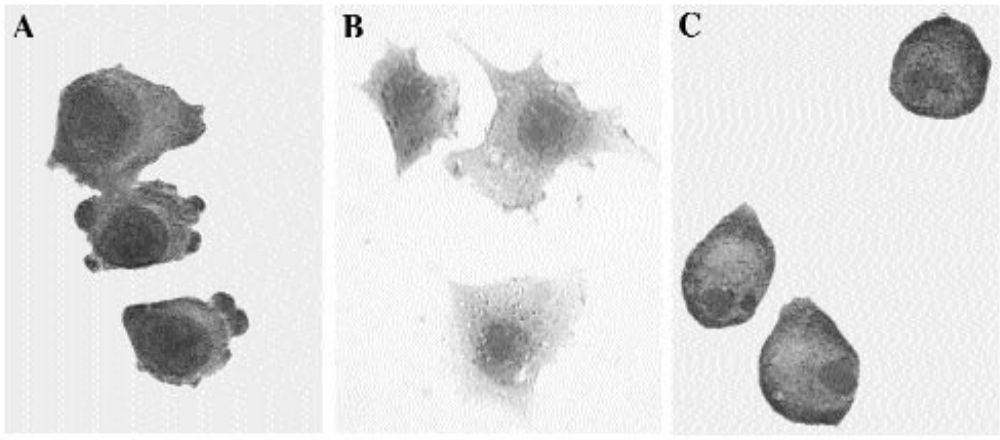

Figure 1. A, Wright-Giemsa staining of MMB1.10; B, MMB2.18; and C, MMB3.19.

did so to a greater degree (>80% positive for both markers) than did MMB2.18 (31.6% and 65.8%, respectively). These morphological and phenotypic results, in addition to consideration of the tumorigenic strategy used for their generation [21,22], determined that the MMB cell lines were all of the macrophage/monocyte lineage, but with distinct features.

Cross-Lytic Potential of MMB3.19-Primed CTL

To determine whether CTL effectors can recognize common determinants expressed by the myeloid tumor lines, B6 mice were presensitized IP with irradiated (30 Gy) MMB3.19 (2 to 3 weeks), and lymph node and spleen T cells were isolated and restimulated in vitro for 5 days in the presence of irradiated (20 Gy) syngeneic splenocytes and irradiated (30 Gy) MMB3.19 cells. These CTL effectors were then depleted of NK cells (by treatment with anti-NK1.1 MoAb and C′) and tested for the ability to lyse MMB1.10, MMB2.18, MMB3.19, and MC57G (a control B6 line) target cells. MMB3.19-primed T cells mediated equivalent specific lysis (45% to 50% at 27:1 E:T ratio) of all 3 MMB target cells at levels well above that of MC57G cells (Figure 2). This cross-lytic potential was titratable and was still evident at the 3:1 E:T ratio.

*Flow Cytometric Phenotypic Analysis of Myeloid Cell Lines**

	Cell Expression, %		
Antibody specificity	**MMB1.10**	**MMB2.18**	**MMB3.19**
FITC control IgG	**0.8**	**1.3**	**1.3**
B7-1 (CD80)	**90.0**	**95.8**	**95.3**
B7-2 (CD86)	**100.0**	**98.0**	**99.8**
H2K^b	**96.2**	**96.6**	**99.0**
H2D^b	**77.2**	**85.5**	**86.9**
H2I-A^b	**89.8**	**31.6**	**81.5**
FITC control IgG	**0.3**	**0.3**	**0.7**
Mac-1 (CD11b)	**99.1**	**96.2**	**83.9**
Mac-2	**99.7**	**98.9**	**99.3**
F4/80	**85.0**	**94.7**	**95.0**
LFA-1 (CD11a)	**92.5**	**65.8**	**90.3**
Macrophage scavenger receptor	**99.6**	**81.1**	**99.9**

*FITC indicates fluorescein isothiocyanate; Ig, immunoglobulin.

Cytokine Production of Cross-Reactive Anti-MMB T Cells

Having determined that the 3 MMB lines share 1 or more common targetable antigens using unfractionated T-cell cytolytic effectors, we investigated whether these lines also expressed 1 or more common antigens capable of cross-stimulating cytokine production by CD4$^+$ T cells. This issue was of particular importance because CD4$^+$ T cells had previously been demonstrated to mediate GVL responses in the absence of GVHD in both syngeneic and allogeneic MMB3.19 models [20]. ELISPOT assays were thus used to detect the frequency of IL-2–, IL-4– and IFN-γ–producing CD4$^+$ T cells from MMB3.19-presensitized B6 mice, after in vitro restimulation with each irradiated MMB line (a 2-day

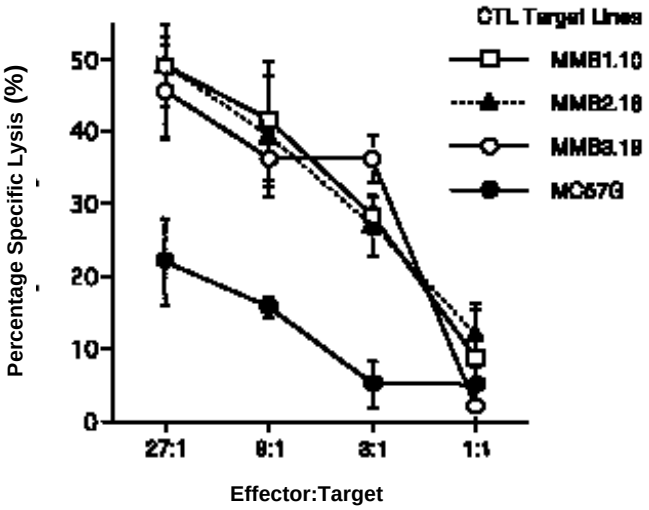

Figure 2. Cross-cytolytic potential of MMB3.19-primed unfractionated T cells in 4-hour ^{51}Cr release assays. MMB3.19-primed T cells were restimulated in vitro with irradiated (30 Gy) MMB3.19 cells and irradiated (20 Gy) splenocytes for 5 days and depleted of natural killer cells before incubation with radiolabeled target cells at the effector:target cell ratios shown. Data are expressed as the mean % specific lysis ± SE and are representative of 2 similar experiments. CTL indicates cytotoxic T lymphocyte.

incubation in the presence of irradiated B6 splenocytes). A high frequency of IL-2–producing cells, indicated by elevated numbers of spots/well, was observed when each of the MMB lines was present in the restimulation cultures, but not in the absence of the tumor cells (Figure 3A). In a similar manner, high frequencies of IL-4– and IFN-γ–producing $CD4^+$ T cells were present in cultures restimulated with each of the 3 MMB lines, but not in those restimulated with the control MC57G cell line (Figure 3B), suggesting that the anti-MMB response is specific for determinants expressed by these myeloid lines. Interestingly, cytokines associated with both helper type 1 (IFN-γ) and type 2 (IL-4) were significantly elevated in the responding antileukemic populations.

Cross-Protective GVL Responses

Based on the in vitro data suggesting that the 3 MMB lines shared a common immunogenic antigen, BMT-GVL assays were performed to investigate whether this cross-reactivity translated into cross-protective in vivo GVL responses. Donor B6 mice were presensitized with irradiated (30 Gy) MMB3.19 cells 2 to 3 weeks before spleen and lymph node harvest. Donor lymphocytes were T-cell enriched, and 2×10^6 MMB3.19-primed or naive T cells were injected intravenously into lethally irradiated (9.5 Gy) recipient mice along with 2×10^6 ATBM. Mice receiving 2×10^6 MMB3.19-primed T cells and rechallenged 1 day later with 1×10^5 MMB3.19 cells exhibited full long-term protection (80% survival; $P \leq .04$) compared with those mice receiving either no T cells (MMB3.19 group) or naive T cells, which succumbed by day 37 with median survival times (MSTs) of 23 and 34 days, respectively (Figure 4C). Similarly, mice receiving the same dosage of MMB3.19-primed T cells and a subsequent challenge of 1×10^5 MMB1.10 cells had significantly prolonged survival (MST 56 days; $P \leq .04$) over those receiving either no T cells or naive T cells (MST 22 and 35 days, respectively) (Figure 4A). However, such a cross-protective benefit was not observed in mice challenged with 1×10^5 MMB2.18 cells (Figure 4B). Groups receiving either MMB3.19-primed or naive T cells experienced similar GVL responses to MMB2.18 leukemia challenge ($P \geq .07$), with MST 47 days.

Because MMB3.19-primed $CD4^+$ T cells were capable of responding by cytokine production to restimulation with all 3 MMB lines in the ELISPOT assays, the GVL survival studies were repeated using MMB3.19-primed or naive $CD4^+$ T cells. As with unfractionated T cells, 2×10^6 $CD4^+$-enriched MMB3.19-primed T cells mediated protection against challenge with either MMB1.10 or MMB3.19 cells (MST 21 and 26 days, respectively) (Figures 5A and 5C). For both of these leukemia challenges, mice given the primed $CD4^+$ T cells experienced significantly prolonged survival ($P \leq .01$) over those receiving naive $CD4^+$ T cells (MST 18 and 21 days, respectively). Mice challenged with MMB2.18 cells also displayed a significant, albeit far less impressive, benefit from transplantation of MMB3.19-primed $CD4^+$ T cells compared with those receiving naive donor cells (MST 67 versus 59 days; $P \leq .03$) (Figure 5B).

To further accentuate the protective effect of MMB3.19-primed $CD4^+$ T cells, syngeneic BMT B6 recipients were challenged with half the number of MMB1.10 and 3.19 cells (5×10^4). The pattern of cross-protection was readily apparent in the mice challenged with MMB1.10 cells (MST 28 versus 19 days for the naive T-cell inoculum; $P \leq .04$) (Figure 6A). Mice challenged with MMB3.19 cells and transplanted with primed $CD4^+$ T cells exhibited enhanced survival over that previously observed (40% versus 0% by day 50) (Figure 6B versus Figure 5C) and over the mice given the naive T-cell inoculum (MST 37 versus 24 days; $P \leq .04$) (Figure 6B).

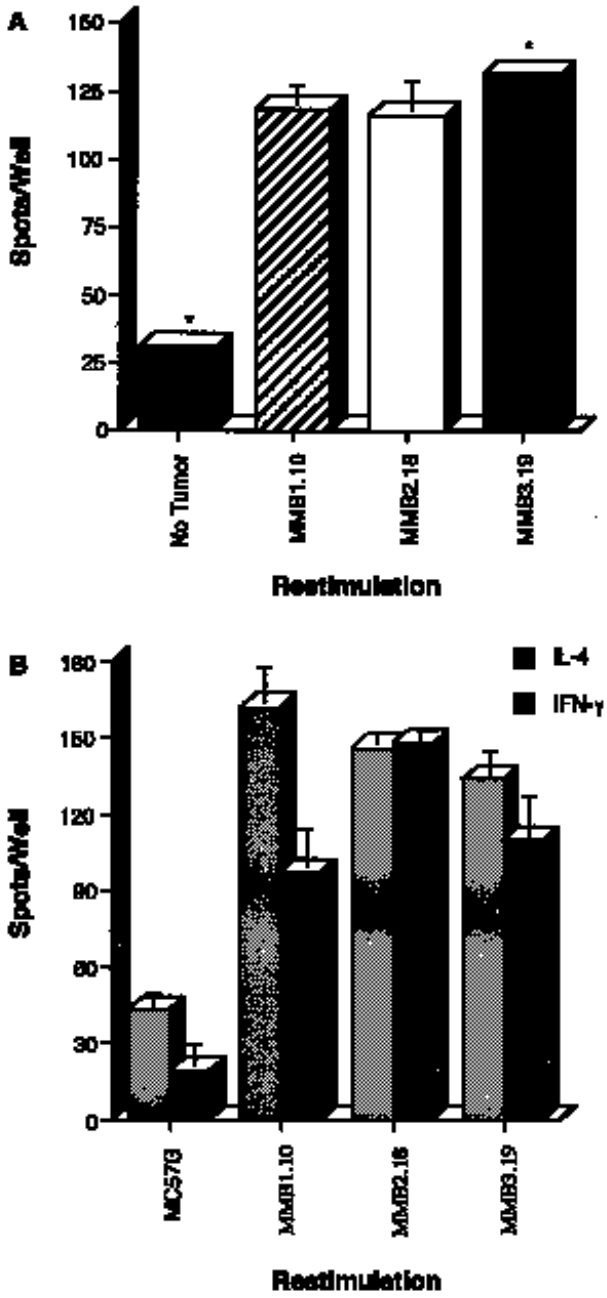

Figure 3. Cross-stimulatory potential of the MMB lines as determined by enzyme-linked immunospot (ELISPOT) assays. MMB3.19-primed $CD4^+$ T cells were restimulated with irradiated (20 Gy) splenocytes ± irradiated (30 Gy) MMB tumors or MC57G for 48 hours and analyzed in interleukin (IL)-2 (A) or IL-4 and interferon (IFN)-γ (B) ELISPOT assays. Results are expressed as the number of spots/well + SE, and the data are representative of 2 similar experiments. *SE ≤1.2 spots/well.

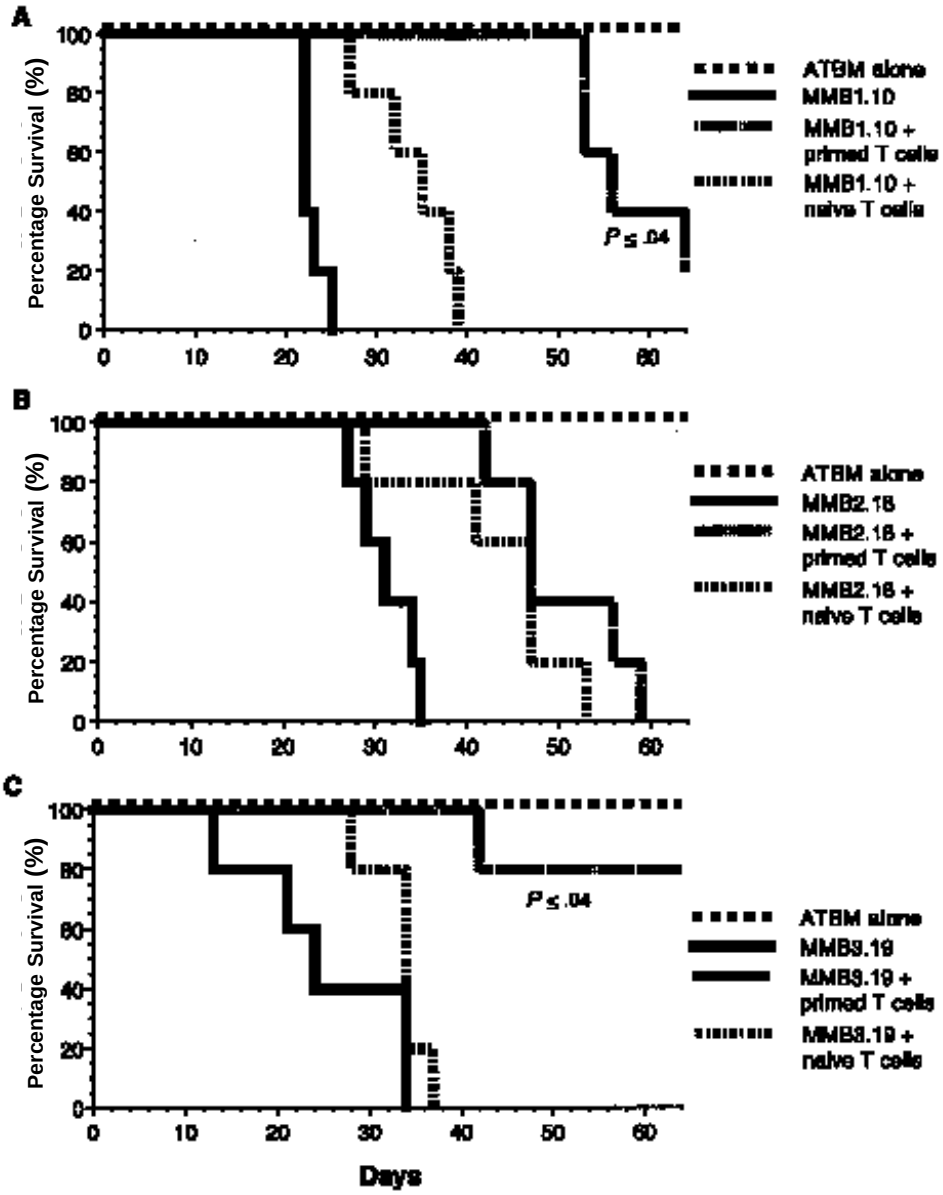

Figure 4. Cross-protective graft-versus-leukemia capacity of MMB3.19-primed unfractionated T cells upon in vivo challenge with the 3 MMB tumor lines. Lethally irradiated recipient B6 mice were injected intravenously with 2×10^6 donor anti-Thy-1–treated (T cell–depleted) bone marrow (ATBM) cells alone or in addition to MMB3.19-primed or naive donor T cells (2×10^6). One day later, mice were challenged with 1×10^5 MMB1.10 (A), MMB2.18 (B), or MMB3.19 (C) cells intraperitoneally in 0.5 mL phosphate-buffered saline (n = 5 for all groups). *P* values represent significance between groups receiving primed versus naive T cells.

To further enhance the weak MMB2.18 GVL response, the donor $CD4^+$ T-cell dosage was doubled (4×10^6) along with the reduction in tumor challenge (5×10^4). In addition, a group of mice that received MMB2.18-primed $CD4^+$ T cells was included to gauge the maximum expected GVL potential. An enhanced level of GVL protection ($P < .03$) was observed in the groups given either MMB2.18- or MMB3.19-presensitized $CD4^+$ T cells (MST 58 and 55 days, respectively) over mice given naive $CD4^+$ T cells (MST 40 days) (Figure 7). The GVL responses between the direct and cross-primed groups were equivalent ($P > .67$), suggesting that the latter antigens were sufficient to generate the maximum effect.

DISCUSSION

Although allogeneic BMT is now recognized as a curative treatment for AML and CML, leukemic relapse remains a major risk factor for patients. The fact that leukemic relapse still poses such a threat can be attributed in part to the lack of knowledge about myeloid leukemia antigens. It has yet to be determined whether common leukemia-specific antigens exist and can be selectively targeted by GVL-reactive cells to enhance antileukemic responses in the setting of allogeneic BMT. Uenaka et al. [15] have identified a radiation leukemia tumor antigen, and others have demonstrated that tumor-specific CTLs can target proteinase 3 [32] and *bcr-abl* junctional peptides [16-18] in vitro. Furthermore, in

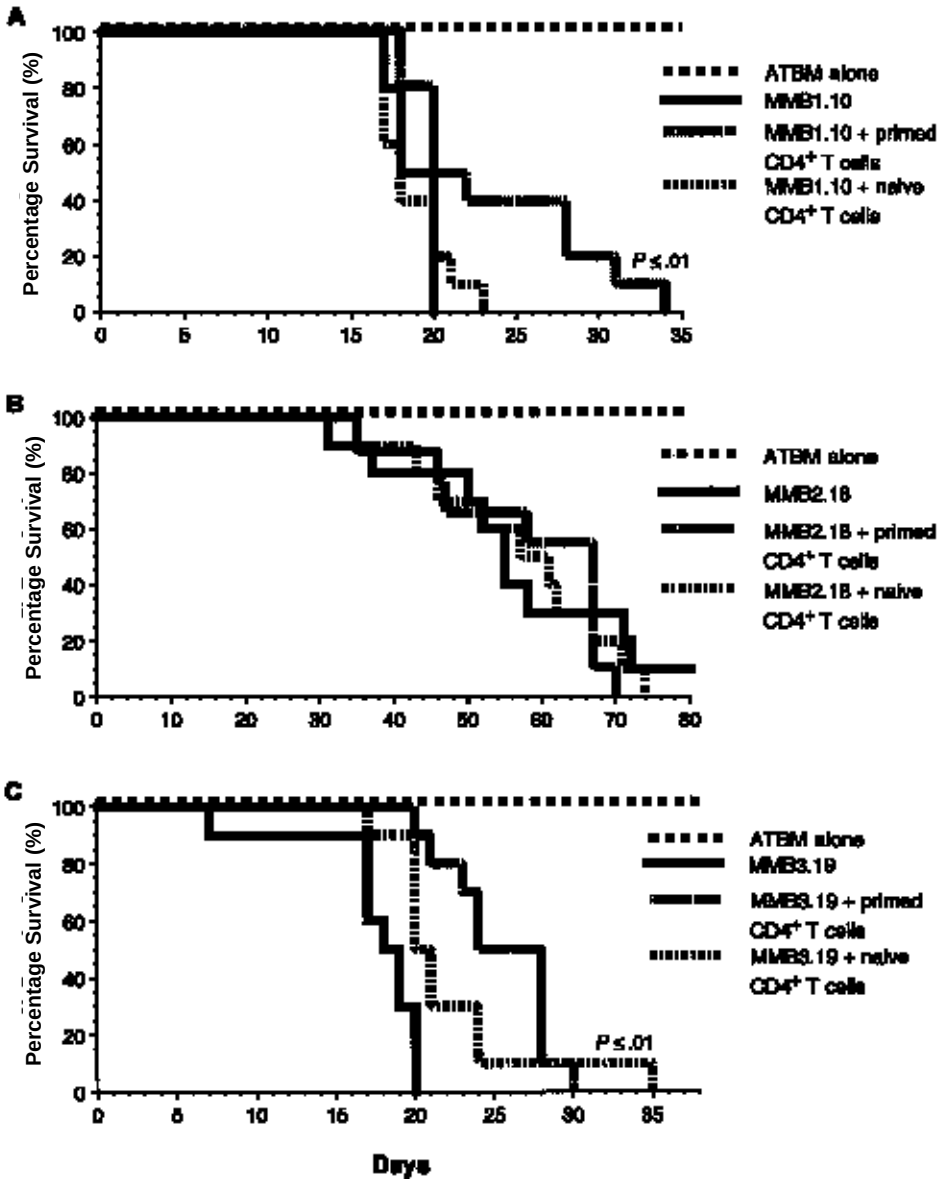

Figure 5. Cross-protective graft-versus-leukemia effect of MMB3.19-primed $CD4^+$ T cells upon challenge with the 3 MMB tumor lines. Lethally irradiated recipient B6 mice were injected intravenously with 2×10^6 donor anti-Thy-1–treated (T cell–depleted) bone marrow (ATBM) cells alone or in addition to MMB3.19-primed or naive $CD4^+$ T cells (2×10^6). One day later, mice were challenged with 1×10^5 MMB1.10 (A), MMB2.18 (B), or MMB3.19 (C) cells intraperitoneally in 0.5 mL phosphate-buffered saline. All groups contained 10 mice (pooled from 2 similar experiments), and the *P* values represent significance between groups receiving primed versus naive $CD4^+$ T cells.

vivo cross-protective immunity has been demonstrated in a subset of tumors derived from murine fetal fibroblasts [33]. However, shared leukemia antigens capable of stimulating cross-protective CTL responses in vivo have yet to be described.

In this report, we studied 3 distinct c-*myc*–derived myelogenous leukemia lines, originating from different B6 mice, to investigate potential shared tumor antigens. These leukemia lines are relevant because they are derived from a common transforming oncogene (as are many human tumors); express no detectable retroviral particles; have undergone secondary, spontaneous genetic alterations; and are of the myeloid lineage (expressing Mac-1, Mac-2, F4/80, LFA-1, and macrophage scavenger receptor). These leukemia lines are similar to spontaneous tumors in that they express an activated form of the normal mouse c-*myc* gene and are thought to have undergone secondary spontaneous mutations necessary for conversion to metastatic tumors [21,22].

In allogeneic BMT, a significant portion of the GVL response may be due to reactivity to allogeneic differences, both MHC and mHA, expressed by residual leukemia cells. In the setting of HLA-matched allogeneic GVL, the relative contributions of mHA-reactive and leukemia-specific antigen–reactive T cells are unknown. Others have demonstrated the existence of mHA-driven GVL responses [34-37]. However, in the current study, to address the possibility of

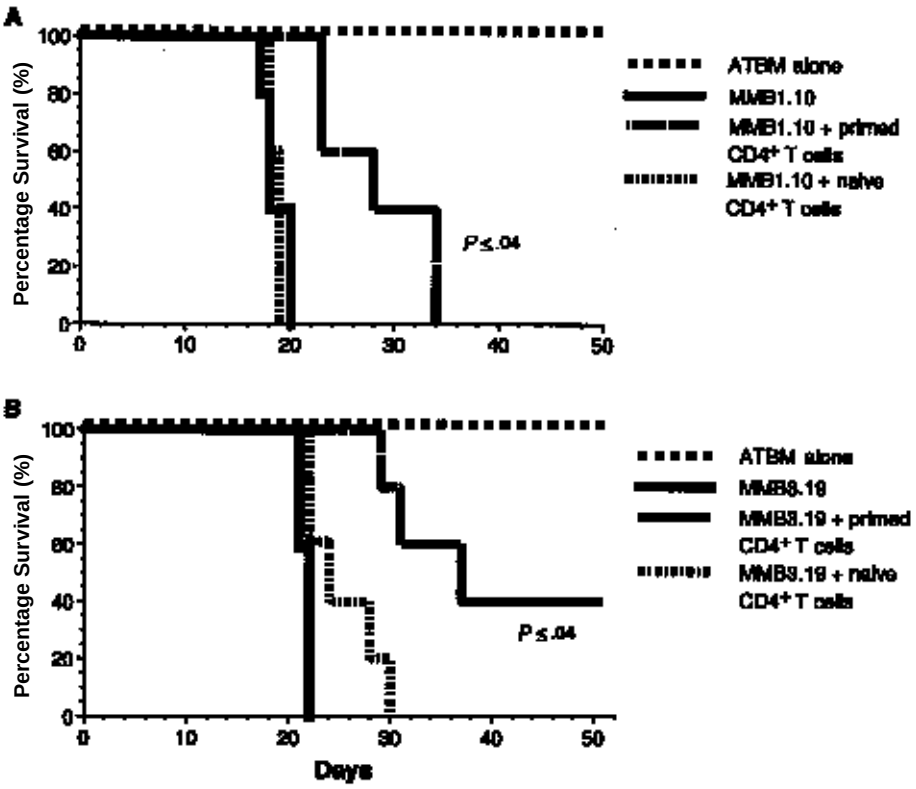

Figure 6. Cross-protective graft-versus-leukemia effect of MMB3.19-primed $CD4^+$ T cells against a lower tumor burden. Lethally irradiated recipient B6 mice were injected intravenously with 2×10^6 donor anti-Thy-1–treated (T cell–depleted) bone marrow (ATBM) cells alone or in addition to MMB3.19-primed or naive $CD4^+$ T cells (2×10^6). One day later, mice were challenged with 5×10^4 MMB1.10 (A) or MMB3.19 (B) cells intraperitoneally in 0.5 mL phosphate-buffered saline (n = 5 for all groups). *P* values represent significance between groups receiving primed versus naive $CD4^+$ T cells.

leukemia-specific antigens shared by similarly derived yet phenotypically distinct myeloid tumor lines, attention was focused on a syngeneic murine BMT model to eliminate any allogeneic contributions to the GVL response. The findings demonstrate that all 3 of the myeloid leukemia lines tested possess 1 or more common leukemia-specific antigens and are capable of cross-immunizing. MMB3.19-primed T-cell populations (unfractionated or $CD4^+$) exhibited reactivity to all 3 tumor lines in both in vitro assays (ELISPOT and ^{51}Cr release cytolysis) and in vivo GVL survival assays. Interestingly, although in vitro experiments indicated equivalent MMB3.19-primed antitumor responses against MMB1.10 and MMB2.18 stimulators, an in vivo cross-protective GVL effect against MMB2.18 challenge was far less evident than the effect against MMB1.10 challenge (Figures 4 and 5). Anti-MMB2.18 responses became more evident when increased numbers of $CD4^+$ T cells and a lower tumor burden were used in the model. In general, there may be several factors responsible for such a difference in GVL activity, including fewer shared antigens (or lower expression levels) on MMB2.18 cells, differential antigen-presenting capabilities or costimulatory potential of the tumor cells, or varying production of inhibitory cytokines. Concerning antigen stimulation capacity, $CD4^+$ T cells from B6 mice presensitized in vivo with irradiated MMB1.10, MMB2.18, or MMB3.19 cells proliferated in vitro equivalently upon restimulation with each of the tumor lines, demonstrating that all lines were potentially cross-stimulatory (data not shown). In addition, semiquantitative reverse transcription–polymerase chain reaction analysis determined that all 3 MMB lines exhibited a similar pattern of messenger RNA (mRNA) expression of inhibitory cytokines—ie, tumor necrosis factor (TNF)-α^+, IL-10^+, and transforming growth factor-β^- (data not shown)—suggesting that they were unlikely to be responsible for the lower level of protection against MMB2.18 challenge. Furthermore, ELISPOT analysis of MMB3.19-primed $CD4^+$ T cells revealed similar patterns of T helper 1 (IFN-γ) and T helper 2 (IL-4) cytokine production when restimulated with each of the 3 tumor lines (Figure 3), suggesting that a differential skewing of the immune response is not a likely reason for the difference in GVL potency against MMB2.18.

An important role for $CD4^+$ T cells in GVL responses has been demonstrated in clinical studies, particularly in the case of CML [38], and is supported by similar findings in murine models [20,39-41]. $CD4^+$ T cells likely mediate GVL effects indirectly by providing cytokine help to $CD8^+$ T cell, NK cell, and other inflammatory cell effectors, or directly via cytotoxicity mechanisms that include perforin/granzyme release, TNF-α release, or Fas/FasL interactions [42-44]. For 2 of the MMB tumors used in the current study (MMB1.10 and MMB3.19), it has recently been demonstrated that $CD4^+$ T cells can effectively use both a perforin and FasL-mediated cytolytic mechanism to resist tumor growth

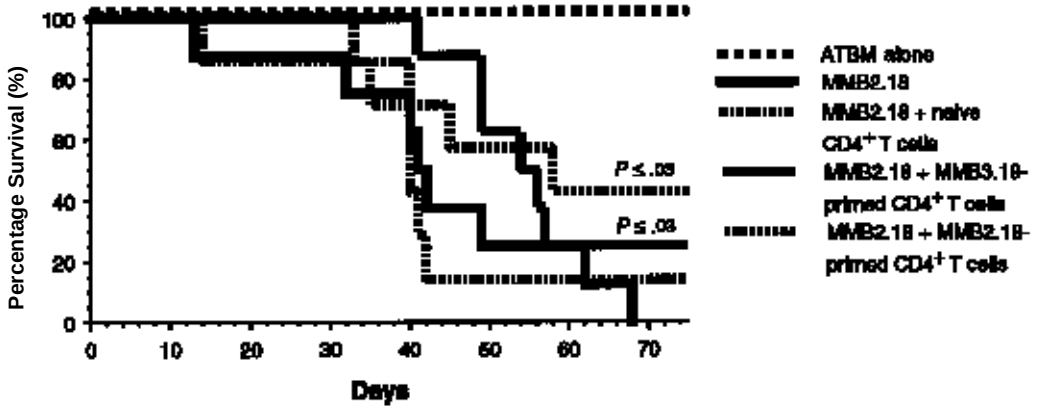

Figure 7. Graft-versus-leukemia protective effect of increased numbers of MMB2.18- or MMB3.19-primed donor $CD4^+$ T cells against MMB2.18 challenge. Lethally irradiated recipient B6 mice were injected intravenously with 2×10^6 donor anti-Thy-1–treated (T cell–depleted) bone marrow (ATBM) cells alone or in addition to MMB2.18- or MMB 3.19-primed or naive $CD4^+$ T cells (4×10^6). One day later, mice were challenged with 5×10^4 MMB2.18 cells intraperitoneally in 0.5 mL phosphate-buffered saline. All groups contained between 5 and 8 mice, and the *P* values represent significance between groups receiving MMB2.18- or MMB3.19-primed versus naive $CD4^+$ T cells.

[45,46]. Yet, despite the GVL potential of $CD4^+$ T cells, few MHC class II–restricted leukemia antigens have been found to date [47,48], perhaps largely because of the relative difficulty in developing $CD4^+$ antitumor T-cell lines. Therefore, efforts must be made to fully appreciate the scope of these responses and to exploit the advantages of this antileukemic immune component.

Given that autologous and syngeneic grafts can be manipulated to mediate GVL effects with and without a concomitant, autoimmune-like GVHD, it is probable that tumor-specific (non–allogeneic-based) antigens do exist [49-52]. However, it is not certain whether antigens representing overexpressed, normally occurring antigens or truly novel antigens arising from unique secondary mutations are responsible for these tumor-specific responses. An example of an overexpressed normal self-protein found in myeloid leukemia cells is proteinase-3 [32], whereas examples of novel mutated genes in leukemia include t(9;22) in CML and t(15;17), t(8;21), and inv16 in AML [53-56]. MMB leukemia lines probably contain overexpressed normal proteins due to the presence of cross-immunogenic determinants and the method of development (introduction of c-*myc*, a potent transcription factor). In regard to this possibility, syngeneic transplants using MMB3.19-primed T cells exhibit no overt pathology as evidence of an autoimmune-like reaction (recipients have normal weight gain, posture, coat appearance, activity level). In addition, ^{51}Cr release assays using MMB3.19-primed CTL effectors revealed specific lysis of MMB3.19 cells but no significant lysis of either B6 concanavalin A or lipopolysaccharide blasts, suggesting that the target antigens were not ubiquitously expressed on other hematopoietic cells. However, we have not ruled out the possibility that novel mutations have given rise to targetable determinants. Our model of myeloid leukemia cross-protection is an important first step in further elucidating the existence of shared tumor-specific antigens. Further studies in this system will enable us to characterize the shared determinants present in these distinct, oncogenically derived myeloid leukemia lines.

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