AD_____

Award Number: DAMD17-03-1-0311

TITLE: Mechanisms of Graft-vs.-Leukemia Against a Novel Murine Model of Chronic Myelogenous Leukemia

PRINCIPAL INVESTIGATOR: Warren D. Shlomchik, M.D.

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, Connecticut 06520-8047

REPORT DATE: July 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

BEST AVAILABLE COPY

20040922 026

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual (1 Jul 2003 - 30 Jun 2004)

4. TITLE AND SUBTITLE
Mechanisms of Graft-vs.-Leukemia Against a Novel Murine Model of Chronic Myelogenous Leukemia

5. FUNDING NUMBERS
DAMD17-03-1-0311

6. AUTHOR(S)
Warren D. Shlomchik, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University School of Medicine
New Haven, Connecticut 06520-8047

E-Mail: Warren.shlomchik@yale.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Our objective is to understand the immunobiology underlying the differential sensitivity of chronic phase and blast crisis CML. Our data thus far support the hypothesis that GVL against mCP-CML can be mediated by redundant processes, and that impairment of an individual pathway is insufficient to prevent GVL. We hypothesize that GVL against BC-CML is less forgiving than that against CP-CML, and that multiple effector pathways must act in concert for effective GVL. In the last year we have created BC-CML in B6 mice and have established the basic features of the model: 1) survival versus cell dose; 2) that GVL requires alloantigen differences; 3) that GVL can be mediated by unfractionated lymph node cells; 4) that GVL can be mediated by purified CD4 or CD8 cells, but that large doses are required. We are currently creating gene-deficient BC-CML and anticipate rapid progress this year in identifying key effector mechanisms and modes of antigen presentation.

14. SUBJECT TERMS
Graft-vs.-Leukemia, immunotherapies, blast crisis, tumor immunology, ag.presentation

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....7

Reportable Outcomes.....7

Conclusions.....7

References.....7

Appendices.....8

I. Introduction.

In this first year of funding, we have established the fundamental aspects of our GVL against murine blast crisis chronic myelogenous leukemia (mBC-CML) model which we anticipate will allow for rapid progress in the following two years. Key research accomplishments are described below. We have also published our work on T cell effector mechanisms of GVL against murine chronic phase CML (mCP-CML) ((1) and appendix). We had a technical problem in making high titer retrovirus, a technique key for this work, that took us several months to resolve. After much effort, we have resolved this problem via the introduction of a new retroviral packaging cell line (PLAT-E cells; (2)). Nevertheless, we are essentially on time for Tasks as defined in the approved "Statement of Work".

II. Body

A. Aim 1 Tasks.

1. Establish murine blast crisis CML (mBC-CML) in B6 mice.

a) Generate appropriate retrovirus.

b) Infect B6 progenitors and transplant into B6 mice

c) Phenotype leukemia

d) Secondary transplants

In our Preliminary Data we showed GVL data against BC-CML in the B10.D2→BALB/c major histocompatibility complex (MHC) identical but multiple minor histocompatibility antigen (miHA) disparate GVHD model. However, this BC-CML only had a marker gene (EGFP) linked to the NUP98/HOXA9 (NH) containing retrovirus. We wanted the ability to separately ensure expression of the p210 gene as well as clinical data suggest that its expression remains important for blast crisis physiology (3). We also proposed doing many experiments in the C3H.SW→B6 transplant model, and as such we needed to create mBC-CML in B6 mice.

To create NH/EGFP and p210/NGFR retrovirus we transfected the BOSC retroviral packaging cell line as described and titered virus on 3T3 cells. At a 1/10 dilution, we were able to infect 6% and 9% of 3T3 cells with each retrovirus, respectively. Procedures for infecting BM are as in the enclosed manuscript. Briefly, to create mBC-CML in B6 mice, B6 mice received 5-fluorouracil (5-FU) and BM cells were harvested 4 days later. Cells underwent spin infection in which 50% of the volume was NH retrovirus and p210/NGFR virus comprised 4%-1% of the total volume. We used relatively low titers of p210/NGFR to minimize the chance that cells would be infected with this virus but not the NH virus. As controls, some BM was infected with only NH or p210 retrovirus. Infected cells were then injected into sublethally irradiated B6 mice. In data not shown, all mice infected with only p210 retrovirus died from mCP-CML. In contrast, mice infected with low titer p210 and higher titer NH developed $EGFP^+NGFR^+$ blasts with little expression of myeloid markers as measured by flow cytometry of peripheral blood. Recipients of BM infected only with NH retrovirus did not develop leukemia during the approximate 60 day observation period as has been reported.

When mice developed palpable splenomegaly, they were sacrificed and spleen cells were analyzed by flow cytometry and frozen for future use. Shown in Figure 1A are flow cytometry results from two representative recipients of p210 and NH infected BM. (each column is data from an individual spleen) . Note that nearly all splenocytes are $EGFP^+/NGFR^+$ with only a minority of cells expressing CD11b. Cells from spleens with the highest numbers and purity of $EGFP^+/NGFR^+$/lineage- cells were used in a limiting dilution secondary transfer experiment. Sublethally irradiated B6 mice received 100,000 to 1000 cells from the primary mBC-CML mice. Greater than 90% of these mice developed mBC-CML, though recipients of fewer cells took longer to develop disease. Shown in Figure 1B is representative flow cytometry of splenocytes from a recipient o 10,000 cells. Note expression of EGFP and NGFR but little to no expression of CD11b, TERR119 and B220. We then froze splenocytes from recipients of the fewest numbers of cells for use in future experiments. .

In our grant submission we showed data with BALB/c mBC-CML created by Gary Gilliland. He used a p210-expressing retrovirus that did not coexpress a surface marker. We have since recreated mBC-CML in BALB/c cells using our p210/NGFR retrovirus (not shown).

2. Establish GVL against B6 mBC-CML

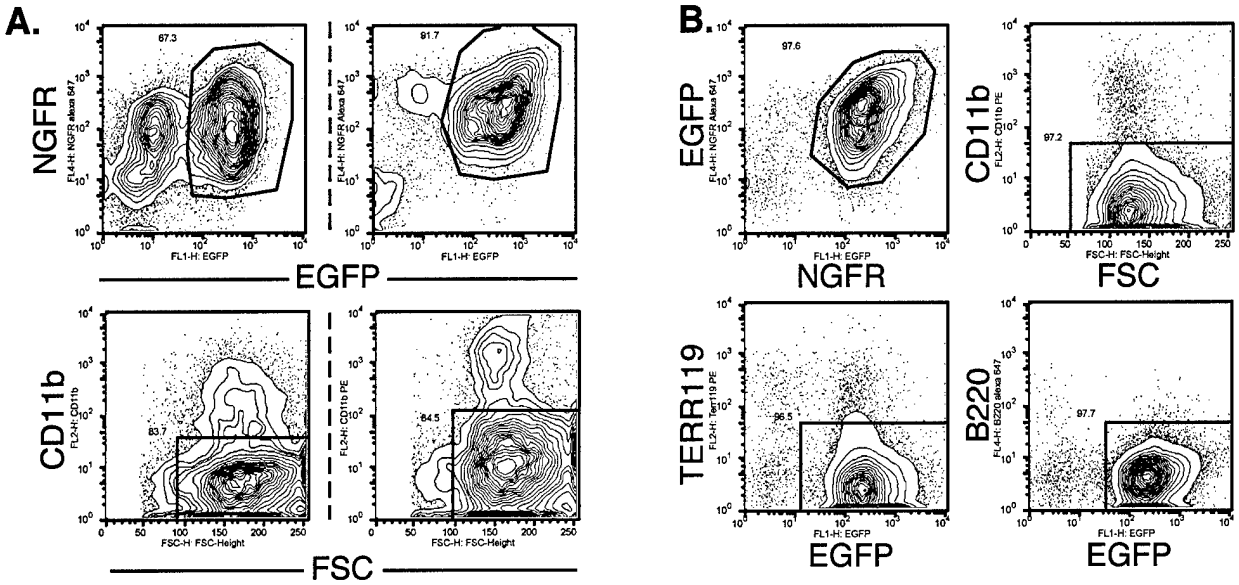

Figure 1. Phenotype of B6 mBC-CML cells. mBC-CML was generated in B6 mice as described in the body of the update. Shown in A (first row) are EGFP (linked to NH expression) and NGFR (linked to p210 expression) expression of splenocytes from 2 representative primary recipients. The second row shows CD11b expression of EGFP⁺NGFR⁺ cells. Each column is from an individual mouse. Primary mBC-CML cells were injected into sublethally irradiated B6 mice. Shown in B is NGFR and EGFP staining or splenocytes from a representative recipient. The upper right panel shows forward scatter and CD11b expression of cells in the gate in the upper left panel. The lower panels show expression of TERR119 and B220 expression versus EGFP. Key to note is that the vast majority of cells do not express lineage markers, consistent with their blast phenotype.

a) Survival Versus Dose of B6 BC-CML cells

We aim to use the C3H.SW (H-2^b)→B6 (H-2^b) model for many of our studies as we have characterized this system very well (see reprints) and due to the availability of key gene deficient mice on B6. Prior to performing GVL experiments, we established the relationship between the number of infused mBC-CML cells and survival. B6 mice were lethally irradiated and reconstituted with T cell depleted B6 bone marrow (BM) with 10^3 (20 mice), 10^4 (20 mice), 10^5 (5 mice) or 10^6 (5 mice) live BC-CML cells. Survival data is shown in Figure

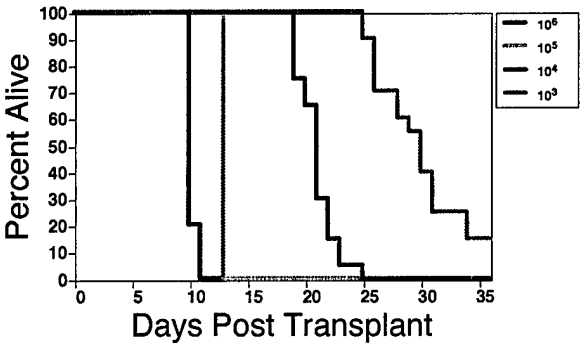

Figure 2. Survival versus dose of mBC-CML. Sublethally irradiated B6 mice received the indicated doses of mBC-CML cells and were followed for survival. All deaths were due to mBC-CML.

2. We chose to perform further experiments with approximately 10^4 BC-CML cells/mouse.

b) GVL mediated by unfractionated LN cells requires alloantigen differences.

To determine the minimum number of LN cells required for GVL and to exclude the possibility that GVL was directed solely against retrovirally transferred genes, we irradiated B6 hosts and performed syngeneic or allogeneic transplants. Irradiated hosts received either T cell depleted B6 BM cells with 5, 10 or 15×10^6 B6 LN cells or received T cell depleted C3H.SW BM and 5, 10 or 15×10^6 C3H.SW LN cells (Figure 3). All recipients of C3H.SW LN cells had prolonged survival and in fact cleared their BC-CML (all deaths were to GVHD). In contrast, recipients of syngeneic B6 LN cells died with similar kinetics to recipients of only T cell depleted B6 BM cells. Thus GVL against BC-CML requires miHA differences.

3. Determine the roles for CD4 and CD8 cells.

4. Determine the minimum number of T cells for GVL.

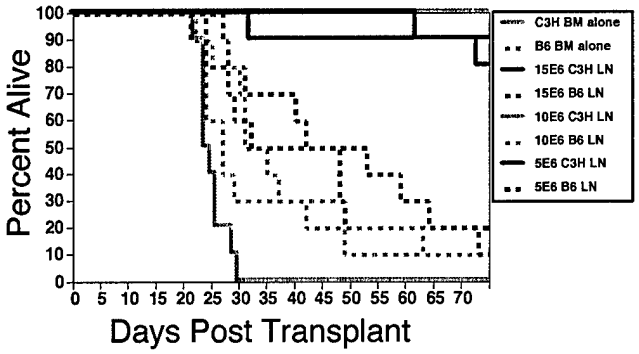

Figure 3. Allogeneic but not syngeneic lymphocytes mediate GVL against mBC-CML. B6 recipients were irradiated and received 15,000 mBC-CML cells. Mice received either T cell depleted B6 BM and graded doses of B6 LN cells or T cell depleted C3H.SW BM and graded doses of C3H.SW LN cells. Shown is total survival. All deaths were from mBC-CML. Key to note is that there was no anti-leukemic effect in the syngeneic transplants and thus GVL absolutely requires alloantigen differences.

a) GVL can be mediated by only CD8 cells.

To ask if purified CD8 cells can mediate GVL against mBC-CML, B6 hosts were irradiated and reconstituted with 15,000 mBC-CML cells, T cell depleted C3H.SW BM, with $4x10^6$, $2x10^6$, $1x10^6$, $5x10^5$ or $2.5x10^5$ purified CD8 cells. One group received no T cells and one group received $5x10^6$ C3H.SW LN cells containing approximately 10^6 CD8 cells and $2.5x10^6$ CD4 cells. Only recipients of $4x10^6$ CD8 cells were completely protected from death by BC-CML (Figure 4; all deaths were due to GVHD; confirmed by a negative assay of peripheral blood prior to death, by the absence of splenomegaly or both). All other deaths were due to BC-CML. These data differ from what we have observed in our GVL against chronic phase CML model (mCP-CML), in which mice that die from mCP-CML die between days 18-20, but that as few as 250,000 CD8 cells provide prolongation of survival and $1\text{-}2x10^6$ cells prevents death from mCP-CML (appendix). Thus this preliminary data suggests that mBC-CML is less sensitive to CD8-mediated GVL than is mCP-CML and this fits with clinical data.

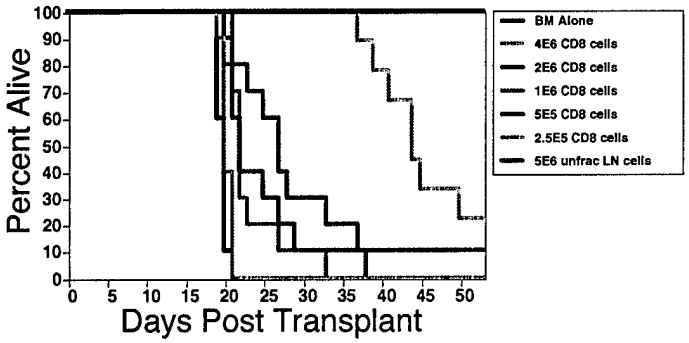

Figure 4. CD8 cells alone can mediate GVL against mBC-CML. B6 mice were transplanted as described in the report body (9-15 mice per group). All recipients of $4x10^6$ C3H.SW $CD8^+$ T cells were free of leukemia, though the majority of mice ultimately died from or were sacrificed due to GVHD.

b) GVL can be mediated only by CD4 cells. To ask if purified CD4 cells can mediate GVL against mBC-CML, B6 hosts were irradiated and reconstituted with 15,000 BC-CML cells, T cell depleted C3H.SW BM and $4x10^6$, $2x10^6$ or $1x10^6$ C3H.SW CD4 cells. One group received no T cells and one group received $5x10^6$ C3H.SW LN cells containing approximately 10^6 CD8 cells and $2.5x10^6$ CD4 cells. We saw prolonged survival in all CD4 recipients as compared to mice that received BC-CML and no T cells (Figure 5). All deaths were due to BC-CML.

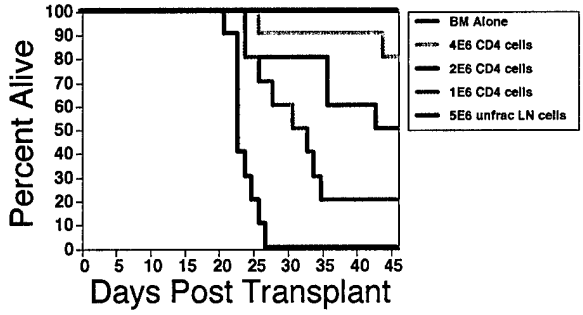

Figure 5. CD4 cells alone can mediate GVL against mBC-CML. B6 mice were transplanted as described in the report body (10-15 mice per group). Prolonged survival was seen in all recipients of C3H.SW CD4 cells in a dose-dependent fashion. All deaths were due to mBC-CML.

B. Current experiments and future directions. Now that we have established the basics of the GVL model in the C3H.SW→B6 strain, we are working on creating B6 gene-deficient BC-CML. As described above, this work was somewhat delayed due to difficulties making high titer retrovirus, a problem which has now been resolved. We anticipate having created the following gene deficient mBC-CMLs by the end of the summer:

MHCII$^-$ (IA$^{b-/-}$), Fas^{lpr}, TNFR1/TNFR2$^{-/-}$ and MHCI$^-$ (β2M$^{-/-}$). Once these leukemias are created we will explore CD4 and CD8 effector mechanisms. We are also breeding the necessary gene deficient donor and host strains for studies on the relative roles of donor and host APCs in this model.

III. Key Results.

A. Establishment of mBC-CML in B6 mice.

B. Established mBC-CML cell dose versus survival curve.

C. Established GVL with lymph node cells in the C3H.SW→B6 strain pairing.

D. Demonstrated that retrovirally encoded proteins were insufficient as target antigens.

E. Established that GVL can be mediated by purified C3H.SW CD4 or CD8 cells.

F. Evaluated survival in recipients of graded doses of CD4 or CD8 C3H.SW CD4 or CD8 cells.

IV. Reportable Outcomes

None yet

V. Conclusions.

In the first year of this application we have established the fundamental aspects of our model as defined in Aim 1 and we are now poised to answer the key biological questions: 1) What are the mechanisms of killing?; and 2) What are the antigen presenting cell requirements?; and 3) How do these compare to what we have established and are continuing to establish for GVL against murine chronic phase CML.

VI. References

1. Matte, C.C., J. Cormier, B.E. Anderson, I. Athanasiadis, J. Liu, S.G. Emerson, W. Pear, and W.D. Shlomchik. 2004. Graft-versus-leukemia in a retrovirally induced murine CML model: mechanisms of T-cell killing. *Blood* 103:4353-4361.
2. Morita, S., T. Kojima, and T. Kitamura. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 7:1063-1066.
3. Sawyers, C.L., A. Hochhaus, E. Feldman, J.M. Goldman, C.B. Miller, O.G. Ottmann, C.A. Schiffer, M. Talpaz, F. Guilhot, M.W. Deininger, T. Fischer, S.G. O'Brien, R.M. Stone, C.B. Gambacorti-Passerini, N.H. Russell, J.J. Reiffers, T.C. Shea, B. Chapuis, S. Coutre, S. Tura, E. Morra, R.A. Larson, A. Saven, C. Peschel, A. Gratwohl, F. Mandelli, M. Ben-Am, I. Gathmann, R. Capdeville, R.L. Paquette, and B.J. Druker. 2002. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 99:3530-3539.

Graft-versus-leukemia in a retrovirally induced murine CML model: mechanisms of T-cell killing

Catherine C. Matte, James Cormier, Britt E. Anderson, Ioanna Athanasiadis, Jinli Liu, Stephen G. Emerson, Warren Pear, and Warren D. Shlomchik

The graft-versus-leukemia (GVL) effect, mediated by donor T cells, has revolutionized the treatment of leukemia. However, effective GVL remains difficult to separate from graft-versus-host disease (GVHD), and many neoplasms are GVL resistant. Murine studies aimed at solving these problems have been limited by the use of leukemia cell lines with limited homology to human leukemias and by the absence of loss-of-function leukemia variants. To address these concerns, we developed a GVL model against murine chronic-phase chronic myelogenous leukemia (mCP-CML) induced with retrovirus expressing the bcr-abl fusion cDNA, the defining genetic abnormality of chronic-phase CML (CP-CML). By generating mCP-CML in gene-deficient mice, we have studied GVL T-cell effector mechanisms. mCP-CML expression of Fas or tumor necrosis factor (TNF) receptors is not required for CD8-mediated GVL. Strikingly, maximal CD4-mediated GVL requires cognate interactions between CD4 cells and mCP-CML cells as major histocompatibility complex–negative (MHC II$^{-/-}$) mCP-CML is relatively GVL resistant. Nevertheless, a minority of CD4 recipients cleared MHC II$^{-/-}$ mCP-CML; thus, CD4 cells can also kill indirectly. CD4 GVL did not require target Fas expression. These results suggest that CP-CML's GVL sensitivity may in part be explained by the minimal requirements for T-cell killing, and GVL-resistance may be related to MHC II expression. (Blood. 2004;103:4353-4361)

© 2004 by The American Society of Hematology

Introduction

The graft-versus-leukemia (GVL) effect has revolutionized the treatment of leukemia and lymphoma.[1-9] In GVL, donor T cells recognize host antigens as non-self and thereby attack neoplastic cells. Chronic-phase chronic myelogenous leukemia (CP-CML) is the prototypical GVL-sensitive neoplasm in which complete molecular remissions are achieved in nearly 80% of patients with CP-CML who receive donor leukocyte infusions (DLIs).[2-4] Despite this success, alloimmune therapy for cancer has 2 principle drawbacks. First, many neoplasms, including CML in blast crisis (BC-CML), are relatively GVL resistant.[2,3,10-22] The basis for this differential susceptibility, even between such closely related leukemias as CP-CML and BC-CML, is unknown. Second, GVL has been difficult to separate from graft-versus-host disease (GVHD), the broad attack by donor T cells on recipient tissues. These 2 problems remain unsolved even though they have been recognized for nearly 50 years.[23]

A major obstacle to overcoming these limitations has been the absence of murine models of clinically relevant GVL-sensitive leukemias. Most murine GVL studies have used cell lines with limited resemblance to human leukemias and even less relevance to CP-CML, which is the most GVL sensitive of human leukemias.[24-45] In addition, most studies have used major histocompatibility complex (MHC)–incompatible models, whereas most human allogeneic hematopoietic stem cell transplantations (alloSCTs) are MHC matched and multiple minor histocompatibility antigen (miHA) mismatched.

A detailed mechanistic understanding of GVL against a clinically relevant murine leukemia would be an important step in understanding differential GVL sensitivity and in developing better strategies for separating GVL from GVHD. To do so, we have adopted a murine model of CP-CML (murine chronic-phase CML; mCP-CML) generated by way of retroviral insertion into murine hematopoietic progenitors of the bcr-abl (p210) fusion cDNA, the defining genetic abnormality in human CP-CML.[46-50] When irradiated mice receive p210-transduced hematopoietic progenitors, a myeloproliferative disease ensues marked by a high peripheral white blood cell (WBC) count and extensive infiltration of bone marrow (BM) and spleen. Most peripheral WBCs are maturing granulocytes with few blasts, whereas the spleen and bone marrow are replaced by myeloid cells in varying states of differentiation. mCP-CML is oligoclonal and is dependent on bcr-abl tyrosine kinase activity.[48,51] A difference between mCP-CML and human CP-CML is that mCP-CML mice succumb to leukemic infiltration of the lung.

A major advantage of this retroviral model is that mCP-CML can be induced in hematopoietic progenitors from any mouse

From the Section of Medical Oncology, Yale University School of Medicine, New Haven, CT; the Section of Immunobiology, Yale University School of Medicine, New Haven, CT; the Section of Hematology and Oncology, University of Pennsylvania School of Medicine, Philadelphia; the Department of Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia; and the Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst.

Submitted November 3, 2003; accepted February 8, 2004. Prepublished online as *Blood* First Edition Paper, February 24, 2004; DOI 10.1182/blood-2003-10-3735.

Supported by grants (R01 HL66279, R01-CA-96943, K08-HL03979) (W.D.S).

C.C.M. and J.C. contributed equally to this study.

Reprints: Warren D. Shlomchik, Yale University School of Medicine, Section of Medical Oncology, PO Box 208032, New Haven, CT 06520; e-mail: warren.shlomchik@yale.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2004 by The American Society of Hematology

strain, including mice genetically deficient in pathways that might be important for GVL sensitivity. Thus, we have been able to study GVL against gene-deficient mCP-CML and in multiple strain pairings. Here, we use mCP-CML to demonstrate T-cell effector mechanisms in GVL against mCP-CML in clinically relevant MHC-matched, miHA-mismatched models.

Materials and methods

Mice

All mice were between 7 and 10 weeks of age. Male or female C3H.SW, B10.BR, and AKR/J mice were obtained from the Jackson Labs (Bar Harbor, ME). B6 and BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). B6 TNFR1/R2$^{-/-}$ mice (TNFR$^{-/-}$) were created by us by crossing TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice (C57BL/6-Tnfrsf1a^{tm1Imx} and B6.129S2-Tnfrsf1b^{tm1Mwm}; Jackson Labs). These mice were screened by way of polymerase chain reaction for both the wild-type and knock-out alleles. RAG$^{-/-}$/Fas^{lpr} mice were generated by crossing B6 RAG$^{-/-}$ and B6-lpr mice (Jackson Labs). Mice were screened by flow cytometry of peripheral blood looking for the absence of B cells, T cells, and Fas expression. In the text, these mice are referred to as Fas^{lpr}. B6 IA beta chain–deficient mice (IA$^{b-/-}$) were obtained from Taconic (Germantown, NY).

Retrovirus production

MSCV2.2 expressing the human p210 cDNA and a nonsignaling truncated form of the human low-affinity nerve growth factor receptor driven by an internal ribosome entry site (Mp210/NGFR) was a gift from Warren Pear. Retroviral supernatants were generated by way of transient transfection of the BOSC ecotropic retrovirus-producing line as described except for use of lipofection instead of calcium phosphate transfection.[52] Briefly, on day −1, 4×10^6 BOSC cells were seeded on 6-cm plates in Dulbecco modified Eagle (DME) with 10% fetal calf serum (FCS). On day 0, the cells were transfected with 7.5 μg Mp210/NGFR using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Thirty-six hours after transfection the media was changed (4 mL). Retroviral supernatants were harvested 12 hours later, filtered through 0.45 μ screens, and stored in aliquots at −80°C. Virus was titered on 3T3 cells as described.[52] Infected cells were enumerated with use of flow cytometry to identify NGFR-expressing cells.

Hematopoietic progenitor infections

To create p210-infected progenitors, recipient strain or gene-deficient mice backcrossed to the recipient strain were injected on day −6 with 5 mg 5-fluorouracil (5FU; Pharmacia & Upjohn, Kalamazoo, MI). On day −2, bone marrow (BM) cells harvested from femurs and tibias were cultured overnight at 2×10^6 nucleated cells/mL in prestimulation media (DME, 15% fetal bovine serum [FBS], 5% WEHI culture supernatant, interleukin-3 [IL-3; 6 ng/mL], IL-6 [10 ng/mL], and stem cell factor [SCF; 10 ng/mL]; all cytokines were from Peprotech [Rocky Hill, NJ]). On day −1, cells underwent "spin infection" with p210-expressing retrovirus. Cells were resuspended at 2×10^6/mL in prestimulation media with the addition of retroviral supernatant, polybrene (4 μg/mL; Sigma, St Louis, MO), and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 100 mm). The final dilution of retrovirus was calculated to be that which would infect 30% of 3T3 cells. BM cells were divided into aliquots in 6-well plates (4 mL/well) and spun in a swinging bucket rotor at 1000*g* for 90 minutes at 37°C. The plates were returned to the incubator for 2 hours. Cells were then harvested and cultured overnight in prestimulation media without polybrene or retrovirus. On day 0, the spin infection was repeated. Cells were harvested, washed, counted, and resuspended in injection buffer (phosphate-buffered saline [PBS], 100 mm HEPES).

Cell purifications

CD8 cells were purified by way of depletion from lymph node (LN) cells. LNs were crushed through metal screens, and red blood cells (RBCs) were lysed with ACK (0.15 M NH_4CL, 1 mM $KHCO_3$, and 0.1 mM Na_2 EDTA). Cells were washed and stained with biotin-conjugated antibodies against CD4, B220, and CD11b. Cells were washed and incubated with streptavidin-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA) and separated on an AutoMACS (Miltenyi Biotec) magnetic cell separator. CD4 T cells were purified from LN by using the same protocol as for CD8$^+$ T cells, except anti-CD4 was omitted and biotin-conjugated anti-CD8 was used in its place. BM was flushed from tibias and femurs, followed by RBC lysis with ACK. BM T-cell depletion was performed with anti-Thy1.2–conjugated magnetic beads (Miltenyi Biotec) and the AutoMACS.

Transplantation protocol

In these experiments, all recipients were wild type. On day 0, B6 and AKR hosts received 900 cGy in 2 450-cGy fractions. BALB/c mice received 800 cGy in two 400 cGy fractions. Recipients were reconstituted with 5 to 7×10^6 T-cell–depleted donor type BM with 7×10^5 or 1×10^6 cells that underwent spin infection, with or without a source of donor T cells. In some experiments, BM cells from gene-deficient mice on a B6 background were infected to generate gene-deficient mCP-CML. Mice were followed for the development of mCP-CML, manifest by increased respiratory rate, hunched posture, and death. In most experiments mice were bled weekly for analysis of WBC counts and the presence of NGFR$^+$ cells by flow cytometry.

Antibodies and flow cytometry

Antibodies used to characterize mCP-CML were Gr-1 fluorescein isothiocyanate (FITC), CD11b FITC, TER119, phycoerythrin (PE), Thy1.2 FITC (all from Pharmingen, San Diego, CA); B220 (clone 6B2, multiple colors; lab conjugated); and biotin-conjugated anti-NGFR (clone 20.4; lab conjugated). Antibodies used for cell separations were anti-CD4 (clone GK1.5; lab conjugated) and anti-CD8 (clone TIB105; lab conjugated). Whole blood was stained with appropriate antibodies, followed by RBC lysis with ACK. Propidium iodide was added to exclude dead cells. Cells were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Statistics

P values for differences in survival were calculated by log-rank Mantel Cox if events occurred in the compared groups or by chi-square test if there were no events.

Results

GVL in 3 different MHC identical, multiple miHA incompatible donor/recipient pairs

We initially tested GVL in the C3H.SW (H-2^b)→B6 (H-2^b) MHC-identical, multiple miHA disparate strain pairing. We chose an experimental design intended to model GVL against residual leukemia that survives lethal conditioning (model is described in Figure 1). B6 recipients were irradiated and reconstituted with Mp210/NGFR-infected B6 BM cells, T-cell–depleted C3H.SW BM, with or without C3H.SW unfractionated lymph node (LN) cells. Addition of 1.5×10^7 LN cells resulted in complete protection from death by mCP-CML (Figure 2). Mice were killed between 5 and 6 weeks after transplantation, and spleens from all LN recipients were free of NGFR$^+$ cells (not shown). Significant GVHD did not develop, as we find in most experiments in this strain pairing when a mix of CD4 and CD8 cells is used.[53]

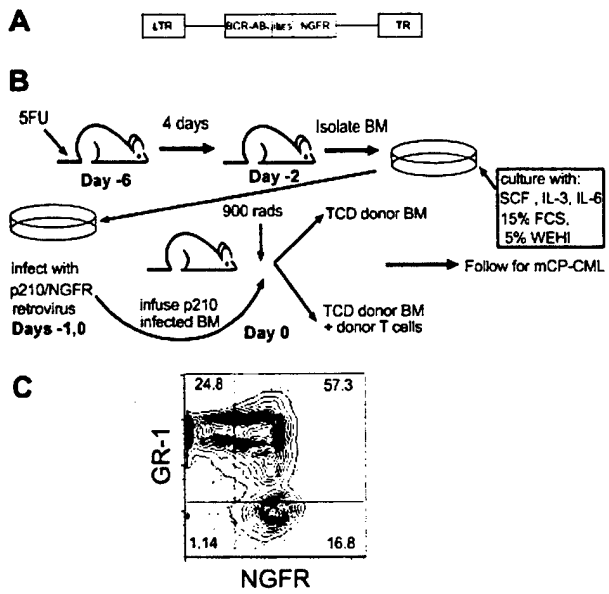

Figure 1. GVL anti-mCP-CML experimental design. (A) Retroviral construct (Mp210/NGFR). LTR indicates long terminal repeat; IRES, internal ribosome entry site. (B) GVL against mCP-CML model. On day −6, mice were injected intravenously with 5 mg 5-FU, and after 4 days BM was isolated and prestimulated (day −2) with SCF, IL-3, and IL-6. Cells underwent spin infection with Mp210/NGFR retrovirus on days −1 and 0. Cells were infused into irradiated syngeneic wild-type recipients along with T-cell–depleted (TCD) BM from allogeneic donors, with or without donor T cells. (C) Representative flow cytometry of peripheral blood from a mouse with mCP-CML.

A key feature of GVL against human CP-CML is that it is effective regardless of HLA type. That is, no individual HLA molecule has been associated with a better or worse outcome after alloSCT. For our GVL model to be representative of human GVL against CP-CML, we should also observe GVL in multiple MHC-matched, miHA-mismatched donor/recipient pairs. We therefore tested GVL in 2 additional strain pairings in which we have studied GVHD: B10.D2 (H-2^d)→BALB/c (H-2^d)[54] and B10.BR (H-2^k)→AKR (H-2^k).[55] GVL was highly effective in both strain pairings (Figure 2). Thus, as in human GVL against CP-CML, GVL against mCP-CML is active on different MHC backgrounds.

p210 and NGFR are insufficient as target antigens

Because both p210 and NGFR are human proteins, it was possible that epitopes from these proteins, and not miHAs, were targeted by donor T cells. In principle, these epitopes would be similar to the epitopes generated around the junction between bcr and abl which would be non-self in humans with CP-CML. Nevertheless, we did experiments to ask whether p210 and NGFR alone would be sufficient for GVL. To do so we performed a syngeneic transplantation in which the only non-self antigens were derived from p210 and NGFR. B6 recipients were irradiated and reconstituted with Mp210/NGFR-infected B6 progenitors, B6 T-cell–depleted BM, with or without unfractionated B6 LN cells. As a positive GVL control, we simultaneously performed a parallel C3H.SW→B6 experiment. Syngeneic LN cells provided no protection from mCP-CML, and all syngeneic recipients died between days 16 and 18 from mCP-CML (not shown). In contrast, all evaluable C3H.SW→B6 mCP-CML/LN survived without the development of mCP-CML. Therefore, even if p210 and NGFR are included among target antigens, at a minimum, miHA differences are absolutely required for GVL. This finding is consistent with the high relapse rate seen in identical sibling transplantations for CP-CML.[56]

GVL can be mediated by CD4+ or CD8+ T cells

To ask whether CD8 cells alone can mediate GVL against mCP-CML, we performed GVL experiments in the C3H.SW→B6 pairing with 3 to 4 × 10^6 purified CD8 cells, a number similar to that contained in the unfractionated LN cell experiments. In 2 independent experiments, no CD8 recipients died of mCP-CML, although some mice died of GVHD without evidence of leukemia (data not shown). To better evaluate the CD8 GVL response, we tested the efficacy of graded doses of donor C3H.SW CD8 cells by using 4 × 10^6, 2 × 10^6, 1 × 10^6, 5 × 10^5, 2.5 × 10^5, or 0 purified C3H.SW CD8 cells (Figure 3). As few as 2.5 × 10^5 donor CD8 cells were able to prolong survival, but most of these mice eventually succumbed to mCP-CML (Figure 3A). All recipients of 2 × 10^6 and 4 × 10^6 CD8 cells completely cleared their leukemia, although 3 of 5 recipients of 2 × 10^6 CD8 cells died of severe GVHD. Absence of leukemia was confirmed in killed mice by flow cytometry of peripheral blood, BM, and splenocytes. Mice that died spontaneously were scored as dying of leukemia if they had a positive assay of peripheral blood at more than 2 weeks after transplantation and an abnormal-sized spleen at necropsy.

Serial flow cytometric analysis of peripheral blood demonstrated that mCP-CML developed in all mice in all groups prior to eradication by donor CD8 cells (Figure 3B). Most of the CD8 recipients had NGFR+ cells in peripheral blood (PB) on day +31. None of 10 recipients of 2.5 and 5 × 10^5 CD8 cells cleared their leukemia, whereas 7 of 10 recipients of 1 × 10^6 and all recipients of 2 × 10^6 or 4 × 10^6 CD8 cells were free of leukemia when killed.

Like recipients of CD8 cells, in 3 independent experiments in the C3H.SW→B6 strain pairing, no recipients of 7 × 10^6 CD4 cells died of mCP-CML, and most mice had no evidence of residual NGFR+ cells (not shown). When we tested graded doses of C3H.SW CD4 cells (6 × 10^6, 4 × 10^6, 2 × 10^6, and 1 × 10^6), as few as 1 × 10^6 cells gave prolonged survival (Figure 4A). However, the only mice that cleared all NGFR+ cells were in the groups

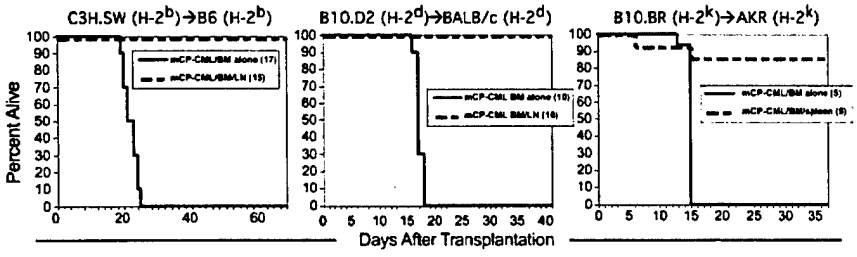

Figure 2. GVL in 3 different MHC-identical, miHA-disparate strain pairings. Transplantations were performed as described in "Materials and methods"; numbers of mice per group are in parentheses. Survival in C3H.SW (H-2^b)→B6 (H-2^b) (left panel; GVL mediated by LN cells), B10.D2 (H-2^d)→BALB/c (H-2^d) (middle panel; GVL mediated by LN cells), and B10.BR (H-2^k)→AKR (H-2^k) (right panel; GVL mediated by spleen cells).

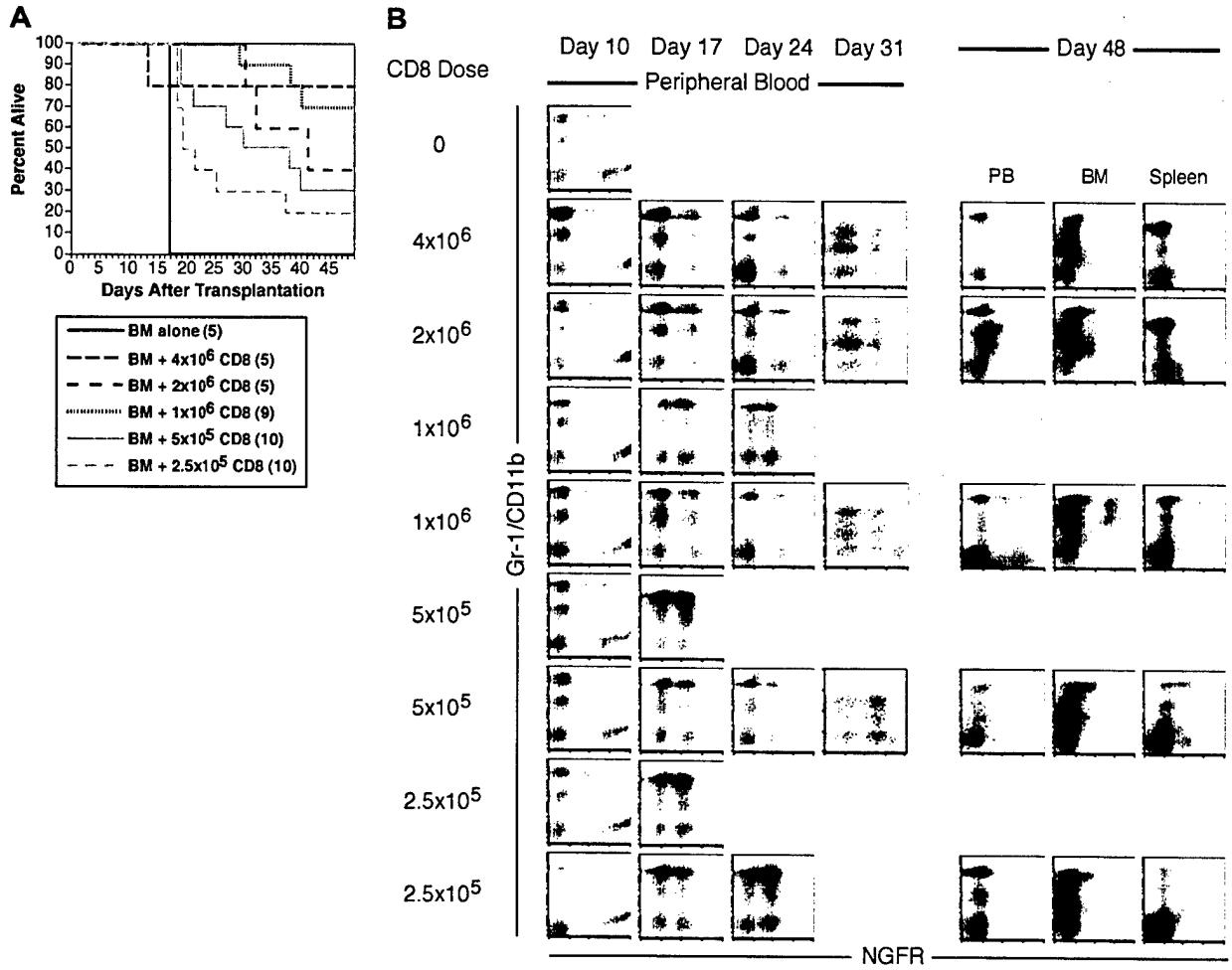

Figure 3. CD8 cells alone mediate GVL. B6 mice were irradiated and reconstituted with T-cell–depleted C3H.SW BM, B6 mCP-CML with 0, 2.5 × 10^5, 5 × 10^5, 10^6, 2 × 10^6, or 4 × 10^6 C3H.SW CD8+ T cells. (A) Survival. $P < .003$ for each CD8 recipient group versus BM alone. (B) Serial flow cytometry of peripheral blood. Mice were bled on days 10, 17, 24, and 31 after transplantation, and cells were stained with antibodies against the myeloid marker Gr-1 (except for day 31 when anti-CD11b was used) and anti-NGFR or an isotype control for NGFR. Shown are superimposed dot plots from anti-NGFR (red dots) and isotype for NGFR (blue dots) stained samples. Single-color dot plots indicate that an isotype control was not available. Each row is a representative single mouse. Results from 2 representative recipients of 1 × 10^6, 5 × 10^5, and 2.5 × 10^5 CD8 cells are shown to capture the types of responses we observed. Flow cytometry of blood, BM, and spleen cells at time of killing is presented for the same mice. Note that mCP-CML develops prior to its elimination by donor CD8 cells.

that received 6 × 10^6 or 4 × 10^6 donor CD4 cells. We also observed CD4-mediated GVL in the B10.D2→BALB/c donor/recipient pair (data not shown). As was the case with GVL mediated by only CD8 cells, all donor CD4 recipients developed mCP-CML prior to eradication by donor CD4 cells (Figure 4B).

CD8-mediated GVL is intact against Fas^{lpr} and TNFR1/R2$^{-/-}$ mCP-CML

Cytotoxic CD8+ T cells primarily kill by way of FasL and perforin/granzyme, although tumor necrosis factor α (TNF-α) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can also mediate target cell death.[57-60] Prior studies into GVL effector mechanisms have relied primarily on reagents that block a pathway (such as infusion of blocking antibodies to TNF-α) or on donor T cells deficient in a killing mechanism (TNF-α, perforin, or FasL-deficient T cells).[28,30,42,43] Both TNF-α and FasL have important roles in regulating T-cell responses.[61-64] Thus, results with these approaches might reflect effects on the development of the T-cell response and not only on the roles of these molecules on T-cell killing. We, therefore, chose to impair death receptors on mCP-CML cells. Irradiated B6 recipients were reconstituted with T-cell–depleted C3H.SW BM and Mp210/NGFR-infected progenitors from wild-type, Fas^{lpr}, or TNFR$^{-/-}$ B6 mice. One group of mice for each of the different mCP-CML types received 1 × 10^6 purified C3H.SW CD8 cells. We deliberately chose a dose of CD8 cells that does not result in 100% leukemia-free survival to minimize the possibility that we would miss an important contribution from an individual cytolytic pathway by infusing an overwhelming number of CD8 cells.

GVL was similar regardless of whether the mCP-CML cells lacked Fas or TNFR1/2 or expressed these molecules (Figure 5). Four of 14 recipients of wt CML and donor CD8 and 1 of 14 recipients of TNFR$^{-/-}$ or Fas^{lpr} died of leukemia; the remaining deaths were due to GVHD. Again, mCP-CML clearly developed in CD8 recipients prior to eradication (Figure 5B). All recipients of TNFR$^{-/-}$ and Fas^{lpr} mCP-CML and donor CD8 cells that survived until killing on day 42 after transplantation cleared all NGFR+ cells from blood, BM, and spleen. Similarly, 5 of 8 recipients of wt mCP-CML and donor CD8 cells completely cleared NGFR+ cells (not shown). Recipients of wild-type, TNFR$^{-/-}$, and Fas^{lpr} mCP-CML without donor CD8 cells died with similar kinetics and spleen weights, suggesting both that comparable numbers of infected

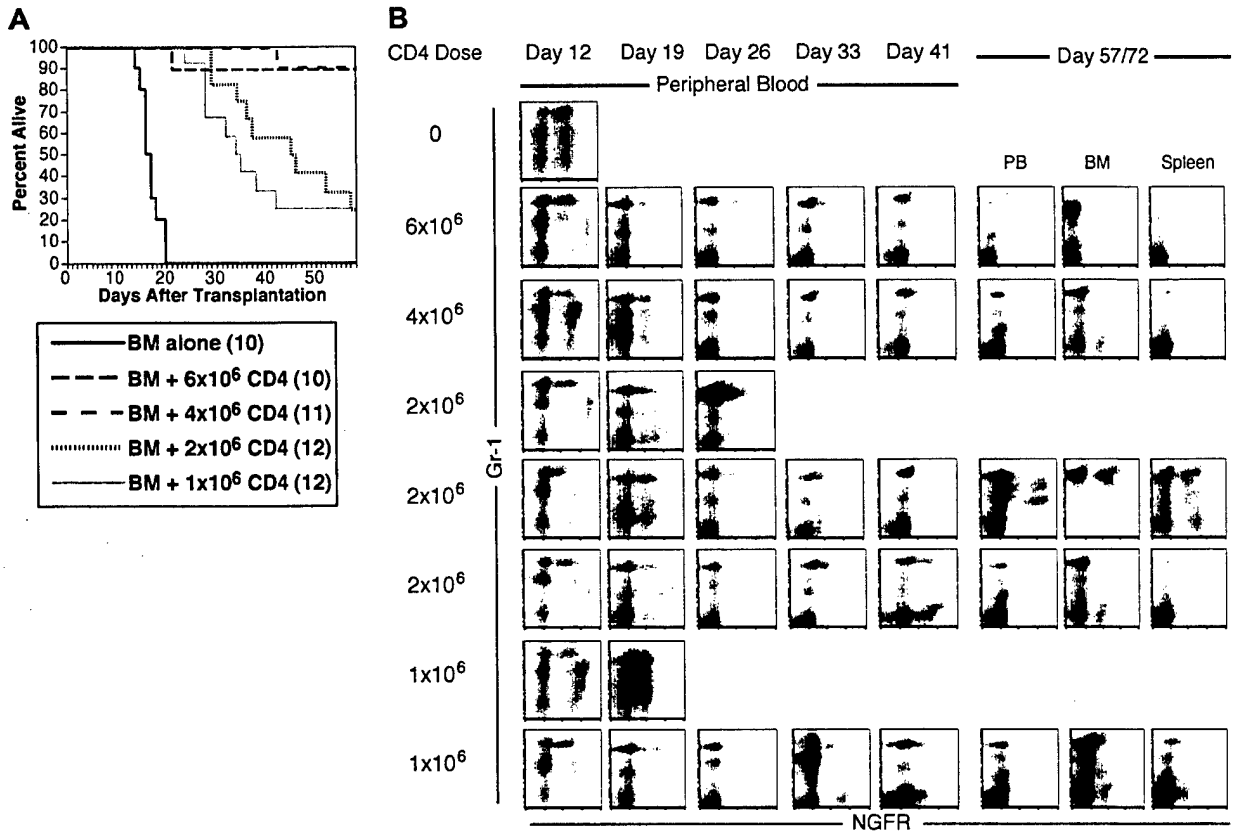

Figure 4. CD4 cells alone mediate GVL. B6 mice were irradiated and reconstituted with T-cell–depleted C3H.SW BM, B6 mCP-CML with 0, 10^6, 2×10^6, or 4×10^6 C3H.SW $CD4^+$ T cells. (A) Survival. $P < .0001$ comparing BM alone versus 1×10^6 CD4 cells analyzed to day 43 after transplantation. (B) Serial flow cytometry of peripheral blood. Mice were bled and cells were stained as described in "Materials and methods." As in Figure 3, staining with anti-NGFR is shown in red, and isotype for NGFR is shown in blue. Isotype staining was not done on the sample with a single-color dot plot. Each row is a single mouse. Three and 2 representative mice are shown for the 2×10^6 and 1×10^6 CD8 doses to capture the types of responses we saw. The middle 2×10^6 mouse nearly clears mCP-CML at day 41, but it returns by day 72 after transplantation. Note that mCP-CML develops prior to its elimination by donor CD4 cells.

progenitors were infused and that the basic biology of mCP-CML was not effected by the gene deletions. This latter point is supported by the similar immunophenotype of gene-deficient and wild-type mCP-CML (Figure 5B).

Optimal CD4-mediated GVL requires cognate interactions with mCP-CML targets and is independent of target Fas expression

CD4 cells could mediate GVL against mCP-CML cells by direct, indirect, or both mechanisms. Cytotoxic CD4 cells (cytotoxic T lymphocytes; CTLs) that kill by way of FasL and perforin/granzyme are well described.[57,58,65,66] Such cells would require T-cell receptor (TCR)–mediated cognate interaction with MHC II-peptide complexes on mCP-CML targets. Alternatively, CD4 cells might act indirectly by way of activation of macrophages that present miHAs or by way of elaboration of cytokines after contacting antigen-presenting cells displaying host miHAs. To distinguish these possibilities, we asked if CD4 cells could mediate GVL against MHC II deficient (MHC II^-) mCP-CML. Because TCRs on CD4 cells recognize peptide antigen presented by MHC II, alloreactive CD4 cells would be unable to interact directly with MHC II^- mCP-CML cells. To create MHC II^- mCP-CML, we infected progenitors from B6 IA beta chain deficient mice ($IA^{b-/-}$), which do not express MHC II.[67] GVL was significantly reduced against MHC II^- mCP-CML in 2 independent experiments (Figure 6), demonstrating that CD4 cells require cognate interactions for maximal GVL and that CD4 CTLs are important effectors. However, a small number of $IA^{b-/-}$ mCP-CML recipients were protected by donor CD4 cells, which suggests that CD4 cells are also capable of indirectly mediating GVL. Our CD4 preparations contained no more than 0.3% $CD3^+CD4^-$ cells; thus, we at most transferred 18 000 CD8 cells, a number unlikely to have mediated GVL against MHC II^- mCP-CML (Figure 3A).

To determine whether FasL-mediated killing is important in CD4-mediated GVL, we asked whether donor CD4 cells could mediate GVL against Fas^{lpr} mCP-CML. Recipient B6 mice were irradiated and reconstituted with T-cell–depleted C3H.SW BM, wild-type, or Fas^{lpr} mCP-CML progenitors, with or without 4×10^6 purified C3H.SW CD4 cells. Donor CD4 cells mediated equivalent GVL against $Fas^{+/+}$ and Fas^{lpr} mCP-CML (Figure 7). Despite the lack of evidence for FasL-mediated killing, mCP-CML cells from spleen clearly expressed Fas (Figure 7). As in prior experiments, serial flow cytometry confirmed that Fas^{lpr} and wild-type mCP-CML developed in all mice prior to eradication by donor CD4 cells (not shown).

Discussion

The 2 principle challenges in improving the efficacy of alloSCT in treatment of malignancy are decreasing GVHD and overcoming the relative GVL resistance of many neoplasms. A detailed understanding of the killing mechanisms in GVL is key for developing strategies to overcome these obstacles, and this was the goal of the work presented.

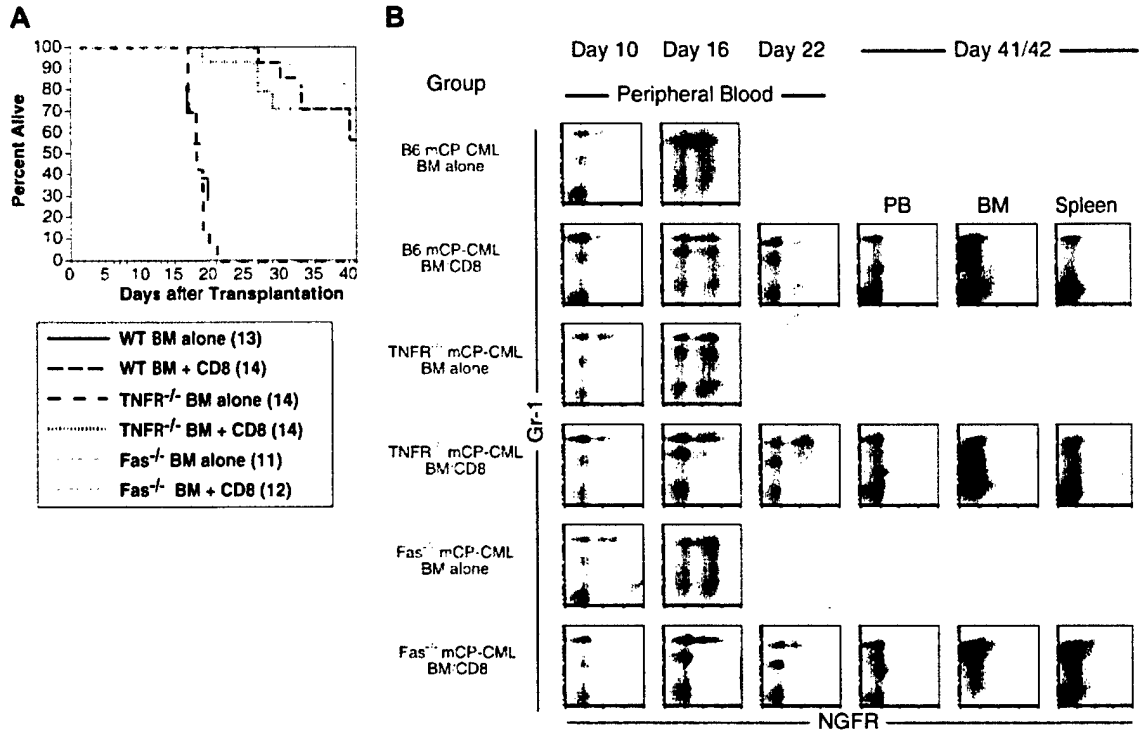

Figure 5. mCP-CML expression of TNFR1/TNFR2 or Fas is not required for CD8-mediated GVL. B6 recipients were irradiated and reconstituted with Mp210/NGFR-infected wild-type, TNFR1/TNFR2$^{-/-}$, or Fas^{lpr} progenitors, T-cell–depleted C3H.SW BM, with or without 10^6 C3H.SW CD8$^+$ T cells. (A) Survival. $P < .0001$ for each CD4 recipient group versus BM alone. (B) Serial analysis of peripheral blood. Each row is an individual mouse. Staining with anti-NGFR is shown in red, and isotype for NGFR is shown in blue. Each row is a single mouse. Note the similarity among wild-type, Fas^{lpr}, and TNFR$^{-/-}$ mCP-CML.

Herein, we describe for the first time GVL against a clinically relevant murine model of CP-CML. There have been numerous murine GVL models in which important observations have been made.[24-45] However, nearly all of those studied GVL against cell lines that shared neither phenotype nor genetic etiology with common human leukemias. In particular, these cell lines do not recapitulate chronic-phase CML, which is the most GVL sensitive of human leukemias. Differences between these cell lines and authentic CP-CML could affect mechanisms and outcome of GVL. Critically, the mCP-CML we used is phenotypically and genotypically an excellent model for human CP-CML.[46-50]

The GVL model described here replicates key features of human GVL against CP-CML, in addition to the use of an appropriate leukemic target. To simulate a clinically relevant situation, our experiments modeled GVL against residual leukemia after lethal conditioning in MHC-matched, multiple miHA mismatched strain pairings. As in human alloSCT, in addition to mCP-CML, hematopoietic reconstitution can be derived from conditioning regimen resistant nonmalignant recipient hematopoiesis and engrafting donor BM. It was important that mCP-CML develop prior to its elimination by alloreactive T cells. Serial analysis of peripheral blood (Figures 3-5) and analysis of cohorts of mice killed at different time points (not shown) clearly confirm that this indeed occurred. Syngeneic transplantations demonstrated that p210 and NGFR were insufficient as target antigens. Thus, as is the case in human identical twin transplantations, p210 expression is insufficient for GVL. There is good precedent for immune competent mice not rejecting syngeneic malignant cells expressing mutant proteins or even model antigens.[68-74] Specific to this work, 32D myeloid leukemic cells that express human p210 are also not spontaneously rejected.[75]

We found effective GVL against mCP-CML in 3 different MHC-compatible, multiple miHA-disparate strain pairings. This is consistent with human GVL data in which no HLA preference

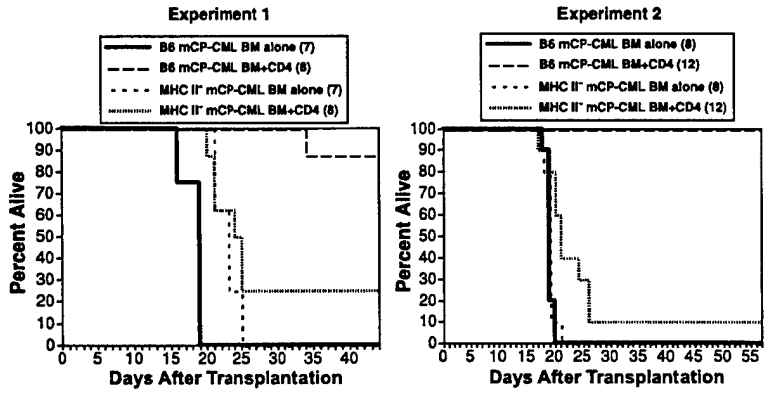

Figure 6. Cognate interactions are required for maximal CD4-mediated GVL. B6 mice were irradiated and reconstituted with T-cell–depleted C3H.SW BM, wild-type, or IA$^{b-/-}$ mCP-CML, with or without 6×10^6 C3H.SW CD4$^+$ T cells. Survival was significantly reduced in recipients of IA$^{b-/-}$ mCP-CML and CD4 cells as compared with wt mCP-CML and CD4 cells. Each panel is an independent experiment. Experiment 1: IA^b mCP-CML BM alone versus IA^b mCP-CML BM/CD4, $P < 0.11$; wt mCP-CML BM/CD4 versus IA$^{b-/-}$ mCP-CML BM/CD4, $P < .0001$. Experiment 2: IA^b mCP-CML BM alone versus IA^b mCP-CML BM/CD4, $P < .02$; wt mCP-CML BM/CD4 versus IA$^{b-/-}$ mCP-CML BM/CD4, $P < .0001$.

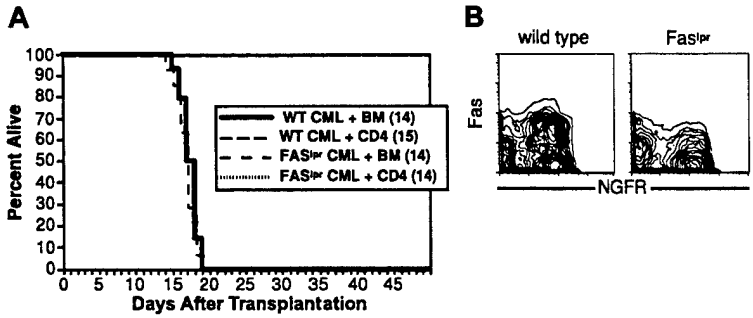

Figure 7. FasL-mediated killing is not required for CD4 GVL. B6 mice were irradiated and reconstituted with T-cell–depleted C3H.SW BM, wild-type, or Fas^{lpr} Mp210/NGFR infected progenitors, with or without 4×10^6 C3H.SW CD4$^+$ T cells. (A) Survival. (B) Fas expression. Note Fas expression in wild-type but not Fas^{lpr} NGFR$^+$ cells.

has been reported and GVL is clearly observed in patients with numerous HLA genotypes. Because different MHC present different peptides, the human data and our murine experiments suggest that no single immunodominant epitope is likely to be required for GVL.

We investigated the T-cell types necessary and sufficient for GVL. Results of human alloSCT with CD8-depleted BM suggested that CD4 cells alone were sufficient for GVL against CP-CML.[76,77] Similarly, CD8-depleted DLI was effective in treating relapsed CP-CML.[76,77] However, in each case, CD8 depletion was incomplete, and significant numbers of CD8 cells were infused. CD4 cells in CD8-depleted DLI might also have provided further help to alloreactive CD8 cells derived from the initial T-cell–replete transplantation, which in turn could have been the direct mediators of GVL. Our results clarify this point by showing that GVL can be mediated by highly purified CD4 cells.

That CD4 cells alone are capable of mediating GVL has implications for recognition as well as killing mechanisms. Surprisingly, maximal CD4-mediated GVL required cognate interactions with mCP-CML cells, as GVL was greatly reduced against MHC II$^-$ mCP-CML. It is possible that engrafting mCP-CML cells are critical because they directly prime naive alloreactive CD4 cells. However, for several reasons we think that the need for cognate interactions is more likely in the effector phase of GVL when the T-cell receptor of CD4$^+$ CTLs bind to target MHC II–peptide complexes. First, it is key to note that in these experiments, both radiation-resistant wild-type host antigen-presenting cells and engrafting donor-derived antigen-presenting cells (APCs) are MHC II$^+$ and are, therefore, available for donor CD4 cell priming.[78,79] Second, alloimmune T-cell activation begins early after transplantation,[80-85] and this activation would be prior to significant engraftment by leukemic cells. Finally, there is no reason to expect that leukemic cells would be more efficient at priming rare alloreactive CD4$^+$ T cells than donor or host dendritic cells in T-cell areas of secondary lymphoid tissues. Rendering cells MHC II$^-$ is the only definitive way to prevent CD4$^+$ T-cell priming. Therefore, to formally exclude a role for mCP-CML–mediated T-cell priming will require a series of experiments that ask whether mCP-CML priming is sufficient (ie, donor and host are MHC II$^-$ and priming can only occur on leukemic cells). This will entail extensive backcrossing of gene-deficient mice; thus, such experiments are beyond the scope of this report.

Despite a dominant role for direct recognition of MHC II on leukemic cells, a small number of mice that received MHC II$^-$ mCP-CML and donor CD4 cells survived, suggesting that CD4 cells can also mediate GVL without cognate recognition of leukemic cells. In these experiments no more than 18 000 CD8 cells could have contaminated our CD4 cell preparations. Because leukemia-free survival was only 20% (Figure 3A) with 250 000 CD8 cells, we think it is unlikely that 18 000 contaminating CD8 cells were responsible for survival. Thus, CD4 cells can promote GVL through multiple mechanisms, possibly including cytokines or macrophage activation, in addition to direct cytotoxicity.

Purified CD8$^+$ T cells alone were also effective, which demonstrates that CD8-mediated GVL is helper T-cell independent as is GVHD in the C3H.SW→B6 strain pairing.[79] Because Fas^{lpr} and TNFR$^{-/-}$ mCP-CMLs were equally susceptible to CD8-mediated GVL, neither pathway by itself is essential for target cell death. Perforin and FasL are principle effector mechanisms of CD8$^+$ CTLs; because Fas-mediated killing is not required, it is likely that perforin/granzyme-mediated killing alone is sufficient. Alternatively, or in addition, TRAIL could be playing a role given recent data showing TRAIL-mediated alloreactive CTL killing of leukemic cell lines in vivo.[60] We hope to address the role of TRAIL in future experiments. FasL and perforin are also thought to be the principle effector mechanisms for CD4 CTLs.[57,58,65,66] That CD4-mediated GVL was intact against Fas^{lpr} mCP-CML demonstrates that FasL-mediated killing is not required and that the perforin/granzyme pathway is likely to be sufficient. As with CD8-mediated GVL, TRAIL may also play a role in CD4-mediated GVL, and this too needs to be examined in the future.

Both FasL and TNF-α are important pathogenic mechanisms in murine GVHD models,[42,43,86-90] and anti–TNF-α therapy already has a role in treating human GVHD. Our results suggest that blockade of FasL/Fas interactions is not likely to affect adversely GVL against CP-CML and may provide a means to deliver GVL with reduced GVHD. TNF-α blockade is similarly unlikely to impair CTL effector function; however, because TNF-α promotes T-cell activation, TNF-α blockade may still weaken GVL.

In summary, our results suggest that CP-CML sensitivity is at least in part explained by the multiple effector mechanisms sufficient for GVL. GVL against mCP-CML could be mediated by either CD4 or CD8 cells and was independent of Fas or TNFR expression, and CD4 cells could kill without directly contacting mCP-CML targets. Thus, GVL might still be effective even if multiple potential effector mechanisms fail because of either properties of the immune response itself (eg, no CD4 alloimmunity) or the absence of major apoptotic pathways in targets. However, unlike CP-CML, some leukemias such as BC-CML are generically GVL resistant, even in the face of GVHD. Alloreactive T cells could be ineffective for multiple reasons. They may fail to traffic to sites of disease, which could play a role in central nervous system relapses. However, GVL sensitive and resistant leukemias are found in blood, bone marrow, and spleen, sites to which T cells normally have access. Loss of a critical immunodominant miHA is unlikely to explain resistance either. We found effective GVL in 3 different MHC identical strain pairings in which the immunodominant antigens are likely to be different; thus, there is a high degree of plasticity in the T-cell response. However, we found that a single mutation—loss of MHC II expression—resulted in substantial

GVL resistance of an otherwise GVL-sensitive neoplasm. Thus, genes that affect sensitivity to killing, like MHC II, could be responsible for de novo resistance to GVL or might be major targets for mutations that render leukemias resistant to GVL. In future studies, by way of generation of additional gene-deficient mCP-CMLs, we plan to investigate these and other potential mechanisms of GVL resistance. We hope that these studies will identify pathways that, if augmented, will overcome GVL resistance and could identify rational ways to maximize GVL without undue GVHD.

References

1. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990;75:555-562.
2. Collins RH Jr, Shpilberg O, Drobyski WR, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J Clin Oncol. 1997;15:433-444.
3. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. Blood. 1995;86:2041-2050.
4. Porter DL, Collins RH Jr, Shpilberg O, et al. Long-term follow-up of patients who achieved complete remission after donor leukocyte infusions. Biol Blood Marrow Transplant. 1999;5:253-261.
5. van Besien K, Sobocinski KA, Rowlings PA, et al. Allogeneic bone marrow transplantation for low-grade lymphoma. Blood. 1998;92:1832-1836.
6. Khouri IF, Lee MS, Romaguera J, et al. Allogeneic hematopoietic transplantation for mantle-cell lymphoma: molecular remissions and evidence of graft-versus-malignancy. Ann Oncol. 1999;10:1293-1299.
7. Champlin R, van Besien K, Giralt S, Khouri I. Allogeneic hematopoietic transplantation for chronic lymphocytic leukemia and lymphoma: potential for nonablative preparative regimens. Curr Oncol Rep. 2000;2:182-191.
8. van Besien K, Champlin IK, McCarthy P. Allogeneic transplantation for low-grade lymphoma: long-term follow-up. J Clin Oncol. 2000;18:702-703.
9. Khouri IF, Saliba RM, Giralt SA, et al. Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. Blood. 2001;98:3595-3599.
10. Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. N Engl J Med. 2000;343:750-758.
11. Gale RP, Horowitz MM. Graft-versus-leukemia in bone marrow transplantation. The Advisory Committee of the International Bone Marrow Transplant Registry. Bone Marrow Transplant. 1990;6:94-97.
12. Cassileth PA, Harrington DP, Appelbaum FR, et al. Chemotherapy compared with autologous or allogeneic bone marrow transplantation in the management of acute myeloid leukemia in first remission. N Engl J Med. 1998;339:1649-1656.
13. Mehta J, Singhal S. Graft-versus-myeloma. Bone Marrow Transplant. 1998;22:835-843.
14. Ueno NT, Rondon G, Mirza NQ, et al. Allogeneic peripheral-blood progenitor-cell transplantation for poor-risk patients with metastatic breast cancer. J Clin Oncol. 1998;16:986-993.
15. Tricot G, Vesole DH, Jagannath S, Hilton J, Munshi N, Barlogie B. Graft-versus-myeloma effect: proof of principle. Blood. 1996;87:1196-1198.
16. Dimopoulos MA, Hester J, Huh Y, Champlin R, Alexanian R. Intensive chemotherapy with blood progenitor transplantation for primary resistant multiple myeloma. Br J Haematol. 1994;87:730-734.
17. Annat J, Churaqui E, Dutartre P, Bruley-Rosset M. Prevention of lethal graft-versus-host disease following allogeneic bone marrow transplantation in mice by short course administration of LF 08-0299. Transplantation. 1996;62:721-729.
18. de la Selle V, Miconnet I, Gilbert D, Bruley-Rosset M. Peripheral tolerance to host minor histocompatibility antigens in radiation bone marrow chimeras abrogates lethal GVHD while preserving GVL effect. Bone Marrow Transplant. 1995;16:111-118.
19. de La Selle V, Riche N, Dorothe G, Bruley-Rosset M. CD8+ cytotoxic T cell repertoire implicated in grafts-versus-leukemia effect in a murine bone marrow transplantation model. Bone Marrow Transplant. 1999;23:951-958.
20. Gahrton G, Svensson H, Cavo M, et al, The European Group for B, Marrow T. Progress in allogenic bone marrow and peripheral blood stem cell transplantation for multiple myeloma: a comparison between transplants performed 1983-93 and 1994-8 at European Group for Blood and Marrow Transplantation centres. Br J Haematol. 2001;113:209-216.
21. Garban F, Attal M, Rossi JF, Payen C, Fegueux N, Sotto JJ, Intergroupe Francophone du M. Immunotherapy by non-myeloablative allogeneic stem cell transplantation in multiple myeloma: results of a pilot study as salvage therapy after autologous transplantation. Leukemia. 2001;15:642-646.
22. Kroger N, Kruger W, Renges H, et al. Donor lymphocyte infusion enhances remission status in patients with persistent disease after allografting for multiple myeloma. Br J Haematol. 2001;112:421-423.
23. Barnes DW, Corp MJ, Loutit JF, Neal FE. Treatment of murine leukaemia with x rays and homologous bone marrow. Br Med J. 1956;2:626-627.
24. Sykes M, Abraham VS, Harty MW, Pearson DA. IL-2 reduces graft-versus-host disease and preserves a graft-versus-leukemia effect by selectively inhibiting CD4+ T cell activity. J Immunol. 1993;150:197-205.
25. Yang YG, Sergio JJ, Pearson DA, Szot GL, Shimizu A, Sykes M. Interleukin-12 preserves the graft-versus-leukemia effect of allogeneic CD8 T cells while inhibiting CD4-dependent graft-versus-host disease in mice. Blood. 1997;90:4651-4660.
26. Teshima T, Hill GR, Pan L, et al. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. J Clin Invest. 1999;104:317-325.
27. Reddy P, Teshima T, Kukuruga M, et al. Interleukin-18 regulates acute graft-versus-host disease by enhancing Fas-mediated donor T cell apoptosis. J Exp Med. 2001;194:1433-1440.
28. Reddy P, Teshima T, Hildebrandt G, et al. Interleukin 18 preserves a perforin-dependent graft-versus-leukemia effect after allogeneic bone marrow transplantation. Blood. 2002;100:3429-3431.
29. Krijanovski OI, Hill GR, Cooke KR, et al. Keratinocyte growth factor separates graft-versus-leukemia effects from graft-versus-host disease. Blood. 1999;94:825-831.
30. Hill GR, Teshima T, Gerbitz A, et al. Differential roles of IL-1 and TNF-alpha on graft-versus-host disease and graft versus leukemia. J Clin Invest. 1999;104:459-467.
31. Pan L, Teshima T, Hill GR, et al. Granulocyte colony-stimulating factor-mobilized allogeneic stem cell transplantation maintains graft-versus-leukemia effects through a perforin-dependent pathway while preventing graft-versus-host disease. Blood. 1999;93:4071-4078.
32. Cooke KR, Gerbitz A, Crawford JM, et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. J Clin Invest. 2001;107:1581-1589.
33. Kim YM, Sachs T, Asavaroengchai W, Bronson R, Sykes M. Graft-versus-host disease can be separated from graft-versus-lymphoma effects by control of lymphocyte trafficking with FTY720. J Clin Invest. 2003;111:659-669.
34. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Sharpe AH, Vallera DA. Opposing roles of CD28:B7 and CTLA-4:B7 pathways in regulating in vivo alloresponses in murine recipients of MHC disparate T cells. J Immunol. 1999;162:6368-6377.
35. Mapara MY, Kim YM, Wang SP, Bronson R, Sachs DH, Sykes M. Donor lymphocyte infusions mediate superior graft-versus-leukemia effects in mixed compared to fully allogeneic chimeras: a critical role for host antigen-presenting cells. Blood. 2002;100:1903-1909.
36. Fowler DH, Breglio J, Nagel G, Eckhaus MA, Gress RE. Allospecific CD8+ Tc1 and Tc2 populations in graft-versus-leukemia effect and graft-versus-host disease. J Immunol. 1996;157:4811-4821.
37. Johnson BD, Drobyski WR, Truitt RL. Delayed infusion of normal donor cells after MHC-matched bone marrow transplantation provides an antileukemia reaction without graft-versus-host disease. Bone Marrow Transplant. 1993;11:329-336.
38. Johnson BD, Truitt RL. Delayed infusion of immunocompetent donor cells after bone marrow transplantation breaks graft-host tolerance allows for persistent antileukemic reactivity without severe graft-versus-host disease. Blood. 1995;85:3302-3312.
39. Johnson BD, Becker EE, Truitt RL. Graft-vs.-host and graft-vs.-leukemia reactions after delayed infusions of donor T-subsets. Biol Blood Marrow Transplant. 1999;5:123-132.
40. Fontaine P, Roy-Proulx G, Knafo L, Baron C, Roy DC, Perreault C. Adoptive transfer of minor histocompatibility antigen-specific T lymphocytes eradicates leukemia cells without causing graft-versus-host disease. Nat Med. 2001;7:789-794.
41. Bortin MM, Truitt RL, Rimm AA, Bach FH. Graft-versus-leukaemia reactivity induced by alloimmunisation without augmentation of graft-versus-host reactivity. Nature. 1979;281:490-491.
42. Tsukada N, Kobata T, Aizawa Y, Yagita H, Okumura K. Graft-versus-leukemia effect and graft-versus-host disease can be differentiated by cytotoxic mechanisms in a murine model of allogeneic bone marrow transplantation. Blood. 1999;93:2738-2747.
43. Schmaltz C, Alpdogan O, Horndasch KJ, et al. Differential use of Fas ligand and perforin cytotoxic pathways by donor T cells in graft-versus-host disease and graft-versus-leukemia effect. Blood. 2001;97:2886-2895.
44. Sato K, Yamashita N, Baba M, Matsuyama T. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. Immunity. 2003;18:367-379.

45. Edinger M, Hoffmann P, Ermann J, et al. Donor CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med. 2003;9:1144-1150.

46. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science. 1990;247:824-830.

47. Pear WS, Miller JP, Xu L, et al. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood. 1998;92:3780-3792.

48. Zhang X, Ren R. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. Blood. 1998;92:3829-3840.

49. Gross AW, Zhang X, Ren R. Bcr-Abl with an SH3 deletion retains the ability to induce a myeloproliferative disease in mice, yet c-Abl activated by an SH3 deletion induces only lymphoid malignancy. Mol Cell Biol. 1999;19:6918-6928.

50. Li S, Ilaria RL Jr, Million RP, Daley GQ, Van Etten RA. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. J Exp Med. 1999;189:1399-1412.

51. Wolff NC, Ilaria RL Jr. Establishment of a murine model for therapy-treated chronic myelogenous leukemia using the tyrosine kinase inhibitor STI571. Blood. 2001;98:2808-2816.

52. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci U S A. 1993;90:8392-8396.

53. Korngold R, Sprent J. Variable capacity of L3T4+ T cells to cause lethal graft-versus-host disease across minor histocompatibility barriers in mice. J Exp Med. 1987;165:1552-1564.

54. Anderson BE, McNiff J, Yan J, et al. Memory CD4+ T cells do not induce graft-versus-host disease. J Clin Invest. 2003;112:101-108.

55. Liu J, Anderson BE, Robert ME, et al. Selective T-cell subset ablation demonstrates a role for T1 and T2 cells in ongoing acute graft-versus-host disease: a model system for the reversal of disease. Blood. 2001;98:3367-3375.

56. Gale RP, Horowitz MM, Ash RC, et al. Identical-twin bone marrow transplants for leukemia. Ann Intern Med. 1994;120:646-652.

57. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol. 2002;2:401-409.

58. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 2002;20:323-370.

59. Pan G, O'Rourke K, Chinnaiyan AM, et al. The receptor for the cytotoxic ligand TRAIL. Science. 1997;276:111-113.

60. Schmaltz C, Alpdogan O, Kappel BJ, et al. T cells require TRAIL for optimal graft-versus-tumor activity. Nat Med. 2002;8:1433-1437.

61. Hill GR, Teshima T, Rebel VI, et al. The p55 TNF-alpha receptor plays a critical role in T cell alloreactivity. J Immunol. 2000;164:656-663.

62. Steinberg AD. MRL-lpr/lpr disease: theories meet Fas. Semin Immunol. 1994;6:55-69.

63. Singer GG, Abbas AK. The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. Immunity. 1994;1:365-371.

64. Ju ST, Panka DJ, Cui H, et al. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature. 1995;373:444-448.

65. Stalder T, Hahn S, Erb P. Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. J Immunol. 1994;152:1127-1133.

66. Ju ST, Cui H, Panka DJ, Ettinger R, Marshak-Rothstein A. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. Proc Natl Acad Sci U S A. 1994;91:4185-4189.

67. Grusby MJ, Johnson RS, Papaioannou VE, Glimcher LH. Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. Science. 1991;253:1417-1420.

68. Wu TC, Huang AY, Jaffee EM, Levitsky HI, Pardoll DM. A reassessment of the role of B7-1 expression in tumor rejection. J Exp Med. 1995;182:1415-1421.

69. Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science. 1994;264:961-965.

70. Huang AY, Bruce AT, Pardoll DM, Levitsky HI. Does B7-1 expression confer antigen-presenting cell capacity to tumors in vivo? J Exp Med. 1996;183:769-776.

71. Sotomayor EM, Borrello I, Tubb E, et al. Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40. Nat Med. 1999;5:780-787.

72. Borrello I, Sotomayor EM, Rattis FM, Cooke SK, Gu L, Levitsky HI. Sustaining the graft-versus-tumor effect through posttransplant immunization with granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing tumor vaccines. Blood. 2000;95:3011-3019.

73. Levitsky HI. Augmentation of host immune responses to cancer: overcoming the barrier of tumor antigen-specific T-cell tolerance. Cancer J. 2000;6(suppl 3):S281-S290.

74. Cui Y, Kelleher E, Straley E, et al. Immunotherapy of established tumors using bone marrow transplantation with antigen gene—modified hematopoietic stem cells. Nat Med. 2003;9:952-958.

75. Zhao RC, McIvor RS, Griffin JD, Verfaillie CM. Gene therapy for chronic myelogenous leukemia (CML): a retroviral vector that renders hematopoietic progenitors methotrexate-resistant and CML progenitors functionally normal and nontumorigenic in vivo. Blood. 1997;90:4687-4698.

76. Champlin R, Ho W, Gajewski J, et al. Selective depletion of CD8+ T lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. Blood. 1990;76:418-423.

77. Alyea EP, Soiffer RJ, Canning C, et al. Toxicity and efficacy of defined doses of CD4(+) donor lymphocytes for treatment of relapse after allogeneic bone marrow transplant. Blood. 1998;91:3671-3680.

78. Shlomchik WD. Antigen presentation in graft-vs-host disease. Exp Hematol. 2003;31:1187-1197.

79. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science. 1999;285:412-415.

80. Sprent J, Miller JF, Mitchell GF. Antigen-induced selective recruitment of circulating lymphocytes. Cell Immunol. 1971;2:171-181.

81. Sprent J, Schaefer M, Lo D, Korngold R. Properties of purified T cell subsets, II: in vivo responses to class I vs. class II H-2 differences. J Exp Med. 1986;163:998-1011.

82. Kosaka H, Surh CD, Sprent J. Stimulation of mature unprimed CD8+ T cells by semiprofessional antigen-presenting cells in vivo. J Exp Med. 1992;176:1291-1302.

83. Gao EK, Kosaka H, Surh CD, Sprent J. T cell contact with Ia antigens on nonhemopoietic cells in vivo can lead to immunity rather than tolerance. J Exp Med. 1991;174:435-446.

84. Korngold R, Sprent J. Negative selection of T cells causing lethal graft-versus-host disease across minor histocompatibility barriers: role of the H-2 complex. J Exp Med. 1980;151:1114-1124.

85. Zhang Y, Louboutin JP, Zhu J, Rivera AJ, Emerson SG. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. J Clin Invest. 2002;109:1335-1344.

86. Hattori K, Hirano T, Miyajima H, et al. Differential effects of anti-Fas ligand and anti-tumor necrosis factor alpha antibodies on acute graft-versus-host disease pathologies. Blood. 1998;91:4051-4055.

87. Graubert TA, DiPersio JF, Russell JH, Ley TJ. Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. J Clin Invest. 1997;100:904-911.

88. Via CS, Nguyen P, Shustov A, Drappa J, Elkon KB. A major role for the Fas pathway in acute graft-versus-host disease. J Immunol. 1996;157:5387-5393.

89. Braun MY, Lowin B, French L, Acha-Orbea H, Tschopp J. Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. J Exp Med. 1996;183:657-661.

90. Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. J Exp Medicine. 1996;183:2645-2656.