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Evidence of B Cell Immune Responses to Acute Lymphoblastic Leukemia in Murine Allogeneic Hematopoietic Stem Cell Transplantation Recipients Treated with Donor Lymphocyte Infusion and/or Vaccination

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These experiments explored mechanisms of control of acute lymphoblastic leukemia (ALL) following allogeneic hematopoietic stem cell transplantation using a murine model of MHC-matched, minor histocompatibility antigen–mismatched transplantation. The central hypothesis examined was that addition of active vaccination against leukemia cells would substantially increase the effectiveness of allogeneic donor lymphocyte infusion (DLI) against ALL present in the host after transplantation. Although vaccination did increase the magnitude of type I T cell responses against leukemia cells associated with DLI, it did not lead to substantial improvement in long-term survival. Analysis of immunologic mechanisms of leukemia progression demonstrated that the failure of vaccination was not because of antigen loss in leukemia cells. However, analysis of survival provided surprising findings that, in addition to very modest type I T cell responses, a B cell response that produced antibodies that bind leukemia cells was found in long-term survivors. The risk of death from leukemia was significantly lower in recipients that had higher levels of such antibodies. These studies raise the hypothesis that stimulation of B cell responses after transplantation may provide a novel way to enhance allogeneic graft-versus-leukemia effects associated with transplantation.

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KEY WORDS: Graft-versus-leukemia, Vaccine, Antibody, Donor lymphoctye infusion, Acute lymphoblastic leukemia, Hematopoietic stem cell transplantation

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is performed for high-risk acute lymphoblastic leukemia (ALL). Relapse of ALL remains the most common cause of treatment failure after transplantation. Although augmentation of graft-versus-leukemia (GVL) effects by withdrawal of immunosuppression and DLI is often effective in treating early relapse of chronic myelogenous leukemia after transplantation, these maneuvers are rarely successful in treatment of ALL [1,2]. The mechanisms of the relative ineffectiveness of DLI and GVL activity in ALL are not fully known. Some of the possibilities include rapid expansion of ALL cells in vivo and poor immunogenicity of ALL cells compared to chronic myeloid leukemia (CML) cells.

Prior work in our laboratory has demonstrated that administration of cellular leukemia vaccines to allogeneic transplant recipients can increase GVL effects without substantial increases in graft-versus-host disease (GVHD) [3,4]. We have also observed that vaccination at the time of allogeneic lymphocyte infusion can result in a significant expansion of antigen specific T cells in vivo [5]. Based on these findings, we hypothesized that vaccination coupled with donor lymphocyte infusion (DLI) might produce more effective control of ALL after transplantation. The reasoning for this was that active vaccination would provide more effective antigen presentation of antigens present on ALL cells, and that the clonal expansion of leukemia-reactive T cells might be more effective in controlling ALL populations that have a rapid expansion rate.

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To address this immunobiologic question, we employed a well-characterized murine model of major histocompatibility complex (MHC)-matched, multiple minor histocompatibility antigen–mismatched transplantation [6], and novel murine pre-B ALL cell lines driven by common human mutations (bcr/abl fusion genes and Ink/ARF locus deletions) [7]. Although many of the minor histocompatibility antigens in this system are known at a genetic and peptide level [6], antigens relatively selectively expressed on the leukemia cells are not. To address this methodologic limitation we exploited sex mismatches, because male HY antigens are known at a genetic and peptide level. By using ALL cells derived from males and using female donors and recipients we were able to use the male HY antigens as models for leukemia-restricted antigens [8].

We discovered that although concurrent vaccination did increase the activity of donor T cells that recognized HY antigens on the leukemias and did have a short-term impact on leukemia expansion, significant survival advantages were not seen by the addition of vaccination to DLI. As we investigated the mechanisms of relapse and immune control of ALL in this model, we discovered that long-term survival after allogeneic transplant and ALL challenge were associated with modest T responses, and surprisingly, B cell responses to leukemia cells.

MATERIALS AND METHODS

Mice

C3.SW mice (Jackson, West Grove, PA) were transplant donors, and C57BL/6 mice (National Cancer Institute, Frederick, MD) were recipients. The mice are MHC antigen matched (H2b) but minor histocompatibility antigen (mHA) mismatched at many loci (H1, H3, H7, H8, H9, H13). C3.SW are H2b and were derived from an 11-generation backcross of C3H against a noninbred H2b donor strain [9].

Cell Lines

NSTY pre-B ALLs were generated from primary marrow cells from INK4A/ARF null mice transduced with a retroviral vector encoding the human p210 bcr/abl cDNA [7,10]. The MSCV-BCR/ABL-IRES-GFP vector was kindly provided by Dr. Richard Van Etten. The neo gene was removed from this vector by digestion with Nco I and Cla I, and the YFP gene was inserted by standard cloning procedure to yield the MSCV-Nup98/HoxA9-YFP vector used in the present study. Retroviral vector plasmids were transfected into phoenix-eco cells (ATCC, Rockville, MD) using lipofectamine 2000 per manufacturer's instructions (10 μg DNA per 100,000 cells in a 6-well tissue culture dish). At 36 hours posttransfection, viral supernatants were collected, filtered, and stored at −80°C. Retrovirus-treated marrow cells (2×10^5) were infused intravenously (i.v.) into irradiated (600 cGy) C57BL/6 recipients, and spontaneous ALLs emerged within 3 weeks. NSTY lines were derived by in vitro culture of splenocytes from these leukemia-bearing mice; no cytokine supplementation was required [11,12]. The male and female acute myeloid leukemia lines (mAML and fAML, respectively) were derived from C57BL/6 male or female mice infused with wild-type syngeneic C57BL/6 marrow transduced in vitro with vectors encoding p210 bcr/abl and NUP98/HOXA9 [10,12]. C1498 (ATCC) is a spontaneous C57BL/6 acute NKT cell leukemia [13]. ASLN cells were derived from a C57BL/6 transgenic mouse created by insertion of a C57BL/6 oocyte of a human p190 bcr/abl cDNA under the control of the immunoglobulin heavy chain enhancer E-mu and the murine Mb-1 promoter [7]. YAC (TIB-160, ATCC) is an A/Sn strain lymphoma line that is sensitive to NK cells and is the conventional target in murine NK cytolysis assays. P815 cells are a DBA/2 strain (H2d) mastocytoma (TIB-64, ATCC).

Reisolation of Leukemia Cells

At time of euthanasia or death, BMT recipient splenocytes were placed in short-term culture to allow outgrowth of relapsing ALL cells. One week later, aliquots of cells were cryopreserved. For subsequent analysis, aliquots were thawed, grown in culture for several days, and then used as target cells in leukemia cell inhibition assays.

Bone Marrow Transplant

Following 800 cGy TBI (given in 2 equal divided doses 14-16 hours apart) and intraperitoneal (i.p.) 5-fluoruracil (0.5 mg) C57BL/6 recipients were infused with 4×10^6 marrow cells plus 10×10^6 splenocytes from normal C57BL/6 mice or C3.SW mice immunized against C57BL/6 splenocytes. They were housed in conventional rooms with food and water ad libitum. From 2 days before BMT until day 14, the water was acidified (pH 2.5) and supplemented with 2 g/L neomycin sulfate (Sigma, St. Louis, MO). We and other investigators have observed that with use of C3.SW donors (with 5-10 $\times 10^6$ T cells in the graft) and C57BL/6 recipients, fatal GVHD is observed in a significant number of recipients over a 6-week period [14]. In experiments in which leukemia progression in vivo was measured, C57BL/6 mice underwent myeloablation with radiation and 5-FU and then were simultaneously infused with 4×10^6 bone marrow cells, 1×10^7 splenocytes, and 1×10^4 NSTY-1 ALL cells. The source of splenocytes varied between experimental groups, with some recipients receiving marrow and spleen cells from C3.SW mice that had been vaccinated against

C57BL/6 splenocytes to enhance alloreactivity, whereas other control mice received cells from C3.SW that had not been vaccinated or from normal C57BL/6 mice.

DLI and Vaccination

Donor lymphocytes were prepared from female C3.SW mice that had been primed against HY antigens by vaccination with irradiated male C3.SW cells. CD90.2 cells were enriched by positive selection from spleen cells using paramagnetically labeled CD90.2 monoclonal antibodies and positive selection columns (Miltenyi Biotech, Auburn, CA). Cells (5×10^6) were suspended in Hanks balanced salt solution and intravenously infused. Vaccinations consisted of 5×10^6 50 Gy irradiated male C57BL/6 spleen cells or male C57BL/6 ALL cells suspended in 0.1 mL complete Freund's adjuvant containing 0.2 μg murine granulocyte macrophage-colony stimulating factor (GM-CSF).

Assessment of GVHD

Animals were judged to have GVHD in vivo if they exhibited extensive hair loss and/or wasting and if at necropsy did not have evidence of leukemia progression (eg, splenomegaly, lymphadenopathy, and/or flow cytometric measurement of leukemia in marrow or spleen). In this strain combination, liver GVHD can be measured fairly objectively by assessment of the number of portal triads with lymphocytic infiltration in liver samples stained with hematoxylin and eosin.

Generation of Alloreactive T Cells

C3.SW mice were vaccinated subcutaneously (s.c.) with 10^7 25 Gy–irradiated splenocytes 2 to 3 times at 2-week intervals. Splenocytes from C3.SW mice immunized against C57BL/6 splenocytes were restimulated in vitro 4 days at a 5:3 ratio at 10^7 cells/mL with 25 Gy–irradiated C57BL/6 splenocytes that express all the known minor histocompatibility antigens. In other experiments designed to generate CTL with a single specificity for 1 minor antigen peptide, C3.SW responder cells were stimulated with syngeneic C3.SW splenocytes preincubated with exogenous minor histocompatibility antigen peptide. Cells were incubated at 37°C in R10S medium (RPMI, 10% fetal calf serum [FCS], 200 mM glutamine, 10^4 U/mL penicillin/streptomycin, nonessential amino acids [1 mL per 100 mL medium], 100 mM Na pyruvate, and 50 mM beta-mercaptoethanol). No cytokines were added to the culture. T cells generated from these cultures were used as effector cells with target leukemia cells, and a flow cytometry–based leukemia cell inhibition assay was used to measure the capacity of the effector cells to inhibit the leukemia target cells.

Leukemia Cell Inhibition Assay

The assay measures the relative size of leukemia target cell populations cocultured for 3 days with potential cytolytic effector cells in microcultures in 96-well plates. Five thousand target cells were placed with effector cells at specified effector-to-target cells ratios. Following 3 days of culture, 4000 fluorescent microbeads were added to each well, and immediately thereafter, the well was harvested and examined flow cytometrically. Regions were defined corresponding to the beads and viable leukemia cells, and the number of events in each region was recorded. The number of viable leukemia cells in a well was calculated using the following formula: (leukemia cells in well) = (leukemia cells counted) × (constant number of fluorescent beads added to well/ number of fluorescent beads counted). The percentage leukemia cells surviving in wells with effector cells was calculated with the following formula: Percentage leukemia cells surviving = (number of leukemia cells in well with effectors/number of leukemia cells in wells without effector cells) × 100. Duplicates or triplicates of each condition were measured.

ELISPOT

Splenocytes (10^6) were added to wells preincubated with interferon-gamma (IFN-γ) capture antibody and stimulated for 48 hours with HY peptides (Uty, Dby, and Smcy) or irrelevant peptide (WPRPQIPP) (5 μg/well) [6]. IFN-γ release was detected with biotinylated anti-IFN-γ antibody (Caltag, Burlingame, CA) and streptavidin peroxidase.

Flow Cytometry

Conventional analytic flow cytometry was performed using a Becton Dickinson FACScan with analysis performed by Cellquest software or WinMDI. Directly labeled monoclonal antibodies for the cell surface markers specified in the text were obtained from Pharmingen (San Diego, CA) or Caltag.

ELISA

U-bottomed 96-well plates were incubated overnight at 4°C with blocking buffer (balanced salt solution with 0.05% Tween, 1 mM Tween 20, and 0.25% bovine serum albumin). After washing, 10^6 leukemia cells or normal spleen cells were incubated for 60 minutes at 4°C in each well with 95 μL flow cytometry buffer (Streck, Omaha, NE) and 5 μL experimental animal serum. Following washing cells were incubated for 60 minutes at 4°C in buffer containing 1:200 dilution of biotinylated goat antimouse immunoglobulin (BD Pharmingen). Following washing, cells were incubated for 1 hour in ice-cold buffer with a 1:1000 dilution of alkaline phosphatase-streptavidin (BD Pharmingen). After washing, cells were incubated in 100 μL of PNPP substrate for 10 minutes. The colorimetric reaction

was then stopped with 25 μL 0.5 M NaOH. Optical density (O.D.) was measured at 405 nm with an ELISA plate reader.

Statistics

Datasets were assessed for normal distribution by Kolmogorov-Smirnov tests. For normally distributed data, 2-tailed *t*-tests were performed to compare means. For data that were not normally distributed, 2-tailed nonparametric Mann-Whitney tests were performed to compare medians. Survival curves were compared with the log-rank test. Association of antibody levels with categoric outcomes in vivo was assessed with chi-square tests. Statistical calculations were performed using GraphPad Prism 4.03 and R 2.5 software packages.

RESULTS

Vaccination Enhances Antigen-Specific T Cell Responses in Transplant Recipients Receiving DLIs

The overall goal of this work was to test the hypothesis that vaccination of the transplant recipient receiving DLI would enhance control of residual acute leukemia. To test this, we used experimental acute leukemias derived from male mice in which immunologically well-characterized male HY minor histocompatibility antigens served as leukemia-specific antigens. Female donors and female recipients were used for transplantation, allowing us to discriminate between allogeneic immune responses that were leukemia specific versus those directed against widely distributed minor histocompatibility antigens.

The first experiments measured the impact of vaccination on antigen specific T cell responses following DLI. One month following allogeneic BMT, female C57BL/6 recipients were infused with splenocytes from female C3.SW donor strain mice. Some groups also received vaccination with male C57BL/6 cells 1 day prior to DLI. Two weeks later, INF-γ ELISPOT assays were performed to measure HY peptide–specific T cell responses. Figure 1A demonstrates that DLI alone or vaccination alone induced negligible HY-specific responses. However, the combination of vaccination with DLI produced significantly greater HY-specific responses in the allogeneic transplant recipients. Similar experiments were performed measuring INF-γ ELISPOT responses to widely distributed recipient strain (C57BL/6) minor histocompatibility antigens (H3, H7, and H13). Consistent with our earlier published findings, vaccination did not induce significant T cell responses to these ubiquitously distributed antigens (Figure 1B).

Combining Vaccination with DLI Does Not Substantially Improve Long-Term Survival Compared to Treatment with DLI Alone

Based on the observation that coupling vaccination with DLI substantially increased the type I T cell responses against the HY antigens on the leukemia cells, we performed experiments in vivo to determine if treatment with vaccine-augmented DLI could exert

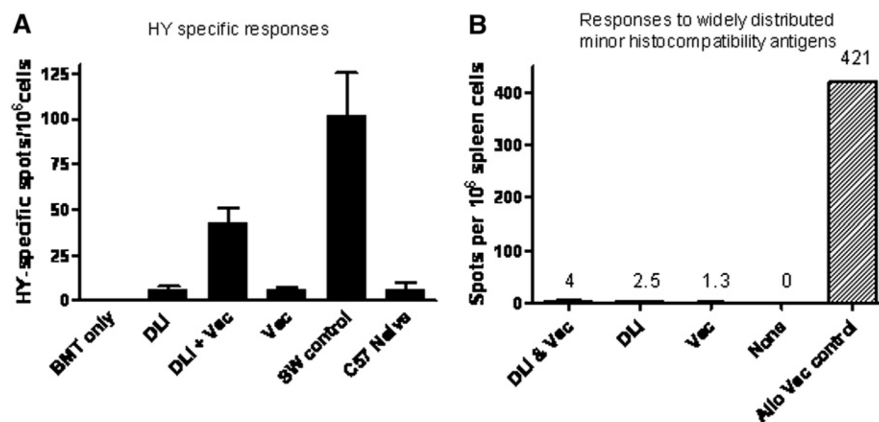


Figure 1. Concurrent vaccination with HY antigen-positive recipient strain cells with allogeneic DLI increases T cell responses to HY antigens but does not increase T cell responses to widely distributed recipient minor histocompatibility antigens. One month after transplantation, some mice were vaccinated with male C57BL/6 spleen cells. One day later, some groups received 5×10^6 CD90.2 selected female strain DLIs. Two weeks later, INF-γ ELISPOT assays were performed using peptide antigens to stimulate spleen cells from the animals. (A) HY antigen specific ELISPOT. HY-specific spots per 10^6 splenocytes represent = (spots in wells with HY peptides) – (spots in wells with irrelevant peptide). Average specific spots are presented for the following transplantation groups: BMT only (n = 7), DLI (n = 12), DLI + Vac (concurrent vaccination and DLI, n = 25), and Vac (vaccination, no DLI, n = 4). Positive control was female donor strain C3.SW primed against male C3.SW (n = 4). Negative control was a normal C57BL/6 female (n = 2). Error bars are standard error of the mean. HY-specific spots in DLI + vaccination group are significantly greater than DLI-only group ($P = .008$ by Mann-Whitney test). DLI + vaccination group is significantly greater than vaccination-only group ($P = .0341$ by Mann-Whitney test). Results are pooled from 5 independent experiments. (B) Recipient minor histocompatibility antigen–specific ELISPOT. Specific spots per 10^6 spleen cells = (spots in response to pooled H3, H7, and H13 minor histocompatibility peptides) – (spots in response to irrelevant peptide). Average and standard error of the mean are displayed. There were no differences between any of the transplant groups (DLI + vaccination n = 9, DLI n = 2, vaccination n = 7, BMT only n = 3). Results are pooled from 2 independent experiments. The positive control was donor strain C3.SW previously immunized with recipient strain C57BL/6 cells.

a biologically significant effect on progression of leukemia. In pilot studies, we performed short-term assays of leukemia growth by measuring the percentage of leukemia in marrow and survival 2 weeks after leukemia challenge and treatment. One month after allogeneic transplantation, mice were injected i.v. with 10^4 ALL cells derived from male C57BL/6 mice. The male HY antigens serve as leukemia-restricted antigens in this model. In our experience this dose of leukemia cells uniformly produces death from leukemia in female mice within 1 month. One day after leukemia challenge, experimental mice were vaccinated with 10^7 irradiated male recipient strain C57BL/6 spleen cells. One day later, they received i.v. infusion of 10^7 CD90.2 selected spleen cells from donor strain C3.SW females that had been sensitized to HY antigens by vaccination with C3.SW male cells. Two weeks later, leukemia burden in marrow was measured by flow cytometry. Animals treated with vaccine-augmented DLI or DLI alone had significantly less leukemia compared to untreated control mice (Figure 2).

Having seen biologically meaningful antileukemia effects in these pilot studies, we performed a large long-term survival study designed to ask whether vaccine augmented DLI led to greater survival than DLI alone. The experiment was designed to have 80% power to detect a 15-day difference in survival. One month following allogeneic transplantation, mice were challenged with 10^4 male ALL cells. One day later, the experimental group was vaccinated with male ALL cells. The following day, mice in both the experimental and control groups received an infusion of 10^7 CD90.2 selected spleen cells from donor strain C3.SW females that had been sensitized to HY antigens by vaccination with C3.SW male cells. Mice did not receive additional vaccinations or DLIs. They were followed for survival for up to 73 days, at which time surviving mice were euthanized. At death, necropsy was performed to assess cause of death. Twenty-two percent of 18 mice treated with vaccination/DLI survived, and median survival was 46 days. In the group treated with only DLI (n = 17), 11% were survivors, and median survival was 37 days (Figure 3A). The differences were not statistically significant. The causes of death were similar in

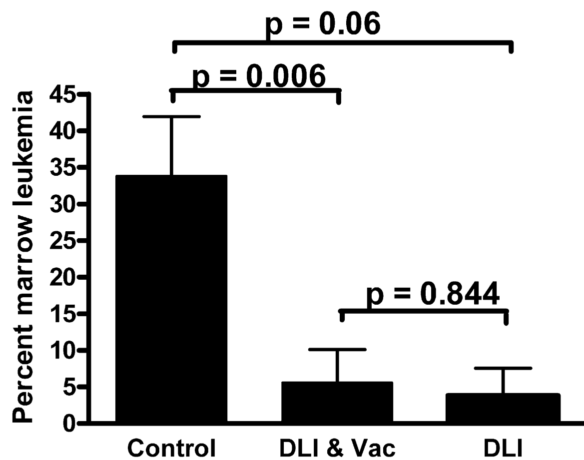
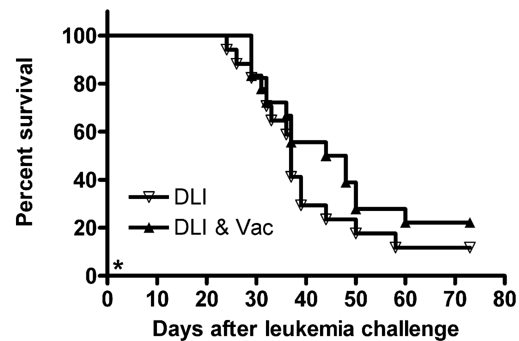


Figure 2. Short-term leukemia burden is reduced in BMT recipients treated with vaccine-augmented DLI. One month after allogeneic transplantation, mice were injected i.v. with 10^4 ALL cells. One day later, the "DLI and Vac" group was vaccinated with 10^7 irradiated male recipient strain spleen cells. Two days after leukemia cell injection, mice in the "DLI" and "DLI and Vac" groups received DLI at a dose of 10^7 cells. Mice were followed daily. Starting at 10 days, some mice appeared moribund. Animals were euthanized when moribund or at 2 weeks after leukemia injection. When animals were moribund or at 2 weeks after leukemia injection, leukemia cell burden was measured by flow cytometry in femur bone marrow. Bars represent average and standard error of the mean for the groups. Control mice (n = 8) received no treatment. "DLI and Vac" mice (n = 10) received vaccine-augmented DLI. "DLI" mice (n = 3) received only DLI. *P* values for comparisons between groups are presented. In marrow, percentage leukemia was significantly less in "DLI + Vac" group (*P* = .006 by Mann-Whitney test).



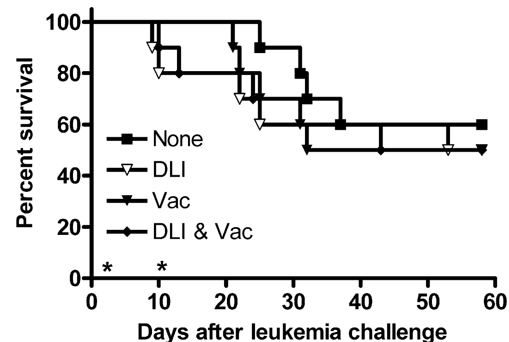


Figure 3. Survival after leukemia challenge in mice treated with vaccine-augmented DLI or DLI alone. (A) Single treatment with DLI and/or vaccine. One month after allogeneic transplantation, mice were infused i.v. with 10^4 male ALL cells. One day later, 1 group was vaccinated with male ALL cells. Two days after ALL cell challenge, all mice received 10^7 CD90.2-enriched spleen cells from HY antigen–primed C3.SW donors as DLI treatment. "*" indicates the time of the treatment with vaccine and DLI. Mice were followed for survival. The modest increase in survival in the DLI/vaccine group was not statistically significant (*P* = .28 by log-rank test; DLI/Vac group n = 18, DLI-only group n = 17). (B) Treatment with 2 courses of vaccine and/or DLI. In an independent experiment, the treated animals received vaccine 1 day after ALL challenge and DLI 2 days after ALL challenge. The treatments were repeated 9 days later, with another treatment with vaccine 10 days after ALL challenge and DLI 11 days after ALL challenge. In this Experiment 2, additional groups were included: a no-treatment group ("None") and a vaccine-only group ("Vac"). There were no significant differences in survival between groups as assessed by the log-rank test.

the 2 groups. In the DLI-only group, 15 mice died; 13 died from leukemia, whereas 2 died of GVHD. Two mice survived, and by body appearance and liver histology did not have active GVHD. In the vaccine-augmented DLI group, 14 mice died; 12 died of leukemia, whereas 2 died of GVHD. Among the 4 survivors, none had body features suggestive of severe GVHD, whereas 2 of the 4 had moderate histologic evidence of liver GVHD.

A limitation of this experiment was that animals were treated only once with vaccine and DLI. Although this experimental design consistently increased the activity of HY-specific donor T cells in vivo, it may have been quantitatively inadequate to control leukemia in a survival experiment. The survival trial was repeated, but transplant recipients were treated twice: once as before and again 9 days later. The transplant recipients were followed for 58 days after leukemia challenge, at which time the survivors were euthanized and examined by flow cytometry for residual leukemia. The outcome was the same (Figure 3B). Approximately half the transplanted animals survived, but there was no difference in survival between the groups. Only 1 of 21 surviving mice had flow-detectable leukemia in the marrow; the limit of detection was 0.1% (data not shown).

Relapsing Leukemia Cells Retain Sensitivity to Antigen-Specific Cytolytic T Cells

Because we had repeatedly observed in these experiments and other experimental models significant enhancement of antigen-specific T cell activity by vaccination coupled with DLI, we were puzzled by the very modest impact concurrent vaccination had on survival. One simple hypothesis for the finding would be loss of leukemia cell sensitivity to cytolytic T cells. This has been observed in numerous tumor immunology systems. Because we had reisolated leukemia cells from many of the animals at time of death, we were able to test this hypothesis. Resistance to antigen-specific T cells can occur if cells cease to express MHC molecules that present the target antigen. In the HY system, the Uty and Smcy antigen peptides are presented by H2-Db. We examined H2-Db expression by flow cytometry but found no reduction in H2-Db in the ALL cells that progressed in vivo (data not shown). Other mechanisms of acquired resistance could be loss of antigen expression or defects in apoptotic responses to cytolytic T cells. We tested these possibilities by examining the leukemias reisolated from animals after allogeneic DLI treatment for sensitivity to cytolytic T cells specific for HY antigens. Leukemia cells were cocultured with HY-specific CTLs, and after 3 days, the number of surviving leukemia cells was measured by flow cytometry. Figure 4 demonstrates that all of the leukemia reisolates remained sensitive to the CTLs ($P < .01$ by Student's *t*-test compared to the insensitive female leukemia antigen-negative control target). There was no difference in sensitivity between the reisolates from the 2 experimental groups (DLI group 29.1 ± 2.9% average ± SEM survival, DLI + vaccine group 30.7 ± 3.2%, $P = .75$ by *t*-test).

Long-Term Transplant Survivors Have Evidence of B Cell Immunity

Although we did not observe a large increase in the efficacy of DLI provided by concurrent vaccination in either long-term survival study, we did see survivors in both experiments. In Experiment 1, 17% of all challenged animals survived without signs of disease until

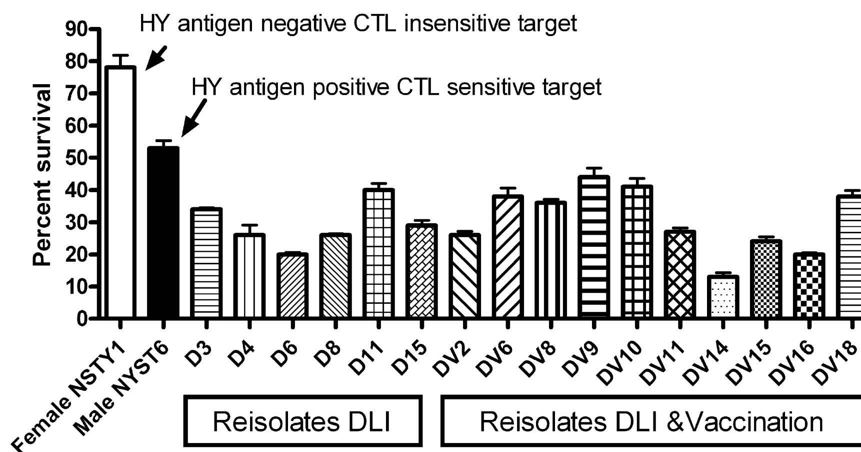


Figure 4. HY-positive male ALLs that exhibited progressive growth in allogeneic transplant recipients treated with DLI with and without vaccination remain fully sensitive to donor cytolytic cells specific for HY antigens. Donor strain cytolytic cells were generated from HY-primed female C3.SW spleen cells restimulated for 4 days in vitro with HY peptides. Cytolytic effector cells were mixed with leukemia cells in triplicate microcultures at effector-to-target ratio of 50:1. Three days later, the number of viable leukemia cells was measured by flow cytometry. The negative control leukemia target was a female recipient strain ALL cell line. The positive control target was the male ALL cell line that was injected into the animals prior to DLI. Leukemia reisolated from animals treated only with DLI are labeled "D#" and those from animals treated with DLI and vaccination are labeled "DV#." In these labels, "#" refers to the unique identifier for an animal in the experiment. "Percent survival" is plotted and was calculated as (number of viable leukemia cells in wells with cytolytic cells)/(number of viable leukemia cells in wells without cytolytic cells). Average and standard error of the mean are plotted.

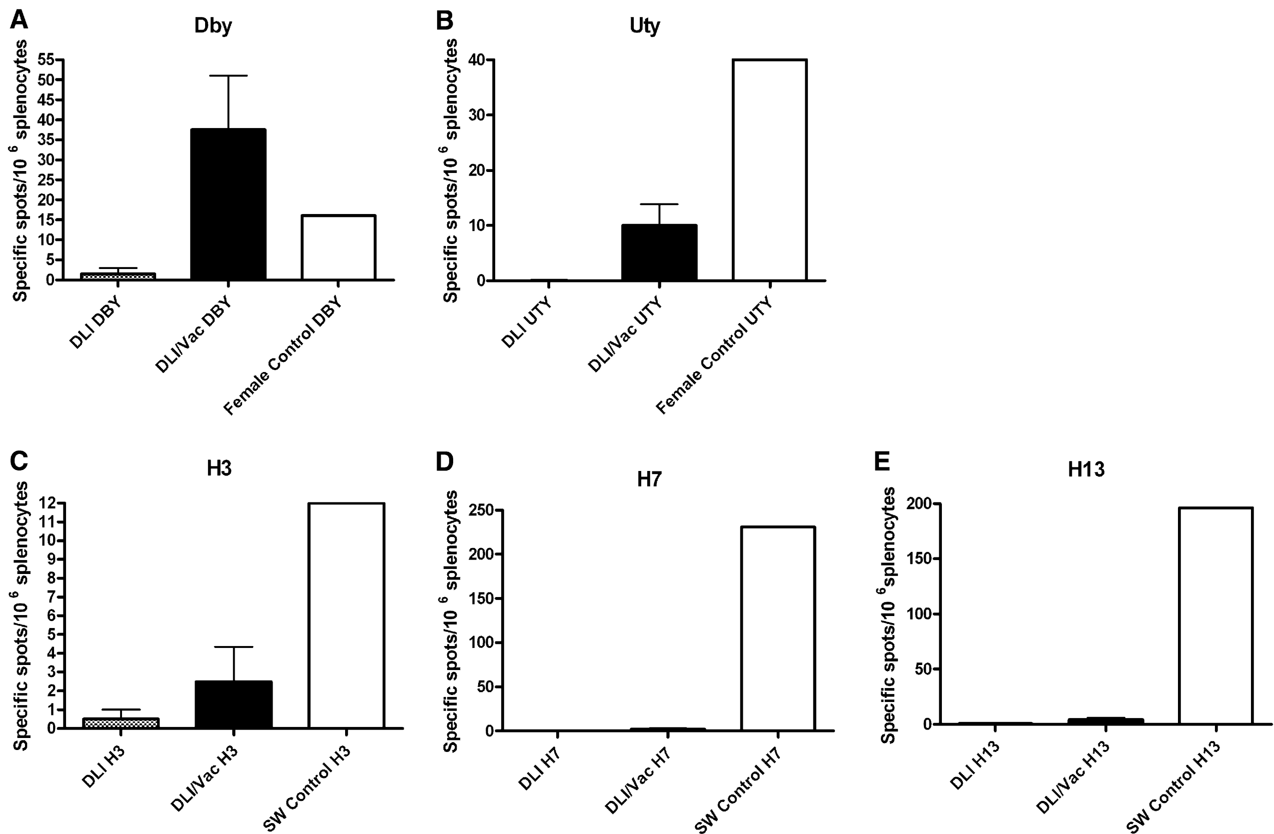


Figure 5. INF-γ ELISPOT responses to leukemia-restricted HY antigens and to widely distributed recipient minor histocompatibility antigens in long-term leukemia survivors. Seventy-three days after leukemia challenge, survivors with no evidence of active leukemia were euthanized, and INF-γ ELISPOT assays were performed on splenocytes of individual animals. In the top panels, responses to HY peptides (A) Dby and (B) Uty are displayed. In the lower panels, responses to the ubiquitously distributed C57BL/6 minor histocompatibility antigens (C) H3, (D) H7, and (E) H13 are displayed. Average and standard errors of the mean for the animals receiving DLI alone and for those receiving both vaccination and DLI are displayed. The positive control for the HY ELISPOTs was a female C57BL/6 primed against male C57BL/6 cells. The positive control for the ubiquitous C57BL/6 minor antigen ELISPOTS was a C3.SW mouse primed against C57BL/6 cells. Dby responses in DLI/vaccine-treated mice (n = 4) compared to DLI-only mice (n = 2) by 2-tailed *t*-test yielded $P = .077$. Similar comparison of Uty responses yielded $P = .082$.

termination of the experiment at 73 days. Spleen cells from all surviving animals were placed in culture to allow outgrowth of minimal residual leukemia. In none of the 6 survivors did we see leukemia emerge in cultures over a 2-week period (data not shown). In Experiment 2 52% of all animals (21 of 40) survived until day 58. Only 1 had leukemia detectable by flow cytometry in their marrow. The dose of leukemia cells used in these experiments is uniformly fatal in normal C57BL/6 mice.

In both survival experiments, we performed some immune function assays to learn if there was any evidence that immunity may have played a role in maintaining the remission of leukemia in the survivors. In Experiment 1, we were unable to detect HY-specific cytolytic T cells in the survivors following 4-day in vitro restimulation with irradiated male spleen cells (data not shown). However, using more sensitive ELISPOT assays, we did observe very modest HY peptide–specific (Uty and Dby) INF-γ release in spleen cells in 4 of 4 animals treated with vaccination and DLI (Figure 5A and B). We did not see similar responses in the 2 surviving animals treated with DLI only. We did not see significant INF-γ responses to the recipient strain minor histocompatibility antigens H3, H7, and H13 the survivors (Figure 5C, D, and E). In Experiment 2, none of the 21 survivors had evidence of either HY peptide–specific or alloantigen peptide–specific INF-γ responses (data not shown).

Our failure to find evidence of a robust T cell memory response in the majority of survivors suggested several possibilities. One possibility was that the T cell response occurred earlier in the experiment and waned to near undetectable levels by the time the surviving animals were assessed 2 months later. Leukemia was either completely eradicated by this wave of T cells or was not completely eradicated, and that animals with residual leukemia relapsed after the T cell response ceased. This interpretation would be compatible with the typical kinetics of a T cell response and the timing of relapse death that started to be observed after day 25. An alternate possibility was that non-T cell responses may have contributed to control of leukemia.

In the first survival experiment at the time of euthanasia, small amounts of serum had been obtained from surviving transplant recipients and pooled. A pilot

experiment was performed to determine if survivors had any evidence of a B cell antibody response that recognized leukemia cells. ALL cells were incubated in dilutions of normal mouse serum or survivors' serum. Fluorochrome labeled antimouse IgG or antimouse IgM was used as a second step, and flow cytometry was performed. Figure 6 demonstrates detection of IgM and IgG binding antibodies at dilutions of 1:40 or greater in survivors' serum. Specificity of the antibodies was explored using a large panel of malignant and nonmalignant cells chosen to help discern whether the antibodies were broadly reactive against hematopoietic cells or restricted to some feature of the NSTY ALL cells used to challenge the mice. The ALL cells were (1) male, (2) GFP positive, (3) contained a human bcr-abl gene, (4) C57BL/6 in origin, and (5) malignant (Figure 7 and Table 1). The pooled serum had broad but not universal reactivity, suggesting that the antibody response was not restricted to 1 of these features.

The pilot study could not address the question of whether the antibodies were induced by alloreactivity alone and/or by the posttransplant vaccination. To further address the question of whether an antibody response that recognized ALL cells could be produced by active graft-versus-host alloreactivity alone, we analyzed another independent experiment in which mice that had undergone allogeneic transplantation in which donors had been previously vaccinated against normal C57BL/6 recipient strain spleen cells to enhance alloreactivity and survived challenge with ALL. These mice had not received posttransplant vaccination against leukemia or normal cells. Eighty-five days after allogeneic transplantation, we collected serum from tail vein sampling from 10 living mice that had survived challenge with 10^6 ALL cells on day 5 after transplantation. Instead of flow cytometry, ELISA was used to detect antibodies because this technique was more sensitive and more quantitative. Figure 8 demonstrates that 9 of 10 mice had evidence of antibodies that bound ALL cells. To test whether posttransplant vaccination could also contribute to an antibody response, an independent experiment was performed in which allogeneic transplant recipients that had not been challenged with live ALL cells were vaccinated against either NSTY ALL or another C57BL/6 acute leukemia C1498. Figure 9 demonstrates that vaccinated mice had antibodies that bound the leukemia cells against which they were vaccinated.

Leukemia-Free Survival Is Associated with Antibody Responses

The first survival study experiments did not shed any light on the question whether the antibody response was related to survival. To address this question in survival Experiment 2, we systematically sampled serum from all transplant recipients at 3 time points: 1 month after transplantation but before challenge with leukemia and treatment, 3 weeks after leukemia challenge and treatment, and at day 58, when all long-term survivors were euthanized. ELISA for antibodies binding NSTY leukemia cells was performed for all samples. In addition, ELISA for antibodies binding normal donor C3.SW and normal recipient C57BL/6 splenocytes was performed on all survivors because we had more serum. Figure 10A demonstrates that leukemia reactive antibodies were significantly greater in survivors compared to those animals that died of leukemia. The survivors' serum exhibited some cross-reactivity with normal C3.SW donor and recipient C57BL/6 spleen cells (Figure 10B).

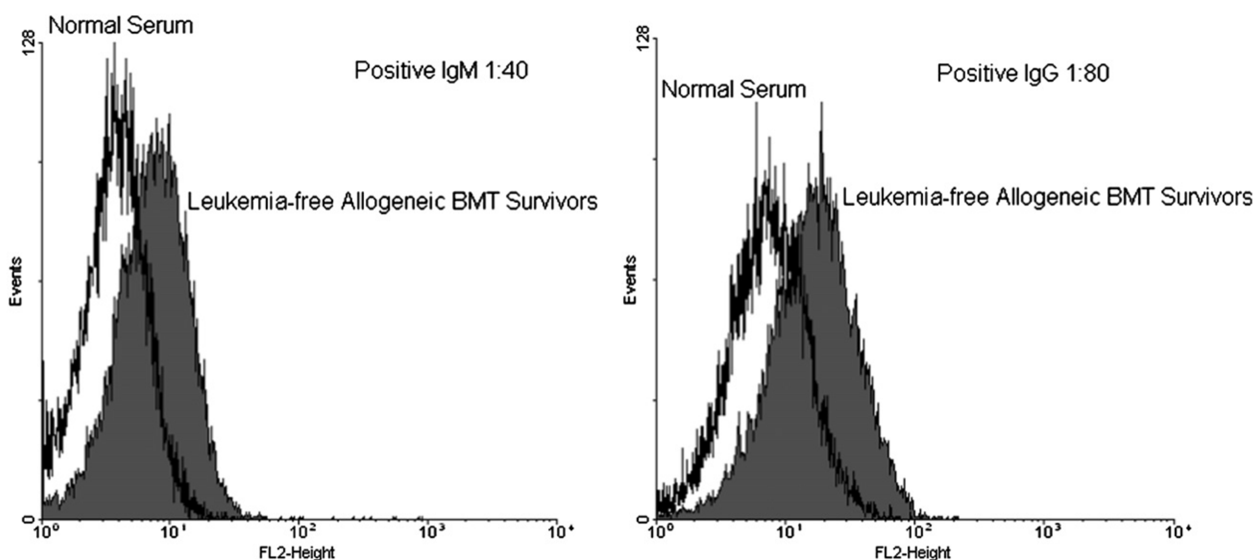


Figure 6. Serum from leukemia survivors contains IgM and IgG antibodies that bind to NYST ALL cells. NYST leukemia cells were incubated with diluted normal pooled mouse serum or serum pooled from mice that were leukemia survivors. PE-labeled secondary antimouse IgM (left panel) or antimouse IgG (right panel) was then used to detect cell surface bound antibody by flow cytometry. IgM antibodies were also detected at dilutions of 1:10 and 1:20. IgG antibodies were also detected at 1:10, 1:20, and 1:40 dilutions.

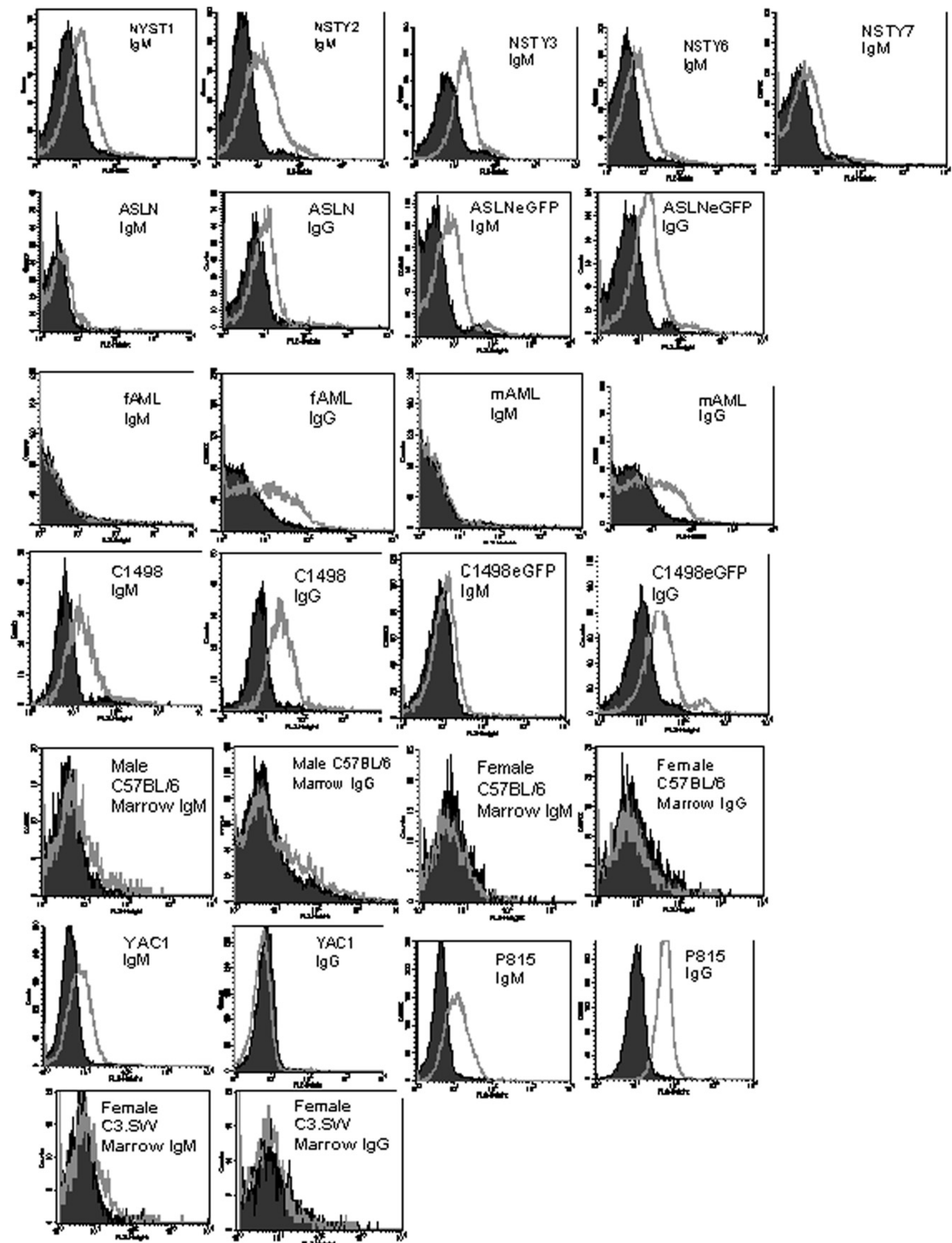


Figure 7. Assessment of cellular specificity of antibody responses in serum from leukemia survivors. Pooled serum from surviving mice diluted to 1:20 was added to a variety of cell types (described in Table 1A) and detected with either PE-labeled secondary antimouse IgM or antimouse IgG. Normal mouse serum diluted 1:20 was used as a control. The shaded histogram represents the normal serum control, whereas the unfilled histogram overlay is the serum from the ALL survivors. Forward and side scatter characteristics were used to gate on viable cells for the leukemia lines. For normal marrow, similar scatter characteristics were used to positively gate on mononuclear cells, whereas CD19-positive normal B cells were excluded from analysis.

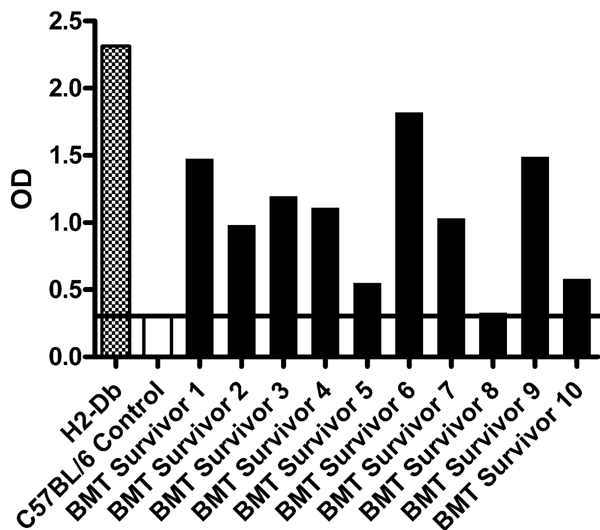


Figure 8. Detection of antibodies that bind an independently derived female leukemia line in animals that had undergone allogeneic transplantation and had survived challenge with leukemia cells. Four days after allogeneic transplantation, mice were challenged with 10^6 female NYST-1 ALL cells. Two months later, serum from individual animals was obtained from tail vein puncture and used at a 1:20 dilution in a cellular ELISA assay designed to detect antibodies on NYST-1. Antibodies were detected by a biotinylated polyclonal goat anti-mouse immunoglobulin followed by a streptavidin-alkaline phosphatase reagent. Optical density of the enzymatic conversion of PNPP is plotted. The negative control is serum from a normal C57BL/6 mouse. The positive control is a 1:100 dilution of a mouse monoclonal antibody specific for H2-Db.

Quantitative analysis on a limited subset of survivor samples demonstrated that the serum concentration of the antibodies was fairly significant, with an average dilution of 1:92, producing 50% maximum O.D. values in the assays. (In comparison, commercial-grade monoclonal antibody directed at H2-Db in a stock concentration of 0.5 mg/mL diluted at 1:750 produced 50% maximum O.D. in the same assays.)

This analysis compared antibody levels from survivors at the end of the experiment to the last sampled antibody level in animals that died of leukemia. We conducted another analysis to address the question of whether antibody levels at the first time point (4 weeks after allogeneic transplantation but before injection with leukemia cells) had any relationship with the risk of later death from leukemia or GVHD. Outcomes for each animal were categorized as alive, dead from leukemia, dead from GVHD, or dead of unspecified cause. Antibody levels for each animal were categorized as above or below the median for the entire experimental population. Chi-square analysis demonstrated a statistically significant relationship between outcome and antibody level at the time of leukemia challenge (Table 2). Few animals with high antibody levels died of leukemia ($P = .0197$). In comparison of death from leukemia with death from GVHD, higher antibody levels were associated with death from GVHD ($P = .0099$). A statistically significant difference was not observed between survivors and death from GVHD; however, the death from GVHD group was small (n = 5), limiting the power of the analysis.

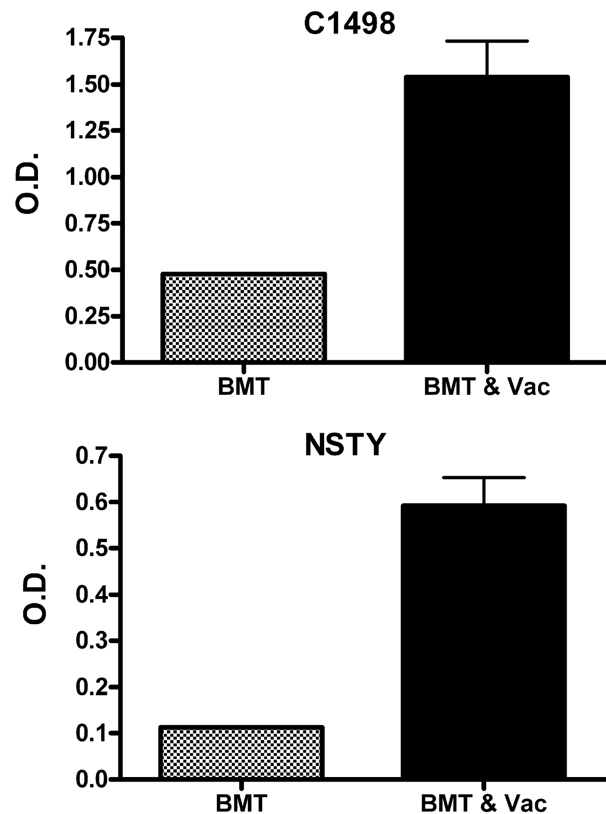


Figure 9. Detection of antibodies binding leukemia lines found in allogeneic transplant recipients vaccinated against C57BL/6 leukemias. Mice that had undergone allogeneic transplantation but had not been challenged with live leukemia cells were vaccinated against either irradiated NSTY or C1498 cells in adjuvant. Serum from individual animals (n = 3) was used in a qualitative ELISA assay for detection of antibodies that bound either C1498 or NSTY. Serum from a mouse (n = 1) that had undergone allogeneic transplantation but had neither been vaccinated nor challenged with viable leukemia was used as a control. Optical density in the ELISA is plotted.

DISCUSSION

Our initial goal was to determine if coupling active vaccination with ALL cells with DLI would improve immunologic control of ALL in recipients of allogeneic HSCT. The basis for this approach was our previous demonstration that coupling of active vaccination at the time of adoptive lymphocyte transfer substantially increased the number of antigen-specific T cells in recipients. We learned that vaccination did increase the frequency of the desired antigen-specific cells in transplant recipients treated with DLI. We also learned that this combined treatment did not increase the frequency of donor T cell responses to ubiquitously expressed recipient minor histocompatibility antigens, or the incidence of clinical GVHD as judged by mortality or histologic assessment of GVHD. However, despite these increases in the activity of

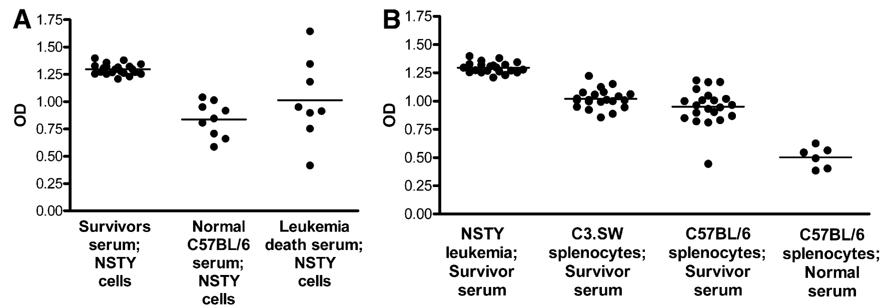


Figure 10. Antibody levels in long-term survivors compared to those in animals dying from leukemia. Serum samples were taken from all animals in survival Experiment 2, 4 weeks after transplantation but before leukemia challenge, 3 weeks after leukemia challenge, and for those animals surviving until day 58, when the experiment was ended. Control serum was obtained from normal C57BL/6 mice. ELISA was performed using NSTY leukemia cells for all samples. ELISA was also performed using C3.SW or C57BL/6 splenocytes for the samples from long-term survivors. All ELISAs were performed using a 1:20 dilution of the serum. (A) Higher levels of antibodies binding NSTY leukemia are present in long-term survivors compared to those present in animals dying of leukemia. Each dot represents a single animal. For survivors, the serum was that at the last sample on day 58. For the animals dying of leukemia, the serum was that last sampled at 3 weeks after leukemia inoculation. Horizontal lines represent the average. Significantly higher O.D. levels are present in survivors compared to those dying of leukemia (*t*-test $P < .0001$), or normal C57BL/6 mice (*t*-test $P = .001$). There was no difference between animals dying from leukemia and normal C57BL/6 mice (*t*-test, $P = .76$). (B) Survivor serum exhibits crossreactive binding with normal splenocytes. ELISA was performed using 1:20 dilutions of survivor serum on NSTY leukemia cells, C3.SW, or C57BL/6 spleen cells. In addition, assays were also performed using normal C57BL/6 serum and C57BL/6 splenocytes. Each dot represents a single animal, and horizontal lines are the averages. O.D. values using survivor serum on C57BL/6 and C3.SW splenocytes are significantly greater than values using normal C57BL/6 serum (*t*-tests, $P < .001$ for each comparison).

Table 1. Binding of Antibodies in Serum of Leukemia Survivors to a Panel of Murine Hematopoietic Cells

A. Characteristics

Cell	Malignant/normal	Male/female	GFP	bcr/abl	Strain	IgM	IgG
NSTY1	leukemia	female	GFP+	bcr/abl+	C57BL/6	+	ND
NSTY2	leukemia	female	GFP+	bcr/abl+	C57BL/6	+	ND
NSTY3	leukemia	female	GFP+	bcr/abl+	C57BL/6	+	ND
NSTY6	leukemia	male	GFP+	bcr/abl+	C57BL/6	+	+
NSTY7	leukemia	male	GFP+	bcr/abl+	C57BL/6	+	ND
fAML	leukemia	female	GFP+	bcr/abl+	C57BL/6	-	+
mAML	leukemia	male	GFP+	bcr/abl+	C57BL/6	-	+
ASLN-GFP	leukemia	female	GFP+	bcr/abl+	C57BL/6	+	+
C1498-GFP	leukemia	female	GFP+	negative	C57BL/6	-	+
ASLN	leukemia	female	negative	bcr/abl+	C57BL/6	-	-
C1498	leukemia	female	negative	negative	C57BL/6	+	+
female C57BL/6 marrow	normal	female	negative	negative	C57BL/6	-	-
male C57BL/6 marrow	normal	male	negative	negative	C57BL/6	-	-
female C3.SW marrow	normal	female	negative	negative	C3.SW	-	-
YAC	leukemia	female	negative	negative	A/Sn	+	-
P815	leukemia	female	negative	negative	DBA/2	+	+

B. Summary

Classification	IgM Positive	IgG Positive
NSTY series ALL	5/5	1/1
Not NSTY series ALL	4/7	7/11
ALL	6/7	3/3
Not ALL	3/9	5/9
BCR-ABL	6/9	5/5
Non BCR-ABL	3/4	3/4
GFP	6/9	5/5
Non GFP	3/7	3/7
Male	2/4	2/3
Female	7/12	6/9
C57BL/6	7/13	7/9
Not C57BL/6	2/3	1/3
Malignant	9/13	8/9
Malignant	0/3	0/3
Totals	**9/16**	**7/12**

Cells were incubated with normal mouse serum or pooled serum from leukemia survivors at a dilution of 1:20, followed by incubation with PE-labeled anti-IgM or anti-IgG. Flow cytometry was then performed, and individual histograms are presented in Figure 7. (A) "Cell" is the cell line or tissue examined. "Malignant/normal" indicates whether the cells were malignant cell lines or normal tissues. "Male/female" indicates the sex of the animal from which the cell line or tissue was derived. "GFP" indicates whether the cells contain a green fluorescent protein gene or not. "bcr/abl" indicates whether the cells contain a human bcr/abl gene. "Strain" indicates the mouse strain from which the cell line or tissue was derived. "IgM" and "IgG" indicate that cell-binding antibodies of this isotype were detected in serum from leukemia survivors. (B) Summary of IgM or IgG reactivity based on features of cells described in (A).

Table 2. Outcomes as a Function of Leukemia-Reactive Antibody Levels

Outcome	Low Antibody	High Antibody	Total
(A) Alive	9	12	21
(B) Dead from leukemia	8	1	9
(C) Dead from GVHD	1	4	5
(D) Dead, cause undetermined	2	3	5
Totals	20	20	40

Chi-square analysis

Comparison	*P*-Value
Overall, all groups	.0487
Alive (A) versus dead from leukemia (B)	.0197
Dead from GVHD (C) versus dead from leukemia (B)	.0099
Alive (A) versus dead from GVHD (C)	.3451
Dead from leukemia (B) versus (A, C, and D)	.0047

GVHD indicates graft-versus-host disease.
Low antibody indicates the antibody level was below the median; high antibody indicates the antibody level was above the median; dead from leukemia indicates animal had exhibited symptoms of acute leukemia prior to death (rear leg paralysis, lymphadenopathy) or at necropsy had massive splenomegaly or >25% marrow leukemia as measured by flow cytometry; dead from GVHD indicates the animal had clinical evidence of GVHD prior to death (weight loss, hair loss) and at necropsy did not have splenomegaly or detectable leukemia in marrow as measured by flow cytometry; dead, cause undetermined was death not meeting the diagnostic criteria for either death from leukemia or death from GVHD.
Chi-square analysis was initially performed upon the entire dataset ("Overall, all groups," 2 × 4 analysis). Having found a statistically significant difference additional 2 × 2 chi-square comparisons were performed between the specified groups.
One month after transplant but immediately before challenge with leukemia and before treatment with vaccination and/or DLI, peripheral blood was sampled. ELISA for leukemia reactive antibody was performed. Median O.D. for all animals was 0.93.

antileukemia donor T cells, we did not see a substantial increase in long-term survival when active vaccination was added to DLI. The failure was not because of acquired resistance to cytolytic T cells by the leukemia cells. These studies do not provide clear mechanistic explanations for the failure to improve acute leukemia-free survival in these experiments. We speculate that the kinetics of acute leukemia population in vivo may have simply outpaced the more limited expansion of antileukemia T cells in vivo.

Although our studies did not show improvements in survival between the experimental and control groups, they did demonstrate that there were a significant number of long-term survivors in both groups. These animals did not have clinical GVHD. We performed studies on survivors exploring the hypothesis that survivors would have evidence of T cell immunity that would have activity against leukemia cells. This hypothesis is compatible with the dominant paradigm in transplantation immunology that donor T cells are the initiators and frequently the mediators of GVL effects. To our surprise, we were not able to detect robust cytolytic activity in these survivors when their splenocytes were restimulated in vitro in conventional cytolytic T cell culture assays (data not shown). More sensitive ELISPOT assays on directly assayed splenocytes demonstrated only modest levels of HY antigen–specific T cell activity in a minority of survivors.

After failing to see evidence of robust long-term T cell immunity in the survivors, we screened the serum for antibodies to leukemia cells. To our surprise, the experiments provided evidence of both IgM and IgG antibodies that bound a variety of leukemia cells. The antibodies were not specific for the leukemia used to challenge or vaccinate the transplant recipients. However, the cross-reactivity did not correlate with single variables such as sex, presence of green fluorescent protein or bcr/abl genes, or strain of cell origin, and thus cannot be dismissed as an artifact. These serum antibodies were likely to have been polyclonal, and thus the observation that reactivity did not neatly segregate with these variables does not rule out the possibility that some of the antibodies may have reacted with epitopes related to these variables. However, the observation that there was evidence in the ELISA assays that the serum antibodies were reactive with nonmalignant C57BL/6 and C3.SW spleen cells suggests the hypothesis that in these transplants, there was generation of some autoimmune or autoreactive antibodies.

In these studies, we observed leukemia binding antibodies in an independent cohort of mice that had undergone highly alloreactive HSCT and had survived challenge with an independently derived ALL line, indicating that active vaccination is not necessary for the development of these antibodies when there is active alloreactivity. However, vaccination after allogeneic transplantation was also shown to induce antibody responses. Thus, both vaccination and active alloreactivity induce antibody responses that bind leukemia cells.

These studies do not answer the question of whether the antibodies we found played a causal role in immune control of leukemia. However, statistical analysis in the second survival experiment did demonstrate a strong correlation between leukemia-free survival and higher antibody levels. There is ample precedent for circulating antibodies having significant impact on normal and

malignant hematopoietic cells. Autoantibodies play a key pathologic role in autoimmune hemolytic anemia, immune thrombocytopenia, and autoimmune neutropenia [15,16]. Monoclonal anti-CD20 has a clear role in the treatment of B lineage lymphomas [17,18]. Antibodies to minor histocompatibility antigens and nonpolymorphic hematopoietic antigens have been identified in some studies of human GVHD [19-22], especially chronic GVHD (cGVHD) [23,24]. Active cGVHD has been associated with evidence of B cell activation [25,26].

In summary, the work described here demonstrates that in a murine model of allogeneic MHC-matched HSCT for high-risk ALL, an allogeneic GVL effect that involves both T cell and B cell responses can be associated with long-term control of leukemia. Our observations in this system indicate that immune therapies based on transient increases in cytolytic T cell activity alone are unlikely to produce dramatic improvements in disease control. However, immune interventions designed to increase both T cell and B cell responses may be worthy of further consideration. Our current work is exploring at a mechanistic level whether the antibody responses we observe in transplant survivors play a significant role in control of leukemia and how B cell responses interact with T cell responses in the posttransplant immune environment.

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