Minor Antigen Distribution Predicts Site-Specific Graft-versus-Tumor Activity of Adoptively Transferred, Minor Antigen-Specific CD8 T Cells

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A B S T R A C T

The clinical success of allogeneic T cell therapy for cancer relies on the selection of antigens that can effectively elicit antitumor responses with minimal toxicity toward nonmalignant tissues. Although minor histocompatibility antigens (MiHA) represent promising targets, broad expression of these antigens has been associated with poor responses and T cell dysfunction that may not be prevented by targeting MiHA with limited expression. In this study, we hypothesized that antitumor activity of MiHA-specific CD8 T cells after allogeneic bone marrow transplantation (BMT) is determined by the distribution of antigen relative to the site of tumor growth. To test this hypothesis, we utilized the clinically relevant male-specific antigen HY and studied the fate of adoptively transferred, HY-CD8+ T cells (HY-CD8) against a HY-expressing epithelial tumor (MB49) and pre-B cell leukemia (HY-E2APBX ALL) in BMT recipients. Transplants were designed to produce broad HY expression in nonhematopoietic tissues (female → male BMT, [F → M]), restricted HY expression in hematopoietic tissues (male → female BMT, [M → F]), and no HY tissue expression (female → female BMT, [F → F]). Broad HY expression induced poor responses to MB49 despite sublethal graft-versus-host disease and accumulation of HY-CD8 in secondary lymphoid tissues. Antileukemia responses, however, were preserved. In contrast, restriction of HY expression to hematopoietic tissues restored MB49 responses but resulted in a loss of antileukemia responses. We concluded that target alloantigen expression in the same compartment of tumor growth impairs CD8 responses to both solid and hematologic malignancies [8,9]. Further, durable GVT responses have been generated, with relative sparing of GVHD, by immunizing donors against MiHA before adoptive transfer [10]. A potential pitfall of this approach, however, is the generation of T cell dysfunction produced by broad expression of minor antigens in alloHSCT recipients. Indeed, expression of a target MiHA in nonhematopoietic tissues has been shown to substantially reduce the efficacy of adoptively transferred T cells against solid tumors [11]. Proposed mechanisms for this phenomenon include chronic and inefficient antigen presentation [12], blockade of functional CD8 memory [13], induction of donor T cell apoptosis [14], and upregulation of negative costimulatory molecules, such as PD-1 [15]. Although these studies have established the influence of minor antigen distribution on the potency of adoptively transferred T cells, the relationship between tissue antigen expression, site of tumor growth, and T cell function has not been directly studied in a single antigen system.

In the present study, we adoptively transferred miHA-specific T cells into alloHSCT recipients mismatched at the male-specific minor antigen HY, expressed on either non-hematopoietic or hematopoietic tissues, and assessed their antitumor efficacy against an HY-expressing epithelial tumor and leukemia. We selected HY as a target antigen because it is endogenous, MHC-restricted, and has been implicated in clinically significant GVHD and GVT effects [16,17]. We demonstrate that broad HY expression produces poor solid and hematologic malignancies [8,9]. Further, durable GVT responses have been generated, with relative sparing of GVHD, by immunizing donors against MiHA before adoptive transfer [10]. A potential pitfall of this approach, however, is the generation of T cell dysfunction produced by broad expression of minor antigens in alloHSCT recipients. Indeed, expression of a target MiHA in nonhematopoietic tissues has been shown to substantially reduce the efficacy of adoptively transferred T cells against solid tumors [11]. Proposed mechanisms for this phenomenon include chronic and inefficient antigen presentation [12], blockade of functional CD8 memory [13], induction of donor T cell apoptosis [14], and upregulation of negative costimulatory molecules, such as PD-1 [15]. Although these studies have established the influence of minor antigen distribution on the potency of adoptively transferred T cells, the relationship between tissue antigen expression, site of tumor growth, and T cell function has not been directly studied in a single antigen system.

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responses to solid tumors that can be improved with hematopoietic restriction of HY. Antileukemia responses, however, are lost with hematopoietic restriction of HY and preserved with broad expression, suggesting that the proximity of target antigen expression to the site of tumor growth predicts the efficacy of adoptively transferred T cells during alloHSCT.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 (B6) CD45.1 (H-2b congenic) mice were purchased directly from the National Cancer Institute-Frederick Animal Production Program (Frederick, MD). Male and female E2a-PBX ALL mice were purchased from Jackson Laboratories (Bar Harbor, ME), MRL/MpJ-FaslRtg/J, CD8 donor lymphocyte infusion [20]. We have previously demonstrated GVHD in additional target organs, including the liver, in male according to an established scoring system [19]. We have previously changes, fur changes, hunched posture, and mobility was performed daily for 7 days. Clinical monitoring for GVHD, including observation for skin disease-related morbidity by gating on live, CD8aþ donor lymphocyte infusion [20].

**T Cell–depleted Bone Marrow Transplantation**

Bone marrow cells were flushed from the tibias and fibulas of female or male B6 CD45.1þ mice using 10% complete mouse media CMM; RPMI 1640 with 10% heat-inactivated fetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, 1% L-glutamine (HyClone, Carlsbad, CA) and 1% N-2-hydroxyethylpiperoxazine-N'2-ethanesulfonic acid buffer (Sigma-Aldrich, St. Louis, MO), passed through a 70-μm nylon filter, and erythrocyte depleted using ACK lysis buffer (Lonza Walkersville, Walkersville, MD). T cells were depleted from donor marrow using anti-CD3 microbeads through automated magnetic cells sorting (AutoMACS, Miltenyi Biotec, Auburn, CA). 3.5 × 10⁶ T cell–depleted marrow was injected in serum-free RPMI via tail vein injection into recipients that were administered lethal irradiation at a dose of 1100 cGy (137 Cs on the source) on the same day. Female or male B6 CD45.1þ HSCT recipients were weighed twice every 7 days. Clinical monitoring for GVHD, including observation for skin changes, fur changes, hunched posture, and mobility was performed daily, according to an established scoring system [19]. We have previously demonstrated GVHD in additional target organs, including the liver, in male recipients of HY-specific CD8 donor lymphocyte infusion [20].

**Adaptive Transfer and Detection of HY-specific CD8 T Cells**

Single cell suspensions of splenocytes from naïve female MoltHAn donors were prepared and 1 × 10⁶ splenic CD8 T cells were intravenously injected on day 7. The delayed adaptive transfer protocol was selected to allow engraftment of donor antigen presenting cells and leukemic cells [21]. Detection of HY-CDS was performed by flow cytometry in the spleen and bone marrow of mice either euthanized at specific weekly time points or for disease-related morbidity by gating on live, CD8aþ CD45.2þ cells. This was a reliable gating strategy because splenocytes from HY-TCR transgenic donors contain negligible numbers of CD8aþ myeloid cells and donor CD8 T cells were isolated from the T cell–depleted transplant would not express the congenic marker. CD45.1. Absolute numbers of HY-CDS were determined by using a live-gated events. If more than 100,000 events were collected, the absolute number of cells was normalized to 100,000 by the proportion (CD8aþ CD45.2þ/live cells – [x/100,000]).

**Ex vivo Sorting and HY Peptide Stimulation of HY-specific CD8 T Cells**

Single cell suspensions of bone marrow were cultured and stained with fluorochrome-conjugated anti-CD8α and CD45.2 antibodies to identify adoptively transferred HY-CDS for flow cytometry-based sorting. Live, CD8aþ CD45.2þ were sorted in PBS,1% BSA in sterile conditions on an LSRII flow cytometer (BD Biosciences). Immediately after sorting, proliferation to HY-peptide stimulation was measured using carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution and flow cytometry. Sorted cells were washed twice in PBS,1% BSA, labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) for 10 minutes at 37°C, placed in culture at a concentration of 1 × 10⁶ cells/ml CMM, and stimulated with 100 μM UTY peptide (Class 1 Dominant HY peptide, WNHHNMDLI) for 72 hours at 37°C. Cells were then harvested, washed twice in FACS buuffer, and prepared for flow cytometry.

**Dendritic Cell Vaccination**

Dendritic cells for vaccines were cultured from male B6 bone marrow as previously described [22]. Dendritic cells were activated with 4 μg/ml anti-CD40 on day 4, collected within 24 hours of activation, resuspended in serum-free media, and intraperitoneally injected at a dose of 1 × 10⁵ cells per recipient at the time of adoptive CD8 T cell transfer.

**MB49 Tumor Challenge**

The MB49 cell line was originally provided by Dr. Edmund Lattime (Robert Wood Johnson Medical School, New Brunswick, NJ). MB49 is a chemically induced uroepithelial carcinoma that expresses the male-specific HY minor antigen [23] and is B6-derived. MB49 was maintained in standard sterile culture conditions (37°C, 5% CO₂) in CMM. Exponentially growing tumor cells were harvested and trypsin and injected at a dose of 1 × 10⁶ cells per recipient into the subcutaneous tissue of the left flank. Tumors were measured in 2 dimensions (length × width) twice every 7 days by digital calipers. Approximate spherical volumes were calculated for each measurement according to (length/2) × (width/2) × (length × width/4) × 4/3π. Tumors were euthanized when the volume reached 2 cm or tumor-related morbidity developed according to institutional protocols. If a mouse was euthanized or found dead, the most recent tumor measurement was carried forward for the remainder of the experiment.

**Pre-B Cell Acute Lymphoblastic Leukemia Challenge**

A male, GFP-conjugated, B6-derived murine pre-B cell acute lymphoblastic leukemia cell line carrying the human E2a-PBX1 transgene by MMLV insertion as previously described [24] was generously provided by Dr. Janet Bijil (Centre de Recherche de l’Hopital Maisonneuve-Rosemont, Montreal, Quebec, Canada). Cells were maintained in culture at 37°C and 5% CO₂, harvested on day 3 in an early exponential growth phase, and prepared as single-cell suspensions. 5 × 10⁵ leukemia cells were infused by tail vein injection in serum-free RPMI at the time of bone marrow transplantation (BMT). Monitoring for leukemia-related morbidity, including hind leg paralysis, wasting, abdominal distention, and poor movement, was performed daily. Leukemia burden in the bone marrow and spleen was measured by flow cytometry in animals euthanized at specific time points or for leukemia-associated morbidity. Animals with clinical findings suggestive of leukemia morbidity but with no GFP þ cells in the marrow were censored from leukemia survival analysis.

**HY-specific Immunogenicity of Male E2a-PBX ALL by Interferon Gamma ELISA**

HY-specific immunogenicity of male E2a-PBX ALL was established by coculturing male E2a-PBX ALL, female E2a-PBX ALL with positive control male dendritic cells with HY-CDS for 72 hours at a stimulator to responder ratio of 1 (1 × 10⁶):1 (1 × 10⁶); Concentrations of INF-γ (pg/ml) from in vitro cocultures were measured by ELISA (Quantikine INF-γ, R&D Systems) in triplicate according to the manufacturer’s protocol and read at 450 nm on a microplate reader (BioRad, Hercules, CA).

**Flow Cytometry**

Flow cytometry analysis for surface molecule expression was performed on an LSR II Fortessa flow cytometer (BD Biosciences, Hunt Valley, MD). The following monoclonal antibodies were utilized for flow-cytometry: fluorescein isothiocyanate, phycocerythrin, peridinin chlorophyll protein complex, cyanine 5.5 (PerCP-Cy 5.5), allophycocyanin, and Pacific Blue–conjugated antibody to mouse B220, CD4, CD8a, CD44, CD45.2, CD62L, PD-1 and Tim-3. Compensation controls and immunoglobulin isotype controls were generated for each experiment using fresh splenocytes.

**Statistical Analysis**

Statistical tests were performed using GraphPad Prism version 4.0b for Macintosh (GraphPad Software, San Diego, CA). For tumor volume measurements, the last tumor volume recorded for each mouse at the time of death was used in calculations of tumor volume at each time point after death. Kaplan-Meier survival curves were created and analyzed using the Wilcoxon rank-sum test to analyze curves. Significant differences between groups in clinical scores, in vivo T cell enumeration, and in vitro assays were determined by unpaired Student t-test. P values less than .05 were considered significant.

**RESULTS**

**Broad HY Expression in Nonhematopoietic Tissues, Expands Adoptively Transferred HY-CDS in Secondary Lymphoid Tissues and Produces GVHD**

We first established whether HY mismatch was sufficient to produce clinically significant allreactivity in our model. Critical to this objective was the ability to enumerate and track antigen-specific CD8 as a function of HY tissue distribution. To this end, we have demonstrated GVHD in additional target organs, including the liver, in male recipients of HY-specific CD8 donor lymphocyte infusion [20].
produced milder, sublethal alloreactivity that allows for quantitative analysis of T cell responses using a delayed infusion platform [26]. In this study, B6 male and females received T cell–depleted B6 female BMT ([F→M] and [F→F] respectively) on day 0, followed by adoptive transfer of $1 \times 10^7$ MataHari (HY-CD8) on day 7. Clinical GVHD scoring and enumeration of HY-CD8 was performed through day +35 to 42 post-BMT. As expected, adoptive transfer of HY-CD8 produced a nonlethal, clinical GVHD in males as defined by clinical score (Figure 1A). HY-directed GVHD in this model manifested primarily with skin changes (scaling, denuded areas), fur changes (ruffling, patchy loss) and decreased activity (Figure 1B) consistent with other murine models of single minor antigen-mismatch alloHSCT [20,27]. Broad expression of HY in [F→M] recipients resulted in the accumulation of large numbers of, CD8a$^+$CD45.2$^+$ HY-CD8, in both the spleen and bone marrow (Figure 1C). As expected, syngeneic female controls with no tissue expression of HY had low but detectable numbers of HY-CD8. Four weeks after adoptive transfer, significantly higher numbers of HY-CD8 could still be detected in [F→M] recipients compared to their [F→F] counterparts (Figure 1D).

Adoptively Transferred HY-CD8 Expand to Broad HY Tissue Expression but Do Not Control a HY-expressing Solid Tumor

Although vaccination against miHA has been shown to cure solid tumors, we have previously observed reduced tumor-specific vaccine responses when alloreactivity generated against minor antigens is not shared by the tumor [20,26]. In the present study, we wished to determine how CD8 function is affected by alloreactivity produced by an antigen shared by the tumor. Again, [F→M] and [F→F] T cell–depleted BMT recipients were given $10 \times 10^6$ HY-

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Adoptively transferred HY-specific CD8 T-cells expand and produce GVHD in allogeneic males. Lethally irradiated B6 male mice received $5 \times 10^6$ T cell (CD3) depleted marrow on day 0, followed by $1 \times 10^7$ HY-specific CD8 by tail vein injection on day +7. Clinical GVHD scoring was performed once weekly. (A) Composite GVHD scores on day +60 after transplantation were consistent with sublethal GVHD, particularly manifested as (B) changes in skin integrity, fur ruffling, and posture/activity, compared with no clinical signs of GVHD in syngeneic female recipients of female BMT. GVHD scoring data represent 4 independent experiments with 6 to 10 mice/group. (C) HY-specific CD8 were enumerated from spleen and bone marrow of transplant recipients by flow cytometry 7 and 28 days after adoptive transfer (day +14 and +35 post BMT) by gating on live, CD8a$^+$CD45.2$^+$ cells. Representative flow plots (left panel) and total numbers of CD8a$^+$CD45.2$^+$ cells per 100,000 splenocytes (right panel, top) or bone marrow cells (left panel, bottom) are shown.
Figure 2. Adoptively transferred HY-specific CD8 cannot control MB49 in male recipients. Experimental [F→M] and control [F→F] B6 mice received T cell–depleted B6 female bone marrow on day 0, followed by adoptive transfer of HY-CD8 on day +7. They were then challenged with \(1 \times 10^5\) MB49, an HY-expressing epithelial tumor, subcutaneously on day +14. Tumor-free survival was followed daily (A), and tumor volumes measured in 2 dimensions twice weekly (B). [F→M] recipients (closed square, solid line) had poor tumor control, similar to [F→F] controls that did not receive HY-CD8 (dotted line, closed triangle). [F→F] recipients of HY-CD8 preserved immune responses. (C) Enumeration of adoptively transferred HY-CD8 in spleen as live, CD8a\(^+\)CD45.2\(^+\) cells 14 days after adoptive transfer showed significant accumulation of HY-CD8 in [F→M] despite poor tumor control, compared to [F→F] tumor bearing (closed circle) or tumor-free recipients. (D) Expression of PD-1 (gray shaded) compared to IgG2k isotype controls (black dotted) was measured on live, CD8\(^+\)CD45.2\(^+\) HY-specific CD8 on day +14 post BMT (at the time of MB49 injection) by flow cytometry. Expansion data and PD-1 histograms are representative of at least 4 independent experiments with 6 to 10 mice/group. (E) Live, CD8\(^+\)CD45.2\(^+\) cells were collected by flow sorting on day +14 post BMT and restimulated in vitro with the Class I immunodominant HY peptide (UTY). Proliferation was measured by CFSE dilution at 72 hours. Naïve female MataHari (HY-specific) splenocytes served as controls.
CD8 on day +7, followed by subcutaneous injection with MB49, an immunogenic [28] HY-expressing uroepithelial tumor, on day +14. [F→M] that did not receive HY-specific adoptive transfer served as controls. All [F→M] mice developed rapidly growing tumors, with 100% tumor-associated mortality by day +35 post-tumor injection (day +42 post BMT) (Figure 2A,B). This survival curve was statistically identical to [F→M] that did not receive HY-CD8. [F→F] recipients of HY-CD8 adoptive transfer; however, they demonstrated preserved antitumor responses with 55 ± 3.7% tumor-free survival (P < .0035). [F→F] that developed tumors did so at a significantly slower rate (P = .039) and had longer overall survival, suggesting improved tumor control (Figure 2B).

Enumeration of CD8+ CD45.2+ HY-CD8 in the spleen on day +21 post-tumor injection (day +42 post BMT) was performed on [F→M] and [F→F] that developed tumors and [F→F] tumor-free survivors in a cohort not included in survival analysis. This time point was selected because it represented both the point of accelerating tumor growth and robust HY-CD8 accumulation in the GVHD studies. Significantly more HY-CD8 accumulated in the spleen (Figure 2C, representative flow plots left panel, total number of HY-CD8 right panel) of [F→M] recipients, compared with syngeneic [F→F] recipients that developed tumor (P = .0042). [F→F] tumor-free survivors had significantly higher numbers of HY-CD8 than their tumor-bearing counterparts, suggesting that adoptively transferred HY-CD8 could expand in response to MB49 and were not deleted in the short term. Interestingly, 100% of HY-CD8 recovered from the spleen of [F→M] recipients expressed PD-1, whereas [F→F] did not express PD-1, regardless of whether they were able to control tumor (Figure 2D).

Poor survival, rapid tumor growth, and PD-1 expression suggested that adoptively transferred CD8 became dysfunctional in [F→M] mice where HY was broadly expressed. To begin to investigate whether HY-CD8 could respond to HY independent of the allogeneic environment, HY-CD8 were isolated by flow sorting live, CD8a+ CD45.2+ splenocytes on day +21 (day +14 postadoptive transfer). An increased HY-CD8 dose of 5 × 10^7 was utilized to improve the sorted yield. The time point selected corresponded to the day of MB49 injection in tumor challenge studies. Sorted HY-CD8 and naïve MataHari control splenocytes were placed in culture, stimulated ex vivo with the class I immunodominant HY peptide UTY, and proliferation measured after 72 hours by CFSE dilution. Under these conditions, both HY-CD8 recovered from [F→F] recipients and naïve HY-CD8 controls proliferated, whereas HY-CD8 from [F→M] recipients, did not (Figure 2E).

**HY-expressing Dendritic Cell Vaccination Cannot Restore Functionality of HY-CD8 Expanded by Broad HY Expression**

To determine whether professional HY antigen presentation could overcome poor immune responses in [F→M] recipients, 1 × 10^5 anti-CD40 activated male DCs were injected intraperitoneally at the time of HY-CD8 adoptive transfer on day +7, followed by MB49 challenge on day +14. DC vaccination completely prevented tumor growth in [F→F] recipients (100% tumor-free survival, Figure 3A), whereas all of the [F→M] developed progressive tumors. As expected, survival curves in unvaccinated controls receiving HY-CD8 were consistent with those shown in Figure 2B.

**Restriction of HY to Hematopoietic Tissues Preserves Functionality of Adoptively Transferred HY-CD8 but Does Not Prevent PD-1 Expression**

To determine whether restricted expression of HY could improve antitumor responses, we transplanted T cell–depleted B6 male marrow into female B6 mice [M→F] and adoptively transferred HY-CD8 on day +7 as described before. No systemic GVHD was observed in [M→F] recipients of HY-CD8 (Figure 4A, top panel) and marrow cellularity was similar at day +60 to [F→M] (Figure 4A, bottom panel), excluding an early immune-mediated aplasia.

When [M→F] were challenged with MB49 at day +14 post-BMT, tumor-free survival was 52.5 ± 4.9% (Figure 4B), similar to [F→F] recipients shown in Figure 2A. Enumeration of CD8a+ CD45.2+ cells in [F→M], [M→F], and [F→F] from

![Figure 3.](image-url) **Figure 3.** HY-expressing dendritic cell vaccination cannot restore antitumor responses in [F→M] recipients of HY-specific CD8 that express HY in nonhematopoietic tissues. Lethally irradiated female or male B6 recipients were transplanted with TCD female B6 bone marrow on day 0, 1 × 10^7 HY-CD8 on day +7 and 1 × 10^6 CD40-activated male dendritic cells were injected intraperitoneally also on day +7. AlloHSCT recipients were then followed for (A) tumor-free survival and (B) tumor growth in [F→F] recipients (closed triangle, solid line) and [F→M] recipients (closed square, solid line), with rapidly growing tumors (closed square, solid line). Unvaccinated [F→M] (closed square, dotted line) and [M→F] (closed triangle, dotted line) that received HY-CD8 served as controls. Survival and tumor volume curves are representative of 3 independent experiments with 8 to 10 mice/group.
Figure 4. Hematopoietic restriction of HY preserves responses to MB49 despite PD1 expression on adoptively transferred HY-specific CD8. To restrict HY to hematopoietic tissues, lethally irradiated female B6 mice received T cell–depleted B6 male [M→F] BMT on day 0 followed by 1 × 10^7 HY-specific CD8 on day +7. AlloHSCT recipients were (A) clinically graded for GVHD and total bone marrow cells recorded after erythrocyte lysis to assess overall marrow cellularity. (B) Recipients were next challenged with MB49 on day +14 and followed for tumor-free survival in [F→M] (closed square) and [M→F] (closed diamond) groups. Data show a single experiment representative of 3 independent experiments with 8 to 12 mice/group. (C) Accumulation of HY-CD8 was measured by flow cytometry as live, CD8a^+^CD45.2^+^ cells 14 days after adoptive transfer via representative contour plots (left panels) and absolute numbers per 100,000 splenocytes (right, top panel) or bone marrow cells (right, bottom panel). (D) PD-1 expression was measured by flow cytometry on HY-CD8 in the spleen and bone marrow of [M→F] and [F→M] recipients 14 days after adoptive transfer. Histograms depict live, CD8a^+^CD45.2^+^PD1^+^ cells (gray shaded) compared to IgG2k isotype controls (dashed line). Data shown are representative of 3 independent experiments. (E) To verify functionality, HY-CD8 were sorted from [F→M] and [M→F] ex vivo 14 days after adoptive transfer, restimulated with UTY peptide, and proliferation measured by CFSE dilution after 72 hours. (F) Expression of PDL-1 (B7:HL), the ligand for PD-1, was measured by flow cytometry in MB49 before subcutaneous injection (gray shaded) and at day +28 in animals that succumbed to tumor-related mortality and shown in this representative histogram.
spleen and bone marrow at the time of tumor challenge (7 days after adoptive transfer, 14 days after BMT) revealed a similar accumulation of HY-CD8 in these tissues (Figure 4C). However, 21 days after tumor challenge (day +42 after BMT), there were significantly higher numbers of HY-CD8 in the spleen of [M→F] compared with [F→M] recipients, regardless of their ability to control tumor (Figure 4C, top panel). Interestingly, numbers of HY-CD8 in the bone marrow remained high in all groups, and were not statistically different between groups (Figure 4C, bottom panel).

Unexpectedly, as shown in Figure 4D, PD-1 was uniformly expressed on CD8a+CD45.2+ HY-CD8 recovered from the spleen and bone marrow of tumor-bearing [F→M], tumor-bearing [M→F], and tumor-free [M→F] (day 21 after tumor challenge, day +42 after BMT), suggesting that PD-1 expression did not correlate with the ability to reject tumor. To determine the ability of adoptively transferred HY-CD8 to respond to HY independent of the allogeneic environment, live, CD8a+CD45.2+ bone marrow cells were flow sorted in a separate experiment 14 days after adoptive transfer, placed in culture, stimulated with UTY for 72 hours, and proliferation measured by CFSE dilution. CD8a+CD45.2+ cells from [M→F] bone marrow did proliferate, whereas those sorted from [F→M] bone marrow did not (Figure 4E) indicating that bone marrow expression of HY impaired the functionality of HY-specific T cells. PDL-1 (B7:H1), the ligand for PD-1, was not expressed on MB49 either before injection or on established tumors in mice (Figure 4F). Collectively, these results suggest

Figure 5. Hematopoietic restriction of HY does not preserve antileukemia responses, which are restored with broad expression of HY. (A) Immunogenicity of a murine, male, GFP-conjugated pre-B cell ALL (E2APBX-ALL) was determined by coculturing 1×10⁶ male versus female E2APBX with 1×10⁶ MataHari splenocytes and measuring interferon gamma production by ELISA at 48 hours. Male dendritic cells and naïve MataHari splenocytes alone served as positive and negative controls, respectively. (B) Overall survival was measured after 1×10⁵ E2APBX-ALL cells were incorporated into T cell–depleted intravenous grafts into ([F→M] solid square), ([M→F], solid diamond) and ([F→F], solid circle) recipients on day 0, followed by adoptive transfer of HY-CD8 on day +7. [F→F] that did not receive HY-CD8 (dotted line) served as negative controls. (C) The quantity of GFP+ cells in the bone marrow was assessed by flow cytometry to measure leukemia burden either at time of death or day +72 post transplantation. (D) Number of bone marrow-infiltrating HY-CD8 (live, CD8a+CD45.2+) on day +7, +14 and +21 after T cell–depleted transplantation and coinjection (intravenously) of 1×10⁵ E2APBX-ALL cells. Data shown are representative of 3 independent experiments.
that PD1 expression on T cells and PD-1 ligation was not required for loss of HY-CD8 tumor control and that PD-1 expression may instead represent a nonspecific activation marker, perhaps because of T cell activating allogeneic effects.

**HY Expression on Hematopoietic Tissues Results in Poor Antileukemia Responses, despite Continued Expansion of HY-CD8 in the Bone Marrow**

We next determined whether hematopoietic restriction of HY could also preserve antileukemia responses against a GFP-conjugated, murine pre-B cell acute lymphoblastic leukemia derived from male mice carrying the human E2A-PBX transgene (E2APBX-ALL) [24]. To establish that this male-derived E2APBX-ALL cell line expressed immunologically significant HY, we cocultured HY-CD8 for 72 hours with male or female E2APBX-ALL in a 1:1 ratio and measured interferon-gamma production by ELISA. Coculture of HY-CD8 with female E2APBX-ALL did not produce detectable interferon gamma production at 72 hours (607 ± 26.1 pg/ml, *P* = .0019, Figure 5A). This confirmed HY-specific immunogenicity of the male E2APBX-ALL cells for incorporation into the alloHSCT model.

For the leukemia challenge study, 1 × 10^6 male E2APBX-ALL were injected intravenously at the time of T cell–depleted BMT on day 0 into [M→F], [M→M], and [F→F] recipients, followed by adoptive transfer of 1 × 10^7 HY-CD8 on day +7. GFP+ leukemic burden in the bone marrow was assessed postmortem by flow cytometry. In contrast to the solid tumor model, [M→M] recipients suffered 100% leukemia-associated mortality by day +7 post BMT at a rate identical to [F→F] recipients that did not receive HY-CD8 adoptive transfer (Figure 5B). [F→M] recipients, on the other hand, had 82.1% ± 5.8% leukemia-free survival. As in the solid tumor model, [F→F] recipients also preserved antileukemia responses after adoptive transfer of HY-CD8, with 62% ± 5.2% survival. Leukemia-associated mortality in [M→F] recipients was characterized by massive splenomegaly and a high burden (64.6% ± 9.7%) of GFP+ leukemia in the bone marrow (Figure 5C) measured by flow cytometry at the time of death. [F→M] survivors had no or very low levels of detectable leukemia on day +72 post BMT (11% ± 0.83%), whereas [F→F] recipients had low levels of leukemia, with 2.2% ± 1.7% GFP+ cells in the marrow (Figure 5C). Achievement of leukemia control by day +21 in [M→M] recipients was associated with a contraction of the HY-CD8 population in the bone marrow (Figure 5D) compared to persistence of HY-CD8 in [M→F] with poor leukemia control. In contrast to the solid tumor model, leukemia challenge was sufficient to expand HY-CD8 in [F→F] recipients, though to a lesser degree.

**Restoration of Leukemia Control by Broad HY Expression is Associated with a Loss of PDL-1 Expression on Leukemia Cells, despite Continued Expression of PD-1 on Marrow-infiltrating HY-CD8**

Next, we determined the PD-1 expression status of bone marrow infiltrating HY-CD8 after leukemia challenge from the time of engraftment (day +7) to the time of either leukemia progression (in [M→F]) or control (in [F→F] and [F→M]). Despite good antileukemia control and contraction of the HY-CD8 pool in [F→M] recipients, nearly 100% of HY-CD8 expressed PD-1 (Figure 6A, top row), and 43.7 ± 4.8 of PD-1+ cells coexpressed the negative costimulatory receptor Tim-3, required for PD-1 induced CD8 exhaustion in some models [29], in [M→F] recipients, where leukemia control is poor, HY-CD8 maintained high levels of PD-1 expression (Figure 6A, center row) until the time of leukemia-associated death. Unexpectedly, by this time, HY-CD8 had lost their PD-1 expression and did not coexpress Tim-3. [F→F] recipients expressed PD-1 in a high percentage of their cells early in engraftment (day +7). Although some [F→F] lost PD-1 expression by day +21, this did not correlate with loss or gain of leukemia control (data not shown) as in the setting of HY allogeneic mismatch.

Finally, we determined whether PD-1 ligand (PDL-1, or B7:HI) expression on HY-E2APBX leukemia cells correlated with maintenance or loss of PD-1 expression on HY-CD8. In 2 independent experiments, we observed that as leukemia control is achieved in [F→M] recipients, PDL-1 expression on live HY-E2APBX leukemia cells is lost, despite continued expression of PD-1 on HY-CD8. Conversely, as leukemia control is lost in [M→F] recipients, the number of HY-E2APBX leukemia cells expressing PDL-1 increases, despite loss of PD1 expression on HY-CD8.

**DISCUSSION**

Endogenous miHA remain ideal targets for adoptive immunotherapy because of their physiologic expression levels compared with antigens generated, or targeted, by exogenous gene modification. Because most miHA are not tumor specific, we hypothesized that the antitumor reactivity of T cells to endogenous antigens is directly affected by the relative expression of that antigen in malignant and nonmalignant tissues. The degree to which the pattern of nonmalignant tissue antigen expression impacts the functionality of miHA-directed responses against tumors that might share the directly impacts which candidate antigens should be selected for adoptive therapy in the clinic. The ability of an experimental system to answer these questions preclinically required (1) controlling the degree of nonmalignant tissue miHA expression; (2) availability of T cells specific for that miHA; and (3) tumors that endogenously express the miHA and are capable of eliciting miHA-specific responses. We devised an experimental system that satisfied these criteria, based on mismatch at the minor alloantigen complex HY.

The male minor antigen complex HY was selected as the model miHA in this system because it has been well validated as a clinically relevant minor antigen in allogeneic transplantation, capable of generating both GVHD and GVLI [30]. HY disparity represents a mismatch at multiple genes, rather than a single MHC-associated locus, and thus may represent a more physiologic, and translatable, approach to antigen mismatch than artificially gene deficient mice [31].
addition, HY is among the few miHA in which the immuno-
dominant peptide complex is known, in mice and in humans,
for which we, and others, have generated clinically significant
immune responses against tumors that endogenously express
HY [18,28]. Although we recognize that because of the sex-
specific expression of HY there are clinical scenarios in
which our data cannot be directly extrapolated, for example,
the occurrence of a male/HY—expressing leukemia in a female
recipient, these scenarios were required as experimental
controls to isolate the key determinants of antigen-specific
T cell responses as a function of minor antigen distribution.
Our observations based on this model, for example, that he-
matopoietic restriction of a miHA may induce local CD8
dysfunction and may not represent an ideal target antigen,
can certainly be extrapolated to the selection of other miHA
complexes being considered for therapeutic use.

We specifically observed that broad expression of HY in
nonhematopoietic tissues produces poor responses to an HY-
expressing solid tumor and is associated with significant
accumulation of adoptively transferred HY-specific CD8 in
secondary lymphoid tissues that express PD-1. This was not
surprising, given the growing body of evidence that chronic,
high levels of viral [32-34] or alloantigen [35] presentation
produce T cell dysfunction. This observation is also
consistent with reports by Meunier et al. [11] that broad
tissue expression of minor antigens can generate large
numbers of dysfunctional CD8 that are more susceptible to
apoptosis. Although our data show that these cells persist in
relatively stable numbers 4 weeks after adoptive transfer, the
fact that they cannot respond to repeat antigen stimulation
ex vivo suggests a more terminal dysfunction that is not
entirely dependent on the allogegeneic environment. Further,
our model indicates that professional HY antigen presenta-
tion in the form of an activated DC vaccine cannot overcome
these effects, an observation that has clinical relevance for
the incorporation of antigen-specific cancer vaccines into
allo-HSCT regimens.

Targeting hematopoietically restricted minor antigens
has been proposed as an ideal strategy to optimize graft-
versus-tumor effects while sparing systemic GVHD [7,36].
Asakura et al. have shown that GVT effects are augmented
when target minor antigens are hematopoietically restricted
and can reverse poor responses induced by T cells exposed to
broadly expressed minor antigens [14]. In addition, Li et al.
have demonstrated that adoptive transfer of memory CD8
from donors vaccinated against a restricted miHA can
enhance GVT effects [10]. On this basis, we reasoned that
hematopoietic restriction of HY might also improve poor HY-
CD8 responses to MB49. Indeed, responses to MB49 were
partially restored and HY-CD8 retained the ability to prolif-
erate to HY ex vivo, despite continued expression of PD-1. We
were able to preserve CD8 activity to a restricted miHA
without prior donor vaccination, suggesting that prudent
selection of minor antigens alone may maximize the benefit
of adoptively transferred allogeneic T cells without addi-
tional manipulations to the donor.

Restoration of responses to MB49 led us to question
whether restriction of HY to hematopoietic tissues would
also confer superior antileukemia responses. However, GVT
effects were profoundly reduced in this setting despite
continued presence of HY-CD8 in the bone marrow and loss
of PD-1 expression, compared with superior responses when
HY was broadly expressed. We, therefore, concluded that
expression of a shared minor antigen in the same tissue
compartment as the tumor (nonhematopoietic for solid
tumors, hematopoietic for leukemia) predicts poor GVT re-
ponses. Conversely, minor antigens expressed outside the
primary tumor compartment (hematopoietic for solid tu-
mors, nonhematopoietic for leukemia) produced durable
GVT responses.

In our model, this location-specific pattern of antigen
expression was the primary determinant of antitumor CD8
activity, rather than degree of clinical GVHD, expansion
of adoptively transferred T cells or expression of PD-1.
Although PD-1 has most recently been associated with
T cell dysfunction and immune regulation, it has also been
described as a marker of T cell activation [37,38] and we
would expect HY-specific CD8 to be activated in the setting
of HY-directed alloreactivity. The fact that PD-1 is not expressed
on CD8+ T cells in the setting of poor antileukemia responses
suggests that PD-1 biology may be uniquely modulated
when alloimmunity is localized to a leukemia-bearing
marrow environment.

Teshima et al. recently observed that PD-1 blockade
partially reverses CD8 suppression induced by epithelial
expression of alloantigen and subsequent CD8 exhaustion
and apoptosis [14]. These work is complemented by the
observation by Schlomchik et al. that PD-1 expression is
increased on effector memory (but not central memory) CD8
capable of tissue homing and expansion but not sustained
GVHD or interferon production, suggesting a repertoire-
dependent functional defect in these cells, transgenic for
a ubiquitously expressed minor antigen [39]. Although we
also observe alloantigen-specific CD8 dysfunction associated
with epithelial expression of HY in the solid tumor model,
our leukemia model suggests that epithelial expression of
alloantigen partially preserves GVL effects and PD-1
expression is insufficient to produce a dysfunctional
phenotype.

Our leukemia challenge model indicates that continued
presence of PDL-1 on lymphoblastic leukemia cells produces
loss of leukemia control when the target miHA is hema-
topoietically restricted, an approach that was sufficient to
reverse epithelial alloantigen-induced CD8 dysfunction in
the solid tumor model. We, therefore, contend that blockade
of PDL-1, alone or in conjunction with PD-1 blockade, would
be required to completely reverse GVVL suppression in this
setting, and studies are ongoing to address this effect. Studies
of PDL-1 blockade have been limited in preclinical acute
lymphoblastic leukemia studies and represent the next
logical step in understanding this phenomenon.

Further, we demonstrate a loss of PDL-1 expression on
leukemia cells, despite ongoing expression of PD-1 on miHA-
specific CD8, in the setting of broad antigen expression. It is
intriguing to consider whether specific signals, from allor-
reactive CD8, the local environment, or both, may be capable
of modulating PD-1/PDL-1 interactions [29,40] to abrogate
immune escape. Our observations regarding the differential
effects of alloantigen distribution, and CD8 reactivity, could
be particularly useful in identifying critical “molecular
switches” in this interaction that could be used to augment
the efficacy of miHA-targeted adoptive therapy for acute
lymphoblastic leukemia while avoiding exogenous gene
modification of T cells.

ACKNOWLEDGMENTS

The authors thank Crystal Mackall and Daniel Fowler for
their critical review of the data and manuscript drafts. The
GFP-conjugated male E2APBX-ALL cell line was generously
provided by Janet Bijil, Centre de Recherche de l’Hopital
Maisonneuve-Rosemont, Montreal, Quebec, Canada. This work was supported by the NIH-T32 Training Grant “Laboratory Training in Pediatric Hematology-Oncology”, Principal Investigator Donald Small, Johns Hopkins University.

Financial disclosure: The authors have no financial conflicts of interest to declare.

Conflict of interest statement: The authors have no relevant financial conflicts or interest to declare.

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