Induction of acute GVHD by sex-mismatched H-Y antigens in the absence of functional radio-sensitive host hematopoietic-derived antigen presenting cells

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ABSTRACT

It is currently thought that acute graft-versus-host disease (GVHD) cannot be elicited in the absence of antigen presentation by radio-sensitive host hematopoietic derived antigen presenting cells (APCs) after allogeneic bone marrow transplantation (BMT). Because clinical data suggest that sex mismatched H-Y antigens may be important minor histocompatibility antigens for GVH responses, we directly tested their relevance and ability to initiate GVHD when presented by either the hematopoietic (host or donor) or the non-hematopoietic derived APCs. H-Y minor antigen incompatibility elicited both CD4+ and CD8+ T-cell driven GVHD lethality. Studies with various well-established bone marrow chimera recipients, in contrast to the current views, demonstrated that in the absence of functional radio-sensitive host hematopoietic derived APCs, H-Y antigen presentation by either the donor hematopoietic derived or the host non-hematopoietic derived APCs is sufficient for inducing GVHD. Our data further suggest that infusion of sufficient numbers of alloreactive donor T cells will induce GVHD in the absence of radio-sensitive host hematopoietic derived APCs.
INTRODUCTION

Antigen presentation on radio-sensitive host hematopoiesis derived antigen presenting cells (APCs) to the alloreactive donor T cells is considered to be obligatory for the induction of acute graft-versus-host disease (GVHD)\textsuperscript{1-6,8-10}. However, under certain conditions, whether clinically relevant minor antigens can induce GVHD in the absence of functional radio-sensitive host hematopoietic APCs is not known\textsuperscript{7,8-9}. Clinical data from MHC-matched BMT demonstrate that male recipients from female donors (F\rightarrow M) are at a greater risk for development of GVHD\textsuperscript{10} and demonstrate H-Y specific alloresponses\textsuperscript{11-14}. These clinical data suggest a strong correlation between H-Y antigen disparity and GVHD. However, in the context of HLA matched clinical F\rightarrow M BMT, the donors are also likely to be mismatched with the recipients at multiple minor antigens. Therefore, whether H-Y disparity alone is sufficient for causing clinical acute GVHD is not known. The experimental evidence for the causative role of H-Y antigens in GVHD and mortality has not been demonstrated. Furthermore the relevance of donor T cell alloreactivity against a single minor antigen and mechanisms of its presentation in causing GVHD are not known\textsuperscript{1-5}. While some studies have suggested that high doses of TCR transgenic T cells can cause GVHD, its severity was limited and was in the context of MHC mismatch or against minor antigens with unknown clinical relevance\textsuperscript{15,16}. Utilizing both, H-Y specific transgenic, and non-transgenic T cells in multiple well-established bone marrow chimeras we demonstrate, in contrast to the existing notion, that presentation of clinically relevant minor H-Y antigen by host radio-sensitive hematopoietic derived APCs is not obligatory for induction of acute GVHD\textsuperscript{3,7,6-9,17}. Our data further suggest that in the absence of radio-sensitive host hematopoietic derived APCs, when sufficient numbers of alloreactive donor T cells are infused, non-hematopoietic derived cells such as endothelial and certain epithelial cells activate alloreactive T cells, might induce GVHD.
MATERIALS and METHODS

Mice: Male and female C57BL/6 (B6, H-2^d^, CD45.2^+^), B6 Ly5.2 (H-2^b^, CD45.1^+^), BALB/c (H-2^d^) were purchased from the Jackson Laboratory (Bar Harbor, ME). B6-background H2-Ab1^−^ mice (B6.129-H2-Ab1^tm1Gru^ N12, CD45.2^+^), β2m^−^ B6 mice (H-2^b^, CD45.2^+^), anti-H-Y TCR transgenic (Tg) mice Marilyn (RAG-2^−^ background, CD4^+^ Tg, H-2^b^, CD45.2^+^, I-A^b^-restricted)^17, Rachel (RAG-2^−^ background, CD4^+^ Tg, H-2^b^, CD45.2^+^, I-A^b^-restricted)^17, and MataHari (RAG-1^−^ background, CD8^+^ Tg, H-2^b^, CD45.2^+^, H-2D^b^-restricted) mice^18 were obtained from Taconic (NIH, Matzinger). All animals were cared for under regulations reviewed and approved by the University Committee on Use and Care of Animals of the University of Michigan, based on University Laboratory Animal Medicine guidelines.

Generation of bone marrow (BM) chimeras: We administered 1,100 cGy total-body irradiation (TBI; ^137^Cs Source) to mice and then injected them intravenously with 5x10^6^ bone marrow cells with 5x10^6^ whole spleen cells from donor mice on day -1. For generating MHC class I-deficient (β2m-KO) BM chimeras, recipient mice were treated with 200 μg anti-NK1.1 mAb (PK136) on days −2 and −1^6. The peripheral blood from sentinel mice were analyzed for donor chimerism at 3 months and found to demonstrate > 98% donor chimerism in all cell lineages. The CD11c^+^ cells in the splenocytes from these animals also demonstrated > 95% donor chimerism.

BMT: BMTs were performed as described before^7. Briefly, splenic T cells were enriched by autoMACS using anti-CD4, −CD8 microbeads (Milenyi Biotec, Bergisch Gladbach, Germany). T cells from bone marrow were depleted by autoMACS using anti-CD90 microbeads. These isolated T cells showed naïve phenotype (CD62L^high^ CD44^low^) and no activated markers (CD69^+^ 1.3-2.2% and CD25^+^ 0.2-0.5%). Recipient animals received...
800-1100cGy total body irradiation (TBI; $^{137}$Cs source) on day-1. They were then injected with T-cell depleted (TCD) BM cells ($5 \times 10^6$) plus splenic CD4$^+$ or CD8$^+$ Tcells from WT-B6, H-Y TCR Tg Marilyn, Rachel or MataHari donors on day 0. For studies in which the recipients were BM chimeras, we induced GVHD 3-5 months after the generation of BM chimera according to a standard protocol as described previously$^7,8$.

**Systemic and histopathological analysis of GVHD:** We monitored survival after BMT daily and assessed the degree of clinical GVHD weekly by a previously described scoring system. We also assessed acute GVHD by detailed histopathological analysis of primary GVHD target organs, as described$^{19}$.

**FACS analysis:** FACS analyses were performed as described before$^7$. The monoclonal antibodies (MoAbs) were used FITC-, PE-, APC-, PerCPcy5.5- conjugated anti mouse CD4, CD8, CD45.2, CD25, CD69, IFN-$\gamma$, Foxp3, granzyme B and CD107a (eBioscience SanDiego, CA). The procedure was performed as described previously$^7$. For intracellular staining (Foxp3, IFN-$\gamma$, Granzyme B and CD107a), the splenocytes were incubated for 5 hours with CD3Ab, CD28Ab and brefeldin A (eBioscience, San Diego, CA). Then, cells were stained for cell surface markers, fixed, permeabilized, and stained intracellular as manufacture’s protocol.

**Cytokine enzyme-linked immunosorbent assay (ELISA):** Concentrations of TNF-$\alpha$, IFN-$\gamma$, and IL-17 were measured in serum by ELISA (BD Biosciences PharMingen, R&D Systems, Inc and Biolegend respectively), in duplicate as the manufacturer’s protocol and read at 450nm by using a microplate reader (Model 3550; Bio-Rad Labs, Hercules, CA).
Immunization, ex vivo expansion and detection of H-Y antigen specific T cells: H-Y antigen specific T cells were expanded as described before. Briefly, 10x10^6 irradiated B6 male splenic cells were injected i.p. into female B6 mice on day 19 before allo-HCT. On day 5 before allo-HCT, B6 female derived splenocytes were harvested and then re-stimulated with B6 male derived BMDCs for 120hrs. On day 0, these expanded H-Y antigen-specific T cells were harvested and isolated with MACS and analyzed H-Y antigen specific T cells with H-Y peptide tetramer (D^b/ WMHHNMDLI (Ut)), made by NIH tetramer core facility, Atlanta, GA).

DC culture and isolation: To obtain DCs, BM cells from male B6 wild-type(WT) were cultured with murine recombinant GM-CSF (PeproTech Inc, Rocky Mill, NJ) for 7 days and isolated as described previously.

Isolation of lymphocytes, endothelial and epithelial cells from liver and small intestine: Hepatic lymphocytes and small intestinal intraepithelial lymphocytes (IEL) were isolated as previously described. Hepatic endothelial cells were isolated by MACS using CD146 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as manufacture’s protocol.

Mixed Lymphocyte Reaction (MLR): For measurement of MLR cultures, splenic T cells were separated by MACS and then cultured with B6 male derived liver endothelial cells or small intestine epithelial cells for 120 hours. Incorporation of ^3H-thymidine (1uCi/well) by proliferating T cells during the final 24 hours of culture was measured by a Betaplate reader (Wallad, Turku, Finland).
Statistical analysis: The Mann-Whitney $U$ test was used for the statistical analysis of *in vitro* data, and clinical scores. We plotted survival curves using Kaplan-Meier estimates and the Wilcoxon rank test was used to analyze survival data. A $p$ value <0.05 was considered statistically significant.
RESULTS

H-Y antigen disparity is sufficient to induce CD8<sup>+</sup> mediated GVHD

We first tested whether disparity across single H-Y will induce acute GVHD. Anti-H-Y monospecific, H-2<sup>b</sup>-restricted T cell receptor transgenic (TCR Tg)<sup>17,18</sup> mice were utilized as donors in MHC-matched (F→M) BMT. We used T-cells from female MataHari mice, an H-Y specific CD8<sup>+</sup> TCR Tg on the B6.RAG.KO background, that recognizes the WMHHNMDLI peptide from the Uty gene, presented by H-2D<sup>b</sup>.<sup>21</sup> Lethally irradiated female and male B6 animals were transplanted with TCD BM from WT female (B6) mice along with 1x10<sup>6</sup> splenic T cells from either WT or MataHari B6 female donors. All of the syngeneic recipients survived, ruling out potential pathogenic effects from lymphopenic expansion of MataHari T cells (Figure 1a). By contrast, all of the allogeneic male recipients that received MataHari T cells died rapidly (Figure 1a) with signs of severe clinical score (day 7 score: MataHariF→ B6M 6±1.3 vs MataHari F→ B6F 0.5±0.3, P<0.05), and GI and hepatic histopathological (Figure 1b) GVHD, with elevated levels of TNF-α, IFN-γ, and IL-17A (Figure 1c). MataHari donor T cells also demonstrated significantly greater expansion (Figure 1d), numbers of IFN-γ secreting cells (Figure 1e), expression of activation markers (CD69, CD25) and enhanced cytotoxicity (CD107a and granzyme B, (Supplementary Figure 1a-c) only in the allogeneic male but not the female recipients. These data demonstrate that this is an allo-specific phenomenon and not a consequence of homeostatic expansion or non-specific activation of H-Y antigen specific T cells in the context of radiation induced cytokine storm.

H-Y specific CD4<sup>+</sup> T cells cause GVHD

To rule out strain dependent artifacts, we evaluated whether H-Y specific T cells from two other different strains of donor mice, Marilyn and Rachel (H2<sup>b</sup>), also induced GVHD only in the allogeneic male recipients. CD4<sup>+</sup> T cells from Marilyn are specific for the H-Y
peptide NAGFNSRNANSSRSS from the *Dby* gene complexed with A^b^, and those of Rachel are specific for A^b^ complexed with an unknown male-specific peptide^{17}. Infusion of CD4^+^ H-Y Tg T cells from either Marilyn or Rachel donors along with BM from WT female B6 donors induced 100% mortality with signs of severe clinical GVHD only in the male recipients (Figures 1f and 1g). Histopathological examination (Supplementary Figure 2a), levels of TNF-α, IFN-γ, and IL-17 (Supplementary Figures 2b-d), expansion of H-Y antigen specific donor T cells (Supplementary Figure 2e) and, IFN-γ^+^ donor T cells (Supplementary Figure 2f) were greater while the Foxp3^+^ T cells were decreased in the allogeneic (Supplemental Figure 2g) males compared to the syngeneic female recipients of Rachel T cells.

**H-Y antigen disparate GVHD results from transplanting sufficient numbers of allo-reactive precursor T cells**

Single minor antigen disparity has been suggested not to be sufficient for causing GVHD^{3}. By contrast, our data suggested that a single H-Y antigen disparity can drive GVHD. We therefore hypothesized that the induction of GVHD by single minor antigen disparity is a function of transferring sufficient numbers of allo-reactive precursor cell. To test this, we next decreased the dose of CD4^+^ (Rachel) T cells 10-fold (0.15x10^6^) and mixed them with (1.35x10^6^) syngeneic male CD4^+^ T cells (~1:9 ratio). All of the female recipients survived, while all of the male animals demonstrated clinical signs of GVHD and mortality (33.3%), albeit less than those that received 1.5x10^6^ Rachel T cells (Figure 2a). The presence of GVHD was confirmed by histopathology in GI tract, liver (Figure 2b) and skin (Figure 2c), the target organs of GVHD. However, transfer of 100-fold less (0.015x10^6^) Rachel T cells did not cause any mortality or significant clinical GVHD (data not shown). Furthermore, similar to polyclonal donor T cells, the ability of these HY-specific T cells to cause GVHD correlated with the intensity of host irradiation
(Supplemental Figure 2h)\textsuperscript{24}. These results suggest that transplanting sufficient numbers of T cell precursors against a single minor histo-incompatible antigen caused GVHD.

**Polyclonal T cells cause GVHD after H-Y antigen mismatched BMT**

To formally rule out the possibility of an artifact from the transgenic nature of the donor T cells, we primed naïve WT B6 female donors with B6 male splenocytes (Figure 3a). The donor B6 female splenocytes were harvested and re-stimulated ex-vivo with BM derived DCs from B6 male animals. The H-Y specific T cells were confirmed with H-Y peptide tetramer ($D^p$/WMHHNMDLI ($Uty$), and $\sim 0.1 \times 10^6$ of these cells were transferred into lethally irradiated B6 male and female hosts along with BM cells from female donors (schema shown Figure 3a-b). The B6 male recipients demonstrated greater mortality, significant clinical severity (Figure 3c) and GVHD specific histopathology of the target organs (Figure 3d). Significantly greater donor H-Y specific T cells were recovered from the representative GVHD specific target organs like the spleen, IEL and livers of the male hosts (Figure 3e).

To further rule out any potential artifact due to ‘priming’ of T cells before BMT, we performed BMT with $2 \times 10^6$ naïve T cells from B6 female donors, which barely caused discernable GVHD in male animals. However, transfer of substantially higher numbers, $15 \times 10^6$, of donor T cells induced significant clinical and histopathological GVHD (Figures 3f and 3g). These results indicate that a single H-Y antigen disparity is sufficient and that epitope spreading is not obligatory for induction of GVHD.

**Expression of H-Y antigens in induction of GVHD**

We then next analyzed the requirement of the expression and presentation of H-Y antigens on either the non-hematopoietic or hematopoietic host tissues for induction of
GVHD. We generated (F→M) or (M→F) BM chimeras such that the H-Y antigens are encoded only by the host non-hematopoietic target tissues, or only by the radio-sensitive host hematopoietic derived cells, respectively (Figure 4a). These chimeras were then used as BMT recipients and injected with female TCD-BM cells plus 1.5x10^6 CD4^+ T cells from WT B6 male or from Marilyn donors (experimental schema shown Figure 4a). The (F→M) recipients that received cells from syngeneic donors survived while 100% of this group of chimeras that received Marilyn CD4^+ T cells died with severe GVHD (Figure 4b and clinical score 8±1 on day 7) and demonstrated significantly greater serum levels of TNFα, INF-γ and IL-17A (Figure 4c). By contrast, only 25% of the (M→F) chimeras that received cells from Marilyn CD4^+ T cells died (Figure 2g) with modest GVHD (score 4.5±0.9 and 3±0.3 vs. 1.5±0 and 1.2±0.2 in syngeneic controls on days 7 and 14 respectively, P<0.05). These data are consistent with previous observations^25 and further demonstrate that minor antigen expression on host target tissues is required for causing severe GVHD.

**Presentation of H-Y antigens by the radiosensitive host hematopoietic derived APCs is not obligatory**

We next explored whether host hematopoietic derived APCs are obligatory for induction of GVHD. We generated [ClassII^-F→ B6M] chimeras utilizing thymectomized B6 male mice (to prevent development of autoimmunity in these mice)^26 and such that the H-Y antigen is expressed on target tissues but cannot be presented to donor CD4^+ T cells by the host hematopoietic derived APCs (schema shown Figure 5a). All of the syngeneic [ClassII^-F→ B6M] chimeras survived while 100% of the animals that received cells from Marilyn donors died after BMT (Figure 5b). These data indicate that in the absence of functional antigen presentation on radio-sensitive host hematopoietic derived APCs, the
H-Y antigen can be efficiently presented by either donor hematopoietic APCs and/or directly by the host non-hematopoietic tissues and cause GVHD.

To rule out strain specific effects and to further determine whether donor T cells respond to minor H-Y antigen when all host (hematopoietic and non-hematopoietic) APCs are incapable of antigen presentation, recipient BALB/c (H2d) male mice were irradiated (800cGy) and injected with TCD BM from either WT or class II-/- B6 along with 0.5x10^6 purified CD4+ T cells from allogeneic Marilyn donors (experimental schema shown Figure 5c). Since the H-Y antigen specific cells from the allogeneic Marilyn B6 donors are H2b-restricted, they can recognize the H-Y antigen from the male BALB/c recipients only when presented by the donor derived H2b APCs. The donor Marilyn CD4+ T cells demonstrated significantly greater proliferation and IFN-γ expression, elevated level of serum IFN-γ only in the BALB/c males that received WT B6 BM (Figure 5d).

**Induction of GVHD in the absence of functional antigen presentation by the donor or radio-sensitive host hematopoietic APCs**

Next, to directly determine whether in the absence of functional donor or radio-sensitive host hematopoietic APCs, the non-hematopoietic host tissues alone can directly present minor H-Y antigen to donor T cells and cause GVHD we designed the following experimental system. We created [β2MG-/-→B6M] chimeras using β2 microglobulin (β2MG-/-) mice such that the H-Y antigen is expressed on target tissues but cannot be presented to donor CD8+ T cells by the radio-sensitive host hematopoietic derived APCs (experimental schema shown Figure 5e). They were transplanted with 1x10^6 CD8+ T cells from MataHari donors along with BM from the β2MG-/- female B6 donors (such that H-Y antigen cannot be presented to donor CD8+T cells by the donor hematopoietic derived APCs). All of the syngeneic chimeras survived while the allogeneic WT chimeras...
died from GVHD (Figure 5f). Importantly the (β2MG<sup>−/−</sup>→B6M) animals that received T cells from MataHari donors along with BM from β2MG<sup>−/−</sup> female B6 animals also demonstrated significantly greater mortality (Figure 5f), clinical GVHD (Figure 5g), expansion of the H-Y-allo-specific T cells (Figure 5h) and GVHD specific histopathology (Figure 5i) when compared to the syngeneic animals.

To further rule out strain and transgenic T cell dependent artifacts we utilized similar [β2MG<sup>−/−</sup>→B6M] chimeras and transplanted them with T cells from either syngeneic B6 (Ly5.1) or MHC disparate BALB/c female donors (schema shown Figure 6a). Donor T cells expansion (Figure 6b) and IFN-γ<sup>+</sup>T cells (Figure 6c) and serum levels (Figure 6d) were significantly greater in animals that received allogeneic BALB/c T cells despite the absence of functional donor or radio-sensitive host hematopoietic derived APCs.

**Antigen presentation by hematopoietic derived APCs is not obligatory but required for induction of optimal GVHD**

To investigate the potential mechanisms for induction of GVHD in the absence radiosensitive host APCs we posited that infusion of sufficient numbers of alloreactive donor T-cell precursors are required for induction of GVHD. We once again utilized the [β2MG<sup>−/−</sup>→B6M] chimeras, as above (Figure 5e), such that the H-Y antigen is expressed on target tissues but cannot be presented to donor CD8<sup>+</sup> T cells by the radio-sensitive host hematopoietic derived APCs. We transplanted ten-fold less (0.1x10<sup>6</sup>) CD8<sup>+</sup> T cells from MataHari donors along with BM from the β2MG<sup>−/−</sup> female B6 donors into the [β2MG<sup>−/−</sup>→B6M] and [B6→B6M]. All of the syngeneic chimeras survived while 30% of the allogeneic WT chimeras died from GVHD (Figure 6e). Importantly the [β2MG<sup>−/−</sup>→B6M] animals that received only 0.1x10<sup>6</sup> CD8<sup>+</sup> T from MataHari donors demonstrated non-lethal GVHD modest clinical severity (Figure 6e). These data suggest that induction of
GVHD in the absence of allo-antigen presentation by the radiosensitive host and donor hematopoietic APCs is less efficient than that induced by the radiosensitive host or donor hematopoietic APCs. Thus minor antigen presentation on host or donor hematopoietic derived APCs, while not obligatory, it induces more robust GVHD severity.

Allogeneic T cells can be activated by non-hematopoietic derived cells

Next, to address which non-hematopoietic cells might be presenting allo-antigens to donor T cells, we analyzed whether host endothelial and epithelial cells would directly stimulate donor T cells. Isolated epithelial cells were analyzed for both epithelial cell specific marker and CD11c+ expression to rule out any possible contamination with professional APCs. The epithelial cells demonstrated expression of epithelial markers without any evidence of CD11c+ expression (Supplementary Figure 4a). The liver endothelial and small bowel epithelial cells from the allogeneic male animals caused significantly greater proliferation of Marilyn T cells when compared to cells from syngeneic female cells (Figures 6f and 6g). The B6 liver endothelial cells also caused greater proliferation of polyclonal CD4+ T cells from allogeneic BALB/c animals demonstrating that this is not merely a response germane to TCR transgenic T cells (Figure 6h). Consistent with their ability to stimulate alloreactive T cells, both the endothelial and the epithelial cells express MHC class II, which is further enhanced by the addition of IFN-γ to simulate an inflammatory milieu (Figure 6 i-j and Supplemental Figure 3). These data suggest that in the context of post-conditioning inflammation, non-hematopoietic cells such as certain endothelial, epithelial and/or other cells might contribute to activation of alloreactive T cells and induce GVHD.
DISCUSSION

Collectively, our data with T cells specific for clinically relevant H-Y antigens and also with polyclonal donor T cells in multiple murine models (summarized in supplementary Table 1), challenge the currently held notions that allo-antigen presentation by radiosensitive host hematopoietic derived APCs is obligatory for induction of GVHD\textsuperscript{6,7}. They suggest that in the context of inflammation, infusion of sufficient numbers of alloreactive donor T cells can induce GVHD even in the absence of radio-sensitive host hematopoietic derived APCs. Although our data did not formally address the role of Langerhans cells (the only professional hematopoietic derived APCs that have been shown to be radio-resistant and survive in these chimeras)\textsuperscript{27}, our data, when taken collectively in light of recent observations by Shlomchik and colleagues\textsuperscript{28} suggest that Langerhans cells and other radio-resistant hematopoietic APCs if any, are unlikely to have contributed to the induction of GVHD.

The previous observations demonstrating an obligatory role for radio-sensitive host APCs is likely a consequence of transplanting insufficient allo-specific T cells\textsuperscript{6,7}. By contrast, our data demonstrate that when sufficient numbers of highly avid and minor antigen specific T cells are transplanted, GVHD can be induced in the absence of radio-sensitive host or donor hematopoietic derived APCs. These observations were germane to H-Y specific and polyclonal T cells across multiple models. Infusion of $5 \times 10^6$ numbers of donor T cells in the MHC matched multiple minor C3H.SW→(β2MG\textsuperscript{−/−}→B6M) model (that was employed in previous reports) also resulted in expansion of donor T cells demonstrating that the induction of GVH responses is likely a function of the transferring appropriate numbers of alloreactive donor T cells (Supplemental Figure 4b).
The putative non-hematopoietic cells that might be present allo-antigens to donor T cells and induce GVHD include endothelial or epithelial cells as suggested by the ability of these cells to activate alloreactive H-Y specific and polyclonal T cells. The ability of endothelial cells to stimulate allogeneic T cells is consistent with observations in humans\textsuperscript{29,30}. However, the direct and/or relative contributions of these cells and the radiosensitive host hematopoietic derived APCs will need further investigation and the development of requisite tools. Furthermore, the site of activation of allo-reactive donor T cells by these putative non-hematopoietic APCs, the requirement of secondary lymphoid organs and/or directly in the target tissues remains to be determined. Nonetheless, our data (Figures 3f and 4e) indicate that the intensity of GVHD severity may be less in the absence of radiosensitive host hematopoietic derived APCs.

The sufficiency of single H-Y antigen disparity in causing GVHD mortality is in agreement with experimental organ rejection observations\textsuperscript{10,18,31} but in contrast to earlier observations in BMT\textsuperscript{2,3}. This could be a consequence of (a) limited expression of the single immune-dominant minor antigen in contrast to the ubiquitous expression of the H-Y antigen\textsuperscript{32,33} and also (b) infusion of insufficient numbers of T cell precursors against the antigen. Nonetheless our data are also consistent with recent observations of the sufficiency of single minor antigen disparity in causing GVHD\textsuperscript{34} and further expand the observation to clinically relevant, H-Y, minor antigens.

Given the previous observations on the requirement of functional radiosensitive host hematopoietic APCs for induction of GVL responses\textsuperscript{8,35,36}, our data raise the intriguing possibility that GVHD and GVL can perhaps be differentially regulated at the level of antigen presentation. While direct allo-presentation by host cells can cause GVHD, direct presentation of tumor specific and allo-antigens by the tumors (that have
developed multitude of tumor intrinsic immune-evasive pathways) may be insufficient for generation of an effective GVL response. It is therefore likely that cross-presentation of tumor antigens is required for optimal GVL. Because cross-presentation of antigens is primarily a function of subsets of professional APCs, it stands to reason that enhancing cross-presentation on the specific professional APC subsets might facilitate an effective GVL response without aggravating GVHD.

Our observations have additional significant clinical ramifications. They demonstrate a causal role for H-Y antigens in clinical GVHD and suggest that identification and tolerizing against relevant immune-dominant minors might prevent GVHD. Our data indicate that GVHD can be induced even in the absence minor antigen presentation by the radiosensitive host or donor hematopoietic derived cells. Because host hematopoietic derived APCs can also negatively regulate GVH responses\textsuperscript{37,38}, our data indicate that strategies that promote the regulatory APC subsets\textsuperscript{39-42}, might be more effective than elimination of hematopoietic derived activating APC subsets\textsuperscript{28,43-45}. Importantly, when taken in light of they indicate that regulating the process of antigen presentation, rather than targeting specific host hematopoietic derived cellular subsets that are capable of activating alloreactive donor T cells might be a more desirable strategy for mitigating GVHD.
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FIGURE LEGENDS:

**Figure 1: Single H-Y antigen specific T cells induce CD8⁺ and CD4⁺ T cells mediated GVHD**

(a) Survival in CD8⁺ mediated GVHD following infusion of 1x10⁶ CD8⁺T cells from MataHari donors. Data are from 1 of 4 similar experiments.

(b-e): GVHD analysis on day +5: Data are from 1 of 3 three similar experiments (b) Histopathological analysis: liver (i, ii) and GI tract (iii, iv) by H-E stain (left panel) and scores (right panel). (c) Serum levels of TNF-α, IFN-γ and IL-17A (N.D stands for not detected). (d) Allo-HY antigen specific donor CD45.2⁺CD8⁺ T cells expansion in spleen. (e) Donor CD45.2⁺CD8⁺IFN-γ⁺ in spleen- left is representative histogram of IFN-γ expression gated by CD45.2⁺CD8⁺ T cells and right is the absolute number of CD45.2⁺CD8⁺IFN-γ⁺ T cells in spleen.

(f) and (g): Survival CD4⁺ GVHD: Data shown are from 1 of 3 similar experiments.

**Figure 2: Donor T cell precursor frequency is critical for induction of GVHD**

Rachel T cells were transplanted along with TCD BM from B6♀ donors into lethally irradiated syngeneic B6♀ or allogeneic B♂ animals. The recipients were monitored for (a) Survival and GVHD score (b) Histopathology scores: Liver and GI tract (c) skin: H & E (left) and score (right). Data are from 1 of 3 similar experiments.

**Figure 3: Polyclonal H-Y antigen specific T cells induce GVHD**

(a-b) Experimental schema: Polyclonal T cells from WT B6♀ mice that are specific for H-Y antigens were generated following in vivo and subsequent ex vivo priming. These T cells were then transplanted along with TCD BM from B6♀ donors into lethally irradiated syngeneic B6♀ or allogeneic B6♂ animals.
The animals were monitored for (c) Survival and GVHD score. Data are from two combined experiments. **p<0.01 compared with female recipients.

(d): GVHD histopathology: scores of liver and GI tract and (d) Lymphocyte infiltration in the spleen, intestine and liver. Data are from 1 of 2 similar experiments.

(f) Unprimed B6♀ T cells induce GVHD in allogeneic B6♂ recipients. CD90⁺ T cells (2x10⁶ or 15x10⁶) donor T cells were harvested from WT B6 female donors and transplanted without priming along with TCD BM into irradiated (11Gy) syngeneic B6♀ and allogeneic B6♂ animals. The animals were monitored for clinical GVHD severity and (g) Histopathological analysis: GVHD damage scores of liver and gastrointestinal (GI) tract 70days after allo-HCT.

Figure 4: Allo-antigen expression and GVHD

(a) Experimental Schema: The [F→F], [F→M] and [M→F] chimeras were generated such that male antigens are either not expressed or expressed only on the target tissues or only on the hematopoietic derived cells. These chimeras were then irradiated and transplanted with Marilyn T cells along with TCD WT B6♀ BM cells and were monitored for (b) Survival and (c) Serum levels of TNF-α, IFN-γ and IL-17A on day 5. Data are from 1 of 2 similar experiments.

Figure 5: Presentation by donor and host non-hematopoietic APCs.

(a) Experimental Schema: B6♂ animals were thymectomized and used for generation of [class II-/♀→B6♂] chimeras. Three months later, these chimeras were irradiated and transplanted with either B6 or Marilyn T cells and monitored for (b) Survival: Data are from two combined experiments.

Donor APCs alone stimulate GVH responses:
(c) Experimental schema: TCD BM from class from either WT or class II-/- B6♀ were transplanted along with WT or Marilyn T cells into lethally irradiated BALB/c males.

(d) Total donor H2Kd+CD4+ T-cell expansion, H2kd+CD4+IFN-γ+ T-cell number and serum IFN-γ concentration from day +7. Data are from 1 of 2 similar experiments.

(e-i) Induction of GVHD in the absence of donor and radio-sensitive host hematopoietic derived APCs.

(e) Experimental Schema: B6Ly5.2 female and male animals were lethally irradiated, treated with anti-NK antibody and transplanted with either WT B6 or β2MG-/- BM. There months later, these chimeras were transplanted with TCD BM from either WT or β2MG-/- B6♀ animals along with MataHari T cells. The animals were monitored for (f) Survival and (g) clinical score. Data from 1 of 3 similar experiments are shown (h) Donor CD45.2+CD8+ T cells expansion in spleen and (i) Histopathology scores of liver and GI tract. Data from 1 of 2 similar experiments are shown.

Figure 6: Allo-antigen presentation and GVHD in the absence of radiosensitive host hematopoietic derived APCs

(a) Experimental Schema: [β2MG-/-♂→B6♀] chimeras were generated as in methods. They were then irradiated with 9 Gy and transplanted with TCD BM from β2MG-/- animals along with T cells from B6♂ or BALB/c♀ donors and analyzed for donor T cell expansion

(b) Total donor CD45.1+CD8+ or H2kd+CD8+ T cells expansion

(c) Donor CD8+IFN-γ+ T cells number and

(d) Serum levels of IFN-γ. Data from 1 of 2 similar experiments are shown.
(e) GVH responses induced by host non-hematopoietic APCs are dependent on T cell precursor frequency. Survival and GVHD score. Data from 1 of 2 similar experiments are shown.

(f-h) Male non-hematopoietic cells can stimulate both H-Y antigen specific T cells and allogenic T cells.

(f, g) Marilyn CD4 T cells against male liver endothelial cells (g) and epithelial cells of small intestine (g). (h) B6 male derived liver endothelial cells can stimulate allogeneic BALB/c T cells. (i-j) Class II expression on liver endothelial cells gated by CD146+ cells. Right-naïve, left- IFN-γ stimulation for 48 hours. (j) Bar graph of Class II expression on liver endothelial cells. Data are from 1 of 3 similar experiments.
Figure 1

(a) % survival over days after BMT for different genotypes and treatments.

(b) Histological analysis of liver and GI tract.

(c) Measurement of TNF-α, IFN-γ, and IL-17A levels at day 5.

(d) Flow cytometry for donor CD8+ T cells and IFN-γ production.

(e) Measurement of IFN-γ levels at day 5.

(f) % survival over days after BMT for different genotypes.

(g) % survival over days after BMT for different genotypes and treatments.
Figure 2

a

b

Day 70

Liver

GI tract

Day 70

skin

Grade 0

Grade 1

P=0.02

P=0.04

P=0.01
Figure 3

a

Day -19

B6 Male splenocytes (10x10^6)

Donor: B6 Female (ip)

1st stimulation (in vivo)

Day -5

B6 Female, whole splenocytes (5x10^6)

+ B6 Male BMDC (2x10^5)

(in vitro)

Re-stimulation

Day -0

B6 Male

Expanded H-Y Ag Tetramer+ T cells (0.05-0.1x10^6)

b

Day -19

WT-B6 (♀) + ex vivo expanded CD90^+ T cells (♀) (n=6)

Day -5

WT-B6 (♀) + ex vivo expanded CD90^+ T cells (♀) (n=13)

Day -0

WT-B6 (♀) + ex vivo expanded CD90^+ T cells (♀) (n=6)

WT-B6 (♀) + ex vivo expanded CD90^+ T cells (♀) (n=12)

WT-B6 (♀) + ex vivo expanded CD90^+ T cells (♀) (n=11)

c

Day 10

WT-B6 (♀) + ex vivo expanded CD90^+ T cells (♀) (n=13)

Day 50

Liver

Score

GI tract

Score

P=0.02

P=0.02

P=0.04

P=0.04

P=0.03

P=0.03

d

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Figure 4

a) BM: B6 (♀) / B6 (♂) / Marilyn (H-Y antigen specific, TCRTg CD4+ (♀))

T cell: B6 B6(♀) / Marilyn (H-Y antigen specific, TCRTg CD4+ (♀))

Chimeras

B6 (♀) / B6 (♂) / Marilyn (♀) / B6 (♀) / Marilyn (♀) / B6 (♀) / Marilyn (♀) / B6 (♀) / Marilyn (♀) / B6 (♀) / Marilyn (♀) / B6 (♀) / Marilyn (♀) / B6 (♀)

b) Days after BMT

% Survival

Days after BMT

- B6 (♀) / B6 (♂) / B6 (♀) (n=3)
- Marilyn (♀) / B6 (♀) (n=3)
- Marilyn (♀) / [B6(♂) / B6(♀)] (n=4)
- Marilyn (♀) / [B6(♂) / B6(♀)] (n=6)

** p<0.05

** p=0.007

9Gy (2nd BMT)

11Gy (1st BMT)

b) TNF-α

Day 5

[pg/ml]

IFN-γ

IL-17A

[pg/ml]

- B6 (♀) / [B6(♀) / B6(♂)] (n=3)
- Marilyn (♀) / [B6(♀) / B6(♂)] (n=3)
- Marilyn (♀) / [B6(♂) / B6(♀)] (n=4)
- Marilyn (♀) / [B6(♂) / B6(♀)] (n=6)

* p<0.05

* p=0.05
Figure 6

a) T cell: B6(♀) or T cell: BALB/c(♀)

BM: β2MG-(♀) T cell: B6/(♀)

or

11Gy (1st BMT)

β2MG-(♂) Ly5.2 (♂)

Chimeras

β2MG-(♀) B6 T cell: β2MG-(♀) Ly5.2 (♀)

β2MG-/(♀) BALB/c T cell: β2MG-/(♀) Ly5.2 (♀)

b) Day 7

Donor CD8+ T cells

p=0.04

[pg/ml]

p=0.03

c) Day 7

Donor CD8+ IFN-γ

p=0.04

[pg/ml]

p=0.03

d) Day 7

Scram IFN-γ

p=0.04

[pg/ml]

p=0.03

e) % Survival

Days after BMT

B6(♀)+MataHariÆ[β2MG-/(♂)ÆLy5.2(♂)] (n=3)

B6(♀)+MataHariÆ[β2MG-/(♂)ÆLy5.2(♂)] (n=6)

β2MG-(♀)+MataHariÆ[β2MG-/(♂)ÆLy5.2(♂)] (n=6)

p=0.13

f) [CPM] 

p=0.01

[CPM] 

p=0.004

[CPM] 

p=0.02

g) [CPM] 

p=0.01

[CPM] 

p=0.004

[CPM] 

p=0.02

h) [CPM] 

p=0.01

[CPM] 

p=0.004

[CPM] 

p=0.02

i) Liver Endothelial Cells

Naive IFN-γ (48Hrs)

Gate P2

isotype

1-Aβ

isotype

1-Aβ

j) [%]

p=0.006

Naive IFN-γ
Induction of acute GVHD by sex-mismatched H-Y antigens in the absence of functional radio-sensitive host hematopoietic-derived antigen presenting cells

Tomomi Toubai, Isao Tawara, Yaping Sun, Chen Liu, Evelyn Nieves, Rebecca Evers, Thea Friedman, Robert Korngold and Pavan Reddy