Mechanisms of antigen presentation to T cells in murine graft-versus-host disease: cross-presentation and the appearance of cross-presentation

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Recipient antigen-presenting cells (APCs) initiate GVHD by directly presenting host minor histocompatibility antigens (miHAs) to donor CD8 cells. However, later after transplantation, host APCs are replaced by donor APCs, and if pathogenic CD8 cells continue to require APC stimulation, then donor APCs must cross-present host miHAs. Consistent with this, CD8-mediated GVHD is reduced when donor APCs are MHC class I−. To study cross-presentation, we used hosts that express defined MHC class I Kb-restricted miHAs, crossed to Kb-deficient backgrounds, such that these antigens cannot be directly presented. Cross-priming was surprisingly efficient, whether antigen was restricted to the hematopoietic or nonhematopoietic compartments. Cross-primed CD8 cells were cytolytic and produced IFN-γ. CD8 cells were exclusively primed by donor CD11c+ cells, and optimal cross-priming required that they are stimulated by both type I IFNs and CD40L. In studying which donor APCs acquire host miHAs, we made the surprising discovery that there was a large-scale transfer of transmembrane proteins from irradiated hosts, including MHC class I–peptide complexes, to donor cells, including dendritic cells. Donor dendritic cells that acquired host MHC class I–peptide complexes were potent stimulators of peptide-specific T cells. These studies identify new therapeutic targets for GVHD treatment and a novel mechanism whereby donor APCs prime host-reactive T cells. (Blood. 2011;118(24):6426-6437)

Introduction

Allogeneic hematopoietic stem cell transplantation (alloSCT) can be a life-saving therapy for hematologic malignancies and acquired or inherited nonmalignant disorders of blood cells. Mature donor T cells in allografts play important roles in alloSCT. They contribute to T-cell reconstitution in the recipient, promote donor hematopoietic engraftment, and mediate an anti–tumor effect called GVL. Unfortunately, donor T cells can broadly target host tissues causing GVHD.1 Because of GVHD, all patients receive some type of prophylactic immunosuppression, either by depleting T cells from the allograft, or more commonly, with pharmacologic agents and that inhibit T-cell function. However, even with pharmacologic immunosuppression, GVHD remains a major cause of morbidity and mortality. Novel approaches are clearly needed.

GVHD is initiated by antigen-presenting cells (APCs) that prime alloreactive donor T cells.1 In MHC-matched alloSCT, donor T cells target minor histocompatibility antigens (miHAs), which are the peptide products of polymorphic genes that distinguish the host from the donor. We previously found that direct presentation of MHC class I–restricted miHAs by recipient APCs is necessary for CD8-mediated GVHD in an MHC-matched, multiple miHA-mismatched model.2 However, although host APCs are key players, there was reason to believe that donor APCs should also play a significant role, as GVHD can begin and persist at times after transplantation when host hematopoietic cells, including APCs, are largely if not completely replaced by donor-derived cells. If, at these later times, alloreactive CD8 cells still need to be primed by professional APCs to be pathogenic, one would predict that donor APCs cross-presenting host miHAs would be important. On the other hand, it is possible that GVHD is maintained by a small number of persistent host APCs.3 In studies to address the role of donor APCs, we previously reported that CD8-mediated GVHD against miHAs is reduced when donor BM, and therefore donor APCs, lack MHC class I.4 One explanation for this result was that donor APCs cross-prime miHA-reactive donor CD8 cells. However, GVHD could have been reduced for a number of other reasons, including reduced survival and antigen responsiveness of donor CD8 cells in an MHC I− environment.5,6 Furthermore, even if cross-priming did occur, we could not determine whether naïve donor CD8 cells were cross-primed or if donor APCs were only capable of stimulating CD8 cells that were previously primed by host APCs.

In this study, we directly address the hypothesis that cross-priming of MHC I–presented miHAs contributes significantly to GVHD. We took advantage of B6 mice that naturally or transgenically express H607 or transgenically express chicken ovalbumin (actOVA).8 Both H60 and OVA contain Kb-restricted immunodominant CD8 targets (LTFNYRNL and SIINFEKL, respectively).9 Importantly, MHC class I–tetramers and intracellular IFN-γ assays can readily detect and quantitate LTFNYRNL and SIINFEKL-reactive CD8 cells. To prevent direct presentation of these peptides by donor APCs, we crossed these mice to B6 Kb−/− mice. We then used these mice as recipients in allogeneic bone marrow transplant (alloBMT) experiments.
Cross-priming was surprisingly efficient, whether antigen was restricted to the hematopoietic or nonhematopoietic compartments. CD8 cells were exclusively primed by donor CD11c+ cells, and optimal cross-priming required they be stimulated by both type I IFNs and CD40L. In studying which donor APCs acquired host miHAs, we made the surprising discovery that there was a large-scale transfer of transmembrane proteins from irradiated hosts, including MHC class I–peptide complexes, to donor cells, including dendritic cells (DCs). Donor DCs that acquired host MHC class I–peptide complexes were potent stimulators of peptide-specific T cells. These studies identify new therapeutic targets for GVHD treatment and a novel mechanism whereby donor APCs prime host-reactive T cells.

Methods

Mice

B6 mice were purchased from the NCI C3H.SW mice (H-2b), CD40−/−, and OT-1 RAG−/− B6 mice were purchased from The Jackson Laboratory and bred at Yale. actOV A mice were derived from and actH60.Kb mice were purchased from Daniel Goldstein (Yale University). The 25-D1 hybridoma,17 which produces an antibody that recognizes SIINFEKL, was obtained from Peter Cresswell (YUSM; lab prepared) and was biotin-conjugated.

In vivo CTL assay

Mice were transplanted as described in the text and were infused with LTNYRNl- or SIINFEKL-pulsed splenocytes, labeled with CFSE or cyan (Invitrogen) respectively. One day after injection, splenocytes were harvested and CFSE− and cyan− cells were enumerated.

Confocal microscopy

Spleens were digested with collagenase and cells were stained with anti–CD11c-PE (H3I-4; BD Biosciences) or isotype controls (for the anti–cytokine antibodies). Analysis was performed on an LSR II (BD Immunocytometry Systems).

Aminis ImageStream analysis

Splenocytes were stained with antibodies against K4 (FITC; SF1-1.1; BD Biosciences Pharmingen), K6 (PE; AF6-88.5, BD Biosciences PharMingen), and anti–Kα-alkaline phosphatase (AF6-88.5; BioLegend), followed by streptavidin-Alexa488 (Invitrogen). The stained cells were dropped onto slides and imaged by confocal microscopy (LSM Confocal Microscope, Carl Zeiss). Images were assembled using ImageJ software (National Institutes of Health).

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and Kb-/→actOVA.Kb-/ (nonhematopoietic OVA) BM chimeric mice. We saw impressive and early cross-priming in both sets of chimeras (representative flow cytometry, Figure 2A; total number of TertSIINFEKL+ cells, Figure 2B). The tetramer data were paralleled by the fraction of cells that produced IFN-γ when restimulated with SIINFEKL peptide (Figure 2D). Thus, both hematopoietic and nonhematopoietic antigens were productively cross-presented.

### Tissue distribution of antigen with Kb, but not antigen alone, controls the magnitude of the T-cell response

Unexpectedly, TertSIINFEKL+ cells were more numerous in actOVA.Kb-/ than in actOVA recipients (Figure 2A-C). TertH60+ cells were also more numerous in actH60.Kb-/ mice than in actH60 mice (data not shown). These data suggested that either exclusive cross-priming was more efficient than was direct priming or that ubiquitous expression of an antigen with its presenting MHC class I molecule blunted the T-cell response. To distinguish these, we compared the accumulation of TertH60+ cells in retransplanted actH60→actH60, actH60→actH60.Kb-/+, and actH60. Kb-/+→actH60 BM chimeras (Figure 3A, representative flow cytometry; Figure 3B, total number of TertH60+ CD8 cells). As was the case in nonchimeric actH60 and actOVA mice, the response against H60 was blunted in actH60→actH60 recipients relative to that in actH60Kb-/+→actH60.Kb-/+ mice. However, relative to actH60→actH60 mice, the number of TertH60+ cells was increased in actH60→actH60.Kb-/+ recipients. Thus, even though hematopoietic LTFNYRNL could be directly presented in both actH60→actH60 and actH60→actH60.Kb-/+ mice, when the presenting Kb was present in nonhematopoietic tissues, the accumulation of TertH60+ cells was reduced.

### Cross-priming does not require CD4 cells

Because responses by naive CD8 cells against directly presented SIINFEKL and LTFNYRNL depend on CD4 help2,21 (and data not shown), we could not reliably use the cross-priming of naive CD8 cells to determine the degree to which cross-presentation itself requires CD4 cells. However, SIINFEKL-reactive CD8 memory cells can be directly restimulated after transfer to actOVA mice in vivo without CD4 help (not shown). We therefore created SIINFEKL-reactive memory CD8 cells and tested whether they required CD4 help to be activated by cross-presentation APCs. Irradiated actOVA.Kb-/+ mice were reconstituted with C3H.SW BM and 5 × 10^5 sort-purified CD44+CD8+ cells from OVA-vaccinated mice (containing ~2.5 × 10^6 TertSIINFEKL+ cells). At day 13, TertSIINFEKL+ cells were nearly 100-fold more numerous in actOVA.Kb-/+ than in control Kb-/+ recipients (Figure 4A, representative flow cytometry; Figure 4B, total number of TertSIINFEKL+ cells per spleen) indicating that cross-presentation does not require CD4 help.

### Naive CD8 cells are cross-primed

Given that SIINFEKL-reactive memory cells were readily cross-primed and that there is sufficient TCR repertoire in memory CD8 cells to cause GVHD in this strain pairing,23 we considered the possibility that memory CD8 cells cross-reactive against H60 or SIINFEKL were being cross-primed and not naive CD8 cells. To test this, we transplanted actOVA.Kb-/+ mice with C3H.SW BM and 10^5 sort-purified naive (CD62L+CD44+) or 3 × 10^5 memory (CD44+) CD8 cells from unmanipulated C3H.SW mice along with 10^6 CD4+CD25 cells. An additional group also received 10^6 naive CD8 cells without CD4 cells. At day 13, TertSIINFEKL+ cells were present in naive CD8 plus CD4 but not memory CD8 plus CD4
recipients (Figure 4C, representative flow cytometry; Figure 4D, percentage and total number of Tet SIINFEKL cells). Tet SIINFEKL cells also expanded in recipients of naive CD8 cells and no CD4 cells, although they were approximately 10-fold fewer than when CD4 cells were also infused, confirming the importance of CD4 help.

Optimal cross-presentation requires CD40L and type I IFN stimulation of conventional DCs

If mechanisms by which APCs cross-present miHAs in alloBMT could be identified, they could be targeted for GVHD treatment.
One approach to investigate critical mechanisms would be to use donor BM from mice deficient in genes that might be involved in cross-presentation in the actOVA, actOVA.Kb−/−, and actH60.Kb−/− mice were irradiated and reconstituted with 6 × 10^6 BM cells, 2.4 × 10^6 CD8 cells, and 10^5 CD4 cells from C3H.SW mice. (A) Representative flow cytometry from day 13, gated on Ly9.1+CD8+ cells. (B) Total number of TetSIINFEKL+ cells per spleen on days 13 and 35. P < .0001, comparing Kb−/− recipients versus actOVA.Kb−/− recipients. P < .0001, comparing actOVA versus actOVA.Kb−/− mice. P = .05, comparing Kb−/− recipients versus other groups except actOVA recipients (by one-tailed Mann-Whitney). Data from actOVA and actOVA.Kb−/− recipients are combined from 2 experiments with similar results. (C) Percentage of CD8 cells that were TetSIINFEKL+ in blood at day 13 (data combined from 3 experiments; P < .0001). Ly9.1+CD8+ splenocytes from mice transplanted as in panel A produced IFN-γ when restimulated with SIINFEKL at day 13 after BMT.

Figure 2. CD8 cells are cross-primed whether antigen is restricted to hematopoietic or nonhematopoietic cells. Kb−/−, actOVA, actOVA.Kb−/−, actOVA.Kb−/−→Kb−/−, and Kb−/−→actOVA.Kb−/− mice were back-crossed to C3H.SW. We therefore determined whether CD8 cells are cross-primed against SIINFEKL when BALB/c (H-2d) mice carrying the actOVA transgene (BALB/c-OVA) are transplanted with B6 BM and CD8 cells, as most gene-modified mice were available backcrossed to B6. Because BALB/c mice are Kd/Kb−/−, only donor-derived B6 APCs can present SIINFEKL. BALB/c-OVA or control BALB/c mice were irradiated and reconstituted with BM and 10^6 CD8 and 10^5 CD4 cells from B6 mice. By day 14, a clear TetSIINFEKL+ population developed in BALB/c-OVA recipients (Figure 5A, representative flow cytometry; Figure 5B, time course). In some experiments, we also saw TetH60+ cells in BALB/c recipients as they are H60− (not shown). Cross-primed CD8 cells produced IFN-γ when restimulated with SIINFEKL (not shown) and selectively killed SIINFEKL-pulsed B6 splenocytes infused on day 13 into transplanted BALB/c-OVA recipients (Figure 5C). Thus, even though most alloreactive donor T cells recognize intact host MHC, there was still substantial cross-priming by donor APCs.

We then used B6 gene-deficient and transgenic mice as BM donors in the B6→BALB/c-OVA model to investigate mechanisms of cross-priming (Figure 5C-E). To test whether donor CD11c+ cells were the key cross-presenting APC, we used CD11c-cre transgenic mice crossed to ROSA26-flox-stop-DTA mice (CD11c-DTA) as BM donors. When cre is expressed, a stop cassette is excised of the DTA sequence, inducing its expression and a profound deficiency of both conventional and plasmacytoid DCs (and data not shown). Strikingly, we observed no TetSIINFEKL+ cells in recipients of CD11c-DTA BM. Given that mouse plasmacytoid DCs have not been shown to cross-present, these data make it likely that donor conventional DCs are the key cross-presenting cell. Cross-priming of naïve CD8 cells is optimal with CD4 help. Surprisingly, however, we found cross-priming to be intact when donor BM was MHC class II−/−. Therefore, even though the CD8 response against SIINFEKL is optimal with CD4 help (Figure 4), CD4 cells need not make direct contact with cross-presenting APCs. Because some polyclonal CD4 cells in
Indeed, in all but one recipient of CD40 priming would be reduced if donor BM was CD40-deficient. Significantly reduced in recipients of IFNAR1 

Although not completely eliminated, cross-presentation was significant in recipients of doublets of C3H.SW and actOV A cells as we used narrow forward scatter and side scatter width gates to exclude these. In contrast, staining in actOV A.Kb 

Donor cells are “cross-dressed” with host-derived transmembrane proteins

After observing cross-presentation in the C3H.SW→actOV A.Kb−/− experiments, we wanted to determine which donor cells cross-presented SIINFEKL. To do so, we stained donor-derived cells in transplanted C3H.SW→actOV A and C3H.SW→actOV A.Kb−/− mice with the 25-D1 antibody, which recognizes Kb-SIINFEKL complexes. We first focused on donor Ly9.1+ (donor-specific) CD11c+ cells, and to our surprise, we only observed significant 25-D1 staining in actOV A and not actOV A.Kb−/− recipients (Figure 6A), even though cross-priming occurred in the latter. The majority of Ly9.1+CD11c+ cells bound 25-D1 such that there was a shift in the entire population, rather than distinct positive and negative subsets. This was unlikely to be the result of doublets of C3H.SW and actOV A cells as we used narrow forward scatter and side scatter width gates to exclude these. In contrast, staining in actOV A.Kb−/− recipients was similar to that in Kb−/− controls. 25-D1 staining of donor cells in actOV A recipients was not limited to DCs but was also observed in CD4 and CD8 cells (Figure 6A). 25-D1 staining on donor cells in actOV A recipients was seen as early as day 3 and as late as day 11 (not shown).

It was possible that either intact Kb-SIINFEKL complexes (present in actOV A but not in actOV A.Kb−/− mice) were being transferred and/or there was free SIINFEKL peptide exclusively in actOV A mice that “pulsed” donor Kb molecules in vivo. We therefore also stained cells in transplanted mice for Kb and observed a similar shift in Kb on donor cells in transplanted control B6 and actOV A mice, but not in either Kb−/− or actOV A.Kb−/− recipients (Figure 6A bottom panel). This suggested that the increase in 25-D1 we observed could have been the result of transfer of intact Kb-SIINFEKL complexes from host to donor. To further test this hypothesis, we infused CFSE-labeled Kb−/− and unlabeled CD45.1 wild-type splenocytes into actOV A, actOV A.Kb−/−, and B6 mice that had been irradiated 5 days earlier. We then gated on CFSE+CD8+ or CD45.1+CD8+ cells and analyzed Kb expression and 25-D1 binding. Both transferred Kb−/− and WT CD8 cells extracted from irradiated actOV A, but not actOV A.Kb−/− or B6 mice, stained with 25-D1 (Figure 6B). Thus, intact Kb-SIINFEKL complexes must have been transferred from host to donor cells.

We also analyzed donor-derived cells early after transplantation in the B6→BALB/c strain pairing (Figure 6C). BALB/c or BALB/c-OVA mice were irradiated and reconstituted with B6 CD45.1 BM. Six days later, mice were scarified and CD45.1+ cells were analyzed for their expression of Kb, Kd, CD45.1, CD45.2, and surface transmembrane OVA (Figure 6C). Most DCs in B6→BALB/c or B6→BALB/c-OVA mice expressed a similar amount of Kb as did DCs in control B6 mice, consistent with their being of B6 in origin. The majority of these Kb+ DCs expressed Kd, although its expression varied over a wide range of fluorescent intensities compared with control BALB/c mice. Expression of host-derived CD45.2 and transmembrane OVA paralleled that of Kd (Figure 6C).
Taken together, these data suggest that donor-derived cells acquired host-derived transmembrane proteins. To better characterize these hybrid cells, we analyzed sorted CD11c+ cells from spleens of B6→BALB/c mice 6 days after transplantation by confocal microscopy (Figure 6D). We observed Kd+Kb+, Kd+Kb−, and Kd−Kb+ cells. Kd appeared to be on the cell surface, and not part of another attached cell, confirming that the donor cells staining for host-specific proteins by flow cytometry were not doublets. Overall, Kd was less uniformly distributed than was Kb.

Kb+Kd+ cells were only present in B6→BALB/c mice, and not in control B6→B6 or BALB/c→BALB/c mice (not shown).

We also analyzed hybrids using the Amnis Image Stream system, which allows high-throughput multichannel fluorescent imaging of a large number of cells.32 BALB/c mice were irradiated and reconstituted with B6 BM and splenocytes containing 10^6 T cells. Six days later, mice were killed and splenocytes were stained with antibodies against CD11c (or CD8; both in Pacific Blue), Kb (PE), and Kd (FITC). Cells were also stained with 7-AAD to exclude dead cells and with the intravital DNA-binding dye DRAQ5. Cells were then analyzed using the ImageStream instrument. To assess the pattern of Kb and Kd staining on CD11c+ cells, we gated on 7-AAD− cells that had a DRAQ5 signal consistent with being single cells (>90% of events). We then gated on CD11c+ cells and analyzed the intensity and distribution of Kb and Kd staining (Kb vs Kd plot, supplemental Figure 1A, available on the Blood Web site; see the Supplemental Materials link at the top of the online article; Kb vs Kd plot; Figure 6E, cell images). As observed by flow cytometry, most Kb+ cells acquired some Kd. Although the distribution of Kb was fairly uniform, in an analysis of ~200 KdKb double-positive cells, Kd signal was more focal, similar to the confocal images. The majority of cells had at least a 3 areas of Kd signal, but a smaller percentage of events had a single and more intense area of staining. Similar data were obtained for CD8+ cells (supplemental Figure 1B). Of note, CD11c+ donor-derived cells in B6→B6 transplants also had focal areas of more intense Kd staining, consistent with donor B6 cells acquiring host-derived Kb.

**Cross-dressed DCs can prime T cells directed against transferred host MHC class I–peptide complexes**

Given that we had observed donor-derived DCs with host-derived MHC in both the C3H.SW→B6 and B6→BALB/c strain pairing, we reasoned that this could be a mechanism whereby donor cells can acquire host MHC-peptide complexes, thereby enabling them to prime donor CD8 cells. The effect would be to cross-prime donor-derived T cells, but without antigen processing characteristic of cross-presentation.33 To test this hypothesis, we determined whether donor-derived DCs that acquire host MHC-peptide complexes can prime CD8 cells specific for the host complex. To set up such a situation, B6 actOVA mice were irradiated and reconstituted with BALB/c BM. Seven days later, sort-purified splenic CD11c+Kb+ cells with the same level of Kd as was present in control BALB/c→BALB/c recipients (Figure 7A) were cultured with CFSE-labeled OT-1 transgenic T cells, which specifically and sensitively recognize Kb-SIINFEKL. Hybrid Kb+Kd+ DCs induced OT-1 division, whereas control BALB/c DCs induced none (Figure 7B). Thus, APCs cross-dressed with host MHC class I–peptide complexes were functional.

**Discussion**

GVHD can develop or persist months to years after transplantation when host hematopoiesis has been mostly if not completely replaced by donor-derived cells. If CD8 cells continue to contribute to GVHD at these late times, then they must be independent of further APC stimulation, be cross-primed by donor-derived APCs, or both. The main goals of our studies were to determine whether cross-priming occurs, and if so, to identify pathways that can be targeted to prevent it.
In the C3H.SW-B6 model, donor-naive CD8 cells mounted strong responses against SIINFEKL or LTFNYRNL when these antigens were exclusively cross-presented by donor APCs. These cross-primed cells were functional effectors that produced IFN-γ and were cytolytic in vivo. Surprisingly, the magnitude and quality of cross-priming responses were similar whether antigen was exclusively nonhematopoietic or hematopoietic, even though one might have predicted that the massive death of hematopoietic cells after irradiation would have provided a more accessible source of antigen.

Little is known about the nature of CD8 responses against nonhematopoietic miHAs in humans or mice as methods for...
Figure 6. Donor cells acquire host transmembrane proteins, including MHC class I-SIINFEKL complexes. B6, actOVA, and actOVA,Kb−/− mice were irradiated and reconstituted with C3H.SW BM, 2 × 10⁶ CD8 cells, and 1 × 10⁶ CD4 cells. Mice were killed 5 days after transplantation. (A) Representative flow cytometry of 25-D1 (top row) and Kb staining (bottom row) of donor Ly9.1+ CD11c+, CD8+, and CD4+ cells. Data are representative of 2 independent experiments with at least 3 mice per group. (B) actOVA,Kb−/− and actOVA mice were irradiated; and 5 days later, WT CD45.1 B6 or CFSE-labeled Kb−/− splenocytes were infused intravenously. Two days later, mice were killed. Shown is 25-D1 staining of CD45.1+ (left panel) and CFSE+CD8 cells (right panel). Note the shift in 25-D1 staining, which occurred only in actOVA recipients. (C-D) BALB/c-OVA (CD45.2) mice were irradiated and reconstituted with B6 CD45.1 BM and 10⁶ CD8 and 10⁵ CD4 cells. On day 5, splenocytes were harvested and stained with antibodies against CD11c, Kb, Kd, CD45.1, and surface OVA. Shown is representative flow cytometry gating on CD11c+ cells. As controls, nontransplanted B6 and BALB/c mice were analyzed. Note that BALB/c-actOVA recipients of B6 CD45.1 BM had a population of CD11c+ cells with a similar intensity of Kb staining as on CD11c+ cells from control B6 mice, but with significant staining for Kd. A similar pattern was seen for CD45.2 and surface OVA (histogram). Data are representative of at least 3 independent experiments. For confocal images, mice were transplanted as in panel C, and on day 6 cells were stained with anti-CD11c-PE and CD11c+ cell were sorted. Sorted cells were stained with antibodies against Kd (biotin followed by SA-Alexa488) and anti-Kb (APC) and then dropped onto slides. Original magnifications ×400. Note cells that express both Kd and Kb (white circles). There were also cells that stained for Kb or Kd only, which are internal controls for staining specificity. No Kb cells were seen when cells from control BALB/c→BALB/c were imaged (not shown). For Amnis image flow analysis (E), BALB/c mice were transplanted as in panels C through D. On day 6, splenocytes were harvested and stained with antibodies against CD11c (Pacific Blue), Kb (PE), and Kd (FITC) in addition to staining with 7-AAD for live/dead exclusion and DRAQ5 to allow isolation of 2N DNA content cells and analyzed on the ImageStream instrument. Shown are representative images from >200 KdKb double-positive CD11c+ cells analyzed in B6→BALB/c recipients and more than 200 CD11c+ cells in B6→B6 and BALB/c→BALB/c syngeneic controls. Results are representative of 2 independent experiments.
identifying miHAs have relied on propagating donor-derived T cells, in most cases harvested from mice or humans post alloSCT, with host-derived hematopoietic cells. Thus, T cells reactive against exclusively nonhematopoietic antigens would not be expanded and their targets not identified. Because T cells can be cross-primed against nonhematopoietic antigens, a complete picture of anti-miHA T-cell reactivity must include an analysis of T cells that target nonhematopoietic antigens, which will require methods for miHA identification that do not rely on the propagation of T cell lines by host-derived APCs.

Cross-priming even occurred in the fully MHC-mismatched B6→BALB/c strain pairing. Although CD8 cells reactive against host antigens presented by donor APCs would not be likely to cause GVHD as host target tissues would lack the restricting MHC and CD8-mediated GVHD requires direct TCR:MHC class I contact, we took advantage of this system to study the mechanisms of cross-priming. Donor T cells were exclusively cross-primed by a CD11c+ cell, most likely a conventional DC, as mouse plasmacytoid DCs have little capacity to cross-present exogenous antigens. To optimally cross-prime naive CD8 cells, donor DCs must be stimulated by both CD40L and type I IFNs. The CD40L:CD40 signal did not require direct cognate recognition of MHC class II on donor APCs by CD8 cells (the most likely source of CD40L) as cross-priming was robust even when donor APCs were MHC class II−. These studies suggest that targeting type I IFNs or CD40:CD40L interactions could inhibit GVHD mediated by cross-primed CD8 cells.

Another surprising finding was how the presence and distribution of Kb-SIINFEKL or Kb-LTFNYRNL, but not the parent proteins without the restricting MHC class I, had a decisive impact on the magnitude of the T-cell response. That this reduction was seen in spleen and LN indicates that suppression probably occurs before T-cell egress into blood and then GVHD target organs. It is possible that, when target MHC-peptides are widely expressed at a high level, even on stromal, endothelial, and hematopoietic cells in secondary lymphoid tissues, reactive T cells are consumed or induced to undergo activation-induced cell death. These data suggest that antigens with a more restricted tissue distribution may generate the largest T-cell responses.

Our findings extend those of Asakura et al and Flutter et al. Flutter et al found in a delayed T-cell infusion model that alloreactive T-cell responses were diminished when antigen was widespread, and used TCR transgenic T cells to track alloreactivity. Asakura et al reported that total donor CD8 expansion and GVLA were augmented when alloantigen was hematopoietically restricted. Because miHA-reactive T cells could not be specifically tracked, it was possible that they were generated equally whether alloantigen/MHC was ubiquitous or only hematopoietic, but that they were functionally impaired. Because we could enumerate polyclonal miHA-specific T cells by tetramers, we were able to show that the major effect of ubiquitous antigen: MHC class I expression was a reduction in the number of miHA-specific T cells.

We had hoped to identify donor APCs that cross-present SIINFEKL using the 25-D1 antibody. Instead, we unexpectedly discovered widespread transfer of host-derived transmembrane proteins to donor cells, including DCs. Confocal microscopy and Amnis imaging confirmed that these hybrids were single cells with relatively large but focal areas of host-derived MHC class I. Importantly, donor DCs that acquired host Kb-SIINFEKL were capable of priming OT-1 cells in vitro. These data suggest that transfer of host MHC-peptide complexes to donor APCs could enhance the early priming of alloreactive T cells, even in the fully allogeneic setting. We were unable to dissect whether such cross-dressing contributes to GVHD, as hosts that would have allogeneic MHC class I-peptide complexes to donate to donor APCs would also be able to directly prime allogeneic T cells.

Transfer of membrane and transmembrane proteins from cell to cell has been described in a number of systems. Exosomes can endow the recipient APC with the ability to prime T cells in contact with targets can acquire target cell-derived MHC in a contact-dependent manner. Exosomes can also be the vehicle for transferring transmembrane proteins, and the relocated MHC can endow the recipient APC with the ability to prime T cells. Most relevant to our studies, the transfer of transmembrane proteins can be potentiated with prior freeze/thaw killing of the donor cells. In our studies, irradiation and cell death, physiologic in the context of alloSCT, were key as we did not see hybrid DCs in stable mixed BM chimeras (not shown), and the microscopy images suggest transfer of relatively large membrane fragments.

In conclusion, our studies make important and novel contributions to the understanding of alloimmune CD8 responses. We have established the extent and importance of cross-presentation and cross-priming against miHAs, and have identified CD40L:CD40 interactions and type I IFNs as therapeutic targets to prevent it. Our studies show that exclusively nonhematopoietic antigens are cross-presented and targeted by alloreactive CD8 cells. We also show how peptide/MHC distribution contributes to immunodominance. Finally, we have identified a novel mechanism whereby donor-derived cells can present host MHC-peptide complexes.
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Authorship

Contribution: X.W. and H.L. designed and performed experiments, analyzed data, and wrote the paper; C.M.-M. and H.S.T. assisted with experiments; N.L. created SIINFEKL-reactive memory T cells; D.R. created B6.H60 and actH60 mice, provided technical advice, and edited the manuscript; and W.D.S. designed experiments, analyzed data, and wrote the manuscript.

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