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

Xiaojian Wang,1 Hongmei Li,1,2 Catherine Matte-Martone,1,2 Weiguo Cui,2 Ning Li,1,2 Hung Sheng Tan,1,2 Derry Roopenian,3 and Warren D. Shlomchik1,2

1Cancer Center and 2Departments of Medicine and Immunobiology, Yale University School of Medicine, New Haven, CT; and 3The Jackson Laboratory, Bar Harbor, ME

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–restricted miHAs. 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 naive 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**

B6-1 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. actH60 mice were purchased from Daniel Goldstein (Yale University School of Medicine [YUSM]) backcrossed to B6 and BALB/c. acT60 and B6.H60 mice (generated by D.R.) were bred at Yale. Kb−/− mice were purchased from The Jackson Laboratory and backcrossed to B6 actOVA, B6.H60, and actH60 mice to create actOVA, Kb−/−, and actH60.Kb−/−, respectively. ActOVA.Kb−/− mice were typed by flow cytometry of blood with antibodies against OVA (ab8389; Abcam) and Kb (AF6–88.5; BD Biosciences PharMingen). B6.Kb−/− were typed for Kb by flow cytometry of splenocytes when mice were harvested for use as BM donors. The depletion of CD11c− cells was confirmed by flow cytometry of splenocytes when mice were harvested for use as BM donors.

**Cell separations**

All donor BM was depleted of T cells using anti-Thy1.2 microbeads and the AutoMACs (Miltenyi Biotec). CD8 or CD4 cells were purified from lymph nodes (LN)s by negative selection as previously described. In some experiments, beads-bound CD8+ T cells were stained with anti-CD62L (Mel-14; BD Biosciences PharMingen), anti-CD44 (EM7; BD Biosciences PharMingen), and sorted into CD62L+CD44+ naive and CD44+ memory populations using a FACSARia (BD Immunocytometry Systems).

**Bone marrow transplantation**

All transplantations were performed according to protocols approved by the Yale University Institutional Animal Care and Use Committee. For experiments in the C3H.SW→B6 strain pairing, B6 background mice were irradiated with 1000 cGy and reconstituted with 107 T cell–depleted BALB/c BM cells.

**Tetramers**

The H-2Kb/LTFNRYNL tetramer conjugated to phycoerythrin (PE) was obtained from the National Institutes of Health tetramer core facility (Atlanta, GA). The H-2Kb/SIINFEKL tetramers were laboratory-prepared as previously described. Biotinylated Kb–peptide complexes were tetramerized by the addition of allophtocyanin-conjugated streptavidin (Invitrogen).

**Peptides**

LTFNRYNL and SIINFEKL were synthesized by the Keck laboratory at YUSM.

**Flow cytometry**

**Intracellular cytokine staining.** Splenocytes were cultured with 100nM SIINFEKL or LTFNRYNL for 5 hours; monensin was added for the final 2 hours. Before permeabilization, cells were incubated with etidium monoaza to allow exclusion of dead cells. Cells were stained with antibodies against CD8, CD229.1 (Ly9.1; 30C7; BD Biosciences PharMingen), CD44, CD62L, permeabilized, and then stained with antibodies against IFN-γ (XMG1.2; BD Biosciences) or isotype controls (for the anti–cytokine antibodies). Analysis was performed on an LSRII (BD Immunocytometry Systems).

**DEC-OVA memory cell generation**

To generate SIINFEKL-reactive CD8+ memory cells, C3H.SW mice were injected with 50 μg anti–DEC205-OVA (laboratory-prepared) and 50 μg anti-CD40 (FGK45; laboratory-prepared). Memory cells were harvested 2 months later.

**In vivo CTL assay**

Mice were transplanted as described in the text and were infused with LTFNRYNL- 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 (HL3; BD Biosciences PharMingen) for 20 minutes, washed once, and resuspended in sort buffer. The sorted CD11c+ cells were stained with anti–Kb–biotin (SF1–1.1; BD Biosciences PharMingen), anti–Kb–allophycocyanin (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).

**Amin ImageStream analysis**

Splenocytes were stained with antibodies against K4 (FITC; SF1–1.1, BD Biosciences PharMingen), K4 (PE; AF6-88.5, BD Biosciences PharMingen), CD11c (Pacific Blue; N418, 5Bioscience), or CD8 (Pacific Blue; TB105, laboratory-conjugated). Cell nuclei were stained with DRAQ5.
(eBioscience), and dead cells were excluded with 7-aminoactinomycin D (7-AAD; eBioscience). Analysis was performed using the ImageStream instrument with 40× magnification (Amnis). The data were analyzed with IDEAS Version 4.0 software (Amnis).

### OT-1 proliferation experiments

At day 6 after transplantation, BALB/c→B6actOVA mice were killed. Collagenase-digested splenocytes were stained with anti-CD11c-PE, anti-K\(^{b}\)-FITC, anti-K\(^{d}\)-allophycocyanin, and CD11c-\(\text{K}^{\text{K}}\text{K}^{\text{K}}\text{K}^{\text{K}}\) cells were sorted. The sorted cells were cultured with 10\(^3\) CFSE-labeled OT1 cells. Cultures were harvested 3 days later, and CFSE dilution of OT-1 cells was assessed by flow cytometry.

### Statistics

Differences in cell numbers or percentages were determined by Mann-Whitney \(U\) test. Comparisons are 2-tailed unless noted to be otherwise.

## Results

### Donor CD8 cells are cross-primed against H60 and OVA

To measure cross-priming, B6.H60 (congenic for \(H60\); H60 expression is mostly limited to hematopoietic cells),\(^{19}\) B6.H60.K\(^{b-/-}\), actOVA.K\(^{b-/-}\) (H60 driven by an actin promoter; expression is ubiquitous but at a low level), and control K\(^{b-/-}\) mice were irradiated and reconstituted with C3H.SW (H-2\(^\text{b}\); H60\(^+\)) BM and 2.4 × 10\(^8\) CD8\(^+\) and 10\(^4\) CD4\(^+\) polyclonal C3H.SW T cells. CD4 cells were infused as priming to H60 is optimal with CD4 help\(^{20,22}\) (and data not shown). Mice were killed on days 8, 14, 22, and 38 after transplantation and donor (Ly9.1\(^+\)) H60-reactive CD8 cells were enumerated using the LTFNYRNL:K\(^b\) tetramer\(^{20}\) (TetH60). TetH60\(^+\) cells were readily found in spleen, BM, liver, and LN (Figure 1A, representative flow cytometry). Tet H60\(^+\) cells were detectable by day 8 in all groups except for K\(^{b-/-}\)-negative control mouse, with a peak percentage in the spleen at day 14 after transplantation (Figure 1B). At day 14, we injected transplanted actH60.K\(^{b-/-}\) mice with a single pulse of BrdU 2 hours before sacrifice to determine the fraction of cycling TetH60\(^+\) cells. Between 21% and 50% of TetH60\(^+\) cells were in division, indicative of active cross-presentation and subsequent activation of LTFNYRNL-reactive CD8 cells (Figure 1C).

We also tested the function of cross-primed H60-reactive cells. They produced IFN-\(\gamma\) when restimulated with LTFNYRNL (Figure 1D), and the fraction of IFN-\(\gamma\)-producing cells was proportional to the frequency of TetH60\(^+\) cells. To assess their cytolytic activity, at day 6 after transplantation, BALB/c→B6actH60 mice were reconstituted with C3H.SW BM and 5 × 10\(^5\) sort-purified CD4\(^+\)CD8\(^+\) cells from OVA-vaccinated mice (containing ~2.5 × 10\(^4\) TeSIINFEKL\(^+\) cells). At day 13, TeSIINFEKL\(^+\) cells were nearly 100-fold more numerous in actOVA.K\(^{b-/-}\) mice than in control K\(^{b-/-}\) recipients (Figure 4A, representative flow cytometry; Figure 4B, total number of TeSIINFEKL\(^+\) 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.K\(^{b-/-}\) mice with C3H.SW BM and 10\(^5\) sort-purified naive (CD62L\(^+\)CD44\(^+\)) or 3 × 10\(^4\) memory (CD44\(^+\)) CD8 cells from unmanipulated C3H.SW mice along with 10\(^4\) CD4\(^+\)CD25\(^-\) cells. An additional group also received 10\(^4\) naive CD8 cells without CD4 cells. At day 13, TeSIINFEKL\(^+\) cells were present in naive CD8 plus CD4 but not memory CD8 plus CD4 by flow cytometry.

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

Unexpectedly, TeSIINFEKL\(^+\) cells were more numerous in actOVA.K\(^{b-/-}\) than in actOVA recipients (Figure 2A-C). TetH60\(^+\) cells were also more numerous in actH60.K\(^{b-/-}\) 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 TetH60\(^+\) cells in retransplanted actH60→actH60, actH60→actH60.K\(^{b-/-}\), and actH60.K\(^{b-/-}\)→actH60 BM chimeras (Figure 3A, representative flow cytometry; Figure 3B, total number of TetH60\(^+\) 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 actH60K\(^{b-/-}\)→actH60.K\(^{b-/-}\) mice. However, relative to actH60→actH60 mice, the number of TetH60\(^+\) cells was increased in actH60→actH60.K\(^{b-/-}\) recipients. Thus, even though hematopoietic LTFNYRNL could be directly presented in both actH60→actH60 and actH60→actH60.K\(^{b-/-}\) mice, when the presenting K\(^b\) was present in nonhematopoietic tissues, the accumulation of TetH60\(^+\) 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 help\(^{20,21}\) (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-presenting APCs. Irradiated actOVA.K\(^{b-/-}\) mice were reconstituted with C3H.SW BM and 5 × 10\(^5\) sort-purified CD44\(^+\)CD8\(^+\) cells from OVA-vaccinated mice (containing ~2.5 × 10\(^4\) TeSIINFEKL\(^+\) cells). At day 13, TeSIINFEKL\(^+\) cells were nearly 100-fold more numerous in actOVA.K\(^{b-/-}\) than in control K\(^{b-/-}\) recipients (Figure 4A, representative flow cytometry; Figure 4B, total number of TeSIINFEKL\(^+\) cells per spleen) indicating that cross-presentation does not require CD4 help.

### Both hematopoietic and nonhematopoietic antigens are cross-presented

That cross-primed CD8 cells could be detected early after transplantation suggested that hematopoietic antigens derived from apoptotic cells induced by irradiation were a key source of antigen. To test this, we used the actOVA system and compared the generation of SIINFEKL-reactive cells in actOVA,K\(^{b-/-}\) mice with that in retransplanted actOVA.K\(^{b-/-}\)→actOVA.K\(^{b-/-}\) (hematopoietic OVA only) and K\(^{b-/-}\)→actOVA.K\(^{b-/-}\) (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 TeSIINFEKL\(^+\) cells, Figure 2B). The tetramer data were paralleled by the fraction of cells that produced IFN-\(\gamma\) when restimulated with SIINFEKL peptide (Figure 2D). Thus, both hematopoietic and nonhematopoietic antigens were productively cross-presented.
recipients (Figure 4C, representative flow cytometry; Figure 4D, percentage and total number of Tet SIINFEKL/H11001 cells). Tet SIINFEKL/H11001 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+, or actH60.Kb+ systems. However, appropriate gene-deficient mice were not available 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-OV A) are transplanted with B6 BM and CD8 cells, as most gene-modified mice are available back-crossed to B6. Because BALB/c mice are Kb+, only donor-derived B6 APCs can present SIINFEKL. BALB/c-OVA or control BALB/c mice were irradiated and reconstituted with BM and 10^5 CD8 and 10^5 CD4 cells from C3H.SW 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 (Figure 4). 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
B6 → BALB/c recipients would be activated by fully allogeneic BALB/c APCs, we reasoned that CD4 cells could be delivering signals in trans to donor B6 DCs without direct TCR/MHC class II contacts. CD40L induced in alloreactive donor CD4 cells seemed a likely candidate molecule.24 We therefore tested whether cross-priming would be reduced if donor BM was CD40-deficient. Indeed, in all but one recipients of CD40 priming would be reduced if donor BM was CD40-deficient. Contrast, staining in actOV A.Kb

In transplanted C3H.SW and actOV A cells as we used narrow positive and negative subsets. This was unlikely to be the result of doublets of C3H.SW and actOV A cells, but rather of distinct subpopulations. The majority of these Kb+ cells were all of B6 in origin. The majority of these Kb+ cells were all of B6 in origin.

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

After observing cross-priming 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.23 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−/− or 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 scarificed and CD11c+ 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).
Figure 4. SIINFEKL-specific but not spontaneous memory T cells are restimulated by cross-presentation APCs. (A-B) K\textsuperscript{b} and actOVA.K\textsuperscript{b\textsuperscript{+}} mice were irradiated and reconstituted with C3H.SW BM and 5 x 10\textsuperscript{5} sort-purified CD8\textsuperscript{+} CD4\textsuperscript{+} CD62L\textsuperscript{+} CD44\textsuperscript{+} K\textsuperscript{d} cells from C3H.SW mice vaccinated 60 days earlier with anti-DEC205-OVA. Mice were killed 13 days after transplantation. (A) Representative flow cytometry (gating on Ly9.1\textsuperscript{+} CD8\textsuperscript{+} cells). (B) Number of Tet\textsuperscript{SIINFEKL} cells per spleen. P = .024. (C) K\textsuperscript{b\textsuperscript{+}} and actOVA.K\textsuperscript{b\textsuperscript{+}} mice were transplanted as in panel A, except that they received C3H.SW sort-purified naive CD8\textsuperscript{+} CD44\textsuperscript{+} CD62L\textsuperscript{+} (1 x 10\textsuperscript{5}) or memory CD8\textsuperscript{+} CD44\textsuperscript{+} K\textsuperscript{d} (3 x 10\textsuperscript{5}) cells, with or without 10\textsuperscript{5} CD4\textsuperscript{+} CD25\textsuperscript{+} T\textsubscript{H} cells. Representative flow cytometry of spleen, LN, and blood is shown in panel C (gated on Ly9.1\textsuperscript{+} CD8\textsuperscript{+} cells). Percentage of CD8 cells that were Tet\textsuperscript{SIINFEKL} and total number of Tet\textsuperscript{SIINFEKL} cells per spleen are shown in panel D. P = .0043, comparing percentage and total number of Tet\textsuperscript{SIINFEKL} cells in recipients of CD4 cells and T\textsubscript{H} versus CD4 cells and T\textsubscript{H} cells. P < .0006, comparing percentage and total number of Tet\textsuperscript{SIINFEKL} cells in recipients of T\textsubscript{H} with or without CD4 cells. Data combined from 2 independent experiments.

Taken together, these data suggest that donor-derived cells acquired host-derived transmembrane proteins. To better characterize these hybrid cells, we analyzed sorted CD11c\textsuperscript{+} cells from spleens of B6 → BALB/c mice 6 days after transplantation by confocal microscopy (Figure 6D). We observed K\textsuperscript{b\textsuperscript{+}} K\textsuperscript{d\textsuperscript{+}} K\textsuperscript{b\textsuperscript{+}} K\textsuperscript{d\textsuperscript{+}} K\textsuperscript{b\textsuperscript{+}} K\textsuperscript{d\textsuperscript{+}} K\textsuperscript{b\textsuperscript{+}} K\textsuperscript{d\textsuperscript{+}} cells 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. BALB/c mice were irradiated and reconstituted with B6 BM and splenocytes containing 10\textsuperscript{6} T cells. Six days later, mice were killed and splenocytes were stained with antibodies against CD11c (or CD8; both in Pacific Blue), K\textsuperscript{b} (PE), and K\textsuperscript{d} (FTTC). 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 K\textsuperscript{b} and K\textsuperscript{d} staining on CD11c\textsuperscript{+} cells, we gated on 7-AAD\textsuperscript{−} cells that had a DRAQ5 signal consistent with being single cells (>90% of events). We then gated on CD11c\textsuperscript{+} cells and analyzed the intensity and distribution of K\textsuperscript{b} and K\textsuperscript{d} staining (K\textsuperscript{b} vs K\textsuperscript{d} plot, supplemental Figure 1A, available on the Blood Web site; see the Supplemental Materials link at the top of the online article; K\textsuperscript{b} vs K\textsuperscript{d} plot; Figure 6E, cell images). As observed by flow cytometry, most K\textsuperscript{b\textsuperscript{+}} cells acquired some K\textsuperscript{d}. Although the distribution of K\textsuperscript{b} was fairly uniform, in an analysis of ~200 K\textsuperscript{b\textsuperscript{+}} K\textsuperscript{d\textsuperscript{+}} double-positive cells, K\textsuperscript{d} signal was more focal, similar to the confocal images. The majority of cells had at least a 3 areas of K\textsuperscript{d} signal, but a smaller percentage of events had a single and more intense area of staining. Similar data were obtained for CD8\textsuperscript{+} cells (supplemental Figure 1B). Of note, CD11c\textsuperscript{+} donor-derived cells in B6 → B6 transplants also had focal areas of more intense K\textsuperscript{b} staining, consistent with donor B6 cells acquiring host-derived K\textsuperscript{b}.

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. 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\textsuperscript{+} K\textsuperscript{b\textsuperscript{+}} cells with the same level of K\textsuperscript{d} 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 K\textsuperscript{b} SIINFEKL. Hybrid K\textsuperscript{b\textsuperscript{+}} K\textsuperscript{d\textsuperscript{+}} 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.SWxB6 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 5. Optimal cross-presentation requires CD40L and type I IFN stimulation of conventional DCs. BALB/c or BALB/c-OVA mice were irradiated and reconstituted with B6 (CD45.2) BM, 10^6 CD8, and 10^6 CD4 cells from B6 CD45.1 mice. (A) Representative flow cytometry of spleen, LN, and blood on day 14 (gated on CD8^+ CD45.1^+ cells). (B) Time course (at least 3 mice/group at each time). At day 14 after transplantation, unpulsed (cyan) or SIINFEKL-pulsed (CFSE) B6 splenocytes were infused and spleens were harvested 18 hours later. Note the selective killing of SIINFEKL-pulsed cells (C). Mice were transplanted as in panel A, except that they received donor B6 BM from WT, MHC class II^−/−, CD11c-cre x ROSA26-flx-stop-DTA, CD40^−/−, or IFNAR1^−/− mice. Recipients were killed on day 14. Shown is representative TetSIINFEKL staining (D; gated on CD8^+ CD45.1^+ cells). Percentages of CD8 cells that were TetSIINFEKL^+ are shown in panel E. Data from 3 experiments combined. P = .02, comparing recipients of WT BM compared with recipients of IFNAR1^−/− or CD40^−/− BM.
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 x 10^6 CD8 cells, and 1 x 10^6 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 10^6 CD8 and 10^5 CD4 cells. On day 5, splenocytes were harvested and stained with antibodies against CD11c, Kb, K^d, 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 K^d. 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 K^d (biotin followed by SA-Alexa488) and anti-Kb (APC) and then dropped onto slides. Original magnifications x400. Note cells that express both K^d and Kb (white circles). There were also cells that stained for Kb or K^d only, which are internal controls for staining specificity. No Kb cells were seen when cells from control BALB/c recipients 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 K^d (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 K^d Kb 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.
toid DCs have little capacity to cross-present exogenous antigens.30 In this study, 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,35 as mouse plasmacytoid DCs have little capacity to cross-present exogenous antigens.30 To optimally cross-prime naïve 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 CD4 T cells (the most likely source of CD40L) as cross-priming was robust even when donor APCs were MHC class II+. This study suggests 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 al36 and Flutter et al.37 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 alloseactivity.37 Asakura et al36 reported that total donor CD8 expansion and GVL 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 alloimmune setting. We were unable to dissect whether such cross-dressing contributes to GVHD, as hosts that would have alloimmune MHC class I-peptide complexes to donate to donor APCs would also be able to directly prime alloimmune T cells.

Transfer of membrane and transmembrane proteins from cell to cell has been described in a number of systems.38-46 T cells in contact with targets can acquire target cell-derived MHC in a contact-dependent manner.38,39 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.41,43,45-47 Most relevant to our studies, the transfer of transmembrane proteins can be potentiated with prior freeze/thaw killing of the donor cells.42 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.
Acknowledgments

The authors thank the Yale Animal Resources Center for expert animal care.

This work was supported by the National Institutes of Health (HL-083072 and AI064343). W.D.S. is a recipient of a Clinical Scholar award from the Leukemia & Lymphoma Society.

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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for X.W. is Institute of Immunology, Zhejiang University, Hangzhou, People’s Republic of China.

Correspondence: Warren D. Shlomchik, Yale University School of Medicine, Cancer Center and Departments of Medicine and Immunobiology, PO Box 20832, New Haven, CT 06520; e-mail: warren.shlomchik@yale.edu.

References

14. Matte-Martone C, Liu J, Jain D, McNiff J, Shlomchik WD. CD8+ but not CD4+ T cells require cognate interactions with target tissues to mediate GVHD across only minor H antigens, whereas both CD4+ and CD8+ T cells require direct leukemic contact to mediate GVL. Blood. 2008;111(7):3884-3892.


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

Xiaoqian Wang, Hongmei Li, Catherine Matte-Martone, Weiguo Cui, Ning Li, Hung Sheng Tan, Derry Roopenian and Warren D. Shlomchik