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Cross-Dressing by Donor Dendritic Cells after Allogeneic Bone Marrow Transplantation Contributes to Formation of the Immunological Synapse and Maximizes Responses to Indirectly Presented Antigen

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The stimulation of naive donor T cells by recipient alloantigen is central to the pathogenesis of graft-versus-host disease after bone marrow transplantation (BMT). Using mouse models of transplantation, we have observed that donor cells become “cross-dressed” in very high levels of recipient hematopoietic cell–derived MHC class I and II molecules following BMT. Recipient-type MHC is transiently present on donor dendritic cells (DCs) after BMT in the setting of myeloablative conditioning but is persistent after nonmyeloablative conditioning, in which recipient hematopoietic cells remain in high numbers. Despite the high level of recipient-derived alloantigen present on the surface of donor DCs, donor T cell proliferative responses are generated only in response to processed recipient alloantigen presented via the indirect pathway and not in response to cross-dressed MHC. Assays in which exogenous peptide is added to cross-dressed MHC in the presence of naive TCR transgenic T cells specific to the MHC class II–peptide combination confirm that cross-dressed APC cannot induce T cell proliferation in isolation. Despite failure to induce T cell proliferation, cross-dressing by donor DCs contributes to generation of the immunological synapse between DCs and CD4 T cells, and this is required for maximal responses induced by classical indirectly presented alloantigen. We conclude that the process of cross-dressing by donor DCs serves as an efficient alternative pathway for the acquisition of recipient alloantigen and that once acquired, this cross-dressed MHC can assist in immune synapse formation prior to the induction of full T cell proliferative responses by concurrent indirect Ag presentation. The Journal of Immunology, 2014, 192: 5426–5433.

Naive donor T cell responses to host alloantigen are critical for driving graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (BMT) (1). GVHD remains one of the key complications of BMT as therapy for hematological malignancy, and despite advances in the field, further insights into disease pathogenesis remain important for design of therapeutic strategies. Donor alloreactive T cells may be stimulated by direct interaction with host-type MHC from either hematopoietic or nonhematopoietic sources (2, 3) or by donor cells presenting processed, acquired Ag via the indirect pathway (2, 4–6). In addition to these two classical pathways, we have been interested in the semidirect pathway of Ag presentation, whereby a donor APC which has acquired MHC of host-type, via “trogocytosis” or “cross-dressing” could potentially stimulate antithost responses from donor T cells (7–9). This phenomenon has been studied with respect to CD8 T cell responses, and data suggest that both naive and memory CD8 T cells can respond to trogocytosed Ag using either proliferation or cytokine production as the measure of functional response (10–13).

Attempts have been made to assess the function of dendritic cells (DCs) bearing acquired MHC II in stimulating CD4 T cell responses (14); however, these studies have been performed using DC populations capable of both indirect and semidirect presentation, making the data difficult to interpret. Dolan and colleagues (15) have demonstrated a low-level of IL-2 production from CD4 T cells in response to cross-dressed Ag derived from a DC-based “tumor vaccine.” The phenomenon of cross-dressing has been observed in mouse models of solid-organ transplantation (16), but its role in alloreactivity has not been assessed. Importantly, CD4 T cell responses to MHC molecules acquired by “cross-dressed” DCs have not been assessed in the BMT setting, and thus, here we investigate the function of host-type allogeneic MHC acquired by donor DCs with respect to its capacity to prime donor T cell responses and contribute to posttransplant alloreactivity.
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I-Ab (AF6-120.1), and Alexa Fluor 647–conjugated anti–I-Ad (39-10-8).

B6.TEa Tg (17) mice, and B6.Rag.

stained as described and fixed in 4% paraformaldehyde prior to the collection.

sort purified on the basis of GFP expression on day 7 post transplant and

Medical Research Institute.

Amnis, EMD Millipore, Seattle, WA), B6.CD11c.GCDL or B6.CD11c.DTR

(BD Biosciences) or MoFlo (Beckman Coulter) instrument. For initial

Fortessa (BD Biosciences). Cell sorting was performed on a FACSAria

and isotype control were purchased from BD Biosciences (San Diego,

Mice were transplanted [total body irradiation (TBI)] on day 0, as previously
described (4, 6). B6.D2F1, B6, and BALB/c background animals received
1100 cGy, 1000 cGy, and 900 cGy total doses, respectively. In the
nonirradiated B6 to B6D2F1 experiments, recipient animals were treated on
day −2 with 1 mg α-NK1.1 (PK136) and 0.5 mg twice weekly until day 21.
Mice were scored weekly according to clinical parameters originally de-
dscribed elsewhere (18). Chimeric mice were generated as previously de-
scribed (2) by transplanting T cell-depleted (TCD) bone marrow into lethally irradiated recipients and allowing hematopoietic reconstitution to occur over a period of 3–5 mo prior to using these animals as transplant recipients.
line with the negative control (Fig. 1D). As expected, given that the I-\text{A}\(^d\) was ubiquitously available (i.e., from both the hematopoietic and the nonhematopoietic compartments) in all recipients, cross-dressing with this molecule was similar in all conditions (Fig. 1D).

Given the hematopoietic source of Ag, we hypothesized that cross-dressing would decrease over time as the supply of recipient hematopoietic cells diminished. Indeed, we observed cross-dressing at high levels early after transplantation (Fig. 2A) and when recipients were conditioned with TBI; cross-dressing with both recipient class I and II molecules was observed for only 1 mo after BMT. Perhaps not surprisingly, in light of the source of acquired Ag, donor DC cross-dressing was significantly enhanced in the absence of myeloablative conditioning (Fig. 2B; MHC class II data shown) and was correlated with prolonged presence of recipient hematopoietic, MHC class II–deficient (B6.I-Ab\(^{-/-}\)) T cells. The cross-dressed DCs bearing their native H-2Db and I-Ab, as well as trogocytosed H-2D\(^d\) and I-\text{A}\(^d\) (levels of cross-dressed I-\text{A}\(^d\) shown in Fig. 2C), effectively stimulated third-party allogeneic H-2D\(^q\) T cells but failed to stimulate H-2Db CD3\(^+\) T cells despite the presence of eGFP expression. As shown in Fig. 3A, recipient-derived I-\text{A}\(^d\) remained on the surface of donor DCs for \(\geq 6\) h after purification, with no appreciable internalization (quantified in Fig. 3B).

To study the functional ramifications of recipient-derived MHC class II (I-\text{A}\(^d\)) on the surface of donor B6 (I-Ab) DCs, donor DCs were sort purified from transplant recipients and used to stimulate syngeneic B6 or allogeneic DBA/1 CD3\(^+\) T cells. The cross-dressed DCs bearing their native H-2Db and I-Ab, as well as trogocytosed H-2D\(^d\) and I-\text{A}\(^d\) (levels of cross-dressed I-\text{A}\(^d\) shown in upper panel, Fig. 3C), effectively stimulated third-party allogeneic H-2D\(^q\) T cells but failed to stimulate H-2Db CD3\(^+\) T cells despite the allogross-self H-2D\(^d\)/I-\text{A}\(^d\) molecules present on the DC surface (Fig. 3C, lower panel).

To test the functional significance of acquired recipient MHC II by donor DCs in vivo, primary syngeneic transplants were performed to generate DCs that were WT and had high levels of native MHC II (as well as acquired MHC), were MHC II\(^{-/-}\) but were cross dressed with B6-derived MHC II, or bore no MHC class II at all. Recipients of these primary grafts were treated with Flt3-L to expand DCs, and thus the resultant CD11c\(^+\) DC population is predominantly CD8\(^+\) (phenotype shown in Fig. 3Dii). To measure the in vivo capacity of these donor DCs to stimulate an allogeneic T cell response, DCs were transferred into irradiated syngeneic, MHC II–deficient (B6.I-Ab\(^{-/-}\)) recipients with naive allogeneic (BALB/c) responder T cells. Despite clear cross-dressing with I-\text{A}\(^d\) within the adoptively transferred DC population (Fig. 3Diii), the DCs failed to initiate a response in the allogeneic BALB/c CD4 T cells (Fig. 3E, 3F).
We next asked whether the process of cross-dressing functioned to regulate T cell responses to alloantigen rather than induce proliferation. To assess this, we performed suppression assays using polyclonal BALB/c CD4+ T cells cocultured with stimulating allogeneic naive B6 DCs in a 10:1 ratio, and added posttransplant DCs that bore native and cross-dressed B6 MHC, cross-dressed B6-derived MHC alone, or no B6-derived MHC II. As shown in Fig. 3, purified posttransplant DCs that bore processed and cross-dressed MHC acted in an additive fashion to promote T cell proliferation (i.e., could improve overall Ag presentation to the BALB/c T cell in a dose-dependent manner), but the DCs with solely cross-dressed MHC neither augmented nor regulated T cell responses. This finding suggests that cross-dressed MHC is not acting as a regulator of CD4 T cell responses.

Having failed to observe a role for cross-dressed Ag in either the promotion or the regulation of responses within a polyclonal CD4 T cell pool, we next sought to directly compare the potency of classical indirect presentation of exogenous recipient Ag within MHC class II with that acquired by cross-dressing using naive CD4 Tg reporter T cells specific for alloantigen. In this model, the I-Ab\textsuperscript{b−/−} I-E\textalpha MHC:peptide (YAe) complex recognized by the TEa TCR Tg T cell is transferred from B6D2F1 recipients to B6.MHC II–deficient (I-Ab\textsuperscript{−/−}) donor DCs. TEa T cells respond to this Ag–MHC complex when generated by classical indirect Ag processing and presentation within donor I-Ab\textsuperscript{b−/−} but could theoretically respond to the acquired cross-dressed YAe complex without any requirement for Ag processing by the donor DCs (Table I). Control DCs were generated by transplanting B6.1-Ab\textsuperscript{b−/−} donor BM into B6.1-Ab\textsuperscript{b−/−} xD2F1 recipients, and used to stimulate Tg TEa T cells. Even at very high T cell/DC ratios (1:1), cross-dressed donor DCs failed to initiate a response in naive allospecific TEa T cells. In comparison, donor DCs capable of classical indirect Ag presentation potently initiated T cell proliferative responses (Fig. 4B). Interestingly, donor DCs that could present only indirect Ag were less potent stimulators of TEa responder T cells than were their counterparts, in which indirect Ag presentation occurred in the presence of cross-dressed MHC on the same DC.

To assess whether the responder CD4 Tg T cells were simply anergic in response to cross-dressed DCs, we supplemented cultures with IL-2 (Fig. 4C). In the positive control setting, in which indirect Ag presentation was taking place, IL-2 supplementation did increase proliferation, but this did not occur in the cultures with cross-dressed DCs, consistent with a lack of stimulation by these DCs, rather than induction of anergy in the responding T cell. To further assess whether cross-dressed MHCs could contribute to the stimulation of T cell proliferation, we added exogenous peptide to the MLC, such that if any functional MHC were present, saturating levels of the exogenous peptide would allow maximal T cell responses without any requirement for intracellular handling of that peptide Ag. Even in these circumstances, with saturating levels of YAe peptide added to the cultures, cross-dressed DCs (dark gray bars) could not induce TEa T cell responses above that seen in the complete absence of the appropriate MHC (black bars) (Fig. 4D), thus confirming that
induction of proliferation is not a functional consequence of cross-dressed foreign MHC class II.

In the primary DC-generating experiments using MHC II–deficient donor BM, we consistently observe lower levels of recipient MHC acquisition than when donor DC are WT (Fig. 4A compared with initial data in Figs. 1A, 2A, 2B). To exclude the possibility that these lower levels of cross-dressing were contributing to the negative results generated, we performed experiments in an additional model, this time using BALB/c donors and B6 recipients as the test group (donor DCs are native BALB/c but bear acquired B6 MHC), alongside B6 → B6 and BALB/c → BALB/c posttransplant DC controls, as well as naive BALB/c, B6, and B6D2F1 DCs. As shown in Fig. 4E, a high level of acquired I-Ab was present on the surface of donor BALB/c DCs; however, even with the addition of exogenous I-E peptide to the MLC, these DCs could not elicit a T cell response above background levels (Fig. 4F).

Given the high levels of cross-dressing seen on donor DCs, and the lower levels of proliferation seen in the experiments in which cross-dressing was absent but indirect presentation was intact (Fig. 4B), we next asked whether this cross-dressed Ag could be used to promote T cell sampling of DC-borne indirect Ag. The formation of an immunological synapse, which requires the dynamic reorganization of the actin cytoskeleton, is a key component of T cell activation (21). It has been proposed that immunological synapse formation may act as a cellular “checkpoint” for full activation, allowing a T cell to assess

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<th>Donor host combinations and resultant mechanism of Ag presentation</th>
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<td><strong>Donor</strong></td>
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<tr>
<td>B6.I-A&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>B6.I-A&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;−/−&lt;/sup&gt;</td>
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in an additional model, this time using BALB/c donors and B6 recipients as the test group (donor DCs are native BALB/c but bear acquired B6 MHC), alongside B6 → B6 and BALB/c → BALB/c posttransplant DC controls, as well as naive BALB/c, B6, and B6D2F1 DCs. As shown in Fig. 4E, a high level of acquired I-A<sup>b</sup> was present on the surface of donor BALB/c DCs; however, even with the addition of exogenous I-E peptide to the MLC, these DCs could not elicit a T Ea T cell response above background levels (Fig. 4F).
the quality and presence of appropriate MHC:peptide complexes on the APC (22). Therefore, to examine whether cross-dressed MHC could contribute to immunological synapse formation, quantitative imaging flow cytometry was used to measure the intensity of phalloidin staining for polymerized/F-actin (f-actin) at the intracellular junction, which is an anatomical marker of the synapse (23). Post-transplant DCs that bore processed YaE complexes or were cross-dressed with YaE complexes were cocultured with naive TEa Tg T cells (Fig. 5A). We observed that the formation of an immunological synapse occurred only when DCs were either cross-dressed in recipient alloantigen or bore both cross-dressed and processed YaE complexes (Fig. 5B). The intensity of f-actin in both groups in which cross-dressing was present was greater than that seen when DCs presented alloantigen by standard indirect means only and were not cross-dressed in addition (Fig. 5B). To further assess the nature of the immunological synapse, LFA-1 was measured at the T cell side of the T:DC interface (Fig. 5C). LFA-1 also aggregated at the site of immune synapses between cross-dressed DCs, DCs bearing cross-dressed and processed Ag, and DCs possessing solely processed Ag. Thus, whereas processed Ag is required for the induction of T cell proliferation, cross-dressed MHC plays an important role in the formation of an immune synapse between donor DCs and T cells after transplantation.

Discussion

We have characterized the phenomenon of donor DC cross-dressing in host-derived allogeneic MHC and examined the functional capacity of these cross-dressed DCs. We have observed the colocalization of recipient type class I and II MHC on donor DCs and we hypothesize that discrete fragments of host cell membrane are the source of the observed trogocytosed host MHC. This may be acquired via cell-to-cell contact or via donor cell contact with the debris generated by dying recipient cells. After identifying recipient hematopoietic cells as the origin of cross-dressed allogeneic MHC, we confirmed that the cross-dressed DCs are maintained in the long term when recipient-derived hematopoietic cells remain present, as they do in the clinical setting in which reduced-intensity conditioning regimens are used. This observation highlights the potential in vivo importance of cross-dressing as a mechanism for supporting DC/T cell contact.
Our results in these MHC class II–dependent assays contrast somewhat with studies in CD8 T cell–based systems wherein cross-dressed Ag appeared sufficient for the induction of proliferative responses (24). Purified cross-dressed DCs bearing MHC class I:SIINFEKL complexes acquired from recipient mice in vivo after BMT could induce low levels of proliferation in naive CD8+ OT-I T cells in vitro, although responses were surprisingly minimal in the positive controls in these studies (which were DCs from nontransplanted B6 animals in which OVA was secreted under control of the β-actin promoter) (24). Furthermore, studies outside the BMT setting have explored the role of cross-dressing in T cell priming and confirm that viral Ag displayed to T cells in this manner contribute only to memory, but not naive CD8, T cell responses (11). An additional study by Smyth et al. (25) has demonstrated that naive Ag-specific (OT-1) CD8 T cell responses can also be generated in response to cross-dressed viral Ag.

The results demonstrating immunological synapse formation between cross-dressed DCs and alloantigen-specific Tg T cells initially appear somewhat paradoxical in light of the functional assays proving that these DCs do not stimulate a proliferative T cell response. For an immunological synapse to develop, binding must occur between adhesion molecules—for example, LFA-1 on the T cell and ICAM-1 on the APC, CD2/CD58, or DC-SIGN/ICAM-3—as these low-affinity transient interactions serve to bring opposing TCR and MHC into close enough proximity to interact (26). The mature immunological synapse is a highly organized structure that contains three domains, known as the central, peripheral, and distal supramolecular activation complexes, which facilitate complete T cell activation if appropriate TCR ligation and costimulatory signals are received (27, 28). The duration of TCR:MHC contact required for full T cell activation is 2–12 h, and in our systems this activation clearly occurs in response to posttransplant DCs displaying native MHC bearing processed peptide, but not in response to MHC acquired by cross-dressing, despite equivalent capacity to form a transient immunological synapse, as measured by f-actin sequestration at the T cell/DC interface.

It has been proposed that CD4 T cells (in contrast to CD8 T cells and NK cells) are able to form an immunological synapse with DCs in an Ag-independent manner, although, consistent with our data, no evidence exists that this alone can lead to robust proliferative responses (29). The results of our study confirm that an immunological synapse can develop in a setting in which T cell proliferation is subsequently absent. This may be due to lack of appropriate intracellular domains or appropriately localized costimulatory or adhesion molecules within the acquired recipient membrane.

Throughout this study we have aimed to delineate the specific role for cross-dressed MHC on donor DCs, with the broader aim of ascertaining the role of this form of Ag presentation in driving GVHD. We have therefore created experimental systems that rely on the use of an MHC II–deficient DC carrying only MHC acquired from recipient cells via cross-dressing, which is obviously a cell that does not exist after BMT, when donor cells are MHC replete. In vivo, following BMT, donor cells bear both native MHC loaded with exogenous peptide and cross-dressed MHC, and it is likely that the cross-dressed MHC facilitates optimal immunological synapse formation that promotes an efficient engage-
The authors have no financial conflicts of interest.

Disclosures
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