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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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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 antihost 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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K.A.M. wrote the manuscript and designed and performed experiments; M.K. and K.H.G. provided key expertise and assistance with experimental design and edited the manuscript; L.L. provided expertise required for the Amnis Image Stream experiments; R.D.K., K.E.L., and B.E.T. assisted with experimental work, animal breeding, and monitoring; K.P.A.M. performed experiments, assisted with experimental design, and contributed to the manuscript; and G.R.H. assisted with experimental design and cowrote the manuscript.

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Abbreviations used in this article: BM, bone marrow; BMT, BM transplantation; DC, dendritic cell; DTR, diphtheria toxin receptor; eGFP, enhanced GFP; Flt3-L, Flt-3 ligand; GVHD, graft-versus-host disease; TBI, total body irradiation; Tg, transgenic; WT, wild-type.

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The incidental observation that donor APCs acquire host MHC after BMT invoked our interest in the phenomenon, and we subsequently sought to formally assess whether this represented an alternate mechanism for these APCs to stimulate donor CD4 T cell responses. We have used various primary transplant systems to generate DC populations that exclusively bear cross-dressed recipient MHC class II, bear cross-dressed MHC II and processed recipient peptide within native MHC II, or are completely devoid of MHC class II. We have then sort-purified DCs from these primary transplants and tested their capacity to stimulate both polyclonal and Ag-specific CD4 T cells, both in vitro and in vivo. We conclude that widespread acquisition of recipient MHC by donor cells (via cross-dressing) after interaction with hematopoietic recipient cells is important for the formation of the immunological synapse, but not, in isolation, for the induction of proliferation. The immune synapse formation, which appears to be augmented by cross-dressed MHC, precedes the induction of full T cell proliferative responses by adjacent MHC:peptide complexes generated by the classical indirect processing pathway.

Materials and Methods

Mice

Female C57BL/6 (B6.WT, H-2D b), B6.Ptprca (B6.Ptprca, H-2Db), and BALB/c (H-2Dd) mice were purchased from the Animal Resource Centre (WA, Australia). The following donor mice (on a B6 background) were bred and housed at QIMR Berghofer Medical Research Institute: B6.MHC II-deficient (B6.H-2Ab/2, used hereafter as I-Ab g/g for clarity) mice, B6.CD11c.DTR, CD45.2 transgenic (Tg) mice [in which the diphtheria toxin receptor (DTR) and enhanced GFP (eGFP) are driven off the CD11c promoter], B6.CD11c.GCDL or B6.CD11c.DTR→B6.CD11c.GCDL (cross-transplanting DCs were generated using the following primary transplants: B6, CD45.1→B6.D2F1 (donor DCs are CD45.1, positive control), I-A b/→I-A b g/B6.dBA/2F1 (negative control), I-A b/→I-A b g/B6.dBA/2F1 (cross-transplanting test group), or B6.CD45.1→I-A b/→I-A b g/B6.dBA/2F1 (donor DCs are CD45.1, processed Ag only test group)]. These cells were sort purified and plated in assays, as described, with TeA Tg T cells (purified on the basis of TCR expression Vα2Vβ6 ± IL-2 (20 U/ml) ± exogenous I-Eα peptide at 10 ng/ml (Na 52–68; sequence: ASFEEAGALANIAVDKA; Mimotopes, Australia). For immunological synapse assays, DCs were cocultured with CFSE-labeled TeA T cells for 2 h. Following coculture, cells were fixed in 1% paraformaldehyde, treated with 0.1% Triton-X to permeabilize (Sigma-Aldrich, St Louis, MO), and stained for f-actin with phalloidin AF647 (Invitrogen, Carlsbad, CA), prior to collection on the Amnis Image Stream. Analysis was performed using the IDEAS software package (Amnis, EMD Millipore).

Statistical analysis

Data are shown as mean ± SEM. SEM was shown, as all the data presented are reflective of both biological and experimental variation. Statistical significance was determined using two-tailed Mann–Whitney U tests because data sets were generally small (n < 10) and a normal distribution cannot be assumed. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). The p values were as follows: *p = 0.05–0.01, **p = 0.01–0.001, ***p < 0.001, ****p < 0.0001.

Results

Using GFP as a definitive intracellular donor marker, we were able to identify the donor DC population and observe the level of surface host MHC using flow cytometry. As shown in Fig. 1A, high levels of cross-dressing with host MHC class I (H-2Db), class II (I-A b), and the recipient congenic marker CD45.1 were observed after surface staining donor DCs with Abs directed against these markers. This phenomenon was seen following both allogeneic (B6,CD11c DTR→CD45.1 expressing B6.D2F1) and syngeneic (B6,CD11c DTR→CD45.1 expressing B6) BMT. Using quantitative imaging flow cytometry, we analyzed GFP-expressing donor DCs and confirmed that recipient MHC class I and II were colocalized (Fig. 1B), as evidenced by the increase in “bright detail similarity” between class I and II compared with class I and nonspecific isotype control (Fig. 1C).

We next sought to formally identify the origin of host MHC for cross-dressing using MHC II-deficient B6 donors and I-A b-deficient or intact BM chimera recipients such that Ag could be acquired from both hematopoietic and nonhematopoietic sources [positive control: wild-type (WT) B6.D2F1→WT B6.D2F1, from either in isolation (B6-I-A b/→I-A b/→D2F1→WT B6.D2F1; WT B6.D2F1→B6-I-A b/→I-A b/→D2F1) or not at all (negative control: B6-I-A b/→I-A b/→D2F1→B6-I-A b/→I-A b/→D2F1). In the setting in which I-A b was available only from host hematopoietic cells, the level of cross-dressing was similar to that seen in the positive control setting, in which I-A b could be acquired from any source. In contrast, when chimeric recipients bore only I-A b on nonhematopoietic cells, a low level of cross-dressing was observed, in
line with the negative control (Fig. 1D). As expected, given that the I-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\(^{+/-}\)) donor grafts. Cross-dressed DCs bearing their native H-2D\(^b\) and I-Ab, as well as trogocytosed H-2D\(^b\) and I-A\(^d\) (levels of cross-dressed I-A\(^d\) shown in Fig. 3D), effectively stimulated third-party allogeneic H-2D\(^q\) T cells but failed to stimulate H-2Db CD3\(^+\) T cells despite the fact that trogocytosed donor DCs invivo, primary syngeneic transplants were performed without reversion to their native H-2Db and I-A\(^d\) molecules present on the DC surface (Fig. 3D, 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–deficient (B6.I-Ab\(^{+/-}\)) recipients with naive allogeneic B6 or allogeneic DBA/1 CD3\(^+\) T cells. In these cross-dressed DCs bearing their native H-2Db and I-A\(^d\), as well as trogocytosed H-2D\(^b\) and I-A\(^d\) (levels of cross-dressed I-A\(^d\) shown in upper panel, Fig. 3C), effectively stimulated third-party allogeneic H-2D\(^b\) T cells but failed to stimulate H-2Db CD3\(^+\) T cells despite the allografted H-2D\(^b\)/I-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–deficient (B6.I-Ab\(^{+/-}\)) recipients with naive allogeneic (BALB/c) responder T cells. Despite clear cross-dressing with I-A\(^d\) within the adoptively transferred DC population (Fig. 3Dii), the DCs failed to initiate a response in the allogeneic BALB/c CD4 T cells (Fig. 3E, 3F).
markers. Representative FACS plots shown from three experiments; expressing BALB/c grafts were tracked until day 65 post transplant. I-Ab histograms shown are gated on donor CD45.1 and I-Ad as definitive donor B6.WT donor BM into B6.I-Ab

To assess this, we performed suppression assays using polyclonal DCs (Table I). Control DCs were generated by transplanting B6.I-Ad donor BM into nonirradiated BALB/c mice. Control I-Ad levels shown in Fig. 3G, purified 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. 3G, 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-A\(^{b}\)I-E\(\alpha\) MHC:peptide (YAe) complex recognized by the TEa TCR Tg T cell is transferred from B6D2F1 recipients to B6.I-Ab donor DCs. TEa T cells respond to this Ag–MHC complex when generated by classical indirect Ag processing and presentation within donor I-Ad 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.I-Ad donor BM into B6.I-Ad xD2F1 recipients, and B6.WT donor BM into B6.I-Ad xD2F1 such that only indirect Ag presentation could take place (i.e., by the WT donor phagocytosing and processing recipient-derived I-E\(\alpha\) molecules into the appropriate peptide for presentation).

Cross-dressed donor DCs, which lack the ability to present Ag by the classical MHC class II pathway (I-A\(^{b}\)I-E\(\alpha\)), were purified by FACS after BMT (level of cross-dressing with I-A\(^{b}\) shown in Fig. 4A [dark gray], compared with native MHC [light gray representing B6 \(\rightarrow\) B6D2F1 DC, dotted line representing B6 \(\rightarrow\) B6. I-A\(^{b}\)-/I-Ad xD2F1 DC, control I-A\(^{b}\) levels shown in lower panel]) 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
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 cross-dressed 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 alloantigen, 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 OV A was secreted under control of the $\beta$-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-
ment of TCRs with processed Ag. Important evidence for this is provided by the experiments performed in which donor DCs can present indirect Ag but do not bear cross-dressed Ag, a situation in which significantly lower levels of proliferation are seen compared with intact DC capable of using both pathways. Prior studies within our group (2, 6) have demonstrated that host hematopoietic cells are the source of allo-MHC for the formation of an immunological synapse, which appears to be required for maximal proliferative responses. We have demonstrated that host hematopoietic cells are the source of allo-MHC for cross-dressing, and in the clinical setting of lower intensity conditioning regimens whereby host-type MHC-bearing cells are likely to be maintained long term, donor DCs are likely to remain cross-dressed for a prolonged period. Thus the process of “semidirect” Ag presentation represents an important pathway for the acquisition of recipient Ag, and although it has minimal capacity to invoke alloreactive T cell proliferation in isolation, it contributes to formation of an immune synapse after allogeneic BMT and is required for maximal responses to indirecly presented allogene.

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