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Novel MHC Class I Structures on Exosomes

Sarah Lynch,* Susana G. Santos,* Elaine C. Campbell,* Ailish M. S. Nimmo,* Catherine Botting,† Alan Prescott,‡ Antony N. Antoniou,§ and Simon J. Powis2*

Exosomes are nanometer-sized vesicles released by a number of cell types including those of the immune system, and often contain numerous immune recognition molecules including MHC molecules. We demonstrate in this study that exosomes can display a significant proportion of their MHC class I (MHC I) content in the form of disulfide-linked MHC I dimers. These MHC I dimers can be detected after release from various cell lines, human monocyte-derived dendritic cells, and can also be found in human plasma. Exosome-associated dimers exhibit novel characteristics which include 1) being composed of folded MHC I, as detected by conformational-dependent Abs, and 2) dimers forming between two different MHC I alleles. We show that dimer formation is mediated through cysteine residues located in the cytoplasmic tail domains of many MHC I molecules, and is associated with a low level of glutathione in exosomes when compared with whole cell lysates. We propose these exosomal MHC I dimers as novel structures for recognition by immune receptors. The Journal of Immunology, 2009, 183: 1884–1891.

Exosomes are small vesicles in the size range 50–150 nm that form by the inward budding of endosomes to generate multivesicular bodies (MVBs) (1–4). A proportion of these MVBs can fuse with the plasma membrane, releasing their internal vesicles to the extracellular environment. A wide variety of cell types has been reported to secrete exosomes, including reticulocytes (5), neurons (6), epithelial cells (7), lymphocytes (8), mast cells (9), and dendritic cells (DC) (10).

Of particular interest, exosomes from immune cell types are replete with many molecules involved in ligand-receptor interactions that can lead to the activation or modulation of various immune responses. For example, peptide-pulsed exosomes from DC can induce tumor rejection in mice (10), while exosomes generated from DC incubated with the immunoregulatory cytokine IL-10 can down-regulate disease activity in a collagen-induced murine model of arthritis (1). This ability of exosomes to act as “cell-free” vaccines has already been tested in phase I clinical trials against melanoma, small-cell lung cancer, and colorectal cancer (11–13).

MHC class I (MHC I) molecules function by binding short endogenous peptides during their assembly in the endoplasmic reticulum (ER) and presenting them to CD8+ T cells, thus efficiently allowing the detection and elimination of virally infected or potentially tumorgenic cells (14). MHC I molecules are composed of an MHC-encoded H chain of ≈43 kDa, a 12 kDa non-MHC encoded β2-microglobulin L chain (β2m), and an 8–9 residue peptide. The recognition of MHC I molecules by the TCR system involves inclusion of the MHC I molecule within immune synapses, with clustering increasing the sensitivity of detection (15, 16).

In addition to being recognized by CD8+ T cells, which is highly peptide- and MHC I-allele specific, it is now known that many MHC I and MHC I-like molecules are also recognized by receptors often found expressed on cells of the NK lineage, including killer cell Ig-like receptors (KIRs) and leukocyte Ig-like receptors (LIRs) (17).

While studying the ankylosing spondylitis (AS)-associated HLA-B27 class I allele in an EBV-transformed B cell line, we discovered that exosomes contained enhanced amounts of MHC I dimer structures. HLA-B27 H chain homodimers have been implicated in the pathogenesis of AS (18–20). However, rather than the partially misfolded HLA-B27 H chain homodimers implicated in AS, we found the exosome-associated HLA-B27 dimers to be fully folded. We demonstrate that cysteine residues located in the cytoplasmic tail domain participate in the formation of these structures. Taken together, these data demonstrate the existence of dimeric MHC I structures on exosomes that may act as a novel ligand for immune receptors.

Materials and Methods

Cell lines and Abs

The human EBV-transformed B cell line Jeshom was obtained from Adam Benham (University of Durham, Durham, U.K.) and Health Protection Agency Culture Collections (88052004), and cultured in RPMI 1640 (Invitrogen) with 20% FBS (Invitrogen). KG-1 cells were obtained from Health Protection Agency Culture Collections (86113406) and cultured in IMDM (Invitrogen) with 20% FBS. KG-1 cells were cultured with 10 ng/ml PMA and 100 ng/ml ionomycin where required. Rat C58 thymoma cells were cultured in RPMI 1640 with 5% FBS. HLA class I negative .221 cells were a gift from Salim Khakoo (Imperial College, London, U.K.) and were cultured in RPMI 1640 with 5% FBS. Human monocyte-derived DCs were generated in DMEM plus 10% FBS from a 20 ml blood donation (obtained after ethical review by the Bute Medical School ethics committee), expanding the 2 h plastic-adherent population in GM-CSF and IL-4 for 4–5 days, followed by stimulation with 100 ng/ml LPS (Sigma-Aldrich) for the indicated times. Abs used were: HC10, recognizing unfolded HLA-B and -C (21); HCA2, recognizing unfolded HLA-A (22); ME1, recognizing folded HLA-B27 (23); BB7.2, recognizing folded HLA-A2 (24); W6/32, recognizing folded HLA-A, -B, and -C (25); V5,
recognizing the V5 epitope tag (26); anti-transferrin receptor (TR), recognizing transferrin receptor; anti-ERp57 (gifted by N. Bulleid, University of Manchester, Manchester U.K.); anti-CD48; anti-transporter associated with Ag processing (TAPI) (gifted by R. Tampé, Frankfurt, Germany); anti-erzin and moesin (gifted by F. Gunn-Moore, University of St. Andrews, St Andrews, U.K.); anti-HLA-B27–FITC (One Lambda); anti-Tsg101 (Santa Cruz Biotechnology); anti-ALIX (Santa Cruz Biotechnology).

Isolation of exosomes

Exosomes were isolated based on published procedures (10, 27). In brief, cells were incubated overnight in either serum-free medium or medium containing 5% FBS or 2.5% exosome-free FBS (pre-spin at 100,000 χ g). The exosomal MHC I dimer signal, determined by immunoblotting with HC10, was identical in all three cultures (data not shown). Culture supernatants were then depleted of cells (1000 χ g, 10 min), alkylated with 10 mM N-ethylmaleimide (NEM), and either filtered or ultracentrifuged at 100,000 χ g for 2 h. Exosomes destined for incubation with live cells were not subjected to NEM treatment. In all other cases, exosome pellets were resuspended in a small volume of PBS with 10 mM NEM, and quantified by spectrophotometry or Bradford assay. Typical yields for Jesthom cells were around 1 μg of exosomes per one million cells cultured overnight. Lower yields were obtained for all other lines used in this study.

Microscopy and mass spectrometry

For electron microscopy, exosomes were placed on a carbon and pifoilomacoated grid, blotted dry, and stained with 3% uranyl acetate in water. Alternatively, exosomes were diluted in water and collected on a carbon film on mica and again stained with uranyl acetate; the film was collected on an uncoated grid. Imaging was performed on an FEI Tecnai 12 TEM (FEI). For immunofluorescence microscopy, cells were fixed in 2% formaldehyde in PBS for 20 min, then blocked and permeabilized with 1% BSA, 0.2% saponin in staining buffer. Cells were stained with FITC-conjugated anti-B27. After extensive washing, cells were resuspended in PBS and mounted on coverslides with Vectashield containing Dapi (Vector Laboratories). Deconvolution microscopy was done using a wide-field optical sectioning microscope, DeltaVision Restoration Imaging System (Applied Precision). For each cell, a z-series of 15 to 45 images at 0.35 μm intervals was captured and processed using homemade deconvolution algorithms (MatLab). Protein identification was performed using the Mascot 2.1 search engine (Matrix Science) against MSDB database. The data was searched with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification. The Mascot search results were accepted if a protein hit included at least one peptide with a score above the homology threshold and the MS/MS interpretation accounted for the major peaks in the spectrum.

Immunoblotting and immunoprecipitation

Cell lysates were prepared by resuspending cells in lysis buffer (1% NP40, 150 mM NaCl, 10 mM Tris (pH 7.3), 1 mM PMSF, 10 mM NEM) for 10 min on ice, then spinning at 20,000 χ g for 10 min. Lysates and exosomes were resuspended in nonreducing or reducing sample buffer as required, heated at 90°C for 3 min, run on 8% SDS-PAGE gels, and transferred to nitrocellulose membranes (BA85, Whatman). Membranes were incubated with the indicated Abs and relevant HRP-coupled second stage reagents and visualized with Femto Western (Perbio) chemiluminescence reagents and a Fuji LAS3000 image analyzer. Immunoprecipitations were performed using cell lysates or exosome lysates (buffer as above), using the indicated Abs and protein G-Sepharose beads (Sigma-Aldrich). Beads were washed in lysis buffer and resuspended in the relevant sample buffer before immunoblotting as above. Two-dimensional gel analysis was performed as previously described (29), using nonreducing sample buffer, followed by immunoblotting as above.

Flow cytometry and glutathione (GSH) assay

Flow cytometry was performed on cells stained with the indicated Abs and FITC-coupled second stage reagents, followed by analysis on a FACScan (Becton Dickinson).

Flow cytometry of exosomes was performed as follows: 30 μg of exosomes in 100 μl PBS were incubated with 20 μl of latex beads (4 μm aldehyde/sulfate, Invitrogen) that had been sonicated for 5 min. The bead/exosome mixture was incubated at room temperature for 15 min, resuspended with 1 ml PBS, and incubated with mixing at 4°C for 1 h. Beads were pelleted at 20,000 χ g for 2 min, then resuspended in 100 mM glycine for 30 min. Beads were washed twice with PBS, resuspended in 0.5 ml PBS, and 50 μl stained with the indicated Abs using standard protocols. Glutathione was measured using the Glutathione Assay Kit (Sigma-Aldrich; CS0260). Samples, quantified by the Bradford method, were deproteinated and total GSH measured in a reaction where GSH causes the reduction of 5,5’-dithiobis(2-nitrobenzoic) acid to the yellow product 5-thio-2-nitrobenzoic acid, which was measured at 380 nm using a FLUO-Ostar Optima plate reader (BMG Labtech). Standard curves were prepared and the amount of GSH in cell lysates and exosome lysates determined. Samples were measured in duplicate.

Mutagenesis

Site-directed mutagenesis was performed using Stratagene Quickchange mutagenesis methodology, using appropriate primers to mutate Cys57 to Ser57, Cys308 to Ala308, and Cys328 to Ala328 in HLA-B*2705 cDNA, or to introduce a stop codon in the cytoplasmic tail domain of HLA-A*0201 cDNA. Mutagenesis was confirmed by sequencing. Mutants, cloned into the pcR3.1 vector (Invitrogen) were transfected into KG-1, 221, or C58 cells by electroporation and selected with 1 mg/ml G418 (Melford). Expression was confirmed by flow cytometry and immunoblotting.

Results

Exosome characterization from Jesthom and KG-1 cells

Exosomes were isolated by either differential centrifugation or by filtration and then centrifugation (with essentially identical results), from the HLA-A2- and -B27-expressing EBV-transformed B cell line Jesthom (30), and the HLA-A30-, -A31-, -B35-expressing DC-like cell line KG-1 (31). Both exosome preparations, when imaged by negative stain electron microscopy, displayed the characteristic exosomal cup-shaped morphology ~100 nm in size (Fig. 1A). Mass spectrometric identification of a number of protein species extracted from a nonreducing SDS-PAGE gel of Jesthom-derived exosomes was also performed, and identified previously reported exosomal species (28) including myosin, TR, annexins 4 and 6, ezrin, moesin, MHC I and II, and CD20 (Fig. 1B). In addition, we identified an MHC I signal in the size region of ~80 kDa, consistent with an MHC I H chain dimer. Furthermore, we also identified the presence of CD48, previously unreported in exosomes, which is a ligand for the NK receptor 2B4 (CD244) (32).

To further characterize Jesthom and KG-1-derived exosomes, we performed immunoblotting of reduced detergent cell lysates compared with exosomes, and flow cytometry of exosomes absorbed to latex beads. The exosome samples did not contain examples of ER-resident species such as TAP1, or the oxidoreductase ERp57, but did contain ezrin, moesin, MHC I, and also ALIX and tsg101, two species highly characteristic of exosomes (Fig. 1C). Flow cytometry confirmed the presence of HLA-A and -B molecules on the extracellular side of Jesthom-derived exosomes using mAbs BB7.2 and ME1, respectively. TR and CD48 were also observed (Fig. 1D). A representative negative control Ab (anti-V5 tag) stained the exosomes only very weakly.

Enhanced MHC I dimer structures on exosomes

As indicated above, the mass spectrometric data indicated the presence of MHC I molecules in a size range consistent with the reported HLA-B27 H chain homodimers implicated in the pathogenesis of ankylosing spondylitis. To further characterize these
putative HLA-B27 dimers on Jesthom-derived exosomes, we immunoblotted nonreduced detergent cell lysates and exosomes with the HLA-B recognizing Ab HC10. Unexpectedly, we observed that the Jesthom-derived exosomes contained a much greater proportion of dimer molecules than the cell lysate, with 50% of the H chain migrating around 80 kDa (Fig. 2A). To confirm this species as an HLA-B27 H chain dimer we examined the sample under reducing conditions, wherein it migrated at the correct monomeric H chain size of 43 kDa (Fig. 2B), indicating it to be a disulfide-linked species. It is also worthy of note that the nonreduced and reduced monomer heavy chains migrate differently in SDS-PAGE, due to the preservation of intrachain disulfide bonds in nonreduced samples, which also affects the apparent migration of the dimer species, again due to preservation of intrachain disulfide bonds. To further determine the composition of these dimeric structures, we examined the sample using nonreducing two-dimensional electrophoresis. H chain dimers should retain an identical isoelectric point (pI) to monomeric H chain, whereas an H chain complexed to another protein would usually alter its overall pI. As shown in Fig. 2C (top), the HLA-B27 signal resolved as monomers and dimers with identical pI, thus indicating them to be authentic HLA-B27 H chain dimers. Additionally, however, we also identified a dimer-sized orphan spot with no corresponding monomer spot (arrowed), which could represent HLA-B27 disulfide linked to another protein. Previous use of this two-dimensional system indicated to us that the other HLA class I allele expressed in Jesthom (HLA-A2) migrated to the left of HLA-B27 (29). We therefore hypothesized that this additional spot may be a disulfide-linked complex of HLA-B27 and HLA-A2 heavy chains. Immunoblotting with the HLA-A recognizing Ab HCA2 (Fig. 2C, middle), which recognizes HLA-A2, also revealed the orphan spot (arrowed) and HLA-A2 dimers. Overlaying separately stained blots of HC10 and HCA2 permitted exact alignment of this spot, and costaining of blots with both Abs also highlighted this same spot (Fig. 2C, bottom). Taken together, the above data strongly suggests that the Jesthom exosomes contain HLA-B27 H chain monomers and dimers, HLA-A2 H chain monomers and dimers, and also a novel population of dimers comprising HLA-A2 and HLA-B27.

**Exosomal MHC I dimers are independent of residue cysteine 67 and are fully folded**

HLA-B27 H chain dimers are disulfide linked through a normally unpaired cysteine residue at position 67 in the peptide groove (18). We therefore tested exosomes from the rat T cell line C58 and

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**FIGURE 1.** Characterization of exosomes from Jesthom and KG-1 cells. A, Electron microscopy of exosomes isolated from culture supernatants of Jesthom (top) and KG-1 (bottom). Size bars 200 nm. B, Mass spectrometric identification of proteins from Jesthom derived exosomes after nonreducing SDS-PAGE. C, Immunoblotting of cell lysates and exosomes from Jesthom and KG-1 cells (nd, not determined). D, Flow cytometric analysis of Jesthom derived exosomes absorbed to latex beads and stained with the indicated Abs. Gray traces are unstained beads. 2° represents second stage FITC anti-mouse IgG alone.

**FIGURE 2.** Detection of MHC I dimers on exosomes. A, Three micrograms of Jesthom cell and exosome lysate was immunoblotted with HC10, revealing enhanced dimer-like structures on exosomes. B, Nonreduced and reduced samples of Jesthom derived exosomes were immunoblotted with HC10. C, Nonreduced Jesthom-derived exosome samples were analyzed by two-dimensional electrophoresis, followed by immunoblotting with HC10 (top), HCA2 (middle), or HC10 and HCA2 mixed together (bottom). Arrows indicate the putative mixed heterodimer of one HLA-A and one HLA-B H chain.
human KG-1 DC-like cells which had been transfected with cDNA constructs for wild-type HLA-B*2705, and a Cys 67-Ser67 mutant (B27.C67S). In both instances, exosomes retained the HLA-B27 H chain dimer signal (Fig. 3A). The previously proposed HLA-B27 H chain dimer structure is thought to involve partial unfolding of the α1 helix, rendering it less well recognized by conformation dependent Abs such as W6/32 and ME1, but more recognisable by HC10 (34). Immunoprecipitation and immunoblotting of Jesthom exosomes lysed in detergent (Fig. 3B) revealed low HC10 signal, but strong signals for both monomers and dimers recognized by the conformation dependent Abs W6/32 (HLA-A, -B, and -C), ME1 (HLA-B, including -B27), and BB7.2 (HLA-A, including HLA-A2). Dimer structures (and monomers) could also be isolated with an anti-β2m specific Ab (data not shown), further confirming the folded nature of the H chain within these structures. We also used immunoprecipitation with the conformation-dependent Ab BB7.2 to confirm the existence of the mixed A2-B27 dimer shown in Fig. 2C. Immunoprecipitation of HLA-A2 from a lysate of Jesthom exosomes was followed by immunoblotting for HLA-B27 with HC10, which revealed a dimer band indicative of a mixed A2-B27 conjugate (Fig. 3D).

Involvement of cytoplasmic domain cysteines and glutathione in exosomal MHC I dimers

If exosomal MHC I dimers are not Cys67 dependent, and are also fully folded, we hypothesized that a disulfide linkage may be forming between unpaired cysteine residues located in the cytoplasmic tail of many MHC I molecules. All HLA-B alleles possess Cys308, which

![FIGURE 3. Exosomal MHC class I dimers are folded MHC class I molecules. A, Exosomes from rat C58 (left) and human KG-1 cells (right), expressing wild-type HLA-B27 or a mutated Cys67 to Ser67 version (C67S) were immunoblotted with HC10. B, Immunoprecipitation of MHC I molecules from a lysate of Jesthom derived exosomes. Samples were immunoblotted with either HC10 or HCA2 as indicated. C, Jesthom exosomes were immunoprecipitated for HLA-A2 (BB7.2) or with an irrelevant control mAb (V5), and immunoblotted for HLA-B27 with HC10. A sample of the input exosome lysate used in the immunoprecipitation is shown (right).](http://www.jimmunol.org/)

![FIGURE 4. A role for cytoplasmic tail domain cysteines and GSH in the formation of exosomal MHC I dimers. A, Sequence of the cytoplasmic tail domain regions of HLA-B27 and HLA-A2, with cysteine residues underlined. B, Immunoblotting of exosomes isolated from the supernatants of Jesthom cells culture with increasing amounts of GSH. C, Similar to B, exosomes were isolated from cells cultured in 50 μM 2-ME. D, Immunoblotting of exosomes isolated from rat C58 cells expressing wild-type HLA-A or a cytoplasmic tail truncation mutant. E, Exosomes isolated from .221 cells expressing HLA-B27 with cysteine to alanine mutations at positions 308 (C308A) or 325 (C325A) were immunoblotted with HC10. F, Total GSH content was measured from 7 μg of Jesthom cell and exosome lysate. Results are representative of two independent experiments.](http://www.jimmunol.org/)
lies on the border of the transmembrane-spanning domain and cytoplasmic domain (Fig. 4A). In addition, some HLA-B alleles possess Cys<sup>325</sup> in the cytoplasmic domain. No HLA-A allele contains Cys<sup>308</sup>, but all have Cys<sup>339</sup> close to the end of the cytoplasmic domain.

The main cellular thiol responsible for maintaining the reducing environment of the cytoplasm is GSH, the intracellular concentration of which can be as high as 10 mM (35). To investigate the role of GSH and the cytoplasmic tail cysteine residues in exosomal MHC I dimer formation, we first raised the external concentration of GSH in the medium up to 10 mM and examined the MHC I dimer signal of exosomes isolated from the culture supernatant. In the presence of 10 mM GSH, MHC I dimers were present in significantly lower amounts (Fig. 4B). Many T cell assays include the presence of 10 mM GSH, MHC I dimers were present in significantly lower amounts (Fig. 4B).

Discussion

We have demonstrated in this study that MHC I molecules can exist in exosomes in a dimeric form, which the evidence suggests consists of two fully folded MHC I molecules attached through a disulfide bond between cysteine residues located in the cytoplasmic domain. We propose that these structures may confer novel immunomodulatory roles for MHC I molecules on exosomes.

The detection of MHC I molecules in dimeric form has been reported on the surface of cells (38). More recently, HLA-B27 dimers have been studied in detail because of their proposed role in the pathogenesis of AS (18, 34). A partially folded, potentially novel H chain dimer has been described which is strongly HC10-dimer positive (Fig. 6A). We further tested this model using the TAP-deficient cell line T2, which expresses only HLA-A*0201 (with Cys<sup>339</sup>) and HLA-B*5101 (without Cys<sup>325</sup>), and its rat TAP-restored derivative (36), denoted T3. T2 has very low levels of HLA-B51, but significant levels of HLA-A2 (Fig. 6B), because of the ability of the latter to bind leader peptides in the ER (37). TAPI and TAP2 restoration results in increased expression of both alleles in the T3 line. Comparisons of cell lysates and exosomes indicated that no HC10 dimers were detected in either T2 or T3 exosomes, but HCA2 reactive dimers were seen weakly in T2 and more strongly in T3 cells (Fig. 6C). Thus, dimer formation follows both possession of cytoplasmic domain cysteine residues, and is enhanced by higher levels of MHC I expression.

We next asked whether human monocyte-derived DC secrete exosomes displaying MHC I dimers. We compared a non-HLA-B27 individual with an HLA-B27-expressing individual. DC were stimulated for up to 72 h with LPS and exosomes isolated and immunoblotted with HC10 (Fig. 6D). Exosomes from HLA-B27-expressing cells (DC exo 2) contained more MHC I dimers and displayed them at an earlier time after LPS activation compared with non-HLA-B27 exosomes. Exosomal MHC I dimers could also be detected in the plasma of a healthy non-HLA-B27 individual (Fig. 6E). Thus, exosomal MHC I dimers occur in vivo as well as in vitro.

Finally, we asked whether exosomal MHC I dimers could be transferred from one cell to another. Jcesthom-derived exosomes, which are strongly HC10-dimer positive (Fig. 6F, input) were incubated for 24 h with KG-1 cells, which have no HC10 reactive dimers (Figs. 3A and 6A). HC10 immunoblotting revealed that dimers were detected in KG-1 cells incubated with exosomes (Fig. 6F), and fluorescence microscopy with anti-HLA-B27 FITC revealed distinct regions where the HLA-B27 positive exosomes accumulated in a representative KG-1 cell (Fig. 6G). Thus, exosomes can transfer novel MHC I structures to cells which are not themselves expressing such molecules.

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**HLA haplotype influences the ability to form exosomal MHC I dimers**

The data presented in Fig. 3A posed a question to our proposed model: why were there no dimers in exosomes from control, non-B27 transfected KG-1 cells? The IMGT/HLA database (www.ebi.ac.uk) confirmed that no HLA-B35 allele (as expressed by KG-1 cells) contains Cys<sup>325</sup>, and would therefore not form HC10-reactive dimers according to our model. This also further indicates that Cys<sup>308</sup> does not contribute strongly to exosomal MHC I class I dimer formation (Fig. 4E). Conversely, the HLA-A31 and -A35 alleles (also expressed by KG-1) do contain Cys<sup>339</sup> and would therefore be predicted to form exosomal MHC I dimers, detectable by HCA2, which proved to be the case (Fig. 6A). We further tested this model using the TAP-deficient cell line T2, which expresses only HLA-A*0201 (with Cys<sup>339</sup>) and HLA-B*5101 (without Cys<sup>325</sup>), and its rat TAP-restored derivative (36), denoted T3. T2 has very low levels of HLA-B51, but significant levels of HLA-A2 (Fig. 6B), because of the ability of the latter to bind leader peptides in the ER (37). TAPI and TAP2 restoration results in increased expression of both alleles in the T3 line. Comparisons of cell lysates and exosomes indicated that no HC10 dimers were detected in either T2 or T3 exosomes, but HCA2 reactive dimers were seen weakly in T2 and more strongly in T3 cells (Fig. 6C). Thus, dimer formation follows both possession of cytoplasmic domain cysteine residues, and is enhanced by higher levels of MHC I expression.

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W6/32 reactive dimers were also seen at low levels (20). Our current study has several unique observations, namely 1) the previously unreported detection of MHC I dimers in exosomes, 2) the prevalence of dimers, wherein they are highly enhanced in exosomes compared with cell lysates, 3) their fully folded nature in preference to unfolded/partially misfolded structures, and 4) their formation by both HLA-A and HLA-B alleles, with the added ability, in at least one instance to form mixed heterodimers between an HLA-A and an HLA-B molecule.

Taking each of the above points in turn, previous studies do not specify the use of nonreduced vs reduced samples in SDS-PAGE. We presume, therefore, that the majority of studies on exosomes to date where MHC I has been detected have involved SDS-PAGE of reduced samples, thus precluding their detection as disulfide-linked structures, whereas in our studies of HLA-B27 we analyze nonreduced samples to preserve such dimers. We were surprised by the dramatic levels of dimers we detected in exosomes compared with cell lysates (Fig. 2).

The folded nature of the exosomal MHC I dimers raises a significant issue. The endosomal route to MVBs, where exosomes are derived from, may alter the repertoire of peptides bound to MHC I molecules, either by removing those bound relatively weakly, or by loading peptides from exogenous sources, thus potentially contributing to cross-presentation. A detailed analysis of peptides eluted from exosomal MHC I molecules is required to address this issue, although the current lack of an Ab to isolate MHC I dimers from monomers currently precludes any discrimination of the peptides from these two structures.

It is also pertinent to ask where these MHC I dimer structures are forming, and the related question as to the role played by GSH. Our data suggest that the low level of GSH in exosomes allows disulfide bonds to form between the cytoplasmic tail residues of MHC I molecules, a situation not found in the strong reducing environment maintained in the cell cytoplasm. We suggest that this normally inhibits the formation of large populations of MHC I dimers of this type in normal cell membranes, but dimers can be induced experimentally by the removal of GSH with diamide (Fig. 5). The GSH assay system we have used here measures total GSH content, by also reducing any GSSG present, thus it is unlikely that exosomes lack reducing capacity due to GSH being sequestered as GSSG. We are currently attempting to determine whether GSH is lost at the stage when MVBs are formed, perhaps because not all of the necessary enzymes required are included when exosomes form, although they do contain at least one key enzyme, GST (40). Alternatively, GSH may be lost after release of the exosomes, when they may equilibrate their level of GSH to the low μM level found in serum and in culture medium (41).
The biological role of the unpaired cysteine residues in the cytoplasmic domains of MHC I molecules remains poorly defined. In this study, we propose they have a key role in the formation of exosomal MHC I dimers. Previous studies have identified that these cysteines can be modified by palmitylation (42, 43). Of great potential interest, in the latter study by Gruda and coworkers (44), mutation of the cytoplasmic tail domain cysteines in HLA-B7 prevented recognition of HLA-B7 expressing cells by a soluble LIR1-Ig fusion protein. It could be speculated that preventing the dimerization of a small population of cell surface MHC I by mutation of the cytoplasmic domain cysteines results in a loss of LIR1-Ig binding. Our current data does not suggest that Cys308 has a major role in exosomal dimer formation, which does not entirely fit with the data from Gruda and coworkers. Cys308 has, however, been identified as forming a transient disulfide linkage with tapasin during MHC I assembly in the endoplasmic reticulum, so remains accessible during at least some part of the lifespan of an MHC I molecule.

In this current study, we have not addressed what biological role exosomal MHC I dimers may have, although we can speculate on two broad possibilities. Firstly, clustering of MHC I molecules is known to enhance their recognition by T cells, and indeed chemically inducing the formation of MHC I dimers enhances their ability to present peptides at low concentrations (15). The reported supine orientation of MHC I molecules in the membrane could also facilitate dimer formation by allowing close proximity of the cytoplasmic domains (45). Secondly, it is also possible that exosomal MHC I dimers may exert their influence by interacting with receptors of the NK cell lineage. Indeed, we report in this study the presence of the NK ligand CD48 on exosomes, indicating that they have the capacity to be recognized by NK cells. In this context we may also be able to compare the exosomal MHC I dimers we describe in this study to those formed by the nonclassical HLA-G class I molecule, which is expressed almost exclusively on trophoblast cells during pregnancy, and which may function to generate dominant immunosuppressive effects to protect a developing fetus. HLA-G has a shortened cytoplasmic domain, with no cysteine residues, and thus cannot form dimers of the type we describe in this study. However, HLA-G is unique among MHC I molecules, as it possesses an unpaired Cys residue, situated on an external loop of the α1 domain, which allows it to form dimers (46). Such HLA-G dimers have been shown to be up to 100 times more efficient at LILRB1 signaling (47). Thus, it is possible that the exosomal MHC class I dimers we describe in this study may be potent ligands for recognition by NK lineage receptors biased toward exosomal MHC I and -B molecules. Of great potential significance, recent observations in a phase I clinical trial of dendritic cell-derived exosomes has shown the significant restoration and activation of NK cells in a cohort of patients with melanoma (48), thus highlighting the ability of exosomes to interact with NK cells.

We have also described in this study that a dimorphism exists in the ability of exosomes to form MHC class I dimers. Although all HLA-A alleles contain Cys38, only a proportion of HLA-B alleles contain Cys38. For example, alleles with Cys38 include HLA-B07, -08, -27, -41, -42, and -44, whereas those without include -B14, -B15, -B35, -B37, -B40, and -B51. Thus, each individual’s HLA haplotype will determine the presence of MHC class I exosomal dimers. In theory dimers may also form between different alleles, for example a heterozygous individual expressing HLA-B07 and -B44 could form exosomal B07-B07 dimers, B44-B44 dimers, and B07-B44 dimers. Furthermore, as we have shown in this study with exosomes from the Jesthom cell line, it is also possible to form dimers between HLA-A and -B molecules (Fig. 2C), though at lower levels, perhaps due to steric constraints caused by the relative positions of the cysteines in the cytoplasmic tails (49). Thus, our data describes for the first time a population of dimeric MHC I molecules on exosomes, which may represent a new range of structures for recognition by the various MHC I oriented receptors expressed on cells of the immune system.

Disclosures

The authors have no financial conflict of interest.

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