Induction of heat shock proteins in B-cell exosomes

Aled Clayton*, Attilla Turkes, Hossein Navabi, Malcolm D. Mason and Zsuzsanna Tabi

Department of Clinical Oncology and Palliative Medicine, Wales College of Medicine, Cardiff University, Velindre Cancer Centre, Whitchurch, Cardiff, CF14 2TL, UK

*Author for correspondence (e-mail: aled.clayton@Velindre-tr.wales.nhs.uk)

Accepted 13 May 2005
Journal of Cell Science 118, 3631-3638 Published by The Company of Biologists 2005
doi:10.1242/jcs.02494

Summary

Exosomes are nanometer-sized vesicles secreted by a diverse range of live cells that probably have physiological roles in modulating cellular immunity. The extracellular factors that regulate the quantity and phenotype of exosomes produced are poorly understood, and the properties of exosomes that dictate their immune functions are not yet clear.

We investigated the effect of cellular stress on the exosomes produced by B-lymphoblastoid cell lines. Under steady-state conditions, the exosomes were positive for hsp27, hsc70, hsp70 and hsp90, and other recognised exosome markers such as MHC class I, CD81, and LAMP-2. Exposing cells to heat stress (42°C for up to 3 hours), resulted in a marked increase in these heat shock proteins (hsps), while the expression of other stress proteins such as hsp60 and gp96 remained negative, and other exosome markers remained unchanged. Stress also triggered a small increase in the quantity of exosomes produced [with a ratio of 1.245±0.07 to 1 (mean±s.e.m., n=20) of 3-hour-stress-exosomes to control-exosomes]. Flow-cytometric analysis of exosome-coated beads and immuno-precipitation of intact exosomes demonstrated that hsps were located within the exosome lumen, and not present at the exosome-surface, suggesting that such exosomes may not interact with target cells through cell-surface hsp-receptors. Functional studies further supported this finding, in that exosomes from control or heat-stressed B cells did not trigger dendritic cell maturation, assessed by analysis of dendritic-cell-surface phenotype, and cytokine secretion profile.

Our findings demonstrate that specific alterations in exosome phenotype are a hitherto unknown component of the cellular response to environmental stress and their extracellular function does not involve the direct activation of dendritic cells.

Key words: Exosomes, Heat shock proteins, B cells

Introduction

Exosomes are a discrete population of nano-vesicles (40-90 nm in diameter) secreted by a variety of cells including B-lymphocytes, T-lymphocytes, dendritic cells, mast cells, epithelial cells, reticulocytes and others (Blanchard et al., 2002; Johnstone and Ahn, 1990; Raposo et al., 1996; Skokos et al., 2001; van Niel et al., 2001; Zitvogel et al., 1998). Currently, there is much discussion about the physiological functions of exosomes, with mounting evidence supporting a role in modulating cellular immunity (Andre et al., 2002; Wolfers et al., 2001; Zitvogel et al., 1998).

Proteomic methods are uncovering the molecular complexity of exosomes purified from different cells or body fluids, and together such analyses are useful for highlighting important cell-type specific differences between exosomes of different sources. They also identify commonly expressed exosome-proteins, such as MHC molecules, tetraspanins, adhesion molecules, cytosolic enzymes and certain stress proteins including hsc70 and hsp90 (Bard et al., 2004; Hegmans et al., 2004; Mears et al., 2004; Pisitkun et al., 2004; Thery et al., 2001; Wubbolts et al., 2003). However, these molecular analyses have essentially been limited to those exosomes produced under steady-state conditions, representing therefore a constitutive exosomal phenotype. It is not yet clear whether the composition – and maybe also the function(s) – of exosomes are dynamic and modified by external microenvironmental factors. So far, only two ways have been described how the extracellular milieu can influence the composition and/or quantity of exosomes that are secreted. First, the inhibition of dendritic cell exosome secretion in response to factors that trigger phenotypic maturation of the dendritic cell (such as LPS) (Kleijmeer et al., 2001; Zitvogel et al., 1998). Second, the activation of T cells (through ligation of the CD3-TCR complex) triggers an elevated exosome secretion with an altered exosomal composition such as higher levels of exosomally-expressed MHC molecules, tetraspanins and adhesion molecules (Blanchard et al., 2002).

Here, we studied whether conditions of cellular stress (specifically heat stress), can modify the quantity and/or composition of exosomes produced by B-lymphocytes. Our data show for the first time a dynamic and highly specific alteration in exosome composition in response to heat stress, resulting in an increased expression of selected heat shock proteins (hsps) (hsp27, hsc70, hsp70 and hsp90) in proportion to the degree of stress. Exosomes purified from either steady-state cells or heat stressed cells were not, however, able to trigger the phenotypic maturation of monocyte-derived dendritic cells. Furthermore, our analysis demonstrates that these stress proteins are encapsulated within the exosome lumen and are not present on the exosome surface.

Overall, our findings indicate that alterations in exotype-phenotype are a component of the cellular response to stress.

The physiological importance of these alterations is not yet clear, but exosomes might enable the elimination of hsp-protein complexes from the cell in a manner that does not directly lead to the activation of dendritic cells.

Materials and Methods

Cell lines

Epstein Barr virus (EBV)-immortalised human B-lymphoblastoid cell lines (B-LCL) were prepared as previously described (Doyle et al., 1994) from healthy donors, designated B-LCL(PC) and B-LCL(LL). An additional EBV-transformed cell line termed IB4 (Hurley et al., 1991) was a gift from Martin Rowe (Infection and Immunity, Wales College of Medicine, Cardiff University, UK). The Jurkat cell line was obtained from the MRC Co-operative (Cell Culture Laboratory, Wales College of Medicine, Cardiff University). Cells were seeded at 2-3 million cells/ml in serum free AIM-V medium, subjected to heat stress (0-3 hours, 42°C in a humidified incubator with 5% CO_2) and cultured for a total of 24 hours before exosomes were isolation. All culture reagents were purchased from Gibco/Life technologies.

Cellular expression of hsps

Twenty-four hours after heat stress, B-cell lines were washed in PBS, fixed and permeabilised (Beckman Coulter Fix/Perm Kit), and stained with a panel of anti-hsp antibodies (10 μg/ml per 10^6 cells in 100 μl). Expression levels were analysed by flow-cytometry (FACScan, BD). The mean fluorescence intensities (MFI) of four cell lines were measured. In addition, cell lysates (plus 2% NP-40 with protease inhibitors) were prepared as described (Clayton et al., 2003), and analysed for hsps by western blot. Densitometric analysis of blots was performed using Quantity One Software (BioRad).

Exosome isolation

Exosomes produced by B cells or Jurkat cells during a 24-hour culture period, were isolated from conditioned culture medium by differential centrifugation and flotation on 30% sucrose-D_2O as described (Andre et al., 2002; Lamparski et al., 2002). This method has been shown to effectively separate exosomes from non-exosomal membrane-vesicles or membranous fragments and other contaminants. Exosome-pellets were resuspended in 100 μl of PBS and aliquots stored at –70°C. Exosome protein was determined by BCA assay (Pierce). Exosomes derived from untreated B cells are referred to as 'control-exosomes', whereas those from heat-treated cells are referred to as 'heat-shocked exosomes'.

Western analysis

Equal volumes of SDS-sample buffer were added to cell lysates or purified exosomes (1-5 μg protein/well), samples were boiled at 98°C for 10 min and separated on 10% acrylamide gels. After transfer to PVDF membranes and blocking overnight (3% milk and 0.05% Tween-20 in PBS), primary antibody (0.5-2 μg/ml) was added for 1 hour, followed by washing and the application of secondary HRP-conjugated antibody (1:26,000 dilution, DAKO). Detection of bands was performed with the ECL$^+$ system (Amersham/Pharmacia).

Analysis of exosome surface molecules by flow-cytometry

Exosomes derived from control or heat-stressed cells were incubated with aldehyde-sulphate latex beads (surfactant free, white, 4 μm, Interfacial Dynamics), as described elsewhere (Lamparski et al., 2002). Exosome-bead complexes were stained with antibodies against known exosome surface markers including CD81 (Pharmingen) and MHC class II (DAKO) as positive controls, or with a panel of hsp-specific antibodies. Specific staining was compared to isotype-matched control antibodies by flow-cytometry.

Immuno-precipitation of intact exosomes

Equal quantities of exosomes from control cells or cells heat-shocked for 3 hours were incubated with a panel of hsp-specific antibodies (1 μg antibody/sample) in a final volume of 100 μl for 3 hours at room temperature. A pan-MHC-class-II antibody was used as a positive control (Dako). Goat-anti-mouse-coated beads (Dynal) at 10×10^6 beads/sample were added. After 2 hours, beads were pelleted (using a magnet) and washed 6 times to remove unbound material. Beads were incubated in SDS-sample buffer and analysed by western blot for the presence of MHC class I.

Monocyte-derived dendritic cells (MDDCs)

Peripheral blood mononuclear (PBM) cells were isolated by histopaque centrifugation (Sigma), and added to 24-well plates (5 million cells/ml/well) in AIM-v for 2 hours. Adhered cells, mainly monocytes, were incubated in AIM-v that contained IL-4 and granulocyte/macrophage-colony stimulating factor (GM-CSF) at 1000 U/ml. Lymphocytes were distinguished from non-lymphocytes, based on a dot-plot of their forward scatter (FSC) and side scatter (SSC) characteristics on a flow cytometer. By day 6, 90% of FSC/SSC-gated cells were MHC class II high, CD14$^-$ by flow-cytometry.

Analysis of the MDDC phenotype and cytokines

MDDCs (day 6) were harvested and seeded into fresh 48-well plates at a density of 5×10^5 cells/ml. Exosomes from control or cells heat-shocked for 3 hours were added (at various concentrations of up to 100 μg/10^5 MDDCs). As a positive control, lipopolysaccatide (LPS, Sigma) was added at 0.1 or 10 μg/ml; as a negative control MDDCs were left untreated. After 48 hours, cells were harvested, fixed (Becton Dickinson Fix/Perm Kit) and stained with FITC-conjugated antibodies against CD83 or isotype-matched control. Cell-free supernatants were analysed for IL-6, IL-10 and IL-12 by ELISA (R&D Duoset Kit).

Antibodies

Mouse anti-human hsp27 (IgG2a, clone F-4), hsp60 (IgG3, clone H-1), hsp70 (IgG2a, clone w27), hsc70 (IgG2a, clone b-6), hsp90 (IgG2a, clone F-8) and LAMP-2 (IgG1, clone H4B4) were from Santa Cruz Biotechnology. Rat anti-human gp96 (IgG2a, clone 9G10) was from Stressgen Biotechnologies. Anti-human CD83-FITC and isotype-matched control were from Immunotech. Antibodies specific for EBV LMP-1 were a gift from Martin Rowe (clone CS1, 2, 3, and 4). Mouse anti-human MHC class I (IgG2a, clone HC10) was obtained from the MRC Co-operative (Cell Culture Laboratory, Wales College of Medicine, Cardiff University, UK). Goat anti-mouse IgG-HRP or rabbit anti rat-IgG-HRP was from DAKO.

Results

Heat stress triggers increased levels of stress proteins in B cells and B-cell derived exosomes

The constitutive expression of hsp90 and/or hsc70 (hsp73) in exosomes derived from antigen-presenting cells (Thery et al., 1999; Wubbolts et al., 2003), reticulocytes (Geminard et al., 2001) and epithelial cells (van Niel et al., 2001) has been reported previously. However, potential alterations in the quantity and/or repertoire of exosomal hsps following stress have not been addressed to date.

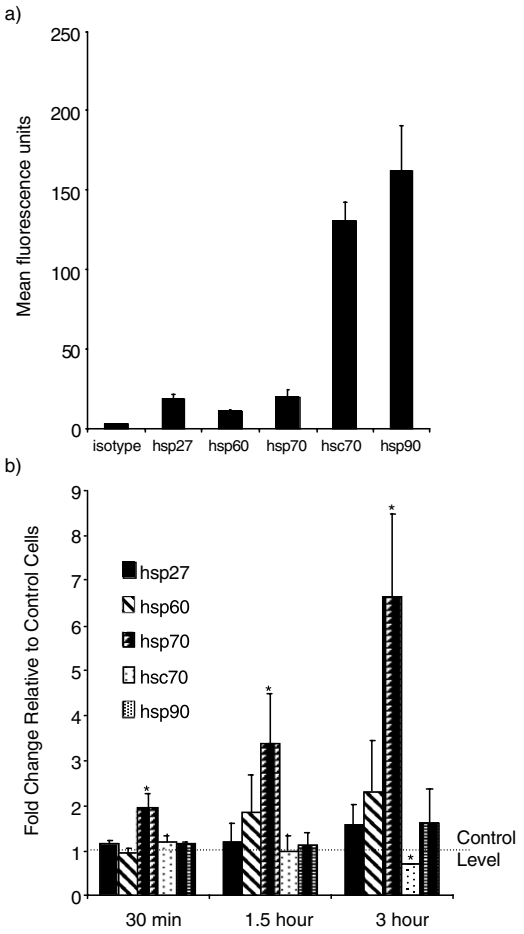


Fig. 1. Constitutive and induced expression of cellular hsps. Non-stressed B-cell lines were washed in PBS, fixed, permeabilised and stained with hsp-specific antibodies or isotype control (as indicated). Levels of hsps were examined by flow-cytometry. (a) The value for the mean fluorescence intensity (MFI) was taken (mean+s.e.m. of four B-cell lines). (b) Altered cellular hsp expression following heat stress. Four B-cell lines were incubated at 42°C for up to 3 hours, incubated for a total of 24 hours at 37°C before hsp expression was analysed by flow-cytometry. The graph is expressed in terms of fold-change relative to non-stressed expression (normalised to a value of 1, and indicated by the dotted line) (mean ± of four B-cell lines).

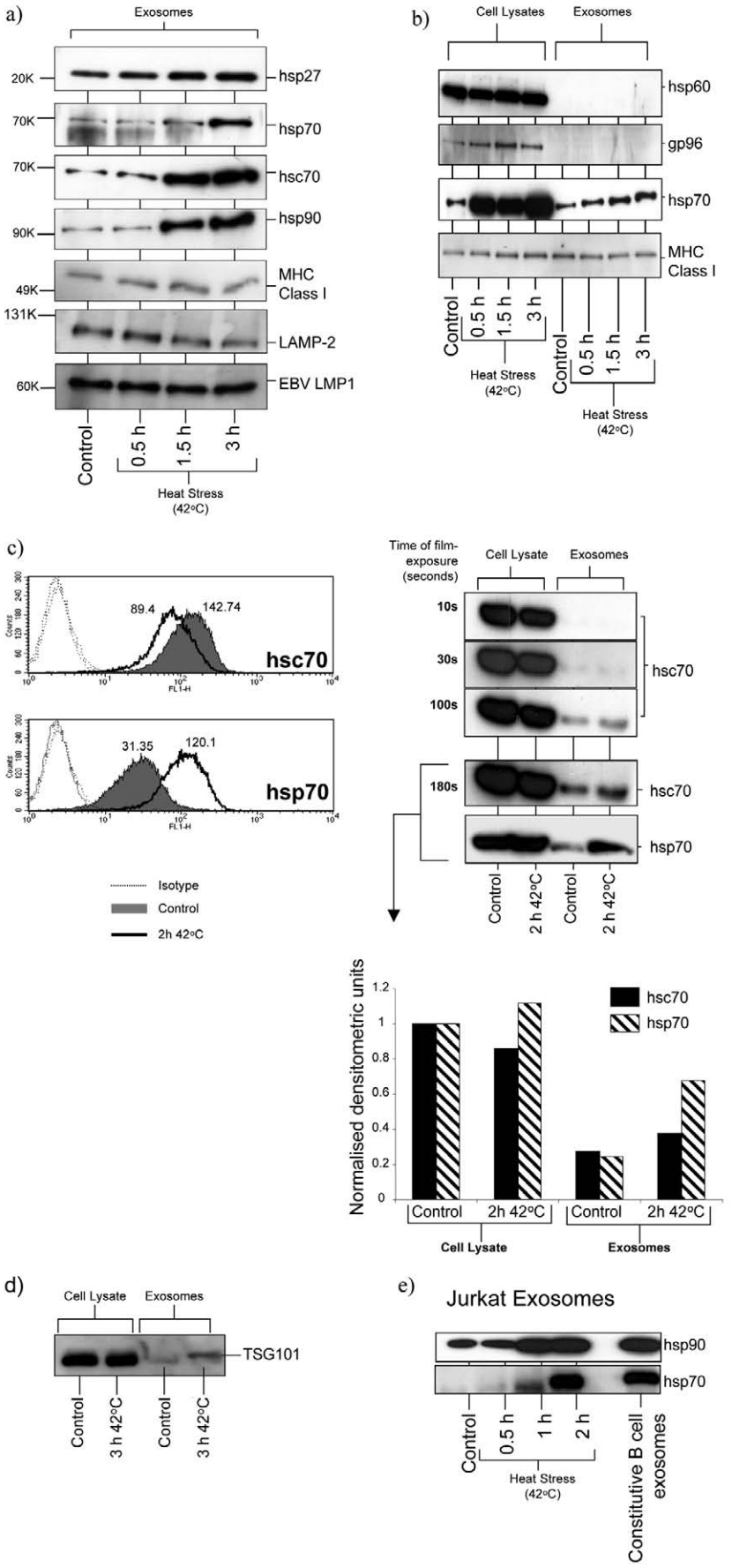
We investigated whether cellular stress leads to altered hsp expression in exosomes and induced hsps by elevated temperature. B-cell lines were exposed to a temperature of 42°C for varying times (up to 3 hours) to generate different levels of non-lethal heat stress. After returning the cells to 37°C for 24 hours, we analysed changes in the levels of hsps, specifically of hsp27, hsp70, hsc70, hsp60 and hsp90, therefore covering most hsp families (Srivastava, 2002). Fig. 1a shows the expression levels of these hsps in normal cells, with high constitutive levels of hsc70 and hsp90, and significantly less (around 7- to 20-fold) expression of hsp27, hsp70 and hsp60. Following only 30 minutes of heat stress, a significant increase of hsp70 expression occurred (Fig. 1b) that rose to approximately sevenfold above its constitutive level after 3 hours. Some increase in cellular hsp27, hsp60 and hsp90 was evident, but this was not statistically significant. In all B-cell lines tested, cellular hsc70 was reduced by approximately 30% compared with its constitutive levels (Fig. 1b).

Exosomes secreted during the 24-hour incubation period post heat-stress, were analysed by western blotting for hsp expression, with each lane containing the same quantity of purified exosome protein (Fig. 2a). A time-dependent (duration of heat stress) elevation in exosomal hsp27, hsp70, hsc70 and hsp90 was evident. However, not all exosome-associated proteins were increased, levels of MHC class I, the EBV-encoded latent membrane protein-1 (LMP-1) and the lysosome associated membrane protein LAMP-2 were unaltered (Fig. 2a). Hsp60 or gp96 were not detected in these samples (not shown), which agrees with previously published reports of exosomes from other cellular sources (Skokos et al., 2003; Wolfers et al., 2001). To demonstrate the selective absence of hsp60 and gp96 from exosomes even under conditions of stress, we compared cell lysates to exosomes by western blot. Although bands for hsp60 and gp96 were seen in lanes containing cell lysates, no bands were evident in lanes containing exosomes. By contrast, the same samples showed a time-dependent elevation in hsp70, evident in both lysates and exosomes, and again MHC class I remained at constant levels (Fig. 2b). Although our flow-cytometric analysis of B cells revealed a reduction in hsc70 levels due to heat stress (Fig. 1b), western blots of exosomes showed a dramatic elevation in hsc70 (Fig. 2a). To confirm these findings, hsc70 levels in cell lysates and exosomes were compared by western blot (Fig. 2c), showing a stress-mediated reduction of cellular hsc70 and an increase in exosomal hsc70. The same samples demonstrated elevated expression for hsp70 in both lysates and exosomes (Fig. 2c). This suggests that a proportion of hsc70 is released from the cell in the form of exosomes after heat stress. Also examined was TSG101, a protein involved in the production of multivesicular bodies (Katzmann et al., 2003) that ultimately give rise to exosomes. TSG101 levels were slightly elevated in stressed exosomes, suggesting that the cellular machinery for exosome assembly can also be activated by cell stress (Fig. 2d). To investigate whether induction of hsps in exosomes is a phenomenon unique to EBV-immortalised B cells, we repeated these studies in cells of a EBV-negative human T-cell line (Jurkat). Exposure of these cells to heat stress, resulted in elevated levels of exosomal hsp90 and hsp70 (Fig. 2d), although the constitutive levels of exosomal hsps was much lower in Jurkat exosomes.

In conclusion, our findings demonstrate that the exosomes secreted during a response to heat stress have a composition that is indeed different from that of steady-state exosomes and, although broadly reflective of changes that occur within the cell, exosomes express a unique and inducible repertoire of selected stress proteins.

Heat stress triggers a small increase in the quantity of exosomes secreted

We next investigated whether heat stress also altered the quantity of exosomes produced by B cells and Jurkat cells. Exosomes were purified from supernatants of the same number of control cells or cells that had been heat-stressed for 3 hours. The total protein of each exosome pellet was measured (by BCA assay) for each preparation, and total protein of heat-stressed cells was compared with that produced by non-



stressed cells (Fig. 3). Multiple preparations of exosomes from three B-cell lines and from Jurkat cells showed only small elevations (less than 1.3-fold) in the number of exosomes produced, with an overall mean ratio of 3-hour-stress-exosomes to control exosomes of 1.245±0.07 to 1 (mean±s.e.m., n=20).

Stress proteins are encapsulated within the exosome lumen

We investigated the location of exosome-associated hsps, present on the outer surface or within the vesicle lumen, because this could have a direct bearing on the function of exosome-associated hsps.

Control and heat-shock exosomes were prepared once, and this stock preparation was used in all the following experiments (Fig. 4). Initial analysis was by western blot where elevated levels of exosomal hsp70 and hsc70 in heat-shock exosomes was confirmed (Fig. 4a). Exosomes were subsequently coupled to aldehyde-sulphate latex beads (Lamparski et al., 2002), and the expression of exosome-surface molecules was analysed by flow-cytometry of the exosome-bead complexes (Fig. 4b). Positive staining for the exosome surface markers CD81 and MHC class I was demonstrated by flow-cytometry, however there was no positive staining for hsp27, hsp70, hsc70 or hsp90. Furthermore, this analysis demonstrated that there was no

Fig. 2. Hsps are specifically enriched in exosomes during stress. B-cell lines were subjected to heat stress (42°C for 0-3 hours), and subsequently incubated for 24 hours at 37°C before exosome and cell lysate preparation. (a) Western blot analysis of stress-dependent (time) hsp expression in exosomes, demonstrating selected elevations in stress proteins but not other exosome markers. (b) Western blot analysis of cell lysates and exosomes, showing the absence of gp96 and hsp60 in exosomes, even after heat stress. Flow-cytometric analysis of hsc70 and hsp70 levels in control IB4 cells or IB4 cells heat-stressed for 2 hours, demonstrating that heat stress reduces cellular hsc70, while hsp70 is elevated (mean fluorescence values of the histogram are shown). Western blots of cell lysates or exosomes from the same experiment were also performed, comparing cell-lysates with exosomes. Various exposure times are shown (10-180 seconds for hsc70). (c) Densitometric analysis (of 180 exposed blots), was performed to confirm decreased cellular hsc70 levels, and increased exosomal hsc70 levels upon heat stress. (d) TSG101, a component of the exosome formation machinery, is only slightly elevated in exosomes following heat stress. (e) Exosomes derived from Jurkat cells subjected to heat stress (42°C for 0-2 hours), also demonstrate elevated levels of stress proteins hsp90 and hsp70, although steady-state levels of hsps in Jurkat exosomes were lower than that in B-cell exosomes.

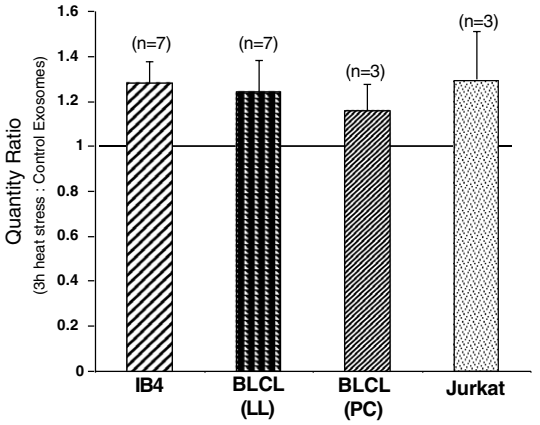


Fig. 3. The quantity of exosome secretion changes following heat stress. Exosomes were purified from culture supernatants containing equal numbers of control or 3-hour heat-stressed cells [IB4, BLCL(LL), BLCL(PC) or Jurkat cell lines]. The exosome pellet of each purification was resuspended in 100 µl PBS, and total protein determined by BCA assay. The graph represents the ratio of control exosome-protein to heat-shock-exosome-protein, where a ratio of 1 (dotted line) would indicate no difference. (Mean ratio±s.e.m. of n purifications.) The overall mean ratio was 1.245±0.07 (n=20), indicating a small elevation (less than 1.3-fold) in exosome secretion due to stress.

difference between control and heat-shock exosomes, indicating that under conditions of stress, the exosome-surface does not become hsp- positive. Beads coated with exosomes underwent several washing steps during antibody staining, which might have resulted in the loss of surface-associated hsp. To confirm that our negative flow-cytometric findings were not due to washing away surface hsp, exosome-beads that had been subjected to washes, but had not been labelled with antibodies were boiled in SDS-sample buffer at 98°C for 10 minutes; the solubilised total exosome proteins were then analysed by western blot (Fig. 4c). There, elevated levels of hsp70 and hsc70 were found in the samples of heat-shock exosomes but not of control exosomes, indicating that exosomal hsps had not been lost during exosome-bead washing steps, and that the detection of elevated hsps levels absolutely required the disruption of the exosome structure (Fig. 4c).

To further support these data, a more sensitive method was

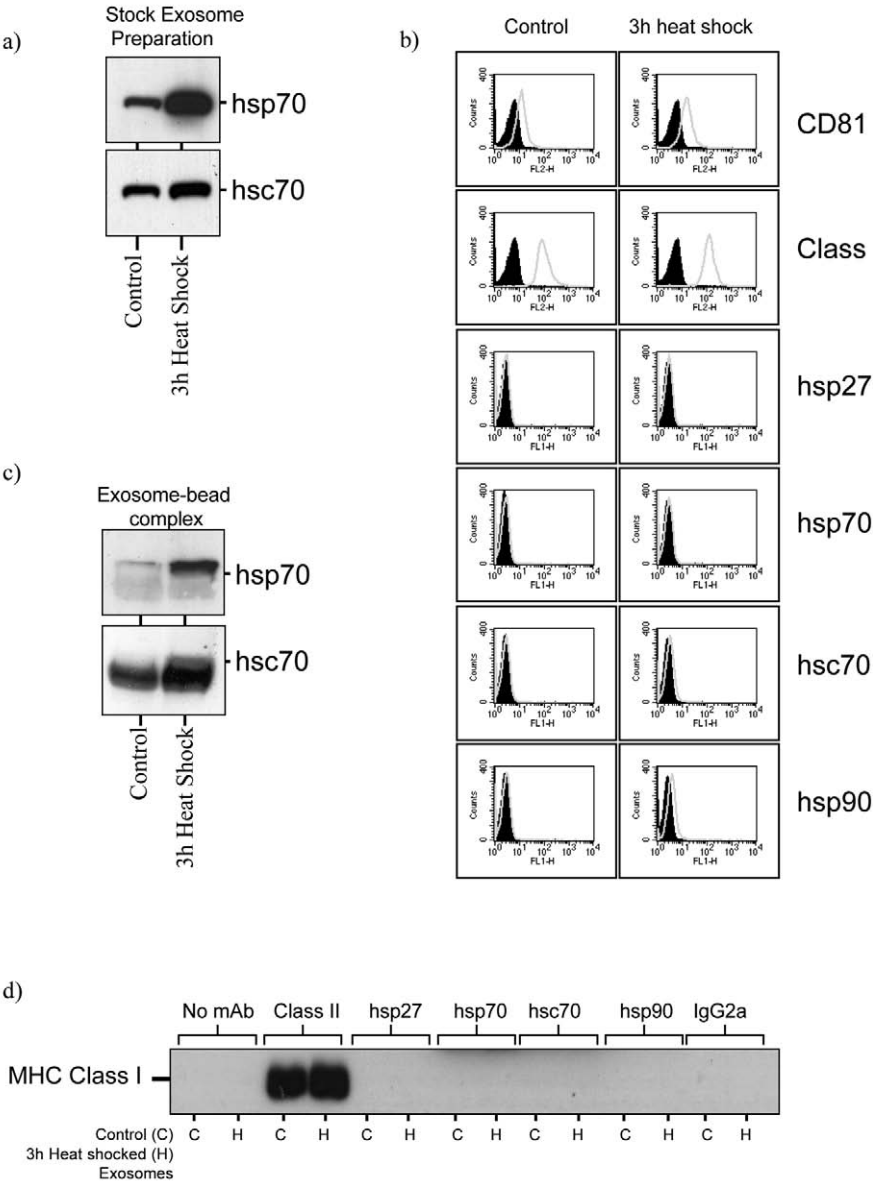


Fig. 4. Hsps are located within the exosome lumen. (a) Purified stock exosomes were analysed by western blot for induced hsp expression. Aliquots of these exosomes were subsequently immobilised onto latex beads and surface-hsp expression was analysed by (b) flow-cytometry; filled histograms represent exosome-bead complexes stained with irrelevant-isotype control antibody, unfilled histogram represent antibody staining as indicated. (c) Remaining exosome-bead complexes, not stained with antibodies, were boiled in SDS-sample buffer and analysed by western blot. (d) The remainder of the initial exosome stock (intact exosomes, not immobilized onto latex beads) was immuno-precipitated with antibodies as indicated and the relative quantity of precipitated exosomes determined by western blot, stained for MHC class I. Although exosomes were effectively precipitated with MHC class II antibodies, no bands were seen with hsp-specific antibodies. These data indicate that hsps are detectable following exosome disruption, but are not present on the exosome surface.

used to detect hsps on the exosome surface. Intact exosomes were precipitated out of solution with antibodies against exosome-surface molecules, and precipitates were analysed by western blot for the presence of MHC class I. Control or 3 hour heat-shock exosomes precipitated equally well with antibodies against MHC class II (Fig. 4d). By contrast, irrelevant isotype-matched antibody was ineffective at exosome precipitation and precipitations with antibodies against hsp27, hsp70, hsc70 or hsp90 were equally unsuccessful at precipitating exosomes. There was no indication that heat stress was a stimulus for locating hsps to the exosome surface, because no difference was seen between control and heat-shock exosomes (Fig. 4d). Together, our data point to a lumenal and not a surface location for hsps in B-cell-derived exosomes.

Stress-modified exosomes do not mature dendritic cells

One possible mechanism by which stress proteins can modulate immunological responses is through activating dendritic cell maturation. This phenomenon is usually associated with the binding of soluble hsps to cell surface signalling receptors such as CD91 (Basu et al., 2001; Singh Jasuja et al., 2000). We examined whether exosomes enriched in hsps are capable of modulating the phenotype of immature dendritic cells towards maturation, by measuring alterations in cell-surface maturation markers (e.g. CD83) and alterations in cytokine secretion.

MDDCs were cultured in GM-CSF and IL-4 from adherent peripheral blood cells, and were at day 6 morphologically and phenotypically still immature. Treatment of day 6 MDDCs with 10 μg/ml LPS, a potent maturation agent, resulted in marked elevation in CD83 protein levels after 48 hours. By contrast, treating MDDCs with control exosomes or heat-shock exosomes, even at doses as high as 100 μg exosomes per 10^5 dendritic cells, had no effect on CD83 protein levels (Fig. 5a). The culture medium of the above MDDCs was also examined for possible exosome-mediated changes in cytokine secretion. There was no significant difference between control and heat-

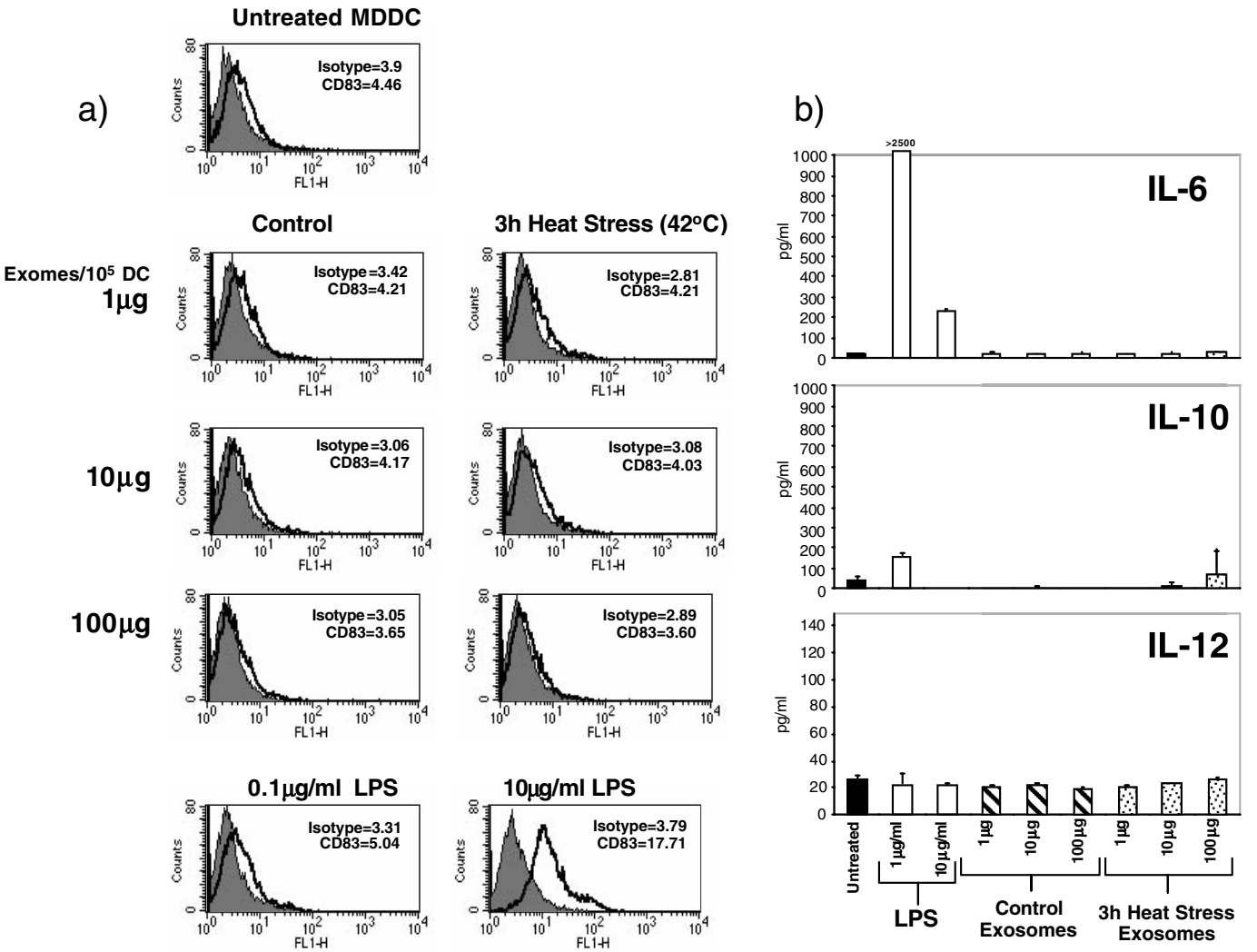


Fig. 5. Stress-derived exosomes do not mature dendritic cells. MDDCs (day 6) were incubated alone, with control exosomes or with 3 hour heat-shocked exosomes (1-100 μg per 10^5 MDDCs), or with LPS (0.1 or 10 μg/ml). (a) After 48 hours, MDDCs were harvested, fixed and analysed for CD83 expression by flow-cytometry. (b) Culture supernatants were taken and cytokines (IL-6, IL-10, IL-12) were measured by ELISA. Graph shows mean+s.d. of triplicate measurements.

shock-exosome-treated MDDCs in the levels of IL-6, IL-10 and IL-12 (assessed by ELISA; Fig. 5b), and the levels of TNFα, IL-1β, IL-10, IL-12, IL-8 and IL-6 at day 3 or day 6 post stimulation (measured by cytometric bead array, data not shown). These data indicate that stress-derived exosomes, like control-exosomes, do not modify the status of maturation of dendritic-cells.

Discussion

Here, we have investigated whether heat stress can affect the molecular composition of nanometer-sized vesicles (exosomes) that are produced by cultured B-cell lines. Our data demonstrates that exposure of B cells to elevated temperature (42°C up to 3 hours) results not only in alterations in expression pattern of cellular hsps, but also results in hitherto unknown changes in the phenotype of secreted exosomes. Specifically, heat stress drives the selective enrichment of certain hsps (hsp27, hsc70, hsp70, hsp90) within exosomes, while others, such as hsp60, remain excluded from exosomes. Other housekeeping exosome proteins, such as MHC molecules and LAMP-2, are unchanged following heat stress. Heat stress also mediates the increased quantity of exosome secretion (approximately 1.2- to 1.3-fold). The functional significance of the release of hsps into the extracellular milieu in the form of exosomes remains unknown.

Stress proteins act to stabilise misfolded proteins, and prevent the formation of protein aggregates under conditions of heat stress. Their presence in the extracellular environment has been proposed to be an indicator of cellular injury, with important roles in inflammation and immunity. Stress proteins act essentially as soluble factors that interact with various cell-surface receptors to drive inflammatory processes (Lamb et al., 1989; Wang et al., 2002) including the maturation of dendritic cells (Basu et al., 2000; Zeng et al., 2003). Extracellular hsps might also act as molecular chaperones, by forming associations with antigenic polypeptides and thus efficiently delivering exogenous antigens to the endogenous antigen-processing-pathway of dendritic cells (Binder et al., 2001; Manjili et al., 2002; Srivastava and Udono, 1994; Zeng et al., 2003).

Exosomes are also thought to offer an efficient mode of transferring antigens, e.g. from cancer cells to dendritic cells, and the notion that certain stress proteins that are expressed by exosomes of diverse sources might play a role in exosome-immunity has attracted much interest. For example, exosomes derived from ascitic fluid of cancer patients were hsp-positive and prove an effective form of antigen for dendritic-cell cross-presentation to $CD8^+$ T cells in vitro (Andre et al., 2002). Similarly, exosomes isolated from several murine tumour cell lines expressed high levels of hsp90 and hsc70, and were immunologically effective as vehicles for delivering antigen to dendritic cells, yet failed to trigger dendritic cell maturation (Wolfers et al., 2001). Recent in vivo studies have shown that co-administration of exosomes with adjuvants (such as CpG) is required to generate optimal immune responses (Chaput et al., 2003). Exosomes may therefore be viewed as an antigen source of insufficient inherent-adjuvant activity to drive alterations in dendritic cell maturation. To date, the only exception of this is a report by Skokos et al., which describes that exosomes of mast-cell origin can alter the dendritic-cell phenotype towards maturation in a CD91-dependent manner (Skokos et al., 2003); however, exosomes from other cellular sources, including B cells, cannot stimulate dendritic-cell maturation. Mast-cell exosomes, which exhibit a different hsp repertoire from B-cell exosomes, might have unique properties such as the ability to express hsp60, to mediate these effects (Skokos et al., 2003). So far however, all these studies have involved steady-state exosomes, and have not addressed the importance of stress-mediated induction of hsp.

The data presented here demonstrate that exosomes from heat-stressed B cells, which express elevated levels of stress proteins, have no effect on the maturation status of MDDCs. There was no difference in the expression of CD83 (or other markers including CCR7 and CCR5, data not shown) in dendritic-cells following exposure to high levels of control or heat stressed exosomes, and there was no effect on cytokine production (IL-6, IL-10, IL-12 or others) by dendritic cells. Our explanation for this surprising lack of stimulatory function lies in the precise location of exosomal hsps. Direct analysis of the exosome surface by flow-cytometry and immuno-precipitation experiments has demonstrated that the hsps lie encapsulated within the exosome lumen even after heat stress (Fig. 4), a stimulus that can often redistribute hsps to, for example cellular surfaces (Feng et al., 2002; Feng et al., 2003). Exosomal-hsps are therefore not directly available for binding to dendritic-cell-surface receptors such as CD91 or other receptors that trigger phenotypic dendritic-cell changes (Basu et al., 2001; Delneste et al., 2002; Ohashi et al., 2000; Wang et al., 2001).

When searching for a possible function for exosome-borne stress proteins, one could imagine that extracellular or intracellular processing of exosomes are necessary to liberate the encapsulated hsps. Intracellular processing, which probably involves exosome-adhesion molecules (Clayton et al., 2004) that bind to the dendritic-cell surface before endocytosis and processing (Morelli et al., 2004), is certainly compatible with earlier studies documenting exosomes as antigen-delivery-vehicles to dendritic cells (Altieri et al., 2004; Andre et al., 2002; Wolfers et al., 2001). The relative potency of stress-derived exosomes in such cross-presentation mechanisms is the subject of ongoing studies in our laboratory.

This work was funded by the Leukaemia Research Fund, Cancer Research Wales, and European Commission Grant QLRT-2001-00093. We are grateful to Fabrice Andre and Laurence Zitvogel, Institute Gustave Roussy, Paris, for their assistance in exosome purification.

References

Altieri, S., Khan, A. and Tomasi, T. (2004). Exosomes from Plasmacytoma cells as a tumor vaccine. *J. Immunother.* **27**, 282-288.

Andre, F., Schartz, N. E., Movassagh, M., Flament, C., Pautier, P., Morice, P., Pomel, C., Lhomme, C., Escudier, B., Le Chevalier, T. et al. (2002). Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* **360**, 295-305.

Bard, M. P., Hegmans, J. P., Hemmes, A., Luider, T. M., Willemsen, R., Severijnen, L.-A. A., Meerbeeck, J. P. V., Burgers, S. A., Hoogsteden, H. C. and Lambrecht, B. N. (2004). Proteomic analysis of exosomes isolated from human malignant pleural effusions. *Am. J. Respir. Cell. Mol. Biol.* **31**, 114-121.

Basu, S., Binder, R. J., Suto, R., Anderson, K. M. and Srivastava, P. K. (2000). Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int. Immunol.* **12**, 1539-1546.

Basu, S., Binder, R. J., Ramalingam, T. and Srivastava, P. K. (2001). CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* **14**, 303-313.

Binder, R. J., Blachere, N. E. and Srivastava, P. K. (2001). Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. *J. Biol. Chem.* **276**, 17163-17171.

Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G. and Hivroz, C. (2002). TCR Activation of human T cells induces the production of exosomes bearing the TCR/CD3/{zeta} complex. *J. Immunol.* **168**, 3235-3241.

Chaput, N., Schartz, N. E., Andre, F., Taieb, J., Novault, S., Bonnaventure, P., Aubert, N., Bernard, J., Lemonnier, F., Merad, M. et al. (2004). Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficientcy prime naive Tc1 lymphocytes leading to tumor rejection. *J. Immunol.* **172**, 2137-2146.

Clayton, A., Harris, C. L., Court, J., Mason, M. D. and Morgan, B. P. (2003). Antigen presenting cell exosomes are protected from complement mediated lysis by expression of CD55 and CD59. *Eur. J. Immunol.* **33**, 522-531.

Clayton, A., Turkes, A., Dewitt, S., Steadman, R., Mason, M. D. and Hallett, M. B. (2004). Adhesion and signalling by B cell derived exosomes: The role of integrins. *FASEB J.* **9**, 977-979.

Delneste, Y., Magistrelli, G., Gauchat, J., Haeuw, J., Aubry, J., Nakamura, K., Kawakami Honda, N., Goetsch, L., Sawamura, T., Bonnefoy, J. et al. (2002). Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* **17**, 353-362.

Doyle, A., Griffiths, J. B. and Newel, D. G. (1994). *Cell and Tissue Culture: Laboratory Procedures*. Porton Down, Salisbury, UK: Wiley.

Feng, H., Zeng, Y., Graner, M. W. and Katsanis, E. (2002). Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. *Blood* **100**, 4108-4115.

Feng, H., Zeng, Y., Graner, M. W., Likhacheva, A. and Katsanis, E. (2003). Exogenous stress proteins enhance the immunogenicity of apoptotic tumor cells and stimulate antitumor immunity. *Blood* **101**, 245-252.

Geminard, C., Nault, F., Johnstone, R. M. and Vidal, M. (2001). Characteristics of the interaction between Hsc70 and the transferrin receptor in exosomes released during reticulocyte maturation. *J. Biol. Chem.* **276**, 9910-9916.

Hegmans, J., Bard, M., Hemmes, A., Luider, T., Kleijmeer, M., Prins, J., Zitvogel, L., Burgers, S., Hoogsteden, H. and Lambrecht, B. (2004). Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am. J. Pathol.* **164**, 1807-1815.

Hurley, E. A., Klaman, L. D., Agger, S., Lawrence, J. B. and Thorley-Lawson, D. A. (1991). The prototypical Epstein-Barr virus-transformed lymphoblastoid cell line IB4 is an unusual variant containing integrated but no episomal viral DNA. *J. Virol.* **65**, 3958-3963.

Johnstone, R. M. and Ahn, J. (1990). A common mechanism may be involved in the selective loss of plasma membrane functions during reticulocyte maturation. *Biomed. Biochim. Acta* **49**, S70-S75.

Katzmann, D., Stefan, C., Babst, M. and Emr, S. (2003). Vps27 recruits ESCRT machinery to endosomes during MVB sorting. *J. Cell Biol.* **162**, 413-423.

Kleijmeer, M., Ramm, G., Schuurhuis, D., Griffith, J., Rescigno, M., Ricciardi Castagnoli, P., Rudensky, A. Y., Ossendorp, F., Melief, C. J., Stoorvogel, W. et al. (2001). Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *J. Cell Biol.* **155**, 53-63.

Lamb, J. R., Bal, V., Mendez-Samperio, P., Mehlert, A., So, A., Rothbard, J., Jindal, S., Young, R. A. and Young, D. B. (1989). Stress proteins may provide a link between the immune response to infection and autoimmunity. *Int. Immunol.* **1**, 191-196.

Lamparski, H., Metha-Damani, A., Yao, J., Patel, S., Hsu, D., Ruegg, C. and Le Pecq, J. (2002). Production and characterization of clinical grade exosomes derived from dendritic cells. *J. Immunol. Methods* **270**, 211-226.

Manjili, M. H., Henderson, R., Wang, X. Y., Chen, X., Li, Y., Repasky, E., Kazim, L. and Subjeck, J. R. (2002). Development of a recombinant HSP110-HER-2/neu vaccine using the chaperoning properties of HSP110. *Cancer Res.* **62**, 1737-1742.

Mears, R., Craven, R., Hanrahan, S., Totty, N., Upton, C., Young, S., Patel, P., Selby, P. and Banks, R. (2004). Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics* **12**, 4019-4031.

Morelli, A., Larregina, A., Shufesky, W., Sullivan, M., Stolz, D., Papworth, G., Zahorchak, A., Logar, A., Wang, Z., Watkins, S. et al. (2004). Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* **104**, 3257-3266.

Ohashi, K., Burkart, V., Flohe, S. and Kolb, H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J. Immunol.* **164**, 558-561.

Pisitkun, T., Shen, R. and Knepper, M. (2004). Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. USA* **101**, 13369-13373.

Raposo, G., Nijman, H. W., Stoorvogel, W., Leijendekker, R., Harding, C. V., Melief, C. J. M. and Geuze, H. J. (1996). B Lymphocytes secrete Antigen-presenting Vesicles. *J. Exp. Med.* **183**, 1161-1172.

Singh Jasuja, H., Scherer, H. U., Hilf, N., Arnold Schild, D., Rammensee, H. G., Toes, R. E. and Schild, H. (2000). The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur. J. Immunol.* **30**, 2211-2215.

Skokos, D., Le Panse, S., Villa, I., Rousselle, J. C., Peronet, R., Namane, A., David, B. and Mecheri, S. (2001). Nonspecific B and T cell-stimulatory activity mediated by mast cells is associated with exosomes. *Int. Arch. Allergy. Immunol.* **124**, 133-136.

Skokos, D., Botros, H. G., Demeure, C., Morin, J., Peronet, R., Birkenmeier, G., Boudaly, S. and Mecheri, S. (2003). Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. *J. Immunol.* **170**, 3037-3045.

Srivastava, P. (2002). Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu. Rev. Immunol.* **20**, 395-425.

Srivastava, P. K. and Udono, H. (1994). Heat shock protein-peptide complexes in cancer immunotherapy. *Curr. Opin. Immunol.* **6**, 728-732.

Thery, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Raposo, G. and Amigorena, S. (1999). Molecular characterisation of dendritic cell-derived exosomes: selective accumulation of the heat shock protein hsc73. *J. Cell Biol.* **147**, 599-610.

Thery, C., Boussac, M., Veron, P., Ricciardi Castagnoli, P., Raposo, G., Garin, J. and Amigorena, S. (2001). Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* **166**, 7309-7318.

van Niel, G., Raposo, G., Candalh, C., Boussac, M., Hershberg, R., Cerf-Bensussan, N. and Heyman, M. (2001). Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* **121**, 337-349.

Wang, Y., Kelly, C. G., Karttunen, J. T., Whittall, T., Lehner, P. J., Duncan, L., MacAry, P., Younson, J. S., Singh, M., Oehlmann, W. et al. (2001). CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. *Immunity* **15**, 971-983.

Wang, Y., Kelly, C. G., Singh, M., McGowan, E. G., Carrara, A. S., Bergmeier, L. A. and Lehner, T. (2002). Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. *J. Immunol.* **169**, 2422-2429.

Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Thery, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., Tursz, T. et al. (2001). Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat. Med.* **7**, 297-303.

Wubbolts, R., Leckie, R. S., Veenhuizen, P. T., Schwarzmann, G., Mobius, W., Hoernschemeyer, J., Slot, J. W., Geuze, H. J. and Stoorvogel, W. (2003). Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J. Biol. Chem.* **278**, 10963-10972.

Zeng, Y., Feng, H., Graner, M. W. and Katsanis, E. (2003). Tumor-derived, chaperone-rich cell lysate activates dendritic cells and elicits potent antitumor immunity. *Blood* **101**, 4485-4491.

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Filament, C., Tenza, D., Ricciardi-Castagnoli Raposo, G. and Amigorena, S. (1998). Eradication of established murine tumours using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat. Med.* **4**, 594-600.