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[4] Abbreviations used in this paper:

APC: antigen presenting cell
CIITA : MHC class II transactivator
IEC: Intestinal epithelial cells
HSA : Human serum albumin
MHC: Major histocompatibility complex
HLA: human leucocyte antigen

Abstract

Background and aims: Intestinal epithelial cells release antigen presenting vesicles (exosomes) bearing MHC class II/peptide complexes stimulating specific immune responses *in vivo*. To further characterize the role of human epithelial exosomes in antigen presentation, their capacity to load antigenic peptides, to bind immune target cells and to induce T cell activation was analyzed *in vitro*. **Methods**: The capacity of exosomes derived from the HLA-DR4 expressing, intestinal epithelial cell line T84, to load the HLA-DR4-specific peptide ^{3}H-HSA 64-76 and to activate a HLA-DR4-restricted T cell hybridoma, was tested in the presence or absence of human monocyte-derived dendritic cells (DCs). Interaction of FITC-labeled exosomes with T cells and DCs was analyzed by flow cytometry and confocal microscopy. **Results**: T84-derived exosomes, enriched in CD9, CD81, CD82 and A33 antigen, were capable of binding specifically HSA 64-76 peptide on HLA-DR4 molecules and of interacting preferentially with DCs. HSA-loaded exosomes were unable to activate the T cell hybridoma directly, but induced a productive T cell activation through DCs. When HSA peptide was bound to exosomal HLA-DR4 molecules instead of in a soluble form, the threshold of peptide presentation by DCs was markedly decreased ($\times 10^{-3}$). **Conclusions**: Exosomes released by intestinal epithelial cells bear exogenous peptides complexed to MHC class II molecules and interact preferentially with DCs, strongly potentiating peptide presentation to T cells. Epithelial exosomes constitute a powerful link between luminal antigens and local immune cells by mediating the transfer of tiny amounts of luminal antigenic information and facilitating immune surveillance at mucosal surfaces.

INTRODUCTION

The intestinal epithelium provides a major interface between exogenous antigens present in the intestinal lumen and the extensive and important lymphoid tissue disseminated throughout the intestinal mucosa. Besides its role in absorption of nutrients and ionic equilibrium, the intestinal epithelium constitutes a barrier restricting the passage of incompletely digested dietary proteins and excluding infectious particles. Sampling of small amounts of luminal content by epithelial cells lining the intestinal lumen is required for the regulation of local immune responses. A small portion of the food antigens reaching the small intestine are endocytosed non-specifically by intestinal epithelial cells (IECs) and processed in their endo-lysosomal system. Their degradation products are released into the serosal compartment in the form of amino acids and peptides of molecular mass compatible with binding to MHC class II molecules (MHC II)[1]. This intracellular processing of exogenous proteins by IECs is in line with their antigen-presenting capacity. In inflammatory conditions in particular[2] but also in basal conditions[3], IECs express MHC II and co-stimulatory molecules[4], allowing antigen presentation to CD4^{+} T lymphocytes, at least *in vitro*. *In vivo*, IEC can occasionally develop projections through pores in the basement membrane[5] but direct contact with CD4^{+} T lymphocytes present in the *lamina propria* is limited. We previously reported that human colorectal carcinoma-derived IEC lines (HT29, T84) secrete exosomes[6] similar to professional antigen presenting cells (APC)[7, 8]. Epithelial exosomes are enriched in MHC II, CD26 and CD63 molecules and display the A33 antigen, an immunoglobulin (Ig)-like molecule[9, 10] specifically expressed in the basolateral membranes of IECs[11]. Epithelial exosomes convey antigenic information and activate humoral and cellular specific immune responses when administered intraperitoneally in mice[12]. The present study was undertaken to better understand the mechanisms through which epithelial exosomes transmit immune information from IEC to intestinal immune cells. Our results indicate that epithelial exosomes are

powerful sensors of luminal antigenic content, interact preferentially with dendritic cells and strongly potentiate antigen presentation.

MATERIALS AND METHODS:

Human intestinal cell lines

Epithelial exosomes were obtained from parental and stably transfected human T84 intestinal cell lines. T84 cell lines were engineered to express constitutively high levels of MHC class II molecules (HLA-DR) by transfection of cDNAs encoding either the HLA-DRB1*0401 (T84-DR4/CIITA) or the HLA-DRB1*1101 (T84-DR5/CIITA) molecules, together cDNA encoding the MHC class II transactivator, CIITA. These cell lines were provided by R.Hershberg[13]. Cells cultured as previously described[6] were seeded (5×10^6) into 75 cm² culture flasks and exosomes were isolated from cell culture supernatants, collected on day 5 and 7 after plating. In some experiments, cells were seeded on cell culture inserts (Falcon®, Becton Dickinson, USA) to collect apical and basolateral epithelial exosomes separately, as previously reported [6].

Preparation of exosomes from intestinal epithelial cell lines and molecular characterization

Cell culture supernatants from culture flasks were pooled and exosomes were isolated as previously described[6]. Each batch of epithelial exosomes was prepared from ten 75cm² culture flasks, giving about 60 µg of exosomes. Exosomal proteins were quantified using the Bradford protein assay (Bio-rad laboratories GmbH). Exosomes collected from culture supernatants of T84-HLA-DR4-CIITA, T84-HLA-DR5-CIITA or wild type T84 intestinal epithelial cells are referred as DR4 exos, DR5 exos and control exos, respectively. In some experiments, T84-HLA-DR4-CIITA or T84-HLA-DR5-CIITA epithelial cells were incubated in the presence of 10 µM (0.6 g/l) human serum albumin (HSA, Sigma Aldrich, France) for 48 h before the isolation of exosomes (HSA^+/DR4 or HSA^+/DR5 exosomes, respectively). HSA^-/DR4 exosomes were obtained in the absence of HSA in the culture medium.

Exosomal protein characterization was performed by Western blot analysis after SDS-polyacrylamide gel electrophoresis under reducing conditions. Blots of T84 intestinal cell lysates and T84-derived epithelial exosomes were incubated for 1 h with mouse monoclonal IgG1 antibodies recognizing tetraspanins CD9, CD81, CD82 (kindly provided by E. Rubinstein) or with the mouse anti-human A33 monoclonal antibody [14]. Primary antibodies were detected using peroxidase-conjugated sheep anti-mouse immunoglobulin (1/10,000) and ECL™ (Amersham Pharmacia Biotech).

Immuno-electron microscopy

Exosomes were placed on formvar carbon-coated EM grids and fixed for 20 min with 2% PFA, 0.2% glutaraldehyde (both from Electron Microscopy Sciences, Fort Washington, USA) in 0.1M phosphate buffer. Free aldehyde groups were quenched with 50 mM glycine in PBS and single immunogold labelings were performed as described[15]. Briefly, tetraspanins (CD9, CD81, CD82) or A33 antigen were labeled using antibodies described above followed by rabbit anti-mouse Ig (Dako, Danemark) and protein A conjugated to 10 nm gold particles purchased from Cell microscopy center (AZU, Utrecht, the Netherlands). Samples were observed under a Philips CM120 Electron Microscope (FEI Company, Eindoven, The Netherlands) equipped with a digital camera Keen View (SIS, Germany).

Immune cells

The EBV-transformed human B cell line (HLA-DR4)*,* kindly provided by S. Zucman (Hôpital Necker-Enfants Malades, France), was cultured in RPMI 1640 Glutamax medium (GIBCO, Life Technologies, USA) supplemented with 10% FCS, 1% sodium-pyruvate, 1% non essential amino-acids, 1% penicillin/streptomycin and 50 mM β-mercaptoethanol. Dendritic cells (DCs) were prepared from monocytes ($CD14^+$ cells) isolated from human PBMC [HLA-DRB1*0401 (HLA-DR4)] or other HLA-DR haplotypes, HLA-DRB1*1001*13 (DR10) and HLA-DRB1*1314 (DR13) by negative magnetic depletion (MACS, Miltenyi Biotec,

Germany) according to the manufacturer's instructions (> 90% purity of CD14^{+} cells). Monocytes were induced to differentiate into immature dendritic cells until day 6 in RPMI 1640 Glutamax medium supplemented with 10% FCS, 4 mM L-glutamine, 1% penicillin/streptomycin, 250 ng/ml GM-CSF and 30ng/ml IL-4 (R&D systems Europe, UK). DCs were collected and resuspended at 10^5 cells/200 µl per well in 96-well culture plates (Falcon, Becton Dickinson, USA) in complete culture medium supplemented with 250 ng/ml GM-CSF and 10 ng/ml IL-4, with or without 100 ng/ml LPS (from *Escherichia coli* 0127-B8, Sigma Aldrich, France) for 40 h, in order to obtain mature or immature dendritic cells (MDCs or IDCs), respectively.

In some experiments, the effect of exosomes on DCs maturation was measured by incubating immature DCs in the presence of 5µg of T84-DR4-exosomes. After 48 hour-incubation, maturation markers (HLA-DR, CD83, CD86) were assessed by flow cytometry. As a control, we used polystyrene polybead® microspheres of 0.1 µm diameter (Polysciences Europe, Eppelheim, Germany) incubated with DCs at various concentrations (10^9 to 10^{11} beads/ml).

As effector cells, we used the HSA 64-76 specific, HLA DR4-restricted T cell hybridoma 17.9 (kindly provided by R.Hershberg), generated after immunization of a DRA*DRB1*0401 transgenic mouse with HSA[16]. This hybridoma was cultured in RPMI 1640 Glutamax containing 10% FCS, 1% sodium pyruvate (GIBCO, BRL), 1% amino acids, 1% penicillin/streptomycin and 50mM β-mercaptoethanol. The T cell receptor of hybridoma 17.9 can interact with human APC expressing HLA-DR4/HSA 64-76 molecular complexes. The activation of the T cell hybridoma, which is fully dependent of TCR activation and does not require co-stimulation, was measured by the secretion of murine IL-2 (Duoset ELISA Development kit®; R&D systems, UK).

Binding of ^{3}H-HSA 64-76 on HLA-DR molecules of epithelial exosomes

HSA 64-76 is a peptide known to bind in the HLA-DR4 pocket and to stimulate the T cell hybridoma 17.9[16]. The direct binding of HSA 64-76 on HLA-DR4 molecules of epithelial exosomes was measured using a tritiated peptide. HSA 64-76 (VKLVNEVTEFAKT) was radio-labeled on the leucine residue. The peptide VK-dehydro-L-VNEVTEFAKT (1 mg) was radiolabeled in the presence of tritium gas and purified, yielding pure VK-3,4[^{3}H$_2$] L-VNEVTEFAKT peptide with a specific radioactivity of 0.37 TBq/mmole. In these conditions, the dehydro-leucine was replaced by a tritiated-leucine and the ^{3}H-HSA 64-76 peptide was strictly similar to the native peptide. As a negative control, we used ^{3}H-gliadin 57-68 (specific radioactivity: 0.40 TBq/mmole), a peptide specific for the HLA-DQ2 pocket but unable to bind HLA-DR4. This peptide was tritiated on a proline residue using the same method[17].

Exosomes (20µg) were incubated for 24 h at 37°C with 25 µM of ^{3}H-HSA 64-76 (total volume 100µl) or ^{3}H-gliadin 57-68, in phosphate buffer saline 0.1M, pH 7.4. Pellets (100,000g 1 h, 4°C) were washed four times with 1 ml PBS. The final supernatant was found to contain a negligible amount of radioactivity and discarded, and ^{3}H-radioactivity bound to pellets (exosomes) was measured using liquid scintillation photometry (Kontron betamatic, France). In some experiments, a 8-mer peptide from glutamic acid decarboxylase (GAD 556-572), known to bind HLA-DR4 molecules{Wicker, 1996 443 /id}, was used at increasing concentrations as a competitive inhibitor (x1, x10 and x25 fold the amount of HSA peptide). Non-specific binding of ^{3}H-HSA peptide was measured using control exosomes derived from the parental T84 cell line that does not express HLA-DR molecules under basal conditions.

Interaction of epithelial exosomes with immune cells

To characterize the binding and internalization of epithelial exosomes by different types of immune cells, FITC-labeled exosomes were incubated with B-EBV lymphocytes, Jurkat T cells, the T cell hybridoma 17.9 or human immature dendritic cells at 4°C (binding) or 37°C

(internalisation). The Fluoreporter FITC protein labelling kit (F-6434, Molecular probes) was used to label exosomes, according to the manufacturer's instructions. Exosomes were labeled (10µg/50µl) and the excess FITC reactive solution was removed by rinsing (x4) in 1ml PBS. The final washing supernatant was used as the negative control in the flow cytometry analysis.

T84-DR4/CIITA and T84-DR5/CIITA-derived exosomes (5µg) were incubated for 3 h at 4°C or for 30 min at 37°C with immune cells at 10^5 cells/well/100 µl in 96-well culture plates. Cells were rinsed twice in 200 µl PBS containing 5 mM EDTA and 5% FCS to remove weakly adherent exosomes and resuspended. Dendritic cells and B cells were then incubated with mouse monoclonal anti-human CD11c and monoclonal anti-HLA-DR (Tü36), respectively. T cell hybridoma 17.9 and Jurkat T cells were incubated with mouse monoclonal anti-human TCRβ chain antibody (H57-597) and anti-human CD3 (SK7) respectively, for 30 min at 4°C. Flow cytometry was performed with a BDLSR®using CELLquest® software (Becton Dickinson, USA).

Confocal microscopy

Binding and internalization of epithelial exosomes by dendritic cells and T cell hybridoma 17.9 was also analyzed by confocal microscopy. FITC-labeled exosomes were incubated with monocyte-derived immature dendritic cells and T cell hybridoma at 4°C (binding) or 37°C (internalisation). Cells were collected by centrifugation on glass slides (cytospin) and fixed with 3.7% formaldehyde. Dendritic cells were labeled with allophycocyanin (APC)-conjugated anti-CD45 (1/5, Becton Dickinson, Le pont de Claix, France) antibody (control isotype IgG1,k) and T cell hybridoma 17.9 was labeled with biotinylated anti-CD3 monoclonal antibody (IgG1, 20 µg/ml, Becton Dickinson) and revealed with streptavidin-APC (control isotype biotinylated mouse IgG1). Preparations were mounted using

Vectashield mounting medium containing propidium iodide (red nuclear staining, Vector Laboratories, France). Microscopic analysis was performed using a LSM 510 ZEISS laser scanning confocal microscope. The percentage of labeled cells was evaluated by analyzing a total of 400 DCs or T cells.

Antigen presentation assays

Direct presentation of HSA peptide to T cell hybridoma 17.9 by HSA-loaded exosomes

The capacity of epithelial exosomes to present HSA peptide 64-76 directly to the T cell hybridoma 17.9 was tested. Increasing amounts (0 to 15µg) of HSA-loaded-exosomes were incubated with 10^5 cells /200µl of the T cell hybridoma 17.9 in 96-well culture plates. After 24 h, T cell activation was measured by assaying murine IL-2 in the culture supernatants. As a positive control for antigen presentation, 5 x 10^4 T84-DR4-CIITA epithelial cells were incubated with HSA protein (10µM), or increasing concentrations of the HSA 64-76 peptide, before incubation with the T cell hybridoma.

Indirect presentation of HSA peptide to T cell hybridoma 17.9 by HSA-loaded exosomes

HSA-loaded exosomes were isolated from culture supernatants of T84-DR4-CIITA or -DR5-CIITA cell lines incubated with 10 µM HSA (HSA$^+$/DR4 exos or HSA$^+$/DR5 exos, respectively). Exosomes isolated from T84-DR4-CIITA cells incubated without HSA are referred as HSA$^-$/DR4 exos. Monocyte-derived immature or mature dendritic cells (IDCs or MDCs), predominantly of the HLA-DRB1*0401 (HLA-DR4) haplotype but also of other HLA-DR haplotypes (HLA-DR10 and DR13), were used as antigen presenting cells. The phenotype of the DCs was analysed by flow cytometry using FITC-conjugated monoclonal anti-CD1a (clone HI149; IgG1,k), FITC- or PE-conjugated anti-CD14 (clone MøP9; IgG2b,k), APC-conjugated anti-CD86 (clone FUN-1; IgG1,k), APC-conjugated anti-CD83

(clone HB15e; IgG1,k) and PE-conjugated anti-HLA-DR (clone TÜ36; IgG2b,k) all purchased from Becton Dickinson. DCs (10^5 cells/ml) were incubated for 48 h in the presence of increasing concentrations of HSA-loaded exosomes (1, 5, 10, 15 µg/200µl) or HSA peptide (0.025, 0.25, 2.5, 10, 15, 25 µM). These cells, referred as immature DCs (IDCs), were rinsed twice before incubation with 5 x 10^4 T cell hybridoma 17.9 cells (final volume 200µl). In some experiments, DCs were incubated for 2h in the presence of increasing concentrations of HSA peptide or exosomes, then LPS was added for 48h before incubation with T cells. These DCs are referred as maturing DCs (I/M DCs). Another group of DCs was incubated with LPS for 48h, rinsed twice and incubated with HSA-loaded exosomes or HSA peptide for 48h before incubation with T cells. These DCs are referred as mature DCs (MDCs). Culture supernatants were collected after 24 h and T cell activation was measured by murine IL-2 release.

Statistical analysis

The results are presented as mean ± standard error (SE). Multiple comparisons (analysis of variance) followed by group to group comparisons were performed using the general linear model procedure of the SAS package (SAS institute, Cary, NC). Non parametric Wilcoxon test was also used. Differences were considered to be statistically significant for values of $p < 0.05$.

RESULTS

Molecular characterization of epithelial exosomes

We have previously demonstrated using mass spectrometry analysis[6] that epithelial exosomes display antigen presenting molecules such as MHC class I and class II molecules as well as the A33 antigen, a marker of the basolateral membranes of intestinal epithelial cells. To further characterize the molecules expressed by epithelial exosomes, the presence of the tetraspanin molecules, CD9, CD81 and CD82 in T84-DR4/CIITA cell lysates and apical and basolateral exosomes was analysed by Western blot. As shown in Fig.1, all tetraspanins are highly enriched in exosomes compared to cell lysates, as also reported for exosomes from immune cells[19]. This enrichment is more pronounced in exosomes released from the basolateral side of epithelial cells. The presence of the intestinal epithelium-specific A33 antigen in epithelial exosomes, which we initially demonstrated using mass spectrometry, was confirmed here by Western blot analysis. The A33 antigen protein was found to be highly enriched in exosomes, particularly basolateral exosomes, compared to cell lysates. Tetraspanins and the A33 antigen were shown, using immunogold electron microscopy (IEM), to be exposed on the external surfaces of epithelial exosomes.

Exosomal HLA-DR4 molecules bind HSA peptide

Exosomes released by T84-DR4-CIITA cells constitutively express HLA-DR4 molecules. We have previously reported that epithelial exosomes derived from this epithelial cell line display MHC class II molecules in a SDS α/β compact form suggesting the presence of functional HLA-DR/peptide complexes[6]. As shown in Fig.2A, ^{3}H-HSA 64-76 binds to control exosomes derived from wild type T84 cells (21057 $\pm$ 3339 cpm), indicating non-specific interaction. However, the binding of HSA peptide to HLA-DR4 (41035 $\pm$ 5689 cpm) and HLA-DR5 (36956 $\pm$ 3669 cpm) exosomes is significantly higher than that observed in control exosomes

(p<0.01 and 0.04, respectively). In addition, the ^{3}H-HSA 64-76 binding is higher than that observed for ^{3}H-gliadin 57-68, a peptide specific of HLA-DQ2 molecules[20]. The relative binding of ^{3}H-HSA peptide to HLA-DR4 and HLA-DR5 molecules is in agreement with their theoretical affinity calculated using the RANKPEP method. The predicted binding of MHC-restricted ligand (the percentile score of HSA 64-76 relative to that of a consensus peptide yielding the optimal score) was 43.7 and 11.4 % for HLA-DR4 and HLA-DR5 molecules, respectively. Evidence of specific binding of HSA 64-76 to HLA-DR4 molecules was provided by an inhibition of 29% and 38% in the presence of increasing amounts (10x and 25x, respectively) of another HLA-DR4 specific peptide (GAD 556-572) acting as a competitor (Fig. 2B). Finally, the HSA peptide binds more efficiently to basolateral than to apical exosomes (Fig. 2C), a result which is consistent with the higher density of MHC class II molecules in basolateral compared to apical exosomes [6].

Preferential uptake of epithelial exosomes by dendritic cells

The capacity of FITC-labeled epithelial exosomes to bind to various types of immune cells such as B cells, T cells and DCs, was tested by flow cytometry. Fig.3A indicates that epithelial exosomes did not bind to Jurkat T cells and bound only weakly to the B-EBV cell line and T cell hybridoma 17.9 at 4°C, whereas a significant binding was observed on immature DCs. Increasing the temperature to 37°C induced a minor interaction of exosomes with the T cell hybridoma 17.9 but the internalization into DCs was more marked.

Furthermore, confocal microscopy indicated that immature DCs were capable of internalization of FITC-labeled exosomes at 37°C (Fig.3B,C) and that the interaction at 4°C was minimal (Fig.3D). The final rinse of the FITC labeling protocol (exos supernatant) provided negative controls (not shown). In contrast, exosomes bound to T cell hybridoma 17.9 at 4°C (Fig.3 G) but they were not internalized at 37°C and remained outside the cells

(Fig.3 E,F). Altogether, 42.9 % of dendritic cells and 43 % of T cells were labeled with FITC-exosomes at 37°C, but only DCs were capable of internalisation.

Lack of direct activation of T cell hybridoma by HSA-loaded epithelial exosomes

As exosomes could slightly bind to the T cell hybridoma 17.9, we tested their capacity to activate T cells directly through HLA DR4/HSA molecular complexes expressed on their surface. HSA-loaded exosomes were not able to induce activation of T cell hybridoma directly, as attested by the absence of IL-2 secretion (Fig. 4). In contrast, positive controls consisting of T84-DR4-CIITA epithelial cells pre-incubated with HSA protein (10µM) or increasing concentrations of HSA-64-76 peptide (0 to 25µM) were capable of activating the T cell hybridoma 17.9, as shown by the significant release of IL-2. Incubating T84-DR4-CIITA cells with pepsin-trypsin-hydrolyzed HSA did not allow T cell activation (data not shown), suggesting that the HSA 64-76 sequence was destroyed in HSA fragments.

Exosomes induce partial maturation of dendritic cells

DCs are initially immature cells capable of internalization and processing of environmental antigens. Under activation conditions, they are converted to mature DCs that have lost their internalization capacity but have up-regulated surface MHC class II- and co-stimulatory-molecules and can present antigens efficiently. The pattern of expression of various surface molecules on immature and LPS- or exosome- stimulated DCs is shown in Fig.5. During their differentiation to immature DCs, monocytes lost CD14 and acquired CD1a, a specific marker of DCs (data not shown). Immature DCs expressed CD1a and HLA-DR but not markers of maturation such as CD83 and CD86 (co-stimulatory molecules). LPS stimulation induced the markers of maturation CD83 and CD86, as expected. Interestingly, epithelial exosomes by themselves induced partial maturation of DCs as shown by the increase in CD83 and CD86 expression. This maturation was not due to LPS-contamination of exosomes, since polymyxin B, a LPS binding agent, did not inhibit the maturation process (data not shown). In addition,

incubation of immature DCs with inert polystyrene beads at high concentration (10^{11} beads/ml) could not induce maturation (Fig.5, last panel) nor could the final supernatant obtained during the exosome preparation and the soluble HSA 64-76 peptide alone (data not shown).

Epithelial exosomes strongly decrease the threshold of HSA peptide presentation by dendritic cells

Taking into account that epithelial exosomes were not capable of inducing T cell activation *per se* but that they could bind to, and were internalized by DCs, we investigated the ability of DCs to present HSA peptide 64-76 via HSA-loaded exosomes. Comparative results of HSA peptide presentation to the T cell hybridoma 17.9, by immature, maturing or mature HLA-DR4 DCs pulsed with soluble HSA peptide 64-76 are shown in Fig 6A. The results obtained with immature and maturing DCs were almost similar, indicating that the maturation process, in the presence of LPS, does not modify the lowest concentration of peptide able to stimulate T cells. Results obtained with immature, maturing and mature DCs pulsed with HSA$^+$/DR4 exosomes, HSA$^+$/DR5 exosomes or HSA$^-$/DR4 exosomes, are shown in Fig.6B,C,D. Immature DCs (IDCs) induced T cell activation in a concentration-dependent manner with a maximal effect at 25µM HSA peptide. The lowest concentration (threshold level) of HSA peptide capable of inducing T cell activation significantly was 2.5µM (IL2 = 1.12±0.31 versus 9.6±2.6 pg/ml in the presence of 0 and 2.5µM HSA peptide concentration respectively, p<0.002). Concentrations of 0.025 µM and 0.25µM induced IL2 release (2.27±0.69 and 3.63±1.44 pg/ml) not significantly different from control (1.12±0.31pg/ml) (Fig.6A). Noticeably, IDCs could activate the T cell hybridoma despite the absence of co-stimulatory molecules since the hybridoma does not require a co-signal for activation and release of IL-2. Meanwhile, MDCs incubated with increasing amounts of HSA peptide did not induce T cell

activation. The weak activation observed at the highest HSA peptide concentration (25μM) might be due to the HSA peptide loading on HLA-DR4 molecules exposed on the external surface of MDCs.

The incubation of HLA-DR4 IDCs cells with 1, 5, 10 or 15 µg HSA^{+}/DR4-exosomes led to the activation of the T cell hybridoma in a concentration-dependent manner (Fig.6B). The HSA peptide concentration equivalent to 1, 5, 10 or 15 µg of HSA^{+}/DR4 exosomes was estimated from the ^{3}H-HSA peptide loading experiments. By considering the specific activity of ^{3}H-HSA peptide 64-76 (0.11×10^{8} cpm/nmoles), we calculated the amount of HSA peptide loaded on epithelial exosomes by taking into account the radioactivity (cpm) bound to HSA^{+}/DR4-exosomes and subtracting the non-specific binding on control exosomes. This calculation indicated that 1, 5, 10 and 15µg of HSA^{+}/DR4 exosomes corresponded to 0.0002, 0.001, 0.002 and 0.003µM HSA peptide, respectively. The results indicate that HLA-DR4 DCs incubated with 15µg HSA^{+}/DR4 exosomes (containing 0.003µM HSA peptide) can activate the T cell hybridoma, whereas an identical concentration of soluble HSA peptide cannot. The threshold levels of HSA peptide capable of stimulating the release of ~15 pg/ml IL-2 by the T cell hybridoma, when presented via HSA^{+}/DR4 exosomes or as soluble HSA peptide, were approximately 0.002µM and 2.5µM, respectively. These results indicate that HSA^{+}/exosomes are one thousand times more efficient at stimulating the immune system than the HSA peptide alone. In the presence of HSA^{+}/DR5 exosomes, a slight activation of the T cell hybridoma was observed (Fig. 6C), suggesting a transfer of HSA peptide from HSA^{+}/DR5 exosomes, capable of binding the HSA peptide, to HLA-DR4 molecules on DCs. In addition, T cell activation was not induced when HLA-DR4 DCs were incubated with HSA^{-}/DR4 exosomes (Fig. 6D). In all experiments, IDCs and I/MDCs behaved similarly, due to the fact that activation of T cell hybridoma 17.9 is independent of co-stimulation via CD86. Finally, when DCs of HLA-DR10 or DR13 haplotypes were incubated with HSA^{+}/DR4 or

HSA$^+$/DR5 exosomes, T cell activation was not observed (Fig.6E, F), a result that strongly suggests that the HSA peptide is presented via DCs, rather than HLA-DR-bearing exosomes.

DISCUSSION

The results of this study reinforce the concept that epithelial cells are capable of releasing antigen presenting exosomes bearing MHC class II molecule/peptide complexes at their surface. Moreover, epithelial exosomes are able to transmit antigenic information to DCs and to highly potentiate immune responses by decreasing the threshold levels of antigen presentation required for activation.

Although M cells overlying Peyer's patches are considered to be a major pathway of antigen sampling in the gut, columnar epithelial cells lining the small intestinal mucosa are also able to endocytose and degrade proteins and to release protein fragments into the circulation[1]. Epithelial exosomes appear to be efficient intercellular communication vehicles allowing protein fragments to escape degradation during transepithelial transport and to transmit immune information to local intestinal immune cells.

The present study reinforces the notion that epithelial exosomes originate from late endosomal compartments, as they are strongly enriched in tetraspanins (CD9, CD81, CD82), like most types of exosomes[21]. Tetraspanins mediate cellular penetration, invasion and fusion events, and they are part of central supramolecular activation complex in APC[22]. They are also involved in signal transduction in immune cells[23, 24], interact with integrins and may be responsible for addressing of exosomes to target cells[25-27]. In addition, and as previously described for murine epithelial exosomes[12], human intestinal epithelial exosomes are highly enriched for the A33 antigen, an immunoglobulin-like protein largely restricted to the basolateral membranes of intestinal epithelial cells and whose function is as yet unknown.

Our *in vitro* experimental approach indicates that HSA peptide 64-76 can bind specifically to HLA-DR4 molecules expressed at the surface of intestinal epithelial exosomes. Indeed, although the ^{3}H-HSA peptide can bind non-specifically to parental T84 cell-derived exosomes that do not express HLA-DR molecules under basal conditions, it binds specifically to the

HLA-DR4 (and to a lower extent to the HLA-DR5) peptide pocket. In addition, competition with GAD 556-572, a peptide with a high affinity for the HLA-DR4 pocket, is capable of shifting the binding of radio-labelled HSA peptide. Finally, the low binding efficiency of gliadin peptide 57-68, a peptide specific for the HLA-DQ2 pocket, to DR4 exosomes, also suggests that HSA peptide binding is specific to HLA-DR4 molecules.

Although epithelial exosomes were shown to expose MHC II /peptide complexes at their surface, they could not, by themselves, induce T cell activation, whereas intestinal epithelial cells themselves could induce such activation. The absence of co-stimulatory molecules on epithelial exosomes[6] was not responsible for this lack of activation, however, since the T cell hybridoma 17.9, restricted for HLA-DR4 molecules, is capable of direct activation through the TCR, even in the absence of co-stimulation.

Although exosomes have been visualized at the surface of follicular DCs by electron microscopy[28], little is known about the capacity of these cells to internalize exosomes. Our results indicate that epithelial exosomes target DCs more efficiently than other immune cells, are internalized by them and induce their partial maturation. Consistent with these favored interactions, exosomes are shown here to induce indirect presentation of exogenous peptides bound to their HLA-DR molecules via DCs. Such indirect presentation is in line with that observed in DC-derived exosomes[29, 30] showing that exosomes can stimulate T cell responses after interaction with DCs. Indeed, DC-derived exosomes can be internalized and processed by immature DCs, for subsequent presentation of exosomal peptides to $CD4^+$ T cells[31]. Exosomes derived from MHC II-engineered rat mast cells also need DCs to efficiently activate T cells *in vitro*[32]. In our study, non-HLA-DR4 DCs were unable to present HSA peptide after incubation with HSA^+/DR4-exosomes. This suggests that DC- but not exosome-HLA-DR molecules are responsible for HSA peptide presentation to T cells. The involvement of DC HLA-DR molecules in antigen presentation is also suggested by the fact that

HSA[+]/DR5 exosomes can transmit HSA peptide to HLA-DR4 DCs, leading to DR4-restricted T cell activation. Taking into account that HSA[+]/DR5 exosomes, potentially bound to the DC surface, cannot trigger the HSA-specific, DR4 restricted, T cell hybridoma directly, a transfer of HSA peptide from exosome to the DC HLA-DR pocket, is suggested. The stronger T cell activation was obtained when exosomes were incubated with IDCs (compared with MDCs), indicating that exosome internalization is required to transmit immune information. Indeed, IDCs have a high endocytic capacity while MDCs have lost this capacity. In MDCs, the membrane expression of MHC class II/peptide complexes and co-stimulatory molecules is up-regulated compared to IDCs, enabling efficient antigen presentation. In our experimental setting, however, IDCs and I/MDCs behaved similarly and IDCs could present the HSA peptide due to their high expression of MHC class II molecules and the fact that we used a T cell hybridoma that did not require a co-stimulatory signal.

Epithelial exosomes induced partial maturation of DCs, as shown by the expression of the maturation markers CD86 and CD83. This maturation process was a specific phenomenon since inert beads of similar size did not induce such maturation. A similar maturation process was also reported in response to mast cell-derived exosomes[32] and exosomal heat-shock proteins (hsp) and their receptors (CD91) were suggested to be responsible for the DC maturation. However, a recent study indicated that at least in B cell-derived exosomes, hsps are located inside exosomes and cannot trigger DC maturation[33].

An important finding reported here is that epithelial exosomes considerably decrease the threshold level of antigenic stimulation required to activate the immune system. Indeed, the HSA peptide concentration leading to T cell activation was strongly decreased (x1000) when antigenic information was delivered by HSA[+]/DR4 exosomes rather than by soluble HSA peptide alone. T cell activation was not observed when HSA[+]/exosomes were incubated with mature DCs, indicating that exosome internalization and processing is necessary for HSA

presentation by DCs and that the binding of exosomes to the surface of mature DCs is not sufficient to induce HSA presentation. Rather, internalization of exosomes within DC endosomal compartments, known to be enriched in MHC II molecules and presenting an optimal microenvironment for peptide loading, may favor peptide exchange between exosomal and DC MHC II molecules.

Altogether, these results suggest that binding of dietary protein fragments to MHC class II molecules in intestinal epithelial cells allows the transfer of significant antigenic information to the local immune system, through the release of exosomes carrying MHC II/peptide complexes. We hypothesize that *in vivo*, epithelial exosomes transmit antigenic information from the intestinal lumen to DCs distributed within the lamina propria. The proposed mechanism for the interaction between epithelial exosomes and DCs is schematically depicted in Fig.7. DCs may internalize epithelial exosomes released from the basal surface of IECs that diffuse towards the lamina propria. This could facilitate the exchange of peptide from HLA-DR molecules on exosomes to HLA-DR-bearing DCs. Due to the very low threshold levels of exosome-linked antigenic material required to stimulate immune cells, epithelial exosomes may constitute a powerful vehicle involved in intestinal antigen presentation.

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Figure legends

Figure 1: Detection of tetraspanins and the A33 antigen in T84-DR4/CIITA cell-derived exosomes.

Western blots indicate that CD9, CD81, CD82 and A33 antigen are expressed by apical (ApE) and basolateral (BasE) exosomes and that all markers are highly enriched in exosomes, especially in basolateral exosomes, compared to cell lysates (CL). Immunogold electron microscopy (IEM) of apical and basolateral exosomes indicates an external, thereby potentially functional, localization of these markers (bars = 50 nm).

Figure 2: Exosomes can load HSA peptide on their HLA-DR4 molecules.

(A) Binding of ^{3}H-HSA 64-76 and ^{3}H-gliadin 57-68 on HLA-DR4-expressing, HLA-DR5-expressing and control exosomes. A significant increase in the binding of ^{3}H-HSA peptide is observed with HLA-DR4-exosomes, and to a lesser extent with HLA-DR5-exosomes, compared to control exosomes (*$p<0.01$ and # $p< 0.04$, respectively). Each result is the mean of 3 to 9 experiments (mean±SE).

B) Competitive inhibition of ^{3}H-HSA peptide 64-76 binding to HLA-DR4 molecules by increasing concentrations of another HLA-DR4-specific peptide (GAD 556-572). Results are the mean of 2 independent experiments.

(C) Differential binding of ^{3}H-HSA peptide to apical and basolateral exosomes. Increased binding of HSA peptide to basolateral, compared to apical, exosomes was observed, consistent with the higher density of MHC class II molecules on basolateral exosomes [6].

Figure 3: Exosomes interact preferentially with DCs

(A) Flow cytometry analysis of the interaction between FITC-exosomes and B-EBV cells, T cells (Jurkat and T cell hybridoma 17.9) or immature dendritic cells (DCs). FITC-exosomes

were incubated for 3 h at 4°C or 30 min at 37°C with immune cells and binding (4°C) and internalization (37°C) were quantified (black line). Control values (shaded area) were provided by the final supernatant from the FITC-exosomes preparation. Binding experiments (4°C) indicate that DCs bind FITC-exosomes more efficiently than other immune cells. Greater uptake of exosomes (37°C) by DCs compared to poorly internalizing B or T cells was observed. One set of data representative of three separate experiments.

(B to D) Confocal microscopy indicates internalisation of exosomes by dendritic cells. Analysis of interactions between FITC-exosomes (green) and DCs (CD45-APC, blue, red nuclei) indicates that DCs can efficiently internalize FITC-exosomes at 37°C (B, C) but not at 4°C (D). The final supernatant from the FITC-exosome preparation provided negative controls (not shown).

(E to G) T cell hybridoma 17.9 was capable of binding FITC-exosomes at 37°C, but no internalisation could be observed.

Results are representative of two independent experiments.

Figure 4: Absence of direct activation of T cells by epithelial exosomes.

Activation of the T cell hybridoma 17.9 was measured by IL-2 release after 24 h incubation with increasing amounts of HSA$^+$/DR4 exosomes. As a positive control for antigen presentation, the T84-DR4-CIITA epithelial cell line was incubated for 24 h with increasing concentrations of HSA peptide or with intact HSA, before incubation with T cell hybridoma. The results indicate that exosomes, in contrast to epithelial cells, cannot activate the T cell hybridoma directly. Data are means ± SE of three separate experiments.

Figure 5: Effect of epithelial exosomes on DC maturation.

Flow cytometry analysis of immature DCs cultured in the absence or presence of LPS or epithelial exosomes. LPS induces the expression of maturation markers such as CD86 and CD83 as expected. Epithelial exosomes also partially induce maturation of DCs as shown by the increased expression of maturation markers. Exosome-induced maturation of DCs was not due to LPS-contamination of exosomes, since the addition to exosomes of polymyxin B, a LPS binding agent, did not inhibit the maturation process. In addition, polystyrene polybead® microsphere of 0.1 µm diameter (similar to exosome diameter) at concentrations up to 10^{11} beads/ml (result shown: 10^{11} beads/ml) did not induced DCs maturation, indicating that the maturation process induced by exosomes is a specific phenomenon.

Blank histograms: antibody labeling, solid histograms: isotype controls.

Figure 6: Epithelial exosomes transmit HSA peptide and potentiate its presentation by dendritic cells.

(A): DR4-DCs present HSA peptide to the T cell hybridoma. Immature, maturing or mature DCs (IDCs, I/MDCs, MDCs) were incubated for 48 h with increasing concentrations of HSA peptide. Immature, maturing and to a much lesser extent mature DCs induce a concentration-dependent T cell activation with a maximal effect observed with 25 µM HSA peptide. The threshold concentration of peptide required to stimulate significantly T cell activation is 2.5 µM.

Data are the means ±SE of 6 independent experiments.

(B): DR4-DCs present HSA peptide loaded on epithelial exosomes to T cell hybridoma. Immature DR4-DCs incubated in the presence of increasing concentrations of HSA[+]/DR4 exosomes (1 to 15 µg) induce a dose-dependent T cell activation (the corresponding HSA peptide concentrations bound to exosomes are indicated in parentheses). Under these

conditions, the threshold of peptide concentration stimulating T cell activation is approximately 0.002 µM. Thus, HSA-loaded exosomes are much more efficient than soluble HSA peptide in activating the T cell hybridoma.via DCs.

(C): DR4-DCs incubated with HSA[+]/DR5 exosomes are able to induce activation of the T cell hybridoma. Increasing concentrations of HSA[+]/DR5 exosomes induce less IL-2 secretion than that observed with HSA[+]/DR4 exosomes, consistent with the lower affinity of HSA peptide for HLA-DR5 compared to HLA-DR4 molecules. This result also indicates that the HSA peptide is transferred from HLA-DR5 molecules on exosomes to HLA-DR4 molecules on DCs. Data are the means ± SE of 6 independent experiments.

(D): DR4-DCs incubated with HSA[-]/DR4 exosomes (prepared in the absence of HSA), are unable to induce activation of T cells.

(E): DR10- or DR13-DCs incubated with HSA[+]/DR4 exosomes do not activate T cells indicating that HLA-DR4 molecules of dendritic cells are necessary to HSA peptide presentation (results are means of two experiments).

(F): DR10-DCs incubated with HSA[+]/DR5 exosomes do not activate T cells.

Figure 7: Hypothetical model of peptide transfer from epithelial exosomes to DCs. Intestinal epithelial cells (IECs) release exosomes bearing MHC class II/peptide complexes that interact with immature DCs. After binding at the DC surface, they are endocytosed and conveyed to multivesicular body (MVB) compartments where peptide exchange from exosome to DC-harboring MHC class II molecules can take place. Peptide-loaded MHC II complexes are then exposed at the DC plasma membrane to trigger activation of CD4^{+} T cells.
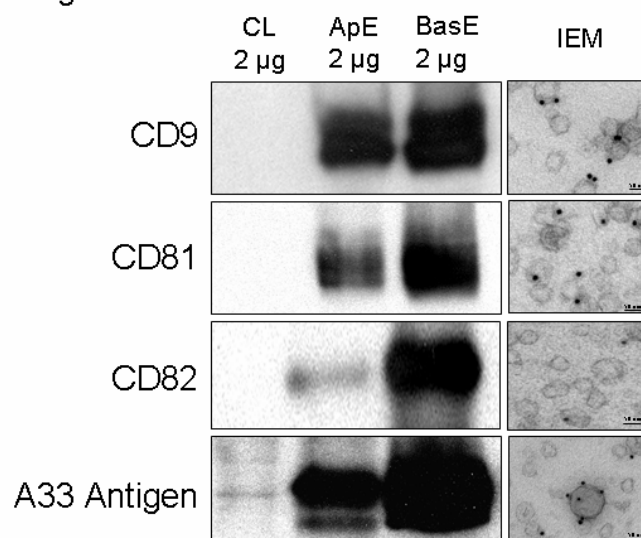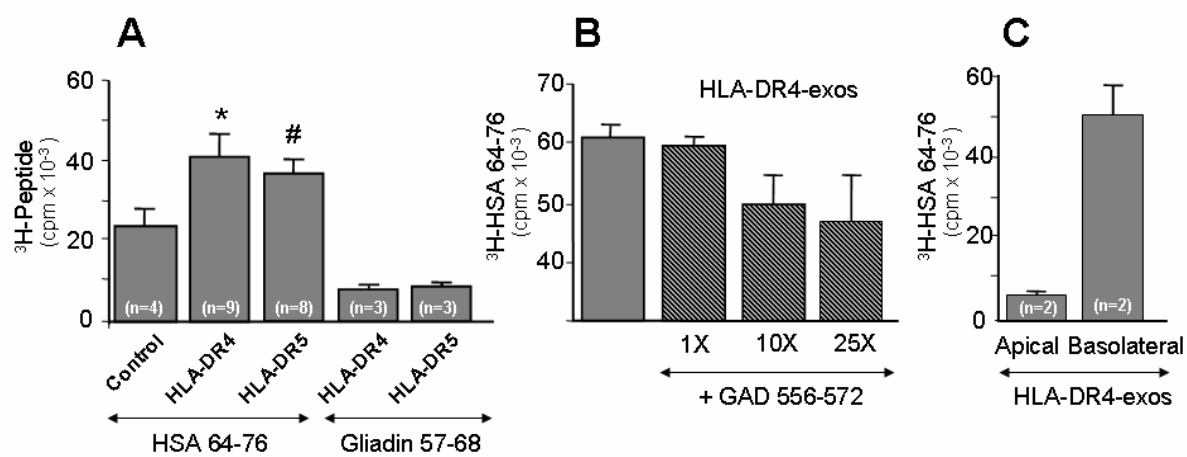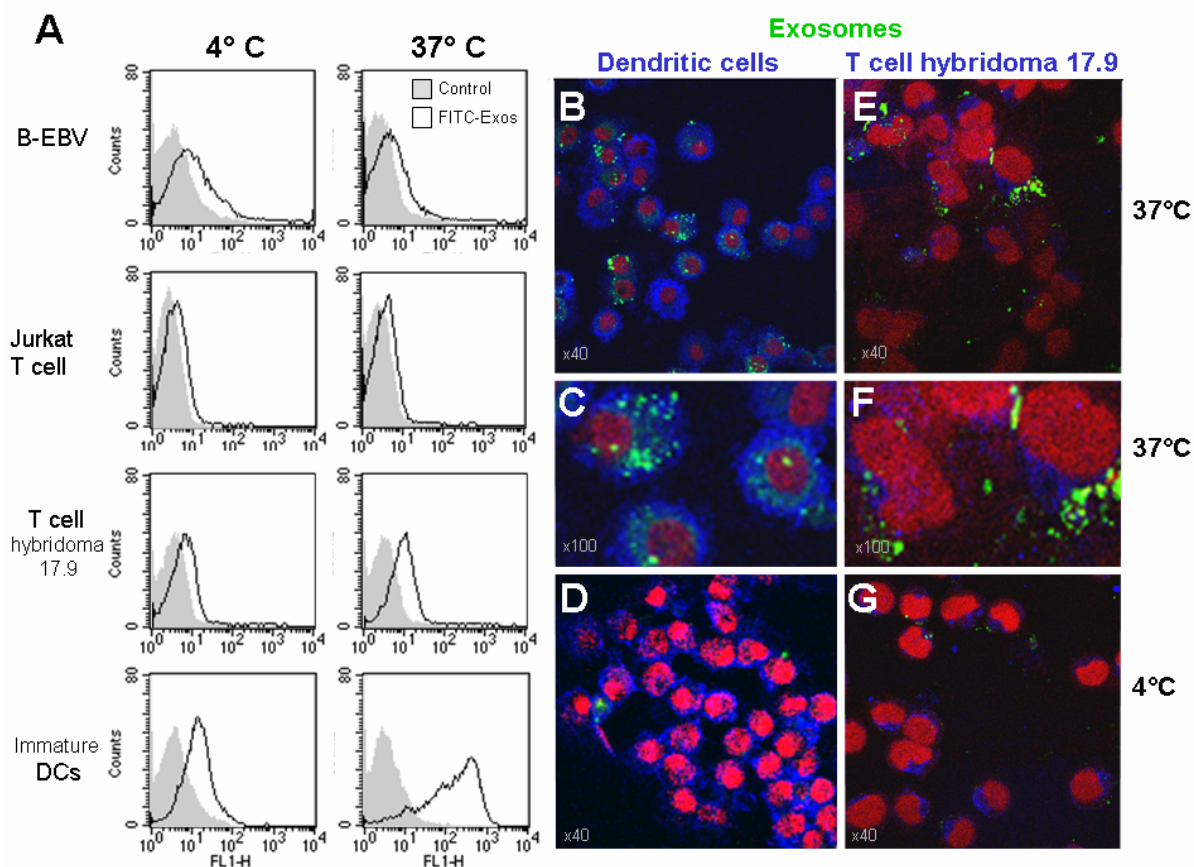
Figure 1

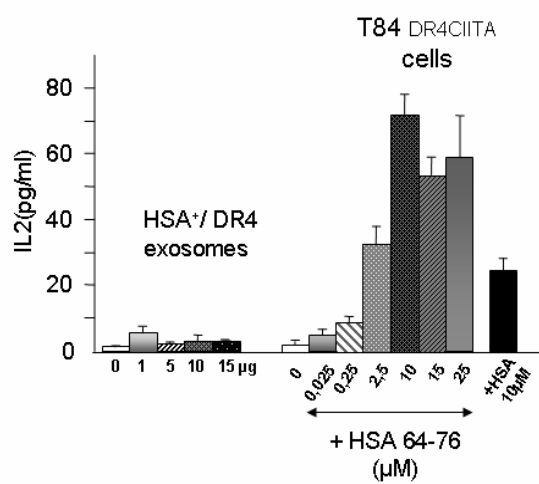

Figure 2

Figure 3

Figure 4

Figure 5

Immature DCs
IDCs

IDCs+LPS

IDCs+exosomes

IDCs+
polystyrene beads
(Control)

Figure 6

Figure 7

Exosome
binding to DC

MHC II
(DC)

Exosome
endocytosis

DC

MVB

Transfer of peptide
to DC MHC II

CD4⁺ T

Lack of exposure of exosomal MHC II/peptide
complexes at DC surface