Immunology and Cell Biology (2011), 1–8

www.nature.com/icb

ORIGINAL ARTICLE

Cross-presentation of epitopes on virus-like particles via the MHC I receptor recycling pathway

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Effective vaccines and immunotherapies against cancer require professional antigen-presenting cells to cross-present exogenous antigen to initiate cytotoxic T-cell responses to destroy tumors. Virus-like particles (VLPs), containing tumor antigens, which can immunize against cancers, are cross-presented by dendritic cell (DC) but the mechanism by which this occurs is not fully understood. Here, we used VLPs, derived from rabbit hemorrhagic disease virus (RHDV) with both murine and human DCs, to elucidate these pathways. We have employed inhibitors to demonstrate that these VLPs are taken up by clathrin-dependent macropinocytosis and phagocytosis before being degraded in acidic lysosomal compartments. VLP-derived peptides are loaded onto major histocompatibility complex I that have been recycled from the cell surface. Antigen-coupled VLPs and murine ovalbumin-specific and human melanoma-associated antigen recognized by T cells (MART-1)-specific CD8$^+$ T cells were used to demonstrate cross-presentation via this alternate, receptor recycling pathway, which operated independently of the proteasome and the transporter-associated with antigen presentation. Finally, we found that cross-presentation of VLPs *in vivo* was not confined to CD8α^+ DC subsets. These data define the cross-presentation pathway for RHDV VLPs and may lead to improved cancer immunotherapies.
Immunology and Cell Biology advance online publication, 11 January 2011; doi:10.1038/icb.2010.161

Keywords: antigen processing; cross-presentation; dendritic cells; virus-like particles

The incidence of cancer is increasing annually and while systemic treatment for some cancers has improved significantly in recent years, non-resectable metastatic disease remains incurable. For other tumors such as melanoma there are no effective non-surgical therapies. Virus-like particles (VLPs) formed from viral capsid proteins are stable, non-replicative and highly immunogenic so they are a safe option for the treatment of cancer patients. They can act as vehicles for the delivery of heterologous antigens to the immune system generating strong cytotoxic T-cell responses. Model tumor antigens chemically coupled to rabbit hemorrhagic disease virus VLP (RHDV VLP)[1] have been shown to delay or prevent the development of tumors *in vivo* using an aggressive model of murine melanoma.[2]

Immune-mediated prevention or regression of tumors is associated with a strong CD8$^+$ T-cell response directed against a variety of defined and non-defined tumor antigens. Dendritic cells (DCs) have a key role in the induction and maintenance of antitumor T-cell responses because of their ability to take up antigens, process them into peptides and present them to CD8$^+$ T cells on major histocompatibility complex (MHC) I in a process known as cross-presentation.

Cross-presentation of exogenous antigens by DC has been shown to operate using three distinct pathways. The first two pathways both utilize the endogenous antigen-processing machinery to generate antigenic peptides. In the endosome-to-cytosol pathway, antigens make their way into the cytosol in which they are degraded by the proteasome before being translocated into the endoplasmic reticulum by the transporter associated with antigen presentation (TAP). Here, the peptides are loaded onto nascent MHC I molecules for presentation to CD8$^+$ T cells.[3,4] The peptide antigens may also be loaded onto MHC I in an intracellular compartment formed through the fusion of a phagosome and the endoplasmic reticulum (ER), which contains the MHC I-loading complex.[5–7] Alternately, endocytosed antigens can be processed independently of the proteasome and the TAP transporter using a pathway whereby antigens are processed into peptides and loaded within the endosomal/lysosomal system.[8,9]

The protection against tumors induced by VLP shown in Peacey *et al.*[2] is thought to be mediated by cross-presentation to CD8$^+$ T cells and several studies have demonstrated that some types of VLP are cross-presented using a proteasome- and TAP-independent mechanism but the mechanism this entails has not been clearly defined.[10–12]

Here, we have translated the observation of VLP cross-presentation previously reported[2] into a more relevant human system and subsequently characterized the pathways used by DC to endocytose VLP coupled to model antigens, process them into peptides and cross-present them to antigen-specific CD8$^+$ T cells. Furthermore, we have explored the potential to utilize human tumor lysates as a source of undefined antigen to couple to VLP to induce tumor-specific CD8$^+$ T-cell responses *in vitro*.

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Received 9 September 2010; revised 7 November 2010; accepted 9 November 2010

RESULTS

RHDV VLP rapidly associate with antigen-presenting cells (APCs) derived from murine bone marrow and spleen

The role of both innate and acquired immune cells in stimulating or suppressing responses effective against tumors has been widely documented with many agreeing that the development of cytotoxic $CD8^+$ T-cell responses are crucial to the destruction of tumors. To identify which immune cells initially associate with RHDV VLP and have the potential to define the outcome of the immune response, we labeled the VLP with a fluorescent molecule, dylight488 (VLP-dylight488) and pulsed these onto cells isolated from murine spleen and bone marrow. After 24 h, the cell populations were analyzed flow cytometrically for their ability to associate with VLP. The greatest amount of VLP was taken up by DC ($CD11c^+$) and macrophages ($F4/80^+$) from bone marrow (Figures 1a and b). B cells ($B220^+$) also took up VLP, but to a lesser degree while NK cells ($NK1.1^+$) did not. Spleen-derived populations of cells did not associate with VLP as effectively although splenic DC, macrophages and B cells did fluoresce positively for VLP.

Uptake into DC is mediated by macropinocytosis and phagocytosis but not receptor-mediated endocytosis

As DCs are known to be potent inducers of cytotoxic T-cell responses through cross-presentation of exogenous antigens, we focused on this cell population and the methods they use to internalize antigens. There is evidence that suggests uptake by phagocytosis or mannose receptor-mediated endocytosis precludes cross-presentation by stabilizing these antigens in early endosomal compartments while uptake by macropinocytosis or scavenger receptor-mediated endocytosis delivers antigens into late endosomes/lysosomes for loading onto MHC I.[13,14] To initially investigate how RHDV VLP are internalized by DC, immature DC were generated *in vitro* from the bone marrow of C57BL/6 mice. DC were cultured with VLP-dylight488 and analyzed by flow cytometry to determine the extent of VLP-dylight488 uptake. Uptake of RHDV VLP by DC occurred rapidly with 95% of $CD11c^+$ DC internalizing RHDV VLP within 1 h while $<5\%$ of control DC cultured at 4 °C took up RHDV VLP (flow cytometric and confocal microscopy data not shown).

To identify the mechanisms involved in RHDV VLP uptake, DC were treated with a range of inhibitors, washed thoroughly then pulsed with VLP-dylight488 for 1 h and analyzed using flow cytometry (Figures 1c and d). The inhibitors used were amiloride, which prevents macropinocytic activity in DC or cytochalasin D, which inhibits phagocytosis. Receptor-mediated uptake of RHDV VLP was assessed using DC treated with heparin while scavenger receptor-mediated uptake was inhibited generally by treating cells with poly I:C. Both amiloride- and cytochalasin D-treated DC were significantly reduced in their ability to internalize VLP while the DC treated with heparin or poly I:C showed no differences compared with the untreated cells. Propidium iodide staining of the DC showed there were no differences in the numbers of dead cells between treated and untreated DC (data not shown). These data indicate that DC rely on macropinocytosis, predominantly, and phagocytosis to internalize VLP while specific receptors have no overt role.

Processing of antigens coupled to RHDV-VLP is TAP and proteosome independent and the derivative peptides are loaded onto MHC-I recycled from the cell surface

The induction of strong cellular immune responses to RHDV VLP with *in vivo* mouse models of melanoma suggests that these VLP are effectively processed and presented by APCs to $CD8^+$ T cells. It is this

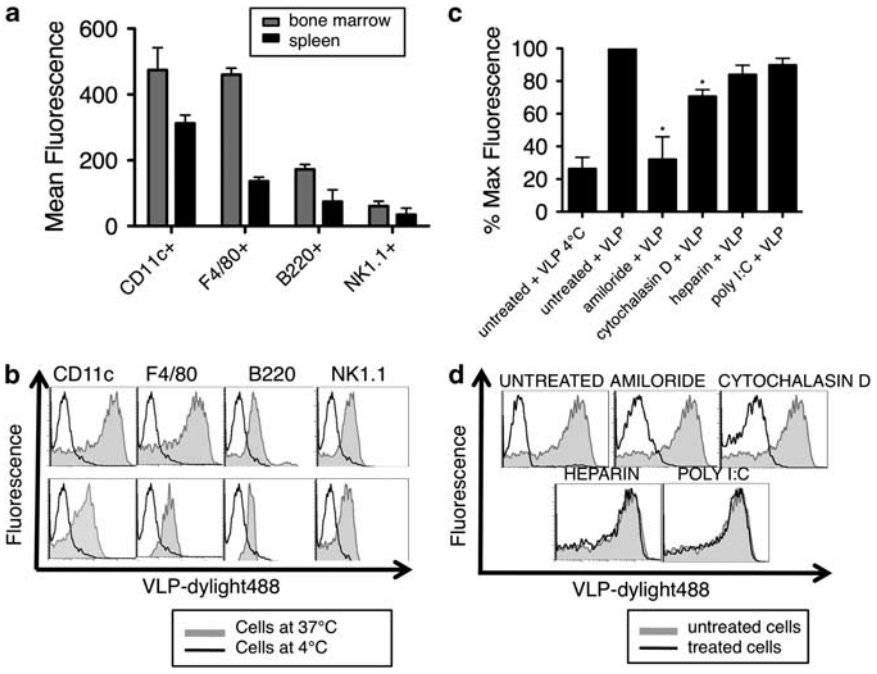

Figure 1 VLP associate with cells derived from murine bone marrow and spleen expressing CD11c, F4/80, B220 or NK1.1. (**a**) Cells isolated from murine bone marrow (grey bars) or spleen (black bars) were pulsed for 24 h with VLP-dylight488 and binding was analyzed by flow cytometry. Background fluorescence of cells at 4 °C was subtracted for each condition. Results show uptake from cells of three mice ± s.d. (**b**) Histograms show cells from bone marrow (top panel) or spleen (lower panel) pulsed with VLP-dylight488 at 4 °C (open curves) overlayed with cells pulsed at 37 °C (shaded curves). (**c**, **d**) Flow cytometric analysis of the mechanism of uptake of VLP by murine $CD11c^+$ bone marrow-derived DCs after 1 h. Bars represent the percentage of VLP uptake relative to the untreated control at 37 °C ± s.d. Histograms show untreated cells (shaded curves) overlayed with cells treated with inhibitors or cultured at 4 °C (open curves). Representative of four independent repeats. * statistically significant differences in the uptake of VLP by treated cells compared with untreated cells ($P<0.01$).

cross-presentation of RHDV VLP that mediates the antitumor effects observed in our previous studies.[2] Here, we firstly demonstrated that murine DC could efficiently cross-present VLP that had either ovalbumin (OVA) or SIINFEKL (the CTL epitope from OVA) coupled to them. These DC were then co-cultured with OT-1 T cells, which recognize this peptide. Both VLP–OVA and VLP–SIINFEKL could induce the antigen-specific activation of these T cells to proliferate (data not shown) and produce interferon gamma (IFN-γ) (Figure 2a). These results indicate that the VLP was being cross-presented on MHC I. Notably, the total amount of OVA delivered on VLP was more than twofold less than OVA delivered alone.

We then elucidated the mechanisms used by these cells to activate naïve MHC class I restricted ovalbumin-specific T cell receptor transgenic (OTI) T-cell responses to exogenous VLP–OVA or VLP–SIINFEKL. DC were treated with a range of inhibitors that block various stages in the cross-presentation and traditional exogenous antigen presentation pathways. The viability of the DC following treatment was determined by propidium iodide staining, which showed no differences in the numbers of dead cells between treated and untreated DC (data not shown). The inhibitors used were lactacystin, which prevents protein degradation by the proteasome, US6 binds the TAP transporter preventing translocation of peptides into the ER while brefeldin A binds adenosine diphosphate (ADP)-ribosylation factor proteins required for sorting and secretion of vesicles. Bafilomycin A1 prevents the acidification of lysosomes and the activation of proteases required for the generation of peptides, while primaquine disengages endosomal trafficking and the recycling of molecules from the cell surface. DC pretreated with brefeldin A, primaquine or bafilomycin A1 and pulsed with VLP–OVA (Figure 2b) or VLP–SIINFEKL (Figure 2c) were unable to activate OVA-specific $CD8^+$ T cells while treatment of the DC with either lactacystin or US6 showed minimal abrogation of the response. This strongly suggests that VLP-antigen conjugates are processed into peptides in acidified lysosomes and are loaded onto MHC I molecules that have been recycled from the cell surface. There is also some requirement for secretion of vesicular proteins as seen with inhibition by brefeldin A treatment. Furthermore, the proteasome and TAP transporter, which are necessary for cross-presentation of OVA are not required for processing and presentation of VLP-antigen conjugates. When DC were pulsed with OVA alone there was a reduction in T-cell activation observed with all inhibitors, indicating that this protein is processed through a variety of routes, as has been described previously[3] (Figure 2b) while the presentation of SIINFEKL peptide alone was not impeded by any inhibitor (Figure 2c) thereby indicating that the peptide does not require processing for effective presentation. To rule out the possibility that sub-optimal doses of inhibitors led to partial inhibition of cross-presentation of OVA and thus the impression of cross-presentation by two pathways, dose response experiments were carried out. No significant decreases in responses were seen with higher concentrations of inhibitors without a concurrent loss of DC viability (data not shown).

Cross-presentation of RHDV VLP by murine DCs generates cytotoxic T-cell responses *in vivo*

Cross-presentation of OVA and cell-associated antigens has been associated exclusively with $CD8\alpha^+$ subsets of DC in mice[15,16] but because of the divergent processing of VLP-antigen conjugate compared with OVA protein alone, this may not be the case for these VLP. To assess this, we utilized an *in vivo* method of cross-presentation.[17] C57BL/6 mice were injected intravenously with cytochrome c, which is taken up by all DC but during cross-presentation by $CD8\alpha^+$ subsets, cytochrome c enters the cytoplasm causing apoptosis, which results in a loss of $CD8\alpha^+$ DC and as a consequence, a loss of $CD8^+$ T-cell

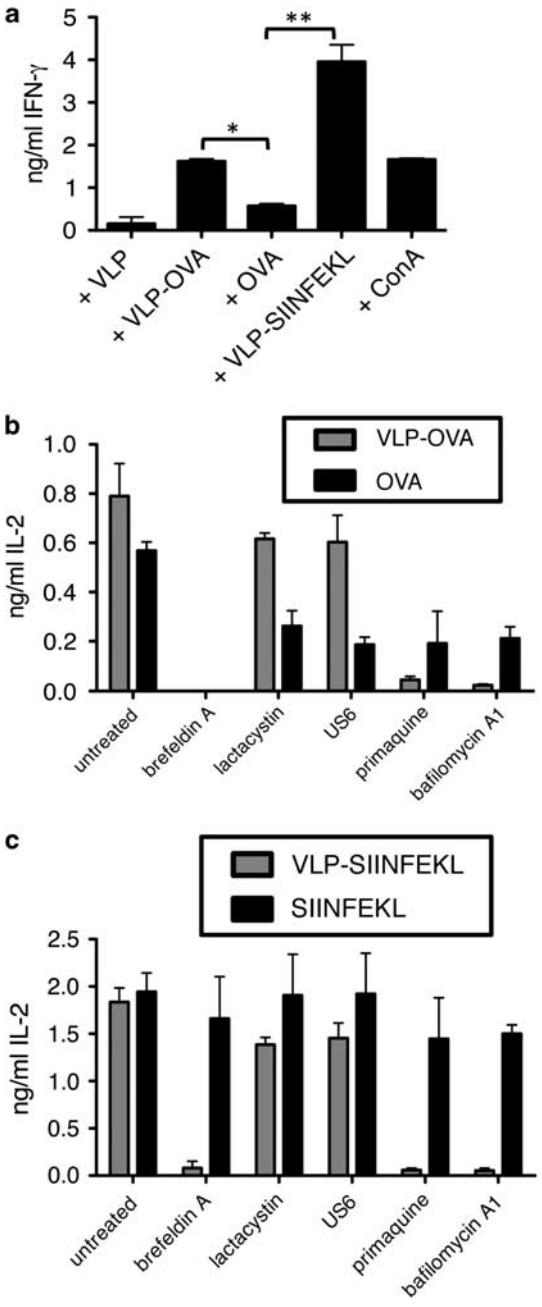

Figure 2 Cross-presentation of VLP by murine DCs generates cytotoxic T-cell responses *in vitro*. (**a**) Murine DCs were pulsed with antigen for 24 h before co-culture with OTI T cells. IFN-γ levels were measured by enzyme-linked immunosorbent assay (ELISA) at 72 h and are representative of the mean of four independent experiments performed in triplicate ± s.d. * and ** statistically significant differences in T-cell stimulation by VLP–OVA or VLP–SIINFEKL-pulsed DC compared with OVA pulsed DC ($P<0.01$ and 0.005). (**b**) Murine DCs, treated for 15 min with inhibitors, were pulsed with VLP-OVA (grey bars) or OVA (black bars) for 24 h before co-culture with OTI T cells. Bars represent the levels of interleukin (IL)-2 produced by OTI T cells after 48 h ± s.d. Data represents four independent experiments performed in triplicate. (**c**) Murine DCs, treated for 15 min with inhibitors, were pulsed with VLP-SIINFEKL (grey bars) or SIINFEKL peptide (black bars) for 24 h before co-culture with OTI T cells. Bars represent the levels of IL-2 produced by OTI T cells after 48 h ± s.d. Data represents four independent experiments performed in triplicate.

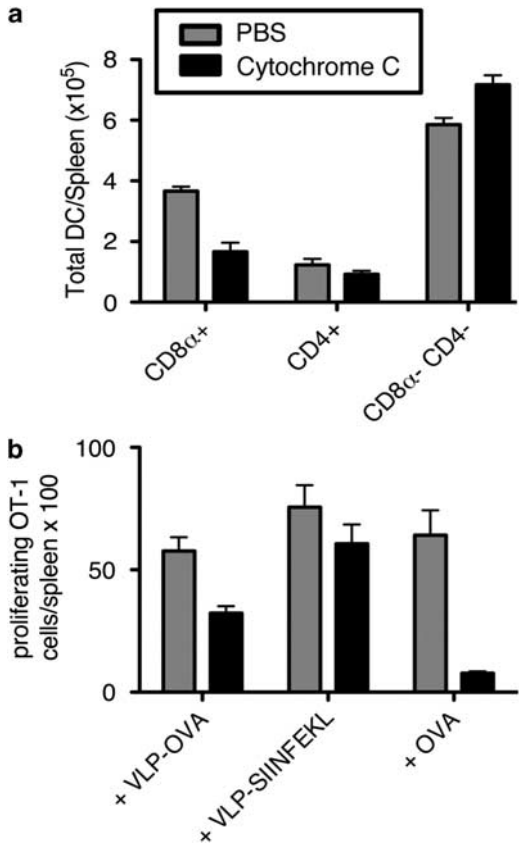

Figure 3 Cross-presentation of VLP by murine DCs generates cytotoxic T-cell responses *in vivo*. (**a**) Flow cytometry of splenic DCs 24 h after intravenous (i.v.) administration of 5 mg of cytochrome c (black bars) or PBS (grey bars). Spleen cells were isolated and stained with fluorescein isothiocyanate (FITC)-CD11c, phycoerythrin (PE)-CD8α and APC-CD4 antibodies. Absolute $CD11c^+$ DC numbers per spleen are shown as mean ± s.d. Data are representative of two mice per group. (**b**) CFSE-labeled OTI T cells were injected i.v. before depletion of DC with three daily doses of cytochrome c or PBS control. VLP–OVA, VLP–SIINFEKL or OVA were administered i.v. and OTI proliferation was measured after 60 h. Grey bars indicate mice treated with PBS while the black bars indicate mice treated with 5 mg cytochrome c. Data shows the proliferating OTI cells per spleen, mean ± s.d. for two mice per group.

activation. Consistent with observations of Lin,[17] we found that the $CD8\alpha^+$ DC were selectively depleted while $CD4^+$ and $CD8\alpha^-CD4^-$ DC populations remained largely unaltered (Figure 3a). Furthermore, we observed that the OTI T-cell responses to OVA in cytochrome c-treated mice were significantly diminished compared with phosphate-buffered saline (PBS)-treated mice suggestive of marked functional changes. Interestingly, we observed no differences in the OTI proliferative responses between cytochrome c-treated and control mice wherein VLP–SIINFEKL was the antigen while only minor decreases were seen in the cytochrome c-treated mice administered VLP–OVA (Figure 3b). These results further support the role of the receptor recycling, antigen-processing pathway of DC in the cross-presentation of VLP and may suggest that the ability of DC to carry out this function *in vivo* is not solely restricted to $CD8\alpha^+$ subsets.

VLP rapidly accumulate inside human monocyte-derived DCs by nonspecific uptake mechanisms

In order to evaluate the effectiveness of the RHDV VLP as a vaccine scaffold for use in humans, it was necessary to translate the initial results we observed in the mouse models into cells derived from humans.

To investigate how RHDV VLPs were internalized by human DC, immature DCs were generated *in vitro* from monocytes of healthy donor peripheral blood mononuclear cells (MoDC). MoDC were cultured with VLP-dylight488 and analyzed over time by flow cytometry to determine the extent of VLP-dylight488 uptake. Uptake of VLP-dylight488 by MoDC occurred rapidly with 93% of $CD11c^+$ DC positive for VLP within 1 h. Only 3% of control MoDC cultured at 4 °C was positive for VLP indicating that active uptake is prevented and the presence of very low nonspecific association with the cell surface (Figure 4a, Supplementary Figure 1). The MoDC continued to accumulate VLP-dylight488 to high levels for 48 h.

To identify the mechanisms involved in RHDV VLP uptake, human MoDC were treated with inhibitors of bulk uptake mechanisms, namely amiloride, cytochalasin D or chlorpromazine, which is an inhibitor of clathrin processing. The amount of VLP-dylight488, measured by mean fluorescence, was significantly reduced by 61, 69 and 64% in MoDC that were pretreated with amiloride, cytochalasin D and chlorpromazine, respectively (Figure 4b). This indicates that DC use macropinocytosis and phagocytosis primarily to internalize VLP-dylight488 and this process is dependent, in part, on the development of clathrin-coated pits. Staining of DC with propidium iodide indicated no differences in the numbers of dead cells between treated and untreated DC (data not shown) confirming that the inhibitors were not having toxic effects.

Next, we assessed whether RHDV VLP were taken up specifically by cellular receptors on DC known to be involved in antigen uptake. Receptor-mediated uptake of RHDV VLP was gauged using DC treated with heparin, anti-CD206 antibody, poly I:C or anti-scavenger receptor B1 (anti-SR-B1) antibody. These results suggest that specific receptors are not involved as indicated by the inability of heparin, poly I:C, anti-SR B1 or anti-CD206 antibodies to impair VLP-dylight488 uptake (Figure 4b).

These data suggest that VLP are efficiently taken up by MoDC through mechanisms, which do not seem to involve specific receptors.

DCs load exogenous VLP-derived antigen onto MHC I recycled from the cell surface to initiate cytotoxic T-cell responses

Cross-presentation of exogenous antigens requires loading of those antigens onto MHC I. VLP were coupled with MART-1 peptide (VLP–MART-1) or lysates derived from Mel888 melanoma cells (VLP–Mel888 lysate; Supplementary Figure 2), pulsed onto MoDC and cultured with MART-1-specific $CD8^+$ T-cell clones (Figure 5a). Human leukocyte antigen-$A2^+$ MoDC pulsed with VLP–MART-1 or VLP–Mel888 lysate markedly stimulated IFN-γ production and degranulation of MART-1-specific $CD8^+$ T cells by 22 and 17%, respectively, compared with 48% when stimulated with the mitogen ConA. Unconjugated lysates were unable to stimulate T-cell responses to the same degree as the lysates coupled to VLP indicating that cross-presentation of VLP by DC is indispensible for superior T-cell reactivation. Contrastingly, the control MoDC pulsed with VLP alone, or MART-1-specific $CD8^+$ T cells pulsed with VLP–MART-1 in the absence of MoDC did not result in the production of IFN-γ or translocation of CD107a to the surface of the T cells (data not shown). These results negate the possibility that activation of the MART-1-specific $CD8^+$ T cells arose through direct activation of the $CD8^+$ T cells with un-processed VLP–MART-1 or free MART-1 and demonstrate that human MoDC can efficiently cross-present RHDV VLP-associated antigens to antigen-specific $CD8^+$ T cells.

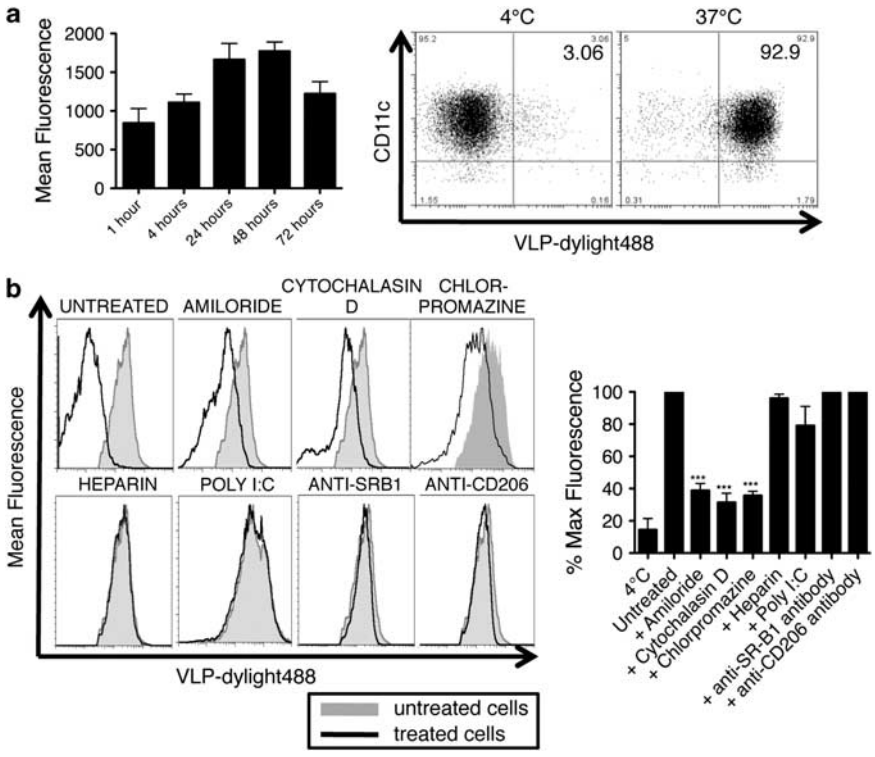

Figure 4 VLP rapidly accumulate inside human monocyte-derived DCs by nonspecific uptake mechanisms. (**a**) CD11c$^+$ monocyte-derived DC were pulsed with VLP-dylight488 at 37 or 4 °C and the levels of uptake by these cells was monitored over time. The bars represent the mean fluorescent intensity of VLP inside CD11c$^+$ DC at 37 with 4 °C value subtracted ± s.d. Dot plots correspond to CD11c$^+$ DC at 37 or 4 °C 24 h after pulse with VLP-dylight488. (**b**) Histograms corresponding to VLP uptake 1 h after pulse, show untreated CD11c$^+$ DC at 37 °C (shaded curves) overlayed with DC treated with each inhibitor or cells incubated at 4 °C (open curves). Bar graph represents VLP uptake by CD11c$^+$ DC, quantitatively as mean ± s.d. for cells from six independent donors. Maximum fluorescence was calculated as a percentage of the fluorescent signal from untreated cells. *** statistically significant differences in the uptake of VLP by treated cells compared with untreated cells ($P<0.001$).

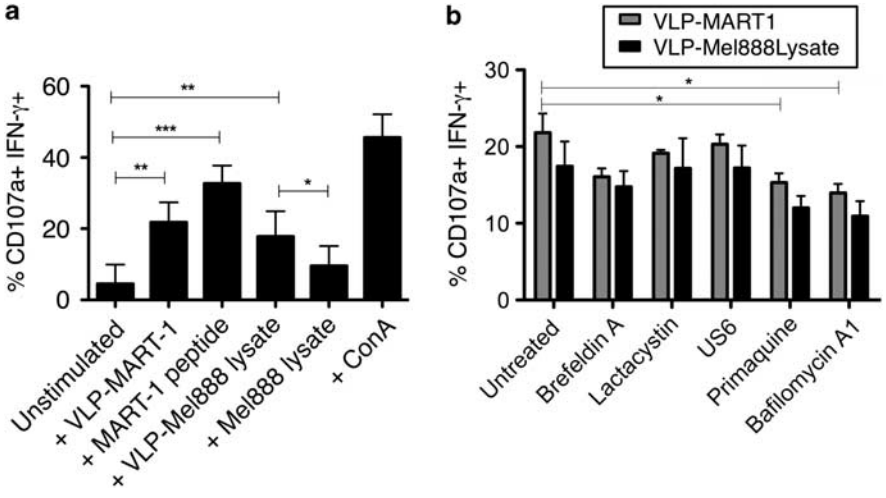

Figure 5 Human monocyte-derived DCs can cross-present VLP coupled to tumor antigens or lysates using a receptor recycling. (**a**) VLP coupled to MART-1 peptide (VLP–MART-1) and VLP coupled to Mel888 lysate (VLP–Mel888 lysate) were pulsed onto human DCs for 24 h before co-culture with MART-1-specific CD8$^+$ T-cell clones for 6 h. Bars show the percentage of T cells positive for both surface CD107a and intracellular IFN-γ, measured using flow cytometry. Data represents mean ± s.d. for cells from five independent DC donors. *, ** and *** statistically significant differences in the T-cell activation between conditions indicated ($P<0.05$, 0.01 and 0.001, respectively). (**b**) MART-1-specific CD8+ T-cell clones were cultured for 6 h with DCs that had been treated with inhibitors for 15 min and pulsed with VLP–MART-1 (grey bars) or VLP–Mel888 lysate (black bars) for 24 h. Bars are representative of data from independent experiments with cells from five individual donors, mean ± s.d. * statistically significant differences in the T-cell activation between conditions indicated ($P<0.05$).

To further elucidate the mechanisms contributing to cross-presentation of RHDV VLP by DC, we treated MoDC with the antigen-processing inhibitors used previously in this study and cultured the cells pulsed with VLP–MART-1 or VLP–Mel888 lysate with MART-1-specific $CD8^+$ T cells. The viability of the DC following treatment was assessed by propidium iodide staining (data not shown). The ability of the treated MoDC to present VLP–MART-1 or VLP–Mel888 lysate to the $CD8^+$ T cells was again measured using IFN-γ production and translocation of CD107a to the T-cell surface (Figure 5b). Following treatment of MoDC with primaquine or bafilomycin A1, the CD8+ T-cell responses to VLP–MART-1 were modestly decreased and this trend was also distinguishable in CD8+ responses to MoDC treated with VLP–Mel888 lysate although here the differences were not statistically significant. The converse was observed with DC treated with lactocytin or US6. Therefore, we have evidence that indicates that VLP-derived peptides are loaded onto MHC I molecules that have been recycled from the cell surface and not onto nascent MHC I molecules in the endoplasmic reticulum as is the case for endogenously derived antigens and that this mode of antigen processing is a common feature of both human and mouse DC.

DISCUSSION

In this study, for the first time, we have elucidated the pathway by which RHDV VLP chemically conjugated with antigen, are processed and cross-presented by DC to $CD8^+$ T cells. This is important because these VLP, carrying a model tumor antigen, have already been shown to significantly inhibit growth of melanoma in mice and they have the potential to become an effective, clinically relevant, immunotherapy. The versatility inherent in the simple conjugation process suggests that these VLP could be tailor made for specific tumors using lysates. More information on the pathway they take for cross-presentation may suggest modifications to this delivery system in order to generate greater antitumor cytotoxicity.

We have confirmed that DC take up VLP more efficiently than other professional APCs, particularly B cells,[18] and that RHDV VLP enter both human and murine DC by clathrin-dependent pinocytosis and phagocytosis. In our assays, we could find no evidence for receptor-mediated acquisition by DC from either species. This differs from the findings from previous studies using hepatitis C virus VLP and human papillomavirus VLP, which reported human DC uptake mediated via a range of receptors including class B scavenger receptors, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC–SIGN), mannose receptors and heparan sulfates.[19–22] This finding may relate to the origin of VLP used in our work as RHDV does not naturally infect mice or humans and it is possible that the lack of receptors enabling efficient entry into host cells contributes to this. Evidence from other studies also show that entry via Fc receptors facilitates cross-presentation in DC,[23] however, we did not investigate uptake via this route this because antibody was highly unlikely to have a role in our *in vitro* assays. It is also unlikely that antibody has a role *in vivo* because of previous work demonstrating that normal mouse serum does not recognize RHDV VLP (unpublished observations).

Internalization of foreign antigens can occur by cell types other than $CD11c^+$ DC that are also reportedly able to stimulate $CD8^+$ T-cell responses through cross-presentation, including plasmacytoid DC[24] (pDC) and neutrophils.[25] These cell types may have a role in aiding the cross-presentation of VLP *in vivo*, particularly pDC that are involved in responses to viral infection, however, it is unlikely that they are the predominant APCs involved in responses to VLP. Additionally, Toll-like receptor ligation can result in differential uptake of antigens[26] and their subsequent cross-presentation to $CD8^+$ T cells.[27] This leaves scope for investigating interactions between pDC and conventional DC in response to VLP because of the stimulatory nature of type 1 IFNs produced by pDC.

Our experiments suggest that VLP are degraded into peptides in acidified lysosomes and as neither proteosomal nor TAP inhibitors prevented the effective presentation of VLP-derived SIINFEKL to peptide-specific $CD8^+$ T cells, we conclude that these peptides do not escape into the cytosol nor do they enter the endoplasmic reticulum. Our evidence suggests that VLP-derived peptides are loaded onto MHC I molecules that have recycled from the surface as seen with inhibition of cross-presentation by primaquine and not from nascent MHC I molecules in the ER as is the case for endogenously derived antigens. However, the lack of T-cell activation seen in which DC were treated with brefeldin A suggests that there is a requirement for nascent proteins other than MHC I in the cross-presentation of VLP. Such accessory proteins could include molecules involved in the regulation of endosomal trafficking because under some circumstances brefeldin A (BFA) can affect the re-cycling pathway.[28] Endosomes recycling from the cell surface containing MHC I:peptide and lysosomes are thought to coincide[29] where presumably the resident peptide is dissociated from these MHC I molecules because of the acidic nature of lysosomal compartments, enabling the newly produced, VLP-derived peptides to replace them. Alternatively, VLP-derived antigens may be contained in antigen storage compartments within DC[30] in which they can associate with recycling MHC I molecules. TAP- and proteasome-independent pathways have been shown to operate for the cross-presentation of endogenous antigens by murine DC[31] and for viral antigens by human pDC,[24] so perhaps this pathway of cross-presentation is more widely utilized than previously identified, although the reason for differential sorting of some antigens into lysosomes and others into the cytosol remains to be clarified. The smaller size of OVA molecules and the presence of carbohydrates such as mannose have been reported to impact on antigen sorting within cells.[3,32] The disparity between the mechanisms of cross-presentation used by DC because of these inherent properties of the antigen being processed would thus account for the differences with processing of VLP–SIINFEKL and VLP–OVA compared with OVA alone.

A substantial amount of evidence has been reported to show that cross-presentation is predominantly carried out by a specific subset of DC despite no differences in antigen capture by different subsets[33] (reviewed by Lin[16]). It was therefore of interest to explore whether RHDV VLP conformed to this pattern. Rather than relying on *in vitro* assays for this component of the work, we employed a powerful, recently published *in vivo* assay to deplete these cells before the introduction of RHDV VLP. Not entirely surprisingly, $CD8\alpha^+$ DC depletion was only partial but despite this there was clearly lower antigen-specific T-cell proliferation in mice immunized with VLP–OVA, indicative of a major role for $CD8\alpha^+$ DC. This result may relate to the presence of the OVA protein as T-cell activation was almost completely ablated in mice treated with OVA alone while the response to VLP conjugated with SIINFEKL was largely unaffected. The highly mannosylated nature of OVA could influence the uptake of VLP by $CD8\alpha^+$ DC thus providing an explanation for differences seen in the DC population-presenting VLP–SIINFEKL and VLP–OVA. Moron[34] also demonstrate that porcine parvovirus-like particles are not presented by $CD8\alpha^+$ DC indicating that cross-presentation through alternative pathways is perhaps more widely spread throughout DC types.

The antigens conjugated to VLP were appropriate for clinical application thus emphasizing the utility of chemical conjugation, which may be a versatile way to customize VLP to individual tumors. We successfully demonstrated that these VLP-activated MART-1-

specific T cells, furthermore, lysate-conjugated VLP were as effective as MART-1 peptide-coupled VLP indicative of the effective coupling of this dominant antigen in the lysate. Interestingly, DC pulsed with a considerably higher quantity of unconjugated MART-I peptide than was present on VLP MART-1 conjugates, did not result in distinguishably higher antigen-specific $CD8^+$ T-cell activation. This mirrors result obtained with murine DC, although to a lesser extent and suggests that VLP can more efficiently target peptides to the cross-presentation pathway than peptides alone. Other groups have shown that melanoma-derived antigens are able to be cross-presented but there are conflicting reports about whether apoptotic and necrotic cells alone are stimulatory[35,36] or inhibitory[37,38] to the development of cytotoxic T-cell responses. This pertains particularly to the manner in which the tumor cells were prepared[39] and their mode of delivery.[40] However, it appears that covalent linking of tumor antigens from melanoma cell lysates to VLP enables the effective delivery to DC whereby they stimulate cytotoxic T-cell responses necessary for antitumor immunity.

We conclude that RHDV VLP enter both murine and human DC via receptor-independent phagocytosis and pinocytosis and are subsequently degraded into peptides with the lysosomal compartment. Our data suggest that peptides from a model tumor antigen and a clinically relevant melanoma antigen, chemically conjugated to RHDV VLP, are loaded onto recycled MHC I molecules and these are transported to the surface wherein they trigger $CD8^+$ T-cell activation. Refinements to the composition and delivery of these VLP conjugates are currently underway to enhance DC activation, MHC I loading and consequent antitumor cytotoxicity.

METHODS

Animals—source, characteristics, Animal Ethics Committee permission

Specific pathogen-free female C57/B6 were sourced from the Department of Animal Laboratory Sciences, University of Otago, Dunedin, New Zealand while OTI transgenic mice were obtained from Dr Sarah Hook, School of Pharmacy, University of Otago, Dunedin, New Zealand. All experimental protocols were approved by the Animal Ethics Committee, University of Otago, New Zealand.

Generation of recombinant RHDV VLP and chemical coupling to peptides

Recombinant Autographa californica Multicapsid Nucleopolyhedrovirus (AcMNPV) containing the VP60 gene of RHDV were used to produce RHDV VLP as described by Peacey.[1] Chemical coupling of defined peptides to VLP was performed as described[1] with an N-terminal Cys residue included on coupled peptides to facilitate chemical conjugation to succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate-treated VLP. VLP were conjugated to N-hydroxysuccinimide (NHS)-DyLight488 as per the manufacturer's instructions (Pierce, Rockford, IL, USA). Unconjugated peptides or dye were removed by extensive dialysis.

Generation of murine bone marrow-derived DCs

Bone marrow was removed from C57/B6 mice, washed in Dulbecco's Phosphate Buffered Saline (DPBS) and cultured for 6 days in Dulbecco's modified Eagle medium (Gibco, Invitrogen, Auckland, New Zealand) supplemented with L-arginine, L-asparagine, L-glutamine and folic acid, 2-mercaptoethanol, penicillin/streptomycin, 5% fetal calf serum (FCS) (Gibco) and 20 ng ml^{-1} granulocyte-macrophage colony-stimulating factor (R & D Systems, Minneapolis, MN, USA).

Generation of monocyte-derived DCs from human peripheral blood

Peripheral blood mononuclear cells were isolated from human leukocyte antigen-$A2^+$ human donors using a Ficoll–Paque separation gradient. The leukocytes were collected and cultured in RPMI (Gibco) containing 2% FCS (Gibco). Non-adherent cells were removed and the remaining cells were cultured for 6 days in RPMI containing 10% FCS, 25 μg ml^{-1} granulocyte-macrophage colony-stimulating factor and 25 μg ml^{-1} interleukin-4 (R & D Systems). Blood was donated by volunteers with the approval of the University of Otago Ethics Committee for Human Participants.

Immunofluorescent analysis of RHDV–VLP internalization

Cells were pulsed with 15 g ml^{-1} VLP-dylight488 for 24 h at 37 or 4 °C (control). Cells were stained with APC-conjugated anti-CD11c (clone HL3), F4/80 (clone CI:A3-1), B220 (clone RA3-6B2) or NK1.1 (clone PK136; Becton Dickinson) and fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowJo software version 8.8.6 (TreeStar, Ashland, OR, USA).

For mechanism of uptake, 10^6 DC were treated with amiloride (5 mM), cytochalasin D (20 g ml^{-1}), heparin (3000 U ml^{-1}), poly I:C (400 g ml^{-1}), chlorpromazine (50 M), anti-SR-B1 antibody (5 g ml^{-1}, clone NB400-104; Sapphire Biosciences, Waterloo, NSW, Australia) or anti-CD206 antibody (5 g ml^{-1}, clone 19.2; Becton Dickinson) for 20 min at 37 °C. The cells were washed three times, re-suspended in RPMI containing 10% FCS and pulsed with 15 g ml^{-1} VLP-dylight488 for 1 h at 37 or 4 °C (control). Cells were stained with anti-CD11c-APC (Becton Dickinson) and analyzed as above. All chemicals were sourced from Sigma (Auckland, New Zealand).

Co-culture of murine DC with splenic T cells

DC (5×10^5 cells ml^{-1}) were treated with the inhibitors lactacystin (20 M), recombinant human cytomegalovirus protein US6 (50 g ml^{-1}), brefeldin A (10 g ml^{-1}), primaquine (50 M) or bafilomycin A1 (1 M) for 15 min, washed in PBS three times, then pulsed with VLP–OVA or VLP–SIINFEKL (50 μg ml^{-1}) or OVA (2 mg ml^{-1}) for 24 h. OTI T cells were added at a DC:T-cell ratio of 1:10. After 48 and 72 h respectively, interleukin-2 and IFN-γ levels were measured by enzyme-linked immunosorbent assay. All chemicals were sourced from Sigma while US6 was purified as described by Kyritsis.[41]

In vivo depletion of DC sub-populations

C57/B6 mice were injected intravenously with 5 mg cytochrome c (Sigma) or PBS and spleens were harvested 24 h later. Single-cell suspensions were stained with fluorescein isothiocyanate-CD11c, phycoerythrin-CD8α (clone 53–6.7) and APC-CD4 (clone RM4-5) and analyzed flow cytometrically.

An assay for *in vivo* cross-presentation was carried out in accordance with the method described by Lin.[17] Briefly, mice were injected intravenously with 10^6 OTI transgenic T cells, labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE), 24 h before three daily doses of 5 mg cytochrome c or PBS. VLP–OVA (100 μg), VLP–SIINFEKL (100 μg), OVA (2 mg) or PBS were administered intravenously in conjunction with the second dose of cytochrome c. Spleens were harvested 60 h after the final cytochrome c injection. The cells were stained with APC-CD8 and PerCP-Vβ5.1 (clone MR9-4) and CFSE dilution was measured flow cytometrically.

Conjugation of Mel888 lysates to VLP

Mel888 tumor cells were grown in RPMI supplemented with 1 % L-glutamine and 10% FCS at 37 °C with 10% CO_2. They were harvested and subjected to three successive rounds of freeze and thaw to create a lysate. The lysates were mixed with a 10-fold molar excess of the heterobifunctional linker sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) in PBS pH 7.3 followed by dialysis to remove unbound chemical linker. The VLP were reacted with a 10-fold molar excess of *N*-succinimidyl *S*-acetylthioacetate (Pierce) followed by treatment with 100 μg hydroxylamine HCl (Sigma). The thiol-activated VLP were passed through a HiTrap desalt column (GE Healthcare, Auckland, New Zealand) to remove unbound *N*-succinimidyl *S*-acetylthioacetate. Equal concentrations of thiol-activated VLP and malemide-activated lysates were mixed together for 1 h. Coupling was confirmed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting using polyclonal rabbit anti-VLP and monoclonal human anti-melan-A antibodies (clone M2-7C10; Serotec, Oxford, UK).

Co-culture of human DC with MART-1-specific $CD8^+$ T cells

DC were pulsed with 50 μg ml^{-1} VLP–MART-1 or VLP–Mel888 Lysate for 24 h with or without the inhibitors described above. The DC were co-cultured at a ratio of 1:2 with $CD8^+$ T-cell clones recognizing the human leukocyte

antigen-A2 restricted MART-1 peptide EAAGIGILTV.[42] Antibodies to surface CD107a (clone H4A3) were added for 6 h after which the cells were stained for intracellular IFN-γ (clone B27) and fluorescence was measured by flow cytometry.

Statistical analysis

Paired two-tailed Student's *t*-tests were performed using GraphPad Prism version 5.0a (La Jolla, CA, USA). Significance was assigned where the calculated *P*-value was 0.05 or less.

ACKNOWLEDGEMENTS

We thank Vivienne Young for her valuable technical expertise and Robert Tampe for his gift of the plasmid encoding US6. This work was supported by the Health Research Council of New Zealand and a Top Achievers Doctoral Scholarship to SW from the Tertiary Education Commission of New Zealand.

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Supplementary Information accompanies the paper on Immunology and Cell Biology website (http://www.nature.com/icb)