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# Vaccine delivery by penetratin: Mechanism of antigen presentation by dendritic cells

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## Author Contributions

GP, VA and DP designed the experiments. DP and C-KT performed the experiments and analysed the data. GP, VA and DP wrote the paper.

## Conflicts of Interest

The authors declare no conflicts of interest.

**Abstract:** Cell penetrating peptides (CPP) or membrane translocating peptides such as, penetratin from *Antennapedia* homeodomain or TAT from human immunodeficiency virus are useful vectors for the delivery of protein antigens or their cytotoxic (Tc) or helper (Th) T cell epitopes to antigen presenting cells. Mice immunized with CPP containing immunogens elicit antigen-specific Tc and/or Th responses and could be protected from tumor challenges. In the present paper, we investigate the mechanism of class I and class II antigen presentation of ovalbumin covalently linked to penetratin (AntpOVA) by bone marrow-derived dendritic cells (BMDC) with the use of biochemical inhibitors of various pathways of antigen processing and presentation. Results from our study suggested that uptake of AntpOVA is via a combination of energy independent (membrane fusion) and energy dependent pathways (endocytosis). Once internalized by either mechanism, multiple tap-dependent or independent antigen presentation pathways are accessed whilst not completely dependent on proteasomal processing but involving proteolytic trimming in the ER and Golgi compartments. Our study provides an understanding on the mechanism of antigen presentation mediated by CPP and leads to greater insights into future development of vaccine formulations.

**Keywords:** *Antennapedia*; cell penetrating peptides; Antp; vaccine; membrane translocating peptide; CPP; cytotoxic T cell epitope; ovalbumin; TAT.

## Introduction

There are many challenges confronting vaccine development for cancer. These include immunosuppression by the tumor microenvironment, identification of appropriate tumor associated antigens (TAA), efficient delivery of vaccine antigen and maintenance of immune responses[1]. It is accepted that vaccines must efficiently target antigen presenting cells (APC), provide a strong adjuvant to break tolerance, utilize multiple TAA that are widely expressed and result both in priming of functional CD4 and CD8 T cells and the induction of tumor-specific antibodies (Ab)[2].

Dendritic cells (DC) are the major APC involved in generating immune responses to cancer antigens. Studies have identified receptors on various DC subtypes that are efficient in generating immune responses[3,4]. There are also multiple approaches reported for the targeted delivery of antigens to some of these receptors expressed on DC and few vaccines are currently in clinical trials[4-8].

Cell penetrating (CPP) or membrane translocating peptides (MTP) are a group of cationic peptides that have the ability to spontaneously enter the cytoplasm of cells[9-12]. These peptides have been used to deliver a number of cargos such as proteins, peptides, DNA, RNA, drugs and virus particles into cells. For these purposes 2 CPP, TAT from the human immunodeficiency virus transactivator of transcription protein and penetratin from the *Drosophila Antennapedia* domain have been used extensively[13]. The *Drosophila Antennapedia* DNA binding domain contains 60 amino acids and consists of 3  $\alpha$ -helices, with internalizing activity mapped to a 16 amino acid peptide, penetratin (RQIKIWFQNRRMKWKK; Antp) within the third helix.

The cell penetrating property of these peptides have been used to deliver antigenic peptide and proteins into antigen presenting cells including DCs for the development of vaccine delivery

systems[13-28]. For this purpose peptides chemically conjugated to protein antigens or synthetic peptides of CPP fused in tandem with cytotoxic (Tc) or helper (Th) T cell epitopes have been used. Mice immunized with these constructs generated antigen-specific CD4, CD8 or mixed responses and were protected from a tumor challenge. Several studies have investigated the immunogenicity of recombinant fusions of antigenic proteins with the TAT CPP either alone or with DC. DC based vaccines have been successfully tested in animal models and some have been tested in human clinical trials, however, the need for leukopheresis and processing leading to extensive costing prevents widespread utility in the clinic[29].

The uptake of CPP has been studied extensively however the mechanism is controversial. This is mainly due to the specific mode of uptake being dependent on the particular CPP, cell type, attached cargo or concentration[9,10,28]. Thus, in the context of CPP in vaccine delivery the mechanism will be dependent if TAT or penetratin is used or if a peptide or protein cargo is used. In addition, uptake in DC will also be different to other cell types. Pouniotis et al. previously reported the uptake and antigen presentation of tandem Antp CPP fused with a Tc cell epitope by murine DC[20]. These studies demonstrated that the peptides were endocytosed via phagocytosis or micropinocytosis in an energy dependent manner and is processed by a proteasome- and tapasin-independent pathway for CD8 epitope presentation. This mechanism for Antp based peptide immunogens was different to TAT based peptide immunogens in that they were tapasin independent but required further processing in *trans*-Golgi and ER compartments[30]. In addition, immunogenicity studies in mice with penetratin covalently linked to ovalbumin and tandem linked to OVA CD4 and/or CD8 epitopes demonstrated OVA-specific class I and class II responses resulting in prophylactic and therapeutic protection of mice from a tumor challenge[21].

The mechanism of Antp linked antigens have not been studied previously and in the current study we investigate the uptake, processing and presentation of AntpOVA in bone marrow-derived DC (BMDC) utilizing a number of biochemical inhibitors of the various pathways. Using the B3Z OVA-specific mouse T cell hybridoma, T cells from OT-I and OT-II mice as readouts of T cell activation we demonstrate that AntpOVA is internalized by a combination of energy dependent endocytotic process and energy independent direct translocation into the cytosol. Once internalized OVA can be processed by multiple tap-dependent or independent pathways with proteasomal or ER/Golgi-mediated proteolytic trimming for Class I presentation.

## Experimental Section

### *Peptides and proteins*

SIINFEKL OVA<sub>257-264</sub> H-2K<sup>b</sup> CD8 T cell epitope (OVA CD8) and OVA<sub>323-339</sub> 16-mer IA<sup>b</sup> CD4 T cell epitope (ISQAVHAAHAEINEAGR, OVA CD4) from ovalbumin were used in the study. Penetratin (Antp) is the 16 amino acid peptide (RQIKIWFAQNRRMKWKK) from *Antennapedia*. All peptides were synthesized by GenScript Corporation (USA) and the purity of the peptides (>95%) was determined by high performance liquid chromatography (HPLC) and mass spectrometry. Ovalbumin protein (OVA) was purchased from Sigma (A2512, VIC, Australia) and endotoxin removed by treatment with triton-X114[31].

### *Conjugation of penetratin to ovalbumin*

Penetratin was conjugated to OVA as described previously[21]. Briefly, OVA [3-5 mg/ml, minimally glycosylated (A2512, Sigma)] in phosphate buffered saline (PBS) was reacted with sulfosuccinimidyl 4-maleimidomethylcyclohexane carboxylate (SMCC), and then reacted with a C-terminal cysteine-modified penetratin. The thioether linked penetratin-OVA (AntpOVA) was dialyzed in PBS and stored at  $-20^{\circ}\text{C}$ .

### *Dendritic cell cultures*

Bone marrow cells from C57BL/6 female or TAP<sup>-/-</sup> mice were cultured at  $10^6$  cells/ml. Petri dishes containing 10 ml of complete RPMI 1640 media [RPMI-1640 media supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 4 mM L-glutamine, 100 units/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin sulphate and 100  $\mu\text{M}$   $\beta$ -mercaptoethanol] (CSL, Australia) with 10 ng/ml of GM-CSF and 10 ng/ml of IL-4. At day 6, cells express medium-high levels of CD11c, CD40, CD80 and CD86 and are characteristic of semi-mature DCs and are approximately 80% CD11c<sup>+</sup>, MHC class II (not shown). DCs were flushed from each dish and washed. DCs were used to assess the kinetics of uptake of AntpOVA by flow cytometry and for pulsing with AntpOVA for 3 hours (h) at  $37^{\circ}\text{C}$ , for all mechanistic studies.

### *Kinetics of AntpOVA uptake*

C57BL/6 bone-marrow derived DC ( $0.5 \times 10^6$  cells in 1 ml media - 10% (v/v) fetal calf serum (FCS)/RPMI 1640) were used. (i) Kinetics of uptake was assessed by adding AntpOVA or OVA alone at various times (5-120 minutes (min)) at fixed concentration (100  $\mu\text{g/ml}$ ) at  $37^{\circ}\text{C}$ . Kinetics of uptake was also assessed by the addition of AntpOVA or OVA alone for a fixed time (60 min) at varying concentrations (10-200  $\mu\text{g/ml}$ ). Uptake of antigen was assessed by flow cytometry and % uptake of total live cells is shown[28]. After incubation, the cells were washed twice with 0.5% (w/v) BSA/PBS, fixed with 2% paraformaldehyde (10 min) and washed again with 0.5% (w/v) bovine serum albumin (BSA)/PBS. The cells were then permeabilized with 0.5% (w/v) Saponin/PBS for 10 min and anti-OVA polyclonal antibody(diluted in saponin/PBS) were added for 45 min at  $4^{\circ}\text{C}$ . After washing the cells with Saponin/PBS 1:50 -FITC-anti-mouse anti-F(ab')<sub>2</sub> in Saponin/PBS was added for a further 45 min at  $4^{\circ}\text{C}$ . Finally, cells were washed twice with 0.5% BSA/PBS. DC were resuspended in PBS and transferred in FACS tubes and analyzed using flow cytometry. Uptake of OVA was calculated by measuring the % surface (non-permeabilised) and % intracellularly stained cells (permeabilised) with results expressed as % uptake = % intracellular-% surface.

### *Mice and immunizations*

C57BL/6 mice were bred and housed at the Biological Research Laboratory at the Austin Research Institute, Heidelberg, Australia. Groups of 4 C57BL/6 mice were immunized either twice (days 0, 14) with either AntpOVA or OVA intradermally at the base of the tail either alone (20  $\mu\text{g}$  per mouse in 50  $\mu\text{l}$ ) or with DC pulsed with OVA or AntpOVA (20  $\mu\text{g/ml}$ ) ( $1 \times 10^6$  pulsed DC/mouse).

### *Immunological assays*

### ELISpot

Spleen cells from immunized C57BL/6 mice were isolated 14 days after the last injection and assessed by ELISpot for IFN- $\gamma$  secretion. Mixed acetate plates (MAIP Millipore) were coated overnight with anti-mouse IFN- $\gamma$  (AN18, 5  $\mu$ g/ml, Mabtech Germany).  $0.5 \times 10^6$  spleen cells/well were added and incubated in 10% FCS RPMI 1640 media in the presence (20  $\mu$ g/ml) of OVA, CD4 epitope or SIINFEKL for at least 18 h. ConA (1  $\mu$ g/ml) or cells alone were used as positive and negative controls, respectively. Cells were discarded and after washing (0.05% Tween 20/PBS) anti-mouse IFN- $\gamma$  antibody-biotin (R4-6A2, Mabtech, CA, USA) was added for 2 h followed by extravidin-alkaline phosphatase (AP) at 0.1  $\mu$ g/ml (Sigma, UK) for 2 h at room temperature. Spots of activity were detected using a colorimetric AP kit (Biorad, Hercules, CA, USA). Cytokine spots were counted with an AID ELISpot reader system (Autoimmun Diagnostika GmbH, Germany). Data are presented as mean spot forming units (SFU) per  $0.5 \times 10^6$  cells  $\pm$  standard deviation of the mean (SD).

### Stimulation of LacZ inducible ovalbumin-specific T cell hybrid

The B3Z T cell hybridoma line contains a gene construct of *E.coli* LacZ reporter gene linked to the nuclear factor of activated T cells (NFAT). Recognition of OVA-K<sup>b</sup> peptide in the context of MHC class I by the TCR results in activation of the enzyme and conversion of the chromogenic substrate that can be measured by absorbance spectrophotometry[32]. DC ( $10^6$  cells) were pulsed with different concentrations of AntpOVA, OVA (20, 50, 100, 200  $\mu$ g/ml) or SIINFEKL (1, 10  $\mu$ g/ml) for 24 h. DC ( $2 \times 10^5$ ) were added to  $10^5$  B3Z cells in 96 well microtitre plates. Following overnight incubation at 37°C, cells were washed with sterile PBS and incubated with chlorophenol red  $\beta$ -D-galactopyranoside (Calbiochem, San Diego, CA) for a further 4 h and optical density was measured at 560 nm using a microplate reader.

### Antigen-specific T cell responses *in vitro*

Purified T cells were obtained from OT-I and OT-II mice using a T cell cocktail (kindly provided by Dr. Mark Wright, Department of Immunology, Monash University). The purity of the population was greater than 85% as identified by CD3 staining by flow cytometry (data not shown). T cells were co-cultured in 96-well plates in the presence or absence of pulsed DC. DC were pulsed with AntpOVA (100  $\mu$ g/ml), SIINFEKL (1  $\mu$ g/ml), OVA CD4 or ovalbumin (100  $\mu$ g/ml) for 24 h. The co-cultures comprised of  $2 \times 10^5$  T cells and  $2 \times 10^4$  DC in a total volume of 200  $\mu$ l. Proliferative responses were assessed from day 1 to day 6 of culture at 37°C. Cultures were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]-thymidine for 18 h, and incorporation of the radionucleotide was measured by  $\beta$ -scintillation spectroscopy using TopCount Gamma Counter (Packard, USA).

### Mechanism Studies

#### MHC class I pathway

The effects of MHC class I processing and presentation of AntpOVA by DC was evaluated by the B3Z.IG7 T cell hybridoma assay[20,32]. DC, EL4 (TAP-competent) and RMA-S (TAP-deficient) cells

were cultured in 10% FCS/RPMI media. Cells were added to 24 well plates (Falcon, BD Biosciences) at  $6 \times 10^5/300 \mu\text{l}$  and pre-incubated for 45 min with various inhibitors.  $\text{NaN}_3$  (0.01-10 mM) (Sigma, USA) and 2-deoxyglucose (0.01-10 mM) (Sigma, USA) deplete cellular ATP activity and energy-dependent processes within cells. Disruption of membrane activities was induced by amiloride (0.006-6 mM) (Sigma, USA) which inhibits membrane cycling and macropinocytosis, cytochalasin D (0.01-10  $\mu\text{g/ml}$ ) (Sigma, USA) which blocks phagocytosis and dextran sulphate (Progen Industries Ltd, Australia) (0.01-10  $\mu\text{g/ml}$ ) which interacts with the positive charges of penetratin and thereby blocks interaction of penetratin with negative charge on the surface of cellular membranes. Nystatin (0.5, 5, 50  $\mu\text{g/ml}$ ) inhibits caveola-dependent endocytosis and filipin III (1, 5, 10  $\mu\text{g/ml}$ ) is a cholesterol depleting agent used to inhibit lipid raft associated endocytosis. Processing via endocytosis was inhibited by chloroquine (0.2-200  $\mu\text{M}$ ) (Sigma, USA), ammonium chloride,  $\text{NH}_4\text{Cl}$  (0.2-200  $\mu\text{M}$ ) (Ajax Chemicals, Australia) and monensin (1-1000  $\mu\text{M}$ ) (Sigma, USA) which all interfere with acidification of endosomal pH vesicles from early to late endosomes and lysosomes. Proteasomal activity was inhibited by lactacystin (0.01-10  $\mu\text{M}$ ) (Calbiochem, USA). Protein transport from endoplasmic reticulum (ER) to the golgi complex was blocked by brefeldin (0.01-10  $\mu\text{g/ml}$ ) (Sigma, USA), furin inhibitor decRVKR-CMK (0.01-10  $\mu\text{M}$ ) (Calbiochem, USA) inhibits endopeptidase activity via the trans-golgi and bestatin (0.01-10  $\mu\text{M}$ ) (Sigma, USA) inhibits aminopeptidase activity in the ER. After the pre-incubation period, AntpOVA was added to cells at 100  $\mu\text{g/ml}$  for 3 h at  $37^\circ\text{C}$  and cells were added to B3Z.IG7 cells as described above. The inhibitors were active and concentrations used were non-toxic[15,20,33]. In all experiments, DC pulsed with OVA CD8 (SIINFEKL) or OVA CD4 (ISQAVHAAHAEINEAGR) in the presence or absence of biochemical inhibitor demonstrated that the inhibitor did not have any effects on the T cells (not shown).

### MHC class II pathway

The effects of MHC class II processing and presentation was evaluated by intracellular  $\text{IFN-}\gamma$  production by purified OT-II transgenic splenocytes. DC were pre-incubated for 45 min with similar inhibitors described for MHC class I mechanism studies. DC were then incubated for 3 h at  $37^\circ\text{C}$  at  $5 \times 10^5/500 \mu\text{l}$  with 100  $\mu\text{g/ml}$  of AntpOVA. The next day, purified OT-II T cells (purification method described above) were added at  $10^6/500 \mu\text{l}$  to each of the DC culture conditions and incubated at  $37^\circ\text{C}$  for 2 h. Three hours before the incubation period was completed, 1  $\mu\text{g/ml}$  of brefeldin was added to the cultures. The ability of DC presenting AntpOVA to MHC class II restricted OT-II T cells was assessed by intracellular  $\text{IFN-}\gamma$  secretion co-stained with CD3. Additional control conditions were also set up to include DC pulsed with SIINFEKL (20  $\mu\text{g/ml}$ ),  $\text{OVA}_{323-339}$  (20  $\mu\text{g/ml}$ ) or no peptide. For intracellular  $\text{IFN-}\gamma$  staining, DC were washed twice with 0.5% (v/v) BSA/PBS and stained with anti-CD3 (Pharmingen, BD, USA) for 45 min on ice, washed twice with 0.5% BSA/PBS then fixed with 2% (w/v) paraformaldehyde for 10 min and washed again with 0.5% BSA/PBS. Cells were permeabilized with 0.5% (w/v) saponin/PBS for 10 min. Anti- $\text{IFN-}\gamma$  (Pharmingen, BD, USA) was added for 45 min on ice, cells were washed twice with 0.5% (w/v) saponin/PBS. After resuspending the cells in PBS, they were transferred into FACS tubes and were immediately analyzed using flow cytometry. Data was analyzed using CellQuest software.

### *Confocal microscopy studies*

AntpOVA was labelled with Alexa Fluro 488 N-hydroxysuccinimide ester as described in manufacturer's instructions (Molecular Probes, Eugene, OR, USA). BMDC ( $5 \times 10^4$  cells) were seeded in eight well chamber slides overnight at 37°C. The next day culture media was aspirated and replaced with 200 µl Hank's Buffered Salt Solution (HBSS) and pulsed with Alexa-labelled AntpOVA for 2 h 37°C. Hoechst nuclear stain (10 µg/ml) was added for 15 min at 37°C. Cells were washed extensively and visualized using a laser scanning confocal microscope. To prevent fixation artefacts all measurements of peptide uptake were performed with living, non-fixed cells.

### *Statistical analysis*

Assays were set up in triplicate. Mean values were compared using an unpaired t-test, one-way analysis of variance or Kruskal–Wallis analysis of variance using Dunn's test for multiple comparisons. Three *p* value thresholds are noted in the text,  $p < 0.001$  (\*\*\*) to indicate a extremely significant difference,  $p < 0.01$  (\*\*) to indicate very significant and  $p < 0.05$  (\*) to indicate a significant difference.

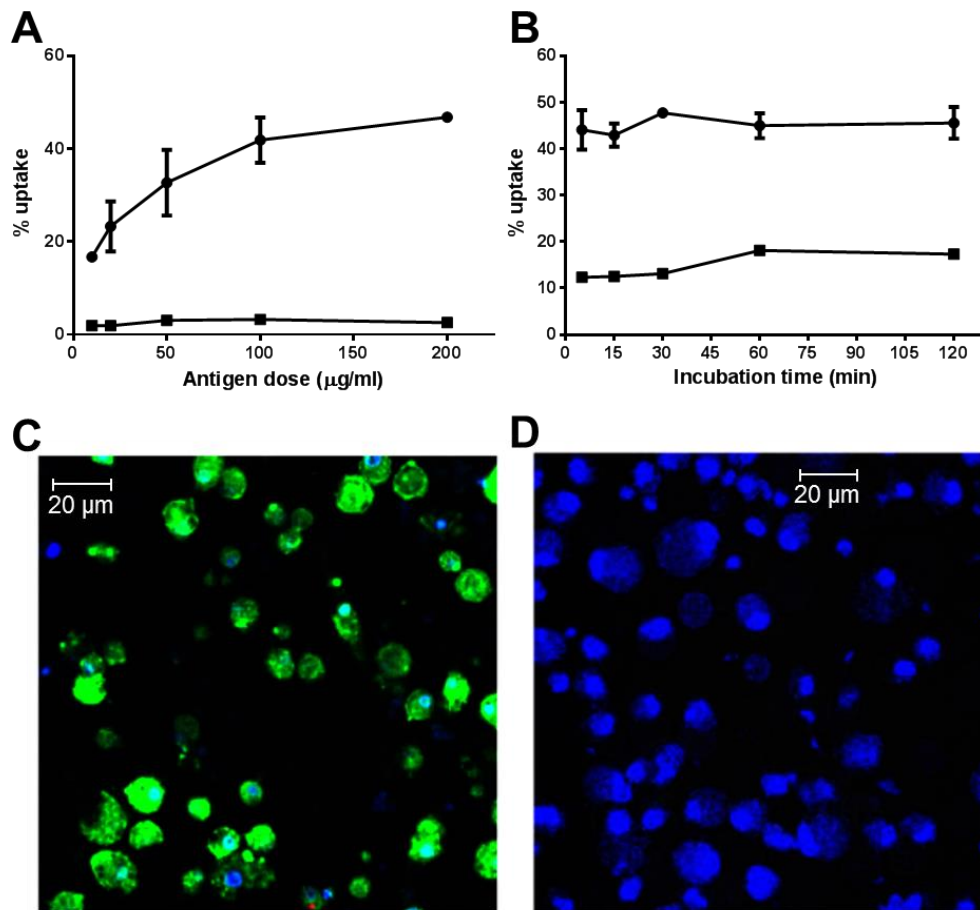
## **Results**

### *Uptake of AntpOVA by DCs*

To analyze the uptake of AntpOVA by APCs, *in vitro* grown DCs (> 80% CD11c<sup>+</sup>) were incubated with titrating concentrations of AntpOVA and compared to OVA alone. DCs were pulsed for 60 min with 10, 20, 50, 100 and 200 µg/ml of AntpOVA compared to OVA alone (Figure 1A). A polyclonal rabbit anti-mouse OVA was used to detect intracellular OVA in permeabilized DC. Uptake of AntpOVA at all concentrations; 100 µg/ml ( $42 \pm 3\%$ ), 50 µg/ml ( $33 \pm 5\%$ ) and 20 µg/ml ( $23 \pm 4\%$ ) were significantly above ( $p < 0.001$ ) uptake of OVA pulsed DC (Figure 1A). Since the uptake of AntpOVA was very efficient in DCs, we set up a time course assay to assess the kinetics of AntpOVA internalization into DCs. DCs were pulsed with 100 µg/ml of AntpOVA or soluble OVA (concentration chosen as it showed the optimal uptake in concentration experiments in Figure 1A) for 5, 15, 30, 60 and 120 minutes (mins) at 37°C (Figure 1B). Intracellular OVA in AntpOVA pulsed DC was detected at all time points at similar levels and at significantly greater levels than in OVA-pulsed DC, which emphasized how efficiently penetratin can transport whole protein into DCs.

The uptake of AntpOVA by DCs was also visualized by confocal microscopy. Alexa 488-labelled AntpOVA was rapidly taken up by DC with accumulation in vacuolar compartments and cytosol (Figure 1C & D).



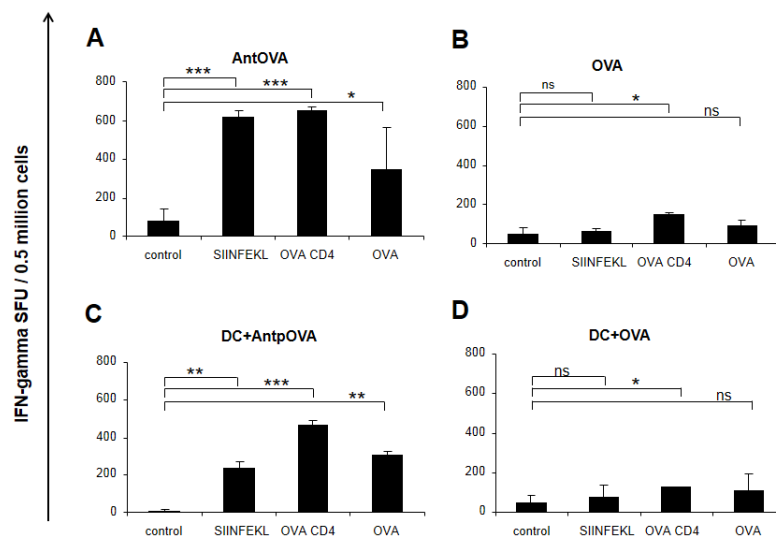


**Figure 1. Uptake and presentation of AntpOVA by dendritic cells.** (A) DC were incubated for 60 min with titrating concentrations of OVA (■) or AntpOVA (●) at 10, 20, 50, 100 and 200 μg/ml or (B) DC were incubated with 100 μg/ml OVA (■) or AntpOVA (●) for 5, 15, 30, 60 and 120 min intervals. Cells were fixed, permeabilized and stained with a polyclonal rabbit anti-OVA followed by FITC-labelled F(ab')<sub>2</sub> anti-rabbit antibody and examined by intracellular flow cytometry analysis. Data is shown as mean±SEM of 2 independent experiments. (C) Confocal imaging of uptake of Alexa Fluor 488-labelled AntpOVA by DC. DCs were incubated with Alexa-labelled AntpOVA (green) in chamber slides for 2 hr, counterstained with Hoechst nuclear stain (blue), washed and visualized by confocal microscopy. (D) Control DC stained with Hoechst nuclear stain only.

#### *AntpOVA can stimulate antigen-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses in vivo*

We further evaluated the potential of AntpOVA to induce CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses *in vivo* [21]. Groups of 4 C57BL/6 mice were immunized with 25 μg soluble OVA, 25 μg AntpOVA, DCs pulsed with soluble OVA at 20 μg/ml or DCs pulsed with AntpOVA at 20 μg/ml. All mice were given 2 intradermal (base of tail) injections, 14 days apart. Sixteen days after the final injection, spleens were removed from mice and antigen-specific IFN-γ responses were detected via ELISpot assay. Mice immunized with AntpOVA showed highly significant IFN-γ responses to SIINFEKL, OVA CD4 and to a lesser extent, whole OVA compared to control mice (Figure 2A). There were no increase in IFN-γ

responses, although some OVA<sub>323-339</sub>-specific responses were detected in OVA immunized mice compared to controls (Figure 2B). In comparison, mice that were immunized with DCs pulsed with AntpOVA showed high IFN- $\gamma$  responses specific to SIINFEKL, OVA<sub>323-339</sub> and whole OVA but not as high as AntpOVA alone (Figure 2C). Similar IFN- $\gamma$  responses were achieved after immunization with DCs pulsed with soluble OVA as seen with soluble OVA alone (Figure 2B & D). Comparing mice that were immunized with or without DCs, the results show that after 2 immunizations, mice immunized with AntpOVA alone gave significantly higher IFN- $\gamma$  responses ( $p < 0.05$ ) via both MHC class I and II to stimulate potent CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses. Moreover, both groups of mice immunized with AntOVA with or without DC showed significantly higher IFN- $\gamma$  responses than the corresponding OVA and OVA+DC treated groups ( $p < 0.05$ ). These results suggest that using penetratin conjugated to protein is immunogenic without the need for *ex-vivo* pulsing of DCs.



**Figure 2. CD8<sup>+</sup> and CD4<sup>+</sup> antigen-specific IFN- $\gamma$  responses induced by AntpOVA.**

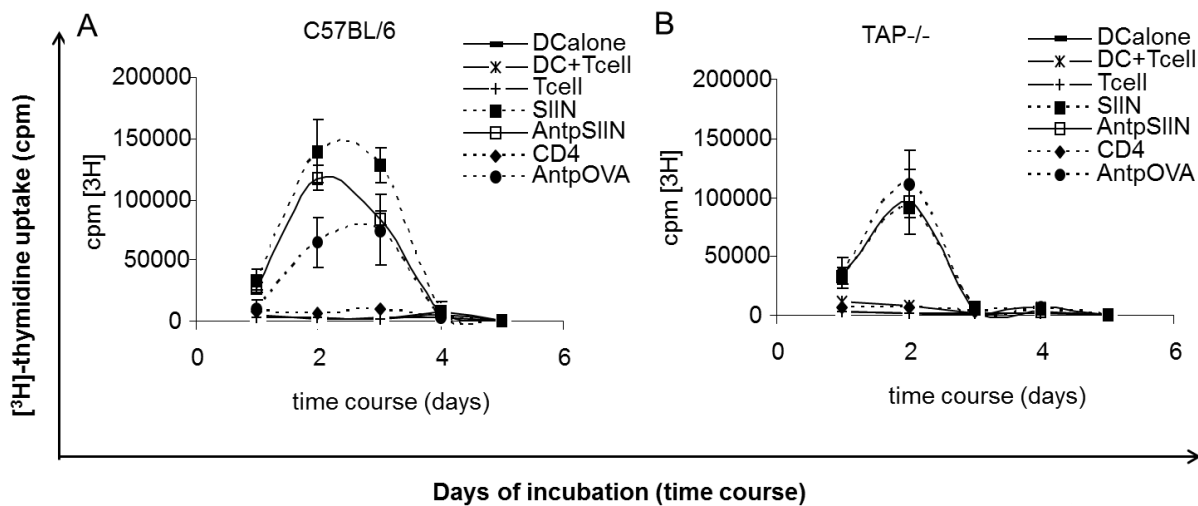
Groups of 4 C57BL/6 mice were immunized with (A) AntpOVA (B) OVA at 25  $\mu$ g per mouse or with DCs pulsed with (C) DC+AntpOVA, or (E) DC+OVA at 20  $\mu$ g/ml. Immunizations were given twice, 14 days apart, intradermally at the base of the tail. Sixteen days post-immunization mice were killed and spleens removed and IFN- $\gamma$  responses using recall antigens (control, SIINFEKL, OVA CD4 peptide or ovalbumin) were assessed via ELISpot assay. Results are shown as spot forming (SFU) per 0.5 million cells. Data is shown as mean  $\pm$  SD from three replicate wells from 4 mice and representative of 2 independent experiments. One-way analysis of variance (ANOVA) was performed and \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; ns, not significant.

#### *Mechanism of AntpOVA antigen presentation*

To study the mechanism of processing and presentation of ovalbumin delivered via penetratin we utilized various inhibitors of MHC class I and class II intracellular trafficking and processing pathways. To demonstrate processing and presentation we utilized 2 assays; (i) the stimulation (proliferation or intracellular IFN- $\gamma$  secretion) of purified CD8+ and CD4+ T cells from T cell receptor (TCR)-transgenic OT-1 and OT-II mice, and, (ii) the stimulation of B3Z T cell hybridoma by AntpOVA pulsed bone marrow derived DCs.

AntpOVA is processed and presented via a TAP independent pathway.

It has previously been shown that the activation of OT-I T cells by AntpSIIN peptide pulsed DC occurs in a TAP-independent manner[20]. To ascertain if the processing and presentation of AntpOVA is TAP dependent, bone marrow derived DC from TAP1<sup>-/-</sup> [34,35] and wild type C57BL/6 mice were pulsed with AntpOVA, OVA or SIINFEKL were used for stimulation of purified OT-1 T cells. Both TAP1<sup>-/-</sup> and wild type DCs were able to stimulate the OT-1 T cells (Figure 3A and B). This was further confirmed when similar results were obtained when pulsed TAP1<sup>-/-</sup> and wild type DC or RMA-S (TAP<sup>-/-</sup>) and EL-4 cells were used to stimulate the B3Z T cell hybridoma (not shown).

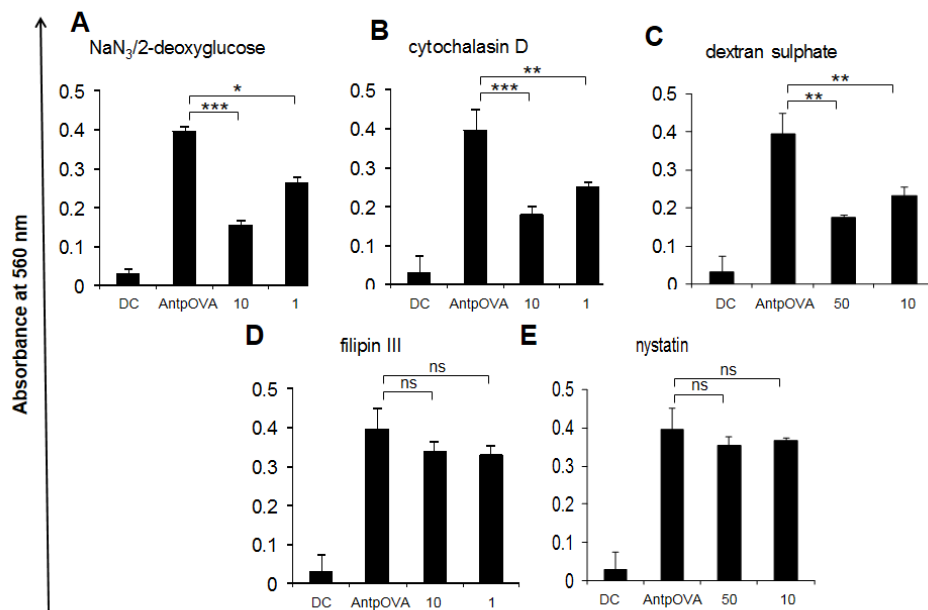


**Figure 3. Tap independent MHC class I processing pathway.** *In vitro* grown (A) C57BL/6 and (B) TAP1<sup>-/-</sup> DCs were pulsed with AntpOVA, SIINFEKL, AntpSIIN and CD4 epitope (343-359) at 20  $\mu$ g/ml for 24 h then added to purified OT-I T cells and cultured for 5 days. Cells were harvested at each time point and [<sup>3</sup>H]-thymidine was measured to assess T cell proliferation. OT-I T cells, DC alone and DCs plus T cells were used in experiments as negative controls. Data presented as mean counts per minute (cpm)  $\pm$ SD from quadruplicate wells and representative of 2 independent experiments.

Uptake of AntpOVA.

Membrane translocating peptides such as penetratin and TAT are known to translocate to the cytosol of cells by forming inverted micelles[36]. It is now becoming apparent that the uptake could also be via endocytosis[12,37]. To ascertain the mechanism of OVA presentation we have used various inhibitors that can prevent uptake and thereby interfering with the processing and presentation

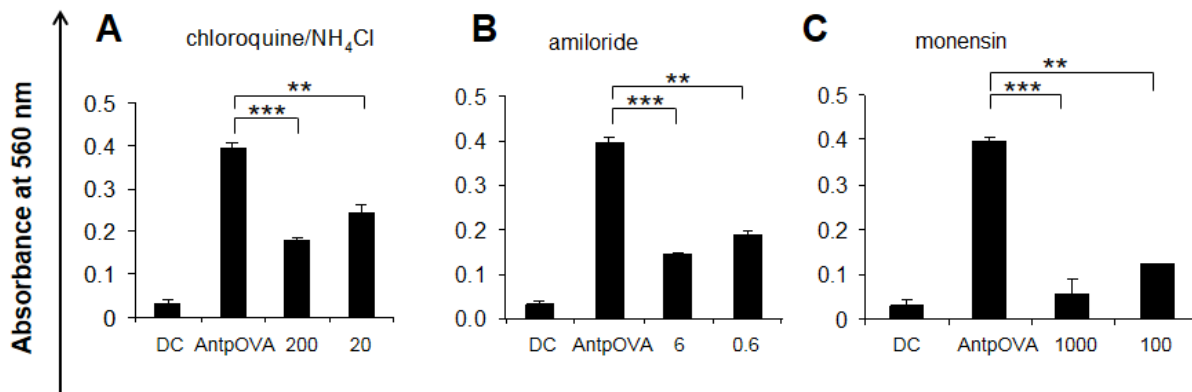
of AntpOVA by DC. Use of sodium azide ( $\text{NaN}_3$ )/2-deoxyglucose known to inhibit cell membrane activities as well as cellular ATP dependent processes, resulted in  $62 \pm 5\%$  reduced stimulation of B3Z cells (Figure 4A)[38]. Cytochalasin D an agent that disrupts cellular actin organization also inhibited the presentation of AntpOVA to B3Z cells by  $56 \pm 6\%$  suggesting that uptake is dependent on an endocytic process (Figure 4B)[39,40]. Studies have indicated that the Antp peptides due to their cationic nature binds to negatively charged receptors and enter the cell by an endocytic process. Dextran sulphate a negatively charged polysaccharide inhibited uptake by  $56 \pm 2\%$  probably by preventing receptor binding by competition (Figure 4C)[41,42]. The uptake of AntpOVA was independent of caveolae as 2 inhibitors of this process, filipin III and nystatin had no effect (Figure 4D, 4E)[43-45].



**Figure 4. AntpOVA internalizes via energy-dependent, caveolin-independent, negatively charged receptors.** DC were incubated for 45 min with various inhibitors (A) sodium azide/2-deoxyglucose (1, 10 mM) (B) cytochalasin D (1, 10  $\mu\text{g/ml}$ ) (C) dextran sulphate (10, 50  $\mu\text{g/ml}$ ) (D) filipin III (1, 10  $\mu\text{g/ml}$ ) and (E) nystatin (10, 50  $\mu\text{g/ml}$ ) followed by incubation with AntpOVA at 100  $\mu\text{g/ml}$  for 3 h and added to B3Z T cell hybridomas for 24 h. LacZ activity in B3Z T cells was assayed by total culture lysates with LacZ substrate CPRG and compared to controls with DC and B3Z cells without inhibitors (DC). The absorbance (560 nm) of culture wells was read after 4 h of incubation at  $37^\circ\text{C}$ . Experiments were performed in triplicate and values are presented as means plus SD with Student's t tests and representative of 2 independent experiments. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; ns, not significant.

Endosomal and lysosomal processing.

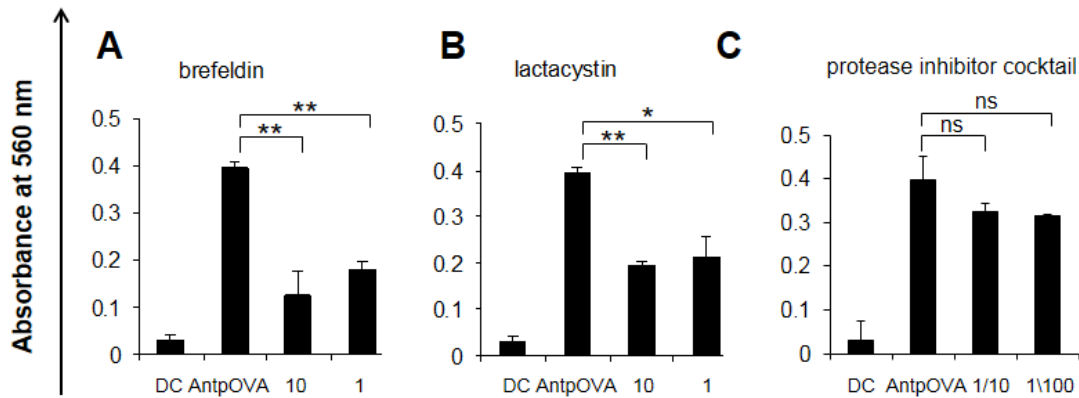
Once AntpOVA is internalized it will be processed in one or more cellular compartments before peptides are loaded onto class I molecules for export onto the membrane. To investigate this aspect we used several inhibitors that affect endosomal/lysosomal acidification, membrane recycling and transport. The possible role for endosomal processing using chloroquine and  $\text{NH}_4\text{Cl}$ , which prevents acidification of endosomes and subsequent proteolysis was investigated[46-48]. After addition of chloroquine and  $\text{NH}_4\text{Cl}$ , stimulation of B3Z was inhibited by  $56\pm5\%$ , suggesting that AntpOVA does present MHC class I specific peptide by initial degradation in the endosomal compartments (Figure 5A). Similarly addition of Amiloride, which interferes with membrane recycling and macropinocytosis, also inhibited the stimulation of B3Z T cell hybridoma by  $69\pm5\%$  (Figure 5B)[49]. Monensin, is a sodium/proton ( $\text{Na}^+/\text{H}^+$ ) ionophore which has multiple roles including interference with golgi transport, acidification of intracellular compartments and protein transfer from endosomes to lysosomes[50,51]. Consistent with the results observed with chloroquine/ $\text{NH}_4\text{Cl}$ , the addition of Monensin led to a reduction in the stimulation of B3Z cells by  $87\pm13\%$  (Figure 5C). However, the greater inhibition by monensin compared to other inhibitors of the endolysosomal pathway would suggest additional inhibition of vesicular transport of loaded MHC class I from golgi to cell membrane.



**Figure 5. AntpOVA is endocytosed and processed in endosomes.** DC were incubated for 45 min with various inhibitors (A) chloroquine/ $\text{NH}_4\text{Cl}$  (20, 200  $\mu\text{M}$ ), (B) amiloride (0.6, 6 mM) and (C) monensin (100, 1000  $\mu\text{M}$ ) followed by incubation with AntpOVA at 100  $\mu\text{g}/\text{ml}$  for 3 h and added to B3Z T cell hybridomas for 24 h. LacZ activity in B3Z T cells was assayed by total culture lysates with LacZ substrate CPRG compared to controls with DC and B3Z cells without inhibitors (DC). The absorbance (560 nm) of culture wells was read after 4 h incubation at  $37^\circ\text{C}$ . Experiments were performed in triplicate and values are presented as means plus SD with Student's t tests and representative of 2 independent experiments. \*\*\*,  $p<0.001$ ; \*\*,  $p<0.01$ .

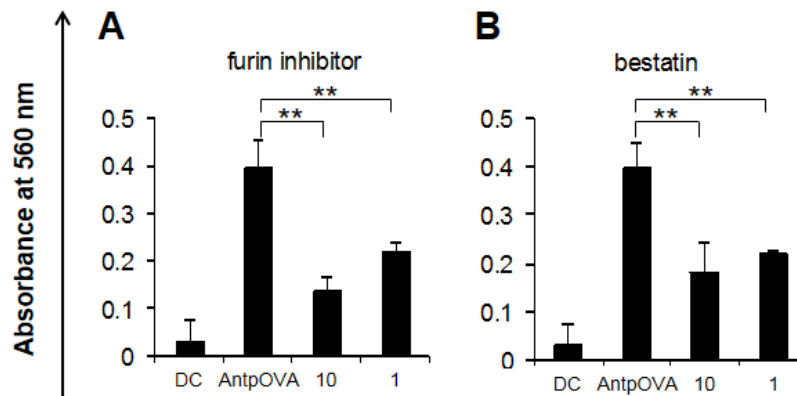
Proteolytic processing and peptide loading.

Brefeldin A, is an inhibitor that prevents the vesicle transport of newly synthesized MHC class I and class II molecules between ER and the Golgi apparatus[48]. Brefeldin A inhibited the presentation of class I peptide by DC to B3Z cells by  $70\pm13\%$  indicating the importance of newly synthesized class I for export of complexed peptide to the surface (Figure 6A).



**Figure 6. Proteolysis and peptide loading of AntpOVA.** DC were incubated for 45 min with various inhibitors (A) brefeldin (1, 10  $\mu\text{g/ml}$ ), (B) lactacystin (1, 10  $\mu\text{M}$ ) and (C) protease inhibitor cocktail (1/10, 1/100 dilution) followed by incubation with AntpOVA at 100  $\mu\text{g/ml}$  for 3 h and added to B3Z T cell hybridomas for 24 h. LacZ activity in B3Z T cells was assayed by total culture lysates with LacZ substrate CPRG compared to controls with DC and B3Z cells without inhibitors (DC). The absorbance (560 nm) of culture wells was read after 4 h incubation at  $37^\circ\text{C}$ . Experiments were performed in triplicate and values are presented as means plus SD with Student's t tests and representative of 2 independent experiments. \*\*,  $p<0.01$ ; \*,  $p<0.05$ ; ns, not significant.

Incubation of DC and AntpOVA in the presence of proteolytic inhibitors did not interfere with the presentation of antigen to B3Z cells confirming that the AntpOVA is not degraded extracellularly and then loaded onto MHC class I molecules on the surface (Figure 6C). The addition of the proteasomal inhibitor lactacystin resulted in significant inhibition ( $55\pm5\%$ ) of antigen presentation (Figure 6B)[52]. Antigens can be further processed by enzymes present in the ER and Golgi compartments after initial proteasome processing. Bestatin and a furin inhibitor also significantly impaired the presentation of antigen by DCs by  $55\pm17\%$  and  $62\pm10\%$  respectively (Figure 7A and B)[53-55].

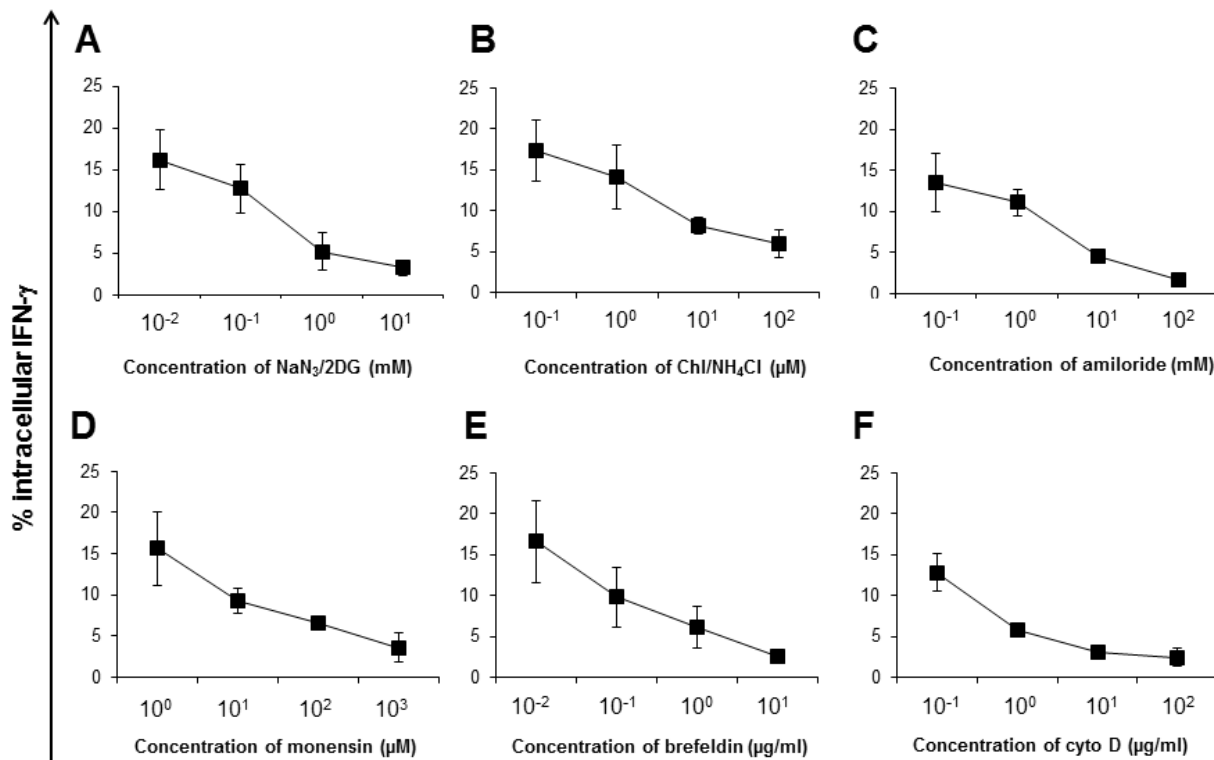


**Figure 7. Proteolysis of Antp in endoplasmic reticulum and Golgi compartments.** DC were incubated for 45min with various inhibitors (A) furin inhibitor (1, 10  $\mu$ M) and (B) bestatin (1, 10  $\mu$ M) followed by incubation with AntpOVA at 100  $\mu$ g/ml for 3 h and added to B3Z T cell hybridomas for 24 h. LacZ activity in B3Z T cells was assayed by total culture lysates with LacZ substrate CPRG compared to controls with DC and B3Z cells without inhibitors (DC). The absorbance (560 nm) of culture wells was read after 4 h incubation at 37°C. Experiments were performed in triplicate and values are presented as means plus SD with Student's t tests and representative of 2 independent experiments. \*\*,  $p < 0.01$ .

In summary, the data indicates that for MHC class I presentation, at least 2 mechanisms of antigen presentation is available for AntpOVA. AntpOVA is taken up by endocytosis, digested in the endosomal compartment and peptides released into the cytoplasm or direct membrane translocation into the cytoplasm. Subsequently AntpOVA or peptide fragments in the cytoplasm are cleaved further in the proteasome or ER and Golgi for loading onto empty class I molecules for export to the cell surface.

#### MHC class II presentation.

As shown in Figures 2 and 3, AntpOVA is processed in DC and presented by MHC class I as well as MHC class II to T cells. To ascertain the mechanism of which AntpOVA is processed and presented by the classical MHC class II pathway we used various inhibitors as described above. Similar to that shown for MHC class I,  $\text{NaN}_3$ /2-deoxyglucose and cytochalasin D inhibited the activation of OT-II cells by DC by 80% and 81% at the highest concentration as measured by inhibition of intracellular IFN- $\gamma$  secretion, confirming that uptake of AntpOVA is an energy dependent process involving endocytic process (Figure 8A, 8F). Class II presentation was also inhibited by  $\text{NH}_4\text{Cl}$ /Chloroquine, inhibitors of endosomal and lysosomal acidification by 72% (Figure 8B).



**Figure 8. AntpOVA specifically targets class II processing pathways.** DCs were incubated for 45 minutes with inhibitors (A) sodium azide/2-deoxyglucose (B) NH<sub>4</sub>Cl/chloroquine (C) amiloride (D) monensin (E) brefeldin and (F) cytochalasin D, followed by incubation with AntpOVA at 100  $\mu$ g/ml for 24 h and added at a 1:10 ratio to OT-II purified T cells for 24 h. Intracellular IFN- $\gamma$  expression was quantitated and used as a measure of T cell stimulation. DCs alone were used as a negative control and DCs pulsed with AntpOVA without any inhibitors at 100  $\mu$ g/ml was used as an internal positive control which resulted in 20% of cells secreting IFN- $\gamma$  (not shown). Data is shown as % intracellular IFN- $\gamma$  expressing cells of gated CD3<sup>+</sup> cells from triplicate wells (mean $\pm$ SD) and representative of 2 independent experiments.

Similar inhibition was obtained with amiloride (87%) and monensin (77%) (Figure 8C, 8D). Furthermore, brefeldin-A also inhibited presentation of MHC class II peptide by DC to OT-II cells by 85% (Figure 8E).

In summary, AntpOVA antigen is processed and presented by the classical class II pathway wherein it is taken up by endocytosis and degraded in the lysosomes prior to its loading to MHC class II molecules and export to the cell surface.

## Discussion

Efficient generation of CD8<sup>+</sup> T cells that recognize endogenously generated peptides presented by cell surface MHC class I molecules is crucial for the destruction of tumor cells or virus infected cells. These peptides are generated from endogenous proteins that are proteolytically degraded by proteasomes and transported to the ER via the TAP proteins to associate with MHC class I molecules and exported to the cell surface[5,56]. For generation of immune responses to an antigen, intracellular



delivery is the first step for access of antigens to the MHC class I and class II antigen processing and presentation pathways. Exogenous antigens are internalized by endocytosis, phagocytosis or pinocytosis and are transferred to the lysosomal compartments where the peptides from the degraded antigen is loaded onto MHC class II molecules and exported to the cell surface[57,58]. DC in particular are proficient in processing exogenous antigens for presentation by class I molecules via the cross-presentation pathway[58]. Most adjuvants have properties or are designed to access one or both of these pathways using a number of mechanisms such as the nature of the particulate (microparticles, VLP, ISCOM)[59-62], having membrane fuseogenic properties (liposomes, ISCOM)[63,64] or cell surface receptor binding (mannan or recombinant antibody)[3,4,6,8].

Membrane translocating peptides such as the peptide derived from the *antennapedia* homeodomain or HIV TAT protein have been used to shuttle proteins or peptides into the cytoplasm of antigen presenting cells[13,14,16,18-20,23,24,26,28,30,37,65]. In these studies synthetic peptides or recombinant peptide cytotoxic T lymphocyte (CTL) epitopes were linked in tandem with the membrane translocating sequence and they were capable of eliciting IFN- $\gamma$ , CTL in mice and protection of mice from a tumor challenge. In addition immunogens consisting of multiple antigen peptides incorporating CD8 T cell epitopes chemically linked to penetratin or fusion peptides of penetratin with CD4 epitopes as well as CD4 and CD8 epitopes in tandem which were immunogenic and generated prophylactic and therapeutic protection in mice[15,21].

In addition to CD8 and CD4 T cell epitope membrane translocating peptides such as penetratin and TAT have been used to carry larger proteins such as ovalbumin and mucin 1 to antigen presenting cells either as chemically linked conjugates or recombinant fusion proteins[14,17,25,65,66]. There has only been one other study where the CPP from HIV TAT was chemically linked to ovalbumin with the aim of generating CTL to SIINFEKL and was also successful[17,21]. The mechanism, subcellular localization and membrane translocation of cell penetrating peptides vary dependent on cargo and cell type[12]. Therefore, in addition to the immunogenicity of peptide (eg. Tc or Th epitopes) or protein (eg. Protein antigens) cargo delivered to dendritic cells by penetratin, the effect on mechanism of antigen presentation is of interest. In the present study we have chemically linked penetratin to ovalbumin for the purpose of studying the OVA-specific Tc and Th immune responses to the conjugate in mice with or without DC and the mechanism of antigen presentation in DCs.

Uptake of OVA with low carbohydrate (mannose) by C-type lectin receptors on DC is inefficient except at higher concentrations (>200 ug/ml) and uptake can be increased by enriching for higher glycoforms or by linkage to mannosylated dendrimers[31]. By utilization of a CPP, AntpOVA was more efficiently internalized than OVA by DCs as measured by intracellular staining with an anti-OVA antibody. At 100  $\mu$ g/ml 44 $\pm$ 3% of the DCs internalized the AntpOVA within 5 min of incubation (Figure 1). When incubated with DCs, AntpOVA was internalised, processed and SIINFEKL presented by class I molecules. This was demonstrated using several methods – (i) stimulation of ovalbumin specific B3Z T cell hybrid, (ii) proliferation of T cells derived from OT-1 mice and (iii) induction of OVA-specific CD8 response *in vivo*. In contrast to CPP fused in tandem to a Tc epitope peptide, AntpOVA conjugates also includes a Th epitope that can be translocated into the APC. The presentation of the CD8 and CD4 epitopes by APC was investigated by incubating AntpOVA pulsed DC with OT-1 and monitoring the proliferative responses. It was demonstrated that

AntpOVA was processed and presented T cell epitopes by MHC class I and class II antigen processing pathways (Figures 2, 3)[21].

To determine whether AntpOVA conjugates were able to generate CD4 and CD8 responses *in vivo* as demonstrated *in vitro*, mice were immunized with AntpOVA alone or AntpOVA pulsed DCs and IFN- $\gamma$  responses measured by ELISpot assays. Splenocytes from mice immunized with AntpOVA and AntpOVA/DC responded by IFN- $\gamma$  production on stimulation with OVA<sub>323-339</sub> (OVACD4) as well as SIINFEKL peptides (Figure 2). The splenocytes from mice immunized with OVA or OVA/DC yielded low levels of INF- $\gamma$  only in response to OVACD4.

Membrane translocating peptides such as penetratin and TAT have been shown to be taken up into cells in a receptor independent and energy independent manner[67]. More recently there have been increasing evidence to suggest that these peptides are taken up by receptor mediated endocytosis and not by a specific receptor but by negatively charged cell surface molecules such as heparin sulphate proteoglycans[68,69]. This interaction is solely due to an ionic interaction between the positively charged CPP and the negatively charged receptor. The mechanism of antigen presentation of TAT- and penetratin-based peptide immunogens have been investigated however, there have been no mechanistic studies with penetratin-based whole proteins such as OVA[16,20,30,37].

From *in vivo* studies with whole ovalbumin protein linked to penetratin it was observed that ovalbumin epitopes access the MHC class I presentation pathway as well as the class II pathway indicating a possible involvement of an endocytic process (Figures 2, 3)[21]. This prompted us to investigate the mechanism of MHC class I and class II antigen presentation using the model systems described herein. The entry of antigen into DC via an endocytic or pinocytosis process was confirmed by the reduction of stimulation of the B3Z cell line when cytochalasin D was used (Figure 4). The inhibition was 56% of the maximum at 10  $\mu$ g/ml in the B3Z assays. Inhibition of the presentation by the negatively charged dextran sulphate further confirms the involvement of a negatively charged receptor (Figure 4). Chloroquine/NH<sub>4</sub>Cl, amiloride and monensin all inhibited antigen presentation indicating endosomal processing prior to presentation (Figure 5). The inhibition with monensin was much higher (87%) compared to with chloroquine/NH<sub>4</sub>Cl (56%) and amiloride (69%) possibly due to the effect of monensin on the Golgi pH interfering with the trafficking of proteins. This observation together with the partial inhibition of uptake by cytochalasin D and azide/2-dG implies that an alternative energy independent mechanism of uptake and subsequent processing is operational. In the presence of the proteasomal inhibitor lactacystin presentation was partially (55 %) prevented indicating a proteosomal independent pathway (Figure 6). The AntpOVA conjugates can also load class I molecules via a TAP independent mechanism as demonstrated using RMA-S cells and TAP<sup>-/-</sup> knockout mice (Figure 3). Interestingly, in contrast to synthetic CPP with Tc epitopes (AntpSIIN) where class I presentation is proteasome dependent, presentation of penetratin-OVA to T cells is proteasome dependent with a proteosomal independent mechanism as well[20]. However, both immunogens can load MHC class I molecules in a TAP independent manner. A furin inhibitor and bestatin also partially prevented antigen presentation (Figure 7) in contrast to the AntpSIIN fusion peptide where there was no effect[20].

From the data it can be concluded that AntpOVA can enter the cytoplasm of cells via direct membrane translocation in an energy independent manner as well as an energy dependent endocytic process possibly via electrostatic interaction with negatively charged membrane lipids or

proteoglycans. At least two pathways of antigen processing for AntpOVA is available. AntpOVA is internalized by endocytosis, degraded in the endo-lysosomes and escapes into the cytosol for further proteolytic processing by the proteasome and loading class I in a TAP-dependent mechanism. Alternatively, AntpOVA can directly translocate into the cytoplasm followed by the proteolysis in the endoplasmic reticulum and Golgi compartments for loading into class I molecules in a TAP-independent mode. Our mechanistic data agrees with that of Lu et al[30] which demonstrates that TAT containing antigens can utilise alternative antigen presentation pathways bypassing the requirement for proteasomal processing and TAP dependence by proteolytic trimming in ER and Golgi compartments[30,70,71].

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