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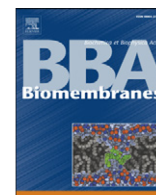

A membrane penetrating multiple antigen peptide (MAP) incorporating ovalbumin CD8 epitope induces potent immune responses in mice

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Cell penetrating peptides (CPP) represent a novel approach to facilitate cytoplasmic delivery of macromolecules. The DNA binding domain of *Drosophila Antennapedia* contains 60 amino acids and consists of 3 α-helices, with internalizing activity mapped to a 16-amino acid peptide penetratin (Antp) within the third α-helix. Here, we report on the use of penetratin to deliver a multiple antigen peptide (MAP) incorporating the immunodominant CD8 epitope of ovalbumin, SIINFEKL (MAPOVACD8). We demonstrate that penetratin linked to the MAPOVACD8 construct either by a disulfide (SS) or thioether (SC) linkage promotes the uptake, cross presentation and subsequent *in vivo* proliferation and generation of OVACD8 (SIINFEKL)-specific T cells. The MAPOVACD8 construct without penetratin is not presented by MHC class I molecules nor does it generate an *in vivo* IFN-γ response in C57BL/6 mice. Moreover, we clearly define the uptake and intracellular processing pathways of AntpMAPOVACD8 SS and SC revealing the majority of AntpMAPOVACD8 is taken up by DC via an endocytic, proteasome and tapasin independent mechanism. We also show that the uptake mechanism of AntpMAPOVACD8 is dose dependent and uptake or intracellular processing is not altered by the type of chemical linkage.

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1. Introduction

Fundamental for an effective vaccine is the delivery of immunogenic antigens to antigen-presenting cells (APC), ensuing processing and presentation, and induction of an immune response [1,2]. Vaccination with peptides incorporating cytotoxic T lymphocyte (CTL) epitopes has proven limited due to the failure for exogenous antigens to be presented efficiently to T cells [2]. There are now several strategies to promote the delivery of antigens to APC, with one such method utilizing the unique translocating properties of cell penetrating peptides (CPP) [3–7].

CPP offer a unique approach for the transport of peptides and proteins into the cytoplasm of cells. The TAT protein from human immunodeficiency virus, penetratin (Antp) from *Drosophila Antennapedia* and the VP22 protein from herpes simplex virus are some of the CPP which have been investigated in vaccine studies for delivery of tumor associated antigens to APC and for the use as a non-viral gene delivery vehicle in DNA vaccines, producing promising results [4,8–10].

It is now clear to overcome some of the limitations of previous peptide vaccines it is necessary to firstly, incorporate both CD8 T cytotoxic (Tc) and CD4 T helper (Th) epitopes to promote the generation of long term immunity and secondly major histocompatibility complex (MHC) restriction must be considered to allow application to the wider population [11,12].

Lu et al. utilized a recombinant protein incorporating the 9-mer TAT peptide linked to multiple epitopes from the protein ovalbumin (OVA) to demonstrate that immunization with multiepitope vaccines incorporating TAT-OVA CD8-CD4 T cell epitopes and the adjuvant CpG results in strong CTL and Th responses and generates an anti-tumor response [13]. Similarly, Dakappagari et al. (2005) utilized a multi-epitope peptide incorporating the CPP Pep-1 with three HLA-A2 restricted epitopes from Her-2/neu to prime CTL responses in mice [14]. However in both cases either furin sensitive (RVKR) sequences or arginine spacers were required to facilitate cleavage for processing and presentation [13,14].

Due to chemical synthesis restraints on the length of linear peptides, the number of Tc and Th epitopes that can be incorporated is limited. Branched multiple antigen peptides (MAP) may help overcome such limitations by allowing the delivery of numerous tumor antigens. Yet there is now evidence that as a result of varying antigen uptake mechanisms and/or differential resistance to the

Abbreviations: Antp, penetratin 16-mer peptide-RQIKIWFQNRRMKWKK; CPP, cell penetrating peptide; CTL, cytotoxic T lymphocyte; OVACD8, immunodominant CD8 peptide from ovalbumin, SIINFEKL; SS, disulfide linkage; SC, thioether linkage

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proteases involved in antigen processing, peptides generated from branched constructs presented to T cells are processed differently to those derived from linear peptides [11,15]. Thus far there have been no reports on the use of CPP to transport branched MAP incorporating CTL epitopes into APC.

Herein we report on the uptake, processing and presentation pathways of a novel asymmetric 4 branched MAP incorporating the H-2K^b CD8 eight-mer epitope SIINFEKL, from the ovalbumin protein. We reveal that linkage of a MAPOVACD8 construct to Antp via a disulfide (SS) or thioether (SC) linkage is endocytosed via phagocytosis or macropinocytosis by dendritic cells in an ATP-dependent manner and processed in a proteasome and tapasin (TAP) independent pathway. Moreover AntpMAPOVACD8 constructs generate potent *in vivo* proliferation and killing and induce antigen-specific IFN-γ secreting T cell responses superior to MAPOVACD8 construct alone.

2. Materials and methods

2.1. Mice and immunizations

C57BL/6, OT-I and OT-II mice, aged 6–10 weeks, were obtained from Walter and Eliza Hall Institute (Vic., Australia) and housed in the animal facilities of the Burnet Institute or RMIT University (Victoria, Australia). For immunization experiments mice were injected three times on days 0, 10 and 17 intradermally (i.d.) at the base of tail with 100 μg peptide. CpG-ODN 1668 (5′-TCC ATG ACG TTC CTG ATG CT-3′) with phosphorothioate linkages was synthesized by Geneworks (Adelaide, Australia) and dissolved in sterile PBS and stored at −20 °C. For tumor protection experiments mice were immunized with 50 μg CpG i.d.

2.2. Peptides

Peptides were synthesized by Genescript Corporation (San Francisco, USA) and purity determined by mass spectrometry. OVACD8 (SIINFEKL) is the ovalbumin H-2K^b CTL epitope 8-mer peptide and penetratin (Antp) is a 16 amino acid (RQIKIWFQNRRMKWKK) *Antennapedia* peptide. MAPOVACD8 is a 4 asymmetric branched (C-Terminal) MAP synthesized using the $(Fmoc)_4Lys_2LysCys\beta Ala$ (Free Cysteine) resin with SIINFEKLKGKGKGKGKG on each branch. The conjugation of MAPOVACD8 to Antp was performed using a disulfide (SS) bond or a thioether (SCH2CO (SC)) bond (Fig. 1) as described below.

For synthesis of the disulphide-linked MAP cysteine modified MAPOVACD8 (0.5 ml, 6.2 mg/ml) in water was reacted with 5,5′-dithiobis-(2-nitrobenzoic acid, DTNB) (0.2 ml, 7.5 mg/ml) in 0.1 M phosphate buffer, pH 7.5 for 3–4 h. The yellow solution was dialysed into PBS overnight. The MAPOVACD8 incorporating the activated disulphide was reacted with Antp peptide (0.2 ml, 5 mg/ml) incorporating a Cysteine at the C-terminal. After 3–4 h the mixture was dialysed into PBS overnight. The concentration of MAPOVACD8 was determined spectrometrically based on the DTNB anion released ($\varepsilon_{412} = 13{,}600\ M^{-1}\ cm^{-1}$). The thioether conjugated MAP was synthesized by reacting MAPOVACD8 (0.5 ml, 6.2 mg/ml) in 0.1 M phosphate buffer, pH 7.5 with the N-terminal bromoacetyl-modified Antp (0.2 ml, 5 mg/ml) for 16 h. The mixture was dialysed overnight to remove free Antp.

The cytotoxicity of the complexes was measured using the lactate dehydrogenase assay [16]. Antp complexes showed no significant cytotoxicity at concentrations of up to 25 μM (not shown).

2.3. Generation of bone marrow derived dendritic cells (BMDC)

Bone marrow cells from C57BL/6 female mice were collected by flushing the tibias of hind legs and treated with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse erythrocytes. Cells were washed and cultured at 5×10^5 cells/ml in 24 well plates with complete RPMI-1640 medium 10% (v/v) heat inactivated fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 100 μM β-mercaptoethanol) with 10 ng/ml each of recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin- 4 (IL-4) (Pharmingen, San Diego, CA). At day 6 cells were >80% CD11c$^+$ which was confirmed by flow cytometry (data not shown).

2.4. Stimulation of lacZ-inducible ovalbumin-specific T cell hybrid

The B3Z mouse T cell hybridoma line contains a gene construct of *Escherichia coli* lacZ reporter gene linked to the nuclear factor of activated T cells. Recognition of the OVACD8 peptide in the context of class I by the T cell receptor (TCR) results in activation of the enzyme and conversion of the chromogenic substrate that can be measured by absorbance spectrophotometry [17]. DC, EL-4 and RMA-S cells (2×10^5 cells) were pulsed with peptides at 5, 25 and 50 μM in 96-well microtitre plates (Falcon, BD Biosciences, North Ryde, Australia) for 24 h at 37 °C. Cells were then washed and 10^5 B3Z cells were added for 18 h at 37 °C. The next day cells were washed with sterile PBS and incubated with chlorophenol red-β-galactoside (Calbiochem, San Diego, CA) (100 μM 2-ME, 9 mM $MgCl_2$, 0.125% NP40, 0.15 mM chlorophenol red-β-galactoside). After 4 h incubation at 37 °C the absorbance was read at 560 nm.

2.5. Enzyme-linked immunosorbent spot-forming cell assay (ELISpot)

Splenocytes from immunized C57BL/6 mice were isolated and assessed by ELISpot for antigen-specific IFN-γ secretion. MultiScreen filter plates (Millipore, Billerica, MA) were coated with 5 μg/mL of anti-mouse IFN-γ antibody (AN18) (Mabtech, Stockholm, Sweden) overnight at 4 °C. Plates were washed six times with sterile PBS and blocked with 200 μl complete RPMI media for 2 h at 37 °C. Spleen cells (5×10^5/well) from immunized mice were added to wells in 100 μl of complete medium and incubated with 20 μg/ml recall antigens for 18 h. Concanavalin A (1 μg/ml) or cells alone were used as positive and negative controls, respectively. Triplicate wells were set up for each condition. Cells were discarded after washing (PBS) and 1 μg/mL biotinylated anti-mouse IFN-γ antibody (Mabtech) was added for 2 h at room temperature. The plates were washed with PBS and 1 μg/ml streptavidin–alkaline phosphatase (Mabtech) added at room temperature for 2 h. Spots of activity were detected using a colorimetric AP-conjugate substrate kit (Bio-Rad Laboratories, Foster City, CA). Cytokine spots were counted with an AID ELISpot Reader system (Autoimmun Diagnostika GmbH, Strassberg, Germany). Data is presented as mean spot-forming units (SFU) per 5×10^5 cells ± standard error of the mean (SEM).

2.6. Tumor protection

Groups of C57BL/6 mice ($n=8$) were immunized i.d. with PBS, AntpMAPOVACD8 SC or AntpMAPOVACD8 SC + CpG on days 0, 10 and 17. Ten days later, mice were challenged with a subcutaneous dose of 2×10^5 B16-OVA cells resuspended in 100 μl sterile PBS. The expression of OVA in B16-OVA tumor cells was confirmed by flow cytometry (data not shown). The subcutaneous growth of the tumor was monitored by measuring the two perpendicular diameters using calipers and the results are expressed as the product of the two perpendicular diameters.

2.7. In vivo maturation

C57BL/6 mice were injected i.d. in the footpad with peptides or LPS (positive control). 18 h later popliteal lymph nodes were isolated and pooled ($n=4$) and stained with CD11c-APC and maturation markers

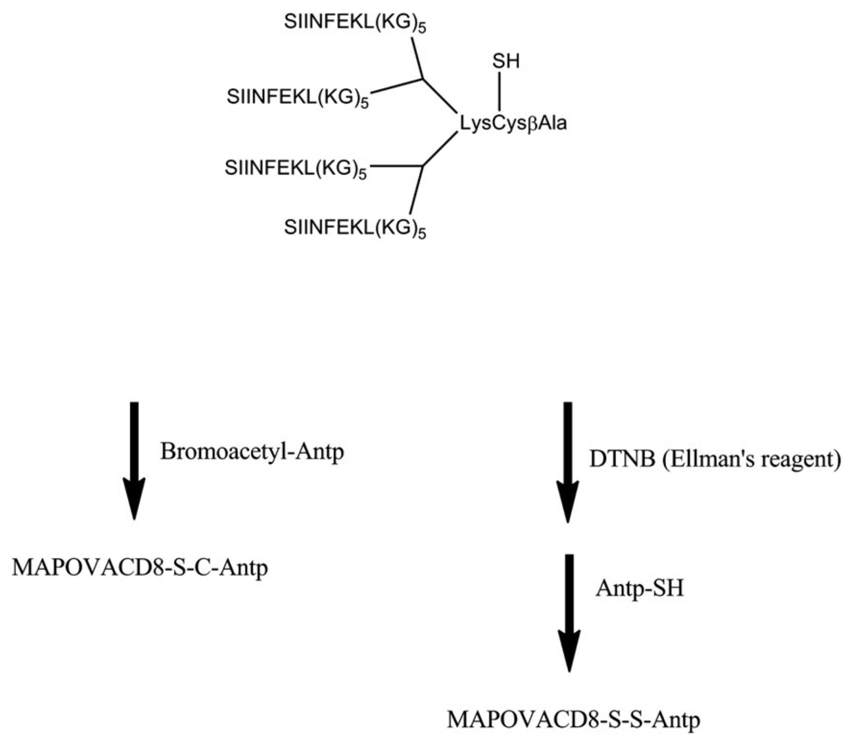

Fig. 1. Conjugation of Antp to a MAP incorporating the CD8 epitope (OVACD8) of the model antigen ovalbumin.

(CD40, CD80, CD86, and MHC class II) assessed by flow cytometry (BDCanto, BD Biosciences, USA). Live cells were gated on PI staining and cells gated on $CD11c^{+\,hi}$ staining.

2.8. In vivo proliferation

Splenocytes from OT-I mice were isolated and purified using a T cell antibody cocktail (rat anti-mouse Gr-1 (RB6–8C5), anti-B220 (RA3–6B2), anti-CD11b (M1/70.15), anti-erythrocyte (TER-119) and anti-MHC class II (M5/114)) (provided by Dr Mark Wright, Burnet Institute). T cells were separated using BioMag anti-rat magnetic beads (Qiagen, Hilden, Germany) (8 beads per cell) at 4 °C for 25 min. Cells bound to the beads were removed by magnetic attraction and washed and counted.

Purified OT-I T cells were resuspended in 0.1%BSA/PBS and labeled with 25 μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) for 10 min at 37 °C. Labeling was stopped with 5× volume of ice cold complete RPMI and cells washed extensively in PBS and CFSE labeling confirmed via flow cytometry. 10^7 CFSE-labeled OT-I T cells were then injected intravenously (i.v.) in 200 μl PBS into C57BL/6 mice immunized 20 h prior i.d. in the base of the tail. Splenocytes were collected 60 h later and stained with APC-conjugated anti-CD3 (BD Pharmingen) for 30 min at 4 °C in 2% FCS/PBS. Cells were then washed and analyzed for co-staining of CD3 and CFSE by flow cytometry.

2.9. In vivo killing

Splenocytes from naïve C57BL/6 mice were isolated and resuspended at 10^7 cells/ml in serum free RPMI and divided into 2 populations, pulsed or unpulsed. Pulsed splenocytes were incubated with 1 μg/ml OVACD8 peptide for 1 h at 37 °C. Cells were washed and resuspended in 0.1% BSA/PBS and labeled with a high concentration (5 μM) (pulsed splenocytes) or low concentration (0.5 μM) (unpulsed splenocytes) of CFSE (Molecular Probes, Eugene, OR) respectively for 10 min 37 °C. Labeling was stopped with 5× volume of ice cold complete RPMI, cells washed extensively in PBS and CFSE labeling confirmed via flow cytometry (data not shown). Peptide-pulsed 5×10^6 $CFSE^{high}$ cells and 5×10^6 unpulsed $CFSE^{low}$ cells were mixed and a total of 10^7 CFSE-labeled cells in 200 μl PBS injected i.v. into mice immunized 8 days prior. After 20 h splenocytes were isolated and analyzed by flow cytometry. Specific lysis was calculated as: {[1 − (ratio $CFSE^{low}/CFSE^{high}$ of naïve mice / ratio $CFSE^{low}/CFSE^{high}$ of immunized mice)] × 100}.

2.10. MHC class I mechanism studies

DC (2×10^5 cells) were cultured in complete RPMI media and incubated for 45 min at 37 °C with the following inhibitors: NaN_3 (10 mM) (Sigma, St Louis, MO) and 2-deoxyglucose (10 mM) (Sigma), amiloride (6 mM) (Sigma), cytochalasin D (10 μg/ml) (Sigma), dextran sulphate (Progen Industries Ltd, Darra, Australia) (10 μg/ml), filipin III (10 ug/ml) (Sigma), ammonium chloride (200 μM) (Ajax Chemicals, Sydney, Australia), monensin (1 mM) (Sigma), lactacystin (10 μM) (Calbiochem), brefeldin A (10 μg/ml) (Sigma), furin inhibitor decRVKR-CMK (10 μM) (Calbiochem), bestatin (10 μM) (Sigma) and chloropromazine (10 μg/ml) (Sigma). AntpMAPOVACD8 SS and AntpMAPOVACD8 SC was added to DC at 5, 25 and 50 μM at 37 °C and added to B3Z T cells as described above. Inhibitors were not removed from the T cell cultures. To determine if inhibitors affected T cells, DC pulsed with OVACD8 and inhibitors were also added to B3Z T cells as controls.

To determine tapasin (TAP) dependence on processing, EL-4 (TAP-competent) and RMA-S (TAP-deficient) cells (2×10^5) were pulsed with 5, 25 and 50 μM peptides at 37 °C for 24 h and T cells added as described above.

2.11. Statistical analysis

Assays were set up in triplicate. Mean values were compared using the two-tailed unpaired *t*-test and ANOVA. Two *p*-value thresholds were used for protection and immunogenicity assays: $p<0.01$ to indicate a highly significant difference, and $p<0.05$ to indicate a significant difference.

3. Results

3.1. AntpMAPOVACD8 conjugates induce specific T cell responses in vitro and in vivo

To assess whether MAPOVACD8, AntpMAPOVACD8 SS and AntpMAPOVACD8 SC are processed and presented by DC to the TCR of B3Z T cells, DC were cultured and pulsed with 5 μM of conjugates. DC were incubated with B3Z T cells for 24 h and the recognition of the OVACD8 epitope on the MHC class I molecule by its specific TCR was assessed via a colorimetric assay. DC alone was used as a negative control. DC pulsed with AntpMAPOVACD8 SS and AntpMAPOVACD8 SC at 5 μM induced presentation to B3Z T cells that was significantly above DC alone (Fig. 2A). However DC pulsed with MAPOVACD8 or Antp peptide alone did not present T cells (Fig. 2A). Thus the conjugation of Antp to MAPOVACD8 allows the uptake, processing and presentation of CD8 T cell epitopes. DC pulsed with OVACD8 is surface-loaded and was used as a positive control.

The ability of MAPOVACD8, AntpMAPOVACD8 SS and AntpMAPOVACD8 SC to induce $CD8^+$ T cell responses *in vivo* was determined using an IFN-γ ELISpot assay. C57BL/6 mice were immunized i.d. with 100 μg AntpMAPOVACD8 SS, AntpMAPOVACD8 SC or MAPOVACD8 on days 0, 10 and 17 and antigen-specific IFN-γ responses measured 14 days later. Mice immunized with either AntpMAPOVACD8 SS or AntpMAPOVACD8 SC generated equal IFN-γ secreting cells which recognized OVACD8 as well as OVA (Fig. 2B, C). The CD4 epitope of OVA ($OVA_{323–339}$) was used as a negative control for induction of

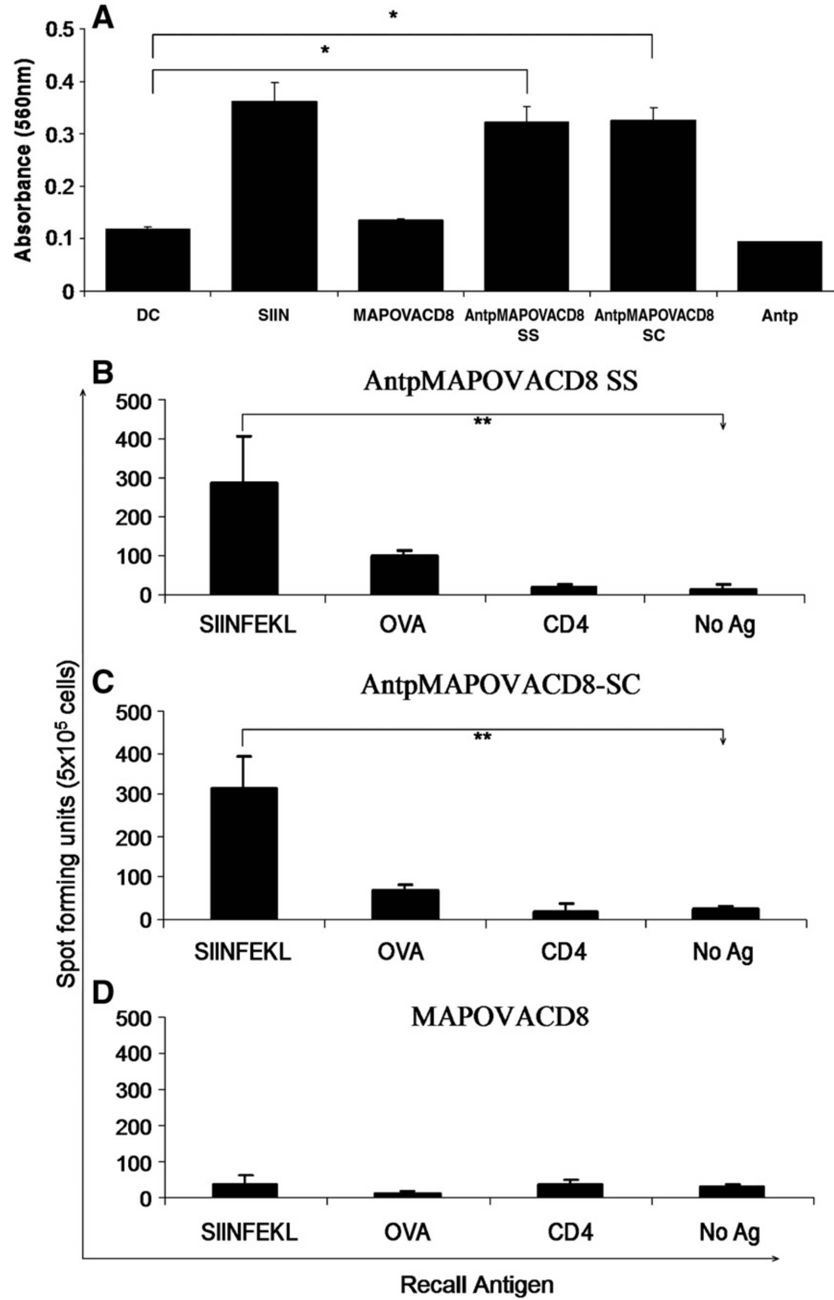

Fig. 2. AntpMAPOVACD8 SS and SC stimulate T cells *in vitro* and *in vivo*. (A) C57BL/6 DC and were stimulated with MAPOVACD8, AntpMAPOVACD8 SS, AntpMAPOVACD8 SC Antp peptide (5 μM) or OVACD8 for 8 h and added to B3Z T cells for 16 h. LacZ activity in B3Z T cells was assayed in total culture lysates with LacZ substrate CPRG. The absorbance (560 nm) of culture wells was read after 4 h incubation at 37 °C. Values are representative of mean ± SEM. (B–D) C57BL/6 mice were injected on day 0, 14 and 17 with 100 μg AntpMAPOVACD8 SS, AntpMAPOVACD8 SC or MAPOVACD8 via i.d. injection. Number of IFN-γ secreting cells was analyzed by ELISPOT assay and results are shown as mean ± SD spot-forming units (SFU)/5×10^5 cells. **$p < 0.01$.

antigen-specific IFN-γ responses. Mice immunized with MAPOVACD8 did not generate IFN-γ secreting cells (Fig. 2D).

3.2. AntpMAPOVACD8 conjugates induce potent in vivo proliferation and killing

To assess the ability of AntpMAPOVACD8 SS and AntpMAPOVACD8 SC conjugates to induce proliferation of T cells *in vivo*, we adoptively transferred CFSE-labeled OT-I splenocytes into mice immunized with AntpMAPOVACD8 SS or AntpMAPOVACD8 SC. Sixty hours after injection with OT-I cells similar proliferation levels of OT-I cells were detected in mice immunized with AntpMAPOVACD8 SS or AntpMAPOVACD8 SC but not control mice (Fig. 3).

The capacity of mice to generate OVACD8 specific killing *in vivo* following a single immunization was assessed. No OVACD8-specific CTL response was detected in the spleen of control mice. In contrast, strong *in vivo* lysis was detected when mice were immunized with either AntpMAPOVACD8 SS (68%) and AntpMAPOVACD8 SC (78%) (Fig. 4).

3.3. Addition of CpG oligodinucleotides with AntpMAPOVACD8 conjugates induces protection against B16-OVA tumor cells

Since disulphide-linked and thioether-linked AntpMAPOVACD8 demonstrated similar immunogenicities the ability of mice immunized with AntpMAPOVACD8 SC to be protected from challenge with OVA-expressing B16 melanoma tumor cells was investigated. In addition we also evaluated whether co-administration with the adjuvant CpG could improve efficacy and enhance anti-tumor immunity. Groups of mice ($n=8$) were injected i.d. with PBS, 100 μg AntpMAPOVACD8 SC alone or 100 μg AntpMAPOVACD8 SC + 50 μg CpG on days 0, 10 and 17. Seven days after immunization mice were challenged subcutaneously with 2×10^5 B16-OVA tumor cells and tumor growth was monitored.

Pre-immunization of mice with AntpMAPOVACD8 SC did not prevent B16-OVA tumor growth in any of the experimental groups (Fig. 5). However, mice immunized with AntpMAPOVACD8 SC with CpG exhibited significantly delayed tumor growth compared to mice immunized with PBS or AntpMAPOVACD8 SC alone (Fig. 5, $p<0.05$).

3.4. Maturation of DCs in vivo by AntpMAPOVACD8 conjugates

To understand the mechanism of induction of potent T cell responses in mice immunized with MAPOVACD8 conjugates, we assessed the ability of AntpMAPOVACD8 conjugates to induce maturation of DCs *in vivo*. C57BL/6 mice were injected i.d. on the footpads and 18 h later popliteal lymph nodes were removed, cells stained with the DC marker CD11c and maturation markers (CD40, CD80, CD86, and MHC class II) were assessed by flow cytometry. Analysis revealed that AntpMAOVACD8 conjugates do not induce maturation of DC (Fig. 6).

3.5. Uptake of AntpMAPOVACD8 conjugates by DC

There are numerous conflicting reports regarding the uptake mechanisms of CPP [18,19]. It is clear that peptide concentration, conjugation methods and cell type are all critical factors. To investigate if AntpMAPOVACD8 SS and AntpMAPOVACD8 SC conjugates are taken up by an energy-dependent mechanism in DC, cells were preincubated with sodium azide and 2-deoxyglucose to deplete ATP, then pulsed with 5, 25 and 50 μM of AntpMAPOVACD8 SS and AntpMAPOVACD8 SC and added to B3Z T cells. Presentation of both AntpMAPOVACD8 SS and AntpMAPOVACD8 SC to B3Z T cells was completely blocked, thus uptake of both conjugates at varying concentrations is via energy-dependant endocytosis (Fig. 7).

To further clarify the endocytic uptake pathway inhibitors of clathrin-mediated and caveolae-mediated endocytosis were assessed. Chlorpromazine and filipin III were incubated with DC followed by pulsing with AntpMAPOVACD8 conjugates for 8 h and presentation to B3Z T cells. Presentation of AntpMAPOVACD8 SS and SC was inhibited by >90% at all peptide concentrations by chlorpromazine, demonstrating uptake is clathrin-dependent. AntpMAPOVACD8 SS presentation was inhibited by 15.8, 99.0 and 99.2% at 5, 25 and 50 μM and AntpMAPOVACD8 SC was inhibited by 41.7, 95.1 and 97.3% at 5, 25 and 50 μM, respectively by filipin III (Fig. 7). Thus endocytic uptake for both SS and SC conjugates is caveolae-dependent at high peptide concentrations, yet caveolae-independent at low concentrations.

To study the role of macropinocytosis in uptake cytochalasin D, an inhibitor of contraction of actin-containing microfilaments which prevents phagocytosis and amiloride, which inhibits the Na^+/H^+ exchange inhibiting phagocytosis and/or macropinocytosis were used. Following pre incubation with cytochalasin D and amiloride, DC were pulsed with 5, 25 and 50 μM AntpMAPOVACD8 SS or SC then presented to B3Z T cells. Presentation of both SS and SC conjugates was prevented by cytochalasin D. Presentation after amiloride treatment of AntpMAPOVACD8 SS was inhibited by 81.9, 83.4 and 88.1% at 5, 25 and 50 μM and AntpMAPOVACD8 SC by 83.4, 87.0 and 87.3% at 5, 25 and 50 μM, respectively.

Overall the uptake of AntpMAPOVACD8 SS and SC is via energy-dependant endocytosis, most likely via phagocytosis and/or macropinocytosis. At a low peptide concentrations uptake is also via negatively charged receptors as demonstrated by inhibition with dextran sulphate for both AntpMAPOVACD8 SS and AntpMAPOVACD8 SC but not for higher concentrations (Fig. 7). Endocytosis is clathrin and caveolae-dependent however at low concentrations uptake is caveolae-independent (Fig. 6). In all experiments, DC pulsed with OVACD8 in the presence or absence of biochemical inhibitor

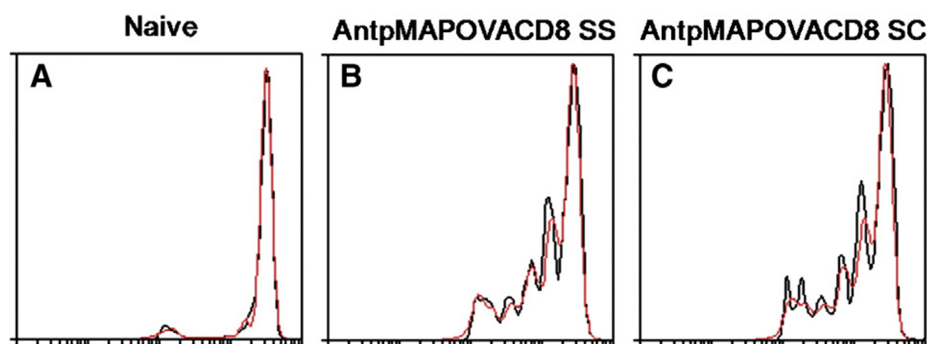

Fig. 3. Measurement of $CD8^+$ T cell proliferation *in vivo* by CFSE dilution. C57BL/6 mice were immunized i.d. with (A) PBS, (B) AntpMAPOVACD8 SS or (C) AntpMAPOVACD8 SC and purified CFSE-labeled OT-I T cells were injected i.v. into immunized mice 20 h later. Splenocytes were subsequently assessed via flow cytometry for CFSE dilution, gated on CD3 T cells. Representative histograms from 2 mice are shown.

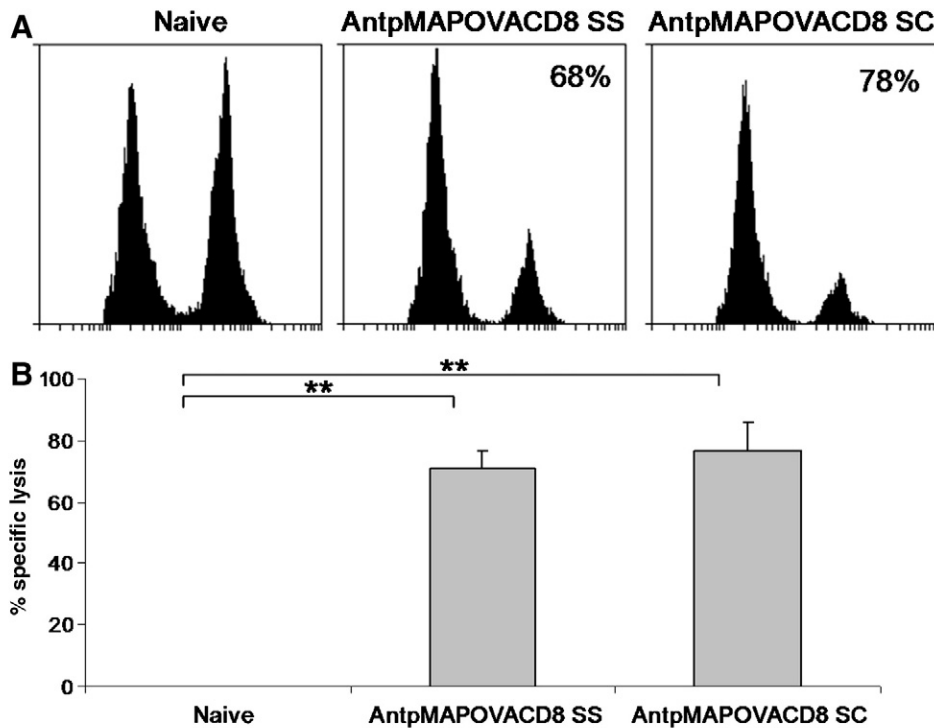

Fig. 4. *In vivo* OVACD8-specific lysis. C57BL/6 mice were immunized i.d. with PBS, AntpMAPOVACD8 SS or AntpMAPOVACD8 SC. (A) The percentage of the OVACD8 specific lysis was determined 8 days after immunization calculated as: $\{[1-(\mathrm{CFSE}^{low}/\mathrm{CFSE}^{high}$ of naïve mice / ratio $\mathrm{CFSE}^{low}/\mathrm{CFSE}^{high}$ of immunized mice$)]\times 100\}$. Representative histograms are shown. (B) Data is presented as mean % of killing ± SEM. $^{**}p<0.01$.

demonstrated that the inhibitor did not have any effects on the T cells (data not shown).

3.6. Presentation and processing of AntpMAPOVACD8 conjugates by TAP-independent MHC class I pathway

The role of endosomal processing was investigated using chloroquine and ammonium chloride, which prevent acidification of endosomes and monensin, a sodium/potassium proton ionophore which interferes with Golgi transport, acidification of intracellular compartments and blocks protein transfer from endosomes to lysosomes. Following pre incubation of chloroquine/ammonium chloride or monensin, DC were pulsed with 5, 25 and 50 μM AntpMAPOVACD8 SS or SC then presented to B3Z T cells. Presentation was inhibited at all peptide concentrations following chloroquine/ammonium chloride or monensin treatment (Fig. 8A–B). This suggests that both AntpMAPOVACD8 SS and SC conjugates are processed via the endosome and lysosome. In all experiments, DC pulsed with OVACD8 in the presence of inhibitor or the absence of inhibitor demonstrated that the inhibitor did not have any effects on the T cells (data not shown). We further investigated the TAP dependence of MHC class I presentation using RMA-S cells, deficient in TAP, pulsed with AntpMAPOVACD8 SS or AntpMAPOVACD8 SC for 1 h and adding to B3Z T cells. EL-4 cells are TAP-competent and were used as a positive control for the experiment. RMA-S pulsed with AntpMAPOVACD8 SS or SC equally presented to B3Z T cells above background responses (Fig. 8C). These results indicate that AntpMAPOVACD8 conjugates at all concentrations are processed and presented by MHC class I molecules via the endosomes and lysosome in a TAP-independent mechanism.

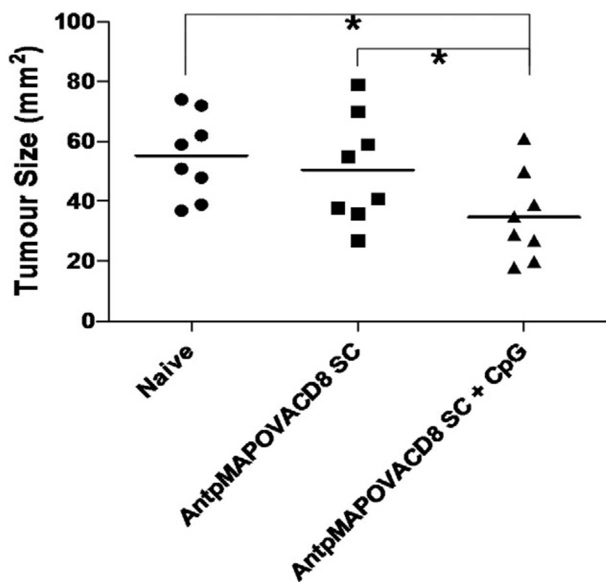

Fig. 5. C57BL/6 mice preimmunized on days 0, 10 and 17 with PBS, AntpMAPOVACD8 SC or AntpMAPOVACD8 SC + CpG then inoculated subcutaneously 7 days after final immunization with 2×10^5 B16-OVA melanoma cells into the abdomen and tumor growth recorded. Data presented 17 days post tumor inoculation.$^{*}p<0.05$.

3.7. Proteolysis and peptide loading of AntpMAPOVACD8 conjugates

Presentation of epitopes on MHC class I molecules can be achieved via degradation in the proteasomes. To examine the processing of AntpMAPOVACD8 conjugates by proteasomes DC were preincubated with lactacystin, an inhibitor of proteasomes. Lactacystin had no effect on the presentation of AntpMAPOVACD8 SS or SC at 5, 25 or 50 μM, thus conjugates are processed and presented by DC without the requirement for proteasomes (Fig. 9). To examine the role aminopeptidase in the ER and furin endopeptidases in the *trans*-Golgi, the inhibitors bestatin and furin inhibitor decRVKR-CMK were added to DCs prior to pulsing with AntpMAOVACD8 conjugates. Neither bestatin nor the furin inhibitor decRVKR-CMK inhibited of presentation to B3Z T cells at any peptide concentration.

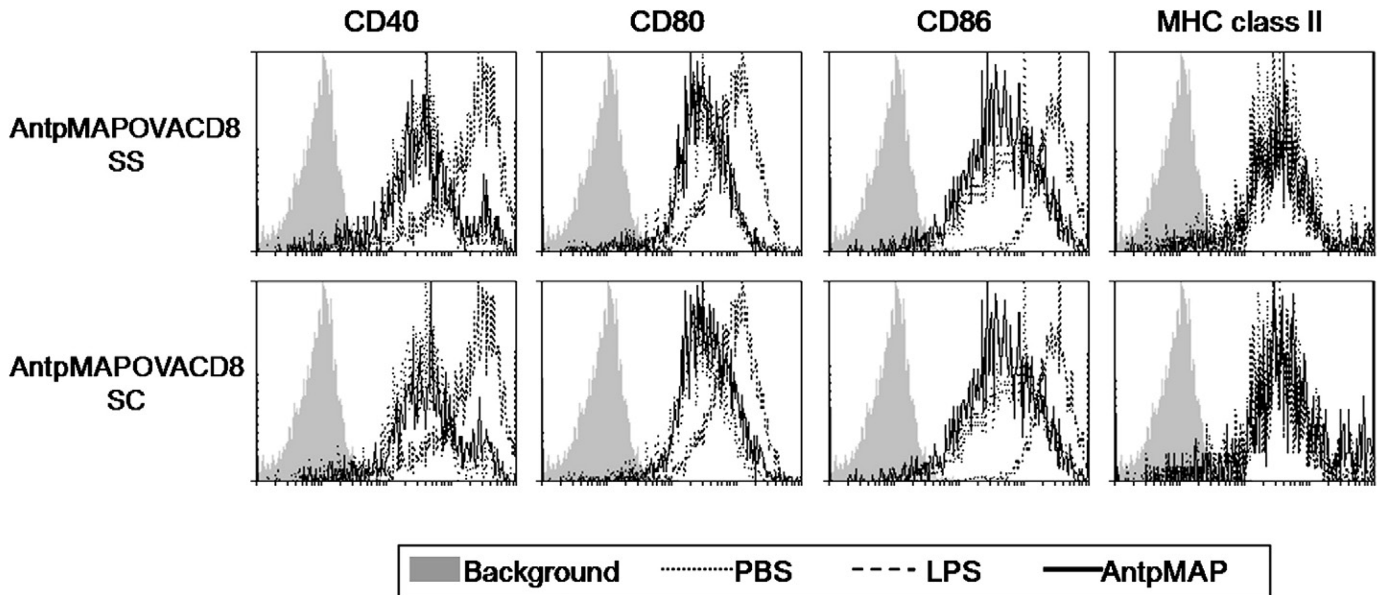

Fig. 6. *In vivo* DC maturation by AntpMAPOVACD8 conjugates. C57BL/6 mice were injected i.d. in the footpad with AntpMAPOVACD8 SS or AntpMAPOVACD8 SC at 100 µg per mouse. 18 h later popliteal LN were isolated and stained with the DC marker CD11c and maturation markers (CD40, CD80, CD86, and MHC class II) assessed by flow cytometry. Representative histogram plots are shown ($n = 3$). Mice injected with LPS were used as positive controls.

Finally Brefeldin A, which inhibits vesicle transport of newly synthesized MHC class I and II molecules between ER and Golgi complex, was pre incubated with DC (Fig. 8). Presentation of AntpMAPOVACD8 conjugates at 5, 25 and 50 µM was prevented, thus it is clear that both AntpMAPOVACD8 SS and SC are not degraded in the proteasome and that furin endopeptidase in the trans-Golgi and aminopeptidases in the ER do not contribute to further trimming. In all experiments, DC pulsed with OVACD8 in the presence of inhibitor

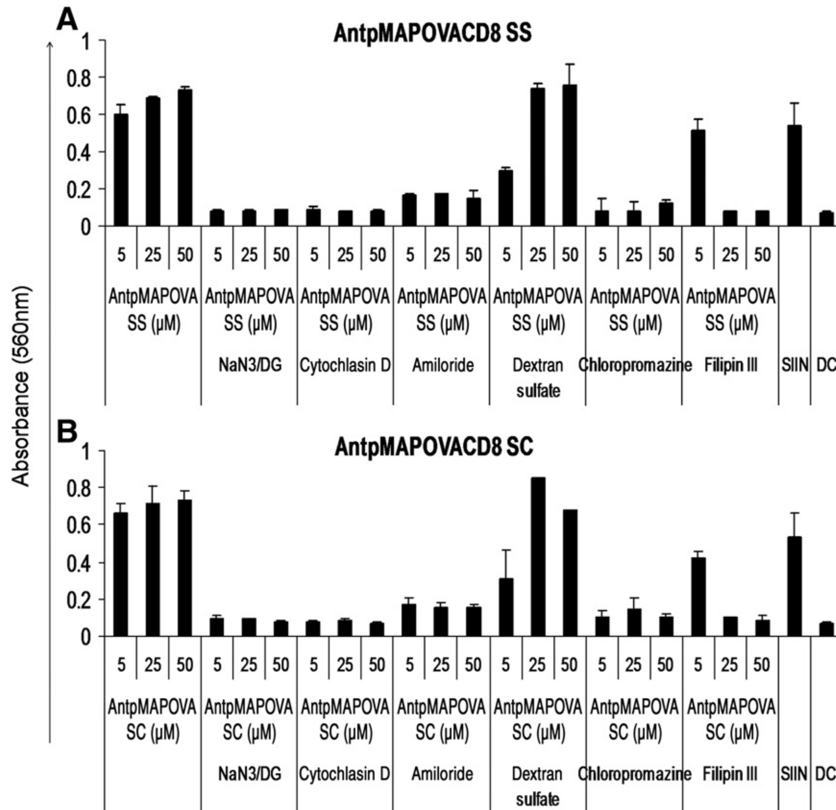

Fig. 7. Uptake and processing pathways of AntpMAPOVACD8 conjugates. *In vitro* grown DCs were incubated for 45 min with biochemical inhibitors of uptake pathways (sodium azide/2-deoxyglucose (10 mM), cytochalasin D (10 µg/ml), amiloride (6 µm), dextran sulphate (50 µg/ml), chloropromazine (10 µg/ml) and filipin III (10 µg/ml)). Cells were then incubated with (A) AntpMAPOVACD8 SS and (B) AntpMAPOVACD8 SC conjugates at 5, 25 and 50 µM. DCs were added to antigen-specific CD8 T cell clones and stimulation was assessed by B3Z assay. Values are representative of mean ± SEM.

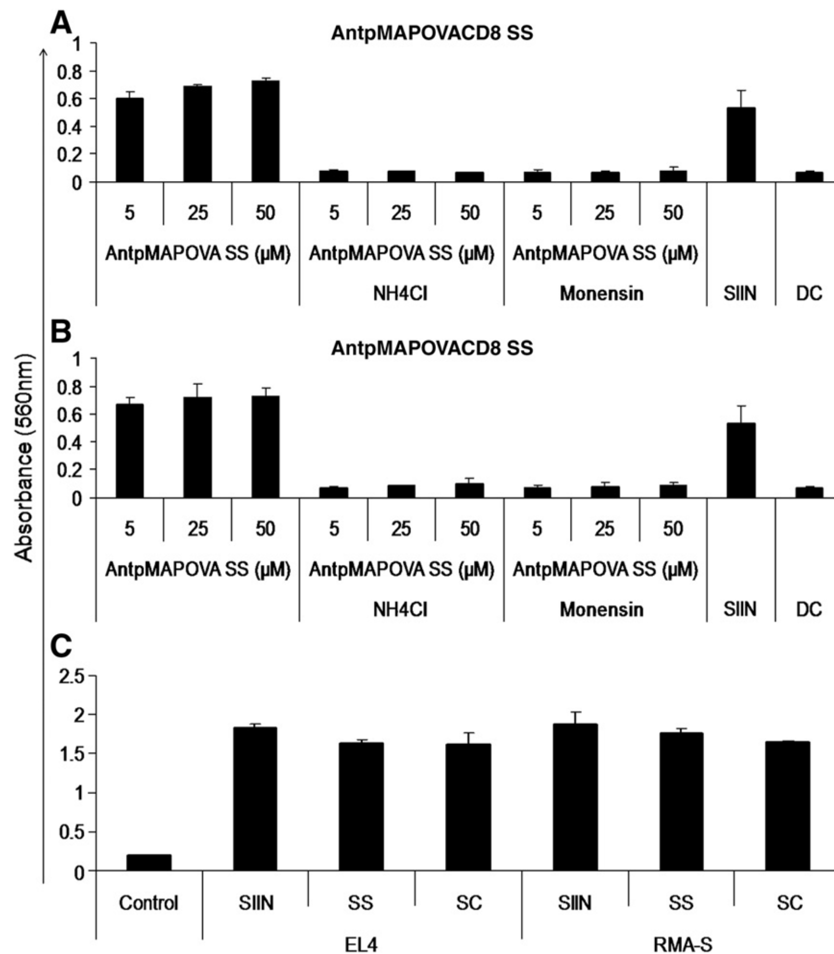

Fig. 8. Presentation and processing of AntpMAPOVACD8 conjugates. *In vitro* grown DCs were incubated for 45 min with inhibitors chloroquine/NH_4Cl (200 μM), and monensin (1000 μM). Cells were then incubated with (A) AntpMAPOVACD8 SS and (B) AntpMAPOVACD8 SC conjugates at 5, 25 and 50 μM. DCs were added to antigen-specific CD8 T cell clones and stimulation was assessed by B3Z assay. (C) EL-4 and RMA-S cells were stimulated with AntpMAPOVACD8 SS and SC (5 μM) or OVACD8 for 8 h and added to antigen-specific CD8 T cell clones and stimulation was assessed by B3Z assay. Values are representative of mean ± SEM.

or the absence of inhibitor demonstrated that the inhibitor did not have any effects on the T cells (data not shown).

4. Discussion

The objective of immunization strategies for cancer is to facilitate the presentation of antigenic epitopes by APCs to T cells and generate a potent immune response. For generation of effector CTL, exogenous antigens must enter the cross presentation pathway and be presented to CD8 T cells in the context of MHC Class I molecules. There is now a wealth of evidence demonstrating the superior cellular delivery of tumor associated peptides or proteins by conjugation to CPPs, ultimately leading to the generation of potent immune responses [4,9,20–22].

It is now clear that the delivery of CD8 and CD4 T cell epitopes is obligatory for the generation of long term immunity [23]. We have previously demonstrated that Antp tandemly linked to OVACD8 was rapidly internalized into the cytoplasm of macrophages, induced peptide specific T cells and protected mice against a subsequent tumor challenge [9]. Moreover both Antp and TAT have been used to deliver to CD8 and CD4 epitopes from OVA generating CTL and Th responses and an anti-tumor immunity [13]. However due to limitations in the synthesis of long linear peptides, the design of branched MAP allow for the inclusion of a greater number of tumor epitopes which would be advantageous for cancer vaccines [11]. The current study validates this approach using a MAP incorporating 4 identical OVACD8 epitopes. A similar approach can be used to deliver MAPs incorporating multiple CD8 and CD4 epitopes from the same or different antigen.

We have now extended on our Antp linear peptide studies to investigate the ability of Antp to deliver a 4 asymmetric branched peptide incorporating OVACD8 (MAPOVACD8) to APCs. We reveal that penetratin (Antp) linked to the MAPOVACD8 either by a disulfide (SS) or thioether (SC) linkage is taken up *in vitro* by DC and enters the cross presentation pathways for MHC class I loading. Moreover mice immunized with AntpMAPOVACD8 SS or AntpMAPOVACD8 SC induced strong $CD8^+$ specific IFN-γ responses shown by ELISpot assay. Whereas the MAPOVACD8 construct without the Antp internalizing sequence is not presented by MHC Class I molecules nor generates antigen-specific IFN-γ response *in vivo*. Mice immunized with AntpMAPOVACD8 SS or AntpMAPOVACD8 SC showed strong *in vivo* proliferation. Likewise mice immunized with AntpMAPOVACD8 SS or AntpMAPOVACD8 SC generated potent and comparable OVACD8 specific *in vivo* lysis.

Our results showing the capacity of CPP to induce a superior immune response via enhanced delivery and presentation is supported by Shibagaki and Udey [22] who compared the immunogenicity of OVA with and without the inclusion of the 11-mer HIV TAT transduction domain (YGRKKRRQRRR). Recombinant TAT-OVA was efficiently transduced into DC and processed by proteasome-dependent pathway for MHC class I restricted presentation to CTL [22]. Furthermore, subcutaneous immunization of TAT-OVA transduced DC induced antigen-specific CTL and Th and protected against a lethal dose of OVA-expressing tumor cell line, EG.7. In contrast, OVA failed to

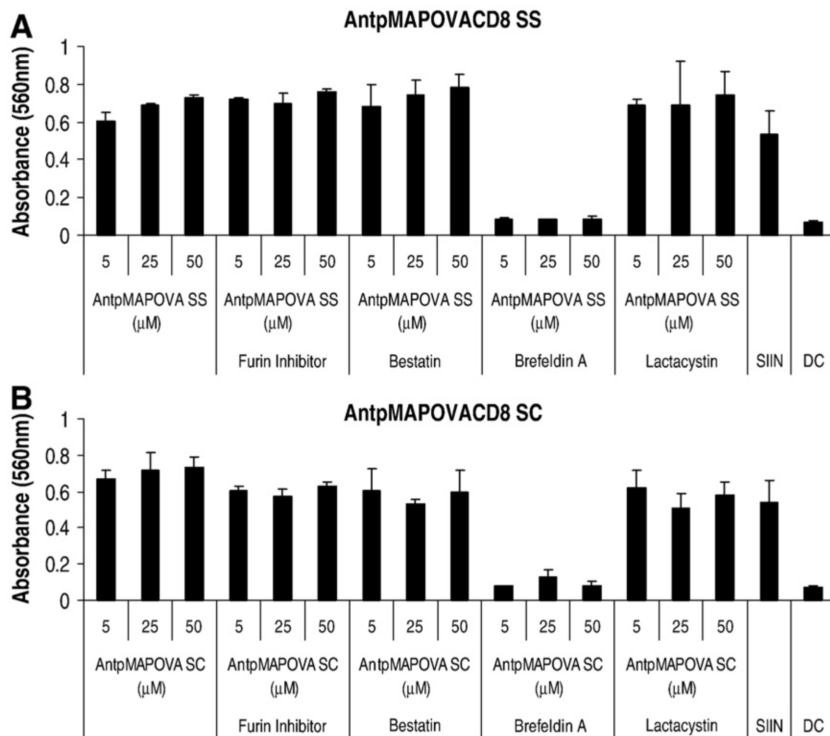

Fig. 9. Proteolysis and peptide loading of AntpMAPOVACD8 conjugates. *In vitro* grown DCs were incubated for 45 min with biochemical inhibitors (furin inhibitor (10 μM), bestatin (10 μM), brefeldin (10 μg/ml) and lactacystin (10 μM)). Cells were then incubated with (A) AntpMAPOVACD8 SS and (B) AntpMAPOVACD8 SC conjugates at 5, 25 and 50 μM. DCs were added to antigen-specific CD8 T cell clones and stimulation was assessed by B3Z assay. Values are representative of mean ± SEM.

prime specific CTL and vaccination with OVA pulsed DC and did not afford the same degree of protection as TAT-OVA DC [22].

Maturation of DCs involves up-regulation of co-stimulatory molecules (e.g. CD40, CD80 and CD86) and pro-inflammatory cytokines (e.g. interleukin (IL)-12, IL-4) for optimal Th1 or Th2 cell priming [24]. However following *in vivo* injection of either AntpMAPOVACD8 SS or AntpMAPOVACD8 SS, isolated lymph node DC did not mature and revealed no change in expression of co-stimulatory molecules including CD40, CD80, CD86 and MHC Class II. Thus the delayed anti-tumor response promoted by the adjuvant CpG may in part be explained by the addition of a danger signal and generation of a more robust T cell proliferation response [7]. In our previous studies we demonstrated that a linear fusion peptide incorporating Antp and the OVA CD8 epitope SIINFEKL (AntpOVACD8) was able to completely protect mice from a lethal B16-OVA tumor challenge [9,25]. However, in the current study utilizing a branched AntpMAPOVACD8 significant tumor inhibition was only apparent when used in combination with CpG. A major difference between AntpOVACD8 and AntpMAPOVACD8 is the purity of peptide. Unlike AntpOVACD8 linear peptide synthesized with purities of greater than 95%, MAP peptide purity range from 60 to 70%. Therefore it is possible that the immunogen AntpMAPOVACD8 is not as potent as AntpOVACD8 and may require higher doses than 100 μg to be more efficacious. In order to overcome the purity issues we are now investigating an alternative synthesis of MAP based on native chemical ligation methods [26,27].

The aim of utilizing CPPs for cancer vaccines is the targeting to intracellular compartments to generate a therapeutic response. The mechanism of internalization of CPPs has gained considerable attention in recent years with varied and conflicting results, arising due to different experimental conditions [28]. The endocytic pathways, macropinocytosis, clathrin-mediated and caveolae/lipid-raft mediated endocytosis, along with interaction of polycationic CPP with negatively charged heparin sulphate proteoglycans are widely implicated in the uptake of CPP [18,29]. We reveal that uptake of AntpMAPOVACD8 SS and AntpMAPOVACD8 SC is via energy-dependant endocytosis. Endocytosis can occur via clathrin-mediated endocytosis, macropinocytosis, caveolae-mediated endocytosis, clathrin-caveolae-independent endocytosis pathways depending on the peptide concentration of the CPP. The majority AntpMAPOVACD8 conjugates are taken up by DC via phagocytosis and/or macropinocytosis and using the inhibitor chlorpromazine we show uptake to occur via clathrin-mediated endocytosis.

No difference between uptake pathways of the disulfide or thioether linked AntpMAOVACD8 construct was seen. However in agreement with others peptide concentration appears to alter the uptake pathway. At low concentrations AntpMAPOVACD8 SS and AntpMAPOVACD8 SC binds to negatively charged receptors, whereas at higher concentrations uptake is independent of negatively charge receptors. Moreover uptake at high concentrations (25 and 50 μM) is caveolae-dependent whereas at 5 μM uptake occurs via caveolae-independent uptake pathway. Both AntpMAPOVACD8 SS and AntpMAPOVACD8 SC are equally inhibited by dextran sulphate, a competitor for negative charges however, uptake of AntpMAPOVACD8 SC is predominantly *via* the caveolar independent pathway.

Studies on the subsequent intracellular trafficking routes of AntpMAPOVACD8 conjugates show proteasome independent processing via the endosomes and lysosome for presentation by MHC Class I molecules with further trimming by furin endopeptidase in the trans-Golgi or by aminopeptidases not required. In addition access to the ER is TAP-independent, as also shown with TAT-OVACD8 and AntpOVACD8 processing studies [9,30]. Studies using the TAT peptide linked to OVACD8 also show a Golgi–ER independent pathway, although linking CPP and the antigen using either the carboxyl or amino terminal end changes the intracellular processing pathways. Linking OVACD8 to the amino terminal (OVACD8-TAT) requires furin endopeptidases in the trans-Golgi for processing. Yet presentation of OVACD8 linked to the carboxyl terminal (TAT-OVACD8) is furin independent [30].

In summary we have shown that the linking of the CPP Antp to an asymmetric 4 branched MAP incorporating the H-2K^b CD8 eight-mer

epitope OVACD8 promotes the uptake and cross presentation and subsequent proliferation and generation antigen-specific T cells. Thus Antp represents a proficient means to deliver branched MAP to generate immunity enabling application with polytope peptides scaffolds. Moreover immunization of antigen with CpG significantly delays tumor growth. We also show that the uptake mechanism of AntpMAPOVACD8 constructs is dose-dependent and neither uptake nor intracellular processing is altered by the type of chemical linkage.

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