Assessment of the enhancement of PLGA nanoparticle uptake by dendritic cells through the addition of natural receptor ligands and monoclonal antibody

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ABSTRACT

Targeting of specific receptors on antigen-presenting cells is an appealing prospect in the production of novel nanoparticulate vaccines. In particular, the targeting of vaccines to dendritic cell (DC) subsets has been shown in models to significantly improve the induction of immune responses. This paper describes the evaluation of natural ligands, mannan and chitosan, and monoclonal antibodies as targeting motifs to enhance uptake of PLGA nanoparticle carriers by bovine DCs. To assess enhancement of uptake after the addition of natural ligands a bovine monocyte derived DC (MoDC) model was used. For the assessment of monoclonal antibody targeting, the model was expanded to include afferent lymph DCs (ALDCs) in a competitive uptake assay. Mannan, proved unsuccessful at enhancing uptake or targeting by MoDCs. Chitosan coated particle uptake could be impeded by the addition of mannan suggesting uptake may be mediated through sugar receptors. Inclusion of monoclonal antibodies specific for the DEC-205 (CD205) receptor increased the number of receptor expressing DCs associated with particles as well as the number of particles taken up by individual cells. These results support the further evaluation of active targeting of nanovaccines to DCs to enhance their immunogenicity in cattle and other large mammalian species including humans.

Keywords: nanoparticle, targeting, mannose, cattle, dendritic cell, DEC-205 antibody

Abbreviations: DC, dendritic cell; MoDC, monocyte derived dendritic cell C; ALDC, afferent lymph dendritic cell; PLGA, poly (lactic-co-glycolic) acid; OVA, ovalbumin; MAN, mannan; mAb, monoclonal antibody

1. INTRODUCTION

Nanoparticulate delivery of subunit vaccines offers a number of potential advantages over conventional approaches including preferential uptake by dendritic cells (DC) and prolonged release of antigen resulting in enhanced T cell stimulation [1]. Furthermore, nanoparticles offer a versatile system that allows for targeted delivery of antigen and adjuvant to the same DC [2]. It is generally acknowledged that DCs are capable of taking up particles with a varied range of physical properties. Studies have shown enhanced DC uptake of particles *in vitro* but few have addressed specific targeting and the uptake of particles by different cell types [3]. A recent study showed liposomes are taken up primarily by CD11c⁺ DCs and macrophages in murine lymph nodes, with the latter being more efficient [4]. Similarly, it was shown that PLGA nanoparticles are more efficiently taken up by macrophages than DC [5]. Since DCs are the primary target of most vaccines, these data suggest that particulate systems may need to be further targeted to specific cell types to achieve optimal induction of immune responses.

Targeting of vaccines to DCs via specific receptors can be facilitated with the use of natural receptor ligands or mAbs specific for DC receptors. Targeting mannose receptors through mannosylation is the most common means of targeting DCs through natural ligands. Indeed protein mannosylation has proved effective at both improving antigen uptake and adjuvanting immune responses [6]. Mannosylation of particles however has yielded variable results both in terms of enhancing particle uptake or DC maturation [7-9].

Targeting of particles to a cell type using specific mAbs was first evaluated in the context of cancer drug delivery [10, 11]. Employing the same principle, nanoparticles targeted to DC using DC-SIGN-specific mAbs generated 10-100 fold increases in the efficiency of

antigen-presentation compared to non-targeted particles [12]. mAb targeting of microparticles to DEC-205 resulted in a two-fold enhancement of immune responses [13]. A second study targeting nanoparticles using DEC-205 specific mAb reported only a 15% increase in uptake over particles coated with an irrelevant isotype matched mAb when targeting immature DCs but a 70% increase when targeting mature DCs [14]. Furthermore, the properties of different particle types such as their size can affect which DC subset becomes associated with the particle and thus the type of immune response evoked [15, 16]. Dendritic cell targeting of biomaterials has been comprehensively reviewed in [17].

Cattle represent an important veterinary species susceptible to a number of infectious diseases for which there is a need to develop new or improved vaccines to support control programmes. Bovine DC subsets have been characterised suggesting that they represent orthologous populations to those described in humans [18, 19]. As such, cattle provide a large mammal model species for the evaluation of DC-targeted nanovaccines, which may be useful in cattle, other animals and humans. As a first step to using the bovine system for the development of novel nanoparticle vaccine systems, this study describes the synthesis of targeted PLGA nanoparticles using both natural ligands and mAb and an evaluation of their effects on uptake by bovine DC *in vitro*.

2. MATERIALS & METHODS

2.1. Isolation of bovine monocytes

All animal work was approved by the APHA Ethics Committee and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood collected from adult male Holstein-Friesian

cattle [20]. Monocytes were purified from PBMC by magnetic activated cell sorting (MACS), using anti-human CD14-microbeads as described by the manufacturer (Miltenyi Biotec).

2.2. Generation of monocyte derived dendritic cell (MoDC)

Monocytes were resuspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (cRPMI) and plated at 2×10^6 cells/well in 24 well Ultra-Low Attachment Tissue Culture Plates (Costar). Differentiation to MoDCs was induced by the addition of recombinant bovine GM-CSF and IL-4 according to the manufacturer's recommended dilution (1:20) (Bovine Dendritic Cell Growth Kit, AbD Serotec, Oxford, UK). Cells were incubated for 6 days at 37°C in a 5% CO₂ humidified atmosphere.

2.3. Bovine afferent lymph dendritic cells

Cells were collected from afferent lymph following pseudo-afferent lymphatic cannulation [21] and cryopreserved in 10% (v/v) DMSO in FBS. Cells were thawed rapidly in a 37°C water bath and washed in pre-warmed cRPMI before use.

2.4. PLGA nanoparticle synthesis

Acid terminated PLGA (Resomer RG502 H, 50:50, Mw 7-17 kDa, Sigma, Poole, UK) was dissolved in ethyl acetate (Sigma) at a concentration of 25 mg/ml to form the oil phase (o). The model antigen ovalbumin (OVA) conjugated Alexa Fluor 488 or Alexa Fluor 647 (Life Technologies) diluted to 5 mg/ml in DPBS formed the first water phase (w₁). 1% (w/v) poly vinyl alcohol (PVA; 98% hydrolysed Mw 13-23 KDa, Sigma) in distilled water was used as a

stabilizer in the second water phase (w_2). To form the first emulsion (w_1/o), w_1 was rapidly added to the oil phase at a ratio of 1:10 and sonicated for 30 sec at an intensity of 14 microns using a Soniprep 150 sonicator (MSE, London, UK). To form the second emulsion ($w_1/o/w_2$), w_1/o was added to w_2 at a ratio of 1:5 and sonicated for 2 min at an intensity of 18 microns. The final emulsion was added to a larger vessel and the solvent was evaporated overnight with stirring (250 rpm) at 37°C. The particles were collected by ultracentrifugation at 40,000 x g for 20 min and washed twice with distilled water to remove excess PVA.

2.5. Particle sizing and assessment of surface charge

The hydrodynamic diameter of the particles was calculated using dynamic light scattering (DLS). Particles were sonicated, dispersed in distilled degassed water and analysed on a Zetasizer 3000 (Malvern Instruments, Malvern, UK) in a disposable cuvette at 25°C with a scatter angle of 90°. Eleven readings were made in triplicate. Zeta potential measurements (20 readings/sample in triplicate) were made using a disposable zeta cell (Malvern Instruments).

2.6. Scanning electron microscopy

Particles were air dried onto poly-L-lysine coated coverslips (BD Biosciences, Oxford, UK). Coverslip were applied to double sided carbon (Agar Scientific, Stansted, UK) attached to a 13mm aluminium stub (Agar Scientific) and sputter coated with gold to a thickness of 15nm using an Emitech K550X Sputter Coater (Quorum Technologies, Ashford, UK). Particles were visualized using a Zeiss Evo LS10 scanning electron microscope (Zeiss, Welwyn Garden City, UK) at an accelerating voltage of 10-20 kV.

2.7. Conjugation of mannan and ovalbumin to PLGA nanoparticles

Ligands were conjugated to the surface of PLGA nanoparticles post-synthesis using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), a carbodiimide cross-linker that activates carboxyl groups for spontaneous reaction with primary amines as schematically illustrated in Fig. 2A. Particles were suspended at 10 mg/ml based on the starting amount of PLGA in 0.1M 2-(N-morpholino)ethanesulfonic acid buffer (MES) (Sigma) adjusted to pH 5.2 with 0.1M hydrochloric acid (HCl). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulphonylhydroxysuccinimide (Sulpho-NHS) (Sigma) were added at a final concentration of 0.1 M, and then mannan (MAN) and OVA (Sigma) were added at a final concentration of 5 mg/ml, unless otherwise indicated. The reaction was carried out overnight with stirring (250 rpm) at room temperature. Particles were collected by centrifugation at 10,000 x g for 2 min and washed twice with Tris buffered saline (TBS). To neutralize reactive groups not utilized in the process and isourea intermediates, particles were resuspended in TBS for 1 hr. The coating of particles was confirmed by staining with concanavalin A-FITC (ConA-FITC; Sigma) or anti-OVA mAb (clone OVA-14, Sigma) followed by APC-conjugated anti-mouse IgG1 secondary antibody (clone X56; BD Biosciences). The particles were washed twice with DPBS and fluorescent staining assessed using a MACSQuant Analyzer flow cytometer. Particles were gated by forward (FSC) and side (SSC) scatter measurements (logarithmic scaling); exclusion of electronic noise was confirmed by running a sample of DPBS. Residual mannose in wash supernatants was quantified using the colorimetric resorcinol assay as described [22]. In brief 20µl of sample or standard mannan was mixed with an equal volume of 6mg/ml resorcinol in water and following this 100µl of 75% H₂SO₄ was added. The reaction was vortexed and heated to 90°C for 30 min. The reaction was allowed to cool for 30 min before absorbance

was read at OD₄₅₀. Residual OVA in wash supernatants was quantified by measuring absorbance at OD₂₈₀.

2.8. Chitosan coating of PLGA nanoparticles

PLGA nanoparticles were coated with chitosan by overnight incubation with 1 mg/ml chitosan salts or chitosan oligomers (all Heppe Medical Chitosan GmbH, Halle, Germany). Unbound chitosan was removed by washing twice in DPBS. The chitosan molecules assessed included the salts; carboxy-methyl chitosan and chitosan HCl. The oligomers included sizes ranging from; <1 KDa, 1-3 KDa, 3-5 KDa and >5 KDa.

2.9. Monoclonal antibody coating of nanoparticles

To preserve conformation and function, mAb was coated onto PLGA nanoparticles using a non-crosslinking adherence based method. OVA-Alexa Fluor 647-loaded PLGA nanoparticles were divided into equal aliquots: 1 mg particles were suspended in 1 ml 0.1 M MES buffer pH 6.2 (Sigma), and either mouse anti-bovine DEC-205 mAb or a relevant isotype control mAb (both AbD Serotec) were added at a final concentration of 25 µg/ml and incubated overnight at room temperature. Particles were washed twice with DPBS and coating was confirmed using FITC conjugated anti-mouse IgG secondary antibody (Sigma) and flow cytometry. Aliquots of antibody coated particles were further coated with 3-5 kDa oligo chitosan as described above.

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184 *2.10. Nanoparticle uptake and inhibition studies*

185 Following 6 days in culture, immature MoDCs were plated at 5×10^4 cells/well in 96-well round-
186 bottom plates (Corning). For the basic uptake assay, PLGA nanoparticles loaded with OVA-
187 Alexa Fluor 488 and matched by polymer weight were added to cells, which were incubated
188 for 60 min at 37°C or 4°C before washing 3 times ($930 \times g$ for 2 minutes) with PBS. For the
189 inhibition studies, cells were pre-treated with; mannan (MAN; 50 µg/ml), polyinosinic acid
190 (poly I; 50 µg/ml) or amantidine (50 µg/ml) (all Sigma) for 30 min at 37°C. Uptake was assessed
191 using a MACSQuant Analyzer flow cytometer. Dead cells were excluded by staining with Near-
192 Infra Red Fixable Live/Dead Cell stain (Life Technologies), and the mean Alexa Fluor 488
193 fluorescence intensity of viable cells used as a measure of uptake.

194

195 *2.11. Afferent lymph dendritic cell / MoDC competitive nanoparticle uptake assay*

196 MoDCs were resuspended in 1 ml PBS with 5% FBS and labelled with carboxyfluorescein
197 succinimidyl ester (CFSE; Life Technologies) [23]. Afferent lymph cells were analysed using a
198 volumetric flow cytometer and the density of afferent lymph dendritic cells (ALDC), defined
199 by a high FSC/SSC profile, was determined. MoDC were mixed with afferent lymph cells at a
200 ratio that ensured approximately equal numbers of MoDC and ALDC in cRPMI. Cells were
201 plated at 5×10^5 /wells in a 96 well round-bottom plate and pulsed with particles (25 µg per
202 well) for 60 min at 37°C. Cells were washed 3 times with cold PBS and stained with Near-Infra
203 Red Fixable Live/Dead Cell stain (Life Technologies) and a pool of anti-bovine CD80 and CD86
204 mAbs (clones IL-A159 and IL-A190) conjugated to Alexa Fluor-405 using the Zenon Mouse
205 IgG1 Labelling Kit (Life Technologies). Analysis was undertaken on a MACSQuant Analyzer flow

cytometer. DCs were identified as viable cells expressing CD80/86. MoDC and ALDCs were discriminated on the basis of CFSE fluorescence and particle uptake was assessed as previously described.

2.12. Statistical analysis

All statistical analysis was performed using GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA). Two-way analysis of variance (ANOVA) followed by a Bonferroni's post-test or unpaired student's t-tests were used for the analysis where indicated. A p value <0.05 was considered statistically significant.

3. RESULTS

3.1. Characterization of PLGA nanoparticle uptake

PLGA nanoparticle formation was confirmed by scanning electron microscopy (Fig. 1A). Discrete particles within the nm size range with a smooth surface morphology were observed, which was confirmed by DLS (Fig. 2I). Furthermore, DLS revealed an acceptable level of polydispersity (<0.2). We used flow cytometry to quantify the internalization of PLGA nanoparticles by bovine MoDC (Fig. 1B). Fluorescent particle uptake was greatly enhanced at 37°C compared to 4°C suggesting an active process was required.

3.2. Natural ligand targeting of nanoparticles to mannose receptors

This study employed a zero length carbodiimide cross-linker to conjugate MAN or OVA to PLGA nanoparticles post-synthesis (Fig. 2A). Analysis of the wash supernatants of MAN- and OVA-coated particles suggested that significant amounts of ligand became bound to the surface of particles (Fig. 2B and 2C). This was confirmed by staining of particles and analysis

by flow cytometry, which showed that ~80% of MAN-coated (Fig. 2D and E) and >90% of OVA-coated (Fig. 2F and G) particles were conjugated. Coating of particles with MAN neutralized the surface charge however OVA coating had little effect on surface charge (Fig. 2H). MAN coating slightly increased particle diameter whereas OVA increased it by approximately 50nm (Fig. 2I).

MoDC uptake of PLGA nanoparticles which were either non-coated or coated with MAN or OVA was assessed in comparison to soluble OVA using flow cytometry (Fig. 3A). Rather than enhancing uptake by MoDC, the net uptake of both MAN- and OVA-coated particles when compared to the non-coated particles was reduced albeit without statistical significance. The net uptake of all particles was lower than that of the soluble protein. To reveal the mechanisms involved in MoDC uptake of coated and non-coated PLGA particles, we assessed particle internalization in the presence of different uptake inhibitors or competing receptor ligands (Fig. 3B-I). The ability of mannosylated particles to specifically target mannose receptors was analyzed using soluble MAN as a competitive inhibitor but this had no inhibitory effect (Fig. 3E and G). Given this unexpected finding, we used flow cytometry and confirmed surface expression of the macrophage mannose receptor 2 (CD206) on bovine MoDCs (data not shown). Furthermore we could demonstrate that mannose blocked nearly 70% of soluble OVA uptake (Fig. 3I), which is in accordance with a previous study using murine DCs [24]. Amantidine, an inhibitor of clathrin-mediated endocytosis, inhibited >80% uptake of all particles and soluble OVA. Poly-inosine, a scavenger receptor inhibitor, had no discernable effect.

Next we looked at the effects of chitosan coatings to enhance PLGA nanoparticle uptake by MoDC (Fig. 4). Coatings were confirmed by assessing changes to the surface charge of particles (Fig. 4A). The positively charged oligomers reduced the negative surface charge

of particles in a manner that correlated with the size of the respective chitosan molecules. The chitosan salts had the expected converse effects due to the slight negative charge bestowed by the carboxylic acid groups on carboxy methyl chitosan and the positive charge from chitosan HCl. Coating of nanoparticles with chitosan impaired particle uptake especially when chitosan salts were used (Fig. 4B). The oligomer coated particles were taken up equally well regardless of molecular weight but still less efficiently than the non-coated particles. We could not rule out the possibility that this was due to the formation of larger particles or aggregates as the sample become extremely poly disperse after chitosan coating. Adherence of chitosan-coated particles to MoDC at 4°C was comparable to non-coated particles (data not shown) suggesting an impairment in active uptake. However, in contrast to the results obtained with MAN- and OVA-coated particles, the co-incubation with soluble mannose reduced uptake of the oligomer chitosan coated particles by between 30-50%; this was not the case with chitosan salts (Fig. 4C).

3.3. Monoclonal antibody coating of PLGA nanoparticles

Finally we assessed whether conjugation of anti-bovine DEC-205 mAb to the surface of PLGA nanoparticles could specifically target this receptor on a bovine DC population. Staining of mAb-coated particles with a fluorochrome-conjugated secondary antibody confirmed coating of the majority of particles (Fig. 5A). To test the selective uptake of these particles, a novel uptake assay was established involving a mixed culture of two DC populations: DEC-205⁻ MoDC (pre-labelled with CFSE to aid discrimination) competing for particles with DEC-205⁺ ALDC (Fig. 5B). Compared to ALDC, there were far more MoDCs associated with particles regardless of the coating (Fig. 5C). Coating with the isotype control or DEC-205 mAb increased the proportion of MoDC that had taken up particles by 21% and 17%, respectively. In the case

of the ALDC, the percentage of cells that had taken up particles was significantly enhanced by the addition of DEC-205 mAb but not with particles coated with the isotype control mAb (Fig. 5C). When assessing particle uptake on a per cell basis, there was a significant increase using DEC-205 mAb coated particles compared to control mAb-coated particles by the ALDC, but not MoDC population (Fig. 5D). Co-coating with both chitosan and mAb reduced overall uptake by almost five-fold nonetheless the trend for DEC-205 mAb to increase uptake in ALDC was conserved (Fig. 5E).

4. Discussion

PLGA nanoparticles offer a malleable system for the targeted delivery of antigens and adjuvants. Enhancing the uptake of nanoparticles by DCs *in vitro* is an important step prior to *in vivo* evaluation. This study firstly confirmed the uptake of PLGA particles by different bovine DC populations demonstrating their versatility for veterinary applications. This study further evaluated specific uptake utilising two of the most popular means of DC targeting: natural ligands and mAb. No enhancement of DC uptake using mannosylated particles was observed, which could be due to the relatively neutral charge of the mannosylated particles produced, since previous studies have found that mannosylation bestows a more negative charge and that may facilitate enhanced uptake [7]. Similar to results obtained with murine DC [24], we found that soluble OVA uptake by bovine MoDC was inhibited by mannose. We therefore coated particles in OVA with the aim of replicating this effect. This could not be achieved, despite the particles retaining a negative charge, and the particle uptake resembled that of non-coated particles. This is consistent with an uptake model in which the particulate nature and factors such as charge and size may play a more important role than the surface ligands. In support of this concept it has been reported that mannosylation increases uptake by cells

301 lacking a defined mannose receptor [25]. In contrast to mannosylation, chitosan oligomer
302 coated particle uptake was partially inhibited by mannose as had previously been described
303 using non-particulate oligo-chitosan that specifically bound the macrophage mannose
304 receptor (CD206) on macrophages [26, 27]. The uptake inhibition of chitosan, as opposed to
305 MAN-coated particles, may be due to the number of ligands available, where MAN was
306 dependent on the reaction between single residues on each of the particle's polymers,
307 chitosan could completely coat the surface due to electrostatic interactions. Chitosan-coated
308 particles were taken up by the DCs to a lesser extent compared with non-coated particles.
309 There are reports stating that chitosan coating enhances uptake although these particles
310 were tested on tumour cell lines which may lack the necessary endocytic pathways to take up
311 PLGA particles to the same extent. Moreover, chitosan is more adherent possibly co-localizing
312 the particle to the cell surface prolonging the window of uptake [28].

313 Finally we used mAb to target particles to the DEC-205 receptor which is expressed on
314 defined bovine DC populations *in vivo*. Two DC populations, MoDCs and ALDC, were employed
315 in a competitive uptake study. A similar model has recently been used to assess mannosylated
316 particle uptake [29]. As only ALDCs express DEC-205 we could evaluate the ability of mAb to
317 target particles to these cells. MoDCs were far superior to ALDC at particle uptake; this is likely
318 due to ALDC representing a maturing population with reduced endocytic capacity. However,
319 mAb targeting specifically increased the number of ALDC taking up particles. Interestingly,
320 coating with irrelevant isotype mAb significantly enhanced uptake of particles by MoDC. This
321 was not due to a decrease in particle surface charge (data not shown) resulting in less
322 electrostatic repulsion and may rather reflect binding of mAb coated particles to Fc receptors.
323 The enhancement of uptake when particles were coated with mAb has previously been
324 described using microparticles and this effect was not observed when coating with BSA [30].

This suggests it is a property of immunoglobulin, and not protein in general, which is responsible for improved uptake and is consistent with our finding that OVA coating did not augment uptake. Compared to MoDCs, DEC-205 mAb coating increased the amount of particles taken up by ALDC on a per cell basis. Whilst the addition of chitosan reduced uptake, specific uptake of DEC-205 mAb coated particles by ALDCs was still observed. This may be due to the chitosan restricting non-specific uptake as previously observed and thus limiting MoDC uptake. However, the use of chitosan in this role complicates the picture as the earlier experiments had suggested that it could bind to mannose receptors on MoDC. Overall our data supports earlier studies showing that the increased affinity of antibodies specific for target cells results in greater binding and uptake when compared to natural ligands [31].

The establishment of the assay and model systems presented in this study provides a solid basis for the further screening and evaluation of DC receptor targeted nanocarriers which could be then be assessed in cattle, as a relevant large animal model for vaccine development. Whilst the enhanced targeting of mAb-coated particles to CD205⁺ DCs is encouraging the predominant uptake by MoDC highlights the challenge faced when delivering particles to the *in vivo* milieu of phagocytes and cellular processes. Further work should evaluate the inclusion of a 'stealth' layer such as polyethylene glycol to make the particles more resistant to non-specific uptake and to further explore the effect of physical parameters on the uptake route of particles in both *in vitro* and *in vivo* models.

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FIGURE LEGENDS

Fig. 1. Assessment of PLGA nanoparticle uptake by bovine MoDCs. PLGA nanoparticles loaded with Alexa Fluor-488-conjugated OVA, as assessed by scanning electron microscopy (A), were incubated with bovine MoDCs for 1 hr at 37°C or 4°C. Flow cytometry was used to quantify the association of MoDCs with particles at 37°C (blue) and 4°C (red) (B). Data are representative of three independent experiments.

Fig. 2. Assessment of the coating of PLGA nanoparticles with sugar or protein motifs. PLGA nanoparticles were coated with mannan (MAN) and OVA using the carbodiimide cross-linker EDC (A). Surface bound MAN (B) and OVA (C) were quantified by determining unreacted ligands in the wash supernatants of particles using a resorcinol assay and absorbance at 280nm, respectively (data represent a single experiment). To assess MAN- and OVA-coating using flow cytometry, non-coated particles (D, F) or MAN- (E) and OVA-coated (G) particles were incubated with ConA-FITC or mouse anti-OVA mAb followed by anti-mouse IgG1-APC secondary antibody to detect mannose residues and OVA, respectively. Data representative of one of three experiments are shown. The effects of MAN and OVA coating on the surface

charge (H) and size of particles (I) were also assessed by dynamic light scattering, data represents readings in triplicate.

Fig. 3. Assessment of the mechanisms of MoDC uptake of PLGA nanoparticles coated with sugar or protein motifs. MoDC uptake of Alexa Fluor-488-conjugated OVA loaded PLGA nanoparticles which were either non-coated (NT) or coated with MAN or OVA was assessed in comparison to soluble Alexa Fluor-488-conjugated OVA (sOVA) by flow cytometry. Uptake of particles was calculated by subtraction of the mean fluorescence intensity (MFI) of cells incubated at 4°C from the values obtained at 37°C (A). To assess the mechanism of uptake, MoDCs were pre-incubated for 30 min at 37°C with a panel of inhibitors; poly-inosine (pl), mannan (MAN), or amantidine (Am). Cells were then exposed to Alexa Fluor-488-conjugated OVA loaded PLGA nanoparticles which were either non-coated (F) or coated with MAN (G) or OVA (H), or to soluble Alexa Fluor-488-conjugated OVA (I), and after 1 hr incubation uptake was assessed using flow cytometry. Representative dots plots are shown for uptake of particles in the uninhibited (B), Am inhibited (C), MAN inhibited (D) and pl inhibited (E) conditions. The percentage inhibition of particle uptake was calculated from the difference in mean fluorescence intensity (MFI) between the uninhibited and the inhibited conditions (F-I). Data presented are the mean of three experiments and error bars represent SEM.

Fig. 4. The uptake of chitosan coated PLGA nanoparticles by MoDC and its inhibition by co-incubation with soluble mannan. Alexa Fluor-488-conjugated OVA loaded PLGA particles were either left non-coated or incubated with chitosan salts; carboxymethyl chitosan (mChitosan) or chitosan HCl (HCl). Alternatively oligomers of chitosan were used and are represented by their respective molecular weights. Coating was confirmed by assessing the

change in surface charge by dynamic light scattering and error bars represent SD of 3 readings (A). To assess uptake, MoDCs were either incubated with particles for 1 hr incubation at 4 or 37°C, and the fluorescence of cells was assessed by flow cytometry. Uptake of particles was calculated by subtraction of the mean fluorescence intensity (MFI) of cells incubated at 4°C from the values obtained at 37°C (B). Alternately, MoDC were pre-incubated with MAN for 1 hr prior to being pulsed with particles and the percentage inhibition calculated (C). The mean of three experiments is shown with error bars representing SEM.

Fig. 5. Assessment of the effect of anti-DEC-205 mAb coating of PLGA nanoparticles to target a specific DC population. PLGA nanoparticle coating with isotype control and anti-DEC-205 mAbs was assessed using flow cytometry following staining with FITC conjugated anti-mouse IgG secondary antibody (A). Bovine ALDCs and MoDC were stained with a DEC-205 (blue) or irrelevant isotype control (red) mAb and analyzed by flow cytometry to confirm that a proportion of ALDC express DEC-205 whereas MoDC do not (B). Co-cultures of ALDC and MoDC were pulsed with OVA-Alexa Fluor 647 loaded PLGA nanoparticles, which were either non-coated (NT), or coated with an irrelevant isotype control mAb (isotype) or with anti-DEC-205 mAb (DEC-205). The proportion of each DC type that had taken up particles was assessed by flow cytometry (C). The relative quantity of nanoparticles taken up by cells of each type was determined by the mean fluorescence intensity (MFI) measurements. To determine the effect of anti-DEC-205 mAb coating, the fluorescence of cells pulsed with anti-DEC-205 mAb coated particles were expressed as a fold increase in uptake over those pulsed with isotype control mAb coated particles. Uptake was compared with PLGA particles coated with mAb alone (D) or coated with 3-5 kDa oligo chitosan and mAb (E). The mean of three experiments

517 is shown, error bars represent SEM and statistical analysis was performed using a 2-way
518 ANOVA (B) or t-test (C, D) ** $p < 0.01$ *** $p < 0.001$.

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Figure 1

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Figure 2

Figure 3

Figure 4

Figure 5