Pharm Res (2011) 28:2288–2301
DOI 10.1007/s11095-011-0459-9

RESEARCH PAPER

Activation of Antigen-Specific T Cell-Responses by Mannan-Decorated PLGA Nanoparticles

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Received: 10 January 2011 / Accepted: 26 April 2011 / Published online: 11 May 2011

ABSTRACT

Purpose Mannosylation of vaccines is a promising strategy to selectively target vaccine antigens to the mannose receptor expressed on dendritic cells (DCs). The purpose of this study was to investigate the effect of mannan (MN) chemically conjugated to poly(D, L-lactide-*co*-glycolic acid) (PLGA) nanoparticles (NPs) on antigen-specific T-cell responses elicited by a model antigen (ovalbumin, OVA) loaded in PLGA-NPs.

Methods *In vitro* T-cell proliferation assay was done to assess the ability of DCs treated with OVA-NPs (±MN decoration) to induce antigen-specific T-cell activation. The efficacy of this vaccination strategy was further evaluated *in vivo*, where T-cell proliferation was performed to evaluate activation of T-cell responses in lymph nodes and spleens isolated from the vaccinated mice.

Results Our results demonstrate that MN-decorated antigen-loaded PLGA-NPs simultaneously enhanced antigen-specific $CD4^+$ and $CD8^+$ T-cell responses compared to non-decorated NPs.

Conclusions MN decoration of PLGA-NPs is a promising strategy for enhancing antigen-specific T-cell responses.

KEY WORDS dendritic cells · Mannan · PLGA · targeting · vaccine

INTRODUCTION

Targeting antigens to mannose receptor (MR) on antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, has been recently recognized as an efficient strategy to improve antigen uptake, processing, and presentation, resulting in enhanced antigen-specific T-cell responses (1). In this context, mannosylation of several antigen delivery vehicles, such as liposomes (2,3), nanoparticles (4), and niosomes (5), for more efficient and specific delivery of antigens to DCs has been examined. Mannan (MN), a polymannose isolated from the cell wall of *Saccharomyces cerevisiae* (6), is one of the natural ligands for MR with a strong binding affinity to this receptor. In addition to its effect on increasing antigen uptake by DCs, MN can act as an adjuvant inducing DC activation/maturation by itself. The exact mechanism relying behind the adjuvant effect of MN is not fully understood, but could be attributed to the ability of MN to interact with Toll-like receptors (TLRs) on DCs (7), resulting in an increase in their immunostimulatory potential.

With this in mind, we have developed MN-incorporated poly (D, L-lactide-*co*-glycolic acid) (PLGA) nanoparticles (NPs) and investigated the effect of MN incorporation on the efficiency of PLGA NPs as antigen delivery vehicles to DCs (8). Polymeric

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NPs made of FDA-approved biocompatible PLGA have been extensively studied as competent vaccine delivery devices (9–12) and shown an exceptional ability to improve vaccine-induced immune responses compared to soluble antigens. Several studies have demonstrated remarkable induction of DC maturation, simultaneous activation of $CD4^+$ and $CD8^+$ T-cell responses, breaking of self-tolerance and induction of potent anti-tumor immunity following co-delivery of antigens along with immunostimulatory adjuvants (such as TLR ligands) in PLGA-NPs (12–15). Incorporation of MN to PLGA-NPs was hypothesized to further facilitate the uptake of NPs by DCs and also enhance DC maturation, which ultimately results in improving the effectiveness of vaccine formulation. Our previous results have shown that both physical adsorption and chemical conjugation of MN into PLGA-NPs result in enhanced NP uptake by bone marrow generated DCs (BMDCs) (8). Decoration of NPs either through physical adsorptions or chemical conjugation also translated to enhanced DC maturation by NPs, as evidenced by up-regulation of cell surface markers (CD40, CD86), secretion of inflammatory cytokines (IL-12, IL-6 and TNF-α) and stimulation of alloreactive T-cell responses by DCs (unpublished data). DCs treated with PLGA-NPs containing either physically adsorbed or chemically conjugated MN have shown a comparable level of cytokine secretion and up-regulation of maturation markers. However, there was a significant increase in the allostimulatory capacity of DCs treated with PLGA-NPs with chemically conjugated MN, compared to DCs treated with PLGA-NPs with physically adsorbed MN. This may be attributed to the higher level of MN incorporation in the former formulation (8).

Based on the aforementioned results, chemical conjugation of MN to PLGA was chosen to develop MN-incorporated PLGA-NPs as actively targeted antigen delivery system. In the current study, a model antigen, ovalbumin (OVA), was formulated in COOH-terminated PLGA-NPs. MN was then chemically conjugated onto the surface of these NPs. The ability of MN-decorated antigen-loaded PLGA-NPs to stimulate antigen-specific $CD4^+$ and $CD8^+$ T-cell responses was further assessed both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6 mice as well as transgenic OT-I and OT-II mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed using 6–12-week-old male mice. All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee (Biosciences, Health Sciences or Livestock) for the University of Alberta.

Reagents

COOH-terminated (capped) PLGA co-polymer (monomer ratio 50:50) molecular weight (Mwt), 7,000 Da, was purchased from Absorbable Polymers International, (Pelham, AL, USA). Mannan Mwt, 35,000–60,000 Da; OVA protein (Grade-V) and poly vinyl alcohol (PVA), Mwt, 31,000–50,000 Da were obtained from Sigma Aldrich Co. (Oakville, ON, Canada). Murine $CD4^+$ and $CD8^+$ negative selection kits were purchased from StemCell Technologies (Vancouver, BC, Canada). Recombinant GM-CSF was purchased from Peprotech (Rocky Hill, NJ, USA). Murine IL-2 and IFN-γ ELISA kit were purchased from E-Bioscience (San Diego, CA, USA). RPMI-1640, L-glutamine, and gentamycin were purchased from Gibco-BRL (Burlington, ON, Canada). Fetal Calf Serum (FCS) was obtained from Hyclone Laboratories (Logan, UT, USA). Micro bicinchoninic acid (BCA) protein assay kit was purchased from BioLynx Inc. (Brockville, ON, Canada). Anti-mouse CD16/CD32, biotinylated anti-CD11c and streptavidin-conjugated phycoerythrin (PE)-Cy5 were purchased from BD Biosciences (Mississauga, ON, Canada).

Preparation and Characterization of Nanoparticles

Plain (Empty) PLGA-NPs were prepared by the double-emulsion solvent evaporation technique. In brief, a primary W/O emulsion was formed by emulsification of the first aqueous phase (100 μL phosphate buffer saline (PBS)) with the organic phase (100 mg PLGA in 1 mL dichloromethane (DCM)). For the preparation of OVA-NPs and OVA/FITC-NPs, 10 mg OVA (in 100 μL PBS) or 5 mg OVA/FITC (in 100 μL PBS) was added to the polymer-DCM solution, respectively. The primary emulsion was further emulsified with a secondary aqueous phase (4 mL of 5% PVA in PBS) to form a secondary W/O/W emulsion. The resulting emulsion was transferred drop-wise to stirring 16 mL of distilled water to allow the removal of DCM by evaporation. After 3 h, the NP suspension was washed three times at 4°C (35,000×g, 15 min) and freeze-dried. Mannan was attached to the freeze-dried NPs by a chemical reaction as described in (8). In brief, a mixture of 100 mg MN, 765 μg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 2.295 mg sulfo-NHS was added to 50 mg of PLGA-NPs and dispersed in 10 mL acidic PBS (pH 5.0). The mixture was then stirred at room temperature overnight. The excess reagents and soluble by-products were then washed away using cold PBS at 35,000×g for 15 min. The NPs were then freeze-dried. Dried NPs were then characterized in terms of average particle size and size distribution using a Zetasizer 3000 (Malvern, UK). Assessment of the level of MN and OVA incorporation in the dried formulations was done using the phenol-sulfuric acid method and the BCA protein assay as

described in details in earlier reports (8) and (16), respectively. MN-decorated empty-NPs, OVA-NPs and OVA/FITC-NPs are given the names of Mannan-NPs, OVA/Mannan-NPs and OVA/FITC-Mannan-NPs throughout the manuscript, respectively. Nanoparticles were prepared by aseptic technique during all the formulation steps. For example, all reagents used were sterilized either by autoclaving or by filtration. Glass and plastic equipments used in the preparation were all sterilized as well. Furthermore, nanoparticle preparation steps (stirring, mixing and suspending) were all done inside biosafety cabinet. All formulations used in the current studies were tested for the presence of endotoxin using Limulus Amebocyte Lysate (LAL) QCL-1000™ (Lonza, Walkersville, MD) as previously described in (13) and were shown to be endotoxin free.

Preparation of Murine Bone Marrow-Derived DCs (BMDCs)

DC primary cultures were generated from murine bone marrow precursors from femurs of wild-type C57BL/6 mice in complete RPMI media in the presence of GM-CSF as described earlier (17). Briefly, femurs were removed and cleaned from the surrounding muscle and fatty tissues. For disinfection, intact bones were put in 70% ethanol for 2 min and then washed with PBS. Afterwards, both ends of the femur were cut with sterile scissors, and the marrow was flushed with PBS using insulin syringe. After one wash in PBS, about $1–2\times10^7$ leukocytes were obtained per femur. Cell culture medium was complete RPMI-1640 (RPMI-1640 supplemented with gentamycin (80 μg/mL), L-glutamine (2 mM), and 10% heat-inactivated FCS). At day 0, bone marrow leukocytes were seeded at 2×10^6 per 100 mm dish in 10 mL complete medium containing 20 ng/mL GM-CSF. At day 3, another 10 mL complete medium containing 20 ng/mL GM-CSF was added to the plates. At day 6, half of the culture supernatant was collected and centrifuged, and the cell pellet re-suspended in 10 mL fresh medium containing 20 ng/mL GM-CSF and put back into the original plate. At day 7, cells were ready for use. The purity of the DC population on day 7 was found to be between 70 and 75% DCs based on the expression of CD11c on the semi-adherent and non-adherent cell populations.

DC Uptake Studies

At day 7 of culture, DCs were treated with either OVA/FITC-NPs or OVA/FITC-Mannan-NPs (1 mg dispersed in 0.5 mL PBS). After an overnight incubation, semi-adherent and non-adherent cells were harvested and washed thoroughly (three times) with cold FACS buffer (PBS with 5% FCS, and 0.09% sodium azide) to remove non-internalized NPs. For flow cytometry studies, a suspension of 250×10^3 DCs in FACS buffer was incubated with anti-mouse CD16/CD32 monoclonal antibody (mAb) for 15 min to block Fc receptors. DCs were then treated with biotinylated anti-CD11c, followed by streptavidin conjugated phycoerythrin (PE)-Cy5. All samples were finally acquired on a Becton-Dickinson FACsort and analyzed by CellQuest software.

In Vitro $CD4^+$ and $CD8^+$ T-Cell Activation Studies

To assess antigen-specific $CD8^+$ and $CD4^+$ T-cell activation induced by PLGA-NPs-treated DCs, two transgenic mouse models were used (OT-I and OT-II, respectively). OT-I $CD8^+$ T-cells recognize $OVA_{257–264}$ (SIINFEKL) in association with MHC class I, whereas OT-II $CD4^+$ T-cells recognize $OVA_{323–339}$ in association with MHC class II molecules. In brief, day 7 DCs were incubated with 1 mg of either OVA-NPs or OVA/Mannan-NPs. Control groups included untreated DCs, DCs treated with either Empty-NPs or Mannan-NPs (with no OVA). After overnight incubation, all DC groups were harvested, irradiated, washed, and plated in 96-well plates. For each group, DCs were plated (in triplicates) in three different cell numbers (20×10^3, 10×10^3 or 5×10^3 DCs/well). These DCs were then co-cultured with either $CD8^+$ or $CD4^+$ T-cells isolated from the spleens of OT-I and OT-II mice, respectively. The number of T-cells/well was kept constant (100×10^3 T cells); DC: T-cell ratios were thus either 1: 5, 1: 10 or 1: 20. The extent of antigen-specific T-cell proliferation was assessed by the addition of ^{3}H-thymidine in the last 18 h of 60 h co-culture. In a separate set of plates, supernatants were collected at the end of the co-culture and assayed for the level of IL-2 secretion by the activated T-cells using ELISA kits as per the manufacturer's recommendation.

Activation of *In Vivo* OVA-Specific $CD4^+$ and $CD8^+$ T-Cell Responses

In these studies, we used wild-type C57BL/6 mice. Four groups (4 mice/group) were immunized twice (subcutaneously (s.c), 2 weeks apart) in the right flank region with 10 mg of either Empty-NPs, Mannan-NPs, OVA-NPs or OVA/Mannan-NPs. All formulations (10 mg of each) were first suspended in 200 μL of sterile normal saline. One week after the last immunization, mice were euthanized; draining inguinal lymph nodes and spleens were isolated and washed three times in PBS. Processing of the isolated lymphocytes and spleenocytes is further described in details below.

After washing, pooled lymphocytes from each treatment group were suspended in complete RPMI media at 1×10^7 lymphocytes/mL. From those lymphocyte cell suspensions, 100 μL (containing 1×10^6 lymphocytes) was added into 96-

well plates containing 1×10^6 spleenocytes isolated from unimmunized mice, functioning as APCs, i.e., ratio of APCs: lymphocytes was 1:1 (all APCs used were irradiated prior to the co-culture). Different APCs/lymphocyte co-cultures were then stimulated (in triplicates) with 20 μM of one of the following peptides: $OVA_{323–339}$ epitope (CD4 test peptide), MUC 1 lipopeptide (irrelevant CD4 epitope), $OVA_{257–264}$ epitope (SIINFEKL, CD8 test peptide) or $TRP2_{180–188}$ (irrelevant CD8 epitope). The extent of antigen-specific T-cell proliferation was assessed by the addition of ^{3}H-thymidine as described above. The ability of our formulations to stimulate *ex vivo* T-cell proliferative responses in lymph nodes was expressed as the stimulation index (S.I.). S. I. was calculated as the ratio of counts per minute (cpm) obtained from lymphocytes isolated from animals immunized with test formulation divided by the cpm obtained from lymphocytes isolated from Empty-NPs immunized animals (upon addition of the same stimulus).

On the other hand, $CD4^+$ or $CD8^+$ T-cells were purified from the spleens of the vaccinated animals using murine $CD4^+$ and $CD8^+$ negative selection kits, respectively, as per the manufacturer's recommendation. Isolated $CD4^+$ or $CD8^+$ T-cells were then suspended in complete RPMI media at 1×10^7 T-cells/mL. From those cell suspensions, 100 μL (containing either 1×10^6 $CD4^+$ or $CD8^+$ T-cells) were plated into 96-well plates containing 0.5×10^6 spleenocytes isolated from unimmunized mice (as APCs) (all APCs used were irradiated prior to the co-culture). Different APCs/T-cell co-cultures were then stimulated (in triplicates) with either test or irrelevant peptides as described above. T-cell proliferation was then assessed by the addition of ^{3}H-thymidine in the last 18 h of the 60 h co-culture. In addition to T-cell proliferative responses, the cytokine secretion pattern of the activated T-cells was assessed by analyzing the level of IL-2 and IFN-γ in the supernatant of the co-culture.

The ability of our formulations to stimulate *ex vivo* T-cell proliferative responses in spleens was expressed as S.I. where S.I. was calculated as the ratio of cpm obtained from $CD4^+$ or $CD8^+$ T-cells isolated from animals immunized with test formulation divided by the cpm obtained from $CD4^+$ or $CD8^+$ T-cells isolated from Empty-NPs immunized animals (upon addition of the same stimulus).

Statistical Analysis

The data obtained in the current studies was subjected to statistical analysis wherever appropriate. Comparisons between two groups were analyzed by unpaired Student's *t*-test. P value of ≤0.05 was set for the significance of difference among groups.

RESULTS

Characterizations of MN-Decorated Formulations

Detailed characterization of the chemical conjugation between MN and PLGA as well as the effect of MN incorporation on particle size distribution and zeta potential of Mannan-NPs (relative to Empty-NPs) were described in our recent manuscript (8). In the current paper, MN decoration of OVA-NPs has resulted in a slight increase in particle size as observed earlier in Mannan-NPs (relative to Empty-NPs without OVA) (8). However, all NPs prepared were still in the preferable range for uptake by DCs (below 500 nm) (18). A detailed characterization of the prepared formulations is given in Table I.

Dendritic Cell Uptake Studies

Incorporation of FITC-labeled OVA into the NP formulations allowed for quantitative estimation of the cellular uptake of NPs by flow cytometry. Treatment of DCs with MN-decorated OVA/FITC-containing NPs (OVA/FITC-Mannan-NPs) has resulted in a slight increase in cellular uptake, compared to DCs treated with the non-targeted formulation (OVA/FITC-NPs). As shown in Fig. 1a, there was a slight increase in the percentages of $FITC^+$ cells (64% *versus* 58%) as well as mean florescence intensity (305 *versus* 295) of the OVA/FITC-Mannan-NPs-treated DCs compared to OVA/FITC-NPs-treated DCs, respectively. Fig. 1b shows the percentage of cells that were positive for both FITC and CD11c (as DC marker). Percentages of $FITC^+$ / $CD11c^+$ cells were 50.4% and 55.1% for OVA/FITC-NPs and OVA/FITC-Mannan-NPs treated DCs, respectively.

Table I Characterization of PLGA-NPs Containing OVA and/or MN

Nanoparticle	Mean diameter ± SD (nm)[a]	Zeta potential ± SD (mV)[a]	MN ± SD (μg/mg NPs)[b]	OVA ± SD (μg/mg NPs)[c]
Empty-NPs	328 ± 30	−36.2 ± 3	NA	NA
MN-NPs	430 ± 45	−46.8 ± 2	365 ± 36	NA
OVA-NPs	372 ± 5	−16.8 ± 4	NA	9.04 ± 0.916
OVA/MN-NPs	406 ± 14	−29.8 ± 7	346 ± 52	10.12 ± 0.86

[a] measured by DLS technique

[b] measured by phenol-sulphuric acid colorimetric assay

NA not applicable

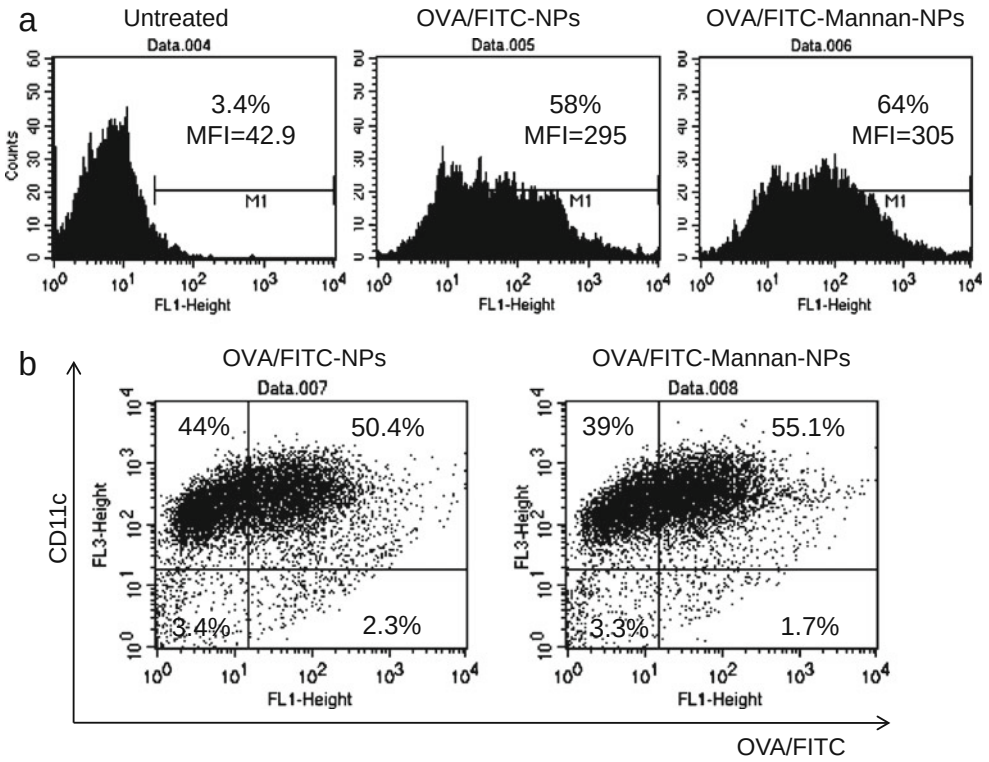

Fig. 1 Effect of mannan decoration on the uptake of OVA/FITC-loaded PLGA-NPs by bone marrow-derived DCs. At day 7 of culture, DCs were treated with 1 mg of either OVA/FITC-NPs or OVA/FITC-Mannan-NPs (1 mg dispersed in 0.5 mL PBS). After an overnight incubation, flow cytometric analysis was performed to assess the uptake of fluorescently labelled particles by DCs. (**a**) Histograms show single color analysis of FL-1 florescence (OVA/FITC). Numbers at the right corners of each histogram represent percentages of positive cells for OVA/FITC and mean florescence intensity (MFI). (**b**) Double color analysis of OVA/FITC (FL-1 florescence) and CD11c as a DC marker (FL-3 florescence). Percentages of OVA/FITC$^+$/CD11c$^+$ cells for test formulations are shown at the upper right corner of each histogram. Percentages of cells that are double negative and single positive for either OVA/FITC$^+$ or CD11c$^+$ are shown in lower left, lower right and upper left corner, respectively. Results are representative of three separate experiments, all of which gave similar results.

Activation of *In Vitro* Antigen-Specific T-Cell Responses

T-Cell Proliferative Responses

As illustrated in Fig. 2, DCs treated with OVA/Mannan-NPs efficiently primed naïve CD4$^+$ (Fig. 2a) and CD8$^+$ T-cell proliferative responses (Fig. 2b). Proliferation of both antigen-specific CD4$^+$ and CD8$^+$ T-cells increased proportionally to the number of plated DCs, with the highest stimulation achieved with the highest DC number/well (20×10^3 DCs/well at DC: T cell ratio of 1: 5). However, the magnitude of CD4$^+$ T-cell proliferation was higher than that of CD8$^+$ T-cells at all ratios tested. The difference between CD4$^+$ and CD8$^+$ T-cell proliferative responses appeared mostly in the presence of the lowest DC number (5×10^3 DCs/well at DC: T-cell ratio of 1: 20), where the extent of CD4$^+$ T-cell proliferation was almost 20-fold higher than that of CD8 T-cells (measured cpm were $53{,}915\pm25{,}987$ and $2{,}727\pm1{,}146$ for CD4$^+$ and CD8$^+$ T-cells, respectively ($p<0.05$, unpaired Student's t test)). On the other hand, treatment of DCs with OVA-NPs has resulted in only marginal priming of CD4$^+$ T-cell responses. This marginal effect was only observed at higher number of plated DC number (at DC: T-cell ratio of 1: 5) and diminished when the number of DCs was lowered (at DC: T-cell ratio of 1:20) Fig. 2a. There was almost no activation of CD8$^+$ T-cells observed in the OVA-NPs-treated group at any of the tested DC: T-cell ratios.

IL-2 Secretion Profile

The profile of IL-2 secretion by activated T-cells in the co-culture supernatant (Fig. 3) was consistent with the pattern of T-cell proliferative responses observed in Fig. 2. OVA/Mannan-NPs-treated DCs induced OVA-specific CD4$^+$ T-cells to secrete large amount of IL-2. The highest amount of IL-2 ($1{,}900\pm13.7$ pg/mL) was detected in the presence of the highest number of DCs (DC: T-cell ratio of 1:5). However, there was still a significant amount of IL-2 detected in the presence of

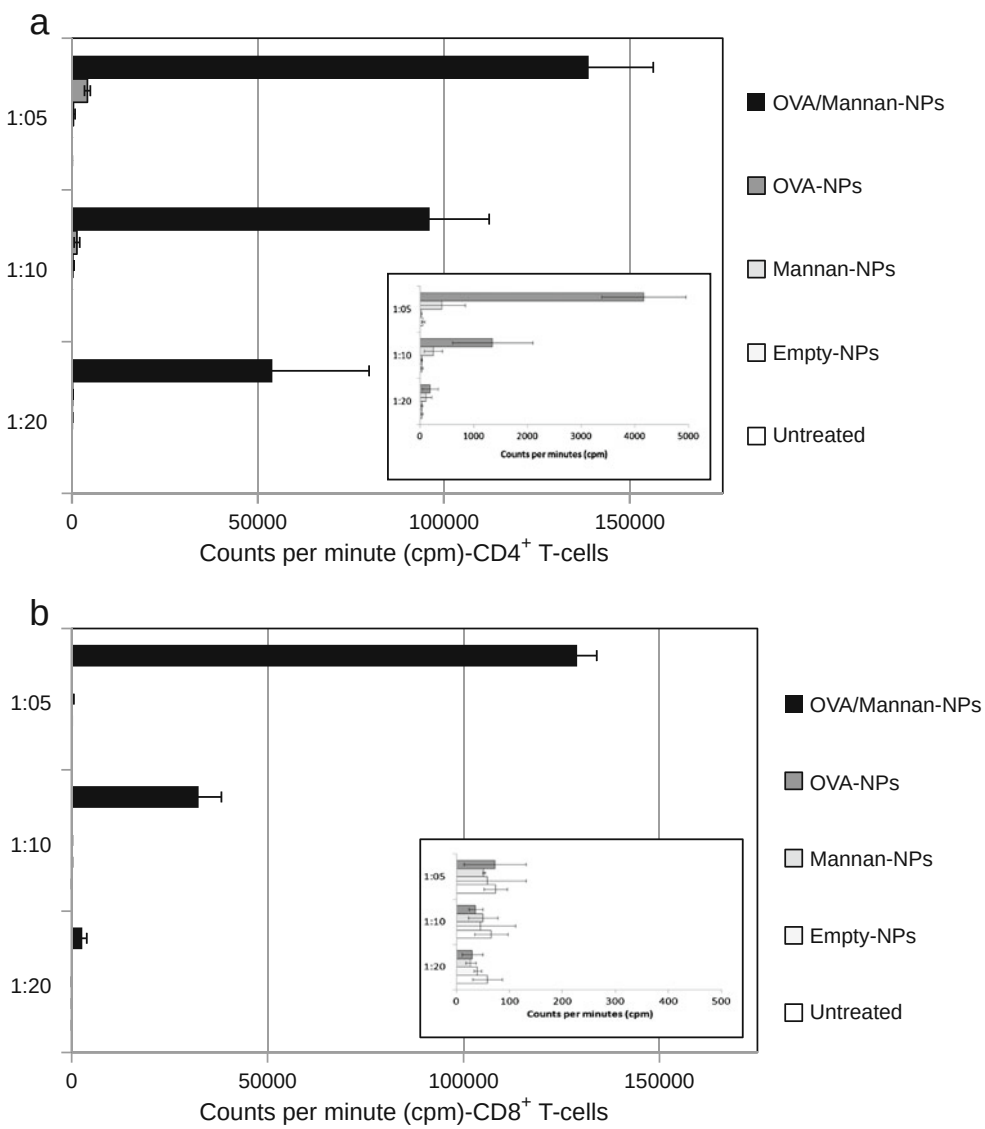

Fig. 2 Treatment of DCs with OVA/Mannan-NPs dramatically enhances primary antigen-specific $CD4^+$ and $CD8^+$ T-cell proliferative responses. Day 7 DCs were incubated with 1 mg of either OVA-NPs or OVA/Mannan-NPs. Control groups included untreated DCs, and DCs treated with either Empty-NPs or Mannan-NPs (with no OVA). After overnight incubation, all DC groups were harvested, irradiated, washed, and plated in 96-well plates. For each group, DCs were plated (in triplicates) in 3 different cell numbers (20×10^3, 10×10^3 or 5×10^3 DCs/well). These DCs were then co-cultured with either $CD4^+$ (**a**) or $CD8^+$ T-cells (**b**) isolated from the spleens of OT-II and OT-I mice, respectively. Number of $CD4^+$ or $CD8^+$ T cells/well was kept constant (100×10^3 T cells); DC: T-cell ratios were thus either 1: 5, 1: 10 or 1: 20. Proliferative responses of $CD4^+$ (**a**) or $CD8^+$ (**b**) T-cells were assessed after adding ^{3}H-thymidine during the last 18 h of a 60 h co-culture. The values are mean of triplicates ± SD. Data shown are representative of three independent experiments (all gave similar results). A graph with a lower scale is embedded into the right corner of each figure to show the readings of treatment groups that showed lower proliferative responses (untreated DCs, and DCs treated with either Empty-NPs, Mannan-NPs or OVA-NPs).

lower number of DCs (1,866±13.7 and 1,546±50.45 pg/mL at DC: T-cell ratio of 1:10 and 1: 20, respectively). In a separate set of experiments, $CD4^+$ T-cells that were co-cultured with lower numbers of DCs (DC: T-cell ratio of 1:40) were still able to secrete high level of IL-2 (data is not shown).

In contrast to the OVA/Mannan-NPs-treated group (1,900±13.7 pg/mL), a much lower amount of IL-2 was detected from $CD4^+$ T-cells that were co-cultured with OVA-NPs treated DCs (184.5±1.5 pg/mL at DC: T-cell ratio of 1:5) (Fig. 3a). This was consistent with the lower level of proliferative responses observed in this group (Fig. 2a) compared to the OVA/Mannan-NPs-treated group. However, in the presence of lower number of DCs (DC: T-cell ratio of 1:10 and 1: 20), DCs treated with OVA-NPs failed to induce any detectable level of IL-2 by OVA specific $CD4^+$ T-cells (Fig. 3a). This was also consistent with the minimal T-cell proliferation observed in the presence of lower numbers of DCs (Fig. 2a).

Fig. 3b shows the pattern of IL-2 secretion by activated antigen-specific $CD8^+$ T-cells. The only group that produced detectable level of IL-2 was $CD8^+$ T-cells that were co-cultured with DCs treated with OVA/Mannan-NPs. The amount of IL-2 detected increased proportionally as the number of DCs/well increased. However, the amount of IL-2 secreted by activated $CD8^+$ T-cells was lower than what was detected by activated $CD4^+$ T-cells at all DC: T-cell ratios tested (Fig. 3a), i.e. 1006.9±21.7, 820.63±21.9 and 278.6±3.05 pg/mL compared to 1,900±13.7, 1,866±13.7 and 1,546±50.45 pg/mL at DC: T-cell ratio of 1:5, 1:10 and 1:20, respectively. This finding is also in agreement with the lower proliferation of $CD8^+$ T-cell (Fig. 2b) in response to OVA/Mannan-NPs-treated DCs, compared to that of $CD4^+$ T-cells co-cultured with the same treatment group.

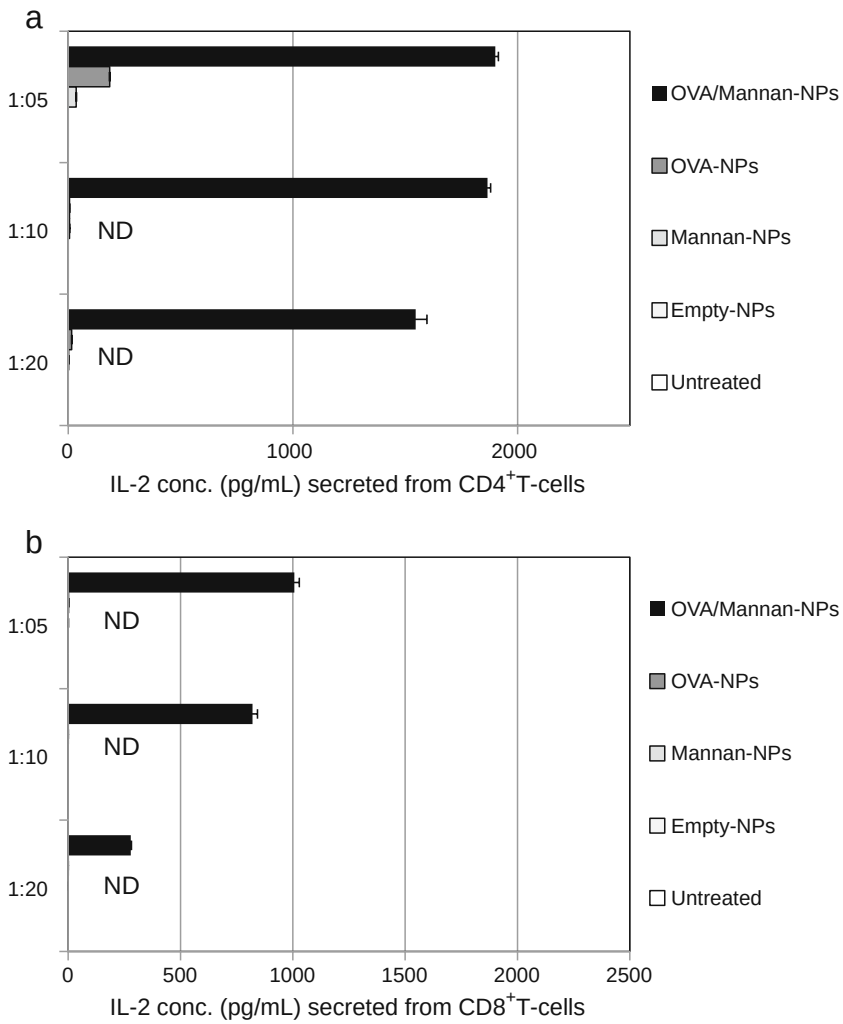

Fig. 3 Cytokine secretion profile from *in vitro* activated $CD4^+$ and $CD8^+$ T-cells. Supernatants obtained at the end of co-culture in the experiment described in Fig. 2 were collected and analyzed for IL-2 secretion. IL-2 secretion by the activated of $CD4^+$ (**a**) or $CD8^+$ (**b**). T-cells were measured using routine ELISA technique per the manufacture's instruction. The values are means of duplicates ± S.D. ND means undetectable level of cytokine.

Activation of *In Vivo* Antigen-Specific T-Cell Responses

Activation of T-Cell Responses in Lymph Nodes

To assess the ability of our test formulation (OVA/Mannan-NPs) to stimulate antigen-specific T-cell responses *in vivo*, wild-type C57BL/6 mice were vaccinated twice (2 weeks apart) with either OVA-NPs or OVA/Mannan-NPs. Control groups included animals that were vaccinated similarly with either Empty-NPs or Mannan-NPs. One week after the last vaccinations, animals were euthanized and their draining lymph nodes were isolated and pooled together. Isolation of $CD4^+$ and $CD8^+$ T-cells from the lymph nodes was not feasible as the total number of lymphocytes harvested (particularly from control groups) was not sufficient to isolate T-cells using EasySep® kits (which require a starting number of at least 50×10^6 lymphocytes or spleenocytes, based on the manufacture recommendations). Therefore, *ex-vivo* T-cell proliferation was performed using the whole lymphocytes as described early in the Materials and Methods section.

As shown in Fig. 4a and b, only OVA/Mannan-NPs-immunized mice showed detectable antigen-specific $CD4^+$ and $CD8^+$ T-cell proliferative responses. Compared to our control group (Empty-NPs immunized mice), there was more than 8-fold increase in the expansion of both $CD4^+$ and $CD8^+$ T-cells in the animals immunized with OVA/Mannan-NPs, as evidenced by S.I. of 8.3 and 8.5 for $CD4^+$ and $CD8^+$ T-cells, respectively (Fig. 4a and b). On the other hand, immunization with OVA-NPs alone (without Mannan) resulted in only marginal $CD4^+$ and $CD8^+$ T-cell proliferative responses, similar to what was observed in the control groups (Empty-NPs and Mannan-NPs immunized mice).

Activation of T-Cell Responses in Spleens

In addition to harvesting draining lymph nodes, spleens of the immunized mice were harvested and pooled. The large number of available spleenocytes from the different treatment groups enabled us to perform both $CD4^+$ and $CD8^+$ T-cell

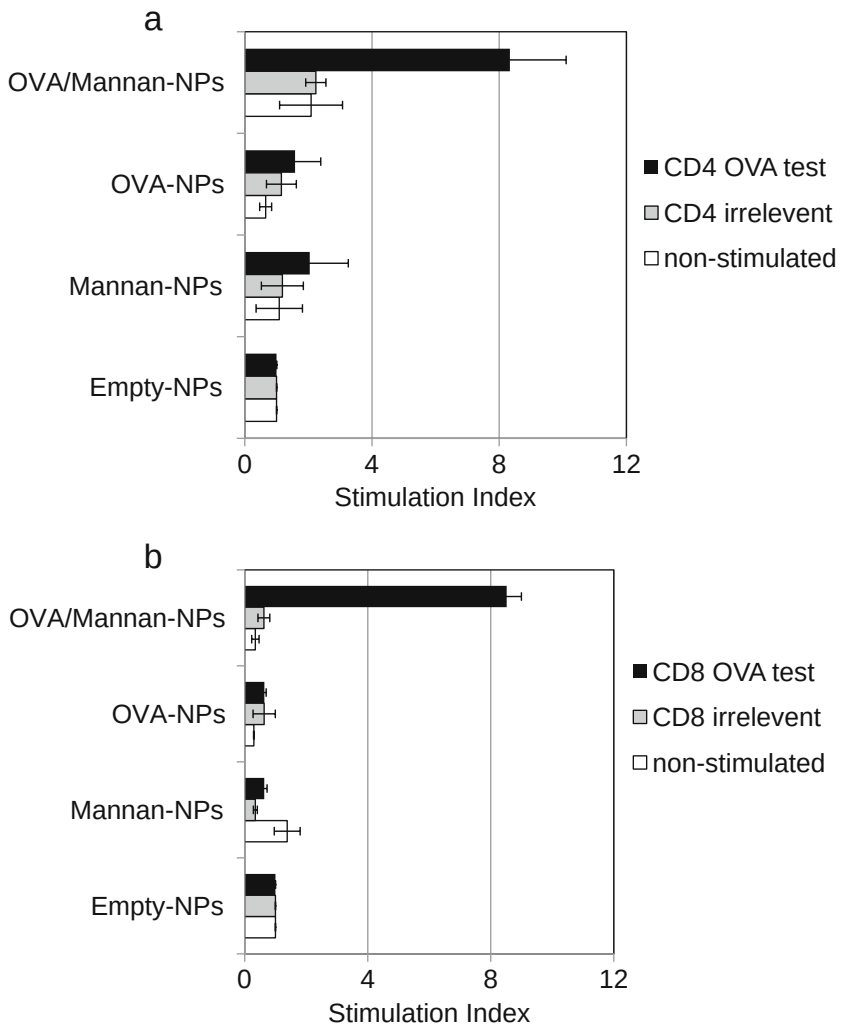

Fig. 4 Immunization with OVA/Mannan-NPs induced $CD4^+$ and $CD8^+$ T-cell proliferation in the lymph nodes of immunized mice. In this set of experiments, four groups of C57BL/6 mice (4 mice/group) were immunized twice (s.c., 2 weeks apart) in right flank region with 10 mg of either Empty-NPs, Mannan-NPs, OVA-NPs or OVA/Mannan-NPs. One week after the last immunization, mice were euthanized; draining inguinal lymph nodes and spleens were harvested, pooled and washed three times in PBS. After washing, pooled lymphocytes from each treatment group were plated with normal spleenocytes (APCs) in a ratio of 1: 1. Different APCs/lymphocyte co-cultures were then stimulated (in triplicates) with 20 μM of one of the following peptides formulations: $OVA_{323–339}$ epitope (CD4 test peptide), MUC 1 lipopeptide (irrelevant CD4 epitope), $OVA_{257–264}$ epitope (SIINFEKL, CD8 test peptide) or $TRP2_{180–188}$ (irrelevant CD8 epitope). The extent of antigen-specific $CD4^+$ (**a**) and $CD8^+$ (**b**). T-cell proliferation was assessed after adding ^{3}H-thymidine during the last 18 h of a 60 h co-culture. Stimulation index was calculated as the ratio of counts per minute (cpm) obtained from lymphocytes isolated from animals immunized with test formulation divided by the cpm obtained from lymphocytes isolated from Empty-NPs immunized animals (upon addition of the same stimulus). The values are means of triplicates ± S.D.

isolation to further characterize T-cell activation pattern induced by our formulations. For the activation of the isolated $CD4^+$ and $CD8^+$ T-cells, T-cells were co-cultured with APCs in the presence or absence of recall peptides as described in the Materials and Methods section. Besides assessing T-cell proliferative responses, the cytokine secretion pattern of the activated T-cells was assessed by analyzing the level of IL-2 and IFN-γ in the supernatant of the co-culture.

Results shown in Fig. 5a demonstrate strong antigen-specific $CD4^+$ T-cell proliferation in the spleens of mice immunized with OVA/Mannan-NPs, as evidenced by stimulation index of 150 when $OVA_{323–339}$ was used as a recall peptide. On the other hand, immunization with OVA-NPs resulted in only marginal $CD4^+$ T-cell proliferative responses, which was similar to what was observed in Mannan-NPs-treated group. For both groups (OVA-NPs and Mannan-NPs-treated groups), the extent of antigen-specific $CD4^+$ T-cell proliferation (in the wells treated with $OVA_{323–339}$) did not differ from what was observed in the control wells (treated with either media alone or with irrelevant CD4 epitope). Compared to other treatment groups, there was higher background observed in $CD4^+$ T-cell isolated from OVA/Mannan-NPs-immunized mice, as evidenced by the non-specific T-cell proliferation in the control wells. Such background may

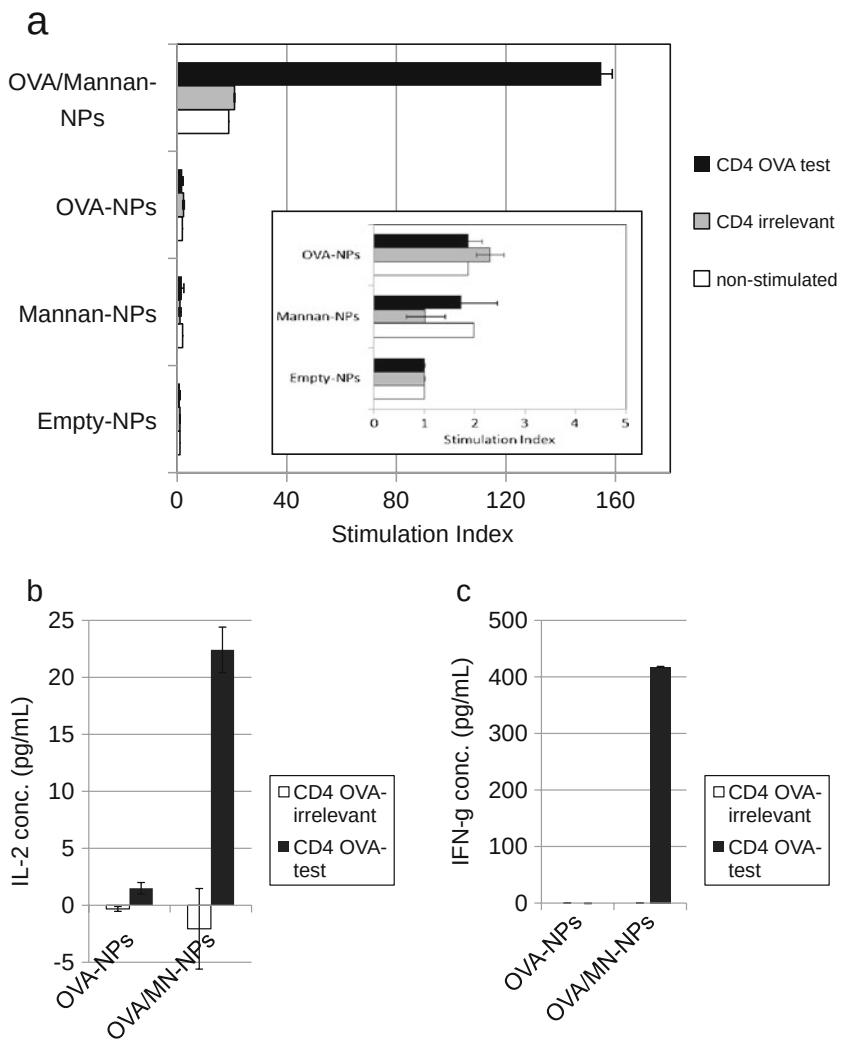

Fig. 5 Immunization with OVA/Mannan-NPs induced potent $CD4^+$ T-cell activation in the spleens of immunized mice. $CD4^+$ T-cells were purified from the spleens of the vaccinated animals (vaccination schedule is described in Fig. 4) using Easysep® $CD4^+$ T-cell negative selection kits as per the manufacture's recommendation. Isolated $CD4^+$ T-cells were then plated into 96-well plates containing normal spleenocytes as APCs (at APC: $CD4^+$ T-cell ratio of 1:2). APCs/ $CD4^+$ T-cell co-cultures from different groups were then stimulated (in triplicates) with either CD4 test or CD4 irrelevant peptides as described in Materials and Methods. (**a**) $CD4^+$ T-cell proliferation was then assessed by the addition of ^{3}H-thymidine in the last 18 h of 60 h co-culture. The stimulation index was calculated as the ratio of cpm obtained from $CD4^+$ T-cells isolated from animals immunized with test formulation divided by the cpm obtained from $CD4^+$ T-cells isolated from Empty-NPs immunized animals (upon addition of the same stimulus). The values are means of triplicates ± S.D. A graph with a lower scale is embedded into the right corner of Fig. 5a, to show the readings of treatment groups that showed lower proliferative responses (Empty-NPs, Mannan-NPs or OVA-NPs). The cytokine secretion pattern of the activated $CD4^+$ T-cells was assessed by analyzing the level of IL-2 (**b**) and IFN-γ (**c**) in the supernatant of the co-culture. For cytokine analysis, only results from OVA-NPs and OVA/Mananan-NPs immunized animals are shown.

reflect non-specific immune activation that may be caused by the test formulation.

Results obtained from analysis of cytokine production profile (Fig. 5b and c) in the co-culture supernatant were in agreement with the results obtained from the $CD4^+$ T-cell proliferation assay (Fig. 5a). The highest level of IL-2 and IFN-γ secretion was observed in $CD4^+$ T-cells isolated from OVA/Mannan-NPs-immunized mice (22.4 ± 3.5 and 417 ± 5 pg/mL, respectively). Consistent with T-cell proliferation results, immunization with OVA-NPs did not induce any detectable level of cytokines in the co-culture supernatant (Fig. 5b and c). The levels of IL-2 and IFN-γ secretion in the other treatment groups (Empty-NPs and Mannan-NPs) were below the detection limit of the ELISA kits (2 and 15 pg/mL, respectively) (data is not shown).

In contrast to the ability of OVA/Mannan-NPs to induce strong activation of $CD4^+$ T-cell response, the extent of antigen-specific $CD8^+$ T-cell activation in the spleens of OVA/Mannan-NPs immunized animals (Fig. 6) was lower (Fig. 5). In fact, results obtained from *ex-vivo* $CD8^+$ T-cell proliferation (Fig. 6) revealed an S.I. of 5.3 ± 0.9 for the $CD8^+$ T-cells isolated from OVA/Mannan-NPs-treated animals,

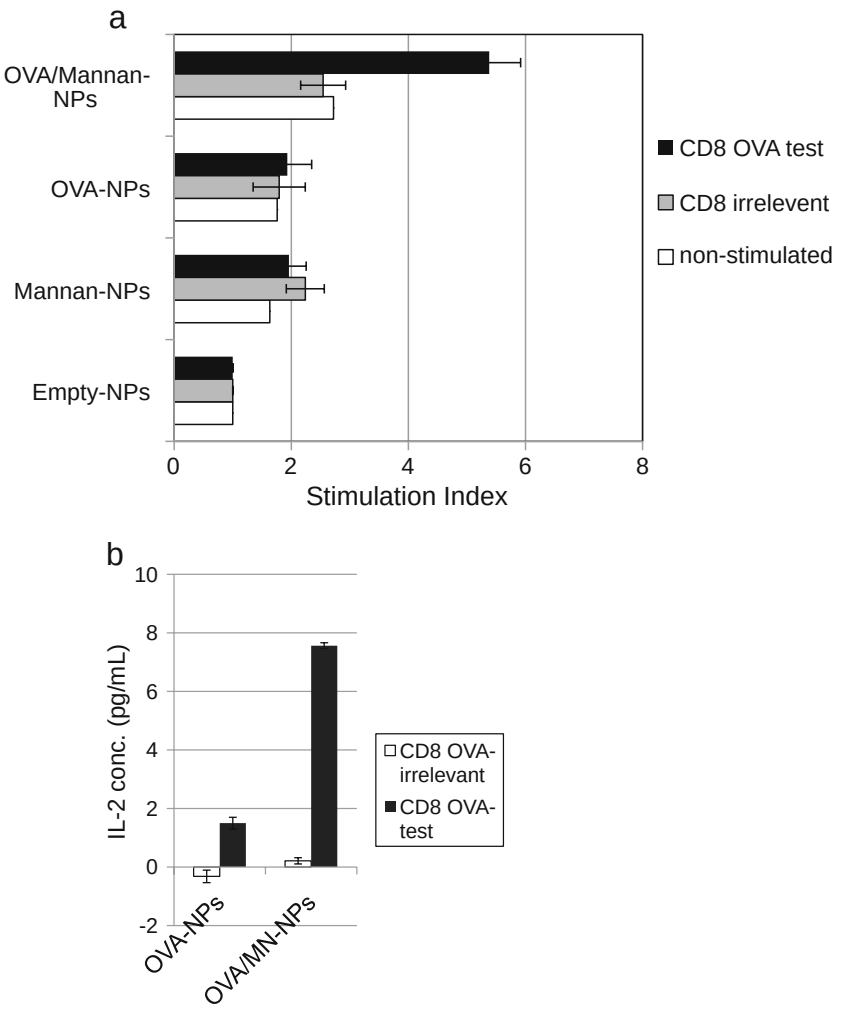

Fig. 6 Immunization with OVA/Mannan-NPs induced modest $CD8^+$ T-cell activation in the spleens of immunized mice. $CD8^+$ T-cells were purified from the spleens of vaccinated animals (vaccination schedule is described in Fig. 4) using Easysep® $CD8^+$ T-cell negative selection kits as per the manufacturer's recommendation. Isolated $CD8^+$ T-cells were then plated into 96-well plates containing normal spleenocytes as APCs (at APC: $CD8^+$ T-cell ratio of 1:2). APCs/ $CD8^+$ T-cell co-cultures from different groups were then stimulated (in triplicates) with either CD8 test or CD8 irrelevant peptides as described in Materials and Methods. (**a**) $CD8^+$ T-cell proliferation was assessed by the addition of ^{3}H-thymidine in the last 18 h of 60 h co-culture. Stimulation index was calculated as the ratio of cpm obtained from $CD8^+$ T-cells isolated from animals immunized with test formulation divided by the cpm obtained from $CD8^+$ T-cells isolated from Empty-NPs immunized animals (upon addition of the same stimulus). The values are means of triplicates ± S.D. The cytokine secretion pattern of the activated $CD8^+$ T-cells was assessed by analyzing the level of IL-2 (**b**) in the supernatant of the co-culture. For cytokine analysis, only results from OVA-NPs and OVA/Mananan-NPs immunized animals are shown.

which was far lower than the S.I. observed for $CD4^+$ T-cells isolated from the same animals (S.I. >150, Fig. 5). $CD8^+$ T-cells isolated from mice immunized with OVA-NPs or Mannan-NPs have shown slightly increased proliferation, compared to $CD8^+$ T-cells isolated from Empty-NPs immunized mice. However, this proliferation was not antigen specific, as the level of $CD8^+$ T-cell proliferation was similar among all wells regardless of the presence or absence of the CD8 OVA recall antigen (SEENFEKL).

The level of cytokine secretion profile by $CD8^+$ T-cells was similar to the level of their proliferative responses. Consistent with the lower *ex-vivo* $CD8^+$ T-cell proliferation (compared to $CD4^+$ T-cells), the level of cytokines produced by $CD8^+$ T-cells was far less than what was produced by the activated $CD4^+$ T-cells. The highest level of IL-2 secretion was observed in the mice immunized with OVA/Mannan-NPs. However, the amount of IL-2 detected by proliferated $CD8^+$ T-cells was 7.5±0.1 pg/mL (Fig. 6b); this is almost one-third the amount of IL-2 secreted by activated $CD4^+$ T-cells isolated from the same animals (Fig. 5b). $CD8^+$ T-cells isolated from OVA/Mannan-NPs-immunized mice failed to produce any detectable level of IFN-γ (data no shown). This is in contrast to the high level IFN-γ secretion (417±5 pg/mL) observed in activated $CD4^+$ T-cells from the same mice (Fig. 5c).

DISCUSSION

The long-term goal of this research is to maximize the efficacy of PLGA-based vaccines. Our research group and others have consistently demonstrated the superior immune responses elicited when immunstimulatory adjuvants (e.g TLR ligands) were co-delivered with antigens in PLGA-NPs, compared to PLGA-NPs containing antigens alone (12–15). Several TLR ligands have been successfully incorporated into PLGA-NPs and/or other vaccine formulations and significantly enhanced the immunogenicity of such vaccines. Examples include Pam(3)Cys (19), poly(I:C) (20), 7-acyl lipid A (12), 3M-019, CpG (21–24) (TLR2, TLR3, TLR4, TLR7 and TLR9 ligands, respectively). Another strategy to improve the efficiency of PLGA-based vaccines is to specifically target the formulation into the endocytic receptors expressed by APCs such as C-type lectin receptors (CLRs). This could be achieved by decorating the vaccine formulation with natural and/or synthetic ligands for these receptors. A recent study by Cruz *et al.* demonstrated that targeting PLGA-NPs to one of the well-known CLRs, DC-SIGN, improved antigen presentation by human DCs and resulted in the activation of antigen T-cell activation with 10–100-fold lower concentrations of antigen compared to the non-targeted formulations (25). MR, another well-known member of the CLR family, has been extensively studied for its capacity to recognize mannosylated antigens and/or mannan conjugates (reviewed in (1)). In previous manuscripts, we have reported on the incorporation of MN in PLGA-NPs by chemical and physical means (8). Our earlier studies have demonstrated a significantly higher level of MN loading (465 ± 36 μg/mg) when it was chemically conjugated onto the surface of PLGA-NPs ($p < 0.05$), compared to formulations containing physically adsorbed MN (139 ± 21 μg/mg) (8). In the current study, we pursued chemical conjugation of MN (the natural ligand for MR) into antigen-loaded PLGA-NPs as means of enhancing the immunogenicity of the incorporated antigen. The impact of MN on enhancing antigen-specific $CD4^+$ and $CD8^+$ T-cell responses was further assessed both *in vitro* and *in vivo*.

MN incorporation resulted in a marginally enhanced cellular uptake, as evidenced by increase in the percentage of cells positive for OVA/FITC (Fig. 1a). Since the particles may be taken up by other phagocytic cells that are present in the bone marrow culture (such as macrophage), double color staining was performed to assess the number of $CD11c^+$ cells (DCs) that were positive for fluorescently labeled formulations. The results showed a slight increase in the uptake of MN-incorporated PLGA-NPs containing OVA/FITC by $CD11C^+$ cell population compared to plain PLGA-NPs, as well (Fig. 1b). It is worth mentioning that the effect of MN in enhancing cellular uptake of MN-conjugated OVA/FITC NPs (Fig. 1) is less than what we have reported in our previous study (8), where TMR-Dextran was used as the florescent probe. This difference may be attributed to the presence of OVA in the formulations in the current studies. In fact, OVA-containing nanoparticles have shown a slight decrease in the negative charge on the surface of nanoparticles (Table I). This is consistent with the findings of Kammona et al. (26), where they observed that the presence of antigen (OVA) partially neutralizes the free anionic surface carboxyl groups on PLGA-NPs. Interestingly, we have recently reported on a significant and positive correlation between zeta potential of PLGA-NPs and DC uptake ($r2 = 0.960$) (8). Therefore, the decreased uptake observed in this study may be attributed to the decreased zeta potential of OVA-containing formulations (~30 mV) (compared to TMR-D-loaded formulations (~50 mV)).

Other factors that can contribute to all the marginal increase in the DC uptake profile may be attributed to the extraordinary capability of DCs to take up particulate matter without specific recognition. In fact, Wattendoef et al. (27) have formulated MN-coated poly (L-lysine) stealth microspheres and studied the effect of graded amounts of MN onto the surface of these microspheres on the efficiency of their uptake by human APCs. Interestingly, their results demonstrated that all microspheres with variable amounts of MN on their surface were internalized with a similar efficiency to unmodified microspheres (without MN) (27). These results suggest that very little MN may be needed to trigger particle internalization (27). The difference between the uptake of MN-decorated and plain PLGA NPs by DCs may be more significant at earlier incubation times (9). In other words, MN decoration may affect the kinetics of PLGA NP internalization by DCs. Alternatively, the fact that MN can be recognized by both MR as well as TLR4 on APCs may imply that recognition of MN by TLR4, and the resultant immune activation may be of a greater relevance to the success of MN-decorated vaccine formulation than the interaction between MN and MR and the resultant enhancement of formulation uptake by APCs. Therefore, our next goal was to assess the potential role of MN as an immunostimulatory adjuvant capable of stimulating antigen-specific T-cell responses when co-delivered with antigen in PLGA-NPs.

The use of OVA-NPs (without MN) has resulted in very weak T-cell responses. There was almost no detectable level of $CD8^+$ T-cell activation in the OVA-NPs-treated group (Figs. 2 and 3). This is consistent with the data in the literature about weak immunogenicity of antigen formulations in the absence of danger signals and/or immunosti-

mulatory adjuvants (12–15). In contrast, treatment of DCs with OVA/Mannan-NPs could stimulate strong antigen-specific T-cell responses (Figs. 2 and 3). The presence of titrated number of OVA/Mannan-NPs-treated DCs resulted in an increase in the extent of T-cell proliferation as well as IL-2 secretion proportionally. Measured $CD8^+$ T-cell responses were significantly lower than that of $CD4^+$ T-cell responses, in terms of both T-cell proliferation and IL-2 secretion. The ability of mannosylated formulations to direct the antigens into MHC class I and class II for stimulation of $CD8^+$ and $CD4^+$ T-cell responses, respectively, have been previously described (reviewed in (1)). Differential activation of either $CD4^+$ or $CD8^+$ T-cell responses have been observed when different forms of MN have been utilized (either oxidized or reduced) (28,29). In brief, oxidized MN has shown to facilitate the endosomal escape of mannosylated antigens from the endosome to the cytoplasm with the subsequent activation of $CD8^+$ T-cell responses (30). In contrast, formulations with reduced MN have remained in the endosome and been subjected to degradation by lysosomal enzymes, with the subsequent activation of $CD4^+$ T-cell responses (30). Results from our *in vitro* studies (Figs. 2 and 3) suggest that the chemical conjugation between MN and PLGA resulted in a preferential activation of $CD4^+$ T-cell-associated immune responses.

The simultaneous activation of OVA-specific $CD4^+$ and $CD8^+$ T-cell responses following vaccination of wild-type mice with vaccine formulations was also investigated (Figs. 4, 5 and 6). Consistent with the *in vitro* results, the *in vivo* vaccination studies revealed the tendency of OVA/Mannan-NP formulation to activate OVA-specific I cell responses, particularly activation of $CD4^+$ T-cells. The majority of activated $CD4^+$ T-cells were found in the spleens (Fig. 5a) rather than lymph nodes (Fig. 4a) of the vaccinated animals. The proliferated T-cells were also capable of producing IL-2 and IFN-γ (Fig. 5b and c). Lower activation of $CD8^+$ T-cells was observed in the spleens from this animal group (S.I = 5.3). Furthermore, the level of cytokine production detected in the expanded $CD8^+$ T-cells was lower than that detected in the activated $CD4^+$ T-cells isolated from the same group (Fig. 6b).

Modification of the physical properties of PLGA can shift the delivery of encapsulated antigens to either the cytoplasm (for MHC I presentation and $CD8^+$ T-cell activation) or to the endosome (for MHC II presentation and $CD4^+$ T-cell activation). In fact, we have previously reported a weak yet detectable antigen-specific $CD8^+$ T-cell response when OVA was delivered alone in PLGA-NPs (16). However, the PLGA used in that study (16) was a capped-type co-polymer (ester terminated). The PLGA used in the current study is an uncapped-type (COOH terminated). The diminished CD8 T-cell responses observed with the current non-targeted formulation (COOH-terminated OVA-NPs) may be explained by two scenarios: either by the effect of polymer structure on the degradation of antigen or the effect of polymer structure on the trafficking of PLGA-NPs inside DCs. Additional studies are needed to directly assess the effect of terminal functional group (ester *versus* COOH) on the intracellular trafficking of internalized PLGA-NPs. In addition, previous studies (31) have shown that cytoplasmic delivery of PLGA content is affected by differences in the molecular weight of PLGA. Small molecular weight PLGA microspheres delivered their content faster than larger molecular weight polymer (31). Other studies have shown that decreasing the particle size (up to 100 nm) can shift the immune responses into $CD8^+$ T-cell-mediated responses (32,33). Alternatively, co-delivery of additional adjuvants (such as TLR ligands) into the MN-decorated PLGA-NPs may further enhance their ability to stimulate $CD8^+$ T-cell-associated immune responses. In fact, a synergism between CLR and TLR ligation has been previously demonstrated, where co-administration of TLR3 and TLR9 ligands (Poly I:C and/or CpG, respectively) with DEC-205 targeted DNA vaccine have resulted in remarkable induction of antigen-specific T-cell responses, compared to non-adjuvanted DEC-205 targeted DNA vaccine (34). A similar synergism between CLR (dectin-1) and TLR (TLR2 and/or 4) ligation was also reported by other groups (35–37). Remarkably, PLGA-NPs can facilitate simultaneous delivery of multiple TLR and/or CLR ligands in the same vaccine formulation. This strategy can result in simultaneous triggering of both TLRs and CLRs with the subsequent induction of optimum vaccine induced immune responses.

In conclusion, our results demonstrate the potential of MN-conjugated PLGA-NPs containing antigen to simultaneously enhance antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. However, stimulation of $CD4^+$ T-cells by MN-conjugated PLGA-NP formulation was more dominant both *in vitro* and *in vivo*. Alteration in the physiochemical properties of PLGA-NPs (decreasing the particle size or reducing polymer molecular weight) and MN (utilization of the oxidized form of MN) may be required to shift the balance to a more favorable $CD8^+$ T-cell response to PLGA-NPs.

ACKNOWLEDGMENTS

The authors would like to thank Elaine Moase for proof-reading the manuscript. The authors would like to acknowledge financial support by research grant from Natural Sciences and Engineering Council of Canada (STPGP 336987). This manuscript is dedicated to Prof.

Dr. John Samuel, our mentor, who passed away in April 2007 and was an expert in the field of cancer immunotherapy. The authors report no conflict of interest.

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