Mannose functionalized plain and endosomolytic nanocomposite(s)-based approach for the induction of effective antitumor immune response in C57BL/6 mice melanoma model

Rajeev Sharma and S. P. Vyas

Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr. H. S. Gour University (A Central University), Sagar, India

ABSTRACT

The goal of present study to assess the antigen specific immunopotentiation effect of mannose functionalized endosomolytic and conventional nanocomposite(s) based combination approach using C57BL/6 mice melanoma model. Endosomolytic and conventional nanocomposite(s) were prepared by double emulsification method. The optimized formulation was extensively characterized for average particle size, zeta potential and PDI of nanocomposite(s) which were measured in range of ≈200 nm, 0.111 ± 0.024, −23.4 ± 2.0 mV, respectively. pH-dependent morphological changes in the surface of MRPRPs and PRPNs were analyzed by using surface electron microscopy at different time intervals. The cellular uptake assessment of developed formulations were followed by using RAW 264.7 macrophage cell lines. Results revealed that after immunizing B16F10 melanoma cells implanted C57BL/6 mice with combination [endosomolytic and conventional nanocomposite(s)] of nanocomposite(s), a significant increase in the interleukins level i.e. IL-2, IFN-γ, IL-12 and IL-6 and OVA Ag(s) specific antibody responses were recorded. Consequently, a strong immunological response was elicited with specific polarization contributing to humoral and activation of CD8⁺ to cellular responses. Finding of histological examination also support the potential of therapeutic outcome. The present approach based on mannose surface functionalization for targeting to antigen presenting cells and pH-dependent prompt endosomal release and escape can be a promising system for efficient cancer immunotherapy.

Introduction

Cancer is a dreadful disease and presently the leading cause of death worldwide. Scientists are continuously exploring new treatment regimen for successful management of this disease. Over the past some years, cancer immunotherapy has emerged as an attractive and alternative option in the area of cancer research. Cancer immunotherapy is thus expected to protect and improve the quality of life of cancer patients because of its ability to induce selective killing of cancer cells without any harmful effects to normal cells and ability to engender immunological memory which could prevent recurrence to tumor [1,2]. Recent advancements in the field of nanotechnology and its integration with the field of immunotherapy have paved ways for improving the therapeutic indices. Immunotherapy refers to therapeutic approaches that treat cancer by involving patient’s own immune system. The immune competent cells targeted immunotherapy as such confers certain benefits including improved therapeutic effect and minimized adverse side effects. A nanotechnology based therapeutic cancer vaccine is generally consisted of a tumor antigens (TAs)/Tumor-associated antigen (TAA)s, an adjuvant and a delivery system [3–6]. Over the past years, for the development of effective nanovaccine, a focus as a concern has been to address certain limitations which include the selection of an appropriate delivery system(s), antigen(s), antibodies and an adjuvant. In addition, the enhanced adjuvanticity, particularly by nano-delivery system(s), appears to be an exciting advantage. In past some years, notable efforts have been made applying nanotechnology-based approaches specifically to target and deliver the antigen(s) to the APCs. Although, these approaches could improve the therapeutic outcome yet an extensive research is needed to explore and develop some effective delivery strategies [7–9].

For the development of effective immunotherapy, certain limitations are to be addressed which include selection of appropriate target antigen(s), antibodies, an adjuvant and delivery system(s). In addition, the enhanced adjuvant activity particularly by nanodelivery system(s) is exiting advantage. In past some years, notable efforts have been made applying nanotechnology-based approaches to specifically target the tumor cells [4,10,11]. Although, these approaches could improve the therapeutic effectiveness yet extensive research are to be needed to explore and develop effective delivery strategies. The challenges in delivery of antigen(s) are not only to deliver them to specific site but also retain their functional and structural integrity as well. In addition, to provoke desired immune response the antigen(s) should be delivered and captured efficiently by antigen presenting cells (APCs) for its processing and presentation to immune competent cells, i.e. T helper cells. In recent years extensive efforts have been made for the development of multifunctional nanocarrier(s) systems that can be used to deliver immune-bioactive(s) more efficiently to the APCs for harnessing a specific immune response.
[12–14]. Design and development of such type of nanovaccine-based strategies improves the protection against large number of diseases. It is also well investigated and documented that surface engineered nanosystem(s) serve both, i.e. as an adjuvant, as well as a carrier for cell specific delivery; to improve the persistence time of antigenic molecules with immune competent cells and following appropriate activation of CD4+ T cells as well as cytotoxic T cells resulting in to a strong immune response. Numerous previous investigations have reported that in vivo targeting to antigen presenting cells (APCs) can be achieved by targeting APCs associated receptors such as mannose receptors (MR, CD206) [15,16], C-type lectin receptors (CTLs) [17–21], and so on. Mannose receptors are generally overexpressed in APCs and have been extensively implicated in targeting of immunogenic molecules to these cells [22–24]. These mannose receptor(s) facilitate the receptor mediated endocytosis of nano-construct(s) which are surface functionalized with mannose moiety. Several studies have evidenced that expression of mannose receptors on APCs, i.e. macrophages, dendritic cells plays a significant role in processing and presentation of antigenic molecules [22,25,26]. In the past some years, there have been numerous studies exploring the concept of pH dependent cytosolic release of immunogens from pH responsive nano-delivery system(s) in order to potentiate the therapeutic effectiveness of the vaccines [27–30].

In the present approach, mannose functionalized release promoter nanocomposite(s) (MRPRPNs; endosomolytic nano-composites) and conventional nanocomposite(s) (PRPNs; non-endosomolytic nano-composites; mannose functionalized nano-composites not containing release promoter) were designed and developed for improving the transient cross-presentation of loaded ovalbumin following their targeted delivery to antigen presenting cells. Here, ammonium bicarbonate was co-encapsulated with OVA Ag(s) in the MRPRPNs as release promoting agent. Thus, the possibility of involvement of both endosomal and cytosolic pathways for MHC-II to MHC-I restricted presentation of OVA Ag(s) results in to strong long-lasting immunological response. It is presumed that mannose surface decoration facilitates the receptor specific binding; to enhance endocytosis and subsequent processing and presentation of loaded OVA Ag(s) in association with both MHC-I and MHC-II, respectively. Our findings revealed that B16F10 melanoma implanted C57BL/6 mice immunized (after tumor establishment) with a combination of both MRPRPNs and PRPNs formulation exhibited a significance increase the level of cytokines, i.e. IL-2, IL-12, IFN-γ, IL-6 and OVA Ag(s) specific antibody responses. The present study, ability of proposed combination-based approach for active targeting to APCs site specific prompt delivery of OVA Ag(s); its cross-presentation and OVA Ag(s) specific immune response have been investigated.

Material and methods

1,2-Disteryl-sn-glycerol-3-phospho-ethanolamine (DSPE) [31,32] and Ovalbumin (OVA) were received as gift sample from Lipoind, Germany and Taj Agro Ltd. (Ahmedabad, India). RPMI -1640 and DMEM (Dulbecco’s modified eagle medium), Tris/HCl buffer and PMSF were obtained from Himedia Laboratories, Mumbai (India). Mouse anti p53 antibodies and β actin were purchased from Cell Singling Technology (CST; USA) and Santa-Cruz Biotechnology (California, USA) respectively. Fluoro Isothiocyanate Labeling Kit and BCA Protein Estimation Kit (KT-31) were procured from Genai Bangalore, (India). RAW 264.7 macrophages cell lines were obtained from National Center for Cell Science (Pune, India). C57BL/6 mice (6–8 weeks old) were obtained from CSIR-Institute of Microbial Technology, Chandigarh Punjab (India). Mouse cytokine estimation kits were purchased from Thermo Fisher Scientific through chemical supplier. The reagents used in SDS-PAGE were procured from BIO-RAD (USA). All other chemicals and reagents were of analytical grades.

Preparation, optimization and characterization of nanocomposite(s)

Mannose surface functionalized release promoter nanocomposite(s) containing ammonium bicarbonate as Ag(s) release promoter and conventional nanocomposite(s) were prepared by double emulsification solvent evaporation method as reported in previous studies [33,34] (outlined in Scheme 1). Various formulation variables and controls were made in order to get MRPRPNs and PRPNs with optimized average particle size, zeta potential, polydispersity index (PDI) and OVA entrapment efficiency. The optimized MRPRPNs and PRPNs formulation(s) were extensively characterized for particle size, zeta potential and PDI using Malvern Zetasizer (Zetasizer-3000HS, Malvern, UK); shape and surface morphological analysis were conducted using High resolution transmission electron microscopy (FEI, Techni G² F-20, S-Twin, the Netherlands) and Field emission-surface electron microscopy (FE-SEM, Fei NANOSEM, the Netherlands). The presence of mannose moiety on the surface of MRPRPNs was assessed by using ligand agglutination assay following reported in previous studies [35,36]. All other methods and related findings are reported in the recently published study [25].

Stability studies

Although, immunologically products are generally stored only in the refrigerated conditions, yet, it may be anticipated and desirable too that antigenic molecules loaded formulations should possess improved stability. Systematically optimized formulations were subjected to stability testing under storage condition at 4 ± 1°C and at room temperature (25 ± 1°C). Evaluation of the samples for size, PDI and % residual OVA content after 15, 30, 45 and 60 days was conducted. Subsequent changes in average nanocomposite(s) size and PDI was measured using Malvern Zetasizer (Zetasizer-3000HS, Malvern, UK). The residual OVA Ag(s) content of the formulation was determined by micro BCA protein estimation assay following instructions given by manufacturer; unentrapped OVA Ag(s) was separated by ultracentrifugation (Z36, HK, Hermle, Laborteknik, Wehingen, Germany) at 22,000 rpm for 30 min at 4°C. The supernatant was collected and diluted with PBS; The OVA Ag(s) content in supernatant was measured by micro BCA assay using UV-spectroscopic analysis (UV 1601 Shimadzu, Kyoto, Japan). The storage stability of OVA Ag(s) was assessed as well; The SDS PAGE was carried out to access the structural integrity of OVA Ag(s) released from nanocomposite(s). The well-established protocol for electrophoresis based study and protocol was followed as reported elsewhere in literature [37,38].

pH-dependent surface analysis

Surface analysis of MRPRPNs and PRPNs was conducted using SEM (Fei NOVA NANOSEM450, the Netherlands) at different pH buffers (5.5 and 7.4) at various time intervals. For surface analysis at neutral pH, the nanocomposite(s) were dispersed in buffer (pH 7.4) and incubated at 37°C under controlled condition. The samples were withdrawn at different time intervals, i.e. 30 min, 60 min.
Further, a drop of sample (20 μL) was transferred on the copper grid coated with carbon material and was kept at ambient temperature (20–25°C) in order to form a thin layer on the grid. Further, the samples were proceeded for gold coating under vacuum and scanned using FE-SEM instrument at a suitable magnification. Likewise, surface analysis of MRPRPNs and PRPNs were conducted at acidic pH (5.5) at different time intervals.

**Assessment of internalization**

The cellular uptake study was carried out by using RAW264.7 mouse macrophages cells; an antigen-presenting cell line, in accordance with the method described in previous studies [39,40]. The RAW 264.7 cells were cultured in six well plate (Coaster, corn-ing Inc., NY, USA) in DMEM medium supplemented with 10% FBS in 5% CO2 humidified incubator (C-170, Binder Gmbh, Germany) for 12 h at 37°C. At 70% confluence, media was removed and washed with PBS. The cells were trypsinized with 1/2 trypsin and after detachment of cells, a FBS containing media was added to inhibit the trypsin action. The cell suspension was then pelleted at 1500 rpm for 2 min and the cell pellet was collected and washed to wash out and remove any cell debris. The cells were resuspended in 1 mL DMEM media and counted using hemocytometer followed by seeding at dilution 5 × 10⁵ cells; the cells were monitored in a six-well plate and incubated for 24 h. Next day, the cells were washed and DMEM media to the cells 50 μL of formulation(s) was added to each well in the plate. The experiment was conducted in replicates by in vitro incubation of RAW 264.7 cells with FITC co-encapsulated MRPRPNs and (FITC-MRPRPNs-OVA), UPNs (FITC-UPNs-OVA) and OVA (1 μg/mL) FITC-OVA. The cells were then incubated for 3 h and cells were washed, trypsinized and resuspended in 500 μL of PBS and analyzed using BD FACS Diva (Biosciences, USA).

**Scheme 1.** Graphically represents the preparation of release promoter-based mannose functionalized nanocomposite(s) and proposed mechanism underlying the induction of antitumor immune responses by combination immunization.

**In vitro cytotoxicity assessment**

The cytotoxicity was determined by using colorimetric MTT assay against macrophage RAW 264.7 cells [41]. Briefly, 1 × 10⁵ cells were cultured in 96-well flat bottom plates (100 μL/well in DMEM) and allowed to grow for 48 h. subsequently, cells were incubated with increasing concentration of MRPRPNs and PRPNs for 24 h (OVA concentration 50 μL/mL). After incubation, the cells layer was washed and incubated with 100 μL of MTT solution (0.8 mg/mL in DMEM) for 4 h at 37°C. The medium then replaced with DMSO and formed formazan crystals were dissolved along with the cell layers and plates were mixed well with glycine buffer, and the absorbance of each well was recorded at 570 nm using microplate reader (Bioired-680, USA) Cells viability was calculated using PBS as control.
**B16F10 induced C57BL/6 mice melanoma model**

The experimental protocol for animal studies as approved by institutional animal ethical committee (GRKIST/406/02/IEAC/37 Reg. No. 1471/PO/a/11/CPCSEA) of GRKIST college of Pharmacy, Jabalpur (Madhya Pradesh, India). The B16F10 cell line (NCCS, Pune, Maharashtra, India) were incubated in a fresh DMEM medium with 10% FBS in 5% CO₂ incubator (C170-230V-R, BINDER GmbH, Germany). This culture flask was taken out without causing any disturbance to media inside the culture flasks and the surface was cleaned with 90% alcohol. The medium was removed and rinsed with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The solution was removed and an additional 1 mL of TPVG solution was added and the flask was incubated at 37°C until the cells were separated followed by addition of fresh medium supplements (pH 7-8); aspirated, dispensed into new flasks and transferred to the CO₂ incubator (5% CO₂) and incubated at 37°C. After third seeding, the cells were cultured for 3 days. Then, the cells were separated from culture media and were transferred to the sterile centrifugation tubes after trypsinization and addition of fresh medium supplements containing FBS; the cell suspension was centrifuged at 2000g for 5 min. The media was carefully decanted from the centrifuge tube and the cells pellet was vortexed to the cells suspended in a fresh medium. Now 20 μL of cell suspension was placed in a neubauer chamber and stained with trypan blue and the viable cells were counted using following equation.

\[
\text{No. of cells counted} = \frac{\text{Total number of squares counted}}{4000 \times \text{dilution}}
\]

A suspension of B16F10 melanomas cells was prepared containing \(10^5\) cells/mL and was injected subcutaneously in C57BL/6 mice under mild anesthesia. The grown tumor became visible after 18 days from the day of challenge.

**Histopathological analysis**

For histopathological examination, control and treated mice were sacrificed, the excised skin samples were collected and rinsed with ringer’s solution. The tissues were then fixed in cornoy’s fluid (3 parts Absolute alcohol:1 part chloroform) for 3 h; followed by transfer to absolute alcohol for dehydration. After dehydration, the tissues were transferred in to a mixture of hematoxylin: absolute alcohol (1:1) and kept for 20 min. These tissues were then transferred to eosin and kept for 40 min. Subsequently, the wax scrapings to xylene was added up to a saturation level and kept for 24 h followed by embedding in paraffin wax. Tissue were sectioned in to \(\approx 5\) μm slices by using a microtome. The sections were observed under microscope (Nikon eclipse E200, Japan) and photographs were captured.

**Analysis of the immune response**

B16F10 melanoma cells bearing C57BL/6 mice (female; after establishment of tumor) were divided randomly in to five groups (n = 3) and were immunized (IV) with OVA loaded formulations (adjusted OVA conc 100 μg) with one of the following regimens: Group 1 = control; Group 2 = Pl-OVA Ag(s) (PBS; pH 7.4); Group 3 = PRPNs-OVA (OVA loaded PRPNs); Group 4 = MRPRPNs-OVA (OVA loaded MRPRPNs), Group 5 = combination of both formulation(s) (MRPRPNs-OVA with PRPNs-OVA; half by half) in order to evaluate antigen specific immune response. The animals of each group were given two booster with a gap of a week after, i.e. on day 7th and day 14th in case of group I to IV while in case group V the booster was given on day 7th using formulation(s) MRPRPNs. However, subsequent booster was based on the combination of both formulation(s) (MRPRPNs & PRPNs). It is noteworthy the group 5 received one additional booster on day 21 thus the first booster are based on MRPRPNs was considered the completion of immunization. The vernier caliper was used to measure the tumor size of melanoma implanted C57BL/6 mice and the tumor volume was calculated using following Equation [42]:

\[
\text{Tumor volume (V, mm}^3\text{)} = 0.5 \times (L \times W^2)
\]

Here, \(L = \) length of tumor (longest dimension); \(W = \) width of tumor. The separate measurements were made in each group of mice to validate the cytokines level. Each sample point an average of three measurements each representing a value from an animal was considered. The mice were sacrificed and splenocytes were isolated and suspended in the RPMI-1640 media and cultured at \(5 \times 10^6\) cells/mL in 96-well plates in the absence and presence of OVA Ag(s). The cells were suspended in fresh RPMI-160 medium supplemented with 10% FBS, 50 mM 2-ME, penicillin (100 μg/mL), streptomycin (100 mg/mL) in 5%CO₂ at 37°C followed by measurement of the cytokines level, i.e. IL-2, IFN-γ, IL-12 and IL-6 using enzyme linked immune sorbent assay [43].

**Assessment of OVA-specific antibody titer**

The systemic immune response was assessed wherein all the samples and standards were made as per manufacturer’s instructions (RioBio, 2016). Briefly, blood samples were collected from melanoma cells implanted C57BL/6 mice after 2, 4, 6, 8 weeks of immunization and serum was obtained containing centrifugation and stored -40°C until analyzed by using ELISA. Findings are recorded as log reciprocal of end point dilution. The end point of titer was expressed as a reciprocal of last dilution that gave an optical density at 490 nm. For the assessment of IgG2a and IgG1 isotypes antibodies; the samples were measured using sigma isotyping kit (Sigma-aldrich Pvt. Ltd., USA) in the sample collated at various time interval of immunization. Results are recorded represented graphically as Figure 7(c).

**Statistical analysis**

The data were expressed as mean standard deviation. Statistical significance was determined using one-way ANOVA analysis of variance using (GraphPad Prism 5.2 software, La Jolla, CA). For comparative analysis between the groups student’s t test was applied.

**Result and discussion**

**Preparation, optimization and characterization of nanocomposite(s)**

The average particle size and zeta potential measured for MRPRPNs and PRPNs 188.4 ± 8.52 nm and -23.4 ± 0.76 mV and 159.8 ± 5.12 nm, -26.5 ± 0.64 mV, respectively. Moreover, poly dispersity index is an evidence of distribution of nano-sized particles and result of PDI being less than 0.5 for all the formulations (MRPRPNs and PRPNs) suggests for a homogenous dispersion of particles. The percent entrapment efficiency of OVA in MRPRPNs and PRPNs determined by using Bradford assay was 60.17 ± 2.41 and 68.93 ± 2.93, respectively. Shape and surface morphology of
nanocomposite(s) were analyzed by using surface and transmission electron microscopic techniques, the findings are reported in our recent published study [25].

**Stability studies**

The findings revealed better stability of nanocomposite(s) at 4 ± 1°C wherein particle size, PDI and % residual antigen content were used as assessment parameters. The integrity of OVA antigen was determined before and after storage using SDS PAGE analysis (Figure 1) which confirms that even on prolonged storage at 4.0 ± 1°C, the antigen integrity in the formulation was retained.

**pH dependent surface analysis**

At pH 7.4, no morphological changes were observed over the surface of the MRPRPNs and PRPNs. However, at acidic pH, i.e. 5.5, the significant changes in the form of pores were visible at the surface of MRPRPNs; with increasing time interval, the density and size of pores appeared to increase (Figure 2). It could be attributed owing to destabilization of MRPRPNs nanocomposite(s) owing to chemical reaction of bicarbonate with acidic environment. At acidic pH, protons (H+5) are infiltrated into the matrix of MRPRPNs followed by their reaction with the co-encapsulated ammonium bicarbonate resulting into the formation of NH3 and CO2. The CO2 and NH3 bubbles so formed on escape created the

![Figure 1. Stability analysis of formulation(s) for (a,b) % residual OVA content (c,d) particle size, (e,f) PDI and at 4 ± 1°C & 25 ± 1°C. (g) Structural integrity assessment of OVA Ag(s) by SDS-PAGE; Lane 1: Mol. Marker Lane 2; before processing OVA Ag; Lane 3: after formulation/processing OVA Ag (released from MRPRPNs).](image-url)
pores in the MRPRPNs thus facilitated the prompt release of loaded OVA Ag(s) at acidic pH \((pH 5.5)\). Therefore, the relatively faster release of OVA Ag(s) was recorded from MRPRPNs compared to PRPNs.

### Assessment of internalization

In the present study, the internalization of FITC co-encapsulated nanocomposite(s) (FITC-OVA, MRPRPNs and PNs) was followed and assessed using RAW 264.7 macrophage cells (as a model antigen presenting cells). The cells were incubated with FITC labeled nanocomposite(s) formulations and analyzed after 3 h. FITC labeled plain OVA in PBS was used as a control. The cells displayed a greater uptake of mannose appended nanocomposite(s) [MRPRPNs] as compared to plain formulation(s) (Figure 3). The maximum internalization and accumulation was recorded for MRPRPNs followed by PRPNs and FITC labeled OVA in PBS. The findings revealed that the percent uptake of FITC labeled OVA antigen was minimum. The maximum cell associated fluorescence was measured in the case of mannosylated release promoter-based nanocomposite(s) [MRPRPNs (OVA + FITC)], i.e. \(\approx 63.05 \pm 2.8\%\) at the end of 3 h. The obtained results convincingly suggest that nanocomposite(s) consisted of PLGA (FDA approved polymer), lipid and mannose were well tolerated and not exhibited cytotoxic effects on the cell viability as indicated by cell proliferation assay.

### Anti-tumor efficiency in experimentally induced mice melanoma model

The antitumor immunological effect after immunization of different OVA loaded formulation(s) is illustrated in Figure 5. Immunization of melanoma bearing C57BL/6 mice combination of both formulation [PRPNs-OVA & MRPRPNs-OVA] showed significant regression \((p < 0.001)\) of tumor as compared to pl-OVA Ag(s) and unmodified/plain nanocomposite(s) [PRPNs]; Following immunization after 4 weeks, the increased in vivo anti-tumor immune response of combination (MRPRPNs with PRPNs) immunization could be attributed to higher cell uptake; prompt endosomal release of encapsulated OVA Ag(s); subsequent presentation and processing of OVA Ag(s) in association of both MHC class-I and MHC class-II respectively, thereby triggering significant cellular and humoral immune responses against implanted melanoma.

### Histopathological analysis

Histopathological observations of tumor bearing, normal and immunized melanoma bearing C57BL/6 mice are shown in Figure 6, Supplementary figure 1 (Control mice). Histopathological examination of melanoma bearing sections showed prominent tumor nodules associated with necrosis. On the other hand, mice immunized with a combination of formulation [MRPRPNs &
PRPNs showed denucleated necrotic cells indicating selected killing of cancerous cells. However, histo-examination after 4 weeks of immunization, observed improvement and most of normal cells appeared to be healthy which might be due to subsequent healing process after programmed cell death of cancerous cells.

Analysis of the immune response

The cytokines levels were quantitatively measured and compared after 4 weeks of immunization with OVA loaded formulation(s) in melanoma bearing C57BL/6 mice. However, in our study we have measured cytokines which include IL-2, IFN-γ, IL-6 and IL-12. The selection of cytokines is mainly to deduce and follow up the involvement of cellular immunity where in IFN-γ signifies the clonal selection of specific antibodies, i.e. production of IgG1 against OVA Ag(s); while IL-2 not only indicates the required activation of CD4+ cells but taken together with IFN-γ it is suggestive of Th1 polarization for immunological consequences. The results demonstrated that the formulation combination-based immunization, i.e. MRPRPNs & PRPNs could induce effective antitumor immune response which could be attributed to uptake of mannosylated nanocomposite(s) by APCs and in case of PRPNs the processing and presentation of OVA Ag(s) in association with MHC-II while in case of MRPRPNs endosomal escape of OVA Ag(s) and its subsequent degradation through ubiquitination followed by its presentation in association with MHC-I (involving cytosolic pathway). However, the mice immunized with pl-OVA Ag(s) there appears poor uptake of soluble Ag(s) by APCs and also its presentation which resulted in to negligible to nil immune response and hence even after immunization the melanoma continued to grow (Figure 7(a–c)). The results showed that combination based immunization approach could significantly increase IL-2, IL-12, IFN-γ and IL-6 level, i.e., 235.78 ± 32.89 pg/mL, 74.24 ± 6.4 pg/mL, 362.72 ± 32.6 pg/mL; and 2.26 ± 0.21 ng/mL, respectively, as compared to OVA Ag(s) solution (IL-2, 32.18 ± 3.40; IL-12, 11.32 ± 1.01 pg/mL; IFN-γ, 48.64 ± 9.6 pg/mL; IL-6, 0.62 ± 0.05 ng/mL), in case of APCs targeted formulation(s) without release promoter and endosomolytic character, i.e. the interleukins/cytokines levels measured were IL-2, 116.32 ± 10.85; IL-12, 24.38 ± 3.03 pg/mL; IFN-γ, 106.14 ± 14.20 pg/mL; and IL-6, 0.78 ± 0.80 ng/mL). The results well commensurate with the findings reported by Yoshizaki et al. [46], Zhang et al. [28]. The level of cytokines critically affects the maturation, activation of APCs as well as helper T cells thus influencing the resultant immune response and its subsequent regulations.

The anti-OVA IgG antibody response produced by various OVA loaded formulations in melanoma implanted C57BL/6 mice was measured after 2, 4, 6, 8 weeks of immunization as illustrated in Figure 7(c). The optimum anti-OVA-IgG antibody titer (log_{10}) was obtained after 6 weeks of immunization with PRPNs, MRPRPNs and combination of both (MRPRPNs with PRPNs), i.e. 2.67 ± 0.18.
2.74 ± 0.24 and 2.98 ± 0.28, respectively. In case of pl-OVA Ag(s) the IgG antibody titer measured 0.37 ± 0.036 which was significantly low compared to IgG level measured after immunization with PRPNs, MRPRPNs and combination of both. Findings further reveal that nanocomposite(s) could elicit combined serum IgG2a/IgG1 immune response (Figure 7(b)). In case of PRPNs and MRPRPNs, the measured IgG2a/IgG1 ratio was 0.72 ± 0.04 and 1.02 ± 0.06 respectively. The elicited immune response was significantly higher than that of plain OVA antigen(s) solution. Nevertheless, the maximum IgG2a/IgG1 ratio was measured in case of immunization(s) with a combination of formulation (MRPRPNs & PRPNs), i.e. 1.64 ± 0.09. Findings indicated that IgG2a/IgG1 ratio was significant in case of combination-based immunization (MRPRPNs & PRPNs). The findings in regard to specific antibodies, IL-2, IL-12 and IFN-γ level suggest that there has been a polarization of Th1 indicating specific immune response against the Ag(s) involving CD8+ T cells as also evidenced by the measured level of IL-6 inflammatory cytokines. Thus, it could be inferred that the formulation capable of Ag(s) processing involving endosomal and cytosolic pathway for MHC-I and MHC-II restricted presentation of antigen(s) could be designed by ameliorating the intrinsic property of nanocomposite(s) as it has been executed in present study by incorporation of release promoter in to nanocomposite(s) which could not only enhance the release of OVA Ag(s) but also it could possibly destabilize the endosome to release the contents in to cytosol APCs. The findings suggest that combination-based immunization, i.e. MRPRPNs & PRPNs is capable of inducing more efficient anti-tumor immune response than conventional or modified nanocomposite(s).

**Conclusions**

To date, various antigen delivery systems, i.e. nanoparticulate and vesicular have been investigated for induction of antigen specific CTLs immune response. These systems might be taken up by antigen presenting cells, i.e dendritic cells, macrophages via endocytosis and may transfer the antigenic peptides into cytosol. Efficient delivery of antigen(s) into cytosol of APCs is a key factor for efficient antigen presentation on MHC class I molecules. Therefore, to ensure antigen presentation in association with MHC class I; the
delivery system(s) should be designed to be endosomolytic in order to deliver the antigen(s) for cytosolic processing by antigen presenting cells. To accomplish the scientific goal, endosomolytic release characteristics and targeted delivery of antigen(s) are necessary for efficient induction of cellular immunity. Thus, in the present approach we have designed and developed antigen presenting cells targeted endosomolytic and conventional nanocomposite(s) based combination therapy for cytosolic and endosomal processing of antigen(s) respectively. Therefore, it has been executed in present study by incorporation of release promoter in to nanocomposite(s) which could not only enhance the release of OVA Ag(s) but also it could possibly destabilize the endosome to release the contents in to cytosol APCs. The findings suggest that combination-based immunization, i.e. MRPRPNs & PRPNs could produce with results more efficient antigen specific immune response compared to conventional or modified nanocomposite(s).
based immunization. The findings showed that mannose surface anchoring facilitated the receptor mediated targeting of OVA Ag(s) loaded nanocomposite(s) to antigen presenting cells. In addition, due to endosomolytic characteristics nanocomposite(s) quickly release OVA Ag(s) into endosome and allowing endosomal escape into the cytosol. Thus, the involvement of both endosomal and cytosolic processing and presentation of loaded OVA Ag(s). Results revealed that after immunizing melanoma cells implanted C57BL/6 mice with combination [MRPRPNs & PRPNs] of nanocomposite(s), a significant increase in the interleukins level, i.e., IL-2, IL-12, IFN-γ and IL-6 level and OVA specific antibody responses were recorded. Consequently, a strong immunological response was elicited with specific polarization contributing to humoral and activation of CD8+ to cellular responses. Finding of histological examination also support the potential of therapeutic outcome. The present approach based on mannose surface

Figure 7. Continued.
functionalization for targeting to antigen presenting cells and pH-dependent prompt endosomal release and escape can be a promising system for efficient cancer immunotherapy. The study further needs to be concluded to find out the effect of negative signals involved in the inhibition of response.

Acknowledgements
The author Rajeev Sharma is thankful for the fellowship support from the Council of Scientific and Industrial Research (CSIR, New Delhi). We also acknowledge to sophisticated instrument Center, Dr. Hari Singh Gour Central University Sagar, India for providing TEM and SEM analysis facility. We sincerely acknowledge Prof. O. P. Katare for his support in carrying out in vivo studies at Panjab University, Chandigarh.

Disclosure statement
No potential conflict of interest was reported by the authors.

References
[1] Conniot J, Silva JM, Fernandes JG, et al. Cancer immunotherapy: nanodelivery approaches for immune cell targeting and tracking. Front Chem. 2014;2:105.
[2] Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med. 2004;10:909–915.
[3] Bolhassani A, Safaian Y, Rafati S. Improvement of different vaccine delivery systems for cancer therapy. Mol Cancer. 2011;103.
[4] Jiang W, Von Roemeling CA, Chen Y, et al. Designing nanomedicine for immuno-oncology. Nat Biomed Eng. 2017;1:0029.
[5] Higgins JP, Bernstein MB, Hodge JW. Enhancing immune responses to tumor-associated antigens. Cancer Biol Ther. 2009;8:1440–1449.
[6] Zhang Z, Guo Y, Feng S-S. Nanoimmunotherapy: application of nanotechnology for sustained and targeted delivery of antigens to dendritic cells. Nanomedicine. 2012;7:1–4.
[7] Caster JM, Wang AZ. Applying nanotherapeutics to improve chemoradiotherapy treatment for cancer. Ther Deliv. 2017; 8:791–803.
[8] Xin Y, Yin M, Zhao L, et al. Recent progress on nanoparticle-based drug delivery systems for cancer therapy. Cancer Biol Med. 2017;14:228.
[9] Sharma R, Mody N, Vyas SP. Nanotechnology-based immunotherapeutic strategies for the treatment of cancer. In: Jana S, Jana S, editors. Particulate technology for delivery of therapeutics. Singapore: Springer Singapore; 2017. p. 83–115.
[10] Smith DM, Simon JK, Baker JR. Applications of nanotechnology for immunology. Nat Rev Immunol. 2013;13:592–605.
[11] Park YM, Lee SJ, Kim YS, et al. Nanoparticle-based vaccine delivery for cancer immunotherapy. Immune Netw. 2013;13:177–183.
[12] Cheung AS, Mooney DJ. Engineered materials for cancer immunotherapy. Nano Today. 2015;10:511–531.
[13] Ranzoni A, Cooper MA. Micro and nanotechnology in vaccine development. Elsevier; 2017.
[14] Qiu H, Min Y, Rodgers Z, et al. Nanomedicine approaches to improve cancer immunotherapy. Wiley Interdiscip Rev: Nanomed Nanobiotechnol. 2017;9(5):1–23. DOI:10.1002/wnnn.1456.
[15] Movahedi K, Schoonooghe S, Laoui D, et al. Nanobody-based targeting of the macrophage mannose receptor for effective in vivo imaging of tumor-associated macrophages. Cancer Res. 2012;72:4165–4177.
[16] Glaflig M, Stergiou N, Hartmann S, et al. A synthetic MUC1 anticancer vaccine containing mannose ligands for targeting macrophages and dendritic cells. ChemMedChem. 2018;13:25–29.
[17] Dasgupta S, Bayry J, Lacroix-Demazes S, et al. Human mannose receptor (CD206) in immune response: novel insights into vaccination strategies using a humanized mouse model. Expert Rev Clin Immunol. 2007;3:677–681.
[18] Kanazawa N. Dendritic cell immunoreceptors: C-type lectin receptors for pattern-recognition and signaling on antigen-presenting cells. J Dermatol Sci. 2007;45:77–86.
[19] Geijtenbeek TBJ, Gringhuis SI. C-type lectin receptors in the control of T helper cell differentiation. Nat Rev Immunol. 2016;16:433–448.
[20] Sharma R, Mody N, Kushwah V, et al. C-Type lectin receptor(s)-targeted nanoliposomes: an intelligent approach for effective cancer immunotherapy. Nanomedicine. 2017;12:1945–1959.
[21] van Dinther D, Stolk DA, van de Ven R, et al. Targeting C-type lectin receptors: a high-carbohydrate diet for dendritic cells to improve cancer vaccines. J Leukoc Biol. 2017;102:1017–1034.
[22] Thoman-Harwood LJ, Kaeuper P, Rossi N, et al. Nanogel vaccines targeting dendritic cells: contributions of the surface decoration and vaccine cargo on cell targeting and activation. J Control Release. 2013;166:95–105.
[23] Keler T, Ramakrishna V, Fanger MW. Mannose receptor-targeted vaccines. Expert Opin Biol Ther. 2004;4:1953–1962.
[24] Apostolopoulos V, Barnes N, Pietersz GA, et al. Metal-organic frameworks: active cancer therapy. Sci Transl Med. 2012;4:78–789.
[25] Yoshizaki Y, Yuba E, Sakaguchi N, et al. pH-sensitive polymeric micelles. J Leukoc Biol. 2017;102:1034–1041.
[26] Liang M, Li Y, Zhang L, et al. PH-Responsive poly(D,L-lactic-co-glycolic acid) nanoparticles with rapid antigen release behavior promote immune response. ACS Nano. 2015;9:4925–4938.
[31] Mitchell JP, Roberts KD, Langley J, et al. A direct method for the formation of peptide and carbohydrate dendrimers. Bioorg Med Chem Lett. 1999;9:2785–2788.

[32] Agrawal U, Chashoo G, Sharma PR, et al. Tailored polymer-lipid hybrid nanoparticles for the delivery of drug conjugate: dual strategy for brain targeting. Colloids Surf B Biointerf. 2015;126:414–425.

[33] Koyamatsu Y, Hirano T, Kakizawa Y, et al. PH-responsive release of proteins from biocompatible and biodegradable reverse polymer micelles. J Control Release. 2014;173:89–95.

[34] Chen L, Mei L, Feng D, et al. Anhydrous reverse micelle lecitthin nanoparticles/PLGA composite microspheres for long-term protein delivery with reduced initial burst. Colloids Surf B Biointerf. 2018;163:146–154.

[35] Woller EK, Cloninger MJ. The lectin-binding properties of six generations of mannose-functionalized dendrimers. Org Lett. 2002;4:7–10.

[36] Sharma R, Gupta U, Garg NK, et al. Surface engineered and ligand anchored nanobioconjugate: an effective therapeutic approach for oral insulin delivery in experimental diabetic rats. Colloids Surf B Biointerf. 2015;127:172–181.

[37] BioRad. Molecular weight determination by SDS-PAGE. Electrophor BioRad Man. Bulletin 6210. 2004;1–4.

[38] Gallagher SR. SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Curr Protoc Essent Lab Technol. 2012;2012:6.1.1–6.1.38.

[39] Haase-Kohn C, Wolf S, Herwig N, et al. Metastatic potential of B16-F10 melanoma cells is enhanced by extracellular S100A4 derived from RAW264.7 macrophages. Biochem Biophys Res Commun. 2014;446:143–148.

[40] Radovic-Moreno AF, Chernyak N, Mader CC, et al. Immunomodulatory spherical nucleic acids. Proc Natl Acad Sci USA. 2015;112:3892–3897.

[41] Asthana S, Jaiswal AK, Gupta PK, et al. Th-1 biased immunomodulation and synergistic antileishmanial activity of stable cationic lipid-polymer hybrid nanoparticle: biodistribution and toxicity assessment of encapsulated amphotericin B. Eur J Pharm Biopharm. 2015;89:62–73.

[42] Miller JP, Egbulefu C, Prior JL, et al. Gradient-based algorithm for determining tumor volumes in small animals using planar fluorescence imaging platform. Tomogr J Imaging Res. 2016;2(1):17–25.

[43] Jiang PL, Lin HJ, Wang HW, et al. Galactosylated liposome as a dendritic cell-targeted mucosal vaccine for inducing protective anti-tumor immunity. Acta Biomater. 2015;11:356–367.

[44] Silva JM, Vandermeulen G, Oliveira VG, et al. Development of functionalized nanoparticles for vaccine delivery to dendritic cells: a mechanistic approach. Nanomedicine. 2014;9:2639–2656.

[45] Engering AJ, Cella M, Fluitsma DM, et al. Mannose receptor mediated antigen uptake and presentation in human dendritic cells. Adv Exp Med Biol. 1997;417:183–187.

[46] Yoshizaki Y, Yuba E, Sakaguchi N, et al. Potentiation of pH-sensitive polymer-modified liposomes with cationic lipid inclusion as antigen delivery carriers for cancer immunotherapy. Biomaterials. 2014;35:8186–8196.