Vaccine nanocarriers: Coupling intracellular pathways and cellular biodistribution to control CD4 vs CD8 T cell responses

Marcela Rincon-Restrepo^{a+}, Aaron Mayer^{a+}, Sylvie Hauert^a, Daniel K. Bonner^a, Edward A. Phelps^a, Jeffrey A. Hubbell^{a,b}, Melody A. Swartz^{a,b} and Sachiko Hirosue^{b*}

⁺ These authors contributed equally to this work

^{*} Corresponding author, sachiko.hirosue@epfl.ch

Author's affiliations

^aInstitute of Bioengineering, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

^bInstitute for Molecular Engineering, University of Chicago, Chicago, IL

Abstract

Nanoparticle delivery systems are known to enhance the immune response to soluble antigens (Ags) and are thus a promising tool for the development of new vaccines. Our laboratory has engineered two different nanoparticulate systems in which Ag is either encapsulated within the core of polymersomes (PSs) or decorated onto the surface of nanoparticles (NPs). Previous studies showed that PSs are better at enhancing CD4 T cells and antibody titers, while NPs preferentially augment cytotoxic CD8 T cells. Herein, we demonstrate that the differential activation of T cell immunity reflects differences in the modes of intracellular trafficking and distinct biodistribution of the Ag in lymphoid organs, which are both driven by the properties of each nanocarrier. Furthermore, we found that Ags within PSs promoted better CD4 T cell activation and induced a higher frequency of CD4 T follicular helper (Tfh) cells. These differences correlated with changes in the frequency of germinal center B cells and plasma cell formation, which reflects the previously observed antibody titers. Our results show that PSs are a promising vector for the delivery of Ags for B cell vaccine development. This study demonstrates that nanocarrier design has a large impact on the quality of the induced adaptive immune response.

Keywords

Vaccine design, antigen presentation, T follicular helper cells, germinal center, dendritic cells

Abbreviated title

Intracellular pathways and cellular biodistribution of vaccine nanocarriers

1 Introduction

Vaccination is undoubtedly a major success in modern medicine and stands as an affordable and safe technology that has allowed the control and eradication of life threatening diseases[1,2]. Yet, today there are pathogens that account for more than a million deaths annually, for which no licensed or fully protective vaccines have been created[3,4].

Development of vaccines based on subunit components has proven to be a safe and cost effective method for eliciting protection against pathogenic infections. It eases safety concerns related to immunization with live attenuated viruses, and it offers the flexibility to target a specific antigen (Ag) as well as to tune the quality of the response by selecting a precise immunostimulatory compound[5]. Still, the ability of subunit vaccines to stimulate an immune response is usually weaker than traditional preparations with whole-attenuated pathogens. One of the approaches used to enhance immunogenicity of subunit vaccines is Ag formulation in biomaterial-based particulate carriers[6–9]. Various studies have demonstrated that Ag loading into synthetic particles enhances uptake and presentation by professional presenting cells, such as dendritic cells (DCs), as well as increases Ag bioavailability by promoting Ag retention in lymphoid organs[10–17].

Our group has recently developed two nanocarriers, nanoparticles (NPs) [6,7,18,19] and polymersomes (PSs)[11,20], composed of the hydrophobic polymer poly(propylene sulfide, PPS), and the hydrophilic polymer polyethylene glycol (PEG). Despite having the same building units, both systems differ considerably in their physical and chemical properties (Figure S1). NPs are carriers of 25-40 nm in diameter, and consist of a rubbery hydrophobic core of PPS, covered by a corona of PEG where Ag can be loaded through diverse chemical ligations. Usually, proteins are attached through a disulfide bond (-ss-), as our laboratory has shown that a reducible bond favors the delivered Ag to be cross-presented to CD8 T cells [7]. PSs instead are constituted of an aqueous core surrounded by a polymeric bilayer of PEG-PPS and have a minimum diameter of 150-175 nm. This conformation allows the loading of hydrophobic compounds into the amphiphilic bilayer, while hydrophilic compounds including Ag can be encapsulated within the aqueous core. These vesicles undergo Ag release *in vitro* in an oxidation-dependent manner, which likely occurs in the conditions found within lysosomes[11].

Interestingly, the differences between the two particulate carriers significantly affect innate and adaptive immune responses. Recently, Stano *et al.*[20] demonstrated that PSs were better at enhancing CD4 and Ag-specific antibody responses, while NPs preferentially enhanced the induction of cytotoxic CD8 T cells. The exact mechanisms by which each particulate carrier enhanced a specific type of response are still unknown. Based on the way Ag is coupled and released from carriers to professional antigen presenting cells (APCs), it is probable that differences in Ag processing and

1 presentation exist at the cellular level[21]. Particle size should also impact lymphatic
2 transport and distribution in lymphoid organs, potentially delivering carriers to specific
3 DC sub-populations[22]. There are various DC subsets in skin draining lymph nodes
4 that are crucial for the initiation of T cell responses in the lymph node (LN)[23], which
5 reside in a unique anatomical localization. For instance, LN CD11b⁺DCs, are located
6 in close contact with cortical and medullary sinuses in draining LNs, and are one of
7 the main subsets associated with MHCII presentation to CD4 T cells[24–27]. On the
8 contrary, CD8⁺ DCs are mainly found in the T cell area, and are responsible for cross
9 presentation of exogenous Ags to CD8 T cells [28]. Consequently, understanding
10 how both nanocarriers interact with immune cells and the nature of their biodistribution
11 in lymphoid organs may shed light on the underlying mechanisms by which NP and
12 PS drove differential T cell immunity.

13 In this study, we utilized the model Ag chicken egg ovalbumin (OVA) loaded onto NP
14 (NP-ss-OVA) and PS (PS-OVA) particulate nanocarriers to investigate the
15 mechanisms by which carrier design generates distinct and potent immune responses
16 after vaccination. We demonstrate that the differential activation of T cell immunity
17 reflects differences in the modes of intracellular trafficking and distinct biodistribution
18 of the Ag in lymphoid organs.

19

20

Materials and methods

Animals

C57BL/6 female mice, age between 8-12 weeks, were purchased from Harlan (Gannat, France). OTI and CD45.1⁺ OTII mice were obtained from an in house colony. All animal experiments were performed under the approval from the Veterinary Authority of the Canton of Vaud (Switzerland) according to Swiss Law.

Cells

Bone marrow derived CD11c⁺ dendritic cells (BMDCs) were generated by adapting the protocol described by Lutz *et al* [29]. Briefly, BM cells were cultured for 8-9 days in the presence of 20 ng/mL of GM-CSF, changing the medium every 3 days. After this time, CD11c⁺ cells constitute the 70-85% of the non-adherent population.

Ovalbumin (OVA)-specific T cell receptor transgenic CD8 T cells and CD4 T cells were obtained from splenocytes of OTI and OTII mice, respectively. T cell populations of interest were enriched from splenocyte cell-suspensions using the EasySep Mouse CD8⁺ and CD4⁺ T cell Enrichment kits (STEMCELL Technologies, Grenoble France), as per instructions provided by the manufacturer.

Materials

All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unless otherwise noted. Endo-free OVA was purchased from Hyglos (Bernried am Starnberger See, Germany). CpG-B ODN 1826 was purchased from InVivogen (San Diego, California). Antibodies for flow cytometry analysis were purchased from eBioscience (Vienna, Austria) or BioLegend (Lucerne, Switzerland) unless otherwise noted. Antibodies for immunofluorescence imaging LAMP1 (clone# D2D11), EEA1 (clone# C45B10) were purchased from CellSignaling (Danvers, Massachusetts). Sandwich ELISAs were purchased from eBioscience (San Diego, California).

Nanoparticle preparation

All chemical reagents were reagent grade and purchased from Sigma-Aldrich unless otherwise stated. Nanoparticles (NPs) were prepared as described previously [6,7,18,19], with some modifications to allow for protein loading into the PEG corona. Briefly, a mixture of 1:3 COOH-PEG / OH-PEG was used to prepared the emulsion polymerization to form NPs. After exposure to air for 2 h, the remaining thiolates were quenched by the addition of 45 mg of iodoacetamide and stirred for 15 min. To functionalize the COOH groups on the NP surface, sulfo-NHS (Life Technologies), Pyridyl disulfide cysteamine (synthesized in house) and 1ethyl-3-(3-dimethylaminopropyl) (EDC) were added to the mixture and let to react for 24 h. For purification, NPs were dialyzed against MQ H₂O for 36 h at room temperature by replacing the water every 6 h, and passed through a filter with a 0.22 µm pore size (Millipore Corporation). The concentration of the loaded pyridyl disulfide was

determined by measuring the absorbance at 340 nm after adding TCEP (ThermoScientific) to the NP solution.

For OVA loading, 500 μ L of NPs were mixed with 286.6 mg of guanidine hydrochloride (GndHCl) to achieve a final concentration of 6 M of GndHCl. The solution was filtered through a 0.22 μ m membrane (Millipore Corporation) and mixed with 50 μ L of OVA at 40 mg/mL. The mixture was left overnight at room temperature, and then purified by size exclusion chromatography through a Sepharose CL-6B column equilibrated in 50 mM sodium phosphate. The concentration of OVA in the fractions obtained after purification was determined by a BCA assay following the manufacturer's instructions (ThermoScientific).

Before and after the coupling, the size of NPs was determined by dynamic light scattering with a Nano Zs Zetasizer (Malvern Instruments). The diameter of NPs varied between 25 nm - 40 nm.

Block-copolymer synthesis and polymersome preparation

All chemical reagents were reagent grade and purchased from Sigma-Aldrich unless otherwise stated. Polymersomes (PSs) were prepared following the original description by Scott *et al.*[11]. Block-copolymers were synthesized using benzyl mercaptan to start the living polymerization of propylene sulfide. The terminal thiolates were capped with PEG-mesylate. For purification, the co-polymers were precipitated two times in cold methanol. The purity and length of the PPS chain were confirmed by gel chromatography and by NMR. Usually, chains of PEG₁₇-bI-PPS_x, containing between x=26-32 PPS units were used to form PSs. An amount of 30-50 mg resuspended in dichloromethane was placed in piranha-etched glass vials and desiccated overnight. For the formation of PSs, the formed thin-film of copolymer was rehydrated with a solution of 5 mg/mL of OVA or fluorescently labeled OVA in 50 mM phosphate buffer, and left rotating for 48 h at room temperature. Afterwards, the solution was extruded at least 4 times through a 0.2 μ m nucleopore track-etched membranes (Whatman) to obtain PSs ranging between 150 nm - 175 nm, with polydispersity values lower than 0.2. Purification of the unloaded Ag molecules was achieved by size exclusion chromatography through a Sepharose CL-6B column. Fractions containing PSs were pooled together and further concentrated through an Ultra-centrifugal filter (Millipore Corporation) with a cutoff of 100 kDa. Final OVA loading was quantified by a sandwich ELISA as described by Stano *et al.*[20].

The size of PSs was determined by dynamic light scattering with a Nano Zs Zetasizer (Malvern Instruments).

Imaging

For imaging studies, OVA was labeled with AF647 NHS at pH 8 for 2 h and purified through a G25 size exclusion column (Sigma-Aldrich, Buchs, Switzerland). Fluorescently labeled OVA (OVA₆₄₇) was used for conjugation on NPs, or incorporation in PSs. NPs were labeled with AF647 C2 maleimide (NP₆₄₇) (Invitrogen, Carlsbad, CA) followed by gel filtration.

All formulations were tested to have negligible endotoxin levels as determined by the HEK-Blue™ TLR4 cell line (InvivoGen, San Diego, CA) performed as recommended by the manufacturer.

Ag distribution after delivery by NP and PS platforms was determined by confocal microscopy where OVA₆₄₇ was imaged against EEA1 or LAMP1 with DAPI as a nuclear counterstain. BMDCs on day 8 were seeded onto poly-D-(lysine) coated coverslips for 2 h at 37 degrees C in 5% CO₂ prior to beginning of experiment. After incubation of the OVA formulations [25 µg/ml] for 2 h in serum starving media, the BMDCs were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, washed, then permeabilized with 0.5% TX100 in PBS for additional 30 min. The samples were then blocked with 0.22 µm filtered animal-free blocker (Vector Laboratories) for 1 h at room temperature. Primary antibodies were applied for 1 h at room temperature or overnight at 4°C in 0.1% BSA + 0.2% TX100 in PBS, then washed prior to incubation with AF488 secondary antibodies (Invitrogen, Life Technologies) in 0.1% BSA + 0.2% TX100 in PBS. ProLong® Gold Antifade Mountant with DAPI was used for mounting the glass covers.

Imaging Analysis

Z-stack confocal images were taken using the Carl Zeiss LSM700 confocal microscope with a 63x oil-immersion objective lens. Images were then deconvoluted with (Huygens Deconvolution software; Scientific Volume Imaging, The Netherlands) prior to processing with Fiji software (National Institutes of Health, Bethesda, MD). Thresholds were determined for each fluorophore with negative controls. Using the specialized colocalization module in Imaris, surfaces for EEA1 or LAMP1 vesicles were defined with 0.1 µm smoothing while spots for NP-ss-OVA₆₄₇ or PS(OVA₆₄₇) were defined at 0.2 µm. The total number of spots within each cell was calculated as well as the total number of surfaces. Using the XT Matlab extension, the number of spots within surface objects was tabulated. The final percentage colocalization is presented as the number of spots within surfaces divided by the total number of spots.

BMDC stimulation, co-culture and analysis

Day 8-9 BMDCs were treated with varying concentrations of OVA formulations and CpG-B for 12 h or 24 h in 96 round bottom well plate. Supernatants were then removed and cytokine secretion was measured by Ready-Set-Go ELISA kits as per the manufacturer's instruction. To setup the OTI or OTII T cell cocultures, 20,000 DCs were seeded in 96 round well format in 100 µl of IMDM with 10% HI FBS + P/S. OVA formulations and CpG-B were added to the wells and incubated for 1 h at 37°C in 50 µl of media, followed by the addition of 100,000 OTI CFSE labeled T cells (5:1=DC:T) or 200,000 OTII CFSE labeled T cells (10:1) for a final volume of 200 µl in each well. The cells were incubated at 37°C, 5% CO₂ for 3 days prior to harvesting of the supernatant. Cells were restimulated with PMA (50 ng/ml) / Ionomycin (1 µg/ml) mix for 2 h at 37°C. Cytokine secretion was stopped with the addition of Brefeldin A at a final concentration of 5 µg/ml for 2 h. The cells were spun down and stained for flow cytometry.

Immunizations and adoptive transfers

All vaccines were administered intradermally (i.d.) into the four limbs. 10 µg of OVA were injected per mouse for all formulations, unless otherwise stated, together with 10 µg of CpG-B (Microsynth). Before the immunizations, OVA was filtered through a 0.22 µm membrane (Millipore Corporation) to remove aggregates. Furthermore, all formulations were tested for LPS contamination using the TLR-based HEK-Blue LPS detection assay (Invivogen).

For adoptive transfers of naive TCR transgenic CD4 T cells, single cell suspensions were prepared from spleens of OTII CD45.1⁺ mice. CD4 T cells were isolated as detailed earlier.

Preparation of lymph node and spleen suspensions

For the identification of stromal cells, lymph nodes were opened with needles and first digested in 750 µL of DMEM (1.2 mM CaCl₂, 2% FBS, 20 mM HEPES, Pen/Strep) containing collagenase IV (1 mg/mL) and DNase I (40 µg/ml). The digestion medium was removed, followed by a second digestion step with 750 µL of DMEM containing collagenase D (1 mg/mL) and DNase I (40 µg/ml), as detailed in [30]. In the remaining experiments, lymph nodes were opened with needles and digested in DMEM (1.2 mM CaCl₂, 2% FBS, 20mM HEPES, Pen/Strep) containing collagenase D (500 µg/mL). After digestion, lymph node cell suspensions were filtered through a 70 µm nylon cell strainer. For spleen, single cell suspensions were prepared by mechanical disruption through a 70 µm nylon cell strainer. Splenocytes were further treated with hypotonic ammonium chloride–potassium bicarbonate buffer to lyse red blood cells.

Flow cytometry

Up to 3x10⁶ cells were plated in 96 well plates and washed once with HBSS for staining with live/dead fixable cell viability reagent (Life technologies). For surface staining, cells were washed once with HBSS supplemented with 0.5% bovine serum albumin (BSA, Sigma) (staining buffer) and resuspended in an antibody cocktail using monoclonal antibodies.

Detection of follicular helper cells T cells was achieved in a 2-step process. First, cells were incubated with CXCR5-biotin (eBioscience) in staining buffer for 30 min, washed once and then incubated with an antibody cocktail with Streptavidin-647 (Biolegend) and the remaining antibodies. For intracellular staining, cells were fixed and permeabilized with the Foxp3 / Transcription Factor Fixation/Permeabilization kit (eBioscience) according to the manufacturer's instructions. After staining, cells were resuspended in staining buffer for analysis by flow cytometry.

Data collection

Flow cytometry was performed using a CyAnTM ADP (Beckman Coulter) or BD LSRII and data were analyzed with FlowJo (Tree Star). We used the non-parametric Mann-Whitney U test or 1-way ANOVA, followed by a Bonferroni post-test to find differences between the groups. A *P* value of less than 0.05 was used as an indicator of statistical

1 significance. All data was analyzed using the GraphPad Prism 5 Software (GraphPad
2 Software Inc., La Jolla, CA).
3
4

Results

NP-ss-OVA and PS-OVA undergo distinct intracellular Ag trafficking in dendritic cells

The intracellular route of Ag is known to play a major role in driving CD4 or CD8 T cell responses[31,32]. Therefore, to understand if intracellular trafficking takes part in the differential T cell activation by the two particulate carriers, we incubated bone marrow-derived dendritic cells (BMDCs) together with PSs and NPs loaded with fluorescently labeled OVA (OVA₆₄₇). We compared the intracellular trafficking of the Ag by assessing the OVA₆₄₇ colocalization with markers associated with endosomal or lysosomal compartments, such as early endosomal antigen 1 (EEA1), or lysosomal-associated membrane protein 1 (LAMP1).

After incubation of PS(OVA₆₄₇) with BMDCs during 2 h, we detected a high colocalization of OVA₆₄₇ within LAMP1 vesicles in the perinuclear space (Figure1, A), but a much lower association with EEA1 (Figure1, B). This was in agreement with previous study from our group, which employed the PS vehicle loaded with calcein dye and demonstrated similar lysosomal association at 4h and 6h[11]. To the contrary, OVA₆₄₇ delivered on NPs appeared intracellularly disperse and colocalized preferentially with EEA1 endosomes (Figure1, C-D). Meanwhile, the NP vehicle alone (NP₆₄₇, no Ag) aggregated in the perinuclear space and exhibited a high degree of LAMP1 colocalization (Figure1, E). This corroborated well with early work showing NP₄₈₈ colocalized in LAMP1 vesicles at 1.5 h[7]. The fact that NP-ss-OVA₆₄₇ colocalized more with early endosomes, while NP₆₄₇ aggregated in late endosomes, suggests that a fraction of OVA₆₄₇ is cleaved from the NP carrier during intracellular trafficking. In contrast, the perinuclear aggregation of PS(OVA₆₄₇) in LAMP1 vesicles indicates that Ag loaded within PS carriers may be precluded from endosomal escape and cytosolic processing at early time points.

Although quantification revealed that the percent of colocalization within LAMP1 compartments for the NP-ss-OVA₆₄₇ group was similar to that found for PS(OVA₆₄₇), the former group had a significantly higher percent of colocalization in EEA1 compartments (Figure1, F). We also observed that the ratio of colocalization between LAMP1 and EEA1 was approximately 1:1 for the NP-ss-OVA₆₄₇ group, while the PS(OVA₆₄₇) displayed a nearly 6:1 ratio of LAMP1 to EEA1 vesicular colocalization (Figure1, G).

Taken together, these data demonstrate that OVA₆₄₇ loading into nanocarriers impacts intracellular Ag routing in DCs. It suggests that Ag loaded within PSs traffics through LAMP1 endosomal compartments via an intracellular route typical of exogenous Ag processing[33]. Furthermore, the differences in the relative distribution of NP-ss-OVA₆₄₇ and NP delivery vehicle alone suggest that a fraction of Ag is cleaved from the carrier in EEA1 endosomes and potentially escapes into the cytoplasm where it can be processed by the machinery required for MHC I presentation (Figure S2).

Figure 1. NP-ss-OVA undergo endosomal escape upon internalization, while PS(OVA) traffic through lysosomes within BMDCs

PS(OVA) and NP-ss-OVA activate differentially CD4 and CD8 T cells in *vitro*.

To understand whether the differential intracellular trafficking of Ag impacts APC activation, we evaluated the ability of NP-ss-OVA and PS-OVA to promote the secretion of cytokines by BMDCs *in vitro*. For this purpose, cells were incubated for 24 h with 0 µg/ml, 5 µg/ml or 50 µg/ml of OVA formulated as NP-ss-OVA or PS(OVA), or given as a soluble form. All the conditions were adjuvanted with 0.05 µM of the Toll-like receptor 9 (TLR9) ligand CpG-B, a short DNA sequence mimetic of bacterial DNA. We selected this adjuvant since the earlier *in vivo* studies that demonstrated the tunable immunity between NPs and PSs employed this TLR9 ligand [20]. We assessed the secretion of molecules such as IL-12 and IL-6, since APCs are known to promote the production of these cytokines in response to this adjuvant[34]. Our results showed that both carriers induced similar activation of BMDCs, except at higher Ag concentration, where more cytokine secretion was observed with the PS-OVA treatment (Figure S3). Notably, similar trends were observed for both PS-OVA and NP-ss-OVA at an earlier time point (12 h) and at varying concentrations of CpG-B (data not shown). These data demonstrated that both carriers, in combination with the TLR9 adjuvant, have the same capability to activate APCs, despite their differences in intracellular Ag trafficking.

We next sought to determine if the observed differences in intracellular trafficking of Ag could in fact lead to differences in MHC presentation and subsequent CD4 or CD8 T cell activation *in vitro*. We employed CD8 T cells derived from OTI mice, which contain a transgenic T cell receptor (TCR) that recognizes an OVA MHCI peptide in the context of the MHCI allele H2K^b. Analogously, CD4 T cells were isolated from OTII mice, which have a TCR that recognizes an MHCII OVA peptide in the context of the MHCII allele I-A^b. For assessing OTI and OTII activation and proliferation, BMDCs were incubated for 1 h *in vitro* with NP-ss-OVA, PS(OVA) or soluble OVA adjuvanted with 0.05 µM CpG-B and immediately after co-culture with CFSE labeled OTI and OTII T cells. Within three days, we observed that the treatment with NP-ss-OVA resulted in superior IFN γ production and secretion by OTI T cells when compared to PS(OVA), as measured by both flow cytometry and ELISA (Figure2, B-D). Interestingly, there was also an increase in the secretion of IL-17 by OTI cells upon NP-ss-OVA treatment (Figure S4). In agreement with these results, NP-ss-OVA also promoted a higher percent of activated OTI cells (CD44⁺CD25⁺) (Figure 2E). On the contrary, OTII T cells displayed an enhanced IFN γ secretion and expression (when analyzed per generation), as well as a higher percentage of activated cells when OVA was delivered within PSs (Figure2, F-I). These trends held across a variety of experiments and formulations with varying OVA concentrations from 3.125 µg/ml to 100 µg/ml (Figure S5) and varying CpG-B concentrations of 0 µM, 0.025 µM, 0.05 µM and 0.1 µM (data not shown). Similar results were also obtained in experiments where cells

were treated with PSs and NPs without any adjuvant (Figure S4). Together, the data demonstrates that there are factors at the cellular level that contribute to the tunable CD8 and CD4 T cell immunity observed in our previous study.

Figure 2. NP-ss-OVA and PS(OVA) promote tunable T cell immunity in vitro

NP-ss-OVA and PS(OVA) display a differential association with subsets of LN resident DCs

APCs are crucial for the initiation of T cell responses. Interestingly, there is an apparent division of labor between various DC subsets within LNs based on their anatomical localization, preferentially activating CD4 or CD8 T cells [23,28,35–38]. We therefore hypothesized that tunable T cell immunity could also be linked to a distinct Ag distribution (and thus DC targeting) upon i.d. immunization. To further test this idea, we examined the early events of Ag acquisition by various LN DC subsets, loading OVA₆₄₇ into NPs or PSs. Mice were vaccinated with both carriers in the footpad of the four limbs, together with CpG-B, and 2 or 36 h later the main draining lymph nodes (brachial and popliteal) were collected and analyzed by flow cytometry (Figure3, A).

Evaluation of LN cellularity upon i.d. immunization revealed that both vaccines drove similar LN expansion, as there were comparable changes in cell numbers 2 h and 36 h post immunization (p.i.) (Figure 3, B). However, we observed main differences in the numbers of cells that were associated with the Ag. The NP-ss-OVA₆₄₇ group displayed twice the numbers of OVA₆₄₇⁺ cells when compared the PS counterpart at 2 h p.i., although by 36 h p.i. the numbers of OVA₆₄₇⁺ cells equalized between both groups (Figure 3, B).

For analysis of DC targeting after 2 h, we distinguished between the main DC subsets based on the expression of MHCII and CD11c. We first excluded CD4⁺CD11c⁻, CD8 CD11c⁻ cells, B cells (B220⁺) and monocytes and macrophages (CD11b⁺CD11c⁻) [25,39–41], and on the remaining cells identified migratory DCs as CD11c^{mid}MHCII^{hi} and resident DCs as CD11c^{hi}MHCII^{mid} (Figure 3, C). There was also a third population that expressed low levels of CD11c and MHCII, which we called CD11c^{low} DCs. This last population may potentially be neutrophils that upregulated CD11c expression early after the immunization.

Next, we evaluated the global distribution of the main DC subsets within the OVA₆₄₇⁺ cells. The majority of Ag was associated with resident DCs and CD11c^{low} DCs, followed by migratory DCs that were already present in the LNs at the time of analysis. Notably, this latest population was significantly more enriched in the NP-ss-OVA₆₄₇ group.

Subsequently, we were very interested in assessing how OVA₆₄₇ was scattered between DC resident subsets, which can be subdivided into CD11b⁺ DCs and CD8⁺ DCs. Interestingly, PS-OVA₆₄₇ displayed a preferential association with CD11b⁺ DCs

1 compared to the CD8⁺ DCs subset. NP-ss-OVA₆₄₇, instead, displayed a more
2 homogenous distribution between CD11b⁺ DCs and CD8⁺ DCs, although OVA₆₄₇⁺
3 cells were more enriched in the CD8 subset (Figure 3, D, left). Although in terms of
4 cell numbers there were not significant differences in the numbers of CD11b⁺ DCs that
5 were associated with Ag between both carriers, we found a significant increase in the
6 number of CD8⁺ DCs targeted by the NP platform (Figure 3, D, right).

7 By 36 h p.i, it was not possible to discriminate between resident and migratory DCs
8 based on MHCII expression, since resident subsets increased the expression of
9 MHCII to levels similar to migratory subsets. We thus analyzed OVA₆₄₇⁺ cells within
10 CD11b⁺ CD8⁻, and CD8⁺CD11b⁻ DCs. There were no differences in Ag association
11 with the CD8⁺CD11b⁻ population between both groups, but we did observed that
12 PS(OVA₆₄₇) were enriched in the CD11b⁺ CD8⁻ subset (Figure 3, E). Interestingly,
13 vaccination with PS(OVA₆₄₇) promoted a higher MCHII expression in LN DCs,
14 suggesting that, *in vivo*, loading of Ag in PSs enhances DC MHCII presentation to
15 CD4 T cells when compared to NPs (Figure 3, F).

16
17 *Figure 3. NP-ss-OVA is homogenously distributed between LN resident CD11b and*
18 *CD8+ DCs, while PS (OVA) display a preferential association with CD11b+ DCs and*
19 *enhance the upregulation of MHCII*

20 **NP-ss-OVA and PS(OVA) are enriched in distinct stromal cells subsets at** 21 **early time points after i.d. immunization**

22 LN stromal cells provide a scaffold for hematopoietic cells and help define the
23 structure of LNs. Moreover, stromal cells provide a highway for peripheral Ag
24 drainage, enhancing the probability of Ag encounter with APCs and its recognition by
25 lymphocytes for the initiation of immune responses [42,43]. Based on this evidence,
26 we rationalized that evaluating early events of Ag association with the stromal cell
27 compartment could give us insights into how Ag distributes inside the LN upon i.d.
28 vaccination.

29 Initially, we sought to compare the relative association of OVA₆₄₇ with the non-
30 hematopoietic (CD45⁻) cell compartment at 2 h and 36 h p.i. The results reflected our
31 earlier observations, as the number of OVA₆₄₇⁺ cells was higher in the NP-ss-OVA₆₄₇
32 at 2 h with respect to the PS-OVA₆₄₇ counterpart, and equalize after 36 h p.i. (Figure
33 4, A).

34 Afterwards, we assessed the LN stromal subsets within the OVA₆₄₇⁺ population. For
35 this purpose, we performed an analysis based on the expression of gp38 and CD31,
36 which classifies CD45⁻ cells as blood endothelial cells (BECs, CD31⁺gp38⁺),
37 fibroblastic reticular cells (FRCs, CD31⁺gp38⁺), lymphatic endothelial cells (LECs,
38 CD31⁺gp38⁻) and double negative cells (DN, CD31⁻gp38⁻) [44] (Figure 4, B). There
39 were striking differences in the subpopulations associated with OVA₆₄₇. In the case of
40 NP-ss-OVA₆₄₇, Ag was found primarily in LECs and, to a lesser extent, in FRCs.
41 Conversely, PS-OVA₆₄₇ associated almost exclusively with LECs. There was also a

small fraction of OVA₆₄₇ in both groups within the BECs and DN cells, but the frequencies were much lower than those observed in the former subsets (Figure 4, B). Instead, at 36 h there was a much more homogeneous distribution of the antigen in both groups, with the FRCs displaying the highest OVA₆₄₇ signal, followed by LECs (Figure 4, B).

Figure 4. NP-ss-OVA associate with fibroblastic reticular cells and lymphatic endothelial cells at early times, while PS(OVA) associates almost exclusively with lymphatic endothelial cells

Encapsulation of antigen in PSs enhances T follicular helper CD4 cell responses and promotes the generation of germinal center B cells and plasma cells

Follicular helper CD4 T (Tfh) cells represent a unique CD4 T cell subset that specializes in providing help to B cells within germinal centers (GCs), where B cells undergo affinity maturation, class switch recombination, plasma cell differentiation, and memory B cell differentiation[45]. Considering that PSs are augmenting CD4 T cells responses, we asked ourselves whether the CD4 Tfh cell subset was also enhanced upon vaccination with PSs.

To analyze the induction of Tfh cell responses, we immunized mice i.d. with PS(OVA), NP-ss-OVA and free OVA, together with 10 µg CpG-B, and at day 9, collected draining LNs and spleen. In order to facilitate the detection of Tfh cells, we adoptively transferred 2x10⁶ congenic (CD45.1⁺) CD4 OTII cells (Figure 5, A).

At this time, PS(OVA) and NP-ss-OVA induced a comparable OTII CD4 T cell expansion, displaying similar counts in both draining LNs and spleen, while soluble OVA displayed almost 100-fold less expansion than OVA associated with the nanocarriers (Figure 5, B). We then analyzed the phenotype of all CD4 T cells (endogenous and adoptively transferred together) after NP and PS immunization. We found that CD4 T cells primed with PS(OVA) had an enhanced activated phenotype, described by a higher frequency of CD44^{hi}CD62L^{low} cells. NP-ss-OVA and soluble OVA displayed instead similar frequencies of activated CD4 T cells (Figure 5, C).

Subsequently, we quantified the frequency of OTII-CD4 T cells that have adopted a Tfh cell phenotype (CXCR5^{hi}PD-1^{hi}Bcl6^{hi}) (Figure 5, D). In LNs, around 15% of the transferred CD45.1⁺ cells in the PS(OVA) group displayed Tfh traits. Much lower values were seen for NP-ss-OVA and soluble OVA. A similar trend was seen in the spleen, although the overall frequencies were much lower. Considering that Tfh cells are positively correlated with GCs[46], we analyzed the phenotype of B cells during the same experiment. In agreement with previous publications[15,46], we found higher frequencies of GC B cells in PS(OVA) immunized mice, with a 2-fold increase over the NP-ss-OVA counterpart (Figure 5, E). We also assessed the frequency of plasma cells, which should be in their peak response in LNs and spleen[47]. Once

1 again, the PS(OVA) group showed an enhancement in the frequencies of plasma cells
2 (IgD^{low} CD138^{hi}) when compared to the other immunization regimes (Figure 5, F). All
3 together, these data suggest that the delivery of Ag within PSs results in
4 enhancement of Tfh CD4 T cells which positively impact important aspects of the
5 humoral response, promoting B cell differentiation into GCs and antibody secreting
6 plasma cells.

7
8 *Figure 5. PS(OVA) immunization enhances the induction of T follicular helper (Tfh)*
9 *CD4 T cells, germinal center cells and plasma cells.*

Discussion

Nanoparticulate-Ag carrier systems are of great interest for the development of subunit vaccines. Understanding their mechanisms in cellular targeting and processing and bridging these mechanisms to the subsequent impact on adaptive immunity is important for effective vaccine design.

It is now well-established that the physicochemical properties of nanocarriers such as their size, geometry, and antigen coupling mode (which affects intracellular trafficking) influence the immune recognition of the vaccine nanocarrier by APCs [22,48–51]. For instance, size can be modulated to tune the trafficking properties of the carrier *in vivo* [52,53]. Reddy *et al* [6] showed that smaller particles (less than 45 nm) drained more efficiently into lymphatics upon intradermal vaccination than ca. 100 nm particles [6]. Antigen coupling can also modify how Ag is recognized by immune cells [7,15]; for example, we previously demonstrated that coupling Ag to the core of PEG-PPS NPs through a disulfide bond that is broken under reducing conditions (as in early endosomes) promoted better Ag cross-presentation to CD8+ T cells compared to when the Ag was coupled to the NP via a non-reducible bond[7].

Our group has developed two nanocarriers that vary both in Ag loading and size. A previous study demonstrated that immunization with PSs enhanced CD4 responses and Ag-specific IgG titers, while NPs enhanced cytotoxic CD8 T cells[20]. Considering the differences in size and antigen loading and composition between these two nanocarriers, here we attempted to elucidate the biological mechanisms by which the designs of these two unique particles were able to drive tunable T cell immunity.

We showed that OVA loaded on NPs via a disulfide link is, upon internalization, partially cleaved from the carrier in early endosomes, allowing for its escape into the cytoplasm and increasing the likelihood of proteasome processing and MHCI loading[54,55]. The disulfide bond by which OVA is conjugated to the PEG corona of NPs is indeed an important factor for allowing endosomal escape and MHCI processing, as our group earlier demonstrated that OVA conjugated through a non-reducible covalent bond led to a diminished MHCI presentation[7], potentially by lowering availability of the Ag in the cytoplasm. Thus, early endosomal reduction appears to favor cytoplasmic trafficking and cross-presentation. We also showed that OVA within PSs displayed higher association with lysosomes. This could account for the polymeric bilayer protecting the cargo from early degradation, and driving it all the way through the intracellular pathway until reaching the lysosomes. In these organelles, the oxidative environment allows for the PSs to disrupt[11] and release their Ag payload, which leads to Ag processing and loading into the MHCII presentation pathway [56].

At the organ level, we observed a higher amount of Ag draining to local LNs after immunization with NPs, when compared to the PSs group. This data suggest that Ag delivered as PS(OVA) has a lower early (2 h) lymphatic uptake rate than as NP-ss-OVA, which could be associated to an increased interstitial resistance associated with the larger particle diameter and effective molecular weight of PSs[8]. At longer time

points (36 h), the overall uptake rates were the same, indicating that the PS(OVA) does indeed arrive in the LN, just not as quickly as does the NP-ss-OVA.

Furthermore, we found a differential distribution of the Ag when delivered by NPs or PSs within the main stromal cell compartments and LN DC populations at earlier times (2 h) after immunization. PS(OVA) was mainly associated with LECs, while NP-ss-OVA was found dispersed between LECs and FRCs. These results might suggest a distinct micro-anatomical localization of the Ag, being retained in lymphatic sinuses when delivered within PSs, whereas reaching the LN paracortical area upon loading into NPs (Figure S6). In line with these results, we demonstrated that Ag delivered on NPs associated more with migratory DCs while Ag delivered in PSs was associated at a lesser extent with this population. Since DC trafficking from the periphery to the draining LN takes 18 h, at this early time, it is unlikely that the migratory DCs had arrived to the LN with Ag captured from the periphery. Rather, the DCs associated with NPs were likely already present at a steady-state level in the LN. Migratory DCs are known to express high levels of CCR7[57], that bind to CCL21 bound to reticular fibers deep in the T cell area. Therefore, migratory DCs are expected to locate mainly in the LN cortex, in close association with FRCs and reticular conduits[24]. Hence, it is likely these cells have accessed the Ag through FRCs upon delivery in NPs, explaining their enhanced association with OVA in our studies.

There were further differences in the association of the nanocarriers with LN-resident DCs subpopulations. After 2 h p.i., PS-OVA was enriched in the CD11b⁺ DC subset, and poorly involved with the CD8⁺ DC subgroup. Interestingly, 2-photon studies have shown that CD11b⁺ LN DCs reside close to the LN cortex, sampling Ag from lymphatic sinuses, where PS(OVA) were mainly found in the stromal cell distribution studies [26]. These might help explain why the CD11b⁺ DCs subpopulation favorably took up this carrier. Notably, CD11b⁺ DCs are related with an enhanced MHCII presentation and are acknowledged by several studies to enhance CD4 responses [24,26–28,37]. Contrastingly, CD8⁺ DCs are located primarily on the T cell area [25,26], in close contact with CD8 T cells. CD8⁺ DCs are acknowledged to be highly efficient in cross-presenting Ag [28,55], and thus the lack of association of PS(OVA) with this population might explain the poor CD8 T cell responses observed upon immunization with this nanocarrier. On the other hand, NP-ss-OVA were more homogeneously scattered through the DC resident subsets, although higher numbers of Ag⁺ cells were observed in the CD8⁺ subset. This data further suggests that NP-ss-OVA might reach the paracortical area of LNs, and consequently cross-present Ag to CD8 T cells. The fact that poorer CD4 responses are observed in the NP immunization compared to PSs, despite similar numbers of cells being associated with CD11b⁺ DCs, indicates that DC targeting does not uniquely account for the tunable immunity observed earlier by Stano *et al.*[20]. Instead, it is a combination of factors, such as intracellular trafficking and biodistribution, that dictates the outcome of the adaptive immune cell response elicited by the nanocarriers.

In addition to differential priming of CD4 and CD8 T cells, we have also demonstrated that PS encapsulated Ag promoted better Tfh CD4 responses, and increased the

1 formation of GCs and plasma cells early during the immune response. These results
2 support the enhanced Ag-specific IgG titers we observed previously[20]. There is
3 evidence that the initiation of Tfh CD4 response relies on DC presentation of the Ag
4 [58,59]. From the vaccine engineering perspective, it would be exciting to understand
5 whether the targeting of a particular intracellular trafficking pathway on DCs favors the
6 formation of Tfh CD4 T cells. Taken together, our data suggest that enhancing
7 lysosomal targeting of nanoparticulate carrier is important to enhance CD4 responses,
8 which translated to Tfh and GC responses. We conclude that Ag encapsulation in PSs
9 is a promising delivery method for vaccines targeting humoral responses for the
10 development of new subunit vaccines.

11 It is clear that Ag formulations have a strong impact on the quality of the elicited
12 immune response. Consequently for particulate vaccine design to target a specific
13 immune response, in addition to the size, physicochemical surface of the carrier and
14 lymphoid organ distribution of the conjugate, consideration of factors such as the
15 route of Ag uptake, method of Ag release, and intracellular trafficking of the Ag,
16 should be taken into account. It is now conceivable to target Ag to specific receptors
17 for trafficking to differential intracellular compartments and change the balance of
18 MHCI or MHCII presentation of the Ag and the adaptive immune response.

1 **Acknowledgments**

2 The authors would like to acknowledge Patricia Corthésy-Henrioud and Yassin Ben
3 Saida for their assistance. The research was supported by Swiss National Science
4 Foundation grant SysX IphD (SXPHI0_142025) awarded to M.A.S. and Sai T. Reddy
5 (ETHZ), and Interdisciplinary project grant (CR23I2_143754) awarded to M.A.S. and
6 Stefan Kunz (CHUV).

7

References

- [1] S.A. Plotkin, S.L. Plotkin, The development of vaccines: how the past led to the future, *Nat. Rev. Microbiol.* 9 (2011) 889–893.
- [2] R. Rappuoli, C.W. Mandl, S. Black, E. De Gregorio, Vaccines for the twenty-first century society, *Nat. Rev. Immunol.* 11 (2011) 865–872.
- [3] S.M. Todryk, A.V.S. Hill, Malaria vaccines: the stage we are at., *Nat. Rev. Microbiol.* 5 (2007) 487–489.
- [4] P. Andersen, J.S. Woodworth, Tuberculosis vaccines--rethinking the current paradigm., *Trends Immunol.* 35 (2014) 387–395.
- [5] P.M. Moyle, I. Toth, Modern Subunit Vaccines: Development, Components, and Research Opportunities, *ChemMedChem.* 8 (2013) 360–376.
- [6] S.T. Reddy, A. Rehor, H.G. Schmoekel, J.A. Hubbell, M.A. Swartz, In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles, *J. Control. Release.* 112 (2006) 26–34.
- [7] S. Hirose, I.C. Kourtis, A.J. van der Vlies, J.A. Hubbell, M.A. Swartz, Antigen delivery to dendritic cells by poly(propylene sulfide) nanoparticles with disulfide conjugated peptides: Cross-presentation and T cell activation., *Vaccine.* 28 (2010) 7897–906.
- [8] D.J. Irvine, M.A. Swartz, G.L. Szeto, Engineering synthetic vaccines using cues from natural immunity., *Nat. Mater.* 12 (2013) 978–990.
- [9] A.E. Gregory, R. Titball, D. Williamson, Vaccine delivery using nanoparticles., *Front. Cell. Infect. Microbiol.* (2013) 3–13.
- [10] A. Bershteyn, M.C. Hanson, M.P. Crespo, J.J. Moon, A. V Li, H. Suh, D.J. Irvine, Robust IgG responses to nanograms of antigen using a biomimetic lipid-coated particle vaccine., *J. Control. Release.* 157 (2012) 354–365.
- [11] E. A. Scott, A. Stano, M. Gillard, A.C. Maio-Liu, M.A. Swartz, J.A. Hubbell, Dendritic cell activation and T cell priming with adjuvant- and antigen-loaded oxidation-sensitive polymersomes, *Biomaterials.* 33 (2012) 6211–6219.
- [12] C. Nembrini, A. Stano, K.Y. Dane, M. Ballester, A.J. van der Vlies, B.J. Marsland, M.A. Swartz, J.A. Hubbell, Nanoparticle conjugation of antigen enhances cytotoxic T-cell responses in pulmonary vaccination., *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) E989–E997.
- [13] A. Stano, A.J. Van Der Vlies, M.M. Martino, M.A. Swartz, J.A. Hubbell, E. Simeoni, PPS nanoparticles as versatile delivery system to induce systemic and broad mucosal immunity after intranasal administration., *Vaccine.* 29 (2011) 804–812.
- [14] M. Ballester, C. Nembrini, N. Dhar, A. de Titta, C. de Piano, M. Pasquier, E. Simeoni, A.J. van der Vlies, J.D. McKinney, J.A. Hubbell, M.A. Swartz, Nanoparticle conjugation and pulmonary delivery enhance the protective efficacy of Ag85B and CpG against tuberculosis, *Vaccine.* 29 (2011) 6959–6966.
- [15] J.J. Moon, H. Suh, A. V Li, C.F. Ockenhouse, A. Yadava, D.J. Irvine, Enhancing humoral responses to a malaria antigen with nanoparticle vaccines that expand Tfh cells and promote germinal center induction, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 1080–1085.
- [16] A. V. Li, J.J. Moon, W. Abraham, H. Suh, J. Elkhader, M.A. Seidman, M. Yen, E.-J. Im, M.H. Foley, D.H. Barouch, D.J. Irvine, Generation of effector memory T cell-based mucosal and systemic immunity with pulmonary nanoparticle vaccination., *Sci. Transl. Med.* 5 (2013) 204ra130.
- [17] D.J. Irvine, M.C. Hanson, K. Rakhra, T. Tokatlian, Synthetic Nanoparticles for Vaccines and Immunotherapy, 115 (2015) 11109–11146.
- [18] A. Rehor, J.A. Hubbell, N. Tirelli, Oxidation-sensitive polymeric nanoparticles, *Langmuir.* 21 (2005) 411–417.
- [19] A.J. Van Der Vlies, C.P. Oneil, U. Hasegawa, N. Hammond, J.A. Hubbell,

- 1 Synthesis of pyridyl disulfide-functionalized nanoparticles for conjugating thiol-
2 containing small molecules, peptides, and proteins, *Bioconjug. Chem.* 21
3 (2010) 653–662.
- 4 [20] A. Stano, E.A. Scott, K.Y. Dane, M.A. Swartz, J.A. Hubbell, Tunable T cell
5 immunity towards a protein antigen using polymersomes vs. solid-core
6 nanoparticles., *Biomaterials.* 34 (2013) 4339–4346.
- 7 [21] I. Routing, S. Burgdorf, A. Kautz, V. Böhnert, P.A. Knolle, C. Kurts, Distinct
8 pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell
9 activation. *Science.* 21 (2007) 612–617.
- 10 [22] V. Manolova, A. Flace, M. Bauer, K. Schwarz, P. Saudan, M.F. Bachmann,
11 Nanoparticles target distinct dendritic cell populations according to their size,
12 *Eur. J. Immunol.* 38 (2008) 1404–1413.
- 13 [23] A. Mildner, S. Jung, Development and function of dendritic cell subsets,
14 *Immunity.* 40 (2014) 642–656.
- 15 [24] M. Sixt, N. Kanazawa, M. Selg, T. Samson, G. Roos, D.P. Reinhardt, R. Pabst,
16 M.B. Lutz, L. Sorokin, The conduit system transports soluble antigens from the
17 afferent lymph to resident dendritic cells in the T cell area of the lymph node,
18 *Immunity.* 22 (2005) 19–29.
- 19 [25] M.Y. Gerner, W. Kastenmuller, I. Ifrim, J. Kabat, R.N. Germain, Histo-
20 cytometry: A method for highly multiplex quantitative tissue imaging analysis
21 applied to dendritic cell subset microanatomy in lymph nodes, *Immunity.* 37
22 (2012) 364–376.
- 23 [26] M.Y. Gerner, P. Torabi-Parizi, R.N. Germain, Strategically Localized Dendritic
24 Cells Promote Rapid T Cell Responses to Lymph-Borne Particulate Antigens,
25 *Immunity.* 42 (2015) 172–185.
- 26 [27] E.J. Allenspach, M.P. Lemos, P.M. Porrett, L.A. Turka, T.M. Laufer, Migratory
27 and Lymphoid-Resident Dendritic Cells Cooperate to Efficiently Prime Naive
28 CD4 T cells, *Immunity.* 29 (2008) 795–806.
- 29 [28] K. Shortman, W.R. Heath, The CD8+ dendritic cell subset, *Immunol. Rev.* 234
30 (2010) 18–31.
- 31 [29] M.B. Lutz, N. Kukutsch, A.L. Ogilvie, S. Rössner, F. Koch, N. Romani, G.
32 Schuler, An advanced culture method for generating large quantities of highly
33 pure dendritic cells from mouse bone marrow., *J. Immunol. Methods.* 223
34 (1999) 77–92.
- 35 [30] M.A.S. Broggi, M. Schmalzer, N. Lagarde, S.W. Rossi, Isolation of Murine
36 Lymph Node Stromal Cells, *JOVE*, (2014) e51803.
- 37 [31] R.A. Petros, J.M. DeSimone, Strategies in the design of nanoparticles for
38 therapeutic applications., *Nat. Rev. Drug Discov.* 9 (2010) 615–627.
- 39 [32] S. Burgdorf, V. Lukacs-kornek, C. Kurts, The mannose receptor mediates
40 uptake of soluble but not of cell-associated antigen for crosspresentation, *J.*
41 *Immunol.* 176 (2006) 6770–6776.
- 42 [33] J.M. Vyas, A.G. Van der Veen, H.L. Ploegh, The known unknowns of antigen
43 processing and presentation., *Nat. Rev. Immunol.* 8 (2008) 607–618.
- 44 [34] J. Vollmer, H. Bellert, In Vitro Effects of Adjuvants on B Cells., *Methods Mol*
45 *Biol.* 626 (2010) 131-148
- 46 [35] R.S. Allan, J. Waithman, S. Bedoui, C.M. Jones, J.A. Villadangos, Y. Zhan,
47 A.M. Lew, K. Shortman, W.R. Heath, F.R. Carbone, Migratory dendritic cells
48 transfer antigen to a lymph node-resident dendritic cell population for efficient
49 CTL priming., *Immunity.* 25 (2006) 153–162.
- 50 [36] A.A. Itano, S.J. McSorley, R.L. Reinhardt, B.D. Ehst, E. Ingulli, A.Y. Rudensky,
51 M.K. Jenkins, Distinct dendritic cell populations sequentially present antigen to
52 CD4 T cells and stimulate different aspects of cell-mediated immunity,
53 *Immunity.* 19 (2003) 47–57.
- 54 [37] G. Iezzi, A. Fröhlich, B. Ernst, F. Ampenberger, S. Saeland, N. Glaichenhaus,
55 M. Kopf, Lymph node resident rather than skin-derived dendritic cells initiate

- specific T cell responses after *Leishmania major* infection., *J. Immunol.* 177 (2006) 1250–1256.
- [38] V. Cerovic, S.A. Houston, J. Westlund, L. Utriainen, E.S. Davison, C.L. Scott, C.C. Bain, T. Joeris, W.W. Agace, R.A. Kroczeck, A.M. Mowat, U. Yrlid, S.W. Milling, Lymph-borne CD8 α (+) dendritic cells are uniquely able to cross-prime CD8(+) T cells with antigen acquired from intestinal epithelial cells., *Mucosal Immunol.* 8 (2015) 38–48.
- [39] W.R. Heath, F.R. Carbone, The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells., *Nat. Immunol.* 14 (2013) 978–985.
- [40] W.R. Heath, F.R. Carbone, Dendritic cell subsets in primary and secondary T cell responses at body surfaces., *Nat. Immunol.* 10 (2009) 1237–1244.
- [41] C. Jakubzick, M. Bogunovic, A.J. Bonito, E.L. Kuan, M. Merad, G.J. Randolph, Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes., *J. Exp. Med.* 205 (2008) 2839–2850.
- [42] S. Hirosue, J. Dubrot, Modes of antigen presentation by lymph node stromal cells and their immunological implications, 6 (2015).
- [43] S.J. Turley, A.L. Fletcher, K.G. Elpek, The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs., *Nat. Rev. Immunol.* 10 (2010) 813–825.
- [44] A. Link, T.K. Vogt, S. Favre, M.R. Britschgi, H. Acha-Orbea, B. Hinz, J.G. Cyster, S. a Luther, Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells., *Nat. Immunol.* 8 (2007) 1255–1265.
- [45] S. Crotty, Follicular helper CD4 T cells (TFH), *Annu. Rev. Immunol.* 29 (2011) 621–663.
- [46] D. Baumjohann, S. Preite, A. Reboldi, F. Ronchi, K.M. Ansel, A. Lanzavecchia, F. Sallusto, Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype., *Immunity.* 38 (2013) 596–605.
- [47] M.K. Slifka, R. Antia, J.K. Whitmire, R. Ahmed, Humoral immunity due to long-lived plasma cells, *Immunity.* 8 (1998) 363–372.
- [48] M.F. Bachmann, G.T. Jennings, Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns., *Nat. Rev. Immunol.* 10 (2010) 787–796.
- [49] L. Kou, J. Sun, Y. Zhai, Z. He, The endocytosis and intracellular fate of nanomedicines: Implication for rational design, *Asian J. Pharm. Sci.* 8 (2013) 1–10.
- [50] H. Herd, N. Daum, A.T. Jones, H. Huwer, H. Ghandehari, C.M. Lehr, Nanoparticle Geometry and Surface Orientation Influences Mode of Cellular Uptake, *ACS Nano.* 7 (2013) 1961–1973.
- [51] H. Kim, T. Uto, T. Akagi, M. Baba, M. Akashi, Amphiphilic poly(amino acid) nanoparticles induce size-dependent dendritic cell maturation, *Adv. Funct. Mater.* 20 (2010) 3925–3931.
- [52] I.C. Kourtis, S. Hirosue, A. de Titta, S. Kontos, T. Stegmann, J.A. Hubbell, M.A. Swartz, Peripherally Administered Nanoparticles Target Monocytic Myeloid Cells, Secondary Lymphoid Organs and Tumors in Mice, *PLoS One.* 8 (2013) e61646.
- [53] S.T. Reddy, A.J. van der Vlies, E. Simeoni, C.P. O’Neil, M.A. Swartz, J.A. Hubbell, Exploiting lymphatic transport and complement activation in nanoparticle vaccines, *Eur. Cells Mater.* 14 (2007) 103.
- [54] P. Cresswell, Antigen processing and presentation., *Immunol. Rev.* 207 (2005) 5–7.
- [55] O.P. Joffre, E. Segura, A. Savina, S. Amigorena, Cross-presentation by dendritic cells, *Nat. Rev. Immunol.* 12 (2012) 557–569.
- [56] C.D. Austin, X. Wen, L. Gazzard, C. Nelson, R.H. Scheller, S.J. Scales, Oxidizing potential of endosomes and lysosomes limits intracellular cleavage of disulfide-based antibody-drug conjugates., *Proc. Natl. Acad. Sci. U. S. A.* 102

- (2005) 17987–17992.
- [57] L. Ohl, M. Mohaupt, N. Czeloth, G. Hintzen, Z. Kiafard, J. Zwirner, T. Blankenstein, G. Henning, R. Förster, CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions, *Immunity*. 21 (2004) 279–288.
- [58] A. Ballesteros-Tato, T.D. Randall, Priming of T follicular helper cells by dendritic cells, *Immunol Cell Biol*. 92 (2014) 22–27.
- [59] C. Shin, J.A. Han, H. Koh, B. Choi, Y. Cho, H. Jeong, J.S. Ra, P.S. Sung, E.C. Shin, S. Ryu, Y. Do, CD8 α (-)Dendritic Cells Induce Antigen-Specific T Follicular Helper Cells Generating Efficient Humoral Immune Responses, *Cell Rep*. 11 (2017) 1929–1940.

FIGURES

Figure 1. NP-ss-OVA undergo endosomal escape upon internalization, while PS(OVA) traffic through lysosomes within BMDCs A-D) Confocal microscopy images of PS(OVA647) or NP-ss-OVA647 (red), and LAMP1 or EEA1 vesicles (AF488, green), after 2 h of nanocarrier incubation with BMDCs. Each image per group contains 1-3 representative cells. Nuclei are counterstained with Dapi (blue). Scale bars correspond to 10µm. E) NP647 vehicle (red) and LAMP1 vesicles (green). F) Percent of PS(OVA647) (n=10), NP-ss-OVA647 (n=10) or NP vehicle colocalization (n=5) with EEA1 or LAMP1 vesicles respectively. G) Relative cellular distribution of NP-ss-OVA647 or PS(OVA647) defined as the percentage colocalization with LAMP1 vesicles divided by the percentage colocalization with EEA1 vesicles. Data are representative of three independent experiments. Error bars represent the SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

COLOR FIGURE

Figure 2. NP-ss-OVA and PS(OVA) promote tunable T cell immunity *in vitro*. BMDCs were incubated with NP-ss-OVA, PS(OVA) or OVA [40 μ g/ml], adjuvanted with CpG-B [0.05 μ M], or left untreated. Cells were then co-cultured during three days with CFSE labeled CD8⁺ OTI or CD4⁺ OTII cells for assessment of cytokine secretion and proliferation. A) Representative FACS plots of IFN γ versus CFSE for OTI or OTII groups treated with PS(OVA) or NP-ss-OVA. B) Frequency of IFN γ ⁺ OTI CD8 T cells as measured by flow cytometry or C) concentration of IFN γ in the supernatant measured by ELISA. D) Frequency of IFN γ ⁺ OTI CD8 T cells vs generation of cell division. E) Percent of activated OTI cells (CD44⁺CD25⁺) assessed by flow cytometry. F) Frequency of IFN γ ⁺ OTII CD4 T cells as measured by flow cytometry or G) concentration of IFN γ in the supernatant measured by ELISA. H) Frequency of IFN γ ⁺ OTII CD4 T cells vs generation of cell division. I) Percent of activated OTII cells (CD44⁺CD25⁺) assessed by flow cytometry. Data is representative of four independent experiments with at least n=3 per group. Error bars represent the SEM. * p < 0.05; ** p < 0.01.

Figure 3. NP-ss-OVA is homogenously distributed between LN resident CD11b⁺ and CD8⁺ DCs, while PS(OVA) display preferential association with CD11b⁺ DCs and enhance the upregulation of MHCII. A) Immunization timeline and vaccination groups. B) Cell numbers of draining lymph nodes of NP-ss-OVA₆₄₇ and PS(OVA₆₄₇) at 2 h and 36 h post immunization (p.i.). *Right*, cell numbers of OVA₆₄₇⁺ cells in draining lymph nodes at 2 h and 36 h immunization p.i.. C) *Left*, gating strategy DC subsets, *right*, percent of DC subsets within the OVA₆₄₇⁺ population at 2 h p.i.. D) *Left*, gating strategy of the lymph node resident DC subsets CD11b⁺DCs⁺ and CD8⁺DCs⁺. Numbers within flow cytometry plots indicate the percent of CD8⁺ DCs or CD11b⁺ DCs of the main resident DC gate. *Right*, cell numbers of CD8⁺ DCs and CD11b⁺ DCs that are associated with OVA₆₄₇ at 2 h p.i.. E) Cell numbers of CD8⁺ CD11b⁻ DCs and CD11b⁺CD8⁻ DCs that are associated with OVA₆₄₇ at 36 h p.i.. F) Mean fluorescence intensity of MHCII in the OVA₆₄₇⁺ DCs at 36 h p.i.. Data are representative of three independent experiments with at least n=3 per group. Error bars represent the SEM. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Figure 4. NP-ss-OVA associate with fibroblastic reticular cells and lymphatic endothelial cells at early times, while PS(OVA) associates almost exclusively with lymphatic endothelial cells. A) Cell numbers of CD45⁺ OVA₆₄₇⁺ cells at 2 h and 36 h post immunization (p.i.). B) *Left*, flow cytometry analysis of CD45⁻ stromal cells within the OVA₆₄₇⁺ population based on the expression of podoplanin (gp38) and CD31. Stromal cells were classified as LECs (gp38⁺ CD31⁺), FRCs (gp38⁺ CD31⁻), BECs (gp38⁻ CD31⁺) and DN cells (gp38⁻ CD31⁻). *Right*, percent of stromal cell subsets in the OVA₆₄₇⁺ population at 2 h and 3 h p.i.. Data are representative of three independent experiments with at least n=3 per timepoint. Error bars represent the SEM. * $p < 0.05$.

Figure 5. PS(OVA) immunization enhances the induction of T follicular helper (Tfh) CD4 T cells, germinal center cells and plasma cells. A) Immunization timeline and vaccination groups. B) Cell counts of CD45.1⁺ OTII cells in lymph nodes and splenocytes 9 days after i.d. immunization with PS(OVA), NP-ss-OVA and soluble OVA. C) Activation phenotype of all CD4 T cells (endogenous + adoptively transferred CD4 T cells) in draining lymph nodes and spleen. D) Identification and quantification of Tfh CD4 T cells by flow cytometry. E-F) *Left*, representative flow cytometry plots displaying germinal center (GC) B cells (upper) and plasma cells (lower) 9 days after immunization with PS (OVA), NP-ss-OVA and soluble OVA. *Right*, Quantification of GCs and plasma cells within the B220⁺ B cell population. Data are representative of two independent experiments with n=5 per group. Error bars represent the SEM. * $p < 0.05$; ** $p < 0.01$.