Published in final edited form as:

J Immunol Methods. 2010 April 30; 356(1-2): 47–59. doi:10.1016/j.jim.2010.02.009.

Using Carbon Magnetic Nanoparticles to Target, Track, and Manipulate Dendritic Cells

Heidi A. Schreiber^{a,b,d}, Jozsef Prechl^{a,d}, Hongquan Jiang^c, Alla Zozulya^a, Zsuzsanna Fabry^a, Ferencz Denes^{c,e}, and Matyas Sandor^{a,e}

Heidi A. Schreiber: hschreiber@wisc.edu; Jozsef Prechl: jprechl@yahoo.co.uk; Hongquan Jiang: hjiang@uta.edu; Alla Zozulya: alzozyula@gmail.com; Zsuzsanna Fabry: zfabry@wisc.edu; Ferencz Denes: denes@engr.wisc.edu; Matyas Sandor: msandor@wisc.edu

^aUniversity of Wisconsin Department of Pathology and Laboratory Sciences, 1300 University Avenue, Madison, Wisconsin 53705, USA.

^bCellular and Molecular Pathology Training Program.

^cUniversity of Wisconsin Center for Plasma-Aided Manufacturing, 1410 Engineering Drive, Madison, Wisconsin 53706, USA.

Abstract

Dendritic cells (DCs) are crucial in the initiation of immune responses and are primary targets in vaccination. Here, we describe fluorescent, carbon magnetic nanoparticles (CMNPs) within the 20–80nm size range that are non-toxic and preferentially endocytosed by DCs. These attributes allow for DC tracing *in vitro*, *ex vivo* and *in vivo*, by both fluorescence and MRI. We show that CMNPs conjugated with an array of proteins are able to induce strong immune responses in mice. The addition of TLR ligand, CpG, to the CMNPs along with protein results in both T cell activation, but also a selective IFN γ response. The magnetism afforded by the CMNPs facilitates a simple DC enrichment *ex vivo* by magnetic means from both secondary lymphoid organs, and sites of chronic inflammation. The magnetic and fluorescent properties of the CMNPs allow for visualization, recovery, and potentially the facilitation of directed DC migration. These particles may support more efficient immunization protocols or new diagnostic assays to characterize functionalities of DCs from patients.

Keywords

Dendritic cell targeting; nanoparticles; vaccine/immunization; Cellular tracing; trafficking; enrichment

1. INTRODUCTION

Both vaccine strategies and therapy towards chronic disease require efficient delivery of antigen to dendritic cells (DCs). DCs, a rare population of white blood cells, play a central role

© 2010 Elsevier B.V. All rights reserved.

Correspondence, Matyas Sandor, 5468 MSC, 1300 University Ave, Madison, WI 53705, Phone: (608) 265-8715, Fax: (608) 262-0846, msandor@wisc.edu.

^dThese authors contributed equally to this work.

^eCo-senior authors

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

in the initiation of immune responses as the only antigen presenting cell (APC) capable of both activating naïve T cells and efficiently initiating a recall T cell response (Banchereau and Steinman, 1998). Based on various environmental cues, the DC can selectively promote these T cells to differentiate into different T cell committed lineages (Kapsenberg, 2003). This ability has made DCs central to immune responses generated in the context of infectious disease, cancer, autoimmunity, transplantation, and allergy (Steinman and Banchereau, 2007). Despite their low frequency during steady-state conditions, DCs can be found in most tissues as tissue-resident DCs, as well as in lymphoid organs (Guermonprez et al., 2002). The primary role of tissue-resident DCs is to sample antigen, mature, and traffic to lymph nodes to initiate a T cell response. It has been suggested that the size of the immunizing antigen affects which APC prefers to sample and process it (Xiang et al., 2006). Bacterial-sized ($> 1 \mu\text{m}$) uptake is favored by macrophages, while viral-sized ($< 100 \text{ nm}$) particles are preferentially phagocytosed by dendritic cells (Fifis et al., 2004a). Additionally, unlike the larger particles, nano-sized particles can efficiently migrate through the lymphatics to reach DCs residing in the lymph nodes (Reddy et al., 2007). Nanoparticle (NP) traffic through the lymphatics further supports the role of DCs as the preferred sampler of nano-sized particles, as DCs utilize the lymphatics as their favored route to the lymph nodes. Targeting DCs as a method of vaccination is a strategy that has been gaining increasing interest (Reddy et al., 2006b; Steinman and Banchereau, 2007; Tacke et al., 2007). An ideal DC-targeting NP would harbor all of the following functionalities together: DC-preferential uptake, antigen and adjuvant conjugation capabilities, *in vivo* imaging, targeted delivery and biocompatibility. Here, we describe the generation of a NP that possesses all of these functionalities. Using Dense Medium Plasma (DMP) technology we have synthesized carbon-based magnetic nanoparticles (CMNPs) by sustaining benzene between two iron electrodes, as previously described in detail (Lee et al., 2007). Unlike many nanoparticle targeting vectors used today, which typically have a polymer matrix of unevenly distributed iron oxides around a metal core, the CMNPs generated by DMP have an even dispersion of iron and iron oxide particles throughout. Furthermore, DMP technology affords the efficient immobilization of liquid phase material onto the surface of the CMNPs, in contrast to the polymerization required by most preexisting nanoparticle delivery systems. Previous studies have demonstrated the effective immobilization of the chemotherapeutic drug, doxorubicin, to these CMNPs, and the subsequent reactivity of the drug on tumor cells (Ma et al., 2004). In this study we demonstrate the immobilization of an array of proteins to the CMNPs and the ease of their detection, both *in vivo* using MRI, and *in vitro* and *ex vivo* with fluorescent detection methods. We further show proficient uptake of the CMNPs and activation of T cells by DCs, both *in vitro* and *in vivo*. The combined ability of CMNPs to reach sites of inflammation, provide a tool for tracing and enrichment, along with the possibility to manipulate their migration *in vivo*, suggests that these CMNPs may provide an affordable, easy, stable method of vaccination and drug delivery.

2. MATERIALS AND METHODS

2.1 PREPARATION OF CMNPS

A detailed description of CMNP preparation was previously described (Ma et al., 2004). Protein conjugation to the CMNPs was done as follows. A 5% protein/CMNP mixture was prepared, Fluorochrome-Avidin-0.6mg:12mg CMNP, purified hen egg lysozyme (HEL) protein, or rat IgG (Avidin-FITC and HEL (Sigma-Aldrich, St. Louis, MO, USA) and Avidin-PE from (Biomed, Foster City, CA, USA) and diluted in a 1mL volume using deionized water. The solution was then sonicated using a UTR200 ultrasound bath (Hielscher USA Inc., Ringwood, NJ, USA) under the following operating parameters: amplitude 60% and cycle 0.5 for 3 min.

Avidin/CMNP complex was vacuum-dried for two days. The dried protein-conjugated CMNPs were resuspended in PBS. 5 minutes prior to use, CMNPs were sonicated in a Ultrasonic

Homogenizer on high for 5 minutes to break up aggregates (Biologics, Inc. Manassas, VA, USA). CMNPs were both dilute and in a 10% Fetal Calf Serum media to help prevent significant agglomeration (Buford et al., 2007). NanoLink Streptavidin Magnetic Microspheres were purchased from (San Diego, CA, USA) and processed according to manufactures protocol.

2.2 BIOTINYLATION OF HEL AND CPG

HEL protein was biotinylated with Biotin-N-hydroxysuccinimide ester (MP Biomedicals Inc. Solon, OH, USA) as follows. HEL protein was reconstituted at 1mg/mL and 1M sodium bicarb solution was added at a 1:10 dilution. 100ug of 1mg/mL Biotin in DMSO was added to HEL protein in DMSO, and incubated at room temperature for 2 hours. Biotin-labeled HEL was dialyzed against PBS for 24 hours with 2 PBS changes. CPG-C oligo was ordered from Integrated DNA Technologies (Coralville, IA, USA). Sequence 5'-TCG TCG TCG TTC GAA CGA CGT TGA T-3' with Biotin.

2.3 MICE

Wild type B10.BR (I-A^k) and 3A9 TCR transgenic mice (Ho et al., 1994) specific for HEL residues 46–61 in the context of I-A^k were purchased from the Jackson Laboratory. CD11c-EYFP transgenic mice on the C57BL/6 background were a generous gift from Dr. Michel C. Nussenzweig (Rockefeller University, NY) (Lindquist et al., 2004). Mice were housed in a pathogen-free facility at the University of Wisconsin Animal Care Unit, according to the guidelines of the Institutional Animal Care and Use Committee.

2.4 CELL ISOLATION AND BONE MARROW-DERIVED DC CULTURE

Isolation of splenocytes, lymph nodes, and granuloma-infiltrating cells was performed as previously described (Hogan et al., 2001). Generation of Bone marrow-derived DC (BMDC) culture was performed as previously described (Zozulya et al., 2007).

2.5 FLOW CYTOMETRY

A total of 10⁶ cells were incubated for 30 min on ice with saturating concentrations of labeled antibodies (Abs) and 40 ug/ml unlabeled 2.4G2 mAb to block binding to Fc receptors, and washed 3 times with staining buffer (PBS plus 1% BSA). Fluorochrome-labeled Abs against CD4 (RM5-4), LFA-1 (7D4), CD69 (H1.2F3), CD62L (MEL-14), LFA-1 (2D7), IFN γ (XMG1.2), along with streptavidin-allophycocyanin, and streptavidin-PE conjugates, were purchased from BD Pharmingen. Anti-CD205 (NLDC-145) and I-A^k (10-3.6) were purified from hybridoma cell supernatant (American Type Culture Collection, Manassas, VA, USA). Anti-3A9 clonotypic Ab (1G12) hybridoma was a gift from P. Allen (Washington University, St. Louis, MO). Cell surface staining was acquired on a FACSCalibur or LSRII (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo (Tree Star, Ashland, OR, USA) software version 5.4.5.

2.6 MICROSCOPY AND IMAGING

2.6.1 IMMUNOFLOURESCENT—Cryosections (5- μ m thick) were cut from tissue embedded in O.C.T Compound (Tissue-Tek Sakura, Torrance, CA, USA) and fixed for 10 minutes in ice cold acetone. Sections were washed 3 times using TBS + 1% BSA and either cover-slipped with Gel/Mount (Biomedex, Foster City, CA, USA) or mounted using ProLong Gold anti-fade reagent with DAPI (Molecular Probes, Carlsbad, CA, USA). Images were acquired on a Bio-Rad MRC-1024 maintained by the W. M. Keck Laboratory for Biological Imaging (University of Wisconsin, Madison, WI), and an Olympus BX40 microscope (Olympus America Inc., Melville, NY, USA) fit with an Olympus Qcolor 3 camera (Olympus America Inc., Melville, NY, USA). The acquired digital images were processed and analyzed using Photoshop CS software (Adobe Systems, San Jose, CA, USA).

2.6.2 SCANNING AND TRANSMISSION ELECTRON MICROSCOPY—SEM images were taken as previously described (Zozulya et al., 2007). TEM images of CMNPs were generated as described. Several TEM gold grids were dipped into a nanoparticle water-suspension for several seconds, the substrates were removed from the suspension and dried under open laboratory conditions. TEM images of CMNPs were generated using a Philips CM200UT TEM.

2.6.3 MRI—MRI images were taken on a whole body horizontal bore imaging Varian 4.7T MRI at the UWCCC Small Animal Imaging Facility (University of Wisconsin, Madison, WI). Mice were anesthetized by ketamine injection and injected with a 100 μ L of 10% CMNP in PBS with 10% FCS i.v. into the tail vein immediately prior to MRI scans.

2.6.4 FERROMAGNETIC RESONANCE SPECTROSCOPY—The experimental parameters for the acquisition of Ferromagnetic Resonance Spectroscopy spectrum of dried C/Fe nanoparticles and data interpretation were done as previously described (Denes et al., 2003).

2.7 INFECTION

Growth and preparation of BCG Pasteur strain (Staten Serum Insititute, Copenhagen, Denmark) was performed as previously described (Hogan et al., 2001). For systemic infection, a non-lethal dose of 1×10^7 CFU dsRED BCG was i.p. injected (dsRED BCG plasmid from Dr. Lalita Ramakrishnan (University of Washington, WA)).

2.8 MAGNETIC ISOLATION

In vitro enrichment for CMNP-containing cells was achieved by exposing a CMNP-pulsed BMDC culture to a Dynal magnet (Invitrogen, Carlsbad, CA, USA). Supernatant was decanted, and magnet-bound cells were washed with PBS and stained. A fraction of cells were collected for staining prior to magnet exposure. Cells were stained with antibodies against DEC205 (NLDC-145) and I-A^k (10-3.6) and analyzed by flow cytometry. To enrich for CMNP-containing cells from the granuloma, mice were i.v. injected with 160 μ g of FITC-CMNPs 5 days prior to a 3 week BCG infection time point. Granuloma infiltrating cells were passed through MACS® Large Cell Separation Columns' (Miltenyi Biotec, Auburn, CA, USA). Columns were used following manufactures protocol. Magnetically enriched cells were washed and prepared for flow cytometry.

2.9 IN VIVO CMNP AND DC TRAFFIC WITH MAGNETIC FIELD

Following anesthesia by ketamine injection, a neodymium ringed-magnet or N52 grade neodymium small round magnet (Applied Magnets, Plano, TX, USA) was positioned to encompass both or juxtaposed to left inguinal lymph node, respectively. For CMNP injection alone, a 3% solution of PE-CMNPs in PBS with 10% FCS was sonicated on high for 5 minutes to break up aggregates. Each anesthetized mouse received 300 μ L of PE-CMNP solution within 5 minutes of sonication. Thirty minutes later inguinal and cervical lymph nodes were pooled and stained with anti-CD11c, and analyzed by flow cytometry for DC accumulation and expression of PE from CMNPs. For injection of NP-loaded DCs, BMDC cultures from CD11c-EYFP mice were generated, and pulsed with 200 μ g per 3.5×10^6 BMDCs of NanoLink particles and 2.5 μ g/mL LPS over night. Bead-containing BMDCs were enriched using a Dynal Magnetic Particle Concentrator (Invitrogen, Carlsbad, CA, USA) and washed three times with sterile PBS prior to injection. 1×10^6 Bead-containing BMDCs in 100 μ L PBS was s.c. injected at the base of the tail. Three hours later inguinal lymph nodes, cervical nodes, and spleen were removed and analyzed by flow cytometry for the presence of CD11c-EYFP cells or fixed over

night in 3% formalin/25% sucrose in PBS prior to freezing down in O.C.T Compound (Tissue-Tek Sakura, Torrance, CA, USA).

2.10 *IN VITRO* AND *IN VIVO* T CELL ACTIVATION

For *in vitro* T cell activation assays, 1×10^6 splenocytes from a HEL-specific Tg mouse (3A9) was plated in a 96-well plate in a 200 μ l volume of complete RPMI + 10% FBS. Biotinylated HEL, HEL conjugated CMNPs or unconjugated CMNPs were added in serial dilutions and incubated for 18hrs at 37°C + 5% CO₂. Purified HEL protein was obtained from Sigma-Aldrich. Adherent cells were dissociated by adding 20 μ l of 20mM EDTA in PBS for 15min, collected, washed and stained with 3A9 clonotypic antibody (1G12), CD69 (H1.2F3), CD62L (MEL-14) and CD4 (RM5-4) for 30 minutes on ice. Cells were washed and ran on a FACSCalibur or LSRII (BD Biosciences, San Jose, CA) and analyzed with FlowJo (Tree Star) software version 5.4.5. For *in vitro* differentiation assay, 1×10^6 splenocytes from a HEL-specific Tg mouse (3A9) were plated in a 96-well plate in a 200 μ l volume of complete RPMI + 10% FBS. Media, 0.2 μ g HEL alone, 20ng of only CpG bound to CMNPs, 0.2 μ g of HEL bound to CMNPs, or 20ng CpG and 0.2 μ g HEL both bound to CMNPs were added to wells and incubated for 3 days. For intracellular cytokine staining, culture was supplemented with GolgiStop (BD Biosciences) in the presence of anti-CD3 Ab for 5 h. After surface staining with antibodies against LFA-1, CD4, and 3A9 clonotypic antibody (1G12), cell suspensions were fixed and permeabilized by Cytotfix/Cytoperm solution (BD Biosciences), followed by staining with anti-IFN γ Ab. Cell staining was acquired using an LSRII (BD Biosciences, San Jose, CA) and analyzed with FlowJo. *In vivo* activation was performed by CFSE (2.5 μ M; Molecular Probes) labeling 3A9 lymph node cells and injecting 500,000 Tg CD4⁺ T cells retroorbitally into wild-type mice. Mice were immunized one day later s.c. at the base of the tail with either 10 μ g of HEL bound to CMNPs or 10 μ g HEL and CpG bound to CMNPs. Six days later inguinal lymph nodes were collected, homogenized and placed in culture for recall and intracellular cytokine staining, as previously mentioned.

3. RESULTS

3.1 Engineered carbon-based nanoparticles are magnetic, and facilitate the conjugation of an array of proteins and tracers

These CMNPs are carbon-based, have an irregular, crystalline-like surface, and are 20–80 nm in size (Fig. 1A). The magnetism was confirmed by ferromagnetic resonance spectroscopy (FMR), which also suggested that the magnetism was due to metallic iron (~0.1%) (Fig. 1B). The large surface area of the CMNPs, along with the plasma-functionalized carboxyl or amino surface, allows for high-capacity binding of proteins. Single or multiple proteins can be conjugated by ester bonds, potentially displaying a complex array of proteins. Figure 1C demonstrates the efficient binding of both antigen, hen egg lysozyme (HEL), and monoclonal IgG proteins. Binding of avidin-FITC (or other fluorescent avidins) allows both for fluorescent monitoring of the particles and the indirect binding of biotinylated proteins to the CMNPs (Fig. 1D). This characterization demonstrates their large surface area, fluorescence, magnetism and ability to present a wide array of proteins.

3.2 CMNPs are preferentially taken up by DCs *in vitro*

To test if CMNPs are preferentially endocytosed by DCs, we fed bone marrow-derived cell cultures, containing both macrophage and DCs, with CMNPs. APCs were distinguished by morphology and cell surface expression of DEC205, CD11c, CD11b and MHCII. By light microscopy and scanning electron microscopy (SEM), we observed that, *in vitro*, DC preferentially endocytose CMNPs (Fig. 2 upper left panel). Importantly, transmission electron microscopy (TEM) images demonstrate that CMNPs within the DCs are contained within endocytic vesicles (Fig. 2 upper right and middle panels), cellular compartments that are

associated with loading antigen-presenting molecules. It is important to note that total dispersion of CMNPs is difficult to achieve under all biological settings. Therefore, prior to *in vitro* culture or *in vivo* injection, CMNPs are suspended in as dilute volume as possible that contains 10% Fetal Calf Serum to aid against agglomeration (Buford et al., 2007). CMNPs are always sonicated within 5 minutes prior to use. The ability to conjugate fluorochromes to the CMNPs demonstrated in Figure 1D enables us to monitor their uptake by DCs (Figure 2 lower panels (both FITC and PE fluorochromes, respectively shown)). The selective uptake and tracing ability alone, make these CMNPs a promising vector for DC targeting.

3.3 Targeting of CMNPs to DCs can be enhanced by conjugation of monoclonal antibodies

To test whether if the selectivity and efficiency of DC uptake of CMNPs could be further promoted, we conjugated anti-DEC205, a DC-specific monoclonal antibody, to the particles along with HEL protein. These dual-conjugated particles, along with CMNPs with HEL alone were pulsed over night in a BMDC culture to test possible differences in uptake. Observation using light microscopy revealed that DEC205/HEL-conjugated CMNPs were endocytosed more readily compared to CMNPs conjugated with HEL alone (Fig. 3A). When quantified, the CMNPs containing both DC-specific antibody and HEL were taken up twice as much compared to particles with only HEL on their surface (Fig. 3B). Therefore, not only does the size of these CMNPs favor DC uptake, but their surface enables the conjugation of an array of proteins that can effectively enhance this cell-specific targeting.

3.4 CMNPs traffic to primary and secondary lymphoid organs in vivo

In order for these CMNPs to be used as a vaccine or therapeutic DC targeting vector, we needed to ensure that they were able to reach various organs. To verify this, mice were i.v. injected with CMNPs and placed in a 4.7T small animal MRI 15 and 75 minutes post injection (Fig. 4A). CMNPs rapidly accumulated in the spleen, followed by kidneys and inguinal lymph nodes. Early accumulation in lymph nodes is necessary for many effective immunizations. Additionally, the ability to detect these CMNPs with MRI makes them a possible image-enhancing agent, allowing noninvasive, easy detection of target cell accumulation. It is also important to note that the CMNPs do not induce inflammation or toxicity following injection. All extracellular particles are cleared after one week (Fig. 4B), demonstrating that the CMNPs are neither toxic nor inflammatory.

3.5 CMNPs preferentially target DCs in vivo and provide a method of DC enrichment

Due to the magnetism of the CMNPs, confirmed in both Figure 1B and Figure 4A, we tested whether these particles could provide an approach for DC enrichment. By using a Dynal magnet, we were able to enrich DCs (MHCII+DEC205+) from a bone marrow culture containing both DCs and macrophage by 6-fold (Fig. 5A). To test whether these CMNPs could also preferentially target DCs *in vivo*, mice were i.v. injected with FITC-CMNPs. Flow cytometric analysis of the spleen demonstrates that FITC+ cells are entirely CD11c+CD11b-, suggesting their ability to selectively target DCs *in vivo* (Fig. 5B). In addition to the spleen CMNPs can also target DCs in often difficult to reach locations, such as sites of chronic inflammation (Fig. 5C). We infected mice with 1×10^7 cfu of dsRed Bacillus Calmette-guerin (BCG), a mycobacteria bovis strain, and 5 days prior to the 3-week acute-infection time point i.v. injected FITC-CMNPs. At 3 weeks post infection fluorescent microscopy of the liver revealed the presence of FITC-CMNPs the primary site of inflammation, the granuloma (Fig. 5C). To test whether CMNP-targeted DCs within these inflammatory lesions could be enriched, liver granuloma cells were isolated and passed through a magnetic separation column (Fig. 5D). Staining with anti-CD11c antibody on both positive and negative fractions shows a dramatic enrichment of CD11c+ cells in the positive fraction. The increased prevalence of CD11c⁻NP-FITC⁺ cells in the positive fraction are likely macrophage, due to the high

predominance of them within granulomas. It is likely that upon CMNP aggregation within the granuloma, the increased size of the aggregates selects for macrophage uptake. Collectively, CMNPs provide a way to target DCs *in vivo* in lymphoid organs and in chronic sites of inflammation, such as granulomas. In addition, their magnetic properties provide a way of isolating DCs back from these different locations. This ability may provide a very useful tool, as it enables us to phenotype a variety of DC populations, test DC functionalities, and identify antigens selected for presentation in such locations.

3.6 Magnetic nanoparticles potentially facilitate directed DC migration using an external magnet

A study by Dames *et al.* has shown that aerosols containing magnetic nanoparticles can be guided to specific regions in a murine lung with the application of an external magnetic field (Dames et al., 2007b). In this study, *in vitro* and *in vivo* uptake of FITC-CMNPs by DCs permitted the DCs to become magnetic when exposed to either a magnet, or filtered through a magnetic separation column *ex vivo*. Using these newly acquired features of the DCs, we tested if *in vivo* trafficking of FITC-CMNP-loaded DCs could be altered by an external magnetic field. To test this, FITC-CMNPs were i.v. injected into anesthetized mice and a ringed neodymium magnet was positioned to encompass the inguinal lymph nodes for 30 minutes (Fig. 6A). Inguinal (magnet exposed) and cervical lymph nodes were removed and single cell suspensions were stained with anti-CD11c. This demonstrates that an exterior magnet was able to enrich PE-CMNPs in the neighboring, magnet-exposed lymph node. To give this technology a new approach, we next attempted to direct the migration of DCs loaded with CMNPs. To do so, we first mimicked the previous set up by injecting mature BMDCs pre-loaded with FITC-CMNPs i.v. into mice that had the same external ring-magnet placed around the inguinal lymph nodes. After 30 minutes the lymph nodes were isolated and analyzed by flow cytometry (*data not shown*). Unfortunately, we observed no DC accumulation in the magnet-exposed lymph nodes. Rather, the majority of the DCs appeared to accumulate in the spleen instead. Several repeats and variations in time, CMNP dose, and external magnet positioning yielded no selective DC accumulation. Since the said study by Dames *et al.* observed three times fewer nanoparticles in the magnet-exposed lung region when the distance of the magnet was moved from 1 to 2 mm away, we considered the possibility that our magnetic field was not optimal. The iron content of the CMNPs presented here is approximately 0.1%, to further test this proof of principle that DC migration can be manipulated with an external magnet, we tried NanoLink™ nanoparticles with a higher iron content and a slightly stronger N52 grade neodymium magnet (Figure 6B). A BMDC culture generated from CD11c enhanced yellow fluorescent protein (EYFP) transgenic mice (Lindquist et al., 2004), in which DCs are identified by their ubiquitous fluorescence was pulsed with NanoLink™. The NanoLink™ particles, though slightly larger than the CMNPs, were readily taken up by the DCs (Fig. 6B red arrows point to particles and YFP fluorescence of same cell). To avoid particle-loaded DC accumulation in the spleen observed when cells were previously injected i.v., we s.c. injected magnetically-enriched, particle-containing BMDCs into the base of the tail of a sleeping mouse that had an external magnet positioned juxtaposed to its left inguinal lymph node (Fig. 6B). The mice were left to sleep with the external magnet for three hours. Approximately half the cases, we observed CD11c-EYFP accumulation in the magnet-exposed inguinal lymph node compared to the opposite inguinal, cervical lymph nodes, and spleen (Fig. 6C). CD11c-EYFP cells containing NanoLink™ particles were easily observed in the magnet-exposed inguinal lymph node by fluorescent and light microscopy (Fig. 6D). Over a year of titrating different combinations of nanoparticles, magnet strength and position, etc., we have not found a consistent method to move DCs *in vivo*. However, despite the inconsistency, the times we were able to achieve selective enrichment suggests that the proof of principle methodology may be possible given intense examination of the mentioned parameters.

3.7 CMNPs deliver antigen and co-stimulation necessary for T cell activation, proliferation, and differentiation *in vitro* and *in vivo*

As previously described, the composition of the irregular, crystalline surface of the CMNPs allows for efficient binding of protein (Fig. 1C). We next tested whether the CMNPs can deliver specific antigen to DCs and subsequently activate their cognate T cells. Biotinylated HEL was bound to avidin-labeled CMNPs. Unbound protein was washed away and HEL-conjugated particles were incubated for 18 hours with splenocytes from a 3A9 HEL-specific (HEL₅₂₋₆₁) T cell receptor (TCR) transgenic mouse. Splenocytes, as opposed to a pure BMDC:T cell culture, were used to test the CMNPs targeting potential under natural DC concentrations. Activation was assessed using fluorochrome-labeled antibodies specific for the 3A9 clonotypic T cell population and early T cell activation markers, CD62L and CD69 (Fig. 7A). Flow cytometric analysis showed an expansion of the TCR-specific CD4⁺ T cell population and a shift from a naïve phenotype, CD62L^{high}CD69^{low}, to an activated phenotype, CD62L^{low}CD69^{high}, when cells were incubated with HEL-bound CMNPs compared to unbound particles. Moreover, when compared to soluble HEL protein, the CMNP-bound HEL resulted in higher expression of CD69 at the same concentrations of soluble HEL protein (Fig. 7B). A ten-fold higher concentration of soluble HEL protein was required to reach 50% activation compared to HEL-bound CMNPs, and considering that the binding of HEL to the CMNPs is not 100% efficient, this difference is likely much larger. This shows that CMNPs can induce an immune response using a lower antigen concentration. However, in order for many vaccines to become biologically effective, certain cues must be delivered to the DC in addition to the antigen to allow it to shape the immune response accordingly. In order to achieve selective Th1 differentiation, we bound biotin-tagged CpG oligos to the particles along with HEL protein, along with media, HEL, CpG-CMNP, and HEL-CMNPs controls (Fig. 7C). These were then incubated for 18 hours with splenocytes from a 3A9 mouse. Cells were washed and stimulated for an additional 5 hours with anti-CD3 in the presence of a protein transport inhibitor, and stained for intracellular accumulation of the cytokine IFN γ . Compared to the media and CpG-CMNP conditions, HEL alone and HEL-CMNPs were able to activate 3A9 cells, as shown by a shift to LFA-1 positive expression. However, the delivery of both HEL protein and CpG resulted in the DC favoring a proinflammatory T cell response, indicated by the production of IFN γ . We next tested if the CMNPs could induce an antigen-specific immune response *in vivo*. For this, 1×10^5 CFSE-labeled 3A9 TCR transgenic CD4⁺ T cells were adoptively transferred via retroorbital injection and one day later HEL alone conjugated CMNPs, or CpG with HEL CMNPs were subcutaneously injected into B10.BR mice at the base of the tail. Six days later cells were stimulated *ex vivo* and stained for intracellular cytokine IFN γ , and activation and CFSE dilution of the transferred cells was also assessed (Fig. 7C). High LFA-1 expression, along with CFSE divisions, indicated that all CMNPs bound with HEL were able to induce both activation and proliferation of the TCR-specific CD4⁺ T cell population (Fig. 7C upper plots). In accordance with the *in vitro* finding, the presence of CpG was necessary to achieve IFN γ production (Fig. 7C lower plots). These data suggest that conjugation of an array of both antigen and adjuvant to CMNPs is an efficient method of immunomodulation.

4. DISCUSSION

As previously mentioned, DCs play a vital role in the initiation and maintenance of adaptive immune responses. Targeting DCs as a method of both therapeutic and preventative vaccination is rapidly gaining interest. However, DC-targeting candidates that are reaching clinical trials are currently dominated by targeting antigen to DC surface receptors through the use of monoclonal antibodies. Despite the specificity this approach provides, some of its limitations include economic feasibility, the difficulty of including multiple antigens, the lack of a DC maturation stimuli, and restrictions on the type of immune response generated from

targeting a single receptor. In order to achieve maturation and activation in the said method, a stimulus requiring systemic administration that poses secondary, unwanted risks. The use of particulate biomaterials as a method of DC targeting overcomes several of these hurdles. Some of these biomaterials include, liposomes, polymer microparticles, particles made of latex, gold, silica or polystyrene, vesicles, micelles, etc (Reddy et al., 2006b; Combadiere and Mahe, 2008). An ideal particular biomaterial would have the ability to selectively target DCs, efficiently deliver antigen, have a feature of traceability, and provide the opportunity for *in vivo* manipulation.

As stated, one feature of nanoparticle vaccination becoming clearer is that size does matter (Kalkanidis et al., 2006; Xiang et al., 2006). Not only are particles in the 20–100nm range able to efficiently migrate through the lymphatics and reach more lymph node-resident DCs compared to larger particles, but are also more able to alter signaling processes involved in regulating cell functions (Reddy et al., 2006a; Reddy et al., 2007; Jiang et al., 2008). Within this nanometer size range, studies have shown that T helper cell differentiation is also influenced by particle size, with beads in the 40–49nm range responsible for IFN γ induction by CD8 T cells and 93–123nm favoring IL-4 production (Mottram et al., 2007). This was demonstrated by the protection and clearance of tumors in mice using polystyrene microspheres 40–50nm in diameter, which induced an IFN γ CD8+ T cell response (Fifis et al., 2004a). The CMNPs presented in our study fall within the 20–80nm size range, ideal for lymphatic migration, targeting DCs, and promoting T helper cell response. Not only were these CMNPs able to reach lymph node and splenic DCs, but were also able to enter sites of chronic inflammation like the granuloma and target resident DCs. As treatment of chronic disease is a difficult hurdle in immunotherapy technology, this latter finding may provide a new tool for influencing the immune response in such locations.

In addition to nanoparticle biomaterials being required to selectively target DCs over other cellular compartments, efficient delivery of antigen is also necessary for effective immunity. In addition to antigen, co-delivery of maturation stimuli to the same DC is advantageous over systemic adjuvant administration. Various nanoparticles bound with antigen exhibited a natural adjuvancy resulting in antigen-specific cellular and/or humoral immunity. These included nanoparticles made of carboxylated polystyrene, methoxypolyethylene glycol-poly(lactide-co-glycolide), Gantrez polymer, and poly(γ -glutamic acid) for the protection or treatment of tumors, foot and mouth disease, hepatitis B, *Listeria monocytogenes*, *Salmonella enteritidis*, and HIV, respectively (Fifis et al., 2004b; Scheerlinck et al., 2006; Akagi et al., 2007; Ochoa et al., 2007; Uto et al., 2007; Wang et al., 2007; Bharali et al., 2008; Greenwood et al., 2008; Wang et al., 2008). Others have achieved adjuvancy by altering the surface of the particles in such a way that stimulated either the complement cascade or TLR signaling by modifying the surface chemistry or ligating CpG or monophosphoryl lipid A (MPLA) to the surface, respectively (Elamanchili et al., 2007; Reddy et al., 2007; Borges et al., 2008). While the previously mentioned particles naturally stimulate specific immune responses, the latter particles and our own CMNPs presented here, are immunologically inert without specific modification. This enables us to modify the CMNPs in such a way as to achieve desired immune outcomes, which make these CMNPs usefully in many contexts. In this study we demonstrate the efficient delivery of antigen to DCs both *in vitro* and *in vivo*, and further show the ability to skew the immune system towards an IFN γ -Th1 response by the co-delivery of CpG. Due to the aviden-generated surface of these CMNPs, biotinylated adjuvants of any nature may be bound to achieve a variety of immune outcomes.

In addition to DC-targeted vaccination and therapy, nanoparticle technology has been increasingly utilized as a means of cellular imaging. Using small particles of iron oxide (SPIO), Baumjohann *et al.* demonstrated that murine BMDC uptake of these particles enabled *in vivo* images of DC migration within the T cell area of the lymph node (Baumjohann et al.,

2006). Using MRI, these same particles have also allowed researches to differentiate metastatic from normal lymph nodes in mice (Wunderbaldinger et al., 2002). Specifically, the field of cancer research has harnessed the variety of applications provided by nanotechnology (Nie et al., 2007). Compared to current cancer-specific probes, the use of magnetism-engineered iron oxide (MEIO) nanoparticles, resulted in a more sensitive and specific MRI detection of small tumors in mice (Lee et al., 2007). The use of fluorescent, semiconductor quantum dot nanocrystals are also proving useful, not only in the field of cancer, but also broadly for imaging in vivo cellular events via fluoresce, TEM, PET, and MRI detection methods (Walling et al., 2009). The CMNPs presented in this study afford *in vitro* and *in vivo* imaging of both the CMNPs, and the cells that contain them, using both fluorescent imaging, TEM, and MRI. In vivo imaging of DCs not only increases our basic understanding regarding DC biology, trafficking, cellular interactions, and involvement in biological events, but in a clinical application it might provide a method of ensuring proper distribution and delivery of a desired drug or immunogen.

In lieu of the ability to image cellular events via nanoparticles, the magnetic property afforded by some nanoparticles enables cellular manipulation and control using a magnetic field. Combined use of magnetic nanoparticles and an external magnetic field is proving useful for *in vitro* gene delivery, *in vitro* cell orientation for the generation of tissue, and drug delivery (Mah et al., 2002; Dobson et al., 2006; Dames et al., 2007a). This nanomagnetic actuation technology has also been applied to Magnetic Fluid Hyperthermia (MFH) therapy. This involves electromagnetic energy used to exclusively heat iron oxide particles within a cell to induce cell death (Gneveckow et al., 2004). This technology is of exceptional interest in cancer research, and is beginning to succeed in killing tumor cells in mice (Hilger et al., 2005a; Hilger et al., 2005b). The delivery of immunotoxins, a toxin-containing protein delivered to target cells by antibody conjugation, is also demonstrating functional use in the elimination of immune cells (Kreitman, 2006). An example an immunotoxin approved for T cell lymphoma is the targeted delivery of diphtheria toxin with anti-IL-2 (Foss et al., 2001). Due to the limitations mentioned earlier regarding monoclonal antibody targeted therapies and the additional usages afforded by these CMNPs, we are currently investigating the selective removal of DCs by delivery of CMNPs coated with the subunit A toxin of Ricin. Ricin has been shown to inhibit human DC maturation at extremely low toxin concentrations and has been considered as a candidate to eliminate DCs in graft transplants in order to prevent rejection (Wiley et al., 1989; Pollak and Blanchard, 2000; Smith et al., 2004).

In order to facilitate the transition of nanoparticle technology into biomedical applications, nanoparticles with advantageous, multi-functional properties must be produced. The CMNPs in this study meet the criteria for a desirable targeting vector on several levels. Not only does their size favor preferential DC uptake both *in vitro* and *in vivo*, but their surface chemistry and large surface area facilitates the binding of an abundant array of proteins and adjuvants, as well as fluorescent tracers. These non-toxic CMNPs can efficiently deliver their antigen array, resulting in a specific immune response skewed in a desired direction. The magnetic and fluorescent properties of the CMNPs allow for visualization, recovery, and potentially the facilitation of directed DC migration. It is known that DC traffic is a highly regulated process and is imperative to their function. Having the ability to influence their migration *in vivo* would allow for DC localization and protein delivery to specific lymph nodes, inflammatory sites or tumors, in a way that allows us to influence the immune response with a high level of specificity.

Acknowledgments

We would also like to thank Toshi Kinoshita for expert histopathology services, and fellow laboratory members for helpful discussion of this work. Special thanks to both Dr. Michel C. Nussenzweig at the Rockefeller University for his generous gift of the CD11c-EYFP mice, to Beth Meyerand and Beth Rauch for their expert assistance with the 4.7T MRI, and Dr. Lalita Ramakrishnan (University of Washington, WA) for her generous gift of dsRED BCG

plasmid. Work supported by National Institutes of Health funding R01-A1048087 and R21-A1072638 (M.Sandor), and Hungarian National Office for Research and Technology - National Scientific Fund K68617 (J.Prechl).

REFERENCES

- Akagi T, Wang X, Uto T, Baba M, Akashi M. Protein direct delivery to dendritic cells using nanoparticles based on amphiphilic poly(amino acid) derivatives. *Biomaterials* 2007;28:3427–3436. [PubMed: 17482261]
- Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245–252. [PubMed: 9521319]
- Baumjohann D, Hess A, Budinsky L, Brune K, Schuler G, Lutz MB. In vivo magnetic resonance imaging of dendritic cell migration into the draining lymph nodes of mice. *Eur J Immunol* 2006;36:2544–2555. [PubMed: 16909432]
- Bharali DJ, Pradhan V, Elkin G, Qi W, Hutson A, Mousa SA, Thanavala Y. Novel nanoparticles for the delivery of recombinant hepatitis B vaccine. *Nanomedicine* 2008;4:311–317. [PubMed: 18640075]
- Borges O, Cordeiro-da-Silva A, Tavares J, Santarem N, de Sousa A, Borchard G, Junginger HE. Immune response by nasal delivery of hepatitis B surface antigen and codelivery of a CpG ODN in alginate coated chitosan nanoparticles. *Eur J Pharm Biopharm* 2008;69:405–416. [PubMed: 18364251]
- Buford MC, Hamilton RF Jr, Holian A. A comparison of dispersing media for various engineered carbon nanoparticles. *Part Fibre Toxicol* 2007;4:6. [PubMed: 17655771]
- Combadiere B, Mahe B. Particle-based vaccines for transcutaneous vaccination. *Comp Immunol Microbiol Infect Dis* 2008;31:293–315. [PubMed: 17915323]
- Dames P, Gleich B, Flemmer A, Hajek K, Seidl N, Wiekhorst F, Eberbeck D, Bittmann I, Bergemann C, Weyh T, Trahms L, Rosenecker J, Rudolph C. Targeted delivery of magnetic aerosol droplets to the lung. *Nature Nanotechnology* 2007a;2:495–499.
- Dames P, Gleich B, Flemmer A, Hajek K, Seidl N, Wiekhorst F, Eberbeck D, Bittmann I, Bergemann C, Weyh T, Trahms L, Rosenecker J, Rudolph C. Targeted delivery of magnetic aerosol droplets to the lung. *Nat Nanotechnol* 2007b;2:495–499. [PubMed: 18654347]
- Denes FS, Manolache S, Ma Y, Shamamian V, Ravel B, Prokes S. Dense medium plasma synthesis of carbon/iron-based magnetic nanoparticles. *Journal of Applied Physics* 2003;94:3498–3508.
- Dobson J, Cartmell SH, Keramane A, El Haj AJ. Principles and design of a novel magnetic force mechanical conditioning bioreactor for tissue engineering, stem cell conditioning, and dynamic in vitro screening. *IEEE Trans Nanobioscience* 2006;5:173–177. [PubMed: 16999242]
- Elamanchili P, Lutsiak CM, Hamdy S, Diwan M, Samuel J. "Pathogenmimicking" nanoparticles for vaccine delivery to dendritic cells. *J Immunother (1997)* 2007;30:378–395.
- Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, McKenzie IF, Plebanski M. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J Immunol* 2004a;173:3148–3154. [PubMed: 15322175]
- Fifis T, Mottram P, Bogdanoska V, Hanley J, Plebanski M. Short peptide sequences containing MHC class I and/or class II epitopes linked to nano-beads induce strong immunity and inhibition of growth of antigen-specific tumour challenge in mice. *Vaccine* 2004b;23:258–266. [PubMed: 15531045]
- Foss FM, Bacha P, Osann KE, Demierre MF, Bell T, Kuzel T. Biological correlates of acute hypersensitivity events with DAB(389)IL-2 (denileukin diftitox, ONTAK) in cutaneous T-cell lymphoma: decreased frequency and severity with steroid premedication. *Clin Lymphoma* 2001;1:298–302. [PubMed: 11707845]
- Gneveckow U, Jordan A, Scholz R, Bruss V, Waldofner N, Rieke J, Feussner A, Hildebrandt B, Rau B, Wust P. Description and characterization of the novel hyperthermia- and thermoablation-system MFH 300F for clinical magnetic fluid hyperthermia. *Med Phys* 2004;31:1444–1451. [PubMed: 15259647]
- Greenwood DL, Dynon K, Kalkanidis M, Xiang S, Plebanski M, Scheerlinck JP. Vaccination against foot-and-mouth disease virus using peptides conjugated to nano-beads. *Vaccine* 2008;26:2706–2713. [PubMed: 18448209]
- Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 2002;20:621–667. [PubMed: 11861614]

- Hilger I, Andra W, Hergt R, Hiergeist R, Kaiser WA. Magnetic thermotherapy of breast tumors: an experimental therapeutic approach. *Rofo* 2005a;177:507–515. [PubMed: 15838755]
- Hilger I, Hergt R, Kaiser WA. Use of magnetic nanoparticle heating in the treatment of breast cancer. *IEE Proc Nanobiotechnol* 2005b;152:33–39. [PubMed: 16441156]
- Ho WY, Cooke MP, Goodnow CC, Davis MM. Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4+ T cells. *J Exp Med* 1994;179:1539–1549. [PubMed: 7909325]
- Hogan LH, Macvilay K, Barger B, Co D, Malkovska I, Fennelly G, Sandor M. Mycobacterium bovis strain bacillus Calmette-Guerin-induced liver granulomas contain a diverse TCR repertoire, but a monoclonal T cell population is sufficient for protective granuloma formation. *J Immunol* 2001;166:6367–6375. [PubMed: 11342661]
- Jiang W, Kim BY, Rutka JT, Chan WC. Nanoparticle-mediated cellular response is size-dependent. *Nat Nanotechnol* 2008;3:145–150. [PubMed: 18654486]
- Kalkanidis M, Pietersz GA, Xiang SD, Mottram PL, Crimeen-Irwin B, Ardipradja K, Plebanski M. Methods for nano-particle based vaccine formulation and evaluation of their immunogenicity. *Methods* 2006;40:20–29. [PubMed: 16997710]
- Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol* 2003;3:984–993. [PubMed: 14647480]
- Kreitman RJ. Immunotoxins for targeted cancer therapy. *Aaps J* 2006;8:E532–E551. [PubMed: 17025272]
- Lee JH, Huh YM, Jun YW, Seo JW, Jang JT, Song HT, Kim S, Cho EJ, Yoon HG, Suh JS, Cheon J. Artificially engineered magnetic nanoparticles for ultra-sensitive molecular imaging. *Nat Med* 2007;13:95–99. [PubMed: 17187073]
- Lindquist RL, Shakhar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, Nussenzweig MC. Visualizing dendritic cell networks in vivo. *Nat Immunol* 2004;5:1243–1250. [PubMed: 15543150]
- Ma Y, Manolache S, Denes FS, Thamm DH, Kurzman ID, Vail DM. Plasma synthesis of carbon magnetic nanoparticles and immobilization of doxorubicin for targeted drug delivery. *J Biomater Sci Polym Ed* 2004;15:1033–1049. [PubMed: 15461188]
- Mah C, Fraites TJ Jr. Zolotukhin I, Song S, Flotte TR, Dobson J, Batich C, Byrne BJ. Improved method of recombinant AAV2 delivery for systemic targeted gene therapy. *Mol Ther* 2002;6:106–112. [PubMed: 12095310]
- Mottram PL, Leong D, Crimeen-Irwin B, Gloster S, Xiang SD, Meanger J, Ghildyal R, Vardaxis N, Plebanski M. Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus. *Mol Pharm* 2007;4:73–84. [PubMed: 17274665]
- Nie S, Xing Y, Kim GJ, Simons JW. Nanotechnology applications in cancer. *Annu Rev Biomed Eng* 2007;9:257–288. [PubMed: 17439359]
- Ochoa J, Irache JM, Tamayo I, Walz A, DeVecchio VG, Gamazo C. Protective immunity of biodegradable nanoparticle-based vaccine against an experimental challenge with Salmonella Enteritidis in mice. *Vaccine* 2007;25:4410–4419. [PubMed: 17434651]
- Pollak R, Blanchard JM. Organ donor or graft pretreatment to prolong allograft survival: lessons learned in the murine model. *Transplantation* 2000;69:2432–2439. [PubMed: 10868654]
- Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA. In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. *J Control Release* 2006a;112:26–34. [PubMed: 16529839]
- Reddy ST, Swartz MA, Hubbell JA. Targeting dendritic cells with biomaterials: developing the next generation of vaccines. *Trends Immunol* 2006b;27:573–579. [PubMed: 17049307]
- Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, Lee LK, Swartz MA, Hubbell JA. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. *Nat Biotechnol* 2007;25:1159–1164. [PubMed: 17873867]
- Scheerlinck JP, Gloster S, Gamvrellis A, Mottram PL, Plebanski M. Systemic immune responses in sheep, induced by a novel nano-bead adjuvant. *Vaccine* 2006;24:1124–1131. [PubMed: 16202487]
- Smith DC, Salio M, Lord JM, Roberts LM, Cerundolo V. Lack of dendritic cell maturation by the plant toxin ricin. *Eur J Immunol* 2004;34:2149–2157. [PubMed: 15259012]

- Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature* 2007;449:419–426. [PubMed: 17898760]
- Tacke PJ, de Vries IJ, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. *Nat Rev Immunol* 2007;7:790–802. [PubMed: 17853902]
- Uto T, Wang X, Sato K, Haraguchi M, Akagi T, Akashi M, Baba M. Targeting of antigen to dendritic cells with poly(gamma-glutamic acid) nanoparticles induces antigen-specific humoral and cellular immunity. *J Immunol* 2007;178:2979–2986. [PubMed: 17312143]
- Walling MA, Novak JA, Shepard JR. Quantum dots for live cell and in vivo imaging. *Int J Mol Sci* 2009;10:441–491. [PubMed: 19333416]
- Wang X, Uto T, Akagi T, Akashi M, Baba M. Induction of potent CD8+ T-cell responses by novel biodegradable nanoparticles carrying human immunodeficiency virus type 1 gp120. *J Virol* 2007;81:10009–10016. [PubMed: 17609261]
- Wang X, Uto T, Akagi T, Akashi M, Baba M. Poly(gamma-glutamic acid) nanoparticles as an efficient antigen delivery and adjuvant system: potential for an AIDS vaccine. *J Med Virol* 2008;80:11–19. [PubMed: 18041033]
- Wiley KN, Clark A, Fox M. The in-vitro inhibition of rat alloantigen presentation by immunotoxins--implications for allografting. *Clin Exp Immunol* 1989;76:132–137. [PubMed: 2525436]
- Wunderbaldinger P, Josephson L, Bremer C, Moore A, Weissleder R. Detection of lymph node metastases by contrast-enhanced MRI in an experimental model. *Magn Reson Med* 2002;47:292–297. [PubMed: 11810672]
- Xiang SD, Scholzen A, Minigo G, David C, Apostolopoulos V, Mottram PL, Plebanski M. Pathogen recognition and development of particulate vaccines: does size matter? *Methods* 2006;40:1–9. [PubMed: 16997708]
- Zozulya AL, Reinke E, Baiu DC, Karman J, Sandor M, Fabry Z. Dendritic cell transmigration through brain microvessel endothelium is regulated by MIP-1alpha chemokine and matrix metalloproteinases. *J Immunol* 2007;178:520–529. [PubMed: 17182592]

Figure 1. Characterization of CMNPs

A, TEM image of CMNPs shows irregular, crystalline-shaped particles with a 20–80 nm size distribution. B, Ferromagnetic resonance spectroscopy confirming magnetism of carbon (0.1% iron) particles. C, Direct conjugation of proteins to CMNPs. Flow cytometric analysis of CMNP conjugated with HEL protein and anti-DEC205 monoclonal mouse IgG specific for DCs (right) or unconjugated (left). D, Flow cytometric analysis of Avidin-FITC conjugated CMNPs (green) compared to non-labeled particles (black).

Figure 2. Dendritic cell uptake of CMNPs

Top, *In vitro* BMDC culture pulsed with CMNPs. Light microscopy (top left- DC with dendrite protrusions and nearby, round macrophage/monocyte-like cell) and SEM (top right) of BMDC culture pulsed with CMNPs over night. Middle, EM images also reveal CMNPs in endocytic vesicles in DCs (arrows point to CMNPs). Bottom, fluorescent microscopy of BMDC culture pulsed with FITC-CMNPs for 30 min at 37°C (bottom left) and CD11c-EYFP BMDC culture pulsed with PE-CMNPs (arrows, bottom right).

B

	% Dendritic Cells with CMNPs	
	HEL	HEL / DEC205
Exp. I	6	16
Exp. II	21	40
Exp. III	5	21

Figure 3. Enhanced targeting of CMNPs to DCs by DC-specific antibodies

A, BMDC culture pulsed overnight with HEL-conjugated CMNPs or HEL/DEC205-conjugated CMNPs. Light microscopy (400 $\times$ magnification (upper) and 1000 $\times$ magnification (lower). B, Quantification of the percentage of DCs with CMNPs from three independent experiments.

Figure 4. CMNP traffic *in vivo*

A, 4.7T MRI scan of mouse 15 and 75 minutes post i.v. injection of CMNPs. Red arrows point to particle accumulation in spleen, kidney and inguinal lymph node. B, Hematoxylin and Eosin staining of formalin fixed tissue samples two days and one week post i.v. injection of CMNPs. Arrows point to extracellular CMNP aggregates.

Figure 5. DC targeting and selective enrichment by CMNPs

A, Selective enrichment of DEC205+MHCII+ DCs from BMDC culture pulsed with CMNPs and exposed to Dynal magnet (bottom FACS panel) compared to non-magnet exposed BMDC-pulsed culture (top FACS panel). B, After 5 days post i.v. injection of FITC-CMNPs, splenocytes were isolated and stained with antibodies recognizing DCs. C, Fluorescent confocal image of liver section taken from a 3 week BCG-infected mouse injected 5 days prior to harvest with FITC-CMNPs. White arrows point to FITC-CMNP extra cellular aggregates and red arrows point to dsRED BCG bacilli. D, Liver granuloma cells passed through Miltenyi magnetic separation column (inset figure). Flow cytometric analysis using anti-CD11c reveals enrichment of DCs using FITC-CMNPs.

Figure 6. Redirecting *in vivo* DC traffic using a magnetic field

A, Sleeping mice were injected i.v. with PE-CMNPs. A neodymium ringed-magnet was positioned to encompass their inguinal lymph nodes for 30 minutes. FACS plots of pooled inguinal and cervical lymph nodes (left and right plots, respectively) show staining with anti-CD11c and expression of PE from CMNPs. B, Light and fluorescent microscopy images of CD11c-EYFP BMDC containing NanoLink particles (red arrows point to particles). N52 grade neodymium magnets were positioned juxtaposed to left inguinal lymph node of sleeping mice. Magnetically enriched bead-containing BMDCs were s.c. injected at the base of the tail and left for three hours. C, Both inguinal lymph nodes, cervical nodes and spleen were removed, and analyzed on flow cytometry for the presence of CD11c-EYFP cells (histogram plots

generated from live cell gate). D, Fluorescent and light microscopy reveals the presence of bead-containing CD11c-EYFP cells in magnet-exposed inguinal lymph node (black arrow points to particles).

Figure 7. *In vitro* and *in vivo* CD4⁺ T cell activation by antigen-conjugated CMNPs

A, 18 hr *in vitro* activation of anti-HEL 3A9 TCR transgenic T cells. FACS analysis staining with 1G12, anti-3A9 clonotypic antibody, and CD4. B, Percentage of CD69 expression on 3A9 T cells after 18 hr *in vitro* activation. C, IFN γ 5 hour recall with anti-CD3 following three day *in vitro* activation of 3A9 splenocytes. Cells were treated with either HEL protein alone, CMNPs conjugated with HEL or CPG alone, or HEL and CPG. D, *Upper plots*, *In vivo* expansion of adoptively transferred CFSE-labeled 3A9 T cells into wild type mice 6 days after transfer and 7 days post immunization of HEL-bound CMNPs (left) and HEL-CpG-bound CMNPs (right). *Lower plots*, intracellular IFN γ cytokine staining following 5 hour recall with anti-CD3 *ex vivo*. Plots shown are from adoptively transferred Tg T cell gate of CD4⁺1G12⁺. Representative plots from two independent experiments with 3–5 mice per group.