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ENGINEERING NANO- AND MICRO-PARTICLES TO TUNE IMMUNITY

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Abstract

The immune system can be a cure or cause of disease, fulfilling a protective role in attacking cancer or pathogenic microbes but also causing tissue destruction in autoimmune disorders. Thus, therapies aimed to amplify or suppress immune reactions are of great interest. However, the complex regulation of the immune system, coupled with the potential systemic side effects associated with traditional systemic drug therapies, has presented a major hurdle for the development of successful immunotherapies,. Recent progress in the design of synthetic micro- and nano-particles that can target drugs, deliver imaging agents, or stimulate immune cells directly through their physical and chemical properties is leading to new approaches to deliver vaccines, promote immune responses against tumors, and suppress autoimmunity. In addition, novel strategies, such as the use of particle-laden immune cells as living targeting agents for drugs, are providing exciting new approaches for immunotherapy. This progress report describes recent advances in the design of micro- and nano-particles in immunotherapies and diagnostics.

1. Introduction

The immune system is a distributed network of cells and lymphoid organs, which play a critical role in providing protection from infectious microbes, and perhaps also in restraining the development of tumors.[1] It is comprised at the organ level of secondary lymphoid organs, including the spleen, nasal-associated lymphoid tissue, Peyer's patches in the gut, and lymph nodes distributed throughout the body. (Primary lymphoid organs, the thymus and bone marrow, are sites where immune cells are generated from stem cells throughout life). At the cellular level, the key components are innate and adaptive immune cells. Innate cells such as macrophages, neutrophils, and natural killer cells provide immediate defense against infections at portals of entry such as mucosal surfaces and the skin.[2,3] By contrast, adaptive immune cells (T-cells and B-cells) reside in lymphoid organs or tissues and are slower to respond to infectious challenges, but can differentiate into long-lived memory cells that provide rapid protection on re-exposure to pathogens.[4]

Manipulation of the immune system by therapeutic interventions is of great interest due to the pervasive role of immunity in health and disease. In many instances, we seek to induce or amplify the normal functions of the immune system. One of the most successful

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biomedical interventions ever devised, vaccination, relies on stimulating immune memory to protect immunized individuals from future encounters with dangerous microbes.[1] However, effective vaccines are still elusive for a number of important infectious pathogens, such as HIV, malaria, tuberculosis, and hepatitis C. Cancer immunotherapies are treatments aiming to stimulate a patient's immune system to attack and destroy tumors, usually in the presence of pre-existing disease; recent successes suggest much promise in this field[5–7] but cancer remains a major challenge in medicine. On the other hand, the immune system can also cause disease itself if immune cells attack healthy tissue (autoimmunity); in this case, therapeutic interventions to restrain immune responses are sought.[8] In addition to therapeutic modulation, there is also a need for strategies to monitor and measure immunity. The disseminated nature of the immune system has made clinical analysis and monitoring of immune function a major challenge, and improved methodologies to track and diagnose the function of the immune system are desperately needed.

Micro- and nano-scale synthetic particles have a major role to play in solving these problems. The complexity of signals regulating proper functioning of the immune system creates a major challenge for therapies based on traditional single-agent bolus drug treatments. Engineered particles are being intensively studied as delivery vehicles and adjuvants for vaccines,[9–12] components of diagnostic systems to analyze ongoing immune responses and immune cell trafficking *in vivo*,[13,14] as systems for the *ex vivo* expansion of therapeutic immune cells for treatment of cancer and infectious diseases,[6,15,16] and as delivery agents for immunotherapy drugs.[17–20] Nanoparticles (NPs) and microparticles (MPs) tailored for these applications are enabling new means to detect and treat diverse conditions with major implications for global health, ranging from cancer to infectious disease to autoimmune disorders. In this progress report, we will summarize recent advances in the design and implementation of engineered particles that can sense, stimulate, or suppress immune reactions by interactions with single cells or with whole tissues/organs, and highlight challenges for which new materials are needed. An exhaustive overview of this rapidly expanding field is beyond the scope of any single review, and we thus aim to highlight areas where particle technologies are already having impact or where new approaches in nano/micro-particle design are ripe for application to problems in tuning or taming the immune system.

2. Shaping immune reactions at the single-cell level: tailoring particle interactions with leukocytes

Cell-cell communication by direct contact and engagement of membrane receptors plays a major role in regulating the functions of immune cells. Perhaps the most important cell-cell interaction in the induction of adaptive immune responses occurs during the activation of T-cells by specialized, rare antigen presenting cells (APCs) known as dendritic cells (DCs).[21] Synthetic particles can be designed to display receptors and co-stimulatory ligands normally expressed on the surface of DCs, thereby mimicking activated APCs and inducing T-cell activation and differentiation. This strategy can be applied in adoptive cell therapies and vaccine development, where engineered synthetic particles may replace APCs and directly interact with T cells to regulate their differentiation and effector functions. Another important aspect in the use of particles as drug delivery agents is the interaction between particles and phagocytic cells, such as macrophages. By modulating particle size, shape, and elastic properties, particle uptake by phagocytes can be tailored at the single-cell or whole organ level, providing the means to optimize drug delivery to particular tissues and organs.

2.1 Synthetic particles as artificial antigen presenting cells

The organized contact between a T-cell and dendritic cell during T-cell activation is known as an immunological synapse (Fig. 1A).[21] DCs have the task of capturing fragments of pathogens from the environment (or from dying infected cells) and physically displaying peptides of foreign material (antigen) to T-cells in the cleft of surface receptors known as the Major Histocompatibility complex (MHC) molecules.[22] Every T-cell expresses a unique T-cell receptor (TCR) and when T-cells contact a DC displaying a cognate antigen, T-cell activation occurs, mediated by the assembly of receptors and secreted factors at the T-APC interface (the synapse). Dendritic cells play a critical role in instructing T-cells to mount the appropriate immune response needed for a particular microbial invader, via the type of co-stimulatory ligands they present to the T-cell and soluble cytokines released at the synapse, which determine the effector functions of the responding T-cell (Fig. 1A).

This crucial role played by DCs in the adaptive immune response has motivated the design of microparticles that can mimic the function of these cells in contact-mediated programming of T-cells, providing a convenient strategy to artificially stimulate T-cell activation *in vitro* or *in vivo* (Fig. 1B). Stimulation of the TCR by surface-anchored antigen/MHC complexes (or antibodies that mimic these natural ligands) rather than soluble ligands is required to properly activate T-cells, and so the simplest forms of MP-based “artificial APCs” (aAPCs) are monodisperse cell-sized polystyrene beads conjugated with immobilized peptide-MHC and costimulatory receptor ligands; such simple DC surrogates are now a common tool in immunology,[15,23,24] and are used in clinical procedures such as adoptive cell therapy, where autologous tumor-specific T-cells are activated and expanded by stimulatory MPs in cell culture prior to infusion into patients to combat cancer.[15,25,26]

More recently, particle engineering has been used to design multifunctional aAPCs that are endowed with more of the physiological features of genuine antigen presenting cells. For example, aAPCs composed of biodegradable poly(lactide-*co*-glycolide) (PLGA) micro- or nano-particles surface-modified with avidin-palmitate conjugates have been generated to anchor peptide-MHC and costimulatory ligands to the particle surfaces (Fig. 1B).[16] Interestingly, when incubated with lymphocytes, ligand-displaying MPs 8 μm in diameter (mimicking APCs in size) stimulated much greater total T-cell activation than NPs (130 nm diameter) displaying the same activation signals.[16] This result might reflect internalization of NPs by T-cells,[27] leading to particle degradation and early termination of TCR signaling. By contrast, T-cells formed stable, tight synapse-like contacts with ligand-displaying microparticles (Fig. 1B). To further mimic cytokine production by DCs themselves[28,29] or helper T-cells in the local microenvironment *in vivo*, aAPCs were loaded with the key T-cell growth factor interleukin-2 (IL-2), which was released over ~1 week in culture. [16] Cytokine-releasing aAPCs stimulated T-cells more strongly than commercial MPs or PLGA particles displaying TCR ligands and costimulatory ligands alone. Interestingly, T-cell expansion stimulated by IL-2-releasing aAPCs was greater than that achieved when T-cells were co-cultured with aAPCs lacking encapsulated cytokine but supplemented with a 10-fold greater total soluble dose of IL-2 added to the medium.[16] This result may be due to the local concentration of the cytokine and IL-2 receptors in the synapse formed between the T-cell and synthetic particle,[30] as IL-2 delivered in a paracrine manner is very efficiently consumed by T-cells[31] and modeling of the T-cell/aAPC interface suggested that the concentration of IL-2 developing in the synapse near an IL-2-releasing particle exceeds that obtained with a 1000-fold-greater concentration of cytokine added to the bulk solution.[16] Furthermore, it was shown that the potent response of T-cells to particle-released IL-2 was dependent on sustained release of the growth factor over time.[16,32]

In addition to their use for expanding T-cells *in vitro*, polystyrene MPs displaying T-cell-activating ligands have also been employed to prime T-cell responses directly *in vivo* with the goal of avoiding costly and laborious *ex vivo* culture procedures.[33,34] Particles prepared from fully biodegradable materials such as the PLGA particles described above will have obvious advantages for translation of such *in vivo* immunostimulation approaches to clinical use. A potentially interesting area for future design of particles that serve as surrogates of APCs is the design of particles that mimic the physical organization of the "mature" immunological synapse. The mature synapse is characterized by a central cluster of TCRs and signaling proteins, surrounded by a peripheral ring enriched in accessory molecules and adhesion receptors (Fig. 1A).[21] Methods developed for the creation of so-called Janus particles[35–37] may be relevant for mimicking this arrangement– for example, patchy anisotropic particles displaying micron-scale patches of one protein surrounded by a second component were synthesized by masking contact points of particles in a colloidal crystal in a reversible manner, followed by modification with proteins (Fig. 1C).[38] Strategies to provide fixed structures mimicking the mature synapse might also be supplemented by the use of particles displaying synapse proteins on MPs coated with fluid lipid membranes,[39] thereby allowing self-reorganization of receptors and signaling molecules during the interaction with T-cells. Such approaches utilizing increasingly refined particle designs may provide the means to tune the differentiation state of T-cells by more closely mimicking the interaction of T-cells with native APCs.

2.2 Engineering particle interactions with phagocytes

2.2.1 Role of particle shape—Much effort has focused on determining how the properties of synthetic MPs and NPs influence binding and internalization by macrophages in the spleen and liver, since this clearance hinders systemic delivery of therapeutics or diagnostic/imaging agents by particles. Early efforts to design phagocytosis-resistant particles focused on engineering surface chemistry to block protein adsorption and complement interactions with the particle surface (a process known as opsonization), since such adsorbed/bound serum components serve as a molecular handhold for phagocyte binding and internalization. The most widely employed strategy for limiting opsonization is to introduce a dense layer of anchored poly(ethylene glycol) (PEG) at the particle surface, which sterically resists protein interactions with the particle.[40,41] Certain zwitterionic polymers can also be used for particle surface modification to achieve even better protection of particles from opsonization via tight water binding at the particle/solution interface.[42]

Recently, it has become evident that surface chemistry is not the only property to play a significant role in dictating the interactions of particles with phagocytes– particle mechanical properties and geometrical shape also have a major influence on the outcome. The latter property has only become clear in the last few years with the advent of powerful new particle fabrication methodologies.[43–46] Using plastic deformation of polymer microspheres and nanospheres embedded in a sacrificial matrix to fabricate MPs and NPs of diverse geometries, it was shown that while spherical particles are readily internalized, highly anisotropic MPs are very poorly phagocytosed along their length by macrophages *in vitro*, due to incomplete formation of actin rings that circumscribe the initial contact point during phagocytosis (Fig. 2A).[47] Needle-shaped polymer particles with aspect ratios of ~10 and a narrow dimension of ~0.5 μm were also found to promote transient (non-toxic) permeabilization of cell membranes,[48] further emphasizing the interplay between particle shape and cellular responses. Particle internalization responses appear also to be size- and/or cell type-dependent, as a recent study reported that internalization of monodisperse PEG hydrogel nanoparticles by transformed epithelial HeLa cells was much more efficient for rod-like, high-aspect-ratio NPs, compared to cylindrical counterparts with similar volume.[49] These contrasting results may stem from different phagocytic/endocytic

pathways triggered by particles of micron vs. submicron size, or distinct uptake pathways employed by professional phagocytes vs. other cell types. Exploiting the fact that phagocytosis of highly anisotopic microparticles is frustrated if the “wide” face of the particle contacts a macrophage, Doshi et al. fabricated polyelectrolyte multilayer polymer discs 4–7 μm in diameter but less than a micron in thickness with a cell-binding hyaluronic acid surface layer on the face of each disc;[50] when mixed with macrophages, these disc-shaped “backpacks” bound to the cells via the flat face of the disc, a configuration allowing strong adhesion to the cells but no internalization of the discs by the phagocyte.[19] By loading the polymer multilayers with relevant therapeutics, macrophages might be exploited as cellular chaperones to carry drug-loaded backpacks to disease sites from the circulation, such as tumors, infection sites, or lymphoid organs (discussed further below).

Does shape influence the fate of particles *in vivo*? When a series of model PS particles coated with anti-ICAM-1 antibodies to target binding to endothelial cells (ECs) were compared, disc-shaped particles with a narrow dimension of 0.1 μm and diameter of 3 μm achieved lower liver uptake, higher lung accumulation (where a large fraction of ECs are located in the pulmonary vasculature), and greater antibody-specific vasculature binding.[51] In relative agreement with these findings, uncoated silica particles of varying geometries (spherical, discoidal, or cylindrical) but nearly identical volumes prepared using lithography showed that discs accumulated to a lower degree in the liver but more in the lungs compared to spheres.[52] By contrast, cylindrical particles showed greater uptake in the liver than either of the other two particle shapes for this particular size. *In vitro* flow chamber studies and computational modeling have suggested that discoidal particles with large contact area with cell membranes pose a significant barrier for phagocytes to polymerize sufficient actin to wrap around particles and “frustrate” the internalization process, thereby evading internalization by the Kupffer cells in the liver and increasing delivery to other organs.[53–56] In contrast, more symmetric spherical and cylindrical particles with a smaller contact area with cell membranes tended to be internalized by Kupffer cells. Finally, studies of irregularly-shaped ~350 nm diam. poly(maleic anhydride)/lipid composite particles showed preferential uptake in the spleen when compared to spherical particles of similar composition and size, which instead showed predominant uptake in the liver.[57] A key aspect of this latter study of splenic particle tropism was the recognition that the murine spleen lacks the sinusoidal structure shared by the spleen of humans, rats, rabbits, and dogs, and thus the analysis was carried out in the latter 3 animal models where the splenic endothelium architecture more closely approximates that of humans. Altogether, these studies suggest that particle shape may be as important as surface chemistry in the design of particles designed to avoid (or target) liver and splenic macrophages.

2.2.2 Role of particle mechanical properties—Red blood cells (RBCs) have been considered as ideal models for designing long-circulating synthetic particles, since the lifetime of RBCs in humans is ~120 days.[58–60] To match the surface chemical composition of RBCs, PLGA NPs were extruded with mouse RBC-derived membranes to coat the former with RBC lipids and proteins.[61] The RBC shell on the NPs greatly increased the *in vivo* circulation half-life of these synthetic particles to 40 h, compared to 16 h for identical particles functionalized with PEG, or bare particles that aggregated in blood immediately. This result suggests that the ability of RBCs to evade the reticuloendothelial system (RES) is partially due to molecular moieties on the RBC surface, such as the cellular recognition protein CD47, which has been shown to inhibit phagocytosis when bound on particle surfaces[62] by providing a “don’t-eat-me” signal for macrophages.[63] However, cell shape and mechanical properties also play an important role in the long *in vivo* half-life of natural RBCs. *In vitro* mechanical measurements have revealed that RBCs lose their deformability as they age, due to shrinking surface area and stiffening membranes.[64,65] It is also known that opsonized rigid particles are preferentially phagocytosed by macrophages over soft

particles by stimulating actin filament assembly required for phagocytosis.[66] Notably, the discoid particles discussed above exhibiting reduced phagocytosis in the liver are reminiscent of RBCs in shape. With these considerations in mind, several groups have actively aimed to produce particles that can mimic the size, shape, and modulus of RBCs to prolong their *in vivo* persistence.

To investigate whether RBC geometry and mechanical properties could be copied to enhance the circulation times of synthetic particles, Mitragotri and colleagues designed polymer MPs with biconcave geometry, which mimicked the key structural and functional features of live mouse RBCs.[67] Polymeric cores were used as a template, upon which layer-by-layer polymer coatings containing drug cargos were applied, followed by chemical crosslinking to stabilize the shell. Dissolution of the template core yielded hollow microcapsules mimicking the size, shape, and elastic modulus of RBCs. These synthetic RBCs could deform and flow through capillaries smaller than their resting diameter, suggesting that these materials may enhance sustained systemic delivery of therapeutics and diagnostic agents *in vivo*. The authors demonstrated three preliminary examples: surface-adsorbed hemoglobin for oxygen delivery, encapsulated iron oxide nanocrystals as imaging contrast agents, and encapsulated heparin as an anti-coagulant. Finally, to study the importance of elastic modulus in RBC circulation, Merkel et al. used lithographic particle fabrication to generate 2-hydroxyethyl acrylate hydrogel particles with tunable stiffness based on crosslinking density, achieving a range of modulus from ~8 to 64 kPa, which spans the reported modulus for RBCs of 26 ± 7 kPa.[68] These swollen hydrogels particles were also designed with a biconcave geometry and approximate dimensions of RBCs, and exhibited low toxicity and endocytosis by cells *in vitro*. When circulated in 3 μm wide microfluidic channels, softer lightly-crosslinked particles were able to deform and pass through repeatedly, while stiffer particles clogged. *In vivo* pharmacokinetics analysis revealed that particles with the lowest elastic modulus had circulation half-lives of 3.6 days with significant accumulation in the spleen, whereas rigid particles of similar shape were rapidly trapped in the lungs after injection.

A final important recent example of the impact of combined shape and elasticity engineering on particle-phagocyte interactions comes from a study by Geng et al. examining cylindrical block micelles.[69] Highly anisotropic flexible filomicelles assembled from block copolymers several microns in length (with micellar diam. < 100 nm) exhibited greatly reduced uptake by macrophages, compared to spherical micelles of the same materials. In conditions mimicking blood flow relevant to systemic therapeutic delivery, these cylindrical particles aligned their long axis with the flow. When the flow-aligned end of a filomicelle made an adhesive contact with the surface of a macrophage, fluid flow provided a shear force on the flexible shaft of the particle sufficient to break the adhesive contact with the cell prior to internalization (Fig. 2B). These experiments highlight the importance of considering particle-cell interactions in the context of the local microenvironment where these interactions will occur (in this case, in blood flow passing through the spleen or liver). When injected intravenously, PEO-PCL filomicelles exhibited increasing circulation time as a function of initial length up to approximately 8 μm, with a fraction of particles persisting in blood even after one week,[69] and this led to improved accumulation of the chemotherapy drug paclitaxel in xenograft lung tumors.[69,70]

3. Nano- and micro-particle vaccines

3.1 Arming and activating antigen presenting cells

Much effort in modern vaccine development has revolved around the design of subunit vaccines, comprised of purified components of pathogens (antigens) to elicit a focused immune response without the dangers associated with live attenuated pathogens. However,

purified subunit antigens are poorly immunogenic, and require formulation with adjuvants, substances that promote the immune response.[71] Synthetic particles packaging antigen and immunostimulatory molecules are of great interest for next-generation subunit vaccines, by mimicking microbes that are themselves nanoparticles (viruses) or microparticles (bacteria), without the complications of toxicity and anti-vector immune responses that are often elicited by recombinant viral/bacterial vaccines.[72] APCs are the primary target of particle vaccines due to their ability to initiate and sustain both cellular and humoral immune responses. In particular, dendritic cells are canonically considered the most potent activators of naïve T cells,[73] although recent studies have suggested that macrophages can also play an important role in T-cell priming by particle vaccines.[74] DCs internalizing particulate immunogens are triggered to process antigens for loading onto class I MHC molecules in a process known as cross-presentation (to prime $CD8^+$ T-cells, important for viral and cancer vaccines).[75,76] In addition, effective crosslinking of B-cell receptors via multivalent display of antigen on particle surfaces can promote the humoral response.[77,78] Particle vaccines are typically injected subcutaneously or intradermally, and subsequently drain through lymphatics to DCs in lymph nodes or are internalized by DCs directly at the injection site. To mount an effective immune response, vaccine particles acquired by APCs must effectively release their antigen cargo and activate these cells to trigger subsequent antigen presentation and T-cell priming.

3.1.1 Engineering synthetic particles for intracellular delivery of antigen—Once antigen-carrying vaccine particles are internalized by DCs or other APCs, the antigen must be released for proteolysis and loading on MHC molecules. Studies of the kinetics of antigen processing in DCs have suggested that following internalization of particle-associated antigens into the phagosomal/endolysosomal pathway, there is a relatively narrow window of time when antigens are productively processed for loading onto MHC molecules before they are fully degraded.[79] Thus, particles have been designed to rapidly release antigen following internalization: Antigen delivery to DCs using acetylated dextran NPs that undergo rapid breakdown at mildly acidic pH characteristic of early endosomes/phagosomes significantly enhanced MHC I presentation of antigen *in vitro*, compared to NPs with slower degradation profiles, demonstrating that the intracellular antigen release rate can be tuned precisely with vaccine particles to promote antigen presentation.[80] A second strategy to enhance antigen processing from vaccine particles is to take advantage of the reducing environment within endosomes for selective antigen release. For example, peptide- and protein-loaded redox-sensitive polymer microcapsules have been prepared by layer-by-layer assembly onto a sacrificial colloidal core, followed by dissolution of the core template. The resulting hollow capsules are stabilized by disulfide linkages that are cleaved in endolysosomes (Fig. 3A).[81,82] Antigens have also been linked to the surfaces of NP carriers by reduction-sensitive disulfide linkages for rapid intracellular release.[83,84] A third approach is to design particles capable of disrupting endosomes to transport antigens directly into the cytosol, where class I MHC antigen processing is normally initiated. This can be achieved by polymer particles containing pH-buffering units that induce an osmotic pressure buildup and disrupt endosomes, a strategy commonly employed in gene delivery.[85] Exploiting this mechanism, particles composed of pH-responsive poly(diethylaminoethyl methacrylate)[86] have been synthesized to enhance the ability of DCs to cross-present particle-bound protein antigens to $CD8^+$ T-cells. Endosome-escaping particles can also be used to deliver mRNA or DNA encoding antigens into APCs.[87] Another physicochemical strategy demonstrated for vaccine delivery to the cytosol uses particles incorporating weak polyacids, which are hydrophilic and water-soluble at neutral pH but become hydrophobic and membrane-lytic when the acid groups are protonated at acidic pH.[88,89] These endosome-disrupting antigen delivery strategies may have additional

adjuvant effects, since disruption of endolysosomes in APCs has been suggested to activate intracellular danger sensors known as inflammasomes that promote DC activation. [90]

3.1.2 Engineering APC activation with particles carrying molecular adjuvants—Natural pathogens are particulate packages of antigens and "danger signals", which trigger APCs to elicit appropriate immune responses and deal with the detected threat (e.g., bacteria, virus, fungus). During natural infection, these activation cues are derived from conserved molecular motifs characteristic of pathogens, such as lipopolysaccharide (a signature of Gram-negative bacteria), unmethylated C-G oligonucleotide sequences (characteristic of bacterial DNA), or single-stranded RNA molecules (characteristic of viral genomes).[91] Activation signals can also be derived from factors associated with tissue stress, such as extracellular DNA-binding proteins (signatures of cell death) or fragmented extracellular matrix polysaccharides (signatures of tissue damage).[92] APCs express cell surface and intracellular receptors to sense these "danger signals." Particularly prominent in vaccine design are adjuvant molecules targeting the Toll-like receptor (TLR) family of pattern-recognition receptors, a group of 10 cell surface and endolysosomal receptors, which control sensing of a variety pathogens[91,93] and promote immune responses leading to long-lived immunological memory.[94] When microbes are internalized by APCs, antigen and activation signals are received simultaneously, and there is evidence that such a physical association of antigen and activating ligands is important for maximal immune responses.[95,96] These observations have motivated many recent studies of particle vaccines incorporating both antigen and danger signals.

Biodegradable NPs composed of PLGA, a polymer used in multiple FDA-approved products, have shown promise for molecular adjuvant/antigen co-delivery in a number of studies. PLGA particles carrying antigen and either lipopolysaccharide,[97] a ligand for TLR-4, or less toxic CpG oligonucleotides (ligands for TLR-9),[98] conferred protection on nearly all vaccinated animals against a live challenge of West Nile virus. PLGA NPs co-encapsulating the TLR4 agonist, MPLA, and tumor antigens activated DCs in lymph nodes and induced anti-tumor immune responses that reduced melanoma tumor burden *in vivo*.[99] As an interesting counterpoint to these studies of antigen/danger signal co-delivery, it has also been shown that extremely potent B-cell responses accompanied by long-lived germinal centers and durable humoral immunity can be triggered following immunization with nanoparticle mixtures, where PLGA NPs containing TLR agonists are mixed with particles containing antigen,[11] a finding consistent with humoral responses measured in other studies of PLGA particle vaccines.[100–102] Understanding the distinct cellular and molecular mechanisms invoked by particle vaccines in these two different scenarios will be a key goal to move this field forward.

A major challenge for non-living subunit vaccines is the generation of strong $CD8^+$ T-cell responses, which may be required for protective vaccines against cancer or intracellular pathogens such as HIV, hepatitis, and malaria. Current licensed adjuvants such as alum elicit weak or non-existent $CD8^+$ T-cell responses.[71,103] The ability of synthetic particles to trigger "cross presentation" of exogenous antigen by DCs to $CD8^+$ T-cells may provide a path for effective T-cell responses to subunit antigens. Recent efforts using particles to co-deliver antigen and TLR agonists in particular is showing for the first time potent T-cell responses to whole protein vaccines previously only observed with recombinant viral vectors. Nordly and colleagues emulsified the TLR-3 agonist polyI:C with the cationic surfactant dimethyldioctadecylammonium and the immunopotentiator trehalose 6,6′-dibehenate, generating stable gel-state multilamellar liposomes with polyI:C densely packed between the bilayers and antigen adsorbed to the surface of the particles.[104] Immunization with these polyI:C/antigen particles elicited impressively high frequencies of antigen-specific IFN-γ-secreting T cells *in vivo*. In a second key recent study, Moon et al. stabilized

antigen-loaded vesicles by the introduction of bilayer-to-bilayer dithiol-crosslinkers in the walls of multilamellar lipid vesicles containing maleimide-functionalized lipids, resulting in interbilayer-crosslinked multilamellar vesicles (ICMVs).[10] ICMV particles with the TLR-4 agonist monophosphoryl lipid A embedded throughout the vesicle walls and antigen entrapped in the particle core elicited massive $CD8^+$ T-cell expansion following *s.c.* immunization, promoted induction of a central memory phenotype believed to enhance recall responses, and augmented cytokine production by responding T-cells. However, as a counterpoint to the strategy of co-delivering strong TLR agonist adjuvant molecules, a third recent study showed that antigen-coated PLGA MPs injected intravenously in the absence of adjuvants prime the immune system to elicit rapid and robust expansion of memory $CD8^+$ T-cells following boosting with viral vectors or other strong booster immunizations.[10] Together with recent clinical trial results showing enhanced survival in lung cancer patients immunized with liposomal vaccines,[105] these data suggest there is reason to be optimistic for the development of fully synthetic vaccines that can elicit protective T-cell responses in humans.

Several recent studies have exploited the chemical nature of TLR ligands to enhance their incorporation into particle vaccine systems. For example, calcium phosphate NPs are biodegradable but also prone to aggregation, which could be avoided by adsorbing negatively charged poly(I:C) or CpG to the outer shell of the particles. Poly(I:C)/CpG-stabilized particles co-loaded with influenza hemagglutinin (HA) peptide were efficiently taken up by murine splenic DCs and induced DC maturation and proliferation of HA-specific T cells *in vitro*.[106] Another study used the phosphate groups on the CpG backbone as reactive groups to crosslink N-trimethyl-chitosan and protein antigen to form a NP vaccine that could be administered non-invasively by the intranasal route.[107] This clever dual usage of CpG as crosslinker and adjuvant translated into serum and mucosal humoral responses that were biased toward the potent IgG_{2a} antibody isotype, and increased T-cell production of IFN-γ, an important effector cytokine. Finally, pathogen-derived polymers can be directly used to form vaccine delivery particles with built-in adjuvant activity. NPs composed of poly(γ-glutamic acid) from bacterial capsules strongly activated and matured DCs *in vitro* and produced both humoral and cellular responses when used as an antigen carrier *in vivo*.[108] These particles exerted their effects through TLR-4 and its downstream signal transducers MyD88 and MAP kinase.

In addition to TLRs, other danger signal receptors such as intracellular NOD-like receptors, DNA and RNA sensors, and extracellular complement pathways can also be exploited to improve the efficacy of particle vaccines. It has been recently found that PLGA particles without any additional adjuvant can activate an intracellular stress-sensing pathway known as the inflammasome in DCs and promote secretion of IL-1β;, IL-18, and IL-1α.[109] PLGA particles carrying antigen and lipopolysaccharide activated both TLR-4 and inflammasomes simultaneously, providing enhanced protection against West Nile virus compared to the conventional adjuvant alum.[97] Activation of inflammasomes is not the only intracellular response pathway that can be triggered by particle vaccines. DCs incubated with NPs of αAl_2O_3 with surface-conjugated antigen, a nanoscale version of the canonical adjuvant alum, promoted very strong proliferation and IL-2 secretion by $CD8^+$ T cells in an *in vitro* cross presentation assay, 10-fold higher than antigen supplemented with other danger signal stimuli.[61] The potency of these alumina NPs was attributed to the activation of autophagy, a cytoplasmic protein and organelle degradation pathway that also sequesters and delivers intracellular pathogens for antigen processing, and is reciprocally regulated by TLR signaling (Fig. 3B).[110] Particle surface chemistry can also be engineered to activate the complement system and functionalize particles with danger signals *in situ*. Poly(propylene sulfide) (PPS) NPs coated with pluronics promoted activation of complement on contact with serum, promoted by the hydroxyl endgroups of the pluronic stabilizer. These

complement-activating particles elicited robust cellular and humoral immune responses *in vivo*.[9] Upon further investigation, it was found that PPS NPs with carboxylated surfaces are even more potent activators of complement C3 after serum exposure, eliciting further amplified immune responses, compared to hydroxylated surfaces.[111]

Altogether, synthetic nano- and micro-particles show much promise as versatile delivery agents that can finely tune antigen delivery and the activation state of APCs, both *in vitro* and *in vivo*. Particle design and development guided by relatively simple rationales (i.e. controlling antigen release rate, co-delivery of danger signals) have produced vaccine particles capable of eliciting very strong humoral and cellular immune responses in small animal models. However, further detailed studies at the interface of materials science and immunology will be needed to define the mechanisms underlying these diverse systems and to permit continued progress driven by rational design rules for augmenting immune responses.

3.2 Targeting particle vaccines to lymphoid organs

3.2.1 Dendritic cells as live vectors for particle transport—As noted above, vaccine particles carrying antigens and/or adjuvant molecules can be engineered for efficient internalization and processing within APCs such as dendritic cells. DCs reside both in lymph nodes and (in lower numbers) in peripheral tissues. In response to local infection, peripheral tissue DCs become activated, phagocytose antigen or infected dying cells from their environment, and migrate to lymphatic vessels, carrying acquired antigen to the draining lymph nodes (dLNs) to prime naïve T-cells and B-cells.[112] Taking advantage of this natural trafficking pattern, DC vaccines based on the injection of autologous, *ex vivo*-activated and antigen-loaded DCs have been recently commercialized in the first-ever therapeutic cancer vaccine (for prostate cancer) approved by the FDA.[113] The *in vitro* preparation of DCs in this approach provides an ideal opportunity to load these cells with vaccine particles aimed to optimally promote DC activation and antigen processing. Further, for clinical assessment and further optimization of this strategy, molecular imaging of particle-laden DCs has proven to be a valuable tool. Recently, development of multifunctional Fe_3O_4-ZnO core-shell NPs was reported, where the iron oxide core served as an MRI contrast agent and the photoluminescent ZnO shell provided a substrate for binding tumor antigens fused to ZnO-binding peptides (Fig. 4A).[13] DCs efficiently internalized these core-shell NPs and were readily visualized *in vitro* with confocal microscopy and *in vivo* with MRI (Fig. 4B). Mice vaccinated with DCs carrying the NP-tumor antigen complex elicited anti-tumor immunity that significantly suppressed tumor progression (Fig. 4C). Simultaneous imaging and antigen delivery by loading DCs with PLGA particles carrying a model antigen, near-infrared fluorophore, and iron oxide NPs has also been reported.[14] These NP-based strategies for combining antigen delivery with diagnostic imaging should provide new insights into the functions of DCs in the context of tumor immunotherapies and normal physiology as well.

3.2.2 Passive targeting of vaccine particles to lymphoid organs—DC vaccines are a labor-intensive and expensive modality best suited to therapeutic vaccines aimed to treat life-threatening conditions. For prophylactic vaccines intended for thousands or millions of people, particle vaccines that can be simply injected into tissues and find their appropriate targets are needed. There are two routes for “passive targeting” of particles to lymph nodes from parenteral injection sites (most commonly, the muscle, intradermal, or subcutaneous (s.c.) tissues): via free diffusion/convection through the tissue to the lymphatics and then to the draining lymph node (dLN), or via cell-mediated transport as macrophages or DCs internalize particles at the injection site and actively transport them to the dLN.[114] Compared to peripheral tissues, lymphoid tissues and organs have a much

larger population of DCs, the key APC for priming naïve T-cells. Thus, recent work in this field has sought to optimize particle properties for maximal direct trafficking directly to LNs.

A critical parameter governing passive targeting is particle size. The extracellular matrix (ECM) of connective tissues is a meshwork of collagens, elastic fibers, and glycosaminoglycans, with a heterogeneous porosity that impacts the transport of even modest-sized macromolecules (albumin, with a hydrodynamic radius of ~3.5 nm, is excluded from 40–50% of the total volume of interstitial fluid in tissue).[115] Hubbell, Swartz and colleagues showed that 25 nm diam. pluronic-stabilized polypropylene sulfide (PPS) NPs were transported to dLNs and acquired by DCs following intradermal injection much more efficiently than particles of identical composition 100 nm in size (Fig. 5A).[9] In independent studies, monodisperse, presumably non-stimulatory polystyrene (PS) particles 20 nm in size or virus-like particles ~30 nm in diam. were shown to drain passively to LNs where they were internalized by LN-resident DCs and macrophages, while larger particles 500–2000 nm in size were primarily transported to dLNs by DC-mediated trafficking.[116] Thus, particles less than 50 nm in diam. appear to effectively target dLNs by passive diffusion/convection. However, the fate of submicron particles with diameters ranging from 100 nm to 500 nm after administration seems less clear. Studies of antigen-conjugated PS particles or viral particles ~200 nm in size have reported accumulation of particles in the subcapsular sinus of dLNs in a pattern suggesting passive draining following *s.c.* injection.[117,118] The discrepancies between these studies of particle size may reflect some initial impact of mechanical flushing of particles into lymphatics during injection, differences in particle interactions with the ECM, distinct ECM/lymphatic organization at different tissue sites used for injection, or other subtle effects of particle composition that are not yet understood.

Local inflammation in the injection site may also impact particle transport over time. Substantial lymphangiogenesis has been reported at both the injection site (a near-doubling of the frequency of lymphatic vessels) and dLNs following immunizations with strong adjuvants, which alters lymph drainage.[119,120] Nanoparticles have been reported to drain to the subcapsular sinus of LNs for at least 8 days following s.c. injection,[116] and lymphatic remodeling in the presence of vaccine adjuvants could sustain or enhance this process. Antigen-loaded multilamellar lipid vesicles 180 nm in diam. co-injected with the TLR-4 agonist monophosphoryl lipid A were found to accumulate in the subcapsular sinus and colocalize preferentially with macrophages at 2 weeks following *s.c.* injection (Fig. 5B).[121] This result contrasts sharply with the result following injection of the same adjuvant and antigen mixed in soluble form, where antigen flushed through the lymph node over a period of a few hours (Fig. 5B).[121,122] Notably, the sustained accumulation of antigen in lymph nodes following particle immunization in this case correlated with greatly enhanced titers, avidity, and durability of the resulting antibody response.[121] Thus, clarifying the role of inflammation in regulating passive and cell-mediated transport of vaccine particles to lymphoid organs will be important for future studies.

An alternative to passive vaccine particle targeting by diffusion/convection from a peripheral injection site is to directly administer vaccines into LNs. While impractical for large-scale prophylactic vaccination, intranodal injections are straightforward outpatient procedures in humans under ultrasound guidance and have been used in clinical trials of anti-allergy and cancer vaccinations.[123–125] Two recent studies have illustrated the potential of intranodal administration to amplify the potency of NP and MP vaccines. When a series of commonly-studied particulate vaccine carriers, including liposomes, N-trimethyl chitosan particles, and PLGA MPs, were administered in mice via s.c., intradermal, intramuscular, or intralymphatic routes, IgG_1 antibody responses were all robustly promoted

independent of the injection site, whereas IgG_{2a} antibody responses depended strongly on the route of administration.[126] Intralymphatic administration of all 3 particulate vaccine formulations significantly increased antigen-specific IgG_{2a} antibody titers in sera, and elicited higher frequencies of IFN-γ-producing splenocytes. These results suggest that sequestration of large amounts of antigen in LNs by intralymphatic administration may increase the duration of interaction between antigen and antigen-specific lymphocytes, promoting "Th1" immune responses that are characterized by elevated IgG_{2a} and IFN-γ production.[126] Intranodal administration of controlled release particles also enhances the effectiveness of adjuvant molecules: non-surgical intranodal injection of soluble antigen mixed with lipid-coated PLGA MPs releasing the TLR-3 agonist polyI:C (Fig. 5C) led to prolonged polyI:C exposure in the LN, increased adjuvant uptake by DCs in the LNs, and greatly increased $CD8^+$ T-cell expansion, cytokine production, and antibody responses.[127] Sustained release of polyI:C into the extracellular LN microenvironment was implicated in this dramatic enhancement in immune responses, as soluble polyI:C that cleared quickly from the LN or PLGA NPs that were rapidly phagocytosed *in situ* failed to elicit similar responses. Thus, manipulation of the local lymphoid environment via direct injection of particle vaccines may offer new avenues to maximize delivery of antigen/inflammatory signals to APCs and induce potent immune responses.

3.2.3 Targeting particle vaccines to dendritic cells with specific ligands and monoclonal antibodies—Preferential delivery of vaccine particles to APCs can be achieved by modifying particles with monoclonal antibodies directed against APC-specific surface receptors. DCs express several C-type lectin receptors, including DEC-205 (CD205), the mannose receptor (CD206), and DC-SIGN (CD209), which have been implicated as promising targets due to their physiological roles in antigen uptake.[128] The DEC-205 receptor is selectively expressed by $CD8\alpha^+$ interstitial, lymphoid, and Langerhans DCs, known for their capacity to cross-present antigens, while CD206 and CD209 are abundant on DCs and macrophages in the LN medulla.[129] Since antigens fused to anti-DEC-205 antibodies have been shown to undergo cross-presentation in DCs much more efficiently than antigens fused to either anti-CD206 or anti-CD209,[130] DEC-205 has been the focus of recent studies to deliver particle vaccines to DCs and promote cellular immune responses. The first demonstration of using anti-DEC 205 to target vaccine particles to DCs resulted in ~3-fold increases in DC uptake of particles and ~2-fold increases in the magnitude of T-cell responses *in vivo*.[131] More recently, DEC-205 targeted, antigen-loaded PLGA NPs were found to induce secretion of IL-10 and IL-5, Th2-associated cytokines, by DCs and T cells, and elicited IgG_1 antibodies in a DEC-205 density-dependent manner, highlighting the importance of the surface density of targeting moieties on the outcome of immune responses.[132] Particle size and antibody-particle linkage chemistry have been shown to be key factors in the efficacy of targeting particles to DCs.[133,134] When PLGA NPs (200 nm) and MPs (2 μm) functionalized with humanized DC-SIGN antibody were used to target human monocyte-derived DCs, the targeting antibody enhanced NP but not MP uptake by DCs.[133] Particle vaccines can also be targeted to CD206 via its natural carbohydrate ligand, mannose. Surface-display of mannose increased particle uptake by DCs both *in vitro* and *in vivo* and enhanced expression levels of co-stimulatory markers, inducing antigen-specific $CD4^+$ and $CD8^+$ T-cell responses.[135–138] A key question for most of these targeting strategies is whether conjugation of targeting agents to particles too large to freely diffuse through the extracellular matrix will be capable of achieving substantial targeting, since particles will only bind DCs they physically encounter and macrophages in the tissue environment will readily engulf particles in a non-specific manner.

3.2.4 Engineering particle vaccines for mucosal and transcutaneous delivery —Mucosal tissues, such as the cervicovaginal, respiratory, and gastrointestinal tracts, are the

most common portals of entry and sites of initial infection for many pathogens, including HIV, herpes simplex virus, and influenza.[2,139] It is generally observed that protection at mucosal surfaces against infectious agents is enhanced by vaccines directly applied to these sites,[140] prompting interest in drug delivery platforms that can deliver vaccines and therapeutics to mucosal tissues. In addition, mucosal sites such as the airways may be an attractive target for needle-free vaccines that might be self-administered. However, the viscous and adhesive mucus layer that lines mucosal surfaces can efficiently trap and rapidly clear foreign molecules and particles, limiting access of particle vaccines to the underlying tissue. Thus, recent efforts have focused on developing nanoparticles that can penetrate mucus layers and deliver cargo materials to the underlining epithelium.

Mucus is a viscoelastic gel that is composed of crosslinked mucin fibers and proteoglycans.[141] Due to the high negative charge and periodic globular hydrophobic regions in mucin fibers, mucus can efficiently entrap particles via polyvalent ionic or hydrophobic interactions. However, an effective strategy to enhance mucus penetration by particles is to shield particle surfaces with PEG. For example, Hanes and colleagues demonstrated that PEGylation of hydrophobic poly(sebacic acid) (PSA) NPs increases their rate of diffusion in human cervicovaginal mucus 250-fold compared to bare particles, representing a reduction in diffusivity of only 12-fold relative to transport of the particles in pure water (Fig. 6A).[142] PEGylation with a sufficient density of 2 kDa M.W. PEG chains allowed particles as large as 500 nm in diam. to penetrate cervicovaginal mucus,[143] whereas insufficient PEGylation[144] or an increase in PEG MW to 10 kDa[145] resulted in mucus-adhesive NPs, due to increased interactions between particles and mucin fibers. These findings were also confirmed in a separate study using PEGylated MPs permeating through reconstituted mucin hydrogels.[146] Similar strategies have been employed to coat anionic PLGA NPs with PEG[147,148] or Vitamin E conjugated to 5 kDa PEG as a surfactant, suggesting the importance of “stealth” layers on particle surface to enhance mucosal particle delivery.[149] PEGylated PLGA NPs were able to penetrate to the underlining epithelial tissue after topical intravaginal application, and maintain a high local particle concentration up to 6 hrs.[148] Besides penetrating mucus, very small PEGylated NPs appear to be effective in crossing airway epithelial barriers, as 30 nm diam. NPs surface-displaying antigen administered into the airways with CpG as adjuvant were readily taken up by ~10% of lung-resident cells, primarily pulmonary macrophages and DCs.[150] APCs with particles migrated to draining mediastinal LNs and cross-presented the antigen, promoting strong systemic and local $CD8^+$ T-cell responses that conferred protective immunity against intranasal flu challenge. Delivery of mucosal vaccines and antiviral therapies may also be enhanced by achieving efficient encapsulation of vaccine cargo in particles. Saltzman and colleagues demonstrated that PLGA NPs achieving very high siRNA loading by pre-complexation of oligonucleotides with spermidine could achieve long-term delivery of siRNA in the vaginal mucosa.[151] Despite not being particularly engineered for mucus penetration, these NPs were dispersed throughout vaginal tissues and sustained gene silencing in areas both proximal and distal to the site of intravaginal application (Fig. 6B).

An alternative to the approach of engineering particles for more efficient penetration of mucus layers is to develop particles that are adhesive to mucus and/or underlying epithelial cells to prevent their premature clearance from the mucosal surface. For example, nanogels composed of pullulan with repeating cholesteryl groups complexed with protein antigen were recently developed to promote mucosal immunity to tetanus toxoid following intranasal application.[152] Modification of the nanogels with a cationic polymer backbone dramatically increased their attachment to the apical membrane of the nasal epithelium via enhanced ionic interactions with the epithelial cell layer (Fig. 6C). These cationic particles acted as an artificial chaperone and remained adhered mostly on the epithelium surface while releasing antigen to the underlining tissues, where > 40% of DCs in the local tissue

internalized the antigen and elicited strong immune responses. Antigen-loaded hydrophobic polyanhydride nanoparticles, which might act in a similar manner by binding to mucus via the hydrophobic domains of mucins, were recently shown to promote robust protection of mice against challenge with the plague bacterium *Yersinia pestis* following intranasal vaccination.[153] Intranasal and pulmonary administration of polyelectrolyte microcapsules[154] and liposomes[155] have also been demonstrated to target antigen to alveolar DCs and macrophages. Finally, another route of administration currently being examined for mucosal immunity is oral vaccination. The fate of anionic polylactide vaccine particles with 200 nm diameter has been tracked using a murine ligated ileal loop and an oral gavage model.[156] Particles initially entrapped in the mucus eventually crossed the epithelial barrier through M-cells, and accumulated in the Peyer's patches, where local B cells and DCs interacted with the particles. These results indicate that particulate formulations may offer a versatile delivery platform for mucosal vaccines.

4. Modifying immune reactions in tumors

The immune system exerts both beneficial and detrimental effects on tumor growth during all stages of cancer. In animal models, natural killer cells and $CD8^+$ T-cells have been shown to remove stressed or damaged cells that may become cancerous, and are especially crucial in preventing carcinomas caused by infectious agents such as human papilloma virus-induced cervical cancer. Cancer immunotherapy, the design of treatments directing the immune system to attack tumors, is gaining increasing interest with recent successes in the licensure of the first therapeutic cancer vaccine[157] and approval of an anti-CTLA-4 antibody that promotes anti-tumor immune responses.[158] Particles designed to deploy immunomodulatory drugs in the tumor environment or systemic lymphoid compartments may provide an important tool for further enhancing antitumor immunity. In addition, strategies leveraging immune cells themselves as delivery vehicles for active targeting of cancer therapeutics to the tumor microenvironment have recently been demonstrated.

4.1 Targeting immunomodulators to tumors with nanoparticles

While progressing primary tumors are often infiltrated by immune cells, these populations are often enriched in myeloid-derived suppressor cells, regulatory T cells, tumor-associated macrophages and other immunosuppressive cells actively recruited and co-opted by the tumor.[159] These co-opted cells secrete factors that fuel tumor growth and metastasis, as well as render tumor-infiltrating T cells ineffective or apoptotic. The genetic heterogeneity of tumors and their exploitation of multiple, redundant signaling pathways to block immune responses suggests that targeting single suppressive pathways will be unlikely to have major therapeutic benefit. Rather, strategies to reverse tumor immunosuppression will need to target "master" immune regulators that govern multiple pathways simultaneously. One promising target for immunosuppression blockade is the transcription factor STAT3, which is expressed by a high frequency of human cancers as well as tumor-associated stromal cells.[160,161] Constitutively active STAT3 in myeloid cells induces secretion of immunosuppressive factors and inhibits production of Th1 cytokines that are essential for anti-cancer immune responses. Thus, therapies aimed to silence STAT3 expression in either tumor or stromal cells may provide beneficial immune responses in the tumor microenvironment. NPs composed of PLGA directly conjugated with JSI-124, a small molecule inhibitor of STAT3, exhibited sustained drug release over one month, and suppressed activation of STAT3 in DCs, while promoting T cell proliferation in a mixed lymphocyte assay *in vitro*.[162] In addition to small-molecule drugs, siRNA, which has recently emerged as a powerful therapeutic modality for specific and effective downregulation of protein expression, can be delivered via NPs in a targeted manner and mediate profound immunomodulation in the tumor microenvironment. Modification of polyethylenimine (PEI) with stearic acid increased siRNA condensation and protected

siRNA against degradation in serum.[163] STAT3 siRNA complexed with stearate-modified PEI reduced the expression level of activated STAT3 in B16 melanoma cells and significantly suppressed tumor growth *in vivo*, by inducing IL-6 production and Caspase 3 activity, while diminishing VEGF secretion in tumors.[163] Toxicity associated with the cationic PEI was subsequently addressed by the same group, who have demonstrated that incorporation of siRNA-PEI polyplexes in PLGA NPs reduces the nonspecific toxicity profile of the polyplexes while maintaining the gene knockdown efficiency by siRNA in DCs (Fig. 7A).[164] Notably, treatment of tumor-tolerized DCs with these NPs blocked STAT3 activation in the cells and restored their function, as evidenced by increased TNF-α secretion and T cell priming (Fig. 7B). When combined with cancer vaccines, suppression-blocking therapies can also synergistically enhance anti-cancer immune responses and suppress tumor growth, as demonstrated in a murine model of breast cancer with anti-HER-2 DNA vaccine and NPs carrying a chemical inhibitor of STAT3.[165] Thus nanoparticles can be effective for targeting immunomodulatory drugs to multiple cell types within tumors.

A major challenge in immunotherapy is the high systemic toxicity often elicited by immunostimulatory cytokines or antibodies.[166] Local immunostimulation in the tumor microenvironment may provide a strategy to avoid such systemic toxicity, and it has been proposed that local treatments amplifying anti-tumor immunity at one tumor lesion may be capable of turning a tumor into its own vaccine, unleashing a systemic immune response that could lead to eradication of distant (untreated) metastases.[167–170] Micro- and nano-particles may be very useful in this context for confining strong stimulatory signals to the tumor or tumor-draining lymph nodes. For example, agonistic antibodies against the costimulatory receptor CD40 expressed by DCs and macrophages can elicit potent anti-tumor immune responses, but systemic administration of anti-CD40 elicits dose-limiting hepatic toxicity and systemic inflammatory effects, which have limited its clinical translation.[171–174] To address this issue, a liposome-based drug delivery platform was used to localize delivery of anti-CD40 and TLR agonists in the tumor microenvironment.[175] Liposome-anchored anti-CD40 and CpG oligonucleotides were synthesized by direct conjugation of anti-CD40 to PEGylated vesicles via maleimide-thiol reaction, followed by post-insertion of lipid-conjugated CpG (Fig. 7C). Liposomes bearing anti-CD40 and CpG on their surfaces suppressed tumor growth in the aggressive, poorly immunogenic B16F10 murine model of melanoma, and delayed tumor progression better than equivalent doses of soluble agonists when injected intratumorally (Fig. 7D). Importantly, unlike soluble antibody or oligos, anti-CD40/CpG anchored on liposomes sequestered these agonists within the tumors and tumor-draining lymph nodes after intratumoral administration. Blockade of drug leakage into the circulation eliminated systemic toxicities associated with these agents, including liver damage, weight loss, and systemic release of inflammatory cytokines (Fig. 7E, F). Thus, nanoparticles can enhance the efficacy of immunotherapy molecules by regulating their biodistribution.

4.2 Targeting tumors with particle-carrying leukocytes

A chaotic vasculature, high interstitial pressure and dense extracellular matrix can prevent efficient extravasation and diffusion of systemically-administered therapeutic particles into tumors.[176] Leukocytes overcome this transport obstacle by recognizing molecular markers of inflammation found on tumor vasculature and actively transmigrating into the tumor bulk. Taking advantage of this intrinsic trafficking behavior, strategies have been recently reported to functionalize live cells with synthetic materials and therapeutic molecules, which are then shuttled into tumors following adoptive cell transfer. This approach can be used to deliver drugs that act on the tumor, stroma, or the functionalized carrier cells themselves. For example, in a two-step pre-targeting strategy, macrophages “fed” gold nanoshells were

shown to infiltrate *in vitro* human breast carcinoma tumor spheroids, including the hypoxic necrotic center– a site devoid of functional vasculature that by definition cannot be effectively targeted by "free" nanoparticles administered systemically (Fig. 8A, B).[177] Irradiation with near-infrared light rapidly heated the engulfed silica-gold nanoshells and caused death of the carrier macrophages as well as bystander tumor cells. This "Trojan horse" approach is interesting not only as a strategy to directly kill tumor cells but also because macrophages that home to tumors are often co-opted by the tumor microenvironment to provide cytokines promoting tumor progression,[178] and thus their simultaneous ablation during irradiation may reinforce the anti-tumor effect of this therapy. In a similar vein, gold NPs were shown to be internalized by human T cells without impairing *in vivo* tissue homing, and increased NP accumulation in tumors by several fold in a xenograft model of lymphoma, compared to free particle injection (Fig. 8C).[179] Another approach is to rely on direct surface conjugation of tumoricidal NPs to tumor-infiltrating cells. As a conceptual demonstration, human mesenchymal stem cells (which can exhibit tumor tropism) were conjugated with polystyrene NPs via a biotin-streptavidin bridge.[180] These stem cells retained their NPs for up to two days and retained their ability to orient towards tumor spheroids *in vitro*. This strategy is generalizable to virtually any combination of tumor-infiltrating cells and therapeutic NPs, and may allow access to tumors deep in the tissue that are not easily penetrated by irradiation.

As described in section 2.1, *ex vivo*-expanded tumor-reactive T-cells are being used to treat metastatic cancer in adoptive cell therapy. Maintenance of the anti-tumor activity of T-cells following infusion of the cells into patients is typically achieved by the systemic administration of adjuvant drugs such as interleukin-2, which stimulate T-cell survival and effector functions.[25] However, such adjuvant drug treatments have significant toxicities that limit their efficacy. As one strategy to overcome this limitation, nanoparticles loaded with adjuvant cytokines were chemically conjugated to the surface of T-cells, enabling tiny doses of adjuvant drugs to be very efficiently provided to their parent lymphocyte while avoiding unwanted systemic exposure to these potent molecules.[17] Key to this approach is stable cell surface binding, so that cytokine cargo released from the nanoparticle can bind to cell surface receptors; internalization of the particle would physically prevent protein drug cargos from accessing their target receptors on the carrier cell.[181] Typically, specific binding to target cells is achieved by functionalizing particles with ligands which will bind to molecules expressed on the surface of the target cell, but often, ligand binding to proteins on the cell surface will trigger endocytosis of the particle.[27,182,183] However, by crosslinking nanocarriers to T-cells via thiol-reactive maleimide groups that conjugate to multiple cell surface proteins, lipid or polymer nanoparticles loaded with protein drugs could be stably attached to the surfaces of T-cells for up to a week, even during cell division (Fig. 8D, E).[17] T-cells thus decorated with cytokine-releasing nanoparticles carried nanoparticles into tumors, at levels greatly exceeding particle entry into tumor sites by passive diffusion/convection from the blood (Fig. 8F). These particle-decorated T-cells exhibited greatly enhanced proliferation *in vivo* compared to T-cells supported by equivalent doses of systemically-administered cytokine, leading to dramatic elimination of established tumors in mouse models of melanoma.[17] Thus, cell engineering with synthetic particles is a potent strategy for enhancing tumor therapy, and might be of interest in other settings of cell transplantation.

5. Nanoparticles for systemic delivery of antiviral, anti-inflammatory, and autoimmune therapies

In addition to vaccine delivery and tumor therapy, tailored nanoparticles are being explored in several additional contexts for treating infectious disease and autoimmunity. We will confine our discussion here to the use of particles to target drugs to immune cells or infected

host cells, and note that a large body of literature has also explored the use of nanoparticles as materials to target therapeutics that act directly on microbes, such as antimicrobial peptides and polymers, a topic outside the scope of this Progress article that has been reviewed elsewhere recently.[184–186] We will also discuss exciting recent studies exploring unexpected novel effects of lymphocyte-targeted nanoparticles in autoimmunity.

5.1 Targeting infections with nanoparticles

The blood-brain barrier (BBB) is a major obstacle in drug delivery to the brain and severely limits our ability to treat many diseases affecting the central nervous system (CNS), including HIV, glioma, and Alzheimer's disease. Targeted delivery of NPs carrying drugs to brain capillary endothelial cells, neurons, and the brain parenchyma have been recently demonstrated using various targeting moieties, including apolipoprotein E,[187] angiopep-2,[188] lactoferrin,[189] sialic acid residues,[190] and trans-activating transcriptor (TAT) peptides[191] with varying delivery efficiencies and therapeutic successes. To address variable BBB penetration by macromolecules or targeted NPs encapsulating drugs, a new strategy based on cell-based targeted delivery to the brain has been recently demonstrated.[18,192] In a murine model of a HIV-1 encephalitis, Gendelman and colleagues administered macrophages that were pre-incubated with NPs encapsulating the retroviral drug indinavir. The particle-pulsed macrophages crossed the BBB and shuttled the drug to HIV-infected subcortex regions, sustaining increased local drug concentration for 14 days and suppressing HIV-1 replication, in comparison to i.v. administration of the drug (Fig. 9).[18,192]

Systemic injection of particles loaded with antiviral drugs may deplete viral reservoirs in infected patients, providing a therapeutic means to control infection. Taking this approach one step further, Shankar and colleagues aimed to "vaccinate" mice against infection using particles loaded with siRNA to downregulate CCR5, a key receptor required for R5-tropic HIV strains to infect immune cells.[193] In a humanized mouse model of HIV, systemic administration of siRNA-loaded liposomes targeted to leukocytes via an antibody against the integrin lymphocyte function-associated antigen-1 silenced CCR5 expression *in vivo* for 10 days, and enhanced resistance to HIV infection as evidenced by reduction in plasma viral load and CD4 T-cell loss. Together, these studies demonstrate that NPs carrying antiviral agents can be delivered to desired tissue targets to control infection in both prophylactic and therapeutic settings.

5.2 Nanoparticle modulation of the innate immune system in autoimmune diseases

Autoimmune diseases are often accompanied by chronic inflammation that fails to resolve and contributes to tissue damage. Thus, strategies to deliver anti-inflammatory drugs selectively to innate immune cells in inflamed tissues and reverse their pathological phenotypes are of great interest in treatments against autoimmune diseases. Recently, nanoparticle-based platforms, such as liposomes and dendrimers, have been utilized as drug delivery carriers for small molecule anti-inflammatory agents[194,195], plasmids encoding for immunomodulatory proteins (OX40-TRAIL)[196], or peptide antigens[197] to suppress production of inflammatory cytokines and ameliorate clinical symptoms in animal models of rheumatoid arthritis and multiple sclerosis (experimental autoimmune encephalomyelitis). Moreover, it has been recently shown that drug-conjugated dendrimers with dual anti-inflammatory and anti-osteoclastic properties can relieve symptoms of rheumatoid arthritis without further exogenous biological or chemical agents.[198] Whereas conventional monoclonal antibodies or soluble cytokine receptors solely target inflammatory cytokines, dendrimers capped with azabisphosphonate were able to inhibit the secretion of pro-inflammatory cytokines by inflammatory monocytes and simultaneously block a signaling pathway essential for their differentiation into osteoclasts, thereby achieving a two-pronged

approach to inhibit inflammation and bone-resorption in two murine models of rheumatoid arthritis. Such strategies to develop therapies based on the innate biochemical properties of polymers on target immune cells should be investigated further toward the development of a clinical therapy against autoimmune diseases.

Several of the targeting strategies based on the intrinsic trafficking of nanoparticles or cellular chaperones discussed earlier have also been used to treat autoimmune and inflammatory conditions. For example, nanoparticle drug carriers administered intravenously will often accumulate preferentially in the spleen and bone marrow (in addition to the liver). Exploiting this innate tropism of NPs, siRNA was targeted to inflammatory monocytes in the spleen and bone marrow to suppress expression of the chemokine receptor CCR2.[199] Downregulation of CCR2 in monocytes with siRNA-NP therapy prevented accumulation of inflammatory monocytes and their differentiation into highly activated antigen-presenting macrophages at the sites of inflammation. Impressively, this single therapeutic approach reduced inflammation in atherosclerotic plaques, decreased infarct size after coronary artery occlusion, prolonged survival of pancreatic islet allografts after transplantation, and suppressed tumor growth.[199] Lastly, the tropism of DCs for lymph nodes as discussed earlier has been exploited to shuttle immunosuppressive drugs to lymphoid tissues in a selective manner.[200] DCs were incubated with NPs pre-loaded with cyclosporine-A, an immunosuppressive agent, and when infused back into animals, NP-carrying DCs migrated to draining lymph nodes, successfully suppressing proliferation of T-cells in the local lymphoid tissue without any significant systemic release of the toxic drug.

5.3 Nanoparticles as targeted therapies in autoimmunity

T-cells are critical effectors that play an important role in protecting the host via their ability to eliminate infected cells or destroy tumor cells, but they can also directly cause tissue damage in autoimmune diseases like type 1 diabetes and multiple sclerosis. Thus, strategies to inhibit T-cell functions are also of great interest. One strategy to suppress autoimmune reactions is to redirect the program of inflammatory T-cells. $CD4^+$ “helper” T-cells differentiate into subclasses that serve different functions in regulating immune responses– Th1 cells that help clear intracellular pathogens, Th2 and Th17 cells that deal with extracellular parasites, and Treg cells (regulatory T-cells) that suppress the effector functions of other T-cells. Th1, Th2, and Th17 cells have all been implicated in different autoimmune diseases. Importantly, it is now known that $CD4^+$ T-cells have the capacity to interconvert between these different effector programs in response to different cytokine cues.[201] This raises the possibility of using drug treatment to convert (for example) disease-causing inflammatory Th17 T-cells into regulatory T-cells, simultaneously blocking the unwanted function of the Th17 cell and introducing a new regulatory cell that may block the inflammatory activity of other effector T-cells in the tissue environment. Nanoparticles offer the possibility to target such identity-altering drugs to T-cell populations. For example, anti-CD4-targeted PLGA NPs loaded with leukemia inhibitory factor, a tolerogenic cytokine, were shown to expand $Foxp3^+CD4^+$ Tregs and prolonged survival of heart allografts between mismatched donor-recipient model *in vivo*.[202] In contrast, NPs loaded with IL-6 exerted the opposite effect, promoting $CD4^+$ Th17 cell development.

A key objective in treating autoimmune disease is the ability to selectively turn off only those T-cells attacking healthy tissue, in order to avoid generalized immunosuppression that cause lead to opportunistic infections. Because the antigen specificity of T-cells is determined solely by each cell’s unique T-cell receptor (TCR), targeting of disease-specific cells has been achieved by using self-antigen peptide-MHC (pMHC) complexes as disease-specific ligands that will bind the TCR of autoreactive T-cells. Notably, the low affinity of TCRs for pMHC (K_D values typically ~1–10 μM), means that multivalent display of pMHC from the surface of nanoparticles is an effective strategy to obtain high avidity, specific

binding to target T-cells.[203] Amphiphilic dendrimers with a hydrophobic core and PEG corona coupled to specific pMHC ligands have been used to deliver the DNA-binding toxin doxorubicin (sequestered in the dendrimer core) to antigen-specific T-cells, showing suppression of T-cell proliferation *in vitro* and *in vivo*.[203] More recently, it has been shown that pMHC ligands can themselves serve as both a targeting agent and the effector molecule to suppress autoreactive T-cells. This approach is based on the concept that naïve T-cells that are triggered through their TCR in the absence of costimulation (critical accessory signals normally provided in parallel by APCs) will be driven to undergo apoptosis or become anergic, i.e. unresponsive to antigen. Such tolerizing signals could in theory be provided by monomeric pMHC, but multivalent display of pMHC ligands on nanoparticles overcomes the low affinity of individual ligands for binding to target cells and may enhance delivery of tolerizing signals to the TCR by virtue of promoting TCR clustering on binding to the T-cell.[204] Santamaria and colleagues tested this concept and discovered that systemic injections of pMHC-conjugated iron oxide NPs could protect mice from autoimmune diabetes, but that rather than anergizing or deleting naïve disease-specific T-cells, the pMHC NPs induced expansion of pre-existing "autoregulatory" memory $CD8^+$ T-cells *in vivo*.[205] These memory cells were naturally produced in diabetic mice as a regulatory response by the immune system seeking to control the autoimmune reaction; injection of pMHC NPs specific for a target diabetes antigen triggered the expansion of these regulatory T-cells, which were then capable of suppressing the autoimmune attack on pancreatic islet cells, essentially providing a boost to the immune systems' own efforts to control the autoimmune disease. An important strength of this approach is that injection of NPs carrying a single type of pMHC (i.e. targeting T-cells reactive to only one disease-related peptide) led to suppression of autoimmune responses against multiple diabetes antigens– meaning that suppression of autoreactivity does not require a different NP for each potential disease antigen. This is critical for a broadly applicable strategy that could be used in the diverse human population (where treatments might need to account for the >2,000 different known MHC molecules in humans) and because the complete profile of autoimmune antigens involved in type 1 diabetes remain unknown. These examples illustrate the power of nanoparticle therapeutics to not only achieve therapeutic success but to also reveal important new aspects of immune physiology regulating disease states.

6. Conclusions and future outlook

Recent progress in the synthesis of multifunctional biodegradable/biocompatible particles has provided new momentum to translate discoveries from basic immunology into novel therapies and diagnostics for numerous diseases, including cancer, infectious diseases, and autoimmunity. As illustrated by the diverse examples discussed here, novel engineered nano-and micro-particles are showing promise as potent adjuvants for vaccines,[9,10,127,152] drug carriers for cancer immunotherapy,[164,175] and as systemic delivery vehicles with circulation times in excess of a week *in vivo*.[68,69]. In parallel with advances in particle design and synthesis, important new ways of *using* engineered particles are coming to light, such as using living cells as Trojan horse chaperones to carry drug-releasing particles into target tissues[18,177] or employing nanoparticles as cell surface modification reagents for cell therapies.[17,180] However, this rapidly moving field is still very young and these early advances also suggest additional new avenues that may be important areas of study in the coming few years.

One avenue ripe for investigation lies at the intersection of advances in the design of long-circulating particles and the design of particle vaccines. Vaccines are traditionally administered by parenteral injection at one or (at most) a few sites, meaning that often only a single lymph node may be involved in priming of adaptive immune responses. This is in contrast to infectious agents, which may systemically disseminate and elicit extremely

potent immune responses by involving many lymph nodes, Peyer's patches in the gut, and/or the spleen. Particles engineered to avoid rapid RES clearance which could release vaccine components systemically might provide a route to "systemic immunizations" that could recruit a large number of lymphoid organs into the immune response with a single injection. Further, future studies providing targeting abilities to long-circulating nano- and micro-particles by displaying monoclonal antibodies or other targeting agents could be used to preferentially target critical antigen presenting cells residing in the spleen, lymph nodes, and bone marrow, while avoiding clearance by the liver. Another parallel question is whether strategies to avoid particle uptake by the RES after systemic administration may be similarly applied to maximize delivery of particles to lymph nodes after parenteral administration. Thus, more studies are warranted to explore the impact of particle shape and modulus delineating the tissue draining patterns of particles and their impact on the immune system.

A second key challenge will be to build on recent strategies of using particles to target drugs or imaging agents to specific immune cells[202,205] or tissues,[9,127] with the goal of systemically modulating the immune system. Leukocytes are attractive targets for targeted particle therapies as they are present at very high concentrations in lymphoid organs that can be accessed by nonsurgical injections, and unlike parenchymal cells of many tissues, they recirculate through the blood. The clinical success of monoclonal antibodies targeting leukocyte-derived cancers is mediated in large part by the ready access of these therapeutics to isolated target cells in the blood (which can be contrasted by the poor ability of antibodies or nanoparticles to penetrate solid tumors). Antibody- or ligand-targeted particles should be capable of delivering immunomodulatory drugs to a majority of target immune cells, especially if particle properties are optimized to provide sufficient circulation half-life to these carriers, enabling either amplification or suppression of specific immune responses. Strategies to target antigen-specific disease-associated leukocytes (e.g., antigen-specific T-cells or B-cells) without triggering negative regulatory or suppressive signals in these cells will be an important challenge to overcome, as the only unique cell surface marker expressed by these cells is their antigen receptor, and as discussed above, nanoparticles that are best suited by virtue of their size for systemic targeting have been found to trigger anergy or regulatory differentiation when decorated with specific antigens that bind to T-cell receptors[205] (a boon for antigen-specific treatments for autoimmunity, but a bane for treatments aiming to bolster immunity).

Altogether, the significant progress made over the past few years at this exciting interface of immunology and materials science strongly suggests that nano- and micro-particles will provide new, effective means to treat and diagnose various diseases in the near future. Tackling these challenges will require continued innovation in materials design, with an increasing focus on problem-specific needs of individual diseases.

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Abbreviations

APC	antigen presenting cell
aAPC	artificial APC
BBB	blood-brain barrier

CNS	central nervous system
DC	dendritic cell
dLN	draining lymph node
EC	endothelial cells
ECM	extracellular matrix
HA	hemagglutinin
ICMV	interbilayer-crosslinked multilamellar vesicle
LN	lymph node
MRI	magnetic resonance imaging
MHC	major histocompatibility complex
MP	microparticle
NP	nanoparticle
pMHC	peptide-MHC
PCL	poly(caprolactone)
PDMS	poly(dimethylsiloxane)
PEG/PEO	poly(ethylene glycol)/poly(ethylene oxide)
PLGA	poly(lactide-co-glycolide)
PPS	poly(propylene sulfide)
PSA	poly(sebacic acid)
PEI	polyethylenimine
RBC	red blood cell
RES	reticuloendothelial system
siRNA	small interfering RNA
s.c	subcutaneous
TCR	T-cell receptor
TLR	Toll-like receptor

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Biographies

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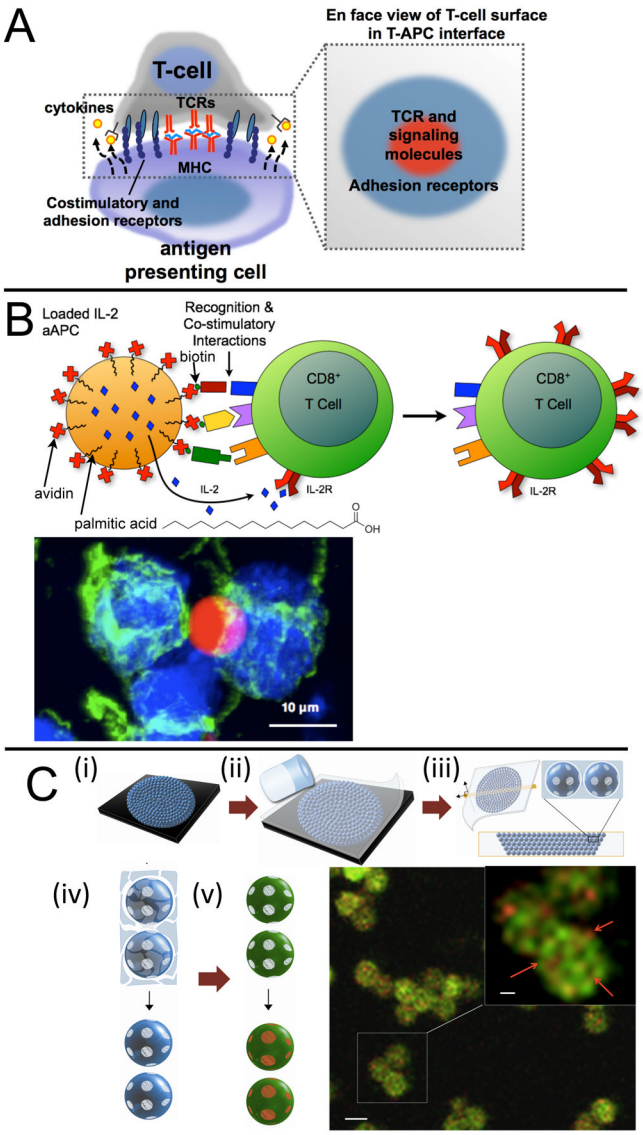

Figure 1. Engineered particles as synthetic antigen presenting cells

(A) Schematic view of key receptor-ligand interactions at the immunological synapse formed between an antigen presenting cell (APC, such as a dendritic cell) and a T-cell during T-cell activation. (B) Upper panel, schematic view of microparticles engineered as artificial APCs (aAPCs), which display ligands and release cytokines to stimulate T-cells. Lower panel, confocal microscopy view of immunological synapse formed between aAPC microparticle (red) and several T-cells (nuclei, blue; actin, green). (C) Fabrication of anisotropic “patchy” protein-coated microparticles by (i) forming colloidal crystals of microparticles, (ii) applying polydimethylsiloxane as a masking agent, (iii) PDMS masking at particle contact points, (iv) separation of particles from the scaffold, and finally (iv) two-step protein coating. Lower right, confocal micrograph illustrates dual protein patterning on patchy microspheres. Panel (B) reproduced with permission from [16]. Copyright 2008, Nature Publishing Group. Panel (C) reproduced with from [38]. Copyright 2011, Wiley.

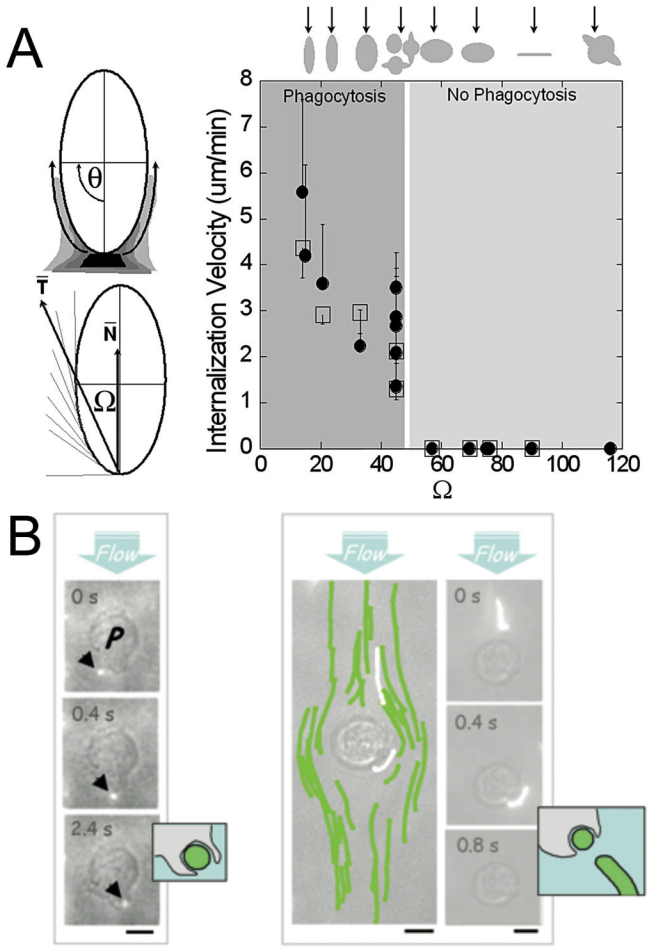

Figure 2. Modulation of particle interaction with phagocytes

(A) The effect of the contact angle between particles and cell membranes on the rate of particle internalization, demonstrating poor phagocytosis of highly anisotropic particles. (B) A flow chamber assay demonstrating rapid uptake of small, isotropic micelles by macrophages, but minimal uptake for long, flexible filomicelles. Scale bars, 5 μm. Panel (A) reproduced with permission from [47]. Panel (B) reproduced with permission from [69]. Copyright 2007, Nature Publishing Group.

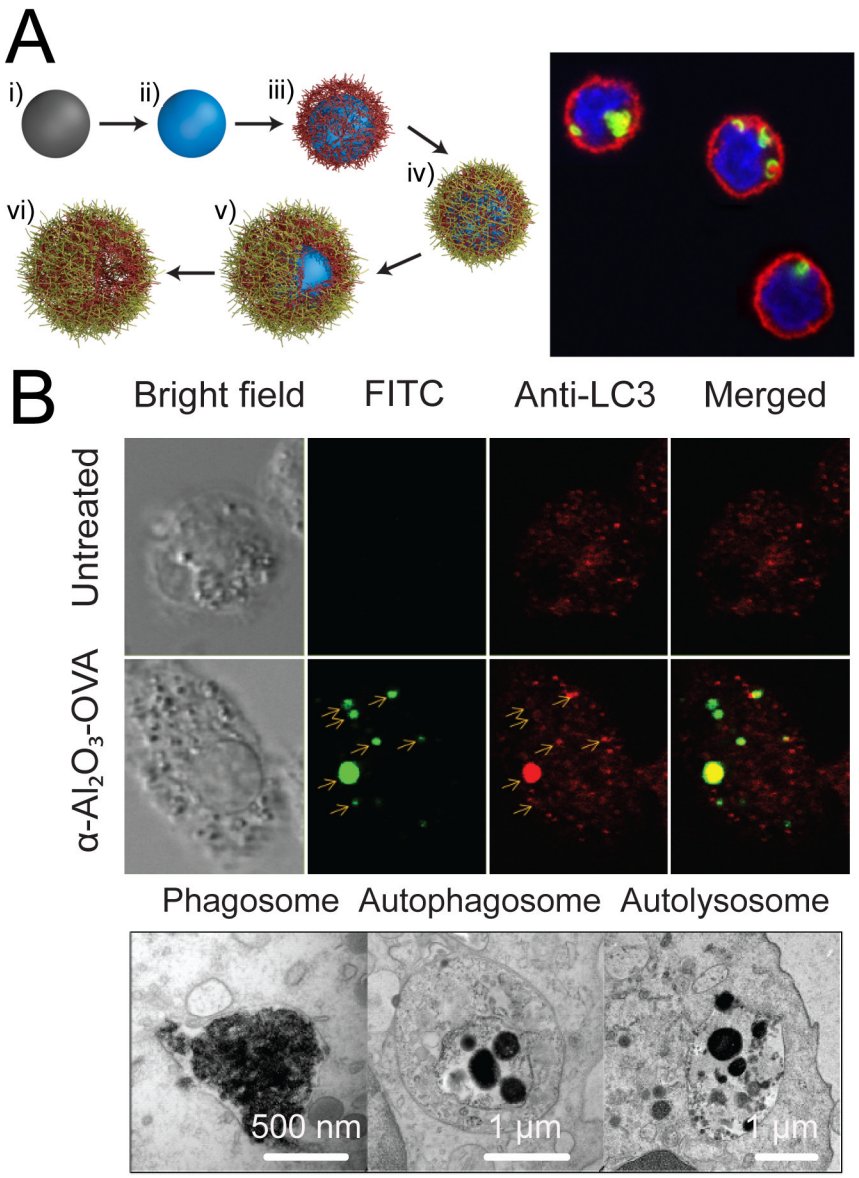

Figure 3. Active targeting of lymphoid organs with particle-carrying leukocytes

(A) Left panel, schematic of layer-by-layer capsule assembly by (i, ii) incubating a colloidal template with antigen, (iii, iv) alternate deposition of interacting polymers to form (v) a multilayered structure, followed by (vi) dissolution of core template. Right panel, confocal image of capsules (green) internalized into dendritic cells (membrane in red and nuclei in blue). (B) Upper panel, confocal images of untreated DCs, and DCs loaded with αAl_2O_3 NPs (green) and stained with antibody against the autophagosome marker, LC3 (red). Lower panel, TEM images showing that internalized αAl_2O_3 NPs are located inside endosomes/phagosomes, autophagosomes, and autolysosomes of DCs. Panel (A) reproduced with permission from [81]. Copyright 2008, Wiley. Panel (B) reproduced with permission from [61]. Copyright 2011, Nature Publishing Group.

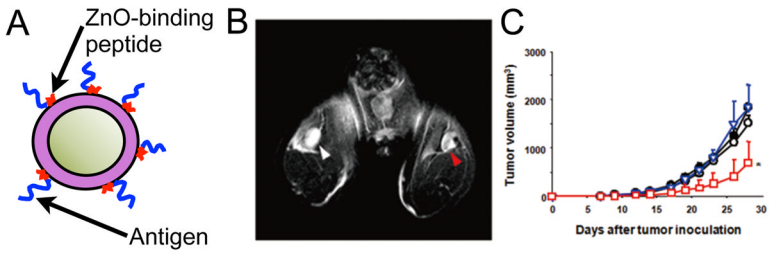

Figure 4. Active targeting of lymphoid organs with particle-carrying leukocytes

(A) Schematic illustration of Fe_3O_4-ZnO core-shell nanoparticles coated with tumor antigens fused to ZnO-binding peptides. (B) *In vivo* MRI image showing accumulation of dendritic cells labeled with nanoparticles in draining lymph nodes. (C) Enhanced suppression of tumor growth after injection of dendritic cells carrying tumor antigen-loaded iron oxide NPs (open red squares) compared to administration with antigen only (open blue triangles), DCs only (filled black circles), or NPs (open black circles). Reproduced with permission from [13]. Copyright 2011, Nature Publishing Group.

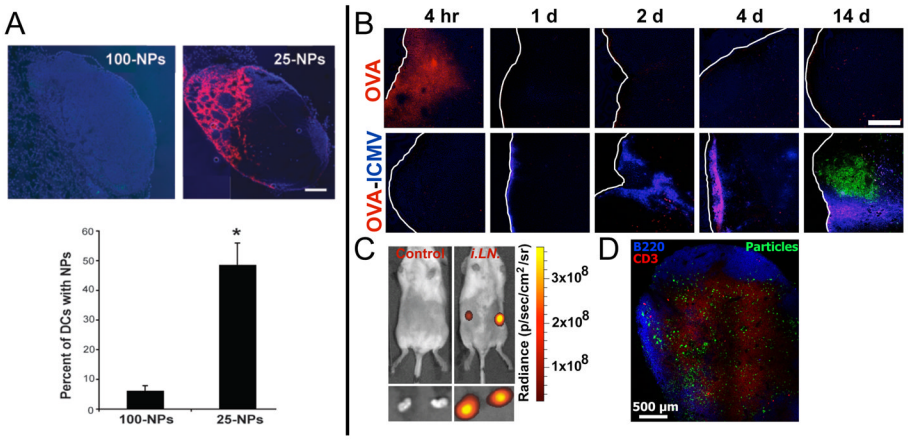

Figure 5. Delivery of vaccine particles to lymphoid organs

(A) Direct draining of sub-50 nm nanoparticles to lymph nodes from s.c. injection sites. Upper panel, histological sections of draining lymph nodes 1 day after administration of poly(propylene sulfide) NPs (red) with mean diameters of 100 nm or 25 nm. Scale bar, 200 μm. Lower panel, percentage of dendritic cells that internalized PPS NPs in the lymph node. (B) Histological section of draining lymph nodes over time after s.c. injection of either soluble antigen (ovalbumin, shown in red) or antigen encapsulated in180 nm diam. multilamellar lipid nanoparticles (blue). Germinal centers in the LN detected by staining with GL-7 antibody on day 14 are shown in green. Scale bar, 10 μm. (C) Intranodal administration of fluorescent PLGA microparticles detected by whole-animal fluorescence imaging (upper panels) or imaging of excised intact lymph nodes (lower panels). (D) Histological section of lymph node following direct intranodal injection of fluorescent PLGA particles (green) with staining for markers of B-cells (B220, blue) and T-cells (CD3, red). Panel (A) reproduced with permission from [9]. Copyright 2007, Nature Publishing Group. Panel (B) reproduced with permission from [121]. Panel (C) reproduced with permission from [127]. Panel (D), courtesy of C.M. Jewell, S.C. Bustamante López, and D.J. Irvine.

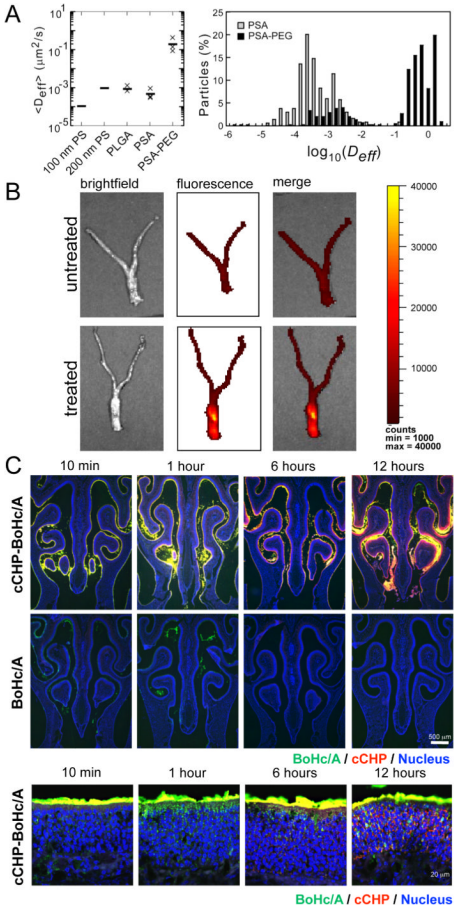

Figure 6. Particles designed to penetrate mucosal barriers

(A) Improved mucus-penetrating ability of poly(sebacic acid) (PSA) NPs after PEGylation as evidenced by increase in effective diffusivity and fraction of particles penetrating human cervicovaginal mucus. (B) Fluorescence image of reproductive tract on day 1 after intravaginal administration of siRNA-loaded PLGA NPs. (C) Mucus-binding particles for intranasal vaccine delivery. Upper panel, wide distribution and attachment of antigen-loaded nanogels on nasal epithelium after intransal administration. Scale bar, 500 um. Lower panel, release and transport of antigen (green) from nanogels (red) into the epithelial layer over time. Scale bar, 20 um. Panel (A) reproduced with permission from [142]. Panel (B) reproduced with permission from [151]. Copyright 2009, Nature Publishing Group. Panel (C) reproduced with permission from [152]. Copyright 2010, Nature Publishing Group.

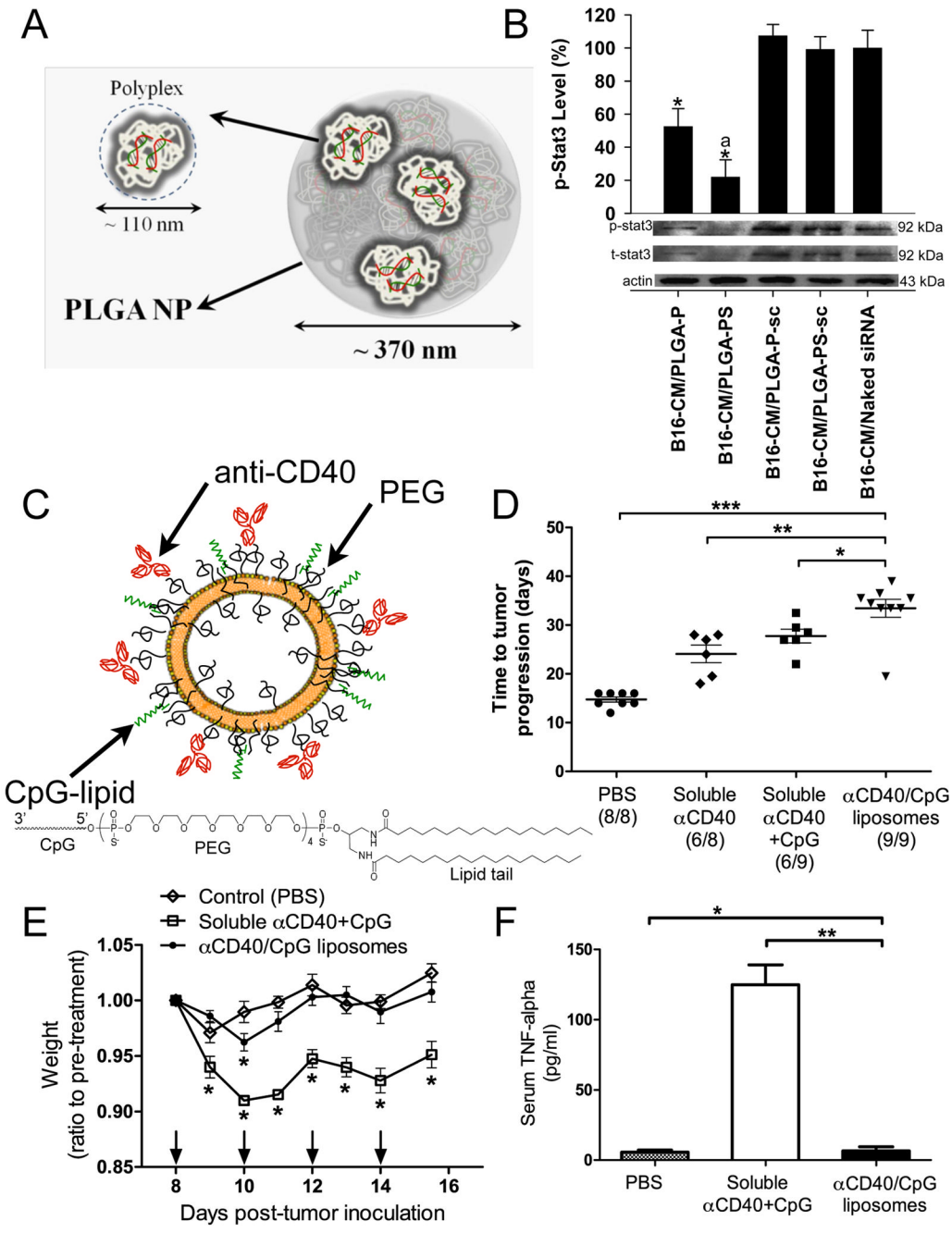

Figure 7. Nanoparitcle delivery of immunomodulatory drugs in tumors

(A, B) Encapsulation of STAT3-sirNA/PEI polyplexes in PLGA nanoparticles to reduce cytotoxicity while maintaining gene silencing activity. (A) Schematic view of polyplex encapsulation. (B) Kockdown of STAT3 in dendritic cells by encapsulated polyplexes, compared to controls with scrambled siRNA (sc) or naked siRNA. (C–F) Blockade of systemic side effects by anchoring immunostimulatory ligands to lipid vesicles for intratumoral injection. (C) PEGylated liposomes displaying aCD40 and CpG were synthesized by surface-conjugation of anti-CD40, followed by post-insertion of CpG-lipid conjugates into the outer leaflet of the vesicle bilayer. Suppression of tumor growth (D) without weight loss (E), liver damage (not shown), or systemic cytokine release (F) after

intratumoral injection of liposomes displaying anti-CD40 and CpG, compared to equal doses of soluble ligands. Panels (A, B) reproduced with permission from [164]. Copyright 2010, American Chemical Society. Panel (C–E) reproduced with permission from [175]. Copyright 2011, Elsevier.

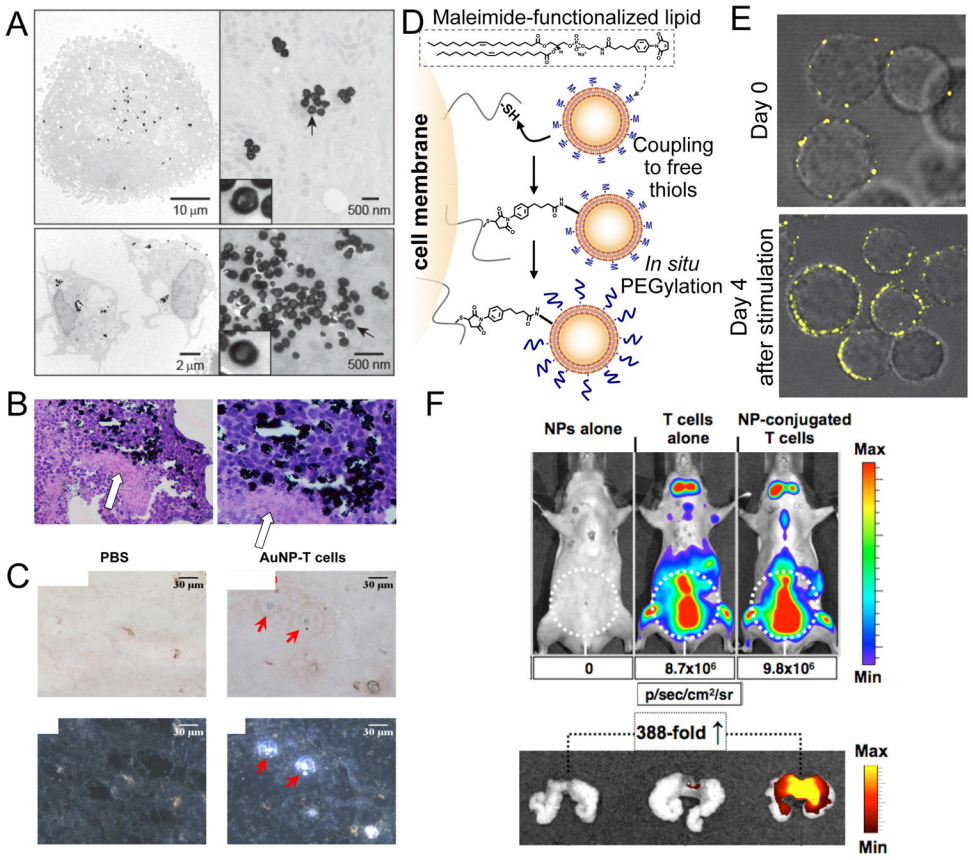

Figure 8. Leukocyte-mediated delivery of nanoparticles to tumors

(A, B) Gold nanoshells transported into tumors by macrophages for photothermal therapy. (A) TEM micrographs of a gold nanoshell-laden macrophage (upper panel) and monocytes (lower panel). Higher magnification views at right show aggregates of nanoshells inside the cells. (B) Histological tissue section of T47D tumor spheroid showing infiltrating nanoshell-laden macrophages (black) within the viable tumor as well as near areas of necrosis (pink staining; white arrow). (C) T-cells can carry internalized gold nanoparticles into tumors. Resected subcutaneous LCL xenograft tumors were analyzed by bright field imaging (top row) and immunohistochemistry for human CD3 expression and dark field imaging (bottom row) to indicate the presence of gold NPs. Red arrows indicate the colocalization of $CD3^+$ T cells and AuNPs within the tumor. (D–F) T-cells carry surface-bound nanoparticles into tumors *in vivo*. (D) Lipid nanoparticles were stably conjugated to the surfaces of T-cells via maleimide-thiol reaction. (E) Nanoparticles remained on the surfaces of T-cells after 4 days of stimulation *in vitro*. (F) TRAMP mice bearing spontaneous prostate tumors were injected with fluorescent lipid NPs alone, Luciferase-expressing tumor-targeting T-cells alone, or luc-expressing T-cells carrying surface-bound NPs. Upper panels, whole-animal bioluminescence imaging of T-cell trafficking to the prostate tumors (dashed circles). Lower panels, fluorescence imaging of dissected intact prostates showing that free NPs achieve no entry into tumor site, while T-cells carry substantial quantities of particles into the tumor. Panels (A,B) reproduced with permission from [177]. Copyright 2007, American Chemical Society. Panel (C) reproduced with permission from [179]. Panel (D,E) reproduced with permission from [17]. Copyright 2010, Nature Publishing group. Panel (F), courtesy of M. Stephan, E. Higham, K.D. Wittrup, and J. Chen.

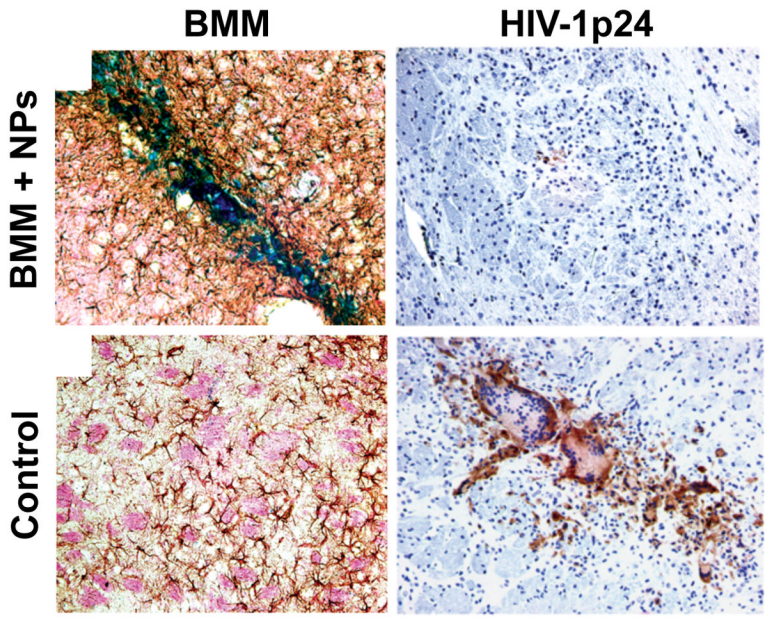

Figure 9. Crossing blood-brain-barrier with particle-carrying leukocytes

Bone marrow macrophages (BMM) loaded with drug-carrying nanoparticles have been used to treat HIV-1 infection in the brain. (Left panels) Histological images demonstrating migration of macrophages loaded with iron oxide NPs (blue staining) into neuroinflammatory HIV-1-infected brain sites (upper panels), but not control brain sections (lower panels). Right panels, dramatic reduction in HIV-1 infected brain sites (detected by staining for HIV p24 protein, blue) after treatment with macrophages carrying NPs loaded with anti-retroviral drugs. Reproduced with permission from [18]. Copyright 2009, American Association of Immunologists.