

Applications of nanotechnology for immunology

Douglas M. Smith^{1,2*}, Jakub K. Simon^{3*} and James R. Baker Jr^{1,3}

Abstract | Nanotechnology uses the unique properties of objects that function as a unit within the overall size range of 1–1,000 nanometres. The engineering of nanostructure materials, including nanoparticles, nanoemulsions or nanotubules, holds great promise for the development of new immunomodulatory agents, as such nanostructures can be used to more effectively manipulate or deliver immunologically active components to target sites. Successful applications of nanotechnology in the field of immunology will enable new generations of vaccines, adjuvants and immunomodulatory drugs that aim to improve clinical outcomes in response to a range of infectious and non-infectious diseases.

Nanometre

(nm). A unit of measurement equal to one billionth of a metre. It is the unit of measurement for many molecular structures that are relevant to immunology.

¹Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan Medical School, 9220 Medical Sciences Research Building III, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109–5648, USA

²Department of Internal Medicine, University of Michigan Medical School, 9220 Medical Sciences Research Building III, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109–5648, USA.

³NanoBio Corporation, 2311 Green Road, Suite A, Ann Arbor, Michigan 48105, USA.

*These authors contributed equally to this work.

Correspondence to D.M.S. e-mail:

smitdoug@med.umich.edu

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Biological molecules such as oligonucleotides, polysaccharides, and proteins that function as antigens, allergens or pathogen-associated molecular patterns (PAMPs) are of nanometre (nm) size^{1,2} (TABLE 1). In this Review we discuss how the size, shape, charge, porosity and hydrophobic properties of a compound influence its effects on the immune response and how nanotechnology can be used to engineer each of these properties (FIG. 1). We review the role of nanotechnology with respect to its applications in the development of vaccines and immunosuppressive agents, and we highlight how the manipulation and control of objects at the nanoscale level (BOX 1) can contribute to our understanding, as well as to our targeting, of the immune response. The field of nanotechnology is vast, therefore including all of the relevant studies is beyond the scope of this Review. However, we provide examples of selected studies that show the broad range of immunological applications of nanotechnology.

Nanotechnology in vaccination

Inactivated vaccines typically incorporate adjuvants, which are substances that enhance the quantity and the quality of the cellular and humoral immune responses generated against the antigens that are included in the vaccine. Nanoparticles provide adjuvant activity by enhancing the delivery of antigens to the immune system or by potentiating innate immune responses (FIG. 2). We discuss some of these nanotechnologies in the section below (FIG. 3; TABLE 2). Some nanotechnologies that improve vaccine efficiency, such as virus-like particles (VLPs) and MF59 (Novartis), have been used for decades, whereas others are still at the early stages of development.

VLP-based vaccines. VLPs are diverse and highly versatile nanoparticles (20–100 nm in size) that have been used in vaccines against infectious diseases and cancer (TABLE 2). More recently, VLPs have been used in vaccines that aim to protect against chronic diseases that have an inflammatory basis, such as hypertension, Alzheimer's disease and rheumatoid arthritis, and also in vaccines against drug addiction^{3,4}. VLPs used in vaccines can be broadly divided into two classes: in one class, VLPs that comprise the viral protein subunits that form the viral capsid in nature; and in another class, synthetic VLPs that are derived by the chemical synthesis of pre-designed subunits as described below.

VLPs can be distinguished from viruses by their absence of genetic material and their inability to replicate or to undergo genetic recombination. More than 20 to 30 different enveloped or non-enveloped VLPs are currently in preclinical and clinical development⁴. VLPs can be produced either without modification or by genetically engineering the viral capsid subunit by bioconjugation of the viral capsid subunit with antigenic peptides or other ligands or by site-directed mutagenesis of the intact VLP to create a functional scaffold for multivalent surface presentation of antigens. Other structures can be incorporated into VLPs, such as Toll-like receptor (TLR) ligands, cell-targeting moieties or other biologically active mediators, to augment vaccine efficacy. In human clinical trials, VLPs loaded with synthetic A-type CpG-oligonucleotides (CpG-ODNs) and peptide epitopes derived from melanoma-associated antigens, such as melanoma antigen recognized by T cells 1 (MART1), have been shown to activate multifunctional central memory T cells and cytotoxic T lymphocytes (CTLs) that produce interferon- γ (IFN γ), tumour necrosis factor- α (TNF) and interleukin-2 (IL-2)⁵.

Table 1 | Biological scale of immunologically relevant materials

Structure	Size	Refs
Molecules		
DNA	1–3 nm	1,2
Polysaccharides	200–1,000 nm	125
Proteins	2–10 nm	126
Receptors		
TLRs	2–10 nm	127
Antibodies	10–15 nm	128
TCRs	10–15 nm	129
Pathogens		
Viruses	10–200 nm	130
Bacteria	0.1–8 μ m	130
Fungi	1–100 μ m	131
Protozoa	1–100 μ m	132
Cells		
Dendritic cells	10–22 μ m	133
Macrophages	10–22 μ m	134
B cells	7–10 μ m	135
T cells	7–10 μ m	136
Neutrophils	8–15 μ m	137
Eosinophils	10–12 μ m	138

TCR, T cell receptor; TLR, Toll-like receptor.

Several of the advantages of using VLPs and other nanoparticles in vaccines are related to the unique nanodimensional size, symmetrical shape, uniformity and stable structure of the assembled nanoparticles, which closely resemble native viruses. These nanoparticles have been used to enable the preferential uptake of vaccine antigens by antigen-presenting cells (APCs). Notably, smaller nanoparticles (25–40 nm in size)⁶ penetrate tissue barriers and traffic to the draining lymph nodes more rapidly than larger nanoparticles (greater than 100 nm in size), which are typically retained by cells at the site of injection and which need to be taken up and trafficked by dendritic cells (DCs) to facilitate their transport to the lymph nodes^{6,7} (FIG. 2). As such, smaller nanoparticles might lead to more effective activation of adaptive immune responses^{7–9}.

In combination with specific cell-targeting strategies, nanoparticles also seem to be more effective than microparticles at enhancing antigen uptake by cells of the immune system; for example, when nanoparticles or microparticles were coated with DC-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209)-specific monoclonal antibody to target them to DCs, the DC-SIGN-coated nanoparticles were effectively targeted to DCs in a mixed population of human blood cells, whereas the DC-SIGN-coated microparticles were endocytosed in a nonspecific manner¹⁰. Furthermore, whereas most extracellular proteins that are taken up by DCs are processed and presented via the MHC class II pathway, the uptake of nanoparticles

such as VLPs promotes DC activation and the presentation of antigens on both MHC class I and MHC class II molecules. Importantly, this leads to the priming of both antigen-specific CD4⁺ and CD8⁺ T cells^{4,11–14} (FIGS 2, 3).

Two prophylactic VLP-based vaccines have been licensed worldwide: one prevents hepatitis B virus (HBV) infection and the associated risk of hepatocellular carcinoma^{15–17}; the other prevents human papillomavirus (HPV) infection and its sequela, cervical cancer¹⁸. The use of a recombinant protein engineered to self-assemble into a VLP in the recombinant HBV vaccine was a major advance in safety, and the plasma-based HBV vaccine has now been replaced¹⁵. However, this first generation of VLP-based vaccines requires adjuvants for optimal immunogenicity in healthy individuals and is not highly effective in high-risk populations such as immunosuppressed patients and elderly individuals. In addition, the HBV vaccines are highly effective as a prophylactic vaccine but ineffective as a therapeutic vaccine. The formulation of HBV vaccines with aluminium (alum) adjuvants is a major limitation with respect to the effective induction of T cell-mediated immunity against chronic hepatitis B, as alum predominantly activates T helper 2 (T_H2)-type immune responses^{19,20}. The HPV vaccine that has more recently been developed uses monophosphoryl lipid A (MPL) added to alum as an adjuvant to induce a T_H1-type immune response and CTLs that are associated with antiviral immunity^{19,21–25}.

Although current VLP-based vaccines are only prophylactically efficacious but have limitations for use in therapeutic applications, these vaccines have an excellent safety record²⁶. Recombinant HBV vaccines have been licensed since 1986 and hundreds of millions of doses have been administered worldwide. Pitfalls of advancing any new technology, such as novel adjuvants that enhance the immune response, include the potential risks of inducing rare, but serious, adverse reactions that only become evident after the evaluation of hundreds of thousands of individuals. Concerns about the induction of autoimmune diseases, including multiple sclerosis, Guillain-Barré syndrome and rheumatoid arthritis, following VLP-based vaccination have been raised but have not been confirmed²⁷. In a systematic review of the association between multiple sclerosis and hepatitis B vaccination, 1 out of 12 studies reported an association. The authors of that review concluded that there was insufficient evidence to modify vaccination schedules and called for additional studies to be carried out²⁷. VLPs and other emerging nanoparticle-based technologies provide new strategies to enhance the induction of potent, long-lasting and potentially broadly reactive (multiclude neutralization) humoral and cellular immune responses that will be crucial to achieve protection and therapy against chronic infections, rapidly mutating pathogens such as influenza and HIV-1, and late-stage malignant diseases as discussed below. These benefits must be balanced against any real or perceived risks so that the use of the vaccine is high and a benefit to public health is achieved.

Nanoparticles

Small particles of non-biological origin typically with at least one dimension that is less than 100 to 1,000 nm; examples include diverse compounds such as dendrimers, carbon fullerenes, solid lipid nanoparticles, nanoemulsions and biodegradable poly(lactide-co-glycolide).

Central memory T cells

Antigen-experienced CD8⁺ T cells that lack immediate effector functions but that are able to mediate rapid memory responses. They also rapidly develop the phenotype and the function of effector memory cells after re-stimulation with antigens. Central memory T cells retain the migratory properties of naive cells and therefore circulate through the secondary lymphoid organs.

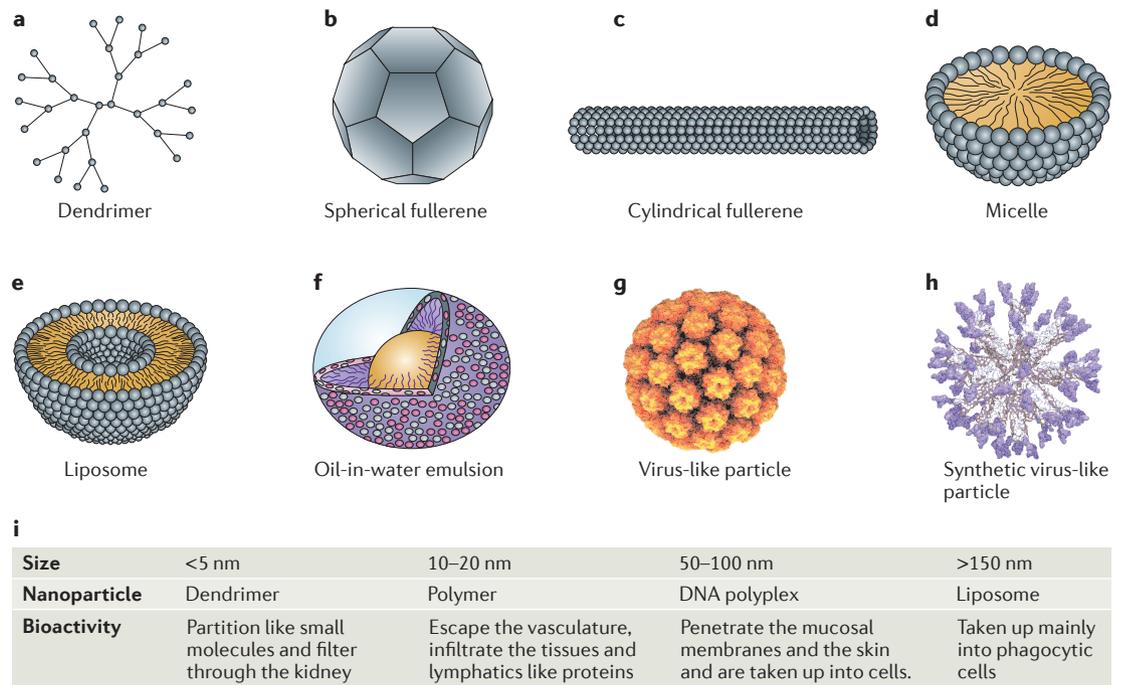


Figure 1 | Examples of nanotechnologies applied to immunoregulation. Nanotechnologies that can be applied to immunoregulation include nanoparticles (parts a–c), nanoemulsions (parts d–f) and virus-like particles (parts g–h). Nanoparticles include dendrimers which branch out (part a), carbon molecules known as spherical fullerenes (part b) and cylindrical carbon molecules known as cylindrical fullerenes (part c). Nanoemulsions incorporate immiscible components such as oil and water that might form amphiphilic molecules such as micelles (part d), liposomes with a lipid bilayer (part e) and oil-in-water emulsions (part f). Virus-like particles are self-assembled structures composed of one or more viral capsid proteins (part g), whereas synthetic virus-like particles are self-assembled from chemically synthesized components (part h). Examples of the relationship between nanoparticle size and bioactivity are shown in (part i).

Synthetic VLPs. The development of fully synthetic VLPs using chemically synthesized lipopeptide monomers²⁸ to enhance nanoparticle assembly and to stabilize the three-dimensional conformational structure of protein antigens provides an intriguing vaccine strategy to stimulate neutralizing antibodies against HIV-1. In contrast to enveloped HIV-1 VLPs, which are limited by low Env density per virion^{4,29,30}, or to the self-assembling peptide nanoparticles (SAPNs) described below, the synthetic approach does not require recombinant DNA technology or the expression and the purification of the monomer proteins from producer cells. Lipopeptide-based synthetic VLPs (20–30 nm in size) have been used to repetitively display a peptide-mimetic epitope derived from the V3-variable loop of gp120 (REF. 31). This

engineered epitope was designed by modelling the stable three-dimensional β -hairpin conformation that is formed after the binding of a broadly cross-neutralizing human monoclonal antibody to the gp120 antigen. This synthetic VLP also incorporated a universal T_H cell epitope³² (this immunogenic peptide promiscuously binds to multiple different MHC class II molecules to improve the induction of T_H cells) and a tripalmitoyl-S-glyceryl cysteine (Pam3Cys) lipid moiety, which induces TLR2 activation. Interestingly, the immunization of New Zealand white rabbits with these synthetic VLPs alone induced the production of neutralizing antibodies against the envelope proteins of multiple HIV-1 strains³¹. Thus, structural vaccinology and other strategies for immunogen design^{29,33}, in combination with repetitive antigen display using nanoparticle-based technologies as a vaccine platform, might enable the induction of responses against poorly accessible but conserved neutralizing epitopes rather than against more readily accessible immunodominant non-neutralizing epitopes expressed on the native gp120 spike²⁹. Responses against these poorly accessible epitopes are required to overcome the extraordinary mutation rate and the diversification of HIV-1 during the course of infection and to prevent viral escape.

The design of synthetic nanoparticles to incorporate lipid moieties for the conformational stabilization of protein antigens, such as the membrane-proximal external region of gp41, is also of great interest as certain

Box 1 | Defining nanotechnology

Definitions of the exact size range that the field of nanotechnology covers have been determined on the basis of size as well as function. The US National Nanotechnology Initiative aims to expedite the discovery, the development and the deployment of nanoscale technology for public benefit, and defines nanotechnology on the basis of size alone, using the range of 1 to 100 nm¹²². Other groups, including the US Food and Drug Administration (FDA), define nanotechnology on the basis of scale and function, using the range of 1 to 1,000 nm, provided that the physical, chemical or biological effects of the material in question are attributable to its dimensions¹²³. The European Medicines Agency (EMA) initially defined nanotechnology in the range of 0.2 to 100 nm in size but has broadened the definition to less than 1,000 nm in size¹²⁴.

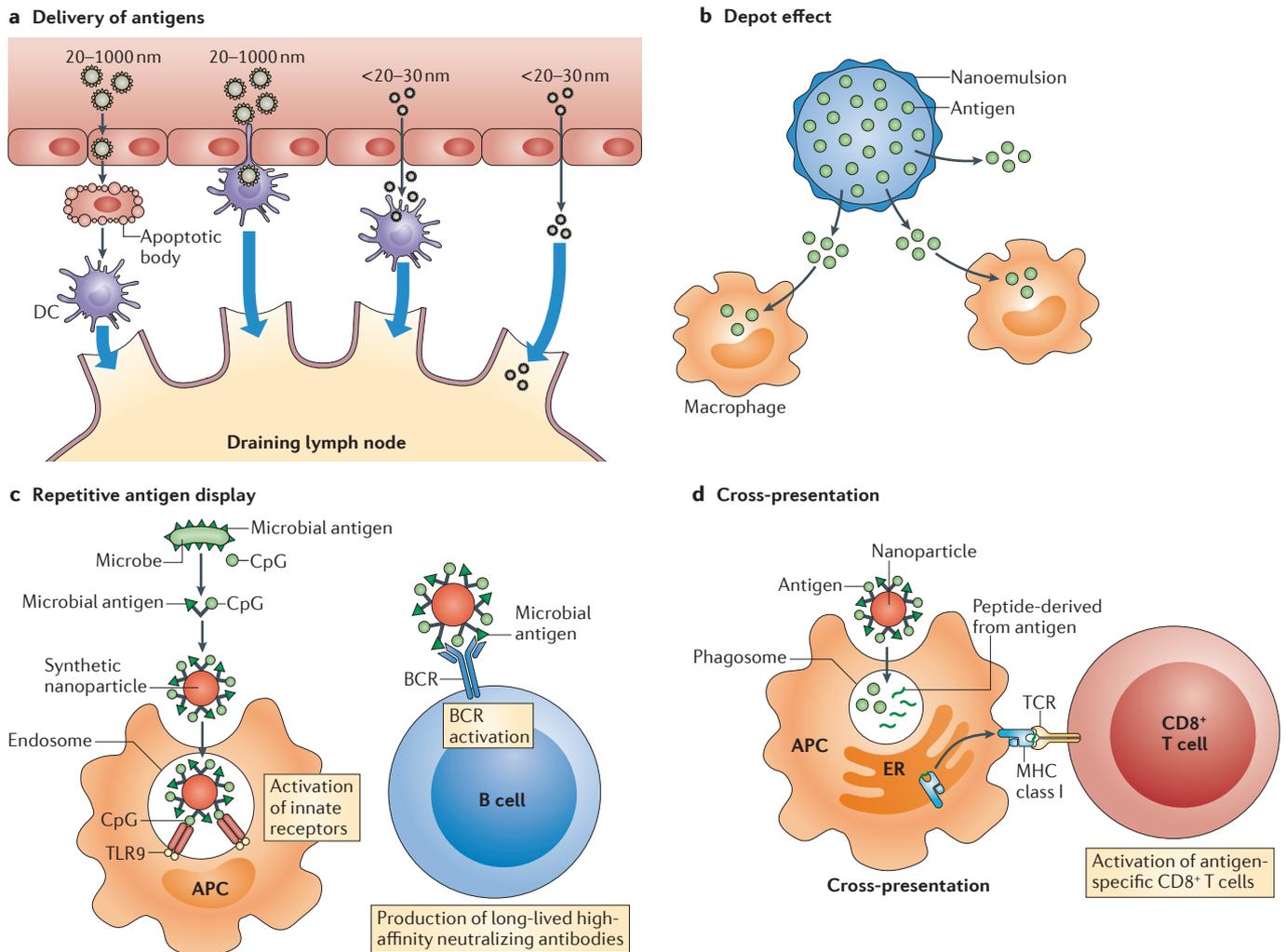


Figure 2 | Mechanisms by which nanoparticles alter the induction of immune responses. The immunostimulatory activity of nanoscale materials has been attributed to diverse mechanisms: the delivery of antigens, including particle size-dependent tissue penetration and access to the lymphatics (part **a**); a depot effect, which promotes the persistence, the stability, the conformational integrity and the gradual release of vaccine antigens (part **b**); and repetitive antigen display in which the spatial organization of the antigens on the particle surface facilitates B cell receptor (BCR) co-aggregation, triggering and activation (part **c**). Additional mechanisms associated with innate immune potentiation include Toll-like receptor (TLR)-dependent and TLR-independent signal transduction (not shown); cross-presentation, which is a mechanism by which exogenously acquired-antigens are processed into MHC class I pathways, caused by the nanoparticle-mediated leakage of antigens into the cytosol after they have been taken up by the phagosome (part **d**); and the release of soluble mediators such as cytokines, chemokines and immunomodulatory molecules that regulate the immune response (not shown). APC, antigen-presenting cell; DC, dendritic cell; ER, endoplasmic reticulum; TCR, T cell receptor.

broadly reactive HIV-1-neutralizing antibodies that interfere with Env-mediated viral membrane fusion have been shown to engage with both membrane lipid and protein components^{34,35} that contribute to native antigenic structure. These interactions potentially enable the generation of improved protective immune responses to the vaccine. Finally, new design strategies for nanoparticle-based vaccines are needed to focus immune responses against conserved CTL epitopes, as cell-mediated immune responses, together with broadly neutralizing antibodies, are associated with the initial control of HIV-1 infection and other viral diseases³⁶.

Nanoparticle-based vaccine carriers. Several different types of nanoparticles have been used either alone as vaccine carriers to entrap antigens or together with other agents, such as humanized DC-specific antibodies¹⁰ or immunomodulators, to enable targeted antigen delivery and the activation of APCs³⁷. Examples of nanoparticles that have been used as vaccine carriers include biodegradable poly(lactide-co-glycolide) (PLGA) nanoparticles, co-polymer hydrogels or ‘nanogels’, cholesterol-bearing hydrophobized pullulan (CHP) and cationic liposomes. Polymers have been used in nanotechnology as nanoparticles, nanospheres and nanocapsules. Vaccine carriers composed of PLGA

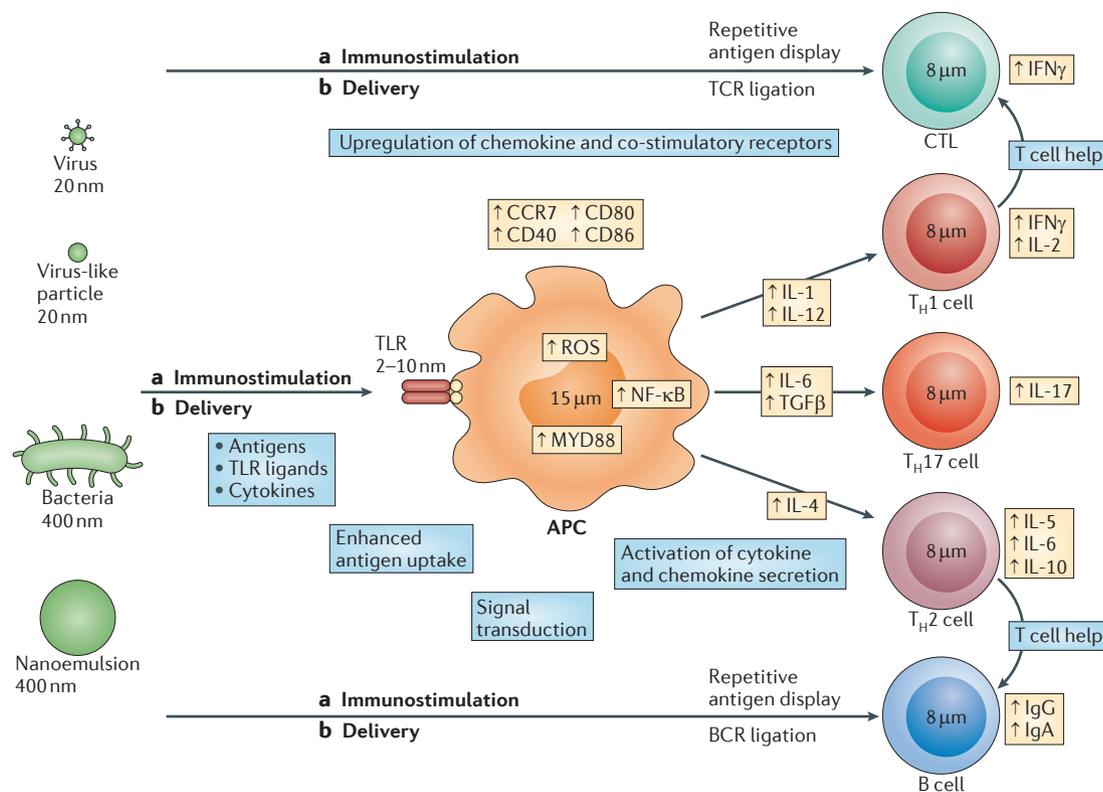


Figure 3 | Nanoscale immune activation. Nanoscale products might have a direct immunostimulatory effect (part a) on components of the immune system, including antigen presenting cells (APCs), B cells or T cells; they might deliver compounds that result in immunostimulation (part b); or they might use both mechanisms at the same time. Direct effects include the upregulation of homing receptors such as CC-chemokine receptor 7 (CCR7) and co-stimulatory molecules including CD40, CD80 and CD86, which results in the enhanced secretion of cytokines and increased B cell and T cell activation. Enhanced delivery of antigens and adjuvants might result in apoptosis or necrosis, which enhances vaccine immunogenicity. The pathways shown are representative examples of how different nanoparticles might activate the immune system. BCR, B cell receptor; CTL, cytotoxic T cell; IFN γ , interferon- γ ; IL, interleukin; MYD88, myeloid differentiation primary-response protein 88; NF- κ B, nuclear factor- κ B; ROS, reactive oxygen species; TCR, T cell receptor; TGF β , transforming growth factor- β ; T_H, T helper; TLR, Toll-like receptor.

have been extensively characterized in animal models and are widely used in clinical applications as a matrix to encapsulate, co-deliver and gradually release active drugs³⁸. In vaccine development, pegylated PLGA (150–200 nm in size) has been used to encapsulate hepatitis B surface antigen (HBsAg), and it promotes the rapid uptake and the endosomal localization of vaccine antigens in DCs, as well as the subsequent production of high titres of antigen-specific antibodies³⁹.

A thermosensitive biodegradable hydrogel consisting of the monomethoxy form of pegylated PLGA (less than 100 nm in size) has recently been used in mice to achieve the subcutaneous delivery of HBsAg at the injection site at the same time as the local and sustained release of granulocyte/macrophage colony-stimulating factor (GM-CSF), which is a crucial cytokine for the survival, the differentiation and the maturation of DCs⁴⁰. This vaccine enhanced the recruitment of DC precursor cells to the site of injection and promoted the maturation and the migration of CD11c⁺ DCs to the local draining lymph nodes. This was followed by the strong induction of HBsAg-specific antibody and T cell responses, even in mice that do not normally generate

immune responses against HBsAg or when low concentrations ($\leq 2 \mu\text{g}$) of HBsAg were used. Importantly, this suggests that this adjuvant could potentially be used in vaccines that incorporate poorly immunogenic antigens or in vaccines for individuals who have an impaired immune system. The ability to protect antigens or other bioactive mediators, to maintain the native conformation of these components at the site of immune challenge and to create a depot effect with the gradual local release of antigens over time is a key advantage for these types of nanoparticles. Biodegradable nanoparticles have also been shown to be safe and biocompatible for use in vaccine technology³⁸.

SAPNs. The unique properties of certain microbial pathogens and VLPs have inspired and guided the systematic modelling, design and manufacture of novel nanoparticles that use combinations of both naturally occurring and synthetically engineered biostructural motifs to optimize immune responses to vaccine antigens. SAPNs constructed from different protein oligomerization domains have been designed to achieve repetitive antigen display⁴¹ (FIG. 2). A customized recombinant

Table 2 | Examples of immunostimulatory nanovaccines

Compound	Size	Medical application	Mechanism	Current use	Refs
Virus-like particles	15–30 nm	Vaccine carrier and adjuvant	Repetitive antigen display, structural or molecular mimicry of virus, particle size-dependent tissue penetration and trafficking to lymphatics, and TLR activation	In humans and animals	4,5,14,15,18,32
MF59 (squalene oil-in-water emulsion)	165 nm	Vaccine adjuvant	Neutrophil, monocyte and DC recruitment, antigen uptake, and the induction of humoral and T _H 1-type immune responses	In humans	48
Nanoemulsion W ₈₀ 5EC (soybean oil-in-water emulsion)	400 nm	Vaccine adjuvant	Antigen uptake by and activation of epithelial cells and DCs, TLR2 and TLR4 activation, local cytokine production, mucosal antibody responses and T _H 1, T _H 2 and T _H 17 cell responses	In humans and animals	57,59,67,68
Poly(lactide-co-glycolide) nanoparticles	100–200 nm	Vaccine carrier and adjuvant when combined with bioactive immunomodulators	Encapsulation for sustained local antigens and co-mediator release	In mice	139,140
Nanogel (cholesterol-bearing hydrophobized pullulan nanoparticles)	30–40 nm	Vaccine carrier or delivery vehicle	Antigen entrapment in a hydrated nanogel matrix for slow release, delivery to APCs and induction of tumour-specific T cells and antibody responses	In humans and mice	141,142,143–145
Cationic liposomes	200–1,000 nm	Vaccine carrier	Encapsulation and targeted antigen delivery or uptake by APCs, and recruitment of monocytes to the injection site	In humans and mice	74,75,76,146
Immune-stimulating complexes	40 nm	Vaccine carrier and adjuvant	Targeting, antigen uptake and activation of DCs	In humans and mice	147,148

APC, antigen-presenting cell; DC, dendritic cell; T_H, T helper; TLR, Toll-like receptor.

construct, which is expressed in *Escherichia coli*, encodes a single polypeptide chain with two different linked protein oligomerization domains, each containing particular coiled-coil heptad repeat patterns that drive the self assembly of the purified monomers into nanoparticles approximately 16 to 25 nm in diameter; these nanoparticles have icosahedral symmetry that is analogous to viral capsids. Thus, SAPNs provide a repeating scaffold structure to enable the conformational presentation of inserted protein epitopes or domains in a highly exposed configuration that protrudes or extends from the surface of the particle after the assembly of the subunits⁴¹. Approximately 180 peptide chains are assembled into a single nanoparticle to form this icosahedral structure. This geometry enables the manipulation of nearly all of the parameters described above as being necessary for VLPs: a scaffold for antigen display and cellular activation that leads to substantial increases in the specific production of high-titre, high-affinity antibodies directed against the inserted linear or conformational antigen epitopes⁴².

SAPNs have been produced with the insertion of a range of different antigens, including the hydrophobic loop-peptide epitope of actin, and can successfully induce the production of antibodies against poorly antigenic phylogenetically conserved determinants⁴³. The incorporation of trimeric coiled-coil epitopes from the surface protein of severe acute respiratory syndrome coronavirus (SARS-CoV) into SAPNs was shown to strongly induce the production of virus-specific

neutralizing antibodies after the immunization of mice with these SAPNs⁴⁴. In addition, the insertion of a tetrameric form of the immunogenic epitope from the external domain of the avian influenza virus matrix protein 2 (M2e) into SAPNs led to the high-density display of oligomeric epitopes in a native conformation and the subsequent reduction of H5N2 virus-shedding after the immunization of chickens with these SAPNs⁴⁵. Furthermore, the integration of a tandem repeat of an immunodominant B cell epitope from the malaria parasite *Plasmodium berghei* circumsporozoite protein (CSP) into SAPNs induced the production of T cell-dependent high-avidity antibodies in mice without the use of an adjuvant. This protected mice against both primary and long-term secondary challenges with live sporozoites without causing parasitaemia⁴⁶. Interestingly, modification of the SAPN vaccine to incorporate both B cell and T cell epitopes derived from the CSP of the human malaria parasite *Plasmodium falciparum* induced the production of both high-titre antibodies and long-lived polyfunctional interferon- γ (IFN γ)-producing central memory T cells, which protected mice against a lethal dose of a transgenic *P. berghei* malaria parasite that expressed the CSP from *P. falciparum*⁴⁴. Finally, the α -helical domain located in the membrane-proximal external region of HIV-1 gp41 was incorporated into a SAPN scaffold to recapitulate the epitopes recognized by pre-existing HIV-1-neutralizing antibodies; however, this approach was unsuccessful in inducing HIV-1-neutralizing antibodies in immunized rats⁴⁷.

Nanoemulsions. Nanoemulsion adjuvants are oil-in-water emulsions that are composed of solvents and surfactants. An example of a nanoemulsion is MF59, which consists of squalene oil in combination with polysorbate 80 (Tween 80) and sorbitan trioleate (Span 85). MF59 has been licensed in Europe as a clinical vaccine adjuvant for influenza and has been intramuscularly administered to tens of millions of healthy adults, elderly individuals and children⁴⁸. The adjuvant activity seems to involve a combination of mechanisms, including an increased cellular uptake of antigens, an enhanced release of cytokines and chemokines, a recruitment of monocytes and granulocytes to the site of intramuscular injection, and an augmented maturation and upregulation of CC-chemokine receptor 7 (CCR7) by antigen-primed DCs, which promotes their migration to the draining lymph nodes^{49,50}.

MF59 has been shown to be a more potent adjuvant than alum for inducing broadly protective humoral and T_H1-type cell-mediated immune responses^{48,51,52}. Furthermore, with respect to vaccination against pandemic influenza viruses (for example, H5N1), M59 promotes protective antiviral immune responses when delivered with relatively low doses of virus-derived antigens. By contrast, the delivery of similar antigen doses in combination with alum results in variable or ineffective antiviral immunity^{48,53}. The clinical experience of using MF59 has been documented in these clinical studies as well as in post-marketing surveillance. Increased reactogenicity, including injection site pain, has been described following administration of MF59 and has been attributed to the increased inflammation that is associated with the enhanced immune response. The ratio of the increased reactogenicity to the benefit of increased immunity remains favourable, especially if enhanced responses are needed, as is the case with pandemic influenza vaccines⁵⁴. Early-stage clinical trials are currently in progress to study the efficacy of MF59 as an adjuvant for vaccines against other viral pathogens, including herpes simplex virus, HBV and cytomegalovirus⁴⁸. Intranasal administration of MF59 enhanced serum antibody responses in naive mice but not in pre-infected mice⁵⁵ or in humans⁵⁶.

The nanoemulsion W₈₀5EC is composed of soybean oil as opposed to squalene and has been shown, both in animal models and more recently in humans, to augment the targeting of vaccine antigens to the immune system and to safely induce potent mucosal, humoral and cellular immune responses after its intranasal administration⁵⁷. The adjuvant activity of this nanoemulsion-antigen mixture is primarily dependent on the retention of a nanodroplet structure in the emulsion and on the positive charge that facilitates binding to negatively charged proteins such as mucin. The nanoscale size and positive ζ -potential of the emulsion enables the penetration of the mucous layer, the binding to cell membranes and the cellular uptake that together mediate the induction of the innate and the adaptive immune responses⁵⁸. Cytokine secretion by ciliated epithelial cells in the nasal mucosa and by cells in the nasal-associated lymphoid tissues (NALT) is

followed by the activation and the migration of antigen-primed DEC205 (also known as LY75)⁺ DCs to the regional lymph nodes⁵⁹. Interestingly, nanoemulsions have been shown to induce necrosis or apoptosis in epithelial cells and to simultaneously activate the surface expression of calreticulin, which is a signal for immunogenic cell death^{60,61}. These observations are consistent with a mechanism by which nanoemulsions rapidly facilitate the engulfment of dying antigen-loaded epithelial cells by DCs and other phagocytic cells in the tissue⁵⁹. Nanoemulsions have broad antimicrobial activity against enveloped viruses, bacteria and fungi⁶²⁻⁶⁵ and can be used to inactivate, as well as to provide adjuvant activity, for vaccine preparations involving intact viruses such as vaccinia virus⁶⁶, respiratory syncytial virus (RSV)⁶⁷ and influenza viruses⁶⁸. Single-antigen formulations, including the 17 kDa outer membrane lipid protein A (OmpA) polypeptide from *Burkholderia cenocepacia*⁶⁹, the anthrax protective antigen⁷⁰, the HIV-1 envelope protein gp120 (REF. 71) and the hepatitis B surface antigen⁵⁸, can also be used as adjuvants.

Nanoemulsions have shown little or no toxicity during extensive testing in multiple species^{58,66,70,71} and no serious adverse effects in humans, although intranasally administered nanoemulsions have only been evaluated in less than 200 individuals so far⁵⁷. This is particularly relevant as the history of intranasally administered adjuvants includes an association of cranial nerve VII palsy (also known as Bell's palsy) with the nasal administration of influenza virosomes together with the *E. coli* heat labile toxin mucosal adjuvant⁷². Although there is no evidence that an intranasally administered nanoemulsion adjuvant undergoes retrograde transport or that it is associated with cranial nerve VII palsy, vigilant monitoring for this side effect and others will continue throughout the clinical development programme and after it has been licensed.

Cationic liposomes. Cationic liposomes (typically ranging in particle diameter from 200 to 1,000 nm depending on the formulation) have been used as vaccine adjuvants and delivery systems to encapsulate, protect and enable antigen uptake with the prolonged activation of professional APCs^{73,74}. The cationic charge and the composition of the lipid components is essential for efficient antigen adsorption to the nanoparticle during the preparation of the vaccine, for retention at the site of injection, for innate activation of immune accessory cells and for vaccine immunogenicity, including the induction of T_H1-type cellular responses^{75,76,77,74}. Different cationic lipids incorporated into these formulations have included quaternary ammonium compounds (for example, dimethyl dioctadecyl ammonium bromide (DDA) and 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP)), cholesterol derivatives (for example, dimethylaminoethanecarbonyl-cholesterol), imidazolium compounds (for example, 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolium chloride (DOTIM)), diC14-amidine-based compounds and other immunostimulants such as trehalose

ζ -potential

A measure of the magnitude of the electrostatic charge or potential (positive or negative) of any particle or nanoparticle, and it is typically expressed in millivolts.

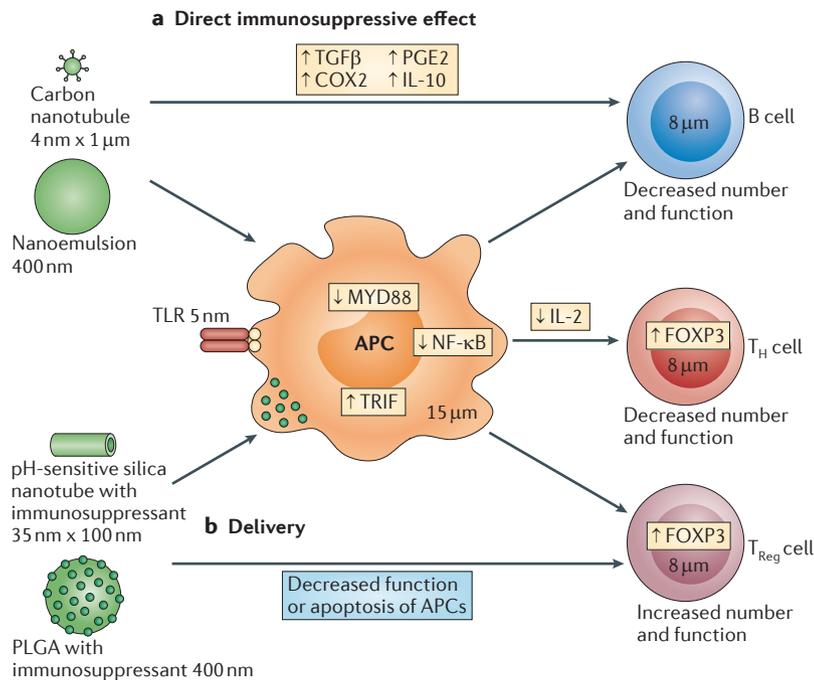


Figure 4 | Nanoscale immunosuppression. Nanoscale products might have a direct immunosuppressive effect (part **a**) on components of the immune response, including antigen presenting cells (APCs), B cells or T cells; they might deliver compounds that result in immunosuppression (part **b**); or they might use both mechanisms at the same time. Direct effects include the upregulation of transforming growth factor- β (TGF β), which results in increased cyclooxygenase 2 (COX2), prostaglandin E2 (PGE2) and interleukin-10 (IL-10), and decreased B cell and T cell activity, as well as apoptosis. The delivery of immunosuppressants results in a decreased response to IL-2 with sirolimus, the downregulation of nuclear factor- κ B (NF- κ B) with steroids and the upregulation of forkhead box P3 (FOXP3), which results in increased regulatory T cell (T_{Reg}) activity when self antigens are presented in a nanoemulsion. The pathways shown are representative examples by which different nanoscale products might suppress the immune system. MYD88, myeloid differentiation primary-response protein 88; PLGA, poly(lactide-co-glycolide); T_H, T helper; TLR, Toll-like receptor; TRIF, TIR-domain-containing adaptor protein inducing IFN β .

dibehenate (TDB), which is a synthetic analogue of trehalose dimycolate that engages a TLR-independent pathway for innate activation⁷⁸. These components have systematically been used to optimize adjuvant activity and to improve the type, the quality and the magnitude of the cellular and humoral responses in different disease models and applications. Cationic liposomes have also been shown to enhance the efficacy and to reduce the systemic toxicity of immunostimulators, including MPL and other TLR ligands^{79–82}. Different cationic liposomes that incorporate immunostimulators and vaccine antigens are currently under evaluation in human clinical trials to test for safety and efficacy against infectious diseases and cancer⁷⁴.

Taken together, these studies show the striking range of applications and the safety of diverse types of nanoparticles (FIG. 1), such as the use of nanoemulsions, nanogels and liposomes to induce potent immune responses against different antigens, including microorganisms and tumour antigens (TABLE 2). Nanoparticles induce a broad spectrum of immune responses, including T_H1, T_H2, and T_H17 cell responses,

and they induce the development of IgG and IgA antibodies, both systemically and locally, depending on the formulation and the route of delivery (FIG. 2). These studies indicate that different nanoparticle compositions can be engineered as desired to enhance the immune response through mechanisms associated with nanoparticle delivery, the controlled release of antigens or the entrapment of immunomodulators in the same location, in addition to the recruitment, the targeting and the uptake of antigens by accessory cells and professional APCs (TABLE 2).

Nanotechnology and immunosuppression

In addition to stimulating and directing the immune response, nanotechnology can be therapeutically used to inhibit the detrimental immune responses that occur in allergy, autoimmunity and in transplant rejection. The immunosuppressive effects of nanotherapeutics are discussed in the following sections (FIG. 4; TABLE 3).

Immunosuppressive effects of fullerenes. Much of the work exploring the direct effect of nanoparticles on the immune response has come from the field of toxicology, in which fullerenes have been shown to have direct immunosuppressive effects⁸³. Fullerenes are molecules that are exclusively composed of carbon and that are commonly used in nanotechnology in the development of items such as electronics, paints and polymer composites⁸⁴. Fullerenes are globally produced in the order of hundreds of tonnes per year⁸⁵. Spherical fullerenes are approximately 1 nm in diameter and can absorb electrons through the benzene rings on their surface, which enables them to function as scavengers for reactive oxygen species (ROS)⁸⁶. When incubated with human mast cells and peripheral blood basophils, spherical fullerenes result in decreased IgE receptor-mediated signalling, decreased production of ROS and decreased degranulation. In a mouse model of anaphylaxis, fullerene treatment prevents histamine release and prevents the reduction in body temperature that normally occurs in mice after allergen challenge⁸⁷.

When fullerene-like structures are manufactured as cylinders they are called carbon nanotubes (CNTs) and they typically have a diameter of approximately 10 nm and a length that can be as much as several micrometres. These structures can be formed as single-walled or multi-walled tubes, both of which have been shown to have direct immunosuppressive effects. Mice exposed to single-walled CNTs by pharyngeal aspiration had increased levels of inflammation in the lungs and enhanced recruitment of DCs, alveolar macrophages, polymorphonuclear cells and lymphocytes. Interestingly, when cultured with stimulatory DCs, both the proliferation and the expansion of splenic T cell populations were decreased in animals that had been exposed to single-walled CNTs. In co-culture experiments, DCs that had been exposed to both lipopolysaccharide (LPS) and single-walled CNTs were less capable of promoting the proliferation and the expansion of T cell populations than DCs that had been exposed to LPS alone⁸⁸.

Table 3 | Examples of immunosuppressive effects of nanotherapeutics

Compound	Size	Medical application	Mechanism	Current use	Refs
Direct					
Spherical fullerenes	1 nm diameter	Allergy	Suppression of mast cell and basophil degranulation	In mice and <i>in vitro</i>	87
Single-walled carbon nanotubes	1–4 nm diameter; 1,000–3,000 nm length	Inhalation exposure	Suppression of DC function	In mice	88
Multi-walled carbon nanotubes	10–20 nm diameter; 5,000–15,000 nm length	Inhalation exposure	Suppression of T cell proliferation and function	In mice	85
Delivery					
Nanocrystals	200 nm	Transplant rejection	Sirolimus	Licensed in humans	92,93
Polyamidoamine dendrimers	1–20 nm	Cerebral palsy, scar formation and gastroenteritis	N-acetyl- cysteine glycosamine	In rabbits	92,95, 96,98
Poly(lactide-co-glycolide)	1–400 nm	Arthritis and autoimmune disease	Betamethasone, bifunctional peptide inhibitors and leukaemia-inhibitory factor	In mice and rats	100–102, 108–110
Carbon nanotubes	3.5 nm diameter and 90 nm length	Induction of apoptosis	Cytochrome c and phosphatidylserine	In mice and rats	91,106, 111,149
Solid lipid nanoparticles	200–400 nm	Transplant rejection	Cyclosporin	In humans	92, 112,113
Liposomes	100–160 nm	Arthritis, coronary artery stenosis and acute lung injury	Liposomal bisphosphanates	In rats, rabbits and pigs	95, 108–110
Liposomes with DC-targeting ligands	50–92 nm	Autoimmune disease	siRNA	In mice	100–102, 106,111
Sterically stabilized phospholipid micelles	15 nm	Rheumatoid arthritis	Camptothecin and vasoactive intestinal peptide	In mice	91,112, 113,149
Nanoemulsions	3–400 nm	Autoimmune thyroiditis	Self antigen	In mice	114

DC, dendritic cell; siRNA, small interfering RNA.

Although the mechanisms responsible for the effect of single-walled CNTs on DC function have not been fully elucidated, more is known about the mechanisms by which multi-walled CNTs affect T cell function. Animals exposed to inhaled multi-walled CNTs for several weeks showed no increase in pulmonary inflammation but they showed systemic immune suppression in the form of decreased T cell proliferation and a lower production of T cell-dependent antibodies⁸⁵. The gene expression of the enzymes cyclooxygenase 2 (*COX2*; also known as *PTGS2*) and prostaglandin E synthase 2 (*PTGES2*) was upregulated in the spleens of multi-walled CNT-treated mice — a response that was abrogated by the administration of a cyclooxygenase 1 (*COX1*) antagonist and in *COX2*-knockout mice. The overall immunosuppressive effect was shown to be mediated by transforming growth factor- β (TGF β) produced by alveolar macrophages⁸⁹.

Fullerenes, which have direct immunosuppressive effects (discussed above), also have the capacity to store and to deliver active drug substances that have immunosuppressive properties. Although spherical fullerenes have not been efficient in the delivery of immunosuppressive drugs such as dexamethasone⁹⁰, carbon nanotubes have been used to effectively deliver compounds such as the pro-apoptotic protein cytochrome *c*⁹¹.

Nanoparticles as vehicles for immunosuppressants. In addition to having direct immunosuppressive effects, nanotechnology can also be used to deliver drugs with known immunosuppressive activity; for example, nanocrystals are used to increase the water solubility and the bioavailability of immunosuppressive drugs that are used to prevent transplant rejection, such as sirolimus^{92,93}. Sirolimus is a triene macrolide that has immunosuppressive effects through the inhibition of IL-2 and other pro-inflammatory cytokines. Owing to its poor solubility in water, the initial formulations of sirolimus were oral solutions with solvent–water mixtures. The currently licensed oral tablet formulation was made available by the use of nanometre-sized crystals generated using nanoscale stabilizers⁹³.

Dendrimers and polymers. Dendrimers are molecules that repetitively branch around a focal point. Similarly to nanocrystals, dendrimers can encapsulate active drugs and in this way they can deliver active drugs to target tissues. In addition, dendrimers and polymers can present targeting ligands⁹⁴. Polyamidoamine (PAMAM) dendrimers are among the best known dendrimers and are composed of a diamine core reacted with methyl acrylate, followed by another diamine, which results in regular radially concentric layers or ‘generations’ that give rise to successively larger dendrimers. PAMAM dendrimers have

been reported to deliver *N*-acetyl cysteine (NAC) across the blood brain barrier. NAC is an antioxidant and an anti-inflammatory agent that is commonly used to treat acetaminophen poisoning and as a mucolytic. On the basis of work showing PAMAM dendrimers to localize to activated microglia and astrocytes in rabbits with cerebral palsy, NAC has been conjugated to a PAMAM dendrimer and intravenously administered to rabbits with cerebral palsy. The administration of NAC with a PAMAM dendrimer but not NAC alone was reported to decrease neuroinflammation and to improve motor function⁹⁵.

Other examples of the use of PAMAM dendrimers in immunosuppression include the development of PAMAM dendrimer glucosamines that have been shown to inhibit TLR4-mediated inflammatory responses and scar tissue formation⁹⁶. Mechanistic studies have shown that the PAMAM dendrimer glucosamine blocks the formation of the TLR4–MD2 (also known as LY96)–LPS complex⁹⁷ by interfering with the electrostatic binding of LPS to MD2 and by blocking the entry of the lipid chains of LPS into the hydrophobic pocket of MD2. On the basis of these findings, the dendrimer glucosamine has been tested in the rabbit ileal loop model of *Shigella* spp. infection and has been shown to decrease IL-6 and IL-8 production⁹⁸ and, as a proof of concept, to decrease inflammation in this model of gastroenteritis.

The effects of dendrimers on TLR7 and TLR8 have also been studied using a dendrimer synthesized from the TLR7 and TLR8 agonist imidazoquinoline⁹⁹. Interestingly, dendrimerization of the small molecule imidazoquinoline⁹⁹ results in a loss of TLR8 activity and a maintenance of TLR7 activity, with an initial dose-dependent increase in cytokine production followed by a decrease. Although the mechanisms by which particular TLRs are ‘turned on’ and ‘turned off’ by dendrimers is not fully understood, it highlights the possibility of molecular manipulation at the nanoscale level with respect to both elucidating and modulating the innate immune response for therapeutic use.

PLGA has been formulated at the nanoscale level and has been used to deliver immunosuppressants including betamethasone¹⁰⁰, bifunctional peptide inhibitors and leukaemia-inhibitory factor (LIF)¹⁰¹. The addition of betamethasone to PLGA resulted in a more sustained anti-inflammatory effect in two animal models of arthritis compared with betamethasone alone¹⁰⁰. Bifunctional peptide inhibitors that simultaneously target MHC class II molecules and intercellular adhesion molecule 1 (ICAM1) have been effectively delivered using PLGA complexes, which results in decreased cytokine production and a suppression of disease in mice with experimental autoimmune encephalomyelitis¹⁰¹. PLGA has also been used to deliver LIF, which results in the upregulation of forkhead box P3 (FOXP3) expression by T cells and an expansion of the regulatory T cell population in mice. This effect was not seen when LIF was administered to mice in the absence of PLGA¹⁰².

The type of polymer and the route of administration might also influence whether there is an enhanced or a suppressed immune response. When negatively charged PLGA was compared to positively charged *N*-trimethyl

chitosan (TMC) both nanoparticles increased the humoral immune responses to ovalbumin when intramuscularly administered; however, only the negatively charged TMC increased the humoral immune responses to ovalbumin when intranasally administered¹⁰³. This is probably due to the electrostatic interaction of the positively charged carrier and the negatively charged mucous and has been shown to occur with other carriers, including liposomes¹⁰⁴ and nanoemulsions. Interestingly, only the negatively charged intranasally administered PLGA induced immunoregulatory responses, which were characterized by enhanced FOXP3 expression in NALT and cervical lymph nodes, by decreased delayed type hypersensitivity responses and by increased IL-10 expression¹⁰⁵. These results show that the type of polymer, its charge and the route of administration can influence whether the resultant immune response is enhanced or suppressed¹⁰⁵.

SLNs, liposomes and micelles. Lipid-based nanotechnology includes solid lipid nanoparticles (SLNs), liposomes and micelles (FIG. 1). SLNs are solid at room temperature and body temperature, whereas liposomes and micelles are liquid at these temperatures. Liposomes are vesicles composed of a lipid bilayer with a hydrophilic centre. Conversely, micelles are composed of amphiphilic molecules arranged as oil in water (a hydrophobic core) or water in oil (a hydrophilic core). An active drug is incorporated and delivered by the carrier, which is chosen on the basis of composition, size and charge^{92,106}. In addition, the carrier might be targeted to specific tissues using specific ligands¹⁰⁷. Examples of liposomes that have been used as carriers to achieve immunosuppression include bisphosphonates, such as clodronate, to deplete pulmonary macrophages in acute lung injury¹⁰⁸ and arthritis¹⁰⁹, as well as alendronate to deplete circulating monocytes in coronary artery stenosis¹¹⁰. Targeting of liposomes to specific cells is exemplified by the delivery of small interfering RNAs (siRNAs) to DCs by surrounding the liposome that is carrying the siRNA with a monoclonal antibody that is specific to DCs. This gene-silencing approach was used to specifically target CD40 expression by DCs and resulted in reduced levels of T cell proliferation in the liposome-treated mice¹¹¹. Micelles have also been used to deliver agents that impair the immune response, including camptothecin^{112,113}. Camptothecin is a topoisomerase inhibitor that was originally used in the treatment of cancer and has more recently been applied to the treatment of rheumatoid arthritis. The formulation of camptothecin in sterically stabilized micelles (SSMs) improved its delivery and had anti-inflammatory effects in a mouse model of arthritis; in this study, vasoactive intestinal peptide was used to target the SSMs to T cells, macrophages and synoviocytes¹¹².

Nanoemulsions and immunosuppression. A final example of how nanotechnology can be applied to suppress an immune response is the use of nanoemulsions to deliver self antigens¹¹⁴. When the nanoemulsion W₈₀5EC is combined with the self antigen thyroglobulin and is delivered to mice, the animals become tolerant to thyroglobulin;

Bifunctional peptide inhibitors

Molecules that are capable of binding both the major histocompatibility complex (the first signal) and a co-stimulatory receptor (the second signal) on an antigen-presenting cell.

this response is characterized by a reduced humoral immune response to thyroglobulin, an upregulation of FOXP3 and TGF β expression and increased T regulatory (T_{Reg}) cell activity. This is particularly interesting, as W₈₀5EC administered with foreign antigens enhances immune responses by increasing antigen delivery as well as by increasing TLR2 and TLR4 activation^{57,115}. The signalling pathways that are activated after TLR4 stimulation include the myeloid differentiation primary-response gene 88 (MYD88) and the TIR domain-containing adaptor protein inducing interferon- β (TRIF; also known as TICAM1) pathways, which result in the production and the release of pro-inflammatory cytokines¹¹⁶. As too much inflammation is detrimental to the host¹¹⁷, the control of TLR4 stimulation is an important immunoregulatory function. Endotoxin tolerance is a mechanism by which immunosuppression occurs in the continued presence of LPS¹¹⁸. In addition, the immunoregulatory molecule TGF β has been described as being increased in endotoxin tolerance, as well as in the aforementioned direct immunosuppressive effects of fullerenes⁸⁹ and in the indirect immunosuppressive effects of nanoemulsion delivery of self antigens¹¹⁴. Another similarity between the signalling mechanisms in nanotechnology-mediated immunosuppression and endotoxin tolerance is a relative increase in TRIF activation and a decrease in MYD88 activation¹¹⁸. This 'TRIF bias' has been described for particular TLR4 agonists such as MPL¹¹⁹, which is an LPS derivative that is used as an adjuvant in numerous vaccines, including licensed recombinant HPV bivalent vaccine¹²⁰.

Summary

Nanotechnology is currently being used to engineer specific immune responses for prophylactic and therapeutic effects. In the future, the use of nanoparticles that have unique immunological properties determined by their size, shape, charge, porosity and hydrophobicity will enable researchers to 'customize' immune responses in new and unexpected ways. Improved protection against the outbreak of pandemic viruses and other emerging or mutating pathogens will require the rapid activation of innate and adaptive immune responses, ideally within hours (for an innate immune response) or days (for an adaptive immune response) after a single priming dose of vaccine. This could be achieved through the absorption of nanoparticles that have unique combinations of antigens and cytokines. It is also possible that passive

immunity through nanoparticle-delivered immunoglobulin genes could produce antibodies specific for pathogens and could provide immune replacement rather than simply immune manipulation by nanotechnology.

In addition, the activation of CTL responses that can target tumours or virally infected cells can be accomplished in several ways using nanotechnology. Nanoparticles could be surrounded by antigens to augment CTL activity or they could be combined with immunomodulators, including cytokines such as GM-CSF, IL-12, IL-15 and FMS-related tyrosine kinase 3 ligand (FLT3L). As many co-stimulatory ligands are now known, multimerizing CD40 ligand (CD40L), glucocorticoid-induced TNFR-related protein (GITR; also known as TNFRSF18) or other activating and blocking ligands could turn lymphocytes on and off with greater precision than the antibodies that are currently being used to help to control autoimmunity and immune responses to allergens or even to transplanted organs. Using antigens with NOD-like receptors, TLR ligands and other microbial pattern recognition systems could induce CTL responses to antigens that normally do not produce this activity. It is also possible that 'suppressive nanoparticles' could be given to relatives of patients with specific autoimmune diseases in order to prevent disease development. Genetic screening for many immune diseases might enable the early correction of some immune defects using nanoparticle gene delivery methods rather than bone marrow transplantation.

In addition to the potential therapeutic applications of nanotechnology, recent advances in nanotechnology-based screening strategies using silicon nanowires in combination with siRNA and transcriptional profiling over time have shown promise for the selective perturbation of the immune response. This therefore has facilitated the identification of crucial points in the molecular network that regulates the immune response¹²¹. These emerging nanotechnologies provide new ways to interrogate complex pathways that control the differentiation of immune cells, including the balance of T_H17 and T_{Reg} cells. This approach might also enable the future design of more effective therapeutics to regulate the immune system and to potentially reduce side effects and inflammation.

In summary, the field of nanotechnology will continue to provide remarkable insights into the nature of the immune response. The application of nanotechnology in immunology might also affect new strategies for the prevention or the treatment of human diseases.

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Competing interests statement

The authors declare **competing financial interests**: see Web version for details.

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Douglas M. Smith, Jakub K. Simon and James R. Baker Jr

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In the version of this Review that was initially published, the reference citations in Tables 1–3 were not accurate. This error has been corrected in the online HTML and PDF versions of the article. *Nature Reviews Immunology* apologizes for this error.