Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns

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Abstract | Researchers working on the development of vaccines face an inherent dilemma: to maximize immunogenicity without compromising safety and tolerability. Early vaccines often induced long-lived protective immune responses, but tolerability was a major problem. Newer vaccines have very few side effects but can be of limited immunogenicity. One way to tackle this problem is to design vaccines that have all the properties of pathogens with the exception of causing disease. Key features of pathogens that can be mimicked by vaccine delivery systems are their size, shape and surface molecule organization. In addition, pathogen-associated molecular patterns can be used to induce innate immune responses that promote adaptive immunity. In this Review, we discuss the approaches currently being used to optimize the delivery of antigens and enhance vaccine efficacy.

The first attempts to immunize against smallpox virus occurred in China and India more than 1,000 years ago[1], when people were inoculated with live, virulent smallpox virus, a process known as variolation. For reasons still not understood, this treatment did not usually result in systemic infection but instead had severe effects only at the site of inoculation. Mortality rates among variolated individuals were reported to be less than 1% rather than the 30% mortality rate that typically occurred during outbreaks of smallpox virus. However, the infection sometimes spread from variolated individuals to naive individuals, resulting in smallpox outbreaks with normal mortality rates. Variolation was therefore a highly risky procedure.

At the end of the eighteenth century, Edward Jenner realized that milkmaids who had acquired a pox-like disease from cows were resistant to smallpox virus infection. He hypothesized that infection with cowpox virus could protect against smallpox virus and, to prove this, he infected the son of his servant with infectious material obtained from a milkmaid and then challenged the child with live smallpox virus through variolation. The boy was resistant to disease and consequently, within a matter of years, the new practice of immunization against smallpox was spread throughout Europe. As the virus used for inoculation was from a cow (*vacca*), the procedure was termed vaccination[1].

Vaccinology has changed greatly since these times. The most recently licensed vaccines are typically recombinant products and are well defined at the molecular level[2]. Vaccine-associated mortalities are now extremely rare events. Unfortunately, for recombinant vaccines, the efficacy profile has also changed. Modern vaccines require two to three initial injections followed by occasional booster injections to maintain protective antibody levels. Indeed, limited immunogenicity and the sometimes great difficulties in inducing antibodies of the appropriate specificity (as, for example, in the case of vaccines against HIV[3]) are major limitations of modern vaccine development. Safety is the foremost requirement for vaccines, particularly for those used for prophylaxis. Therefore, a crucial challenge for vaccine development is to design and produce vaccines that are safe but, at the same time, induce potent and long-lasting immune responses. Indeed, to increase immunogenicity without compromising safety and tolerability is the holy grail of the vaccine industry.

Two approaches can be taken to increase vaccine immunogenicity. The first is to develop vaccines based on live pathogens that have been rendered as safe as recombinant proteins, and the second is to produce recombinant proteins as immunogenic as live pathogens. The first approach is challenging because the more

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Particulate adjuvant
An adjuvant that forms small particles or droplets and entraps the antigen.

attenuated (that is, less virulent) a pathogen becomes, the less immunogenic it is. Conversely, the second approach is hampered by the fact that increased immunogenicity of recombinant protein antigens usually leads to increased reactogenicity (that is, more side effects).

For non-replicating antigens, vaccine delivery systems that attempt to mimic various properties of pathogens[2] and thereby increase immunogenicity are often used. There are numerous prominent and successful examples; the influenza virus vaccine Inflexal (Crucell) is a virosome that incorporates the influenza virus protein haemagglutinin into liposomes. Another example is the hepatitis B virus vaccine Engerix B (GlaxoSmithKline Biologicals), which has properties of both virus-like particles (VLPs) (BOX 1) and liposomes in that the vaccine particles incorporate lipids during the self-assembly process in yeast. A vaccine delivery platform that most closely mimics the properties of viruses is VLPs (BOX 1). Gardasil (Merck & Co.) and Cervarix (GlaxoSmithKline) are licensed VLP-based vaccines against human papillomavirus (HPV) that exemplify this approach. VLPs can also be used to display antigens that are unrelated to the VLP itself and can therefore be used to induce antibody responses against chosen epitopes[4,5].

Currently, there are various other delivery systems in development. Examples include nanoparticles and microparticles, which have dimensions that are similar to those of microbial pathogens. Such particulate delivery systems can be made from various materials, such as polylactide co-glycolide (PLG), immunostimulating complexes (ISCOMs) (a mixture of cholesterol, phospholipids and *Quillaja* saponins), chitosan (a chitin-derived polyaminosaccharide), polyanhydrides, hyaluronic acids, starch, proteins or synthetic materials such as polyethylene glycol and polystyrene[6,7].

The success of most, if not all, prophylactic vaccines is based on the induction of long-lived T helper cell-dependent IgG responses[8]. Hence, the main focus of classical vaccine development is the optimization of B cell responses. By contrast, therapeutic vaccines for the treatment of chronic infections or cancer rely on the induction of robust pro-inflammatory $CD4^+$ and $CD8^+$ T cell responses. Hence, for prophylactic and therapeutic vaccines different design strategies are often used.

The induction of protective immune responses involves several key steps. These include antigen uptake and processing by antigen-presenting cells (APCs), activation of APCs for effective T cell priming and activation of B cells. This Review describes the ways in which recombinant vaccines can be made more effective, by optimizing each of these steps through harnessing properties of pathogens (FIG. 1). By designing vaccines that mimic the size, geometry, kinetics and molecular patterns of viral antigens, it may be possible to induce immune responses that are as potent as those induced by viral infection but without the associated risks.

The role of size in vaccine design

The effect on antigen uptake and processing. The dimensions of vaccine antigens vary greatly[2,9] (FIG. 2). The smallest (<10 nm) are protein or viral subunit antigen vaccines. Often, such antigens are formulated with adjuvants (such as alum and Freund's adjuvants) to form larger particles or aggregates. Supramolecular particulate antigens, such as VLPs and nanoparticles, are larger (20–200 nm). Small liposomes, such as virosomes, are approximately 100–200 nm. Antigens presented in the context of microparticles, liposomes, water in oil emulsions (Freund's adjuvants), oil in water emulsions (MF59 adjuvant), mineral salts (alum adjuvants) and whole-cell vaccines are the largest (100 nm–20 μm).

The crucial APCs for the induction of T cell responses are dendritic cells (DCs) and probably macrophages[10,11], which are both highly specialized to take up and process antigen. Uptake of antigens by DCs, trafficking of the DCs to the lymph nodes and triggering of DC maturation are key steps in the generation of potent immune responses and need to be evaluated for the various vaccine delivery systems.

Uptake of antigens by APCs depends on several antigen-associated properties, such as size, shape, surface charge, hydrophobicity and hydrophilicity, as well as receptor interactions[12]. The size of antigens is an important factor for their efficient uptake by APCs. Particulate antigens, such as whole-cell vaccines, virosomes and VLPs, or antigens formulated in particulate adjuvants, such as liposomes and microparticles, have large surfaces that have charged, hydrophobic or receptor-interacting properties. This leads to better interaction of APCs with particles than with soluble proteins. Many pathogen surfaces are highly repetitive and thereby allow efficient binding of natural IgM antibodies through high-avidity interactions, leading to the recruitment and activation of complement component 1q (C1q) and the classical pathway of the complement cascade. Furthermore, pentraxins (such as C-reactive protein, serum amyloid P component and pentraxin 3) bind to pathogen surfaces through poorly understood mechanisms. Binding of pentraxins, in particular the long pentraxin 3, also

Box 1 | **Virus-like particles**

Virus-like particles (VLPs) are self-assembly systems that spontaneously form virus-shaped particles following expression of one or several viral proteins. Typically, VLPs have an icosahedral or rod-like structure. The symmetry of VLPs usually reflects the symmetry of the original virus. In most cases, it is the viral envelope proteins that assemble into VLPs, but viral core proteins may also form VLPs, as is the case for the hepatitis B virus core proteins. VLPs cannot replicate owing to the absence of replicases and nucleic acids encoding viral proteins. Some VLPs, however, spontaneously assemble around RNA or DNA fragments; for example, VLPs derived from the bacteriophage Qb. Qb envelope proteins assemble into icosahedral VLPs following expression in *Escherichia coli*. Each VLP contains 180 subunits and a random selection of different RNA species derived from *E. coli*. The particles can be disassembled and will spontaneously reassemble in the presence of polyanionic structures. Hence, the RNA (which is a ligand for Toll-like receptor 7 (TLR7) and TLR8) may be replaced, for example, by CpG-containing oligodeoxynucleotides (which are ligands for TLR9) or polyGlu (which is not known to bind TLRs). This allows manipulation of the TLR stimulus that is given together with the VLPs.

All VLPs have highly repetitive surfaces and therefore induce potent antibody responses. Antigens displayed on VLPs assume a similar immunogenicity as the underlying particle. VLPs may therefore be a potent platform to induce antibody responses against antigens of choice.

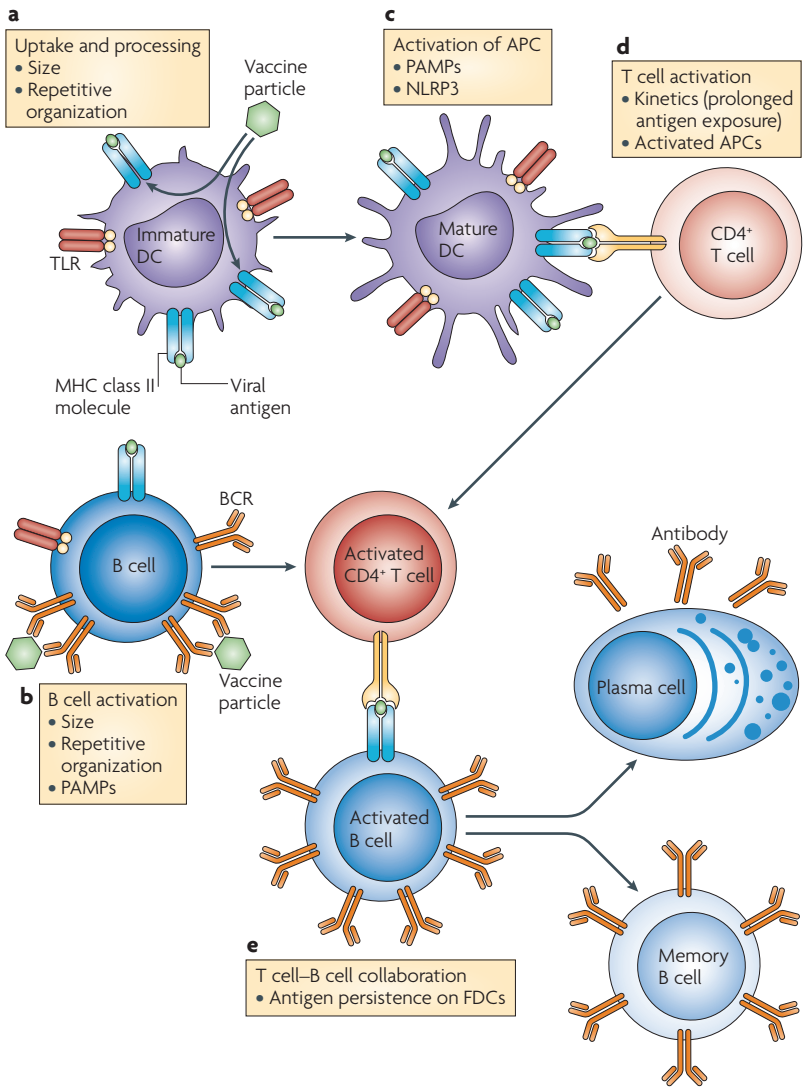

Figure 1 | **Key steps during the generation of protective immune responses.** **a** | Antigen processing is facilitated if antigens are particulate and have a repetitive surface organization, which increases phagocytosis and the ability to activate complement and recruit other molecules of the innate humoral immune system. **b** | B cell activation is also facilitated by antigens that have a repetitive surface organization (through cross-linking of the B cell receptor (BCR) and activation of complement), that are 20–200 nm in size (which allows them direct access to the lymphatic system) and that contain pathogen-associated molecular patterns (PAMPs). **c** | Activation of antigen-presenting cells (APCs) is facilitated by the recognition of PAMPs by Toll-like receptors (TLRs) or other pattern-recognition receptors (such as NOD-like receptor family pyrin domain-containing protein 3 (NLRP3)), or by other mechanisms. **d** | T cell activation is facilitated by the prolonged presence of antigen through depot-forming adjuvants or perhaps vaccination regimens. **e** | T cell–B cell collaboration is essential for the generation of antibody-producing plasma cells and memory B cells but not much is known about the factors that influence this interaction. It is likely that factors that increase persistence of antigen on follicular dendritic cells (FDCs) would be beneficial. DC, dendritic cell.

causes activation of C1q. In addition, pentraxins bind to Fc receptors, further facilitating the uptake of pathogens[13]. The same molecules of the innate humoral immune system react with hydrophobic surfaces[14], as found on some microparticles and nanoparticles used in vaccines. In addition, artificial hydrophilic surfaces, such as those of pluronic-coated nanoparticles, activate the alternative, rather than the classical, pathway of the complement cascade. This also results in increased uptake by DCs and macrophages[15]. Adjusting the hydrophobicity or hydrophilicity of a particle[14] may thereby allow manipulation of their capacity to trigger the complement cascade and become opsonized[14]. It is interesting to note that the concept of hydrophobicity may also explain why fully denatured proteins, which have exposed hydrophobic regions, unexpectedly induce potent T and B cell responses[16,17]. In summary, in contrast to small protein antigens, which are inefficiently taken up and presented by APCs, pathogen-sized particles and protein aggregates may efficiently be taken up by APCs[18,19].

Following uptake by APCs, antigens will reach the endosomal–lysosomal compartments, where they are degraded into peptides. These peptides are then loaded onto MHC class II molecules and are subsequently transported to the cell surface for stimulation of $CD4^+$ T helper cells.

Through the process of cross-presentation, the vaccine antigen can also be presented by MHC class I molecules to induce the priming of $CD8^+$ T cell responses, an important feature for therapeutic vaccines against cancer and chronic viral infections[20]. Soluble protein antigens are usually only inefficiently cross-presented, but particulate antigens that are a similar size to, or larger than, viruses efficiently reach the MHC class I pathway without intracellular replication[19]. Hence, cross-presentation of peptides derived from particulate antigens occurs much more readily than cross-presentation of peptides derived from soluble antigens[19,21]. Furthermore, cross-presentation of MHC class I-associated peptides derived from particulate antigens occurs with similar efficiency as presentation of MHC class II-restricted peptides[22]. Processing of particulate antigens that are 20 nm–3 μm in diameter has been documented and there is no clear indication that there are preferred sizes within this range[19,21,23,24]. It is therefore possible that APCs have evolved to effectively process any antigen with dimensions that are similar to pathogens, ranging from viruses (20–100 nm) to bacteria and even cells (in the micrometre range). So, generating particulate vaccines may enhance the uptake, processing and presentation of the antigens by professional APCs.

The effect on antigen transport. Vaccines are injected subcutaneously or intramuscularly. However, adaptive immune responses are mainly induced in the secondary lymphoid organs. So, the transport of antigens through the lymphatic system from the peripheral tissues to lymphoid organs is important for vaccine design. The lymphatic system regulates tissue fluid balance by allowing the transport of fluids and serum components that leak from blood capillaries into the interstitial space back to the blood. It is also the route through which peripheral immune cells and antigens or pathogens enter the secondary lymphoid organs. Lymph fluids are propelled through lymph vessels and ducts by a system of small peristaltic pumps, contraction of adjacent skeletal

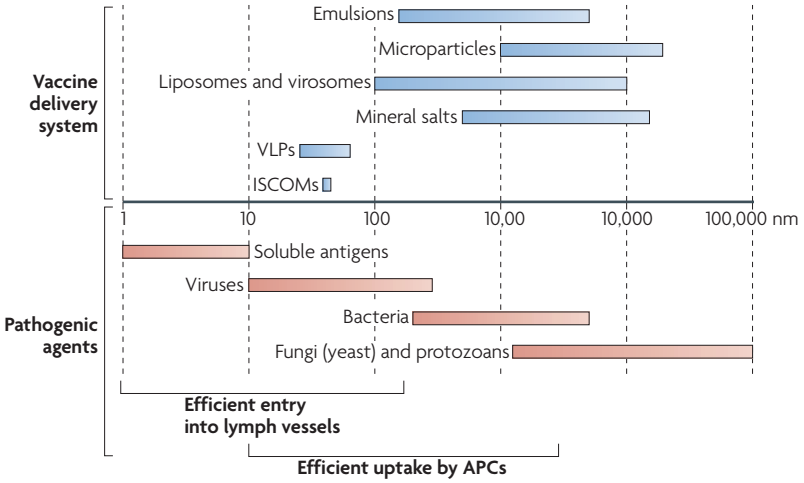

Figure 2 | **The sizes of adjuvant delivery systems and pathogenic agents.** The size ranges of various adjuvant delivery systems and the dimensions of different pathogenic agents are indicated on a nanometre log scale. The range of particle sizes that allows most efficient uptake by antigen-presenting cells (APCs) and the size of macromolecules that most efficiently enter the initial lymphatic vessels are shown. ISCOMs, immunostimulating complexes; VLPs, virus-like particles.

muscle and arterial pulsation[25]. Initial lymphatic vessels are blind-ended structures that are lined with overlapping endothelial cells. These cells function as flap valves that ensure unidirectional transport of solutes and small particles along the lymphatic vessels[26]. Initial lymph vessels are 10–60 μm in diameter, whereas larger lymphatic vessels can be up to 2 mm in diameter. The size of lymph vessels is therefore large enough to transport particles and cells of up to several μm. As lymph fluid is actively pumped, also against gravity, it is evident that the free exchange of water and macromolecules between the lymph and the interstitium must be regulated. Indeed, several factors influence the entry of macromolecules into the initial lymphatic vessels, and size is an important determinant. Molecules of 20–200 nm efficiently enter the lymphatic system, with an optimal size being ~40 nm[26–28]. The distal initial lymphatic vessels are partially open structures that have gaps and clefts between the endothelial cells. By contrast, the more complex proximal vessels are essentially impermeable to proteins that are more than 70 kDa (corresponding to a dynamic radius of 5–6 nm)[29]. Consequently, both cells and macromolecules that are smaller than 100–200 nm enter the lymphatic system at the distal sites of the lymph vessels[30].

By contrast, particles that are larger than 200–500 nm do not efficiently enter lymph capillaries in a free form[15,31]. Instead, large particles need to be carried into the lymphatic system by specialized cells[32], such as DCs, which can squeeze through openings between overlapping endothelial cells[33] (FIG. 3).

The size of vaccine delivery systems also affects the kinetics of lymph drainage. Nanoparticles of less than 200 nm reach the lymphoid organs directly through the lymph drainage within hours of injection, whereas the transport of particles that are larger than 200–500 nm requires DCs, and it takes approximately 24 hours for them to arrive in lymph nodes[32]. As discussed below, free versus cell-associated drainage of vaccine antigens has a crucial effect on the targeting of cell populations by the differently sized particles.

The effect on targeting B cells. The finding that size determines whether particles freely drain to lymphoid organs or arrive at these sites in association with cells has major consequences for vaccine design and immune cell targeting. As antigens with virus-like dimensions (and smaller) most efficiently reach the lymph nodes in a cell-free state, vaccine delivery systems with similar dimensions will facilitate direct interaction of antigens with follicular B cells.

There are several reasons why the direct interaction of antigens with B cells is important for the optimal induction of effective antibody responses. First, vaccine antigens should be in an authentic native configuration for B cell activation, which can be achieved when antigens access B cell follicles in secondary lymphoid organs in a native state. Second, cross-linking of B cell receptors (BCRs) is a strong activation signal for B cells and is facilitated by repetitive structures, such as those presented on viral surfaces or viral particles[34–36] (see later). The presentation of antigens that have a repetitive structure is therefore optimal when such antigens directly access the lymph node. Third, viruses and many vaccine delivery systems contain Toll-like receptor (TLR) ligands, which can enhance antibody responses. It has recently been shown in mice that the effect of antigen-associated TLR-based adjuvants is mediated by activation of TLR4, TLR7 or TLR9 expressed by B cells, indicating that direct interaction between particles and B cells is necessary[37–41].

In this context, it is interesting to understand how antigens reach B cells in the lymph nodes. Soluble proteins can directly reach the B and T cell regions through conduits[42,43]. In addition, follicular B cells may interact with antigens that diffuse through small pores in the subcapsular sinus[44]. By contrast, larger antigens, such as viruses and immune complexes, are initially captured by subcapsular sinus macrophages and subsequently shuttled to B cells, which transport the antigens to follicular DCs (FDCs) located in B cell follicles[45–47]. Alternatively, DCs have also been shown to capture antigen and activate extrafollicular B cells in T cell areas[48,49]. Another mechanism was found to be important for immunity to influenza virus: subcapsular sinus macrophages prevented viral dissemination, while viral antigen was transported into B cell follicles by DCs bound to DC-SIGN-related protein 1 (SIGNR1; also known as CD209b)[50]. In addition to SIGNR1, complement receptors or DC-specific ICAM3-grabbing non-integrin (DC-SIGN)[46,50] can also be involved in the transport and presentation of antigen on the cell surface. Alternatively, antigen may be stored in non-degrading intracellular compartments and subsequently recycled to the cell surface[49,51].

This cell-associated transport of native antigens has mainly been described in the context of antigen transport from the subcapsular sinus to the B cell follicles in lymph nodes. There is little direct evidence

Cross-presentation
The ability of certain antigen-presenting cells to load peptides that are derived from exogenous antigens onto MHC class I molecules. This property is atypical, because most cells exclusively present peptides from their endogenous proteins on MHC class I molecules. Cross-presentation is essential for the initiation of immune responses to viruses that do not infect antigen-presenting cells.

Immune complex
A complex of antigen bound to antibody and, sometimes, components of the complement system. The levels of immune complexes are increased in many autoimmune disorders, in which they become deposited in tissues and cause tissue damage. To effectively activate complement and Fc receptors, more than one antibody needs to be present in a complex.

Subcapsular sinus macrophage
A specialized macrophage that resides in the subcapsular sinus of lymph nodes and is efficient at capturing antigen from the lymph.

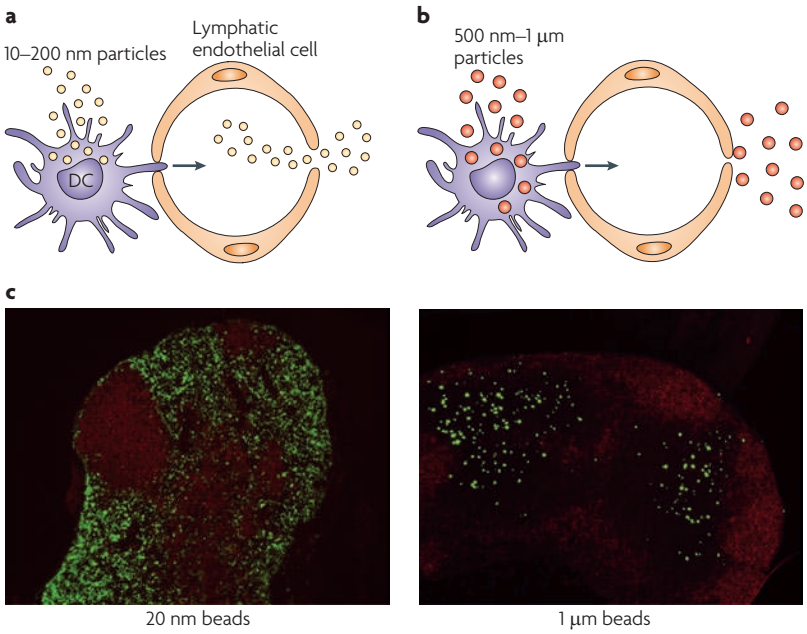

Figure 3 | **Entry of particles into initial lymphatic vessels and localization in the draining lymph node. a** | Particles with a size range of 10–200 nm enter the initial lymphatic vessels from the interstitial space by directly diffusing through lymphatic endothelial cell junctions. Particles of this size can also enter the lymphatic vessels after being endocytosed by dendritic cells (DCs), which then enter the lumen of the vessel by intravasation. **b** | Particles that are larger than 200 nm diffuse from the interstitial space into the lumen less efficiently with increasing size. Particles that are 500 nm–1 μm are too large to pass through endothelial cell junctions and are transported into the lymph vessel following uptake by DCs. **c** | Frozen sections of popliteal lymph nodes that were isolated from mice 48 hours after injection with either small (20 nm, left panel) or large (1 μm, right panel) green fluorescent nanoparticles and stained with B220-specific antibody to depict B cell areas (red). Small particles are distributed in the subcapsular sinus, as well as in the cortex and paracortex in close proximity to B cell follicles. By contrast, large particles are excluded from the subcapsular sinus and B cell regions and can only enter the paracortex and the medulla of the lymph node, where DCs and T cells reside. Images in part **c** are reproduced, with permission, from REF. 32 © Wiley-VCH GmbH & Co. KGaA (2008).

that either Langerhans cells or dermal DCs that migrate from the skin to the lymph node have a similar function. Nevertheless, as DCs have been reported to shuttle infectious viruses, such as HIV virions, from the periphery to lymphoid organs[52], it is also possible that this could occur for other particulate antigens. Dedicated receptors, such as DC-SIGN, SIGNR1 and complement receptors, may facilitate this process[46,50,53]. Immune responses induced by whole-cell vaccines may benefit from such a mechanism, as bacteria express carbohydrates and other ligands recognized by complement, pentraxins and scavenger receptors on DCs[13]. Similar considerations may apply to particles that activate molecules of the innate humoral immune system. Nevertheless, although it cannot be excluded that such vaccines are transported to lymph nodes by DCs in the native form to directly interact with B cells, it is clear that the ability of vaccines to drain to lymph nodes independently of cellular transport greatly influences direct antigen exposure to B cells. Indeed, a comparison of the distribution of small and large nanoparticles inside lymphoid organs after peripheral injection reveals that small particles can access all areas of the lymph node, including the subcapsular sinus and the B cell area[32]. By contrast, large particles are restricted to the T cell regions, indicating that exposure of B cells to antigens is restricted if large particles are used for immunization (FIG. 3). Thus, direct drainage of antigen to lymph nodes may not be a prerequisite for the induction of B cell responses, but it facilitates and enhances the interaction of B cells with their cognate antigen and associated TLR ligands.

In this context, it is interesting to consider how classical adjuvants, such as alum and incomplete Freund's adjuvant, affect lymph node drainage of antigens. Surprisingly, and consistent with an important role for direct drainage of native antigens into lymph nodes, these adjuvants have very little influence on the kinetics and amount of antigen that drains into the lymph nodes. Thus, although these adjuvants may form a local depot at the injection site, most of the antigen drains into lymph nodes within hours[54].

The effect on targeting DCs. Although activation of B cells by antigen is important for the induction of antibody responses, concomitant activation of antigen-specific T helper cells is equally important, as antibody responses remain short-lived in the absence of T helper cell-dependent class switching and the generation of long-lived plasma cells (FIG. 1). DCs, the most potent inducers of T cell responses, therefore also need to be loaded with antigen. $CD8^+$ lymphoid DCs and plasmacytoid DCs (pDCs) are two important types of DC that are essentially restricted to lymphoid organs[55–57]. Both cell types have important properties that should be considered when designing vaccines: $CD8^+$ DCs are essential for cross-presentation of exogenous antigens[58–60], and pDCs are an important source of type I interferons[56,61].

As only $CD8^+$ DCs and not other DC subsets can cross-present antigens, at least in mice, it is essential that non-infectious vaccines target these cells if they are to induce cytotoxic T lymphocytes (CTLs). For this purpose, 20–200 nm particles, which directly reach the lymph nodes, are preferable to larger particles that need to be transported by skin-resident DCs. Indeed, 40 nm polystyrene beads have been shown to be the optimal size for cross-priming in mice[62], and proteins delivered in Iscomatrix adjuvant (CSL) particles have been shown to induce $CD8^+$ T cell responses in humans[63]. Furthermore, although $CD8^+$ lymphoid DCs and $CD8^-$ myeloid or conventional DCs are differentially positioned in lymph nodes, VLPs and nanoparticles are efficiently taken up by both DC subsets[32,60]. However, it is only the $CD8^+$ DCs that can cross-present particle-derived antigens[60]. One possible reason for this difference may be that the processing machinery of the two types of DC is different, a notion that is consistent with the recent finding of a specialized endosomal cross-presentation compartment in human DCs[64] and with selective expression of the GTPase RAC2 in $CD8^+$ DCs controlling phagosomal alkalinization[65].

Follicular DC
(FDC). A cell that has a dendritic morphology and is present in lymph nodes. These cells display on their surface intact antigens that are held in immune complexes, and B cells present in the lymph node can interact with these antigens. FDCs are of non-haematopoietic origin and are not related to dendritic cells.

Langerhans cell
A professional antigen-presenting dendritic cell localized in the epidermis of the skin.

Whether activation of monocyte-derived DCs enables cross-presentation remains a contentious issue. Stimulation of monocyte-derived DCs with defined TLR7 and TLR9 ligands failed to promote cross-presentation[60]. By contrast, strong inflammatory conditions, as found during viral infection, may promote cross-presentation by conventional DCs[66]. Whether these findings can be translated into vaccines that are more potent at inducing cross-priming but have an acceptable side effect profile still needs to be determined. So, vaccine delivery vehicles that are sized in the nanometre rather than the micrometre range may be preferable to induce $CD8^+$ T cell responses by cross-priming.

Much recent work has focused on targeting vaccines to DCs together with TLR9 ligands. CpG-containing oligodeoxynucleotides (CpG-ODNs) — the prototype TLR9 ligands — are widely used in vaccinology to enhance B and T cell responses to recombinant antigens[67]. In mice, CpG-ODNs are powerful adjuvants, particularly if they are linked to an antigen, presumably because the same cells that present the antigen are simultaneously activated by the TLR9 ligand[40,68,69]. In mice, TLR9 is expressed by all DC subsets, but in humans its expression is restricted to pDCs[70]. In humans, Langerhans cells and dermal DCs that take up CpG-ODN-linked antigen in the periphery do not respond to the CpG-ODNs and, so, do not mature. Only pDCs in the lymph nodes can respond to the CpG-ODNs. Therefore, if the goal of a TLR9 ligand-based adjuvant is to activate DCs, the ligand needs to reach lymph nodes to be taken up by pDCs. As discussed above for $CD8^+$ DCs, the ability of small particles with nanometre dimensions to directly enter lymph nodes and be taken up by pDCs favours their use in combination with TLR9 ligands, instead of large particles with micrometre dimensions[32]. Moreover, pDCs optimally respond to A-type CpG-ODNs rather than B-type CpG-ODNs; however, B-type CpG-ODNs have most commonly been used in vaccine design, as they are chemically stabilized and not easily degraded by DNAses[67]. Thus, results obtained in mice using CpG-ODNs as an adjuvant may be misleading and difficult to translate to the human setting. We have recently developed an A-type CpG-ODN-containing VLP vaccine displaying melanoma-specific melan-A peptides. In mice, the 30 nm sized vaccine reached draining lymph nodes and was taken up by pDCs. Vaccination of patients with melanoma led to the induction of multifunctional $CD8^+$ T cells with measurable *ex vivo* activity[71]. Thus, nanoparticles loaded with A-type CpGs may indeed be candidates for the development of CTL-inducing vaccines for use in humans.

The effect of size on lymph drainage might also have consequences for the delivery of conventional pharmaceutical drugs. By attaching or packaging drugs in large particles, phagocytic cells outside of the lymphatic system may be targeted. By contrast, drugs delivered by small particles can be used to target cells that reside in lymph nodes. For example, such small particles could be packaged with A-type CpG-ODNs to target pDCs for the production of type I interferons as a potential therapy for chronic virus infection. Thus, the current use and development of nanoparticles (such as ISCOMs, chitosans, small liposomes and VLPs) as adjuvants may be extended to small-molecule delivery systems.

Harnessing the geometry of viruses

The crucial effector molecules that are induced by most classical vaccines are antibodies. A key feature of previous successful recombinant and inactivated or attenuated vaccines has been their ability to activate both B cells and cognate T helper cells. Most viral surfaces[72], as well as bacterial structures such as the flagellum[73], consist of one or a few proteins and consequently are highly organized and repetitive in nature. Both the innate and adaptive immune systems have evolved to recognize such highly repetitive structures[72]. Indeed, antigen organization and repetitiveness could be viewed as a pathogen-associated geometric pattern similar to pathogen-associated molecular patterns (PAMPs)[34,72]. Most importantly, highly repetitive surface patterns efficiently cross-link BCRs, delivering strong activation signals to the B cell. It has been shown that 15–20 hapten molecules that are spaced 5–10 nm apart is an ideal geometry for optimal B cell activation[74] and is similar to the average spacing of viral coat proteins[72]. This also holds true for peptides that are conjugated to nanoparticles, in which 60 epitopes displayed per particle at a spacing of 5–10 nm were found to be sufficient for optimal antibody responses[36,72].

For the induction of self-antigen-specific antibody responses, repetitive and appropriately spaced presentation of antigens is also important, as non-repetitive self antigens usually fail to break B cell unresponsiveness[34,75]. This is an important feature for therapeutic vaccines that aim at inducing self-antigen-specific antibody responses for the treatment of chronic diseases[76]. Repetitive viral surfaces are also ideal for the activation of complement[77], which further facilitates B cell activation through engagement of the CD19–CD21 complex[78]. Concomitant cross-linking of the BCR and CD19–CD21 reduces the number of BCRs that need to be engaged for B cell activation[36,79,80] and also induces the expression of the transcription factors B lymphocyte-induced maturation protein 1 (BLIMP1) and X-box-binding protein 1 (XBP1)[81], which are crucial for the differentiation of long-lived plasma cells[82].

Natural IgM antibodies bind strongly to repetitive surfaces through multivalent, high-avidity interactions[8]. Together with complement, tightly bound natural antibodies facilitate the trapping of particles in lymphoid organs and also the uptake of particles by DCs and other APCs required for T helper cell priming[83]. It might not be a coincidence that important components of the innate humoral immune system — such as C1q, pentraxins, ficolins and collectins — are multimeric, potentially allowing high-avidity interactions with repetitive pathogen surfaces[13]. So, repetitive structures enhance antibody responses by efficiently cross-linking BCRs, by fixing complement to lower

Depot
An antigen depot refers to antigen persisting at the site of injection, causing prolonged exposure of the immune system to the antigen. Many adjuvants induce antigen depot formation.

Plasmacytoid DC
A cell that has a plasma cell-like morphology and produces high levels of type I interferons after exposure to viruses.

CpG
A DNA motif rich in non-methylated CG motifs that is mainly found in bacterial or viral DNA and is recognized by Toll-like receptor 9.

Hapten
A molecule that can bind antibody but cannot elicit an immune response by itself. Antibodies that are specific for a hapten can be generated when the hapten is chemically linked to a protein carrier that can elicit a T cell response.

CD19–CD21
Co-stimulatory molecules for B cells. CD21 binds complement degradation products, and CD19 is the signal transduction molecule for CD21.

thresholds of B cell activation and promote long-lived plasma cell differentiation, and by allowing binding of natural antibodies to stimulate DC-mediated priming of T helper cells.

Mimicking the kinetics of viral antigens

Optimizing T cell responses. Antigen load and kinetics are crucial for the establishment of long-lived, potentially protective T cell memory. Too little antigen exposure, as is the case during HPV infections, fails to result in a protective T cell response[8]. By contrast, overwhelming infections, such as those caused by high doses of lymphocytic choriomeningitis virus (LCMV) in mice or sometimes by hepatitis B or C viruses in humans, lead to T cell exhaustion, abrogating immunity[84–86]. Whereas high doses of LCMV induce T cell exhaustion, low doses of the same virus induce potent and long-lived T cell responses in mice. It is often assumed that rapid initial replication and subsequent elimination of the virus to very low levels is the key for the induction and maintenance of protective memory[8,87].

It is therefore important to consider the kinetics of viral replication in vaccines designed to induce T cell responses. A possible way to prolong exposure of antigen to the immune system may be to use appropriate vaccination regimens. Rather than giving a single dose of vaccine in a depot form, vaccines might be injected weekly or even daily. Such prolonged antigen exposure has been shown to have a pronounced effect on immune responses in mice. Indeed, it has been shown that prolonged antigen exposure results in co-stimulation-independent T cell responses[88,89], as well as in the development of improved long-lived protective T cell memory in mice[90,91]. Because such regimens are complicated, they would not be feasible for prophylactic purposes but might be suitable for therapeutic vaccines. Such an approach would have the advantage of being able to simulate the kinetics of antigen load typically occurring during viral infection by starting vaccination with low doses, going through a peak dose followed again by lower doses. At least in mice, such a regimen has been shown to induce more potent T cell responses than when the same total amount of vaccine was given in several equal doses[91]. Thus, it may be possible to mimic viral antigen kinetics by using optimized vaccination regimens.

For prophylactic vaccines, however, vaccination schedules must be simple. In addition, a schedule of injections given a month or more apart is usually preferred for the induction of protective antibody responses. This is probably due to the fact that the immune system can generate its own antigen reservoirs in germinal centres to keep B cells stimulated and generate strong antibody responses. In the germinal centres, B cells proliferate and differentiate into memory B cells and plasma cells[92]. Importantly, antigen persists in these germinal centres on the surface of FDCs either in the form of immune complexes or bound to complement receptors[93–95]. In this way, the immune system generates a small depot of antigen that maintains the B cell response for several weeks. This is sufficient for the induction of memory B cells, as well as for the generation of long-lived plasma cells. If the antigen reservoir is prematurely eliminated, by depletion of FDCs for example, the B cell response terminates and no long-lived memory B cell or antibody responses are generated[96]. The ability of the immune system to form its own antigen depot may explain the success of inactivated viral vaccines, which fail to replicate to high levels in the host but nevertheless induce sustained B cell responses[97].

Enhancing vaccine efficacy with adjuvants: depot formation and activation of APCs. Adjuvants, in particular aluminium-based adjuvants in humans, and incomplete and complete Freund's adjuvants in mice, have been used for almost 100 years. A key feature of these adjuvants is their ability to create an antigen depot, resulting in prolonged exposure of the antigen to the immune system. Many adjuvants currently in development, and indeed aluminium salts in common use, attempt to exploit this phenomenon. Such adjuvants are referred to as depot-forming adjuvants. Mineral salts (aluminium hydroxide (Alhydrogel) or phosphate (Adju-Phos)), emulsions (Montanide, AS04, ISA-51 and ISA-720) and protein precipitates (IC31) form antigen depots, leading to a prolonged antigen release[98–100].

It is interesting to note that the above-described antigen retention by FDCs should render B cell responses generally independent of additional antigen depots created by adjuvants. However, it is important to emphasize that vaccine-induced IgG responses are T helper cell dependent[8] (FIG. 1), and so the well-documented depot effect of these adjuvants is probably required to boost T helper cell responses rather than to influence B cells directly.

In addition to forming antigen depots, modern adjuvants are now designed to contain substances that activate the innate immune system — in particular, TLR ligands[101]. The mechanism of action of TLR ligands may be rather complex. In the case of TLR ligands packaged into particles, it has been shown that CpG-ODNs and RNA act directly on B cells rather than DCs[38,40,41]. Specifically, in the presence of TLR ligands packaged into VLPs, antibody responses were higher overall and dominated by IgG2a, IgG2b and IgA isotypes. By contrast, for antigens mixed with TLR ligands, the situation seems different, and TLR ligands might mainly promote B cell responses indirectly by activating DCs[102].

Physical linkage of TLR ligands with the antigen, rather than creating a simple mixture, may be a way to reduce side effects while maintaining immunogenicity. By co-delivering antigen and an innate stimulus, only the cells that interact with the antigen become activated by the TLR ligand. This allows more focused activation of the innate immune system, which is usually responsible for acute vaccine-induced side effects. Indeed, CpG-ODNs mixed with CTL-inducing nanoparticles caused severe side effects in mice, including splenomegaly. By contrast, if the CpG-ODNs were packaged into the nanoparticles, strong CTL responses were induced in the absence of measurable side effects[69,103].

Germinal centre
Transient, highly organized multicellular structure present within B cell follicles that is essential for the generation of memory B cells and long-lived plasma cells, as well as for affinity maturation of antibodies.

It has recently been shown that a subset of T helper cells known as T follicular helper cells is required for optimal IgG responses[104,105]. Currently, little is known about vaccination strategies that favour the induction of this particular T cell subset. However, it has become clear that TLR ligands, in particular TLR7, can overcome the requirement for T follicular helper cell-derived interleukin-21 (IL-21) in IgG responses[106]. Specifically, VLPs devoid of RNA, the natural ligand for TLR7 and TLR8, generally failed to induce IgG responses in the absence of IL-21, whereas VLPs containing a TLR7 ligand induced largely normal IgG responses in the absence of this cytokine[106].

It is interesting to note that repetitive structures, such as VLPs or haptens coupled to dextran, are highly immunogenic in the absence of additional TLR-mediated stimuli[74,75]. Nevertheless, the presence of TLR4 ligands might further increase the response, as evidenced by the fact that Cervarix, which is based on HPV-derived VLPs, is used in combination with alum and monophosphoryl lipid A (MPL), which is a ligand for TLR4 (REF. 107).

Vaccines using MPL or MF59 (an oil-in-water emulsion based on squalene) are thought to generate a pro-inflammatory environment, facilitating the generation of immune responses[99]. Interestingly, although MF59 has been administered to millions of people, it is currently not known how this pro-inflammatory response is generated[108]. Alum has recently been shown to activate NOD-like receptor family pyrin domain-containing protein 3 (NLRP3), and it has been suggested that this ability to stimulate the inflammasome is essential for the adjuvant activity of aluminium salts[109–111]. However, more recent evidence did not support an important role for NLRP3 in aluminium salt-mediated enhancement of immune responses[112,113]. Other studies have shown that optimal immune responses induced by aluminium adjuvant-containing vaccines required an appropriate level of adsorption of the antigen to the aluminium salts; too strong an adsorption inhibited the generation of immune responses[114,115]. So, aluminium salts may aid immune responses through several pathways, including activation of NLRP3 and depot formation, and the contribution of each pathway may vary with antigen, the presence of TLR ligands and the dose used for immunization.

Interestingly, aluminium salt-based adjuvants are among the weakest adjuvants known — despite their ability to activate the NLRP3 pathway. This may question whether the inflammasome is a good target for adjuvant development. In contrast to TLR ligands, which signal the presence of pathogens, the inflammasome may instead be important for signalling endogenous stress[116]. Hence, activated inflammasomes may not promote protective immune responses. It is interesting to note in this context that it has previously been shown that other endogenous stress or 'danger' signals fail to support T helper cell differentiation in the absence of additional TLR ligands[117]. Hence, signals directly associated with pathogens rather than endogenous stress signals hold promise for new-generation adjuvant development.

Another important aspect of adjuvants that is not well addressed is their ability to render antigens in a form that makes them susceptible to phagocytosis by APCs. Large macromolecular adjuvants such as Alhydrogel can be viewed as a crude way to render soluble antigens particulate, thereby enhancing their uptake by DCs[118]. In an analogy to the 'immunologist's dirty little secret' (REF. 119), one may call this the 'manufacturer's dirty little secret', as aggregation status, homogenicity and exact three-dimensional conformation of the antigens, for example, are difficult to assess once they have been adsorbed to Alhydrogel.

In summary, adjuvants in current use enhance protective antibody responses mainly by enhancing T helper cell responses through depot formation, rendering antigens particulate and, in some circumstances, activating DCs through stimulation of TLRs and perhaps other innate receptors, such as NLRP3. In addition, TLR ligands may act directly on B cells, particularly if conjugated to or associated with the antigen.

Concluding remarks

The key properties of viruses that are responsible for eliciting potent immune responses may be used as a framework for rational vaccine design. These important immunogenic properties of viruses include their size, geometry, an ability to induce innate immunity with appropriate conditioning of the adaptive immune responses and an ability to replicate, leading to characteristic antigen kinetics and distribution. New-generation vaccines aim to harness these properties.

To induce potent immune responses, vaccines of viral size are preferable, as only particles in the nanometre range can reach lymph nodes and directly interact with B cells. The antigenic epitopes should be displayed on the nanoparticles in an ordered and highly repetitive way to optimally activate B cells and fix components of the innate humoral immune system (natural antibodies, complement and pentraxins). To further enhance antibody responses and induce more protective IgG2a (mouse) and IgG1 (human) antibodies, vaccines should be co-delivered with particular TLR ligands. To ensure maximal B cell stimulation together with minimal non-specific side effects, TLR ligands should be linked to or packaged into the vaccine particles.

Nanoparticle-sized vaccines are also preferable for the induction of T cell responses, as size limits the ability of particles to reach the appropriate DC subsets in lymphoid organs. Linkage of the particles to TLR ligands will facilitate licensing or activation of DCs, which is essential for the induction of T cell responses. Nevertheless, the induction of strong T cell responses in humans even with nanoparticle-based vaccines has remained difficult and it remains to be established whether a combination of different innate stimulators or creation of long-lived antigen exposure with new depot-forming formulations or vaccine regimens may be more successful.

In summary, vaccine carrier systems that mimic the size, geometry, replication kinetics and PAMPs of viruses may be one possible way to optimally harness viral properties without the risks associated with infection.

T follicular helper cell
A specialized T helper cell found in B cell follicles. These cells require interleukin-21 for their development and are specialized in providing help to B cells.

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