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Modulating the immune system through nanotechnology

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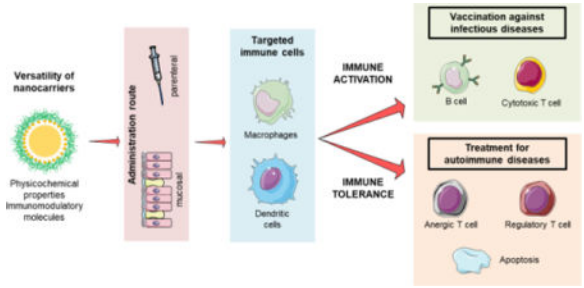
Abstract

Nowadays, nanotechnology-based modulation of the immune system is presented as a cutting-edge strategy, which may lead to significant improvements in the treatment of severe diseases. In particular, efforts have been focused on the development of nanotechnology-based vaccines, which could be used for immunization or generation of tolerance. In this review, we highlight how different immune responses can be elicited by tuning nanosystems properties. In addition, we discuss specific formulation approaches designed for the development of anti-infectious and anti-autoimmune vaccines, as well as those intended to prevent the formation of antibodies against biologicals.

Graphical abstract



Keywords

nanotechnology; immune system; tolerance; stimulation; autoimmune disease; vaccine

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1. Introduction

The modulation of the immune system is the base of new and promising therapies for some of the most prevalent and/or severe diseases of our time, such as cancer, HIV, and type 1 diabetes. The development of treatments based on this modulation is a field in expansion, where the contribution of nanotechnology is growing exponentially [1–3]. Based mainly on the molecular principles that govern the interaction between pathogens and immune cells, the use of nanotechnology represents a new way of communication with the immune system. Both, the composition and the physicochemical characteristics of nanocarriers, can influence their interaction with immune cells. By mimicking the size of microorganisms (bacteria and viruses) and incorporating key molecules involved in immune processes (TLR agonists, cytokines, etc.), nanocarriers can be taken up by the immune cells and modulate their responses. Besides, the use of nanocarriers decorated with targeting moieties can favor their preferential access to specific immune cell populations [2,4–9]. Importantly, the tunable nature of nanotechnology offers the possibility of reinforcing the desired aspect of immunomodulation, which maybe (i) the activation of the immune system in order to generate an immune response against a specific antigen, or (ii) the induction of immunotolerance against antigens and immunoactive drugs. The first option improves the chances of controlling infectious diseases that do not respond well to traditional vaccines, such as HIV or tuberculosis, among others [10–12]. The second, and less explored option, refers to the development of vaccines against autoimmune diseases as well as the targeted administration of immunomodulatory drugs [13–15]. The capacity of nanotechnology to elicit different responses comes from its versatility, gained through the specific combination and meticulous choice of its molecular components, and from the physicochemical properties of the nanosystems.

In this review, we first summarize how nanotechnology may help reaching the desired cell population, and achieving its specific modulation. Then, we offer an overview of the role that nanotechnology has played in the development of new vaccines against infectious diseases, followed by an analysis of its contribution to the treatment of autoimmune diseases. Finally, recent achievements to fight antidrug antibodies are summarized.

2. Access of nanostructures to target cells

For a nanovaccine to be effective, it first needs to access the tissues where the target cells are present. Depending on the administration route, different physiological barriers must be overcome to reach these cells. Thus, nanoparticles (NPs) should be specifically engineered to go preferentially to the target tissue from the site of administration.

2.1. Routes of administration of nanocarriers intended for immunomodulation

Although immune cells are distributed throughout the body, the key cells involved in immunity are concentrated in the lymphoid tissues. Hence, targeting these tissues facilitates the access to immune cells and, consequently, increases the efficacy of administered nanovaccines. Lymphoid tissues are directly accessible through the mucosal surfaces, such as airways, the intestinal tract, or the vagina, although a more straight way to target them is

by parenteral injection. The way antigens reach the lymph nodes (LN), following different modalities of administration is illustrated in Fig. 1.

2.1.1. Mucosal administration—Following mucosal administration, a needle-free and appealing route for vaccination, it is possible to induce both, mucosal and systemic, immune responses [16]. The mucosa-associated lymphoid tissues (MALT) are connected to the mucosal environment through the M cells, which are specialized in the transcytosis of microorganisms and particulate components [3,16]. The activation of mucosal resident T and B cells can be of great importance for an efficient mucosal vaccination [17,18]. This is the reason our group and many others have explored the potential of nanocarriers for the transport of antigens across different mucosae in order to reach a proper stimulation of the immune system. Moreover, the administration of nanocarriers through mucosal routes has also been investigated for tolerance generation [19].

In order to elicit an adequate response, NPs need, first, to overcome the mucus layer that covers the mucosal surfaces. Then, once in contact with the epithelium, nanocarriers are transported either by M cells or by regular epithelial cells [20–22]. NPs can also be internalized by paracellular transport if their composition includes components that can open tight junctions [23]. Moreover, it has also been described that dendritic cells can take up NPs by extending their dendrites into the lumen [24,25].

The specific physiology of the mucosal surfaces is different throughout the body and, hence, the optimal properties for the nanocarriers to cross them may also be different. Initially, bioadhesive nanosystems were thought to be a promising strategy to facilitate the interaction of nanocarriers with the mucus layer and a number of strategies have been described for that purpose [26,27]. For example, Nochi *et al.* developed adhesive cationic nanogels made of cholesterol-modified pullulan that were able to increase the survival rate of mice after intranasal vaccination against tetanus and the botulin neurotoxin [28]. However, it was also observed that if the systems were retained in the mucus by high adhesive forces, they could be soon eliminated by the clearance mechanisms. This disadvantage led to the engineering of nanocarriers with mucodiffusive properties that would allow them to cross the mucus layer and reach the epithelium. Nowadays, a precise balance between mucoadhesive and mucodiffusive properties is believed to be critical for the effectiveness of nanocarriers delivered through mucosal routes. For good mucodiffusion properties, it has been reported that particle size should be smaller than the mucus mesh size [29]. Although there are studies where microparticles (MPs) showed better results than NPs after oral administration [30,31], in general, the recent trend has been to consider that NPs perform better than MPs [32–37]. In this regard, our group reported that the transport of pegylated polylactic acid (PEG-PLA) NPs across the nasal mucosa was higher than that of MPs. Furthermore, the smaller micrometric sizes (1 and 5 μm) also crossed the epithelium more efficiently than 10 μm particles, with no significant differences between 1 and 5 μm [38]. Interestingly, based on recent *in vivo* data, very small nanometric sizes (30 nm) may not be as effective as larger ones (200 nm) [39].

Besides the particle size, other nanocarrier's features may also have important consequences for mucopermeation. For example, in 1998, our group described for the first time that the

presence of a PEG coating in NPs made of PEG-PLA had an important role in increasing their transport rate through the nasal [40] and intestinal epithelia [41]. Furthermore, other authors have described that the presence of an adequate PEG coating allows particles with a size in the range 200 – 500 nm to penetrate across the mucus [42,43]. In brief, we may conclude that the size and composition of the nanocarriers, and notably the surface composition, may influence the particle transport across mucosal surfaces.

2.1.2. Parenteral administration—Intramuscular, subcutaneous, and intradermal administrations are the main routes of vaccination. Following these modalities of administration, and depending on their physicochemical properties and composition, NPs can drain directly to the closest lymph node, or stay in the injection site and attract migratory dendritic cells or macrophages. Overall, the main conclusion drawn from several reviews in the literature is that sizes up to 100 nm are able to self-drain to the nearest lymph node, being the drainage usually inversely proportional to the particle size [3,9,34,44–47]. However, very small particles (< 10 nm) can directly drain to blood capillaries [48] and those that reach the lymph nodes have shown limited retention [49]. With regard to the surface charge, some authors have indicated that the drainage of negatively charged NPs to the LN is facilitated by their repulsion with the negatively charged extracellular matrix. This repulsion acts as a driving force moving NPs to the lymphatic system [50–52]. On the other hand, cationic nanosystems tend to form a depot after parenteral administration, being taken up by peripheral and migratory APCs or slowly draining to LNs [53]. Nevertheless, this charge effect may be counterbalanced by the appropriate adjustment of the particle size. For example, Zeng *et al.* showed that 30 nm cationic micelles were able to self-drain to the closest lymph nodes [54]. Similarly, Kim *et al.* have reported that both small cationic and anionic poly(γ-glutamic acid)-based nanosystems (30–60 nm) were able to self-drain to the closest lymph node [55]. Finally, the presence of PEG on the surface of the nanocarriers, that usually renders their surface charge close to neutrality, has a positive effect in the drainage to the LN [56–59]. This does not necessarily translate into a higher interaction with immune cells [51,56,60], as the degree of pegylation and the PEG molecular weight may have an impact on the NP opsonization [8,61].

In the case of intravenous (IV) administration, it has been found the possibility to generate a tolerogenic effect by antigen-loaded nanocarriers [62,63]. The hypothesis to explain this result is that NPs delivered by this route are mainly accumulated in the liver and engulfed by Kupffer cells, which are essential for the elimination of apoptotic cells and other debris from the blood, mechanism associated with the maintenance of peripheral tolerance [64]. In this situation, Kupffer cells and liver dendritic cells were shown to have an increased expression of PD-L1 in their surface, which contributes to a higher tolerance [65].

Overall, the conclusion from the reported studies is that the final outcome of the nanocarriers is determined by the simultaneous influence of their properties including particle size, surface charge, shape, hydrophobicity and stiffness, among others (Fig. 2).

2.2. Targeted cell populations in immunomodulation and immunological responses

The immune system is comprised by circulating cells, which are in charge of capturing peripheral antigens (monocytes, macrophages and dendritic cells) and more static cells, such as B and T cells. All these cells are targets of interest for immunomodulation depending on the desired type of response (Fig. 3).

In order to generate a biased immune response, two different approaches can be followed, and they involve (i) the design of nanocarriers that can reach preferentially one subset of immune cells, either by passive or active targeting. For this purpose, nanocarriers features, i.e. particle size, surface charge or shape, can be modulated in order to facilitate their passive access to immune cells; however a good discrimination between cells can only be achieved through the use of active targeting ligands. (ii) The use of adjuvants that modify the response given for a specific immune cell subset. These immunomodulatory molecules may mimic pathogen-associated molecular patterns (PAMPs), which are bacterial cell wall components, viral RNA, and CpG DNA. These molecules activate different receptors that will lead to cellular or humoral immune responses. Similarly, different cytokines and other immunomodulatory molecules, such as rapamycin, vitamin D3 or phosphatidylserine (PS), can be loaded into nanosystems to induce tolerogenic responses.

Based on this scheme, the different targeted populations in immunomodulation are monocytes, macrophages and dendritic cells (DCs). **Monocytes and macrophages** are one of the most common phagocytic cells in the body and represent the first innate defense line. They can be either circulating or resident in tissues, clearing pathogens and apoptotic cells. These cells are able to drain to the injury site, attracted by chemokines, and, hence, they have an important role in presenting antigens and releasing cytokines that modulate the immune response. In addition, they mediate inflammatory processes, which are relevant in a large variety of inflammatory diseases as well as in tumor growth and metastasis.

Several *in vitro* studies have been conducted in order to determine the characteristics of NPs and MPs that are key for the passive targeting to macrophages. All these studies have shown that both, particle size and shape, may influence the internalization efficiency by macrophages (Table 1A). In this sense, in the past and mainly based on *in vitro* studies, it was assumed that particles in the micrometric range were well recognized by macrophages [66–72]. Nevertheless, recent studies have questioned this assertion and the current tendency is to believe that NPs can be very efficiently taken up by macrophages [73,74]. On the other hand, regarding the influence of the surface charge on the uptake of NPs by macrophages, several *in vitro* and *in vivo* studies have shown different results. Indeed, while in some cases cationic nanosystems were taken up by macrophages at a greater extent than neutral and negative ones [73,75–78], in others, the negative charge was preferable for an efficient uptake [79–84]. For example, Nakanishi *et al.* reported that positive multilamellar vesicles elicited stronger cellular and humoral immune responses both *in vitro* and *in vivo* than neutral or negative systems [78]. On the contrary, Fromen *et al.* observed that after a pulmonary instillation of anionic and cationic PRINT hydrogels, negative nanosystems were engulfed in a greater manner by pulmonary macrophages [79]. More examples of these somehow contradictory results are summarized in Table 1A.

The studies above-mentioned highlight the lack of a clear conclusion on the best way to target macrophages through the modification of nanocarrier's particle size and surface charge. Furthermore, their composition is probably an important factor dictating such interaction. To improve this, some authors attempted an active targeting to specific macrophage receptors (Table 1B). For example, iron-oxide NPs coated with IgG, were shown to be taken up by monocytes and macrophages in a much higher extent than the uncoated ones [85]. Other authors have found that targeting the mannose receptor was a way to enhance the interaction of liposomes with tumoral macrophages after IV administration [86].

In other studies intended to induce tolerance, authors have taken advantage of the specific expression of folate receptor β in activated macrophages in inflamed joints. For example, folate-functionalized dendrimers showed an increased joint accumulation after IV injection in collagen-induced arthritis (CIA) mice model [87,88]. Similarly, the specific recognition of dextran by scavenger receptors was explored to develop an anti-inflammatory therapy. Namely, dextran NPs containing dexamethasone were used to target pro-inflammatory macrophages from obese patients [89]. On the other hand, hyaluronic acid, has been proposed as a way to specifically interact with the CD44 receptor, found in lymphocytes, among other cells [90,91]. In addition, it has been recently reported that low molecular weight hyaluronic acid exhibits immunostimulant properties [92] and that these properties can be related to the ability of hyaluronan fragments to activate TLR2 and TLR4 [93,94]. Furthermore, recent reports have also claimed the capacity of hyaluronic acid to polarize tumor-associated macrophages from M2 towards a M1 anti-tumoral subtype [95], although further investigation is still needed to determine the impact of these studies.

Dendritic cells (DCs) are the most important antigen-presenting cells (APCs) and have a key role in the modulation of the immune system [96]. As illustrated in Fig. 3, DCs internalize antigens from their surroundings, process them in endosomes/lysosomes and present the resulting peptides through the class II major histocompatibility complex (MHC II), leading to a specific $CD4^+$ T cell activation and proliferation [97]. On the other hand, if the antigens are found in the cytosol of DCs, as in the case of intracellular infections, the peptides will be presented by class I MHC (MHC I) to naïve $CD8^+$ T cells, activating cellular responses. In some cases, external antigens can be translocated from endosomes to the cytosol and, thus, be presented via MHC I, process known as cross-presentation [3,98]. This phenomenon is of great importance in antitumor and infectious disease vaccination where a potent cellular response is required [99]. In both cases, besides antigen presentation, a co-stimulation of T cells through cytokines or co-stimulatory signals is normally needed [45].

Significant attempts have been made to passively (Table 2A) or actively (Table 2B) target DCs using nanocarriers (Fig. 4). DCs have a high phagocytic capacity similar to that of macrophages, however, unlike them, DCs preferentially ingest small virus-size particles [72,100,101]. Therefore, a way to passively target DCs is through the reduction of the nanocarriers' size. On the other hand, it is also known that providing nanocarriers with a positive surface charge enhances the chances for them to interact with DCs and macrophages [75,79,101–103]. Nevertheless, irrespective of the influence of size and surface charge in the

specific uptake of particles by dendritic cells, it seems clear that the most effective approach to precisely target DCs would be providing the nanocarriers with specific targeting ligands (Table 2B) [104]. For example, Cruz *et al.* systematically studied this possibility by functionalizing pegylated poly(lactic-co-glycolic) acid (PEG-PLGA) NPs with antibodies to target either CD40 (TNF-α family receptor), CD11c (integrin receptor) or DEC-205 (C-type lectin receptor) receptors. All NPs contained an antigen (OVA) and TLR3 and 7 agonists, but only those with a specific ligand showed increased $CD8^+$ T cell activation, both *in vitro* and *in vivo* [105]. The targeting of the mannose receptor has also been reported as a strategy to increase the activation of DCs *in vitro* and *in vivo* [106,107].

2.2.1. Cellular responses—In order to fight some infectious diseases (i.e., HIV, malaria) or other diseases, i.e. cancer, the stimulation of a powerful cellular response is necessary. In this context, a correlation between NP size and its ability to favor cross-presentation has been reported [3,9]. In general, studies have shown that smaller sizes enhance cross-presentation and Th1 responses [108–110]. It has been hypothesized that this effect might be related to the capacity of the these NPs to self-drain to the lymph nodes and thus, directly interact with resident $CD8^+$ DCs [111], and also to the specific uptake pathway they follow for internalization. Regarding their uptake, it has been described that particles with sizes similar to virus are endocytosed by DCs through a internalization route that facilitates endosomal escape and drives cellular responses [112–114]. Also, as mentioned above, active targeting to DC205, CD40 or CD11c has shown higher $CD8^+$ T cell activation [105].

As previously discussed, to drive cellular immunity, DCs need to present antigens on MHC I. To achieve this cross-presentation, the antigen has to be present in the cytosol of DCs, thus favoring endosomal escape of the antigen is a requirement for achieving a cellular response (Fig. 4D, Table 2C). This endosomal escape can be promoted by the disruption of the endosome membrane, as discussed by several authors [115,116]. Keller *et al.* showed how pH-responsive micelles significantly enhance cytotoxic T lymphocyte responses, in comparison to micelles without these properties [117]. This effect was achieved because the forming polymers are protonated at endosomal pH which allows them to interact with the membrane and disrupt the endosome [117,118]. The same tendency was reported in the case of pH-sensitive liposomes, cationic liposomes and bioreducible linkages [119–122]. Other example of cross-presentation and increased cytotoxic T lymphocyte activity has been shown by the ISCOMATRIX adjuvant, both in preclinical and clinical studies, due to a rapid antigen translocation from the endosome [123,124].

With regard to the use of adjuvants, toll-like receptors (TLRs) are extensively used for immunomodulation (Fig. 4A, Table 2C). More specifically, for Th1 biased responses, endosomal TLRs (TLR3, 7, 8 and 9) are an interesting target. These receptors recognize bacterial and viral genetic material, thus their activation will trigger a cellular response, as would a viral or intracellular-bacterial infection. In addition, the combination of nanotechnology and adjuvants has shown a great $CD8^+$ T cell activation with a decrease in the toxicity associated with these molecules [125–127]. Furthermore, since it is known that pathogens normally express several PAMPs at the same time, the combination of several immunomodulatory molecules can further enhance the elicited immune response [7,9,128].

An alternative procedure to generate cellular responses is a direct targeting to $CD8^+$ T cells (Fig. 4E). For this, some authors have employed the so-called artificial antigen presenting cells (aAPCs), which present in their surface major histocompatibility complex molecules and also specific cell markers for T cell recognition and activation [129]. Using this strategy with paramagnetic particles and quantum dots, an increase in $CD8^+$ T cell activation and a decrease in tumor growth were observed [130]. Later on, ellipsoidal PLGA nano-aAPCs were developed, and were shown to be more efficient than the spherical ones in driving $CD8^+$ T cell activation [131].

2.2.2. Humoral responses—Since B cells are in charge of antibody production, a sustained activation of these cells is crucial to guarantee humoral responses. This is the mechanism of action by which most vaccines on the market led to long-lasting antibody responses. Normally, B cell activation is driven by both, the direct interaction of the antigen with the B cell receptor (BCR) and the co-stimulation by $CD4^+$ T cells [132,133].

Some authors have suggested that the location of the antigen on the NP's structure may influence the resulting humoral response (Table 3). For example, Temchura *et al.* observed that calcium-phosphate NPs with the antigen covalently attached to their surface, led to a substantial increase in B cell activation *in vitro*, in comparison to the soluble antigen [134]. Similarly, Moon *et al.* showed that the display of the antigen onto the surface of multilamellar vesicles provided an enhanced humoral response as compared to the antigen encapsulated [135]. In agreement with these data, several reports showed that the covalent conjugation of the antigen to liposomes could generate stronger antibody responses as compared to those obtained for other types of antigen association (Table 3) [136–146]. Nevertheless, the number of studies on the importance of the linking process of the antigen to the nanocarrier is very limited and it requires further exploration.

With regard to the influence of the size on the humoral responses of antigens associated to NPs, it has been reported that for some specific compositions micrometric sizes have a tendency to preferentially generate Th2 responses, in comparison to smaller sizes [112–114]. The mechanism behind this behavior could be related to the uptake pathway. It has been described that for sizes bigger than 500 nm the internalization and processing route of the antigen lead to a more efficient presentation by MHC II, generating stronger humoral responses [113,114].

Another possibility to favor humoral responses could be the administration of TLR2 agonists, since these are able to generate Th2-biased responses [147,148]. Similarly, the activation of surface TLRs (TLR2 and TLR4) showed that they can efficiently inhibit $CD8^+$ T cell activation [149].

2.2.3. Tolerogenic responses—In autoimmune diseases, the generation of tolerance is needed to control the immune response developed against self-antigens. During the last years, different nanotechnology-based approaches have been explored with regard to their capacity to generate tolerogenic profiles (Fig. 5).

The debris produced during apoptosis, a process of programmed cell death, are eliminated by APCs. The APCs present the processed antigens within a tolerogenic environment, without activating immune responses [150]. Mimicking this environment, nanocarriers can follow debris elimination routes and take advantage of this process to generate tolerance. For the uptake of apoptotic debris, scavenger receptors play the main role in apoptotic signal recognition and debris endocytosis [151]. The incorporation of these apoptosis signal molecules, such as phosphatidylserine (PS), in the nanocarrier composition may enhance its uptake in APCs and allow for a tolerogenic antigen presentation. For example, in one experimental approach, 50 % of mice treated with antigen-loaded PS liposomes could be prevented from acquiring type 1 diabetes (T1D) [152]. Also, experiments show that MARCO-targeted polystyrene MPs follow the debris elimination route, and help to present the antigens loaded in a non-inflammatory way [62]. Interestingly, not only the presence of PS, but also its geometrical surface disposition was found to play a role in tolerance induction. For example, Roberts et al. observed that PLGA NPs displaying a nanorod-presentation were more efficient at inducing tolerogenic responses than the spherical ones [153].

Furthermore, the loading of immunomodulatory molecules in nanocarriers has been shown to help APCs to achieve a tolerogenic state. Molecules such as rapamycin, dexamethasone or vitamin D3 may be co-encapsulated with antigens inside nanocarriers, and promote its presentation in a tolerant environment in APCs [154–156]. Moreover, the delivery of nucleic acids coding for modulatory cytokines has been explored with the goal of inducing tolerogenic profiles in immune cells [157,158].

Finally, the association of antigen-MHC complexes (pMHC) on the surface of iron oxide NPs has been shown to expand autoregulatory T cell memory in different animal models. Indeed, Tsai *et al.* showed that pMHC class I-coated NPs triggered massive expansions of autoregulatory $CD8^+$ T cells, and these cells were able to suppress polyclonal autoimmune responses by selectively targeting autoantigen-loaded APCs in the target tissue and draining lymph nodes [159]. On the other hand, another report showed that the use of pMHC class II-coated NPs expanded disease-specific regulatory $CD4^+$ T cells.[160].

3. The potential of nanotechnology for vaccination

During the last decades, great efforts have been made to develop systems capable of generating protective immune responses against a variety of antigens. In this section, we present an overview of the work done for specific antigens, such as HIV, malaria or hepatitis B. In this context, it is important to mention that most vaccines currently on the market are based on the generation of humoral protection, which has turned out to be inefficient for some infectious diseases and for cancer, where a strong cellular response is needed. In these particular cases, nanotechnology might be a promising solution. Another article of this special issue is focused on the application of nanotechnology for cancer treatment, which is out of the scope of this review.

The first evidence of the potential of nanotechnology for vaccination was reported 30 years ago by Birrenbanch and Speiser. These authors showed that polyacrylamide NPs could work

as adyuvants as they were able to increase the immune response against human IgG and tetanus toxoid after subcutaneous administration to guinea pigs [161]. Years later, Preis and Langer proposed the idea of “single-dose vaccines” based on the possibility to control the release of proteins from polymeric beads [162]. These results were the foundation for the development of controlled antigen delivery systems and nanovaccines.

The development of nanotechnology-based vaccines with a more translational perspective started in the early 90s when the World Health Organization (WHO) proposed the initiative of developing a single-dose vaccine for **tetanus toxoid**. From this point on, many studies with PLGA-based microsystems were conducted [163]. Unfortunately, despite their good antigen release profiles, a certain protein denaturation was observed due to the pH acidification caused by the degradation of the polymer. To solve this problem different approaches were considered, among them, the use of a protective oil-core surrounded by a PLGA shell or the inclusion of poloxamer 188 to prevent interaction between polymer and antigen [164,165]. At the same time, the potential of nanometric size systems started to gain importance. Almeida *et al*. developed 500 and 800 nm PLA microspheres for nasal administration of tetanus toxoid with promising results [166]. Later on, our group found that the pegylation of PLA was essential in order to enhance the stability and penetration of the NP across mucosal surfaces [167]. Indeed, the results from experiments using PEG-PLA NPs, did show an increase in the access of the associated antigen to the blood circulation and LNs [40]. Moreover, high and long-lasting anti-tetanus Ig titers were reported with these nanosystems, due to their ability to cross the nasal epithelium [37,168]. Subsequently, more hydrophilic polymers were explored with regard to their ability to transport antigens across mucosal surfaces. In particular, our group pioneered the development of chitosan NPs as alternative candidates for the development of nanovaccines, especially for those administered through mucosal routes [169]. Our studies concluded that the intranasal administration of chitosan NPs loaded with tetanus toxoid resulted in an increase in the humoral and mucosal responses, in comparison to the results obtained with the administration of the free antigen or even with those obtained when the antigen administered was associated to alum [167,170].

As previously mentioned, many studies have tried to develop nanotechnology-based vaccines against a large number of diseases. These diseases include hepatitis B, malaria or HIV, among others, as reported in the following lines.

Our group has also been involved in the development of nanoformulations of the recombinant **hepatitis B** surface antigen (rHBsAg). In particular, rHBsAg was associated to chitosan NPs and administered by the intramuscular route. The results showed an IgG immunogenic response that was higher than the one observed for the control alum formulation [171]. The same antigen was also adsorbed on chitosan-based nanocapsules [172], a system that was also pioneered by our group [173]. These nanosystems are composed of an oily core surrounded by a chitosan shell, where the protein is adsorbed. After intramuscular administration of rHBsAg attached to chitosan-based nanocapsules, an important antibody responses as well as a more balanced Th1/Th2 profile were obtained [172].

The tendency in the last years has been to design nanosystems that combined the intrinsic targeting properties of nanocarriers with the encapsulation of adjuvants. In this regard, we combined the mucoadhesive properties of chitosan with the adjuvants squalene and imiquimod (TLR7/8 agonist). The results of the intranasal administration of this system showed that the co-encapsulation of antigen and adjuvants was key to generate enhanced and long-lasting IgG levels [174]. More recently, a layer-by-layer approach was evaluated to encapsulate the rHBsAg. This approach consisted on coating the rHBSAg viral particles with a cationic polymer (protamine or polyarginine), followed by an anionic layer of poly(I:C). These nanostructures were able to elicit a more balanced Th1/Th2 ratio after intranasal and intramuscular administration [175].

The development of an effective vaccine against **malaria** has also attracted a lot of attention in the last decades. In 2015, GSK licensed a vaccine under the name of Mosquirix™, that contains the circumsporozoite protein of *Plasmodium falciparum* and the liposome-based adjuvant AS01, composed by monophosphoryl lipid A (MPLA) and the saponin QS-21 [176]. This new vaccine has shown good safety profiles and an efficacy rate of 50 % [177], leaving the door open for new improved systems. In this regard, some critical advances have been made thanks to the use of nanotechnology. For example, Moon *et al.* developed two different formulations of the VMP001-malaria antigen. One of them consisted of PLGA NPs with a phospholipidic coating [178], and the other one of multillamellar vesicles[135], both of them carrying the malaria antigen on the surface. The subcutaneous administration of both formulations in the presence of adjuvant MPLA led to strong humoral and cellular responses, as well as a more balanced Th1/Th2 profile [135,178].

The design of an **HIV** vaccine is another global challenge, since this disease kills over 1 million people per year according to the World Health Organization. Currently, the most promising vaccine undergoing clinical trials is based on the combination of a viral vector expressing the group antigens (Gag) and the protease (Pro), together with the HIV gp120 envelope recombinant glycoprotein adsorbed onto alum, which has demonstrated a 31 % efficacy [179]. These results highlight the importance of continuing the search for new HIV nanovaccines. The major obstacles for an HIV vaccine are the choice of an effective immunogen and the development of a nanosystem able to generate a potent immune response. The above-mentioned multilamellar vesicles developed by Moon *et al.* were also evaluated as a potential carrier for the antigen consisting of the envelope glycoprotein (Env) gp140 trimers. This new composition resulted in Th1/Th2 balanced profiles and increased titers against the antigens [180]. A similar strategy based on displaying HIV trimers on the liposomes surface in order to target B cells has been adopted by other authors, showing positive results in terms of neutralizing antibodies responses [181–183]. On the other hand, Hanson *et al.* co-administered two liposomal formulations, one of them displaying an Env-derived peptide and encapsulating a T-helper peptide, and another one loaded with cyclic di-GMP. Their results showed enhanced $CD4^+$ and $CD8^+$ T cell responses and high-titer and durable humoral responses in mice. However, the immune sera did not neutralize HIV [184]. More recently, Kasturi *et al.* reported enhanced protection of non-human primates against up to 12 low-dose intravaginal challenges with SIVsmE660. Interestingly, these results were achieved using PLGA-based NPs loading TLR 4/7/8 ligands as adjuvants, in a physical

mixture either with the soluble immunogens Env and Gag or displayed in virus-like particles [185].

Important efforts have also been devoted to develop a nanovaccine against ***Chlamydia trachomatis***, an intracellular bacterium that infects over 100 million people annually. For example, Stary *et al.* reported positive results for NPs made of a triblock copolymer (PLGA-polyhistidine-PEG) and functionalized with the TLR7/8 agonist resiquimod. These nanoparticles exhibited a pH-dependent surface charge, that switched from slightly negative (at pH: 7.4) to positive (at pH below 6.5). This positive charge allowed the adsorption of the NPs to the antigen (inactivated *Chlamydia trachomatis* bacteria). The formulation was then administered subcutaneously, nasally or intravaginally to mice and, in all cases, strong systemic memory T cell responses were generated. However, only mucosal vaccination effectively protected against a challenge with *Chlamydia trachomatis* [186].

As a consequence of all these efforts, some NP-based adjuvants have already reached the market. This is the case of MF59, AS03, or the previous mentioned AS01. MF59 is a 160 nm nanoemulsion of squalene, Tween®80 and Span®85 that is part of an Influenza vaccine, commercialized mostly in Europe since 1997 by Novartis under the name of Fluad® [187]. AS03 is also a nanoemulsion-based adjuvant, composed of squalene, tocopherol and Tween®80, property of GSK. Currently, this adjuvant can be found in the pandemic influenza vaccine Prepandrix™, approved in 2008 [188]. Also, Epaxal® and Inflexal® V are two virosome-based vaccines for hepatitis A and influenza, respectively, that are commercialized in some European countries [189].

In addition to these adjuvants and vaccines, a great number of nanoformulations for vaccine delivery are currently in clinical trials and they are illustrated in Table 4.

Taking into consideration the huge efforts made in this field at the research level, it is expected that, in the near future, new nanovaccines will land in the market, and provide hope for defeating devastating illnesses of our generation.

4. The potential of nanotechnology for immunomodulation of autoimmune diseases

As previously mentioned, immunomodulation is a desirable strategy to avoid exacerbated immune responses against ubiquitous molecules, such as self-proteins and, hence, it is of particular interest for the treatment of autoimmune diseases. In autoimmune diseases, autologous proteins are recognized as non-self-antigens by the immune system, leading to the generation of autoreactive T and B cell clones. Currently, the treatment of this kind of diseases is symptomatic and relies on the use of classical anti-inflammatory drugs as well as immunosuppressive therapies. Unfortunately, these therapies are unspecific and lead to significant side effects (Table 5). Due to the complex regulatory network of the immune processes, moving from these therapies to targeted and specific treatments has been found to be an important challenge in biomedical research. In that sense, nanotechnology offers the possibility of the specific delivery of the drug/antigen to the desired cell population, as well as the co-delivery of the targeted drugs with adequate immunomodulatory molecules.

Furthermore, nanotechnology offers the possibility to protect the drug from degradation, increasing its half-time life.

In this section, we discuss recent advances in nanotechnology regarding immunomodulation to fight against autoimmunity. First, we present the role of the nanocarriers used to enhance the response of immunosuppressant drugs. Next, we focus on more specific approaches evaluating the potential of nanotechnology for antigen-specific therapies in autoimmune diseases with known self-antigens. From the delivery point of view, the common feature of these strategies is that the target cells are the immunocompetent cells.

4.1. Nanomedicines for the treatment of inflammatory diseases

Inflammation is a common immune process that helps the body to eliminate injury related debris, such as microbes, toxins, and necrotic cells. This mechanism is triggered by extracellular signaling factors that attract plasma proteins, immune cells and phagocytes. This inflammatory response could be either acute or chronic. Chronic inflammation usually lasts longer and leads to complications due to tissue degeneration [219]. The chronic inflammatory diseases include autoimmune diseases and auto-inflammatory diseases. In the case of autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis, type 1 diabetes, lupus or multiple sclerosis, T cells are thought to be the main triggers of the disease process. Different cytokines, such as TNFα, play a role in maintaining these autoreactive T cells. On the other hand, auto-inflammatory diseases, such as sepsis, gout or type II diabetes, are mainly mediated by innate immune system effectors, such as macrophages, the complement cascade, and cytokines such as IL-1β [220,221]. In these chronic diseases, the targeted treatment of inflammatory conditions could be considered as an immunomodulatory approach, slowing down disease progression and ameliorating the symptoms by changing the immune response, both directly (using immunosuppressant drugs) or indirectly (using anti-inflammatory drugs). This section focuses on different nanotechnology-based therapies developed for the treatment of inflammation in autoimmune diseases.

Immunosuppressant molecules are frequently used for the treatment of chronic inflammation. The many drugs available on the market for the treatment of inflammatory conditions have shown limited success in controlling disease symptoms due to their non-targeted biodistribution. Moreover, the immunosuppressant therapy is normally associated to off-target organ side effects and systemic toxicity, exacerbated by frequent and long-term dosing. Nanoencapsulation of immunosuppressive agents has been shown to increase the therapeutic success of those drugs based on the principle of passive or active targeting. The targeted delivery of these molecules, mainly to macrophages in the inflammation site, has led to the reduction of their side effects and also to improve their action on the inflammatory signaling routes mediated by immune cells, which can be consider also as immunomodulation. This has been widely reviewed in the literature for pathologies as inflammatory bowel disease, rheumatoid arthritis, or systemic lupus erythematous [15,222,223] (Fig 6).

4.1.1. Inflammatory bowel disease—Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the digestive tract, including ulcerative colitis (UC) and Crohn's disease (CD). UC is confined to the colon, whereas CD can affect any region of the gastrointestinal tract, being the terminal ileum and the colon the most commonly affected areas. Recent research has shown that genetic susceptibility, external environment, intestinal microbial flora and immunological profile are all involved in the pathogenesis of IBD, but the specific causes remain unknown [224]. Current treatments are symptomatic for the induction of remission in acute episodes and avoiding relapsing events. Conventional drugs, including 5-aminosalicylic acid (5-ASA), corticosteroids, immunosuppressant drugs, and anti-TNFα agents are the main treatments today. Depending on localization and activity of the inflammation, these drugs are administered topically, systemically or in combination.

In the case of IBD, colon targeted delivery of immunosuppressive agents is desirable to avoid side effects. For the delivery of small immunosuppressive molecules, polymeric NPs have been widely explored and reviewed in the literature [223,225]. Apart from immunosuppressive drugs, nanotechnology-based siRNA delivery directed to APCs is another approach that has been explored for resolving inflammation in IBD [226,227]. For example, chitosan and its derivatives have been investigated for the siRNA delivery in the colonic region due to its mucoadhesive properties. In one case, chitosan-PLGA NPs were tested orally for the delivery of an antisense oligonucleotide to block NF-κβ factor in an induced-colitis model. The results showed that chitosan-PLGA NPs were selectively accumulated in inflamed tissue and improved the clinical scoring [228]. Similarly, galactosylated trimethylchitosan NPs loaded with a siRNA against mitogen-activated protein kinase (MAPK) showed good *in vivo* efficacy in induced-colitis mice model after oral administration [229]. Finally, the local delivery of anti-inflammatory peptides or protein antagonists of immune receptors in the inflammation site is a promising approach for the *in situ* modulation of immune effector cells. For example, the colonic delivery of an alginate-chitosan hydrogel (double oral gavage procedure for *in situ* gelation) containing KPV peptide-loaded PLGA NPs to an induced-colitis mice model, resulted in a marked amelioration of the inflammatory symptoms. In fact, a considerably lower dose of peptide (12,000-fold) compared to the free peptide, led to a similar therapeutic efficacy. This effect was explained taking into account the better access of the peptide-loaded NPs to the target epithelial and immune cells [230].

4.1.2. Rheumatoid arthritis—Rheumatoid arthritis (RA) is a chronic autoimmune disorder that primarily affects joints. RA is characterized by synovial inflammation and swelling, autoantibody production as well as cartilage and bone destruction [231]. It has been proposed that the course of the RA development follows a three-step process. Autoimmunity starts to develops in genetic-susceptible individuals, with the presentation of autoantibodies in serum [232]. In a second step, there is an expansion of reactive immune cells that leads the infiltration of inflammatory cells in the joints as a prelude of the chronic inflammatory response. Finally, the patient presents a chronic joint inflammation promoted mainly by macrophages, which constitutes the major hallmark of the third phase of the disease [233]. The systemic delivery of immunosuppressant molecules, both classic small drugs and anti-TNFα antibodies are the main current treatments (Table 5) [231].

The design of nanotechnology-based approaches in RA is focused on increasing the retention time of small immunosuppressive drugs in the joint [222]. For that purpose, a wide variety of nanocarriers have been tested and extensively reviewed in the literature, including polymeric NPs, liposomes, solid-lipid NPs and polymeric micelles [222,234]. Moreover, nanotechnology- based gene therapy has also been explored for the treatment of RA. As in IBD, this therapy is focused in siRNA knockdown of TNFα [226]. Also, the encapsulation of pDNA encoding for IL-10 was widely explored. As an example, Jain *et al.* showed effective macrophage repolarization from M1 to M2 phenotype in adjuvant-induced arthritis (AIA) mice model after intraperitoneal administration of IL-10-encoding pDNA-loaded alginate NPs [235]. Regarding protein delivery, different anti-inflammatory proteins have been explored. This is the case of self-assembled NPs composed of metracrylate-based copolymers loaded with an IL-1 receptor antagonist (a protein implicated in blocking pro-inflammatory signals). This system was able to maintain the biological activity of IL-1 receptor antagonist *in vitro* and prolong its retention in rat stifle joint following intra-articular administration in healthy rats [236]. Another example is the nanocomplex of etanercept with succinylated pullulan-g-oligo (L-lactide) polymer. After two months of fortnightly subcutaneous injection of this nanocomplex to a collagen-induced arthritis (CIA) rat model, no cartilage erosion and a depletion of the synovial inflammation were observed [237].

4.1.3. Systemic lupus erythematosus—Systemic lupus erythematous (SLE) is a chronic autoimmune disease characterized by loss of tolerance to self-antigens and production of numerous autoantibodies, due to its heterogenic and non-organ specific origin [238]. The most common treatment strategies are NSAIDs, antimalarial drugs and oral glucocorticoids. Immunosuppressive medications are used to control serious lupus activity that affects major organs (Table 5).

Nanotechnology-based therapies for the treatment of SLE have been reviewed recently [15]. In the following lines, we highlight some of the most significant works in this field. Look *et al.* developed a liposomal system with a gel-like core containing cyclodextrins surrounded by a lipid bilayer for the delivery of anti-inflammatory agents. Following intraperitoneal administration of this system loaded with mycophenolic acid in a murine lupus model, it was found an increased 2 – 3 months the mean survival time, and this was attributed to the preferential accumulation of the system in DCs [239]. The same group also found that the DCs immunosuppression achieved with this new system was more significant than for the one observed for PLGA NPs loaded with the same drug [240]. In another example, methylprednisolone-loaded liposomes were administered subcutaneously in a murine lupus model and the results showed a reduced the mortality for this group of mice, as compared to that of the group treated with the free drug [241]. Attempts have also been made to treat lupus with gene therapy approaches. For example, following intraperitoneal administration of siRNA anti-MAPK1 (a protein implicated in the pro-inflammatory signaling cascade) loaded into PEG-poly(L-lysine) NPs, in a murine model of lupus nephritis, a significant amelioration of the renal damage was observed [242].

To summarize, different nanotechnology approaches were developed for the treatment of the inflammation in autoimmune diseases (Fig 6). This offers the possibility of controlled and

targeted release of immunosupressive drugs, which would avoid the systemic effects of the drugs currently on the market. Furthermore, the change from invasive administration routes (IV) to more patient-friendly ones (mucosal) can also be accomplished by nanotechnology, thus increasing patient compliance.

4.2. Nanovaccines for the treatment of autoimmune diseases

Apart from the symptomatic treatment using anti-inflammatory and immunosuppressive drugs, nanotechnology can contribute with more specific treatments for autoimmune diseases. In this sense, antigen-specific therapies seem a good option to prevent self-antigen recognition that would lead to the activation of auto-reactive T or B cell clones.

The best-known disease-specific self-antigens are: myelin in MS, insulin in T1D, and collagen in RA. Loss of tolerance towards self-antigens is often thought to be the result of both genetic and environmental risk factors, including exposure to infection by particular pathogens, molecular mimicry of endogenous antigens, or bystander activation [243]. However, the molecular mechanisms behind the autoimmune process are not well understood yet. Furthermore, in most of the cases, the self-antigens involved in the physiopathology of the disease remain unknown, limiting these therapies to illnesses with known self-antigens. In a healthy situation, T lymphocytes can distinguish between different antigens with high specificity; however they cannot discriminate between self or non-self-antigens. Central tolerance process occurs in the thymus during the first years of life. During this process, thymic epithelial cells expose in their surface a great variety of self-antigens to T cells. Normally, the T cells that recognize those antigens are eliminated to prevent self-reactivity [244,245]. Besides central tolerance process, peripheral mechanisms regulate these self-reactive T cells if they reach the bloodstream. However, in the case of patients with autoimmune disorders, these peripheral mechanisms fail and the self-reactive T cells stay and cause damage [246].

Different mechanisms for maintenance of peripheral self-tolerance have been proposed. Most of them include DCs and regulatory T cells as the main modulators of self-reactive T cell response [247,248]. The molecular signals in the microenvironment drive DCs homeostasis and function, especially regarding cytokines production and surface expression of co-stimulatory molecules. Differences in the microenvironment can lead to phenotypical changes in DCs, promoting T cell anergy, T cell depletion and regulatory T cell proliferation after immune synapsis formation and antigen recognition [249]. This regulatory T cell expansion promotes the suppression of specific self-reactive T cell clones by different mechanism [248]. Within this context, the “holy grail” of immunotherapy in autoimmune diseases would be the development of antigen-specific treatments targeted to dendritic cells. This approach could maintain the functionality of the immune system whereas specifically blocking the self-reactive T cells which are pathogenic in autoimmune diseases. For this purpose, different protocols where developed during the last decades for the induction of specific tolerance [250].

Trying to simulate the process elicited in allergy treatment, high doses of soluble antigen were injected in order to induce anergy or activation-induced cell death after T cell re-stimulation in autoimmune diseases [251,252]. Unfortunately, although promising result

based on this strategy were obtained [253,254], in others, a hyper-sensitivity reaction was observed after the administration of the soluble antigen [255,256]. These contradictory results can be explained by the fact that soluble peptides can induce specific tolerance, but cannot block polyspecific responses in the case of epitope spreading, which is the situation that exists in autoimmune diseases [257].

Based on the high amount of foreign antigens present in food and the general lack of immune reaction against them (except in the case of food allergies), the mucosal administration of soluble antigens has also been explored to induce tolerance. This is thought to happen by different mechanisms dependent on antigen dose. Low-dose of self-antigen is processed by antigen presenting cells in the gastrointestinal tract, promoting the activation of regulatory T cells. On the other hand, high doses of antigens seem to cross the gastrointestinal barrier and promote anergy once in systemic circulation [258]. Studies in animal models led to promising results in terms of blocking disease progression [259–261], however, so far, these results did not translate to a clinical set-up [262].

Nanotechnology is a promising approach to improve vaccination strategies to treat autoimmune diseases. It offers the ability of specific targeting and association of multiple antigens capable of inducing tolerance before epitope spreading happens. Most of the nanotechnology-based strategies are focused on the delivery of self-antigens to DCs, taking advantage of natural peripheral tolerance mechanisms mediated by this cell type (Fig 5). In the next lines, we will summarize and discuss the latest and most relevant nanotechnology-based approaches in antigen-specific therapy against different autoimmune pathologies (Table 6).

4.2.1. Multiple sclerosis—Multiple sclerosis (MS) affects around 2.3 million people worldwide, and is the second most common cause of disability in young adults. MS is a central nervous system disorder of autoimmune origin, in which encephalitogenic T cells are involved in damaging the myelin, promoting inflammation, and triggering neuronal and axonal damage [263]. Some self-antigens are known to be related with the pathology, including myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), and proteolipid protein (PLP) [264,265]. The most common treatments for MS are interferon β (IFNβ), glatiramer acetate (GA), and the monoclonal antibody natalizumab, known as disease-modifying therapies (DMT). These treatments are unspecific for MS and often have serious side effects, such as opportunistic infections and tumors [266–268]. As previously indicated, the ideal treatment should be antigen-specific and DCs-targeted, to avoid systemic immunosuppression. In addition, the co-administration of antigen and immunomodulatory molecules using nanotechnology is now emerging as a new therapeutic option for the treatment of MS.

Most of the systems developed for MS treatment are based on PLGA and designed for the co-delivery of the antigen and immunomodulatory molecules such as rapamycin or IL-10. Following subcutaneous or intra-nodal administration in experimental allergic encephalomyelitis (EAE) mice model, it was found that these systems were able to successfully inhibited the progression of the disease [154,269,270]. In another report it was described that a new antigen-coupled PLGA formulation induced liver-dependent tolerance

in a relapsing-remitting EAE mice model after IV administration [63]. Similarly, Carambia *et al.* showed that antigen-coupled poly(maleic anhydride-alt-1-octadecene) polymeric NPs induced also liver-dependent tolerance in an EAE mice model, providing effective control of the disease with a single IV administration due to the efficient induction of regulatory T cells [271].

The ionic complexation of antigenic peptides, or their DNA encoding sequences, and immunomodulatory molecules is nowadays presented as a new nanotechnology approach to induce tolerance. For example, Yuan *et al.* developed self-assembled NPs using a plasmid encoding for the co-inhibitory receptor B and T lymphocyte attenuator (BTLA) as immunomodulatory signal and MOG antigen modified with the cell penetrating peptide Tat49-57. When DCs pretreated with these NPs were administrated intraperitoneally to an EAE mice model, a decrease in the spinal cord inflammation and inhibition of specific T cell proliferation were observed [272]. A similar approach involved the complexation of arginine-modified MOG antigen and GpG oligonucleotide, an antagonist of TLR9. Studies in EAE mice model showed an improvement in the progression, severity, and incidence of the disease [273,274].

Recently, PS liposomes were also tested as peptide carriers for MS therapy. Liposomes loaded with MOG peptide were administered intraperitoneally (2 boosts) in EAE mice model and the result of this treatment was a decrease in the clinical score and the incidence of the disease [275].

4.2.2. Type 1 Diabetes—Nowadays, 415 million people worldwide have diabetes [276]. Diabetes mellitus is a pandemic group of disorders where insulin metabolism is altered. Within this group, type 1 diabetes (T1D) is considered a chronic autoimmune disease caused by the destruction of β-cells located in the Langerhans islets by the immune system, causing the loss of insulin production in pancreas [277]. Human and murine models have been extensively used to study the pathophysiology of the disease. Results from these studies have shown that the destruction of β cells occurs in a cell-mediated manner, requiring both $CD4^+$ and $CD8^+$ T cells and macrophages [278,279]. Most well-known antigens recognized by T cells in T1D are preproinsulin, glutamic acid decarboxylase and islet-cell antigen-2 [280]. The standard treatment for T1D is the subcutaneous injection of insulin to maintain normoglucemia. As an alternative, antigen-specific treatments aim to avoid the underlying autoimmune response, treating the disease at its origin [250]. The main barriers for the design of antigen-specific approaches are: the complexity of T1D autoantigens map, and the specific targeting to the immune cells involved in disease onset and progression. Nanotechnology offers the possibility of specific targeting and loading multiple antigens at the same time, with or without immunomodulatory molecules. The recently developed nanotechnology-based treatments for T1D are summarized below.

PLGA NPs were explored for the delivery of self-antigens in combination with immunomodulatory molecules, in the treatment of T1D. For example, Lewis *et al.* developed a dual-sized PLGA MPs formulation, where non-phagocytosable MPs (30 μm) were loaded with chemoatractive cytokines and phagocytosable MPs (0.5 – 2.5 μm) were loaded with insulin B peptide and vitamin D3. This approach relies on the assumption that the large MPs

releasing chemokines stay in the injection site and help to attract immune cells and, hence, to enhance the phagocytosis of small MPs. Once in the APCs, the small MPs deliver the antigen with vitamin D3 for a tolerogenic presentation to the lymphocytes. Using this approach, 40 % of mice were protected from T1D development when the combination of both MPs was injected subcutaneously twice [156]. A different approach involved the use of human denatured insulin-loaded PLGA MPs included in a hydrogel with chemoatractive cytokines. After 3 subcutaneous injections, 40% of non-obese diabetic (NOD) mice were protected [281].

Liposomes containing PS were also developed for the delivery of insulin antigens. As mentioned above, PS was selected as it works as an “eat me” signal in apoptotic cells that can promote the presentation of the antigen with the secretion of tolerogenic cytokines such as PGE_2. According to the results, around 50% of NOD mice did not develop diabetes after intraperitoneal liposomes administration [152].

4.2.3. Rheumatoid arthritis—As we stated previously, RA is a long-term autoimmune disorder that primarily affects the joints. Currently, RA treatment is focused on easing the symptoms, or slowing the course of the disease by using immunosuppressant drugs such as corticosteroids or anti-TNF antibodies [231] (Table 5). Although most of the novel approaches for RA treatment are focused on the targeted delivery of immunosuppressant drugs or tissue regeneration, antigen-specific approaches are also being explored as a promising treatment at the onset of the disease through the downregulation of the underlying autoimmune processes, although the number is still limited [19,282,283]. Self-antigens, such as collagen derived peptides, were found to be RA triggers in different animal models [231], and mucosal administration of collagen peptides were found to ameliorate the progression of the disease in patients [284].

Among the different nanotechnology approaches to treat RA, there is the attempt described by Kim *et al.* based on the oral administration of PLGA NPs loaded with both whole type II collagen (CII) and CII derived peptides. The results showed a reduction of the severity of arthritis after a single oral administration to CIA mice and this positive effect was associated to the accumulation of the CII-loaded PLGA NPs in the Peyer’s patches [19]. Similarly, using CII-derived peptides modified with PEG, Lee *et al.* developed peptide-loaded PLGA NPs for oral administration. They found that a single administration of the encapsulated PEG-conjugated peptides to healthy DBA/1 mice was able to increase both the rate of IL-4^+ $CD4^+$ cells and of IL-10^+ $CD4^+$ cells, which could be a promising approach for inducing tolerogenic phenotypes by the oral route [282]. Moreover, liposomes were also explored in antigen-specific therapy for RA by Capini *et al.*. In a methylated BSA-induced arthritis model, they showed that, after subcutaneous administration of methylated BSA and lipophilic NF-κβ inhibitors (Bay11-7082, curcumin, or quercetin) co-encapsulated in liposomes, all of the combined formulations diminished the score of disease symptoms, compared with untreated mice [283].

In summary, nanotechnology-based antigen-specific approaches are promising for autoimmune diseases therapy and offer the possibility of controlled and targeted release of self-antigens. In addition, the possibility of loading immunomodulatory agents gives

nanosystems the ability of enhancing tolerance generation. However, more research is needed for antigen identification and for a better understanding of what causes an autoimmune disease. These studies could help us elucidate the multiple factors that are involved in both epitope spreading and autoimmune response processes. Therefore, multiple antigen approaches could be the best option for efficiently blocking disease progression. To this end, nanotechnology could be a very valuable tool in combining multifactor therapy.

5. The next challenge in immunomodulation: overcoming antidrug antibodies

We are in the era of biologicals, also named as biodrugs or biotherapeutics. The development of the recombinant DNA technology starting in the early 70s and the introduction of recombinant insulin in the market have laid the foundations for the use of biomolecules as therapeutic agents [285,286]. Nowadays, biomolecule-based therapies for a huge variety of diseases are already being used clinically, or are in trials, which shows the great potential of biotherapeutics [287,288]. Linked to this development of biodrugs, one of the major safety concerns that needs to be assessed during preclinical and clinical trials is undesired immunogenicity [289].

One of the first approaches to address unwanted immunogenicity is to measure the formation of antidrug antibodies (ADAs). ADAs recognize different epitopes in a recombinant molecule and bind to them, causing different outcomes in the pharmacological activity of the drug, depending on their neutralizing potential. ADAs formation and their effect on biotherapeutics have been extensively reviewed due to its direct relation with immunogenicity and treatment efficacy [290]. In the case of replacement therapies, ADAs formation could result in cross reactivity with endogenous proteins and, thus, cause severe adverse effects. One highlighted example is erythropoietin, a hormone required for red blood cell development which is used as treatment for anemia in patients with chronic kidney disease [291]. A few clinical subjects were found to develop pure red cell aplasia after erythropoietin infusion, due to ADAs formation and subsequent endogenous erythropoietin recognition [292]. In this case, several factors affected the protein immunogenicity including those depending on product-formulation (leakage of polysorbate 80 from the rubber stoppers of the syringes) and administration (change from IV to subcutaneous administration route) [293–295].

There are multiple factors that influence the immunogenicity of biodrugs and formation of ADAs. Originally, a bacterial or fungi origin of the recombinant proteins could cause immunogenicity, due to the differences in sequence and structure of biomolecules between species. Although nowadays the use of humanized or fully-human biodrugs has greatly contributed to reduce this risk, immunogenicity associated to the aggregation of biodrug molecules and other factors is still a major concern for the optimum exploitation of these modern drugs [296,297]. The association between biodrug molecules upon injection has been thought to be a natural way to enhance antigen processing and presentation in the cells [298,299]. On the other hand, the presence of impurities could also be part of the problem. Finally, the administration route [300,301] and patient-related issues, such as genetic

predisposition to ADAs formation or cytokine pattern, could impact immunogenicity [302,303].

Today, there is not a single standard therapy available for ADAs formation. The most frequent therapy is the administration of a prolonged immunosuppressive regimen as in the case of “Pompe disease”, a lisosomal storage disorder [304,305]. Nevertheless, this approach could enhance opportunistic infections and other complications due to systemic immunosuppression. The ideal treatment to avoid ADAs effects would be drug-specific: achieving tolerance to the delivered biotherapeutic molecule, and maintaining its safety and efficacy without systemic immunosuppression. In this field, nanotechnology is emerging as a new approach where a biotherapeutic agent can be specifically delivered together with an immunosuppressive drug, avoiding systemic immunogenic effects and increasing the treatment efficacy. For this purpose, DCs are the usual target, due to their important role in antigens presentation and also because of their relevance in the fate of T cells [247,306]. Indeed, as indicated above, it has been described how the uptake of rapamycin by DCs promotes the differentiation of T cells towards a regulatory phenotype [307]. Furthermore, rapamycin encapsulation, both in PLGA NPs and MPs, enhances the tolerogenic activity of DCs [308,309].

Within this context, it is worthwhile to mention the formulation activity of Selecta Biosciences, in terms of inducing immunotolerance through the use of nanotechnology. They co-administered the coagulation factor VIII together with the immunosuppressive agent rapamycin loaded into PLGA NPs and observed promising *in vivo* results in terms of maintaining the efficacy of the biologic entity in an hemophilia A mice model [154]. In their search for a more universal approach, they explored the interest of co-administering rapamycin-loaded PLGA NPs together with different proteins (OVA, pegsiticase, adalimumab) and the results showed durable inhibition of ADAs formation [310]. Currently, patients are being recruited for a phase II clinical trial for the evaluation of the co-administration of rapamycin-loaded PLGA NPs with free pegsiticase: a pegylated uricase enzyme implicated in the metabolization of uric acid, that is currently administered for the treatment of hyperuricemia and chronic gout [311].

Despite this original work there is still a limited understanding of the processes that underlie the immunogenicity of biotherapeutics and further research is needed to determine how nanocarriers could modulate immune mechanism to promote tolerance. All this knowledge will help us come up with a rational design of nanotherapeutic agents with better performance for tolerance generation.

Conclusions

In the last decades, nanotechnology has shown an important potential in the immunotherapeutic field. The modulation of a broad variety of immune processes can be achieved with nanotechnology, with promising results not only *in vitro* but also *in vivo*. In this review, we have analyzed a significant number of nanotechnology-based formulations for immune activation and tolerance generation. In both cases, the nanocarrier composition and its physicochemical properties have shown to play a crucial role in achieving the desired

immune outcome. Despite the knowledge generated, it is quite risky at this point to correlate the physicochemical characteristics of the nanocarriers with their capacity to modulate the immune system. Regarding the composition of the nanocarrier, the use of ligands for specific cell receptors is expected to substantially increase the targeting to a particular subset of immune cells. Besides, the use of immunomodulators could be a useful strategy to effectively polarize the immune response.

As a consequence of all the nanotechnology-based approaches for immune modulation in preclinical studies, a high number of nanoformulations for vaccine delivery and tolerance generation is currently being tested in clinical trials, and a few of them have already reached the market. Indeed, thanks to the significant efforts made in this field at the research level, essential advances have been made to treat diseases like HIV, tuberculosis, type-1 diabetes or multiple sclerosis, among others. A deeper knowledge from the immunological point of view will help to rationally design and engineer new nanosystems that are expected to contribute to find a cure for some of the most threatening illnesses of our time.

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Highlights

- Nanocarriers can be designed to target specific immune cells
- Nanovaccines may help fighting diseases that are elusive to traditional vaccines
- Nanocarriers can bias the immune response from humoral to cellular
- Autoimmune disease treatments can be improved with nanotechnology-based approaches
- The use of nanocarriers may help to avoid ADAs formation against biotherapeutics

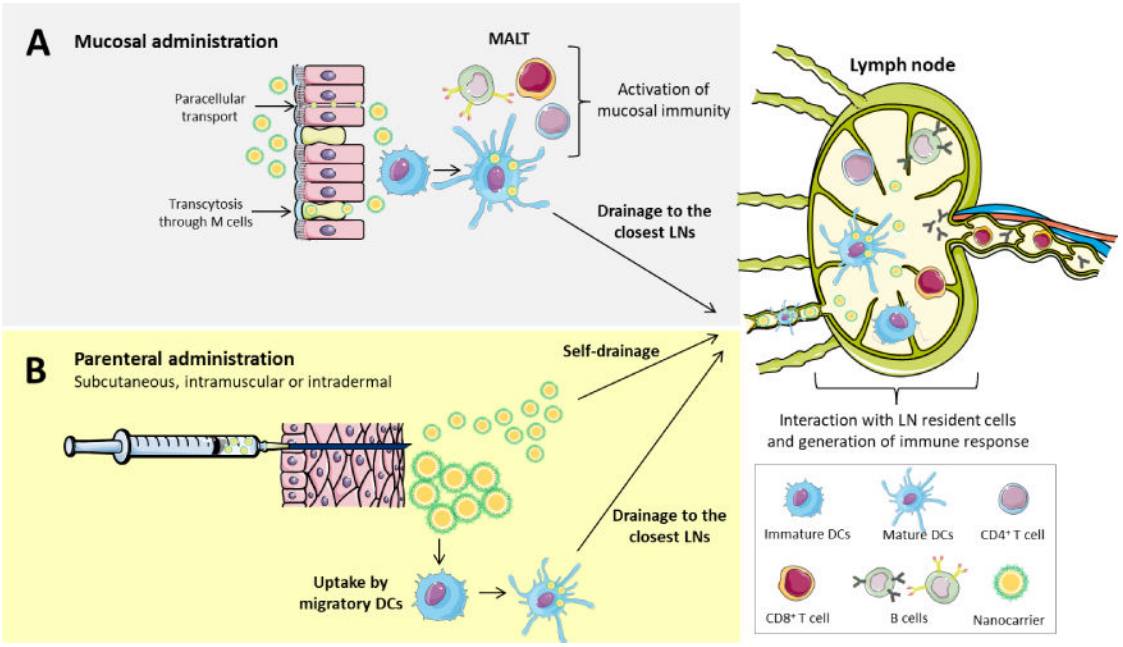

Figure 1. Vaccine administration routes

The main administration routes for vaccines are mucosal and parenteral. (A) Mucosal administration refers to the administration mainly through the nasal, oral or vaginal routes. In all these cases, nanocarriers need to reach the mucosal-associated lymphoid tissues (MALT). This can be principally achieved either by a paracellular or transcellular across the microfold (M) cells. At the level of M cells or underneath the epithelium, nanocarriers will encounter the resident dendritic cells and activate them, generating a mucosal immunity while, at the same time, some dendritic cells will drain to the closest lymph node and activate a systemic immune response. (B) Parenteral administration includes subcutaneous, intramuscular or intradermal injection of the nanosystems. The nanocarriers are deposited in the interstitium, where they can have two different fates: self-drain to the closest lymph node or be taken up by migratory dendritic cells, which then will migrate to the closest lymph node.

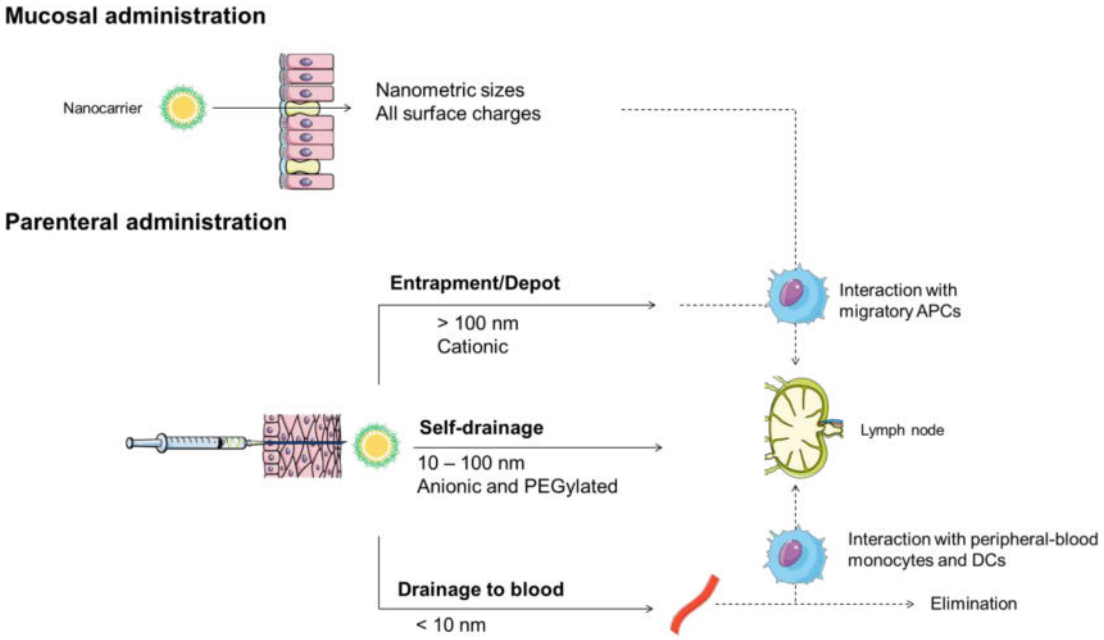

Figure 2. Summary of the influence of the physicochemical properties of nanocarriers (particle size and surface charge) in the fate of the nanosystems after administration

Both particle size and surface charge play an important role in the outcome of nanosystems once administered, either by mucosal or parenteral routes.

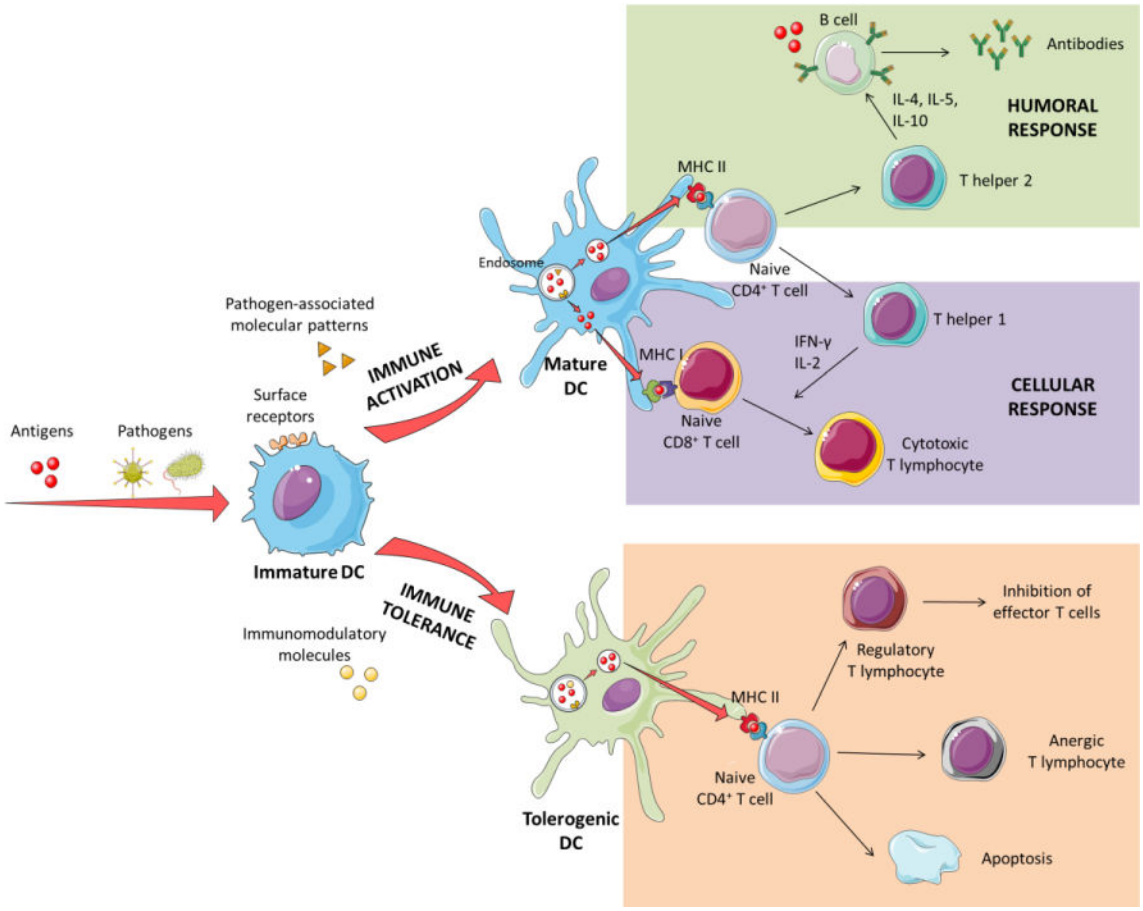

Figure 3. Immune cell network

Schematic overview of the generation of different immune responses by dendritic cells. Antigens, pathogens and other molecules are taken up by immature dendritic cells. In the case of pathogens or systems expressing pathogen-associated molecular patterns (PAMPs), their internalization by dendritic cells leads to their presentation by class II major histocompatibility complexes (MHC II) to naïve $CD4^+$ T cells, which activate T helper cells (Th). Th2 cells produce IL-4, IL-5 and IL-10, which stimulate B cells to produce antibodies against the antigen. At the same time, antigens themselves can interact directly with B cells and activate them. Antigens can also be found in the cytosol of dendritic cells, which allows them to be presented by class I major histocompatibility complexes (MHC I), directly activating cytotoxic T lymphocytes. In this case, Th1 cells produce IFN-γ and IL-2, which favor cellular activation and hence, cytotoxic T cell responses.

In the case of antigens presented in the absence of co-stimulatory molecules, or in the presence of immunomodulatory molecules for tolerance, dendritic cells are driven to a state of immune tolerance. In this state, dendritic cells can inhibit T cell activation by different mechanisms. Different stimuli, such as IL-10 or PD-L1 can cause T regulatory cells proliferation that, at the same time, can inhibit effector T cells. Furthermore, the absence of co-stimulatory surface molecules can lead to an unresponsive state in T cells known as anergy. Finally, co-stimulatory Fas-signaling in the immune synapsis can lead to T cell apoptosis and deletion.

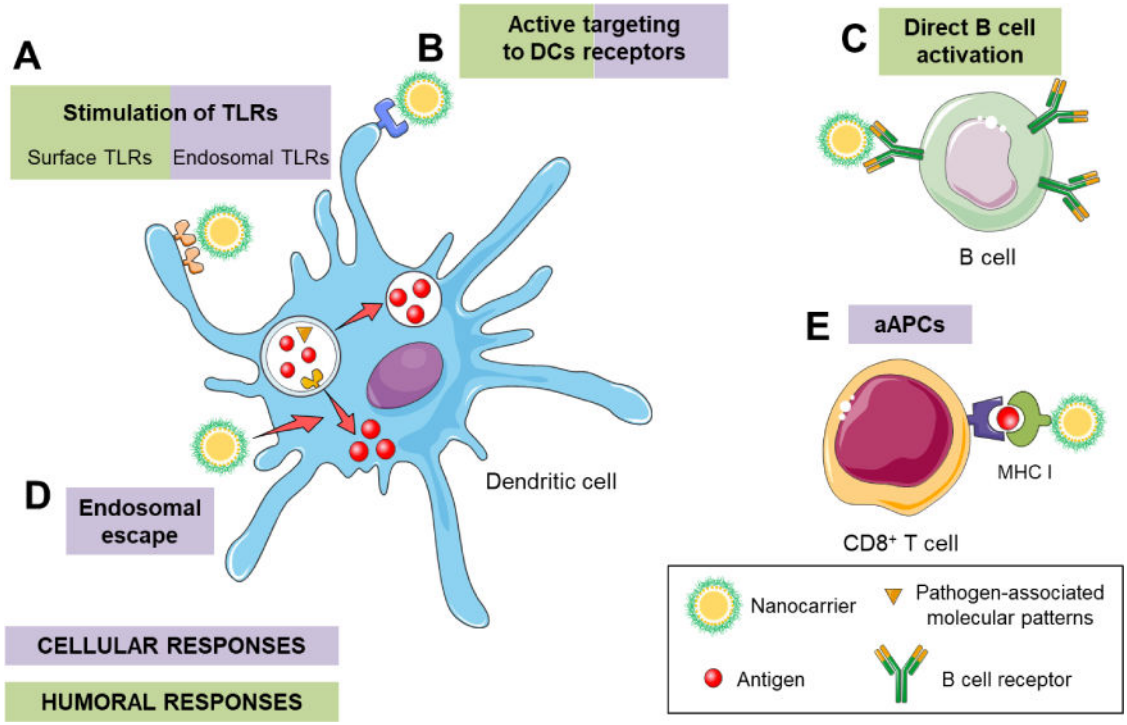

Figure 4. Nanotechnology-based approaches to modify the immune response to enhance humoral or cellular responses

Nanocarriers can drive both humoral and cellular responses, depending on their features and composition. (A) Nanocarriers can deliver toll-like receptor (TLR) agonists that can activate surface or endosomal receptors, driving humoral or cellular responses, respectively. (B) Decorating nanocarriers with antibodies against specific receptors of dendritic cells (DCs) (e.g., CD40, CD11c, DEC-205, mannose, etc.) can activate these cells. (C) The direct targeting to B cells can stimulate them and, thus, favor antibody production and humoral responses. (D) Nanocarriers with properties that promote endosomal escape of the antigens, favor cellular responses. (E) A direct activation of $CD8^+$ T cells through artificial antigen presenting cells (aAPCs) stimulates cytotoxic T lymphocytes.

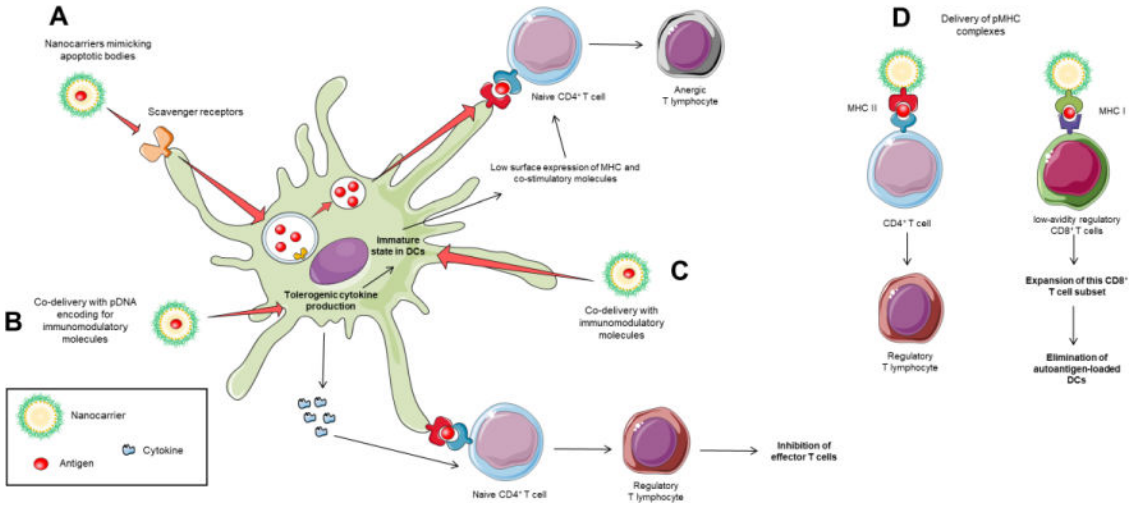

Figure 5. Nanotechnology-based antigen-specific approaches for tolerance generation

Strategies for antigen-specific tolerance mediated by dendritic cells (DCs) modulation through nanotechnology. (A) Nanocarriers mimicking apoptotic bodies may follow the debris elimination process, where self-antigens presentation induces regulatory T cells. (B) The co-delivery with pDNA encoding for tolerogenic cytokines, i.e. IL-10, may enhance its expression and induce regulatory T cells and anergy. (C) Using immunomodulatory molecules may promote the maintenance of immature state of DCs, while presentation of antigens with low surface density of major histocompatibility complexes (MHC) and costimulatory molecules may promote T cell anergy. (D) The delivery of peptide-MHC (pMHC) complexes to T cells may expand memory T cells with regulatory capacity.

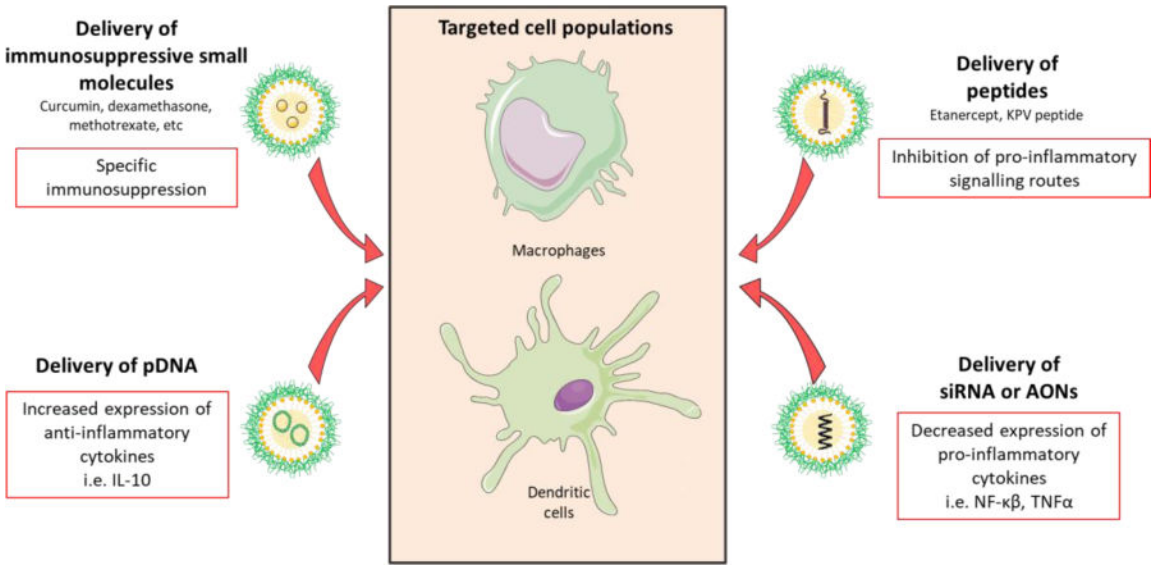

Figure 6. Main strategies for nanotechnology-based anti-inflammatory treatment of autoimmune diseases

There are four approaches of nanotechnology-based treatments depending on their cargo. First, the delivery of small immunomodulatory molecules has been extensively explored in the suppression of the inflammatory activity of macrophages and dendritic cells (DCs). Second, the delivery of anti-inflammatory peptides has been used in different pathologies. Finally, gene therapy strategies include: pDNA delivery for the expression of anti-inflammatory cytokines, and siRNA or antisense oligonucleotide (AONs) delivery for the downregulation of pro-inflammatory molecules expression.

Table 1A

Summary of the different strategies considered for a passive targeting to monocytes and/or macrophages

Studied feature	Composition	Particle size	Surface charge	Key results	Ref.
Particle size	Non-functionalized polystyrene	0.9, 1.9, 2.3, 3, 4.3, 5.7, 9 μm	n.d.	≈ 2 – 3 μm more readily phagocytosed *in vitro* than smaller (≈ 1 μm) and larger particles (≈ 6 μm)	[68]
	Polystyrene and PLGA	1, 2, 3.2, 6.4, 10.1 μm	−87.6 mV/− 9.7 mV	≈ 6 μm polystyrene particles and ≈ 3 μm PLGA particles were the most efficiently phagocytosed particles *in vitro*	[70]
	PLGA	1.5, 3.3, 6.1, 10 μm	n.d.	More efficient *in vitro* delivery of the cargo achieved with ≈ 3 μm particles than with larger (≈ 6 – 10 μm) and smaller ones (≈ 1 μm)	[71]
	Carboxylated polystyrene	0.02, 0.04 and 1 μm	Anionic	48 h after *in vivo* administration, more 1 μm particles were co-localized with macrophages in the draining LNs than the smaller particles (0.02 – 0.04 μm)	[72]
	Carboxymethyl chitosan grafted and chitosan hydrochloride grafted	149.2 – 157.3, 300.7 and 456.5 nm	− 38.4 to − 13.2 mV; + 14.8 to + 34.6 mV	Larger NPs were more efficiently taken up *in vitro*	[73]
	Carboxylated polystyrene	0.5 – 4.5 μm	Anionic	Small-particles group (0.5, 1 and 2 μm) was internalized *in vitro* at a higher rate than the group of larger particles (3 and 4.5 μm)	[74]
Shape	Non-functionalized polystyrene	Axis from 1 to 12.5 μm	n.d.	Better phagocytosis *in vitro* in alveolar macrophages for MPs that allow a lower contact angle (ellipsoids and disks)	[66]
Particle size and shape	Non-functionalized polystyrene	0.5 – 3 μm	n.d.	Particles with the longest dimension of 2 – 3 μm showed the maximum attachment to macrophages *in vitro*	[69]
Surface charge	Carboxylated polystyrene covalently coated with BSA or PLL	1 – 4.5 μm	− 58.3 to − 18.4 mV; + 39.6 to + 49.7 mV	Cationic particles were better taken up than anionic particles by macrophages *in vitro*	[75]
	Carboxylated polystyrene covalently coated with BSA, PLL, IgG or PEI	1 μm	− 21.1 to − 0.8 mV; + 38.2 to + 45.7 mV	Cationic MPs were better taken up by macrophages than negative particles *in vitro*	[76]
	Carboxymethyl chitosan grafted and chitosan hydrochloride grafted	149.2 – 157.3; 300.7 and 456.5 nm	− 38.4 to +34.6 mV	Positively charged NPs were more efficiently taken up than negatively charged ones *in vitro*	[73]
	DOPC/DODAP and DOPC/DOPS liposomes	120 nm	Cationic, neutral and anionic	Positively charged liposomes were better taken up than negative and neutral liposomes by rat macrophages *in vitro*	[77]
	PC/Chol/SA, PC/Chol/PA, PC/Chol multilamelar vesicles	n.d.	− 18.6 to + 8.9 mV	Positively charged liposomes showed a higher uptake rate by macrophages *in vitro* and better immune responses *in vivo* than neutral and negative liposomes	[78]
	PRINT hydrogel, derived from HP_4A	80 nm × 80 nm × 320 nm	Cationic and anionic	After pulmonary instillation, anionic NPs were more efficiently taken up by macrophages than cationic NPs	[79]
	Cyanoacrylate NPs coated with dextran or diethylaminoethyl-dextran	200 nm	+ 30 mV/− 20 mV	Anionic NPs showed a higher internalization by macrophages *in vitro*, and also higher anti-inflammatory properties than the cationic ones	[80]
	DSPC/DODAB/Chol or DSPC/DSPG/Chol liposomes	180 – 190 nm	− 29, 0.1 and + 25 mV	Anionic liposomes were more effective than neutral ones *in vivo* and *in vitro*. Cationic liposomes were more potent, but	[81]

Studied feature	Composition	Particle size	Surface charge	Key results	Ref.
				this was associated with a higher cytotoxicity of the forming polymers	
	Neutral, carboxylated and aminated polystyrene	500 nm	− 50, − 0.5 and + 40 mV	Anionic particles decreased the infiltration of inflammatory monocyte-derived macrophages *in vivo* to a larger extent than cationic or neutral NPs of the same size	[83]

BSA: bovine serum albumin; Chol: cholesterol; DODAB: dimethyl dioctadecyl ammonium bromide; DODAP: 1,2-dioleoyl-3-dimethylammonium propanediol; DOPC: 1,2-dioleolyl-sn-glycero-3-phosphatidylcholine; DOPS: 1,2-dioleolyl-sn-glycero-3-phosphatidylserine; DSPC: 1,2-distearoyl-sn-glycero- 3-phosphocholine; DSPG: distearoyl-phosphatidylglycerol; HP4A: tetra(ethylene glycol) monoacrylate; IgG: immunoglobulin G; LN: lymph node; MP: microparticle; n.d.: not determined; NP: nanoparticle; PA: L-a-dimyristoyl phosphatidic acid; PEI: polyethylenimine; PC: egg phosphatidylcholine; PLGA: poly(lactic-co-glycolic) acid; PLL: poly-ι-lysine; PRINT: particle replication in non-wetting templates; SA: stearylamine

Table 1B

Summary of the different strategies followed for an active targeting to monocytes and macrophages

Ligand	Nanosystem	Key results	Ref.
IgG coating	SPIO	Higher *in vitro* uptake and sustained distribution in lymphoid tissue, in comparison to non-coated SPIO	[85]
Mannosylation	Liposomes	Functionalized liposomes accumulated in tumor-associated macrophages better than in other lung areas	[86]
Folate	Dendrimer (G5)	High *in vitro* internalization by macrophages in a receptor-specific manner and great *in vivo* anti-inflammatory properties	[88]
Dextran	Dextran conjugates	After peritoneal administration, larger conjugates selectively associated with macrophages of the adipose tissue	[89]

G5: generation 5; IgG: immunoglobulin G; SPIO: superparamagnetic iron-oxide nanoparticles

Table 2A

Summary of the different strategies followed for a passive targeting to dendritic cells

Studied feature	Composition	Particle size	Surface charge	Key results	Ref.
Particle size	Carboxylated polystyrene	0.02, 0.04 and 1 μm	n.d.	48 h after *in vivo* administration, smaller sizes (0.02 – 0.04 μm) were found in a higher amount in DCs, in comparison to 1 μm particles	[72]
	Carboxylated polystyrene plain or coated with PLL, PA, PrS, TT or WGA	0.1, 0.5, 0.9 and 4.5 μm	− 66.9 to + 41.4 mV	Particles smaller than 0.5 μm had better *in vitro* uptake by DCs	[101]
Surface charge	PC/PG/Chol, PC/PS/Chol, PC/TAP/Chol liposomes	180 to 279 nm	− 54.2 to + 44.2 mV	Positively charged liposomes had a greater interaction with DCs *in vitro* than anionic ones	[102]
	Carboxylated polystyrene covalently coated with BSA or PLL	1 – 4.5 μm	− 58.3 to − 18.4 mV; + 39.6 to + 49.7 mV	Cationic particles uptake by DCs *in vitro* was higher than for anionic particles	[75]
	PRINT hydrogel derived from HP_4A	248 to 285 nm	− 38 to + 45 mV	Pulmonary administration of cationic NPs elicited stronger antibody responses than negative NPs administration, which correlates with a higher uptake *in vitro* of the former by DCs	[103]
	PRINT hydrogel derived from HP_4A	80 nm × 80 nm × 320 nm	Cationic and anionic	After pulmonary administration cationic NPs, rather than negative ones, preferentially associated to lung DCs	[79]
	Carboxylated polystyrene plain or coated with PLL, PA, PrS, TT or WGA	0.1, 0.5, 0.9 and 4.5 μm	− 66.9 to + 41.4 mV	Positively charged particles were better internalized by DCS *in vitro* than negative ones, especially when they were of micrometric sizes	[101]

Chol: cholesterol; DC: dendritic cell; HP_4A: tetra(ethylene glycol) monoacrylate; n.d.: not determined; NP: nanoparticle; PC: dimyristoyl phosphatidylcholine; PG: dimyristoyl phosphatidylglycerol; PA: L-a-dimyristoyl phosphatidic acid; PLL: poly-ι-lysine; PRINT: particle replication in non-wetting templates; PrS: protamine sulphate; TAP: dimyristoyl trimethylammoniumpropane; TT: tetanus toxoid; WGA: wheat germ agglutinin

Table 2B

Summary of the different strategies adopted for the active targeting to dendritic cells

Ligand	Nanosystem	Key results	Ref.
Ab for CD40, CD11c, DEC-205	PEG-PLGA NPs	Only active targeting improved $CD8^+$ T cell activation *in vitro* and *in vivo*	[105]
Mannosylation	PLGA NPs	More efficient *in vitro* uptake of NPs by DCs with chemically conjugated mannan than for plain mannan-adsorbed NPs	[106]
	PLGA NPs	Mannose functionalization stimulated Th1 bias responses, decreasing tumor growth, both in prophylactic and therapeutic treatments	[107]

Ab: antibody; NP: nanoparticle; PEG-PLGA: pegylated poly(lactic-co-glycolic) acid; PLGA: poly(lactic-co-glycolic) acid; Th1: T helper 1

Table 2C

Summary of the different strategies followed in order to facilitate the endosomal escape of antigens in dendritic cells

Mechanism	Critical feature	Nanosystem	Key results	Ref.
Membrane disruption	pH-responsive diblock copolymers	Polyacrylic micelles	pH-responsive micelles caused a higher increase of $CD8^+$ T cell responses *in vitro* and *in vivo* than non-pH-responsive controls	[117]
Fusion with the membrane	pH-sensitive poly(glycidol) polymers	EPC/DOPE/polymer liposomes	Modified liposomes elicit stronger cellular responses than unmodified systems *in vivo*	[119]
Unknown	Positive lipids (DOTAP or DC-Chol)	DOTAP/Chol/DSPE-mPEG, DC-Chol/DOPE/DSPE-mPEG, EPC/Chol/DSPE-mPEG liposomes	Liposomes with cationic lipids, but not with anionic ones, increased cross-presentation and $CD8^+$ T cell activation *in vitro*	[120]
Membrane disruption (?)	Disulfide crosslinking of the gel	Bioreducible alginate/PEI nanogels	Humoral and cellular responses where enhanced *in vitro* by the bioreducible nanogel in comparison to the non-reducible one	[121]
Unknown	Disulfide bond to nanocarrier	Propylene sulfide NPs	More efficient cross-presentation of the antigen when attached by a reducible link rather than by a non-reducible one	[122]
Unknown	ISCOMATRIX adjuvant	ISCOMATRIX + antigen (OVA or *E. coli* protein)	ISCOMATRIX adjuvant allowed a rapid translocation of the antigen from lysosomes to the cytosol and a greater cross-presentation *in vitro*, in comparison to immune complexes	[123]
Activation of endosomal TLR3 or TLR9	Poly(I:C), CpG or plasmid DNA	Liposome-Ag-nucleic acid complexes	Complexation of TLR agonists showed an increased $CD8^+$ T cell activation independent of $CD4^+$ T cell help, in comparison to liposomes without TLRs. Also, both prophylactic and therapeutic effects were achieved in two different mice models	[125]
Activation of endosomal TLR3	Poly(I:C)	Cationic adjuvant system (CAF01), composed of DDA and TDB	Immunization with OVA + DDA/TDB/poly(I:C) elicited stronger and longer $CD8^+$ T cell responses in mice than CAF01 alone. In addition, less	[126]

Mechanism	Critical feature	Nanosystem	Key results	Ref.
			inflammatory side effects were observed than when administering poly(I:C) alone	
Activation of endosomal TLR7/8	Resiquimod	Temperature-responsive self-assembling particles, based on resiquimod anchored to HPMA or NIPAM scaffolds	Particle formation was key to diminish systemic toxicity and to generate Th1 bias responses, high antibody titers and $CD8^+$ T cell activation *in vivo*	[127]

Ag: antigen; CSF21: cationic adjuvant system; Chol: cholesterol; DC-Chol: 3β-[N-(N′,N′-dimethylaminoethane)- carbamoyl] cholesterol; DDA: dimethyldioctadecylammonium; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP:, 2-dioleoyl-3-trimethylammonium-propane; DSPE-mPEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; EPC: egg phosphatylcholine; HPMA: hydrophilic N-(2-hydroxypropyl) methacrylamide; NIPAM: N-isopropylacrylamide; NP: nanoparticle; OVA: ovalbumin; PEI: polyethylenimine; poly(I:C): polyinosinic–polycytidylic acid; TDB: trehalose 6,6′-dibehenate; Th1: T helper 1; TLR: toll-like receptor

Table 3

Summary of the different antigen attachments used for activation of humoral responses

Nanosystem	Covalent attachment	Key results	Ref.
CaP NPs loading HEL or BSA	Maleimide bond	NPs were absorbed to B cells in an antigen-specific manner *in* vitro, inducing their activation	[134]
ICMVs with malaria antigen	Maleimide bond	ICMVs with antigen conjugated and encapsulated elicit stronger *in vivo* humoral responses than MVs with encapsulated antigen alone	[135]
DLPC/Chol, DOPC/Chol, PC/Chol, DMPC/Chol, DPPC/Chol, DSPC/Chol liposomes	Diazotisation	No difference in immune responses were found when comparing encapsulation to surface-conjugation of the antigen	[136]
DMPC/Chol/DPPE liposomes	Pyridyldithio propionic acid	Covalent linkage of antigen increased both IgG and IgM responses, while encapsulation only elicit IgG responses	[137]
PC/SA/Chol liposomes	Diazotisation	Conjugation of antigen to the surface elicit longer and stronger antibody responses than encapsulated or free antigen	[138]
DMPC/Chol/DPPE liposomes	Diazotisation	More rapid and prolonged responses obtained with antigen surface linkage than encapsulation	[139]
PC/PS/Chol liposomes	Palmitylation of the peptide	Incorporation of antigen conjugated to palmitic acid showed stronger humoral responses *in vivo* than liposomes with the free antigen	[140]
PC/PG/PE/Chol liposomes	Maleimide bond	Conjugation of the antigen to SUV or LUV showed greater responses *in vivo* than encapsulation, with the best responses for SUV observed with the antigen coupled and MPLA encapsulated	[141]
PC/Chol liposomes	n.d.	Surface conjugation of antigen increased the antibody levels faster, while entrapment of the antigen showed stronger secondary responses	[142]
DMPC/Chol/DPPE liposomes	Diazotisation	Surface conjugation elicited longer responses *in vivo* than encapsulation, also presented a different Ig profile	[143]
DMPC/Chol/DPPE liposomes	Diazotisation	Both conjugation and encapsulation elicited strong humoral responses *in vivo*, but conjugation generated a greater blastogenic response	[144]
DMPC/DMPG/Chol/LA liposomes	Diazotisation	A high surface display of the antigen generated better humoral responses *in vivo*	[145]
DSPC/Chol/DMPG/MPLA liposomes	Peptidic bond	Physical association was needed for T cell activation, and only surface conjugation induced strong antibody responses	[146]

BSA: bovine serum albumin; CaP: calcium phosphate; Chol: cholesterol; DLPC: dilinoleoyl phosphatylcholine; DMPC: dimyristoyl phosphatidylcholine; DMPG: dimyristoyl phosphatidylglycerol; DOPC: dioleyl phosphatylcholine; DPPC: phosphatylcholine; DPPE: dipalmitoyl phosphatidylethanolamine; DSPC: distearoyl phosphatylcholine; HEL: hen egg lysozyme; IgG: immunoglobulin G; IgM: immunoglobulin M; MPLA: monophosphoryl lipid A; MVs: multilamellar vesicles; n.d.: not determined; LA: lipid A; LUV: large unillamelar vesicles; NP: nanoparticle; PC: egg phosphatylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; SA: stearylamide; SUV: small unillamelar vesicles

Table 4

Summary of some relevant nanovaccine-delivery systems that are being evaluated in clinical trials

Name/Company	Nanocarrier	Disease	Vaccination route	Clinical Phase	Ref.
SELA-070/Selecta Biosciences	Synthetic Vaccine Particles (SVP™)	Smoking cessation and relapse prevention	Parenteral (SC)	Phase I	[190, 191]
MAS-1/Nova Immunotherapeutics Limited	Nanoparticular emulsion-based adjuvant	Seasonal Influenza	Parenteral	Phase I	[192, 193]
FluGem®/Mucosis BV	Bacterium-like particles	Influenza	Mucosal (IN)	Phase I	[194, 195]
SynGem®/Mucosis BV		RSV	Mucosal (IN)	Phase I	[194, 196]
VCL-HB01/Vical Inc	Vaxfectin® adjuvant: cationic lipid-based liposomes	HSV-2	Parenteral (IM)	Phase II	[197, 198]
ASP0113/Vical Inc	Poloxamer CRL1005+ DNA	CMV in hematopoietic cell transplant patients	Parenteral (IM)	Phase III	[199, 200]
		CMV		Phase II	[199, 201]
HBV003/Vaxine Pty Ltd	Advax: D-inulin MPs	Hepatitis B	Parenteral (IM)	Phase I/II	[202, 203]
FLU003/Vaxine Pty Ltd		H5N1 Avian Influenza	Parenteral (IM)	Phase I	[202, 204]
R21 + Matrix M1/University of Oxford & Novavax	Antigen + Matrix M™: saponin-based particles (saponins, synthetic Chol and phospholipids)	Malaria	Parenteral (IM)	Phase I/II	[205, 206]
RSV F Vaccine/Novavax	RSV F Vaccine: recombinant F-proteins from RSV that self-assemble to form NPs	RSV	Parenteral (IM)	Phase III	[207, 208]
RSV F Vaccine + Matrix M/Novavax	RSV F Vaccine + Matrix M™	RSV	Parenteral (IM)	Phase II and III	[205, 208, 209]
LV305/Immune Design	LVR305: Antigen-specific ZVex® vector (hybrid, reengineered virus designed to carry genetic information of a tumor antigen)	Non-small cell lung cancer, Melanoma and Sarcoma	Parenteral (ID)	Phase I	[210 – 212]
CMB305/Immune Design	LV305 + G305(GLA adjuvant system)	Sarcoma, Melanoma, Non-small cell lung cancer and Ovarian cancer	Parenteral (ID and IM)	Phase I	[210, 211, 213]
JVRS-100/Juvaris Biotherapeutics Inc	JVRS-100: cationic lipids/non-coding DNA plasmid complexes	Leukaemia	Parenteral (IV)	Phase I	[214, 215]
1790GAHB/GlaxoSmithKline	GMMA: outer membrane particles from bacteria	Dysentery	Parenteral (IM)	Phase I	[216, 217]
CTH522-CAF01/Statens Serum Institut	CAF01: cationic adjuvant system composed of DDA and TDB	Chlamydia trachomatis	Parenteral (IM)	Phase I	[126, 218]

Chol: cholesterol; CMV: cytomegalovirus; DDA: dimethyldioctadecylammonium; GLA: glucopyranosyl lipid A; GMMA: generalized modules of membrane antigen; HSV-2: Herpes Simplex Virus - 2; ID: intradermal; IM: intramuscular; IN: intranasal; MP: microparticle; NP: nanoparticle; RSV: Respiratory Syncital Virus; SC: subcutaneous; TDB: trehalose 6,6′-dibehenate

Table 5

Current and most used treatments for selected autoimmune diseases

Disease	Treatment	Administration route	Mechanism of Action
Multiple sclerosis	IFNβ	SC/IM	Balances the expression of pro- and anti-inflammatory agents in the brain Reduces the number of inflammatory cells that cross the blood brain barrier
	Glatiramer acetate	SC	Strong promiscuous binding to MHC molecules and consequent competition with myelin antigens for their presentation to T cells
	Natalizumab	IV	Blockade of α4 integrin and consequent inhibition of immune cells extravasation
	Immunosuppresive agents	Oral/IV	Blockade of immune response at different levels
Type 1 diabetes	Insulin injections	SC	Decrease of glucose levels
Rheumatoid arthritis	NSAIDs	Oral	Inhibition of the synthesis of prostaglandins and thromboxanes
	Corticosteroids	Oral/intra-articular	Regulation of genes related with inflammation and suppression of immune response
	TNFα antagonists	SC/IV	Blockade of either TNFα or its receptor
	Disease-modifying anti-rheumatic drugs (DMARDs)	Oral/SC/IV	Slow down disease progression by different mechanisms
Inflammatory bowel disease	Aminosalicylates	Oral	Modulation of gene expression and consequently inhibition of cyclooxygenase and NF-κβ and its downstream signals
	Corticosteroids	Oral	Regulation of genes related with inflammation and suppression of immune response
	Immunosuppressive agents	SC/IV	Blockade of immune response at different levels
	TNFα antagonists	SC/IV	Blockade of either TNFα or its receptor
	Antibiotics	Oral	Decreasing concentrations of bacteria in the gut lumen Altering the composition of intestinal microbiota
Systemic lupus erythematosus	NSAIDs	Oral	Inhibition of the synthesis of prostaglandins and thromboxanes
	Antimalarial drugs	Oral	Altering lysosome stability Suppressing antigen presentation Inhibiting prostaglandin and cytokine synthesis Influencing both TLR signaling and leukocyte activation
	Corticosteroids	Oral	Regulation of genes related with inflammation and suppression of immune response
	Immunosuppressive agents	SC/IV	Blockade of immune response at different levels

IM: intramuscular; NSAID: nonsteroidal anti-inflammatory drug; MHC: major histocompatibility complexes; SC: subcutaneous; TLR: toll-like receptor

Table 6

Most representative polymeric and lipidic nanocarriers for antigen-specific tolerance generation in autoimmunity

Nanocarrier type	Loaded molecule	Administration characteristics	Animal model	*In vivo* results	Ref.
PLGA NPs	IL-10 or MOG antigen	SC, co-administration of both systems	EAE	Inhibited disease development No vaccination delayed disease onset	[269]
	Rapamycin and PLP antigen	SC and IV	R-EAE	Delay in disease onset Complete inhibition of relapse episodes (IV)	[154]
	PLP antigen covalently linked	IV	R-EAE	Prevention of disease onset Complete inhibition of relapse episodes	[63, 65]
	CII	Oral	CIA	Peyer's patches accumulation for longer time Reduced plasma levels of CII-antibodies Reduced incidence of arthritis	[19]
	PEG-CII derived peptides	Oral	Healthy	Expansion of IL-4^+ and IL-10^+ $CD4^+$ T cells	[282]
PLGA MPs	Rapamycin and MOG antigen	Intra-nodal	EAE	Permanently reduction of disease onset and severity	[270]
	Vitamin D3 and Insulin B, or TGF-β1 and GM-CSF	SC, co-administration of both systems	NOD	Disease onset prevention in 40 % of mice treated Increase in survival time from 19 weeks to 24 weeks	[156]
Liposomes	PS and insulin peptides	IP	NOD	Reduced incidence of T1D Delay in disease onset	[152]
	PS and MOG peptide	IP	EAE	Reduced clinical score Delay in disease onset	[275]
	Methylated BSA and NF-κβ inhibitors	SC	AIA	Reduction in joint swelling severity scores	[283]
Nano-complexes	pDNA encoding for BTLA and MOG antigen	IP injection of pre-treated DCs	EAE	Delay in disease onset Reduction of disease severity	[272]
	GpG and arginine-modified MOG antigen	SC	EAE	Reduced clinical score In some cases mice remained asymptomatic for the duration of the study (24 days)	[273, 274]

AIA: adjuvant-induced arthritis; BSA: bovine serum albumin; BTLA: B and T lymphocyte attenuator; CIA: collagen-induced arthritis; CII: type II collagen; DC: dendritic cell; EAE: experimental allergic encephalomyelitis; MOG: myelin oligodendrocyte protein; NOD: non-obese diabetic; PEG: pegylated; PLGA: poly(lactic-co-glycolic) acid; PLP: proteolipid protein; PS: phosphatidylserine; IP: intra-peritoneal; R-EAE: relapsing-EAE; SC: subcutaneous