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Review

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Harnessing nanoparticles for immune modulation

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Recent approaches using nanoparticles engineered for immune regulation have yielded promising results in preclinical models of disease. The number of nanoparticle therapies is growing, fueled by innovations in nanotechnology and advances in understanding of the underlying pathogenesis of immune-mediated diseases. In particular, recent mechanistic insight into the ways in which nanoparticles interact with the mononuclear phagocyte system and impact its function during homeostasis and inflammation have highlighted the potential of nanoparticle-based therapies for controlling severe inflammation while concurrently restoring peripheral immune tolerance in autoimmune disease. Here we review recent advances in nanoparticle-based approaches aimed at immune-modulation, and discuss these in the context of concepts in polymeric nanoparticle development, including particle modification, delivery and the factors associated with successful clinical deployment.

Introduction

Nanotechnology is revolutionizing many aspects of modern medicine, including diagnostics and therapeutics [1,2]. The first nanoparticle (NP) therapy was approved by the FDA in 1989. Subsequently, numerous NP therapies have been approved, most of which have focused on optimizing the safety and pharmacokinetic properties of small-molecule agents and hormones [2,3]. More recently, our increasing knowledge of the cellular subsets and regulatory roles of various members of immune system, combined with the emergence of safe, biocompatible nanoparticle platforms, is catalyzing the development of complex, highly adaptable, and programmable NP therapies that are predicted to revolutionize the standard of care of numerous disorders. For example, NPs may be engineered to specifically target cells of the mononuclear phagocyte system (MPS) for the purposes of restoring peripheral immune tolerance or to regulate aberrant monocyte activities during severe inflammation [2,4–7]. Five-hundred-nanometer NPs with negative zeta potential can be harnessed to target circulating monocytes, reducing their potential for causing immune pathology in numerous experimental disease models including West Nile virus (WNV) encephalitis, myocardial infarction, and inflammatory bowel disease (IBD) [8]. The combination of such NPs with specific autoantigens can also be used to restore peripheral immune tolerance in autoimmune models including experimental autoimmune encephalitis (EAE) [5–7,9]. In addition, NPs maybe utilized to mop up extraneous circulating inflammatory mediators.

The functional outcome of NP immune modulation depends on numerous factors that are intrinsic to NPs, such as composition, size, and charge, as well as extrinsic factors such as route of administration. These concepts and how they relate to manipulating immune responses are the primary focus of this review.

Immunological considerations in therapeutic particle design and utilization

NP design

NPs are particles sized between 1 and 1500 nm. They can be made from almost any compound, including poly(amino acids), polysaccharides and poly(alpha-hydroxy acids) as well as non-degradable compounds such as gold, silver, carbon, iron, and silica. The ability to synthesize NPs from biocompatible and biodegradable polymers such as poly-lactide-co-glycolide (PLGA) has revolutionized the use of NPs in the field of immune modulatory therapeutics and this will be the focus here. NPs can be engineered to deliver, alone or in any combination, small-molecule drugs (including immune suppressants and chemotherapeutic agents), proteins (hormones and antibodies), peptides (for vaccine or immune tolerance purposes), DNA (as part of gene therapy approaches), miRNAs, and even machinery to target clustered regularly interspaced short palindromic repeat (CRISPR) components for gene-editing purposes. It is now clear that the physiochemical characteristics of unadorned NPs can also alter immune responses independently of any associated active pharmaceutical ingredient [8].

A primary function of NPs involves the delivery of a specific cargo and numerous methods have been developed due to the challenge associated with the efficiency of

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encapsulation and the properties of this cargo. A straightforward approach is to chemically conjugate the desired active molecule to the particle. Peptide antigens have been chemically conjugated to NPs using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC or ECDI) [6,9], which attaches the amine on the target to a carboxylic acid on the particle. Various chemistries, such as Click chemistry or Michael-type addition, are available and their use is based on the chemical groups within the polymer and on the cargo molecule. Alternatively, the active molecule can be incorporated into the particle directly. Using PLGA to exemplify the approach, if the cargo is either soluble in an organic solvent or stable in a crystalline form when dispersed in an organic solvent [10], the encapsulation can be accomplished using a water/oil single-emulsion method. For delivery of water-soluble molecules, these are incorporated into polymeric NPs using a water–oil–water double-emulsion method [11]. For the double-emulsion method, the aqueous drug is initially dispersed within the dissolved polymer solution and then a second emulsion is formed with an aqueous solution containing an emulsifying agent.

Size and shape

The downstream immunological outcome of NP therapy is strongly influenced by the mechanism of cellular uptake. NPs entering cells via pathways that allow access to the cytosol have different immune-modulating capabilities from those taken up via phagocytosis [12–14]. Size and shape influence biodistribution and the mechanism of particle uptake [15,16], but studies using a broad range of standard cell lines (HeLa, CHO, Caco-2, and MCF-7) and NPs (derived from gold, polystyrene, polymer, silicon, titanium, and iron oxide) show that the ideal size and shape for particle uptake depends on the cell type [16,17]. In non-phagocytic cell types, NP <100 nm diameter are most efficiently taken up via caveola- or clathrin-mediated processes [15,16]. For professional antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), uptake is impacted more by shape than size, with spherical NPs having more favorable uptake kinetics than rod-shaped NPs, irrespective of NP size [18]. Targeting NPs to phagocytes is a critical aspect of any therapy attempting to manipulate the immune response and is discussed further below.

Size also affects NP toxicity [19]. Nanoparticles <100 nm in diameter tend to interact with cellular organelles, including the mitochondria and nucleus, and these interactions can trigger cellular respiratory and gene toxicity in cells [20]. This risk is reduced with increasing NP size, presumably because larger NPs tend to initiate phagocytosis, which effectively isolates particles from the more sensitive cytoplasmic environment.

Charge

NP charge is a dynamic physicochemical characteristic, with the particle microenvironment, including the protein corona, all capable of altering the surface charge of NPs. Generally speaking, NP charge can be modified by increasing the number of carboxyl (negative charge) or amine (positive charge) groups on the surface of the NP. Studies using different NP charges have clearly shown that this factor influences both uptake mechanisms and downstream immune outcomes. For instance, relative to anionic particles, cationic NPs appear to be taken up more readily via clathrin-mediated processes [21]. Furthermore, positively charged antigen-loaded NPs are significantly more effective at stimulating Th1 responses after either intradermal or mucosal (pulmonary) inoculation, whereas anionic particles stimulate T and B cell responses poorly under similar conditions [22,23]. The ability of cationic particles to stimulate Th1 responses has been associated with preferential DC uptake of such particles and the propensity of cationic particles to regulate positive costimulatory molecules [24]. However, cationic NPs may also alter mitochondrial and endoplasmic reticulum function, triggering the production of reactive oxygen species and proinflammatory cytokines, as well as cell death [25–27]. These events may underlie the adjuvant effects of cationic NPs, but attempts to harness this phenomenon clinically will need to address the consequences of any off-target toxicities.

Anionic NPs, by contrast, have been associated with little to no toxicity [20]. Furthermore, NPs with a charge below −30 mV have been found to have anti-inflammatory properties and when combined with antigen can induce antigen-specific immune tolerance [6,8,9]. This phenomenon is associated with the ability specifically to target scavenger receptors such as MARCO on monocytes and macrophages [6,8,9].

Stiffness and fluidity

Stiffness also affects the biological impact of NPs. NPs made of rigid materials may be associated with increased potential for embolism, while flexible polymer-based NPs that can more easily deform may gain better access to tissues during the complex vascular changes associated with inflammation. The fluidity of NPs, too, affects the ability of antigen-loaded NP to stimulate immune responses. Thus, intramuscular, solid-phase, antigen-containing liposome immunization elicits a more robust Th1/Th17 response than similarly administered fluid-phase liposomes [28]. The stimulatory ability of solid-phase particles is proposed to result from the formation of an immobilized antigen particle depot, similar to that observed for traditional oil-in-water emulsions and aluminum adjuvants [29,30]. This results in a prolonged supply of antigen for APCs and is also associated with upregulation of positive costimulatory molecules such as CD80, which support efficient T cell priming [28]. By contrast, intramuscularly injected fluid-state liposomes are rapidly removed, do not appear to stimulate positive costimulation, and are much less capable of stimulating a T cell response [28]. Whether the intramuscular fluid liposome–antigen combination induced peripheral immune tolerance was not tested, but intraperitoneal (IP) administration of fluid OVA-decorated liposomes can induce antigen-specific IgE non-responsiveness [31,32]. While this was argued to occur in a T cell-independent fashion, the increased levels of IgG after IP administration suggest immune deviation [33]. Notwithstanding this, these findings highlight the importance of understanding the contribution of fluidity and stiffness in NP-mediated manipulation of immune outcomes.

Factors associated with particle opsonization

Based on *in vitro* studies with fetal bovine serum, a protein corona forms around NPs in a two-step process. In the first step, high-affinity proteins rapidly bind to NPs to form a primary corona. In the second step, proteins of lower affinity bind either directly to the NP or to the proteins in the primary corona forming a secondary corona [34–37]. Constituents of the protein corona are thus inevitably impacted by the protein content of the serum and thus by the homeostatic or immune responses that regulate it. The above studies show that proteins with high abundance, such as albumin, comprise a significant proportion of the primary corona [34,38]. However, we have found that NPs with different charges bind significant amounts of less-abundant proteins in the plasma of WNV-infected animals [39].

In vivo the formation of a protein corona has been associated with little to no impact on NP delivery and function [8]. However, proteins binding to NPs could feasibly alter NP charge or mask functional groups important for NP targeting to certain receptors and/or enhance clearance of NPs by phagocytes [27,36,40]. Therefore, NP engineering efforts have focused on the development of techniques to reduce this phenomenon [41]. Many of these methods have been informed by experience gained from increasing the half-life of biologic therapies [42]. Commonly used processes include coating the NP's surface with polymeric ethylene glycol (PEG) or its low molecular weight derivative polyethylene oxide (PEO) [43]. PEG increases surface hydrophilicity, resulting in improved circulating NP half-life due to reduced serum protein binding [43]. Unlike PEG, the use of PEO to develop 'stealth' NPs has been associated with potential toxicity issues. Like, PEG, PEO forms a mushroom- or brush-like configuration on the surface of NPs. Unlike PEG, however, PEO may activate the C1q-dependent complement pathway in its mushroom-like configuration, whereas the brush-like configuration can trigger the lectin-specific complement cascade [44]. Interestingly, although NP coatings may prolong NP serum half-life, cells of the MPS still appear to be involved in the primary clearance mechanism for intravenously infused NPs [43]. Although these observations highlight the possibilities for modulating NP surfaces to reduce opsonization events, they also serve as a potent warning that such changes can dramatically alter the biocompatibility of NPs.

NP administration route and organ localization

The biodistribution of NPs has been examined in many studies, but widely varying conditions have resulted in diverse datasets, often making specific conclusions difficult (Figure 1). For example, studies often fail to perfuse animals before tissue examination [45–49], making it difficult to discriminate between NPs in the bloodstream and NPs adherent to the endothelium or NPs that have gained access to the organ parenchyma. In non-perfused animals after intravenous (IV) NP administration, the high intravenous NP content in organs with large blood volumes makes assessment of biodistribution extremely difficult by most modalities and the accurate evaluation of NP interactions with specific cells in the tissue virtually impossible (D.R. Getts and N.J.C. King, unpublished).

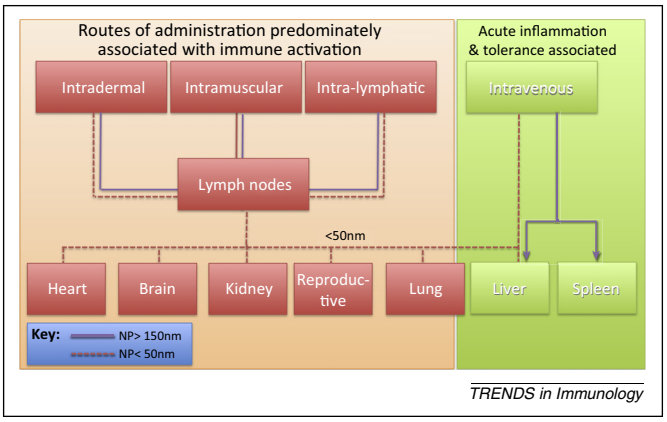

Figure 1. Size and administration heavily impact the biodistribution of nanoparticles (NPs). The underlying immunological niche of each physiological site determines the downstream outcome of NP administration. For example, the skin is associated with immune induction, whereas intravenous delivery is associated with immune tolerance induction.

NPs introduced into the body are generally perceived as foreign and the innate immune mechanisms that protect against invasive pathogens usually recognize, remove, and potentially degrade NPs rapidly. Subcutaneously administered NP are exposed to a highly specialized immune environment containing numerous migratory antigen-presenting subsets as well as relatively sedentary structural cells such as fibroblasts, all in the context of the local lymphatic drainage system. Similar to mucosal and intramuscular sites, the lymphatics carry APCs to local lymph nodes where processing and containment of antigenic material occurs. Small particles (<200 nm) may drain freely from subcutaneous, muscular, and even mucosal sites of application to local lymph nodes [50]. However, particles >200 nm in size must be phagocytosed by local or blood-derived phagocytes before transport to the draining lymph nodes. Both freely draining and APC-associated NPs arrive in the subcapsular sinus of the lymph node. This region is rich in CD169-expressing macrophages [51], which in the spleen are important for the coordination of CD8+ dendritic cells and their stimulation of effective CD4+ and CD8+ T cell responses against invading pathogens [52]. Subcutaneous delivery of NPs is very effective for stimulating immune responses to numerous T and B cell antigens [50,53]. Once there, NPs rarely escape the lymph node, which generally contains foreign antigenic material to limit the spread of invasive pathogens. Only ultrafine NPs (30 nm in size) seem to circumvent this sequestration [54]. However, this size of NP is associated with increased potential for oncogenesis and toxicity.

Intravenously administered NPs theoretically have access to the entire body; however, their biodistribution is strongly influenced by size [48,55,56]. For example, intravenously infused nanoparticles of diameter <5 nm can pass through the glomeruli and be detected in the urine [57]. Except where vascular integrity is disrupted, intravenously delivered NPs >100 nm in diameter cannot cross endothelial barriers such as the blood–brain barrier or the endothelial lining of the heart, reproductive organs, and gastrointestinal tract [48,55,56]. These particles, irrespective of surface modifications, are predominately engulfed

by circulating monocytes and cells of the MPS that line the fenestrated endothelium of the spleen and liver. The latter cells are essential for the daily filtering and phagocytosis of apoptotic cells and other debris from the blood [48,55,56] and these cells are critically associated with the maintenance of peripheral tolerance. The delivery of NPs via the IV route, in the absence of adjuvant, promotes immune tolerance and provides an avenue to modulate circulating inflammatory monocytes [6,8,9].

It is clear that the route of administration of NPs impacts both the organ distribution and the possible downstream immune outcomes. This point has been acknowledged by the FDA immunogenicity assessment guidance on therapeutic protein therapies [58]. In this document the authors note that, relative to other administration routes, IV administration of proteins and protein-containing therapies is associated with reduced risk of immune stimulation and cytokine-release syndrome-like events [58].

Therapeutic immune manipulation: beyond vaccines

The potential for NPs to optimize vaccination has been examined extensively [2,59,60], as has the delivery of immune-suppressive agents by NPs [61,62]. This horizon has recently expanded to include the use of NPs to control immune pathology associated with severe inflammatory responses as well as in the restoration of peripheral immune tolerance. These novel avenues are discussed in greater detail below.

Addressing acute inflammatory responses with NPs

The innate immune response is essential for host survival. However, abnormal or overactive acute inflammatory responses can result in immune pathology. This can result from cytokine-release syndrome, such as that caused by some biological therapies, or from aberrant innate immune cell function associated with numerous infectious agents, including Ebola, influenza, flaviviruses, and MERS as well as associated syndromes such as acute encephalitis syndrome and severe acute respiratory syndrome (SARS) [63–65].

Removal of inflammatory mediators and specific toxins

Overproduction of cytokines and other inflammatory mediators is a common occurrence in poorly regulated immune responses [63–65]. This phenomenon can be severe or life-threatening if left unchecked. Treatment options are limited, with large-dose steroids the most frequently prescribed treatment. Recently, extracorporeal filters such as the cytosorb, used during dialysis, have been effective in sepsis [66,67]. However, this treatment modality is limited by the need for expensive dialysis machinery. NPs may revolutionize this approach as NPs may be engineered for *in vivo* use, with surface modifications or the attachment of antibodies or other moieties, to bind specific toxins and/or proteins before being safely digested by liver and spleen phagocytes. In recent rodent proof-of-concept studies, infused highly negatively charged 'immune-modifying NPs' (IMPs) can absorb certain blood proteins, including S100 family and heat shock proteins, before finally being removed and destroyed by cells of the mononuclear phagocyte system [8,39,68]. Furthermore, this mechanism may also be used to capture and concentrate certain circulating proteins. IMPs have been shown to bind Annexin 1. The accumulation of Annexin 1 and its presentation to particular leukocyte subsets can have broad immune outcomes. For example, Annexin 1-loaded NPs may reduce neutrophils via induction of apoptosis [69] and/or promote T cell activation [70].

Targeting inflammatory myeloid cells

In humans, monocytes express CD14, from $CD14^{lo}$ to $CD14^{hi}$, as well as CD115 (the CSF-1 receptor) and the integrin CD11b. As the major monocyte population in normal blood in humans, $CD14^{hi}$ $CD16^{-}$ defines 'classical' or inflammatory monocytes [71]. In mice, this population is identified by its $Ly6C^{hi}$ $CD43^{lo}$ CCR2+ $CX3CR1^{lo}$ phenotype [72,73]. By contrast, a much smaller, $CD14^{lo}$, $CD16^{hi}$ 'nonclassical' or patrolling monocyte subset comprises some 10% of blood monocytes in humans and is defined as $Ly6C^{lo}$ $CD43^{hi}$ $CCR2^{lo}$ $CX3CR1^{hi}$ in mice [72,73]. $CD14^{hi}$ $CD16^{hi}$ and $Ly6C^{hi}$ $CD43^{hi}$ subsets define an intermediate group in humans and mice, respectively. Comparative RNA profiling suggests that human intermediate monocytes are functionally closest to mouse classical monocytes [74,75]. Work in many areas, including infectious disease, hypoxia, autoimmunity, and cancer, shows a definable wave of inflammatory monocyte recruitment into inflamed tissues expressing CCL2 and other chemokines. Once in the target tissue, these cells may differentiate into different effector cells, including DCs, macrophages, and even cells with a microglial phenotype [4,76], depending on the prevailing soluble factor milieu. In some cases these cells have important housekeeping functions (i.e., to digest and clear tissue debris before tissue remodelling); however, in many cases they express high levels of NO via induced NOS2 expression, as well as increased levels of NADPH oxidase, cathepsins, and myeloperoxidase, all of which may demonstrably contribute to further tissue damage in numerous conditions, including experimental autoimmune encephalomyelitis, myocardial infarction, and viral encephalitis [8,77,78]. Such monocyte management has recently emerged as a unique potential treatment modality to limit damage associated with the acute phase of inflammation in numerous disease indications.

The circulation serves as the primary conduit for monocytes to travel from the bone marrow/spleen throughout the body to sites of inflammation, making these highly susceptible to being targeted via intravenously administered NP. One approach involving the combination of siRNA with liposomes has shown some promise in animal models [78]. However, we have recently shown that NPs derived from a broad array of biodegradable and non-degradable compounds and molecules, without traditional active pharmaceutical ingredients (APIs) or other additional immune-stimulating agents, are able, through targeting of precise scavenger receptors, to reduce circulating inflammatory monocytes. Specifically, IMPs (i.e., NPs with a highly negative surface charge) bind with high specificity to inflammatory monocytes, marking them for sequestration by the spleen and thereby preventing migration to sites of inflammation and subsequent differentiation and participation in pathogenic immune responses [8]. Sequestered

monocytes either undergo caspase-3-mediated apoptosis or differentiate into CD11b+ CD11c+ CD103+ DCs [8,39]. While the role of these CD103+ CD11b+ DCs remains to be completely defined, IMP treatment is associated with the expansion of a short-lived, TIM3-expressing regulatory T cell (Treg) population [39] (D.R. Getts *et al.*, unpublished). The emergence and subsequent disappearance of these Tregs correlates with treatment efficacy in EAE models [39]. This monocyte management approach is effective in numerous models of inflammation (Table 1) and Phase 1/2 clinical studies are planned for 2016.

NPs for immune tolerance

Autoimmunity results from the breakdown of peripheral tolerance, an active immunological process that usually controls self-reactive T and B cells. The 'Holy Grail' of autoimmune therapy is the restoration or induction of robust peripheral immune tolerance in only those cells causing disease. The use of apoptotic cells to induce tolerance was uncovered originally in 1979 [79] and has shown promise in an early clinical study [80], However, the use of cells has numerous manufacturing, cost, and patient accessibility issues. The serendipitous discovery that negatively charged NPs target specific scavenger receptors and localize to the same regions of the spleen, similar to apoptotic cells, catalyzed the development of a synthetic cell replacement that could carry antigen for immune tolerance induction [6,8,81]. Subsequently, several NP-based approaches for the induction of immune tolerance that function via alternative mechanisms have emerged. Some focus on harnessing the natural capabilities of NPs to deliver antigen in a tolerogenic fashion, while other approaches deliver antigen combined with small-molecule immune suppressants or target aryl hydrocarbon receptors to change DC phenotype [5–7,9]. Below is a brief discussion of the mechanisms through which NPs have been shown to induce tolerance, with a focus on harnessing NP delivery of antigen without additional immune suppressants or modulating agents.

NP development for tolerance

The induction of immune tolerance using NPs, at least in rodent models, depends on several factors. First and foremost, NPs have to be loaded with the correct antigen and targeted to APCs capable of regulating T cell function concurrently with supporting the induction and/or expansion of Tregs.

The ability of antigen-loaded NPs to inactivate, with exquisite specificity, autoreactive T cell clones depends on the delivery of antigens that, when processed by APCs, will be directly recognized by autoreactive T cells [5–7]. The primary hurdle for antigen-specific tolerance approaches remains the identification of disease-associated antigens and their epitopes. Thus, in many autoimmune diseases, the epitopes associated with disease remain to be defined. Identification is further complicated in multiple sclerosis (MS), for example, by the fact that the appropriate target antigen may change over time due to epitope spreading. Nevertheless, in some disorders, including celiac disease, MS, and type 1 diabetes, numerous antigens have been identified [80,82–84]. Another potential strategy, which both harnesses the natural mechanisms that may have initiated autoimmunity and obviates the need for knowing the precise epitopes, may be the use of full-length proteins. Encapsulation of full-length proteins may allow host APCs to liberate numerous non-defined epitopes from autoantigens, similar to the process that presumably underlies the initiation of the disease.

The appropriate targeting and subsequent tolerogenic stimulation of certain populations of APCs is crucial for immune tolerance induction. Incorrect targeting can result in immune stimulation. For example, antigen-loaded NPs that are designed to target CD40, DEC205, or CD11c induce robust production of IL-12 and type 2 interferon as well as CD8+ T cell proliferation [85], whereas particles that target MARCO+ macrophages in the liver and spleen induce tolerance [6]. This highlights the fact that APC populations are not created equal, with some populations better equipped for tolerance induction and others equipped for immune induction. Germane to this, splenic and liver macrophage populations express numerous scavenger receptors, which during homeostasis play an important role in the recognition and recycling of dying leukocytes, red blood cells, and other debris on a daily basis [86–88]. Importantly, these activities occur without triggering inflammation or breaking peripheral immune tolerance. Tolerogenic IMPs (TIMPs) were designed to take advantage of the MARCO-targeting ability of highly

Table 1. IMP efficacy in severe inflammation models.

Condition	Animal model	Species	Outcome	Refs
Acute myocardial infarction	Temporary LAD occlusion Complete LAD occlusion Complete LAD occlusion	Mouse Mouse Rat	Reduced inflammation, reduced infarct size Increased function	[8]
Kidney ischemia	Temporary renal artery occlusion	Mouse	Reduced tubular necrosis and increased function	[8]
Stroke	Temporary carotid artery	Mouse	Reduced inflammation	Unpublished
MS relapse	EAE SJL (relapsing–remitting) EAE C57BL/6 (progressive disease)	Mouse Mouse	Reduced symptoms, reduced inflammation and demyelination	[8]
IBD	DSS colitis	Mouse	Reduced symptoms, reduced inflammation, rapid recovery of the colon	[8]
Acute encephalitis syndrome	WNV encephalitis Japanese encephalitis	Mouse Mouse	Increased survival Increased survival	[8]
Spinal cord injury	Spinal cord crush	Mouse	Increased mobility, reduced inflammation	Unpublished

negatively charged NPs [8,89]. The intravenous administration of antigen-encapsulated IMPs results in biodistribution similar to that of apoptotic debris [6,81], with particles homing to MARCO+ macrophages in the liver and spleen (Figure 2) [6,8,9,81]. More importantly, one course of TIMPs results in tolerance induction through T cell anergy and the parallel induction of Tregs [90]. This mechanism has subsequently been shown to be applicable to the induction of long-lived tolerance in numerous Th1-, Th17-, and even Th2-mediated rodent disease models (Table 2).

The inherent immunological behavior of lymph nodes, especially the phenomenon historically known as 'lymph node shutdown' makes it very difficult to target liver and spleen populations using non-intravenous routes of administration. In the context of TIMPs, IP administration results in only partial tolerance, while subcutaneous and oral delivery fail to induce regulation [5,6,9]. In some cases, subcutaneous administration can even result in sensitization and/or disease exacerbation [5,6,9]. The lymph nodes appear to be devoid of inherently 'tolerogenic' macrophage populations. This may be unsurprising, since most epithelial surfaces are consistently being challenged by opportunistic and other pathogens and, as a potential defense mechanism, epithelial and lymph node APC populations tend to express higher levels of costimulatory molecules than APCs elsewhere (D.R. Getts *et al.*, unpublished). There have been attempts to utilize NPs that combine antigen and immune-suppressive agents such as rapamycin to alter DC phenotype *in vivo* and potentially induce tolerance [5,6,9]. Rapamycin has been known for over two decades to have 'tolerogenic' effects when combined with antigen [91,92] and the adoptive transfer of rapamycin-treated DCs can promote the long-term survival of skin grafts in rodent transplant recipients [93]. In the context of NPs, Haddadi and colleagues showed that rapamycin-loaded PLGA NPs preferentially bound to DCs and induced an immature 'tolerogenic' phenotype [61]. More recently, Maldonado and colleagues showed that biweekly subcutaneous injections of pegylated PLGA rapamycin and

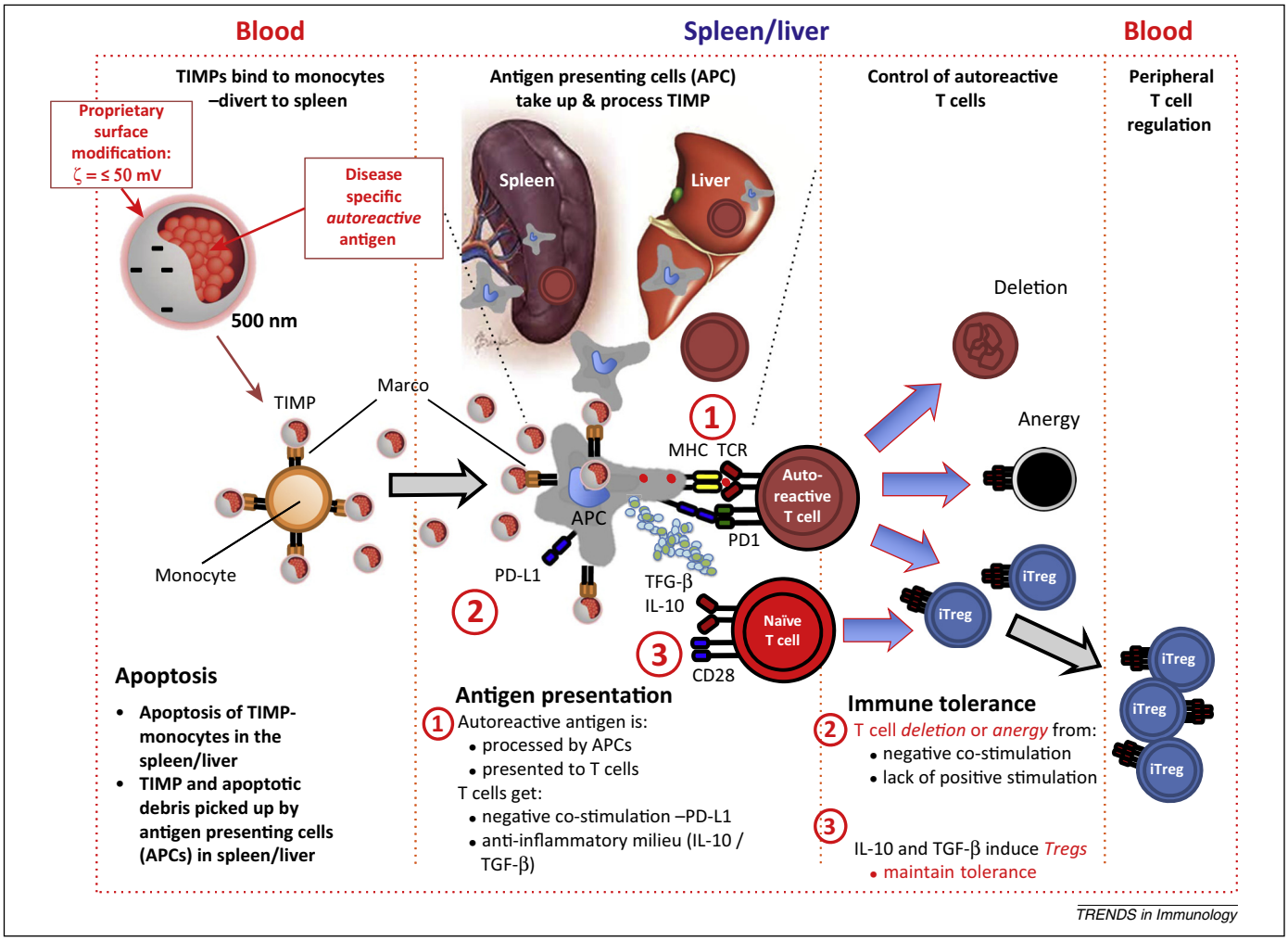

Figure 2. Proposed mechanism of action tolerance induction using toleragenic immune modifying nanoparticles (TIMPs). The induction of tolerance using TIMPs requires that the antigen-loaded particles must be delivered intravenously. The particles themselves are then taken up via a scavenger receptor-mediated processes, with MARCO shown to be involved in particle uptake and tolerance induction [6]. MARCO is expressed on circulating inflammatory monocytes as well as marginal zone macrophages. Based on TIMP size (500 nm), phagocytosis is likely to be the primary uptake mechanism. On uptake, these macrophage populations produce IL-10 and TGF. These factors have numerous immune regulatory functions including modulating the level of PD-L1 on antigen-presenting cells (APCs). When integrated, these APC responses coordinate the regulation of autoreactive T cells via three predominant pathways. In the context of activated autoreactive T cells, the upregulation of negative costimulatory molecules on APCs, including PDL-1 and CTLA-4, promote autoreactive T cell anergy and apoptosis. With naïve T cell regulation resulting from TCR stimulation (signal 1), without regulatory T cell induction. Although the precise temporal contribution of each regulatory mechanism requires further examination, evidence suggests that tolerance is the result of early anergy, with regulatory T cells (T_{REGS}) playing a major role in long-term tolerance maintenance.

Table 2. NP immune tolerance platform efficacy.

Disease model	Antigen	Refs
IMP + antigen		
MS (EAE)	$PLP_{139–151}$, $PLP_{178–191}$, $MOG_{35–55}$	[6,9,79,81]
Type 1 diabetes (BDCA2.5, NOD)	Insulin-associated antigens and P31	[101]
Allergic airway (whole OVA)	Ovalbumin	[102,103]
Celiac disease (gliadin model)	Whole gliadin	(Unpublished)
Food allergy (Th2 mediated)	Whole peanut extract (WPE/SEB)	[102,103]
Allogeneic islet grafts	Alloantigen	[47]
Xenogeneic islet grafts	Xenoantigen	[104]
Gene therapy	Tolerance to vector/CD8 tolerance	(Unpublished)
Encapsulated rapamycin + antigen pegylated PLGA NP		
Model antigen (OVA)	Ovalbumin	[5]
MS (EAE)	$PLP_{139–151}$	[5]
Hemophilia A	Factor V peptides	[5]
Gold NPs conjugated with antigen and 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester		
MS (EAE)	$MOG_{35–55}$ $PLP_{139–151}$	[7]

$OVA_{323–334}$ NPs significantly reduced the production of OVA-specific IgG [5]. However, it should be noted that, with repeated OVA exposure, titers were observed to increase over time in treated mice [5]. Using similar particles, although loaded with $PLP_{139–151}$, the authors also showed that subcutaneous injection could reduce the incidence of EAE; however, unlike intravenous infusion, protection was not 100% [5,94]. Another strategy that focuses on altering DC phenotype is the administration of NPs that agonize the ligand-activated transcription factor aryl hydrocarbon receptor [7,95–97]. The activation of aryl hydrocarbon receptor on DCs supports their differentiation into tolerogenic APCs, which supports the expansion of Tregs [95–97]. Using the $MOG_{35–55}$ or $PLP_{39–151}$ EAE model, pegylated gold NPs loaded with the aryl hydrocarbon receptor agonist 2-(1′*H*-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester and $MOG_{35–55}$ or $PLP_{39–151}$ were tested for their ability to ameliorate disease [7]. While not tested subcutaneously, weekly IP or IV administration of these NPs successfully resulted in disease management [7].

An alternative approach to delivering NP–antigen cocktails to APCs involves the direct targeting of specific T cell populations associated with disease. For example, it has been shown that polyclonal Tregs can be expanded using NPs coated with leukemia inhibitory factor [98]. A more antigen-specific approach includes using polymeric or monomeric peptide MHC (pMHC), which may selectively delete T cells expressing the cognate T cell receptor that recognizes the pMHC complex. These have shown efficacy in experimental models of transplantation and type 1 diabetes; however, clinical translation is complicated by the requirement of having exquisite knowledge of each autoreactive epitope [99,100]. Nevertheless, the field is rapidly progressing toward clinical translations of NP use for treatment of various disease states using the inherent properties of NPs to direct antigen uptake to particular APC subsets for the induction of immunity or tolerance.

Concluding remarks

The use of NPs as immune-regulating agents has only recently emerged and many unanswered questions remain. In the future, the development of novel biocompatible polymers, as well as our increased understanding of the precise interplay between the physiochemical characteristics of NPs and their associated downstream immune interactions, is likely to result in further expansion of the field. In the next 3–5 years, several clinical studies are expected to begin testing the ability of highly negatively charged PLGA NPs to reduce severe inflammation in myocardial infarction and acute encephalitis syndrome [8]. Furthermore, evidence suggests that clinical testing of NP tolerance platforms in areas such as pemphigus vulgaris, types 1 diabetes, MS and even celiac disease is likely to occur in the next 2–3 years (http://www.courpharma.com; http://selectabio.com). Moving forward, questions surrounding the most appropriate NP formulation and safety will need to be addressed. Further barriers, such as antigen identification in the context of autoimmunity treatments, also remain. At the very least, these studies will inform future NP approaches. If successful, however, these therapies will revolutionize the treatment of many diseases and place NP-based therapies at the forefront of immune-modulating therapeutics.

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