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Targeted nanotherapy for induction of therapeutic immune responses

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Nanotechnology permits the design of therapeutic devices with defined structure and molecular composition. Modular designs employing surface-bound ligands provide specific homing devices for loaded cargo, and biocompatible and biodegradable constructs provide surrogate temporary microenvironments. We first present a case for developing 'smart' modular constructs as immunogenic vaccines to prime immune memory against specific pathogens where current vaccines fail. Second, we argue that nanotherapeutic intervention can harness pivotal molecular pathways recently discovered to regulate lineage development between pathogenic TH17 cells associated with autoimmune disease, versus tolerogenic regulatory T cells (Treg). Underpinned by molecular mechanisms that enable exquisitely specific responses in adaptive immunity, targeted nanodevices designed to stimulate either immune aggression or immune tolerance signify the birth of a new era in therapeutics.

The promise of nanotherapeutics

Nanotechnology holds great promise in therapeutic medicine by enabling three highly desirable therapeutic aims: (i) targeted drug delivery (for example, cytotoxins to cancer cells where relatively high doses of drug can be focused upon the tumor to reduce off-target side effects); (ii) exploitation of endogenous regulatory systems, such as harnessing of the adaptive immune response towards immune self-tolerance in treatment of autoimmune disease; and (iii) creation of an artificial transient microstroma as a supportive niche for endogenous repair following trauma or in age-related degenerative diseases. Moreover, because nanotherapeutic devices are defined in structure and molecular composition, and biodegradable where appropriate, safety standards are readily met.

In this article, we illustrate the progress and future promise of nanotherapeutic medicine, focusing on the field of the adaptive immune response. In this context, nanotherapy not only capitalizes on the exquisite specificity of antibodies to direct the nanodevice but also harnesses the power of the T lymphocyte to regulate either immune tolerance or immune-mediated destruction. Once the therapy is guided towards a desired lineage, that lineage will be maintained by endogenous regulation in subsequent responses to the target antigen. Such exploitation of the immune response capitalizes on both immune memory, especially relevant in vaccination, and immune surveillance throughout the host tissues. We present two scenarios where the use of nanotherapy is anticipated, firstly in the development of designer, multimodular vaccines, and secondly in providing surrogate microenvironments to guide autoimmune tolerance.

Part I: 'Smart' modular nanoconstructs as immunogenic vaccines

Despite successful vaccination programs in some regions of the world, the global risk of life-threatening disease following pathogenic infection remains high for at least two reasons: (i) a lack of access to proven vaccines, especially in underdeveloped countries; and (ii) a lack of vaccines against dangerous infectious agents, including those that cause highly pathogenic hepatitis C, dengue fever and West Nile fever. Even for less pathogenic viruses that

Glossary

Avidin: tetrameric biotin-binding protein. Avidin-coated nanoparticles bind biotinylated antibody for cell-specific targeting.
Capped mRNA: nascent mRNA with 5′ cap structure of 7-methylguanylate (m7G) that prevents premature mRNA degradation by 5′ exonucleases before protein translation. Regulates nuclear export.
Foxp3: Forkhead box P3 transcription factor required for development and function of Treg cells. Lack of Foxp3 results in death from overwhelming autoimmune disease. Encoded on the X chromosome.
IL-6: interleukin 6, belonging to the IL-6 cytokine family that signals through the signal transducing receptor gp130. Released by T cells and macrophages and is linked to inflammatory diseases.
LIF: leukemia inhibitory factor, an IL-6 cytokine family member that regulates growth and development. Associated with 'stemness' and required for embryo implantation. Released by $CD4^+$ T cells upon primary activation.
Mycophenolic acid: immunosuppressive drug that inhibits *de novo* purine synthesis required for proliferation of B and T lymphocytes.
NANOG: a transcription factor critically involved in self-renewal of undifferentiated stem cells; required to maintain pluripotency. In stem cells NANOG is a direct downstream target of LIF signaling.
Nanoparticle: ultrafine particulate units sized between 1 nm and ~100 nm. Specific nanoconstructs designed to carry cargo (e.g. drugs or cytokines) underpin nanotherapeutic devices.
PLGA: poly(lactic-co-glycolic acid) copolymer approved by the FDA for therapeutic devices, being biodegradable and biocompatible. Devices include sutures, grafts, prosthetics and implants in addition to microparticles and nanoparticles.
Rapamycin: a macrolide immunosuppressant also known as sirolimus. Closely related in structure to FK506 (tacrolimus) but having a different mode of action. Requires receptor binding to FKBP12 to become biologically active.
RORγt: orphan nuclear receptor and transcription factor that controls differentiation of TH17 cells. Required for development of lymphoid tissue inducer (LTi) cells that guide lymphoid development within the fetus, and later in the adult intestine.

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Box 1. Key variables for immunization

Vaccine antigen: the first variable is the form of the antigen itself. This can be inactivated or attenuated whole organisms; purified proteins and peptides; or DNA-encoded antigens. Given that pathogens continually emerge and change (e.g. HIV and influenza viruses), vaccine systems that can be rapidly and efficiently modified are required to counter the evolving antigens. Successful conventional vaccines have used live attenuated microorganisms where a single vaccine dose effectively elicits protective humoral and cellular immunity, as for the Sabin polio vaccine or the smallpox vaccine. However, live attenuated vaccines have problems, including the need for refrigeration that is not possible in many areas of major clinical need in underdeveloped nations. A further issue in the use of live attenuated vaccines is the danger that the attenuated pathogen may revert to a more pathogenic form.

Adjuvant: non-specific immune adjuvants are a second variable to consider [2,3]. Adjuvants emerged as a means to enhance the immunogenicity of purified individual antigens given that once purified, isolated antigens may be less immunogenic compared with whole pathogens or crude extracts. Adjuvants also address the issue of stimulating both the innate and adaptive arms of the immune system in the context of the antigen subunit [4,5]. Typical adjuvants include bacterial products, toxins or other molecules that augment specific immunity. The inclusion of pathogen-associated molecular patterns (PAMPs) on nanosystems is an emerging technology that blurs the line between the vaccine carrier and the role of an adjuvant, as these systems function simultaneously as both an adjuvant and an antigen carrier. Figure I

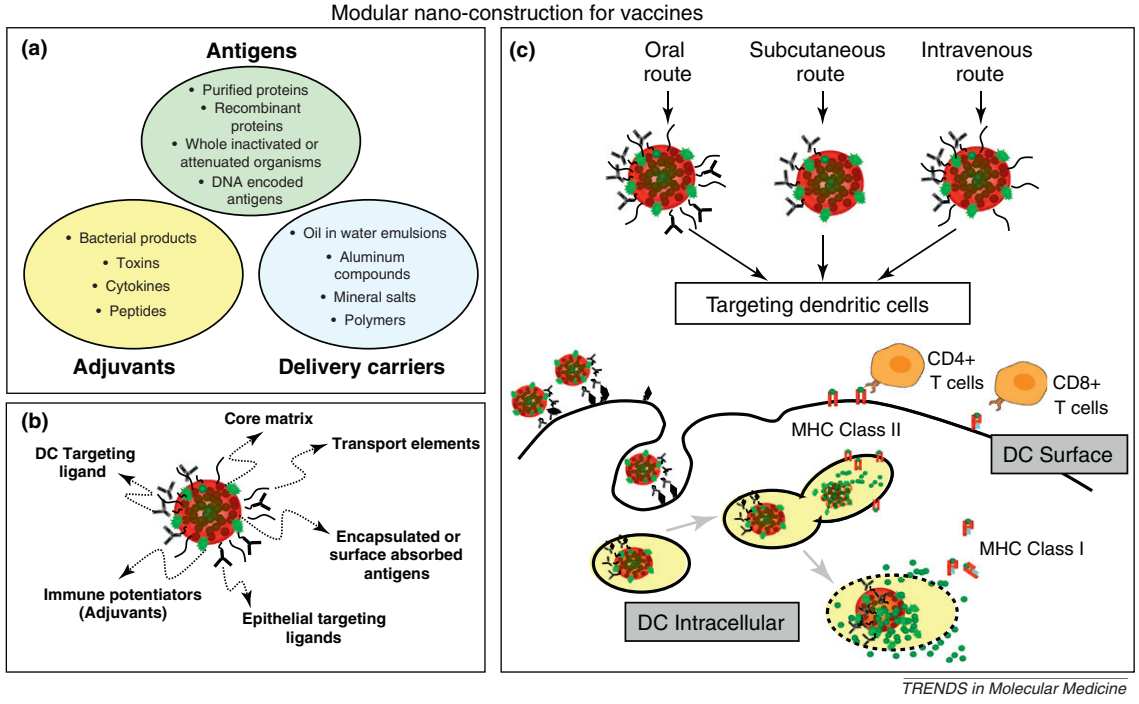

Figure I. Basic components and modular nanoconstruction for vaccines
(a) Major components of vaccines. **(b)** A schematic of a modular vaccine nanoparticle. A core matrix encapsulating an antigen is modified with dendritic cell (DC) targeting ligands, immune potentiators, epithelial targeting or endothelial elements that facilitate transepithelial cell uptake or enhanced adhesion to the endothelium, and protective transport elements that maintain bioparticle integrity during transit. **(c)** Schematic of nanoparticle vaccine administration by various routes and mode of DC intracellular antigen delivery. For oral delivery, particles need to be protected from harsh degradative conditions in the gastrointestinal tract by coating with protective elements. To evade the reticuloendothelial system during intravenous delivery, a protective mechanism is also needed. Particles that bind DCs are taken up and then prime MHC class II molecules in endocytic compartments for the activation of $CD4^+$ T cells and the subsequent helper-mediated antibody response. Particles may also break free from the endocytic compartment and deliver antigen to the cytosol to prime MHC class I molecules that activate the cytoxic $CD8^+$ T cell response. Abbreviations: MHC, major histocompatability complex.

are widespread and life-threatening to immunocompromised patients, including Epstein–Barr virus and cytomegalovirus, no protective vaccines exist. The risks are further compounded by global warming plus population migration that decrease physical boundaries to the transmission of infectious agents. There is an urgent need for global access to current vaccines to limit disease spread and for new vaccine development. Biomedical engineering promises rapid vaccine production using modular platforms that will be adaptable across a range of targets.

Learning from the foe

The motivation for nanoconstructs as vaccine systems stems from the idea that several components important for vaccine efficacy can be rationally assembled, optimized separately, and incorporated into a single vehicle to effect a potent immune response. These components may act additively or synergistically but in the end will work cooperatively to affect the desired immune response. Indeed, viruses and some bacteria are in essence highly evolved nanovehicles optimized to modulate the magnitude and direction of an immune response. Key lessons can be learned for constructing an effective vaccine by considering some of the key variables used by such organisms for modulating immune responses (Box 1) [1–5].

Molecular dynamics of vaccine engagement with the immune system

To achieve optimal stimulation to a given antigen, a formulation is needed that delivers the correct amount of

Box 2. Advantages of nanoparticles and the nano-LIF device

Nanoparticles have several, multifaceted advantages for delivery of therapeutics. In addition to their nanoscale size, nanoparticles based on biodegradable materials can be utilized to solubilize a concentrated payload of drug, improve drug stability and bioavailability, and extend drug or gene effects through sustained delivery (Figure I). The use of biodegradable, biocompatible materials reduces the risk of unwanted toxicities and adverse effects. Biodegradable nanoparticles have proven to be versatile platforms for the delivery of a large variety of compounds and have been well studied for the delivery of small molecules, proteins and nucleic acids, including small inhibitory RNAs (siRNAs). Recent advances in the design and engineering of nanoparticles have included modification of the surface to improve stability and circulation throughout the body, biospecific targeting against cellular ligands or extracellular matrix components, and incorporation of diagnostic imaging agents.

Solid, biodegradable nanoparticles offer distinct advantages over liposomes or other carriers that do not control release of the cargo. First, by varying the morphology and polymer composition of the particle, one can effectively optimize a variety of controlled release characteristics, allowing for moderate constant doses over prolonged periods of time. There have been a variety of materials used to engineer solid nanoparticles, both with and without surface functionality. Perhaps the most widely used are the aliphatic polyesters, specifically the hydrophobic PLA [poly(lactic acid)], the more hydrophilic PGA [poly(glycolic acid)] and their copolymer PLGA [poly(lactide-co-glycolide)]. The degradation rate of these polymers, and often the corresponding drug release rate, can vary from days (PGA) to months (PLA) and is easily manipulated by varying the ratio of PLA to PGA. Second, the physiologic compatibility of PLGA and its homopolymers PGA and PLA have been established for safe use in humans; these materials have a history of over 30 years in various human clinical applications including drug delivery systems. Finally, PLGA nanoparticles can be formulated in a variety of ways by altering parameters such as size, morphology, charge, surface functional groups, and agent release profile, all of which can improve drug pharmacokinetics and biodistribution to target tissue by either passive or active targeting.

These advantages are exemplified by the proposed nano-LIF device, as described in [36] and illustrated in Figure Id. The LIF cytokine is embedded in a PLGA matrix produced as avidin-coated nanospheres that hold 100–400 ligands per particle. The entrapped LIF retains bioactivity, and total release occurs over 7 days at 37 °C, providing a sustained, paracrine-type release. Cellular targets are selected by choosing the appropriate biotinylated antibody, such as anti-CD31 for targeting to vascular endothelial cells. The particles are stable for months when freeze-dried at −80 °C. Room temperature stability has not been tested but is probably limited by the stability of the encapsulated cytokine. Experiments show that these nano-LIF constructs provide sustained low-level release of LIF to the immediate environment of the targeted cell. CD4-targeted nano-LIF (i) opposed IL-6-driven Th17 development; (ii) expanded donor-specific Treg *in vivo*; (iii) prolonged survival of vascularized heart grafts in mice; and (iv) expanded FOXP3^{+}CD4^{+} T cell numbers in a non-human primate model *in vitro* [36]. Nanoparticle-mediated LIF delivery was critical to efficacy as neither unloaded targeted nanoparticles nor soluble LIF recapitulated the efficacy of cytokine-loaded nanoparticles.

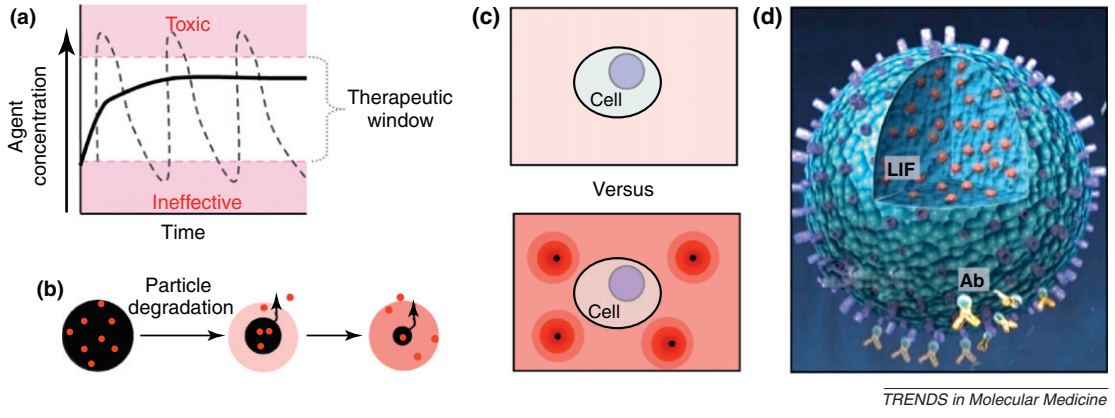

Figure I. **(a)** Most traditional delivery methods (e.g. oral or intravenous) result in tidal drug concentrations with high peak concentrations postadministration followed by clearance and need for frequent re-administration. Sustained release (solid line) seeks to maintain therapeutic concentrations from less frequent doses. **(b)** This profile can be achieved by the local release of agent from biodegradable particles. As particles degrade over time, encapsulated agent is released and made bioavailable. **(c)** Owing to limits imposed by systemic toxicities, agent concentration at the target site may be suboptimal (pale pink). By contrast, particle-mediated localization of agent allows for focused, concentrated delivery to the target (dark pink), resulting in improved efficacy and lower systemic exposure. **(d)** Cartoon of the nano-LIF device showing the encapsulated cytokine leukemia inhibitory factor (LIF; red spheres in cut-away) in the interior of the particle and the biotinylated targeting antibody (Ab; yellow) tethered to the surface by avidin.

antigen in a repetitive or sustained manner, both to the appropriate immune cells, and to the appropriate compartments within those cells (Box 1, Figure Ic and Box 2). Thus, a designed delivery vehicle, which can function as an adjuvant, should target the vaccine antigen (cargo) for combined delivery of both antigen and immunopotentiating molecules to specific cells of the immune system.

Problem areas

Traditional methods for increasing vaccine effectiveness have focused on coadministration of adjuvants, predominately montanide polymers or colloidal alum. However, these have a limited capacity to adsorb many antigens and greatly limited immunostimulatory properties [6]. Unpredictable risks arise due to the live attenuated vaccine itself as well as potential allergic side effects to the adjuvant [6–8]. Moreover, it is the T cell response that is essential for inducing lasting antiviral immunity. These various aspects, coupled with the difficulties inherent in manufacture, storage, and transport, emphasize the need for new and rational cellular targeted approaches to vaccine design [9–12].

Vaccine delivery that exploits dendritic cells (DCs) is enabled by nanotechnology

The outcome of the T cell response to cognate antigen ultimately depends on signals received from both the antigen-presenting cell (APC) and the microenvironment. APCs include B lymphocytes, macrophages and DCs, with

DCs being the most potent [13,14]. Importantly, DCs express receptors for various microbial and inflammatory products, and respond to antigen in different ways through a process of 'DC maturation' that depends on the nature of the pathogen encountered (virus, bacteria, or protozoan). The information is transmitted to T cells by altered patterns of cytokine release at the time of antigen presentation in lymph nodes, regulating the type of T cell response elicited. Thus, by targeting DCs it is possible to enhance antigen delivery as well as eliciting antigen responses in general, and to qualitatively control the nature of the immune response, depending on the desired vaccination outcome, as reviewed in [1,15–17].

Designer vaccines

Elucidation of the basic features of DC biology has provided an important tool chest for engineering vaccines with potent therapeutic outcomes [14,15,18]. The designer vaccine concept involves the flexible addition and subtraction of antigen, adjuvant, immune potentiators, molecular recognition, and transport mediation elements, as well as intracellular uptake mediators that target DCs and control many of the variables that are important in optimizing an effective vaccine delivery system. Adaptor proteins that facilitate the attachment to an antigen core of a cocktail of modular units that perform different functions are attractive for that purpose. A schematic of the modular functions and the vaccine administration routes in which they play a dominant role is shown in Box 1. This modular nanoscale vaccine device is attractive for several reasons: (i) it offers flexibility to target different cells, enabling optimal tissue selection and priming for antigen presentation; (ii) it offers flexibility to deliver a wide variety of clinically important antigens; and (iii) it enables the assembly of different combinations of modules designed to achieve a broad-spectrum, potent vaccine response. Recently reviewed in [17] is a full discussion of the use of biodegradable nanoparticles that target pathogen recognition receptors, and their status in vaccine clinical trials.

DC vaccines for tolerance

In addition to promoting immunity, immature DCs have been shown to induce immune tolerance [19,20] as they are poor immune stimulators [21,22]. For example, rapamycin-treated DCs have been shown to downregulate alloimmune responses both *in vivo* and *in vitro* [21,23]. Nanoparticles targeting DCs may thus be used to promote tolerance for vaccination against autoimmune and alloimmune disease states. Studies have shown that the immunosuppressant rapamycin can be encapsulated within nanoparticles [24], which may ameliorate some of the potential systemic side effects of rapamycin treatment. Rapamycin is a macrolide that potently inhibits translation of eukaryotic capped mRNAs, leading to suppression of responses to growth factors, including T cell responses to interleukin 2 (IL-2) and consequential blockade of T cell activation. Systemic effects of rapamycin include impaired wound healing, whereas possible side effects include thrombocytopenia and hyperlipidemia. Accordingly, focused drug delivery via a nanoparticulate formulation provides an attractive therapeutic route. Recent research has demonstrated that biodegradable nanoparticles encapsulating mycophenolic acid (NP-MPA) were significantly better in prolonging allograft survival compared with a 1000-fold higher dose of the soluble drug. In particular, administration of these particles can induce the upregulation of programmed cell death ligand-1 (PD-L1) on DCs, and the allograft prolonging effects of NP-MPA were, in part, mediated by PD-L1 [25].

The potential of nanoscale devices for targeted immune therapy is the subject of Part II of this article, where the principles of modular design outlined above are applied to the delivery of immunoregulatory cytokines.

Part II: Targeted therapy for autoimmune disease

Treg: the guardians of 'self'

In the adaptive immune system, a fine line between autoimmune self-tolerance versus autoimmune self-destruction has evolved around a master switch termed 'Foxp3' (see Glossary). Foxp3 is a transcription factor essential for differentiation of the regulatory T cell (Treg) lineage of T lymphocytes. Treg function as guardians of 'self', actively suppressing autoimmune attack. A lack of Foxp3 results in a lack of Treg, leading to lethal autoimmune self-destruction in both mice and humans, illustrating their vital role in survival. Autoimmune diseases arise when autoantigen-specific Treg populations become inadequate in protecting their target. The burden of autoimmune diseases is immense, as illustrated in a March 2011 report on the global state of autoimmune diseases (ADs), where it is noted that 'Of the 50 million Americans [USA] living and coping with ADs, more than 75 percent are women. AD is one of the top 10 leading causes of death of women under the age of 65. It encompasses more than 100 diseases, including type-1 diabetes, multiple sclerosis, rheumatoid arthritis, Crohn's disease and lupus. It is responsible for more than $100 billion [USD] in direct health care costs annually.' (see http://www.aarda.org). The report goes on to observe that 'Research programs that seek to find common mechanisms among groups of autoimmune diseases may provide a more reasonable and effective way forward. . .'. We propose that use of targeted nanotherapy to promote Treg function – by harnessing recently discovered regulatory switches – will progress this aim.

Complexity is underpinned by simple switches revealing novel therapeutic targets

The seemingly ever increasing myriad of factors involved in regulation within the immune system are at last revealing core pathways that initiate T cell lineage development. The realization that two lineages – the inflammatory TH17 and the tolerogenic Treg – have common ancestry and are reciprocally regulated opens the way for rational therapeutic approaches to divert lineage progression towards Treg. A wealth of literature, including excellent reviews, exists that describes the molecular and cell biology of both TH17 and Treg cells, and we refer the reader in the first instance to works by Ziegler and Buckner [26] as well as Littman and Rudensky [27]. In this article, we discuss novel aspects of regulation and emphasize the importance of the *in vivo* microenvironment when considering therapy. Here antigen-specific T cell cohorts might be guided towards tolerance by a surrogate therapeutic stroma designed for the

paracrine delivery of regulatory factors. As such, cytokine-loaded nanoparticles provide a simple means to promote the Treg lineage *in vivo*: complex strategies such as viral-mediated delivery or attempts to manipulate endogenous gene expression are not required. Although not discussed here, future scenarios include the converse strategy of developing nanodevices able to break antigen-specific tolerance for treatment of diseases where target-specific Treg afford protection either to pathogens such as *Leishmania* [28] or to cancer cells [29].

Central to therapeutic considerations is functionality *in vivo*, and although defined *in vitro* models are an invaluable source of information, by definition they fail to encompass the *in vivo* microenvironment where extracellular regulatory cues arise. Use of *ex vivo* models more closely represents the *in vivo* state and have revealed novel core regulatory elements including leukemia inhibitory factor (LIF) [30], a stem cell cytokine known to play a role in promoting epigenetic plasticity including access to NANOG [31]. The link between LIF and tolerance was discovered in a full mismatch heart allograft model where only 12 factors proved to be tolerant-specific out of some 30 000 screened. Two were directly linked to LIF, one being LIF itself and the second an E3 ligase, MARCH-7, subsequently shown to regulate protein levels of the LIF receptor (LIF-R) [32–34].

In vivo the supportive niche acts as an important reservoir of growth factors including matrix-bound latent transforming growth factor β (TGFβ), basic fibroblast growth factor, and LIF. In the immobilized form, certain growth factors have enhanced potency [35], an effect that may be recapitulated by nanoparticle-mediated delivery [36]. During T cell activation, incoming signals are qualified by a combination of signal strength [37], cytokines and growth factors, stromal environment, and redox potential [38], all of which influence epigenetic plasticity towards lineage-specific gene expression profiles. Epigenetic DNA modifications are regulated by the TET enzymes [39] (first discovered in lymphocytes [40]), with promoter suppression occurring via cytidine methylation to 5-methyl cytidine (5-MC). Currently, considerable excitement follows the discovery that in addition to repression of promoter activity, derepression may occur, involving 5-MC hydroxylation to 5-hydroxyMC and subsequent demethylation [39], enabling dynamic tuning of specific genes during development. At the histone level unmasking of epigenetically silenced genes can be induced by modifying chromatin structure, as occurs for genes encoding STAT-3 and LIF-R in mouse spleen cells treated with FR901228, a histone deacetylase inhibitor [41]. Transcript profiles are subject to further regulation by microRNAs and a direct link between Treg microRNA profiles and Foxp3 was first described by the Merkenschlager group [42].

Against a background of emerging appreciation of mechanisms involved in epigenetic plasticity, many groups are encouraged to design new treatments for immune-based diseases. We argue that by generating surrogate 'microstroma' targeted to specific cell types or domains *in vivo*, nanotechnology offers a unique opportunity for therapy-mediated recovery of regulatory self-tolerance in autoimmune disease.

Switch mechanisms control TH17 versus Treg

The TH17 lineage plays an important role at epithelial/mucosal barriers by preventing crossing of pathogens. However, when inappropriately activated, TH17 immunity exacerbates autoimmune inflammatory destruction of tissues; this destruction can be seen in several diseases, including inflammatory bowel disease, rheumatoid arthritis, psoriasis, multiple sclerosis, and juvenile diabetes [43]. TH17 activity represents a major barrier to bone marrow transplantation [44]. Treg oppose the action of TH17 cells and, as previously introduced, the transcription factor Foxp3 orchestrates and maintains the Treg cell lineage [26,27]. It is understood that the lineage-specific transcription factor for TH17 is RORγt, although it is important to note that TH17 also expresses high levels of RORα, which contributes to maintenance of the TH17 genotype (RORα and RORγt are splice variants of the *RORA* gene). Neither Foxp3 nor RORγt are mutually exclusive over the other in the presence of the activated form of TGFβ, although in the additional presence of IL-6, RORγt becomes dominant [26]. Two recent reports identify seemingly different molecular switch mechanisms for Treg versus TH17 fate determination. We propose that, in fact, the two are intrinsically coupled, as presented below and in Box 3.

(i) The metabolic switch via HIF-1α

Initially quiescent, the naive T cell becomes dramatically altered upon activation when new bioenergetic demands arise during massive clonal expansion. These demands result in a metabolic switch to glycolysis and increased anabolic activity. The redox responsive transcription factor HIF-1α is induced and Shi *et al.* [38] have shown that HIF-1α plays a direct role in TH17 versus Treg fate determination. Accordingly, HIF-1α levels provide a lineage-specific metabolic checkpoint: blockade of glycolysis, or lack of HIF-1α, inhibits TH17 development and promotes generation of Treg. Because induction of HIF-1α requires signaling through mTOR (mammalian target of rapamycin), and because mTOR is a central regulator of metabolism that is sensitive to inhibition by rapamycin, these new findings help explain why rapamycin – an immunosuppressive macrolide in wide clinical use for organ transplantation – may promote differentiation of the Treg lineage [45].

(ii) The pivotal switch via LIF versus IL-6

Activation of $CD4^+$ T cells also results in early release of LIF and induction of cell surface LIF-R, irrespective of eventual lineage outcome, and it has been proposed that activated naive $CD4^+$ T cells enter an early, relatively undifferentiated state linked to LIF signaling [46]. Indeed, it has been further argued that Treg persist in this undifferentiated state by continued LIF activity that is unique to Treg and is linked to Foxp3 expression [47].

Both LIF and IL-6 signal through the STAT-3 pathway and the recent finding that IL-6 is a microenvironmental cue for TH17 development [48] was at odds with the earlier report that LIF is linked to transplantation tolerance [30]. This apparent discrepancy led to a head-to-head comparison of LIF versus IL-6 in CD4 T cell activation with the discovery of a previously unrecognized molecular mechanism of

Box 3. Model of LIF-R/IL-6-R competition at the Treg/Th17 axis

In naive CD4$^+$ T cells, activation through the T cell receptor (TCR) initiates a series of stages leading to lineage differentiation towards either Treg or TH17 cells (Figure Ia). Progressive stages of the model are outlined below, together with options for therapeutic modulation.

1. Initial strength of the activation signal qualifies outcome, with suboptimal activation biasing differentiation towards Treg development [37].

Therapy: reduce primary TCR signal by, for example, inducing activation in the presence of non-depleting anti-CD4.

2. Early release of LIF and expression of both gp130 and gp190 at the T cell surface [34] results in the cell becoming receptive to LIF.

Therapy: create a niche of immobilized LIF at T cell surface using nano-LIF targeted to CD4.

3. A phase of massive proliferation where cell cycling is associated with progressive epigenetic changes in DNA and chromatin. The cells become responsive to IL-6. A 'metabolic switch' occurs where metabolic demand results in glycolysis and induction of HIF-1α, with the presence of HIF-1α favoring differentiation towards TH17 cells [38].

Therapy: treat with rapamycin to inhibit glycolysis as well as nano-LIF targeted to CD4 to oppose IL-6.

4. 'LIF/IL-6 switch': there is a crucial intrinsic link between the LIF/IL-6 switch and the metabolic (IL-6/HIF-1α) switch, where LIF-R gp190 expression is pivotal. This is outlined in more detail in Figure I panels (b) and (c), where schematics of a LIF/LIF-R autocrine loop and the IL-6/IL-6-R autocrine loop (low gp190) are shown. The ratio of b:c determine Treg/TH17 outcome as follows. (b) The Treg lineage progresses if LIF signaling predominates with induction of Foxp3. HIF-1α and RORα are repressed, and gp190 transcription and protein levels are maintained, perpetuating the LIF signaling pathway. Epigenetic remodeling establishes stable expression of Foxp3, LIF and gp190 in response to subsequent stimulation, creating a feedback loop for endogenous tolerance to the specific target antigen [58]. (c) The TH17 lineage progresses if an IL-6/STAT-3/HIF-1α autocrine loop becomes established. gp190 transcription is suppressed by HIF-1α, whereas MARCH-7 transcription is greatly amplified via RORα binding to the MARCH-7 promoter. The outcome is loss of gp190 transcripts (HIF-1α-mediated) as well as degradation of gp190 protein by MARCH-7.

Therapy: nano-LIF targeted to CD4 to oppose IL-6 signaling as well as to promote stable LIF signaling and Foxp3 expression.

Therapy: develop agents to modulate MARCH-7.

Approach to break tolerance: create an IL-6-rich niche targeted to CD4 using nano-IL-6 to break Treg-induced tolerance, as in the case of tolerance to cancer cells or to Leishmania.

Autoimmune disease

In autoimmune disease, it is envisaged that natural population dynamics will provide opportunities to guide new recruits of T cells towards Treg as they mature through stages of plasticity. The improved clinical outcome of patients with multiple sclerosis (MS) following lymphodepletion and homeostatic recovery with emergence of MBP-reactive Treg [57] is proof-of-concept that self-tolerance can be regained and becomes stable in patients suffering from autoimmune disease, and a role for LIF is now confirmed [55,56].

Therapy: lymphodepletion followed by nano-LIF targeted to CD4.

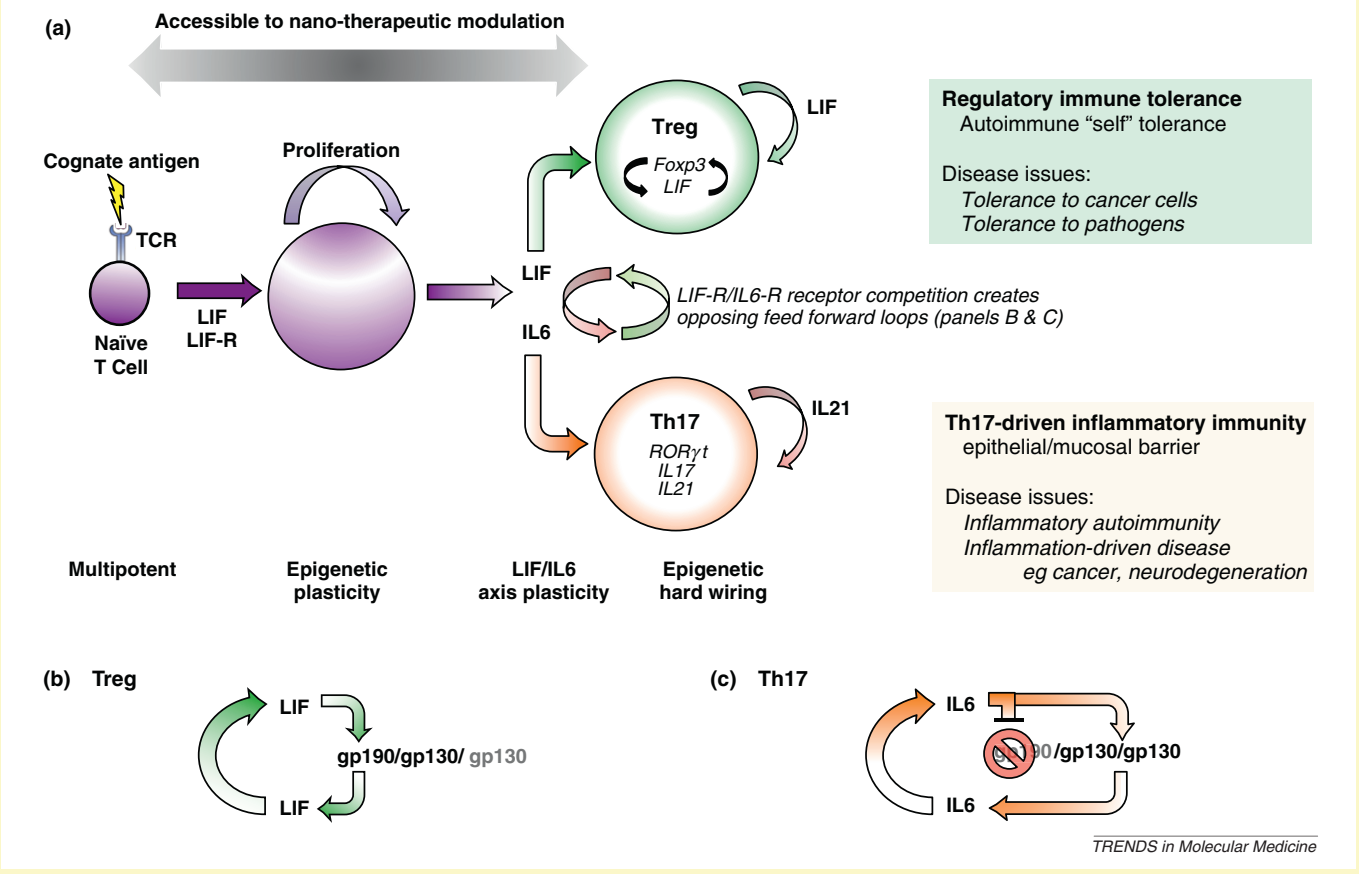

Figure I. Panels **(a–c)** illustrate the axis driving differentiation of T cells towards either the regulatory T cell (Treg) or Th17 lineage.

opposing feed-forward loops for Treg (LIF) versus TH17 (IL-6) lineage progression [32]. Notably, each cytokine – LIF or IL-6 – actively suppressed the effects of the other, indicating a fundamental pivotal switch.

The mechanism of the LIF/IL-6 axis appears to involve gp190, which is the LIF-specific receptor subunit of the LIF-R heterodimer gp190/gp130. IL-6 was discovered to inhibit transcription of gp190 [32], thereby (i) suppressing

LIF-R levels to inhibit LIF-signaling and (ii) promoting a proportional increase in IL-6-R expression (gp130/gp130 homodimers) within the membrane. Thus, Treg/TH17 lineage outcome is directly qualified by the levels of LIF-R versus IL-6-R on the T cell surface.

Two switches or one? A unifying role for gp190

Given the evidence of two switches for Treg/TH17 lineage determination, we asked if there is there any link between the IL-6/HIF-1α axis and the LIF/IL-6 axis? Literature searches revealed that in mouse embryonic stem cells, HIF-1α represses gp190 transcription by binding to the hypoxia response element located in the *gp190* gene promoter [49]. This raises the possibility that IL-6-mediated repression of gp190 in T cells is via HIF-1α. Such a model requires a direct link between IL-6 and HIF-1α, and indeed such a link is already known in glioblastoma cells where an IL-6/STAT-3/HIF-1α autocrine loop occurs [50] and is suggested for T cells, as IL-6 was required for the metabolic induction of HIF-1α [38]. Thus, HIF-1α provides a hypothetical mechanism by which IL-6 opposes LIF in the LIF/IL-6 axis that determines Treg versus Th17 cell fate.

A link to this unifying hypothesis that is centered on gp190 lies with the E3-ligase MARCH-7, which is able to degrade gp190 protein [32]. IL-6 causes induction of MARCH-7 transcripts in T cells, and importantly the promoter of the *MARCH-7* gene has two RORα binding sites. Additionally, RORα is induced by IL-6. Thus, a further compounding mechanism by which IL-6 opposes LIF is via degradation of gp190 protein following increased MARCH-7 E3-ligase activity. In either situation – suppression of gp190 transcription by HIF-1α or degradation of gp190 protein by MARCH-7 – a LIF/LIF-R autocrine loop is able to oppose the HIF-1α/IL-6 autocrine loop. Overall, there is a strong case for gp190 being pivotal to the mechanism by which LIF, gp190, IL-6, and HIF-1α coordinate in the regulation of Treg/TH17 lineage maturation (see Figure I in Box 3).

Epigenetic plasticity is permissive for stable recovery of self-tolerance

Early attempts to publish the opposing functions of LIF and IL-6 in T cells suffered from the assumption that both cytokines would have similar effects as they are both members of the same cytokine family and both activate the STAT-3 pathway. This assumption not only failed to appreciate the option of LIF signaling through the phosphoinositol-3-kinase pathway but also ignored the fundamental role of epigenetic regulation: owing to lineage-specific epigenetic profiles, LIF-driven STAT-3 response genes (Treg) differ from IL-6-driven STAT-3 response genes (TH17). Epigenetic plasticity in T cell lineage development, where inheritable changes can be guided by therapy, is permissive for maturation of stable populations of endogenous autoantigen-specific Treg.

Notably, LIF supports tissue repair at physiological doses, being a naturally occurring cytokine that plays a major role in tissue homeostasis by supporting tissue stem cells and progenitor cells. Targeted delivery of LIF is of huge value in this respect, compounding the value of promoting Treg-mediated immune tolerance. In marked contrast, although the drug rapamycin also promotes Treg, it acts to suppress growth factor-driven signaling pathways and thus inhibits endogenous reparative ability within tissues and will impede engraftment of cell and tissue grafts.

Multiple sclerosis (MS): a role for Treg

Within the nervous system both TH17 cells and the cytokine IL-6 are pathogenic and linked to autoimmune and degenerative diseases. Modeling of cumulative genetic risk for MS in a genome-wide association analysis revealed IL-6 as the sixth highest risk factor [51]. In marked contrast to IL-6, LIF is a neuropoietic cytokine for neural stem and precursor cells [52], and plays key roles in neuroprotection, axonal regeneration, and prevention of demyelination. The therapeutic potential of targeting LIF in MS has been discussed in two recent reviews [53,54]. This is confirmed in a study by Cao *et al*. [55] who discovered that therapeutic stem cell transplantation for MS can be replaced by LIF therapy. This study has been discussed in light of earlier work showing that LIF opposes IL-6-driven TH17 lineage differentiation [56]; notably, experimental design issues prevented Cao *et al.* observing LIF-mediated induction of Treg, their test model being already fully polarized to Treg and thus insensitive to further effect of LIF.

In the clinic, endogenous repair mechanisms mediated by Treg have been identified as part of a partial cure for MS [57]. Following therapeutic depletion of mature lymphocytes using alemtuzumab (CamPath1-H) sustained clinical benefit was found. By contrast, matched patients treated with interferon β-1 suffered a steadily progressive disease. Endogenous repair was linked to homeostatic recovery of a T cell population responsive to myelin basic protein that releases neuropoietic cytokines when stimulated. These patient-derived reparative T cells are thought to be of the Treg lineage and directly involved in the endogenous cure.

Nanoparticulate LIF as a therapeutic

The evidence described above points to LIF as underpinning self-tolerance. Yet, as a therapeutic, exploitation of LIF has been dogged by the major problem that soluble LIF is rapidly degraded by serum proteases. The option of viral-mediated delivery is unattractive owing to patient exposure to viral vector or foreign protein, in addition to the risk of uncontrolled, high doses of LIF which are toxic. To overcome these issues, nanoparticulate, or 'nano-LIF', has been developed [36] (see Figure Id in Box 2). Nano-LIF proved to be efficacious in promoting Treg in experimental models [32,36] enabling exploitation of LIF to harness the recently discovered molecular switches in the Treg lineage.

Concluding remarks

We have presented two cases for nanotechnology uniquely enabling therapies targeting the immune system. The bioengineering approach to modular vaccine constructs is well advanced, with a wide variety of encapsulated cargos in vehicles constructed as adjuvants and decorated with targeting antibody to mimic pathogenic infection and elicit aggressive inflammatory immunity plus immune memory. Given the tiered range of modules for construction,

Box 4. Outstanding questions and future perspectives

General

- *In vivo* trafficking of nanodevices must be studied and optimized to provide efficacious delivery. Mapping kinetic distribution profiles following delivery via various routes including intravenous, intraperitoneal, intranasal, intramuscular, and subcutaneous. Here, homing and tissue distribution will be qualified by particle composition.
- Safe means of access to specific structures, such as joints or the central nervous system, needs to be determined.

Nanoconstructs as vaccines

- Nanodevice vaccines must be constructed to ensure that when eliciting an aggressive immune response to a pathogen, only the local microenvironment is subject to endogenous cytokine release to avoid systemic inflammatory toxicity.
- Is repeated vaccination efficacious?
- Nanodevice vaccines must be constructed to avoid depot accumulation of inflammatory nanodevices, so as not to jeopardize self-tolerance.

Nano-LIF therapy for autoimmune disease

- It will be important to ensure that induced Treg are specific for autoimmune antigens.
- Can a primed TH17 response become suppressed by Treg?
- Can particles be designed to cross the blood–brain barrier?
- Does lymphodepletion combined with nano-LIF therapy reverse autoimmunity and promote repair?

optimization will ultimately lead to novel vaccine devices tailored to specific pathogens that are safe, robust, and standardized for dose.

Our second example focuses on nanodevices capable of regulating immune tolerance. The proven principle of access to genes encoding lineage-specific transcription factors afforded by nanoparticle-mediated delivery of extracellular cytokines [32,36] is of wide importance within the advancement of medical devices. Furthermore, because LIF is the pivotal cytokine for Treg differentiation and the nano-LIF device carries intrinsic reparative and regenerative properties beneficial to many cell types, the therapeutic benefits in the treatment of autoimmune diseases will be compounded [57]. Of more immediate clinical relevance, the nano-LIF device has great potential as a topical agent for psoriasis and as an *ex vivo* treatment to favor engraftment in regenerative cell therapies, such as in transplantation of bone marrow, pancreatic islets, and dopaminergic cells.

Transient where appropriate, exquisitely specific, and having the ability to provide a temporary stromal-like microenvironment, targeted nanotherapy carries promise of a paradigm shift in the future of immune-based medicine (Box 4).

Conflict of interest

The authors have no conflict of interest.

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