IMMUNOENGINEERING

Engineering Approaches to Immunotherapy

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As the science of immunology grows increasingly mechanistic, motivation for developing quantitative, design-based engineering approaches has also evolved, both for therapeutic interventions and for elucidating immunological pathways in human disease. This has seeded the nascent field of "immunoengineering," which seeks to apply engineering analyses and design approaches to problems in translational immunology. For example, cell engineers are creating ways to tailor and use immune cells as living therapeutics; protein engineers are devising new methods of rapid antibody discovery; biomaterials scientists are guiding vaccine delivery and immune-cell activation with novel constructs; and systems immunologists are deciphering the evolution and maintenance of T and B cell receptor repertoires, which could help guide vaccine design. The field is multidisciplinary and collaborative, with engineers and immunologists working together to better understand and treat disease. We discuss the scientific progress in this young, yet rapidly evolving research area, which has yielded numerous start-up companies that are betting on impact in clinical and commercial translation in the near future.

IMMUNOLOGY, MEET ENGINEERING

Bioengineering has, for many years, involved at least some consideration of immunology, as the therapeutic success of drug delivery vehicles, synthetic implants, and engineered tissues has required the development of strategies to avoid immune detection or rejection. For example, polymers comprising poly(ethylene glycol) (PEG), to which proteins adsorb poorly (*1*), have been used to coat implant surfaces (*1*) and cells (*2*) and create biomaterial matrices (*3*) that substantially escape immune detection. More recently, however, growing numbers of bioengineers are shifting focus toward immunotherapy and embracing a broad range of immunology-related problems. This has followed the recent explosion in immunology discoveries, including the identification and characterization of antigens in numerous chronic and autoimmune diseases, bringing closer the seemingly limitless therapeutic potential of antigen-specific immunotherapy to treat chronic viral diseases, autoimmunity, allergy, and cancer.

Thus, the nascent field of immunoengineering aims to provide new approaches to our understanding, application, and therapeutic manipulation of immunology. Broadly speaking, immunoengineering is generally more synthetic, quantitative, and translationally oriented, applying engineering analysis toward goals of developing new molecular and cellular immunotherapies—for example, with biomaterial-based nanoparticle vaccines, in situ cell surface engineering strategies, and antibody engineering. Additionally, immunoengineering contributes to basic immunology, including system-level descriptions of immune responses and evolutionary biology of T cell receptor (TCR) and B cell receptor repertoires. Although most translational strategies are still in preclinical stages, the potential impact of engineering on the future of immunotherapy is enormous. Engineers are equipped with analytical tools to describe complex dynamic systems; to quantify, control, and optimize outcomes; to develop synthetic mimics; and to solve practical and technical problems—all of which will be needed to facilitate translation on a broad scale. Furthermore, as trends continue toward protein and peptide drugs, pharmaceutical companies will require more immunology expertise in their engineers and chemists. Indeed, several undergraduate and graduate bioengineering programs have injected more immunology training into their curricula to meet the anticipated future needs of pharmaceutical and biotechnology industries. (It is likely that these curricula, at the same time, are helping to define those future needs.)

This review highlights some of the emergent and more translational areas of immunoengineering, although most research projects are still in preclinical, experimental stages. This review does not address the exciting but well-developed and very broad topic of antibody engineering; instead, we refer the reader to several excellent reviews (*4*–*7*).

NANOMATERIALS IN VACCINES

Subunit vaccines consist of recombinant and synthetic components designed and formulated to induce adaptive (antigen-specific) immune responses. These biosynthetic alternatives to inactivated pathogens aim to provide better safety and efficacy profiles, greater ease of manufacture, and favorable storage conditions. Rapid progress is being made with subunit vaccines against important pathogens, for example, for protection against hepatitis B virus (HBV) and human papilloma virus (HPV) in the clinic (with the approved vaccines Fendrix and Gardasil, respectively) and tuberculosis in a guinea pig model (*8*). Still, further technological breakthroughs are needed to improve cellular immune responses, especially for therapeutic vaccination in the case of cancers and chronic viral diseases such as HBV and HPV in infected patients, where current vaccine technologies are ineffective. Moreover, engineering technologies may open up new routes of vaccine administration, such as needle-free intradermal administration as well as mucosal administration, the latter to induce protective immunity at the surfaces of the nose, lung, vagina, and rectum. Central to these approaches are synthetic nanomaterials, which are being developed to carry antigen to anatomical and subcellular locations and to co-deliver biomolecular adjuvants (Table 1).

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Delivering antigen to anatomical and subcellular locations

Recently, immunoengineers have begun developing nanomaterials to deliver and potentiate the action of antigen as well as adjuvant molecules (discussed below). Nanomaterials serve to deliver antigen to antigen-presenting cells (APCs) in target tissues (Fig. 1), to direct antigen to particular subcellular compartments within APCs, and to co-deliver adjuvant molecules to the same APC populations (*9*). Promising synthetic nanomaterial designs and functions are illustrated in Fig. 2. These materials offer special features of size, for penetration of interstitial and mucosal barriers to access APCs most efficiently, and release of their antigen payload after endocytosis, targeted to particular subcellular compartments. How the materials' designs yield these functions is elucidated below and exemplified in Table 1.

One level of vaccine delivery is to the optimal tissue and cellular target (Fig. 1, A to C); here, nanomaterials are making great progress. Antigens in the periphery, for example, after intradermal injection, can be taken up by patrolling dendritic cells (DCs), which may or may not become activated by danger signals as they travel to the lymph node via lymphatic vessels (Fig. 1, A and B). Delivered antigens or nanomaterials, depending on size, can also be targeted directly to lymphatics. Upon injection, sufficiently small particles (<~100 nm, depending on tissue and injection pressure) are carried by the interstitial flow that exists between the blood lymphatic capillaries with velocities of roughly 0.1 to 1 μm/s (or much higher immediately after injection) through the tissue's network architecture into the draining lymphatics, where they can be collected by APCs in the lymph nodes with remarkable efficiency (*10*, *11*) (Fig. 1C). In mucosal tissues, lipid antigens drain exclusively into lymphatic vessels.

Upon reaching the lymph node (Fig. 1C), free antigens or nanomaterials proportionally target different cells depending on size and opsonization (*12*), as well as on other features that remain poorly understood. Some lymph-borne solutes will bypass the lymph node through the subcapsular sinus (SCS), entering the blood circulation where they accumulate primarily in the liver and spleen—tissues that are also targeted when nanomaterials are administered intravenously. Some materials, particularly larger (>70 kD) or opsonized antigens, are taken up by subcapsular macrophages that can transfer the materials to nearby DCs (Fig. 1C). Smaller antigens flow through conduits that allow them to be directly sampled by B cells and functionally immature APCs, which can then interact with naïve and regulatory T (T_{reg}) cells to initiate adaptive immune responses or maintain peripheral tolerance (Fig. 1D). Nanomaterials have also been designed to target B cell germinal centers using a surface-bound antigen that binds and clusters the B cell receptor, thus inducing strong humoral immune responses (*13*) (Fig. 1E).

In addition to the lymph nodes, targeting nanomaterials to mucosal surfaces, such as the nasal cavity and the lungs, offers particular promise to induce mucosal immunity for protection against pathogens transmitted via the mucosae (for example, orally, respiratory, or sexually). Nanomaterials can be targeted to lymphoid tissues in the nasal cavity (*14*) and by APCs in the lung parenchyma (*15*) to induce potent humoral and cellular immunity and therefore to protect against viruses, such as influenza (*15*), or bacteria, such as tuberculosis (*16*) (Fig. 1F). To date, to our knowledge, such studies with nanomaterials have only been performed in mouse models.

Nanomaterials can even target antigen to particular subcellular locations (*9*). Classically, extracellular antigens are collected by APCs

Table 1. Examples of material design concepts being developed at the preclinical stage for vaccination.

Target tissue	Desired functionality	Material implementation	References
		Nanomaterials	
Draining lymph nodes and mucosal lymphoid tissues	Material homing to APCs	Nanomaterials (<100 nm) that can penetrate tissue barriers after intradermal or intratracheal administration; antigen may be conjugated to the surface or entrapped inside	(*10*, *11*, *14*–*16*, *18*)
Mucosal surfaces	Sustained mucosal release of antigen	Cationic nanomaterials that bind to mucus	(*27*)
Subcellular	Harnessing cross-presentation pathways via rapid endosomal degradation	Reduction-sensitive linkages between antigen and nanomaterial	(*19*)
		Lipase-sensitive encapsulation of antigen within nanomaterial	(*18*)
Subcellular	Direct cross-presentation via cytoplasmic access	pH-sensitive materials that destabilize the endosomal membrane	(*20*)
		Vesicle-forming block copolymers that destabilize by oxidative hydrophilization of the hydrophobic block	(*21*)
		Scaffolds	
Within tissues	Prolonged tissue presence; APC homing to the material	Poly(lactide-*co*-glycolide) (PLGA) scaffolds as depots embedded with inflammatory cytokines and adjuvant molecules; antigen is entrapped and released over time	(*33*, *34*)

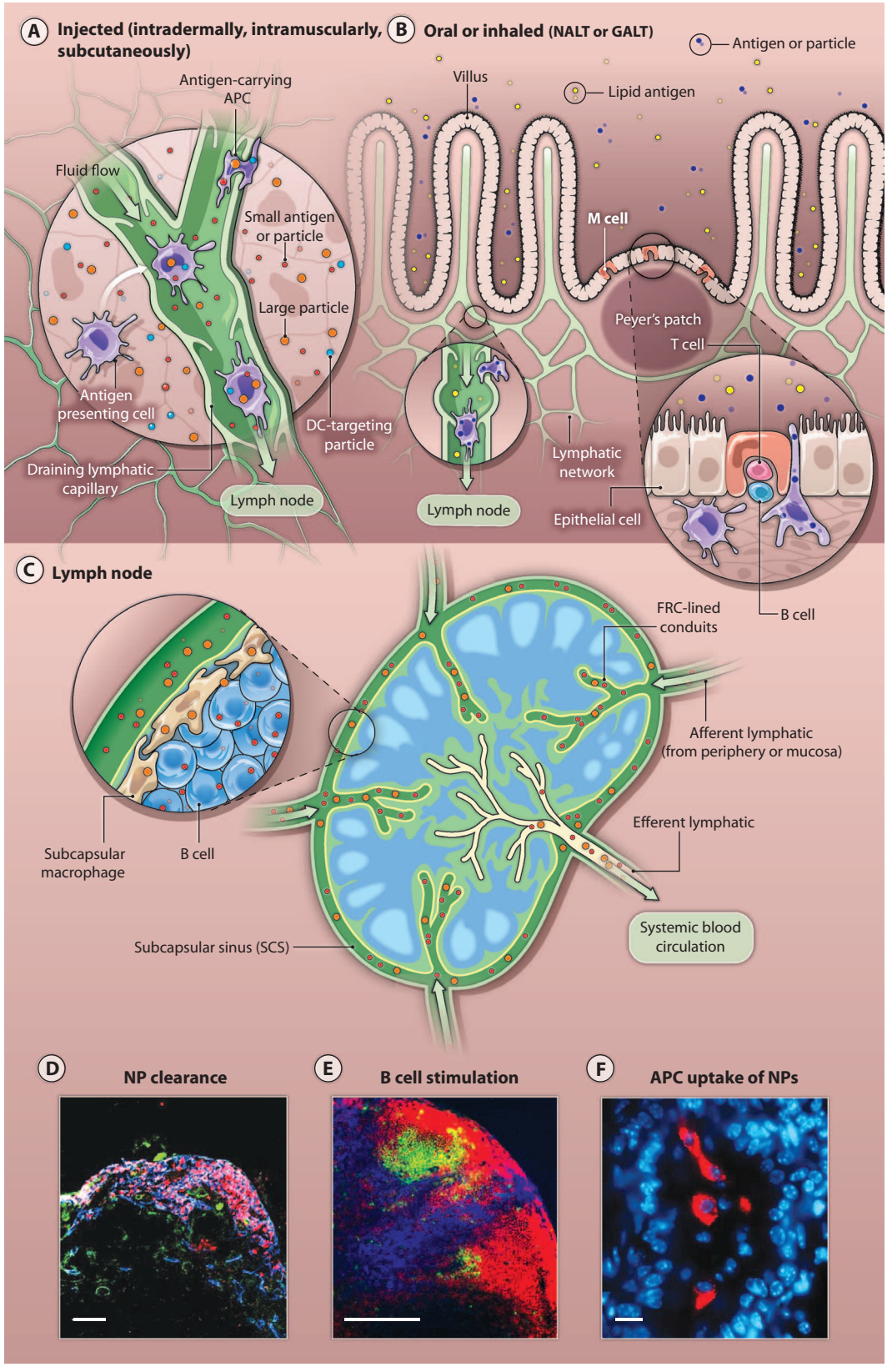

Fig. 1. Tissue and cell targeting for antigen delivery in nanoparticulate subunit vaccines. (**A**) Nanomaterials injected into most tissues flow into draining lymphatic capillaries or are taken up by tissue-resident APCs. Small particles (red, <100 nm) more easily penetrate the extracellular matrix and thus distribute more broadly than larger particles (orange), which are retained more in the tissue. Interstitial APCs can be exclusively targeted by particles that are >500 nm or decorated with targeting antibodies (blue). Upon entering lymphatic vessels, cells and antigens travel to the draining lymph node. (**B**) Inhaled or consumed antigens or nanomaterials mostly target the nasal- or gastrointestinal-associated lymphoid tissue (NALT or GALT, respectively), which include Peyer's patches. Because of the tight epithelial barrier, most antigens and particulates are taken up by immune cells, where they then traffic to the lymph node. Ingested lipids and lipid antigens are taken up directly into the lymphatics, or lacteals, contained in the villi of the small intestine. (**C**) Antigens, particulates, and immune cells from the afferent lymphatics encircle the lymph node in the SCS and enter different areas according mainly to size. Subcapsular macrophages take up large particles (>7 nm) or opsonized antigens (orange), whereas smaller antigens flow into conduits (red). Some of the lymph-borne particles will flow into efferent lymphatics and eventually enter the systemic blood circulation. FRC, fibroblastic reticular cell. (**D**) Polymeric nanoparticles (<50 nm, red) are rapidly cleared after intradermal injection and drain into the lymph node, where they are taken up by APCs surrounding the lymphatic endothelium (blue) and T cell stroma (green). Scale bar, 200 μm. (**E**) Multilamellar lipid vesicles (180 nm, blue) were drained into the lymph node, after which germinal centers (green) formed nearby, generating B cells (red) specific to the antigen borne by the vesicles. Scale bar, 200 μm. Reproduced from (*13*), with permission. (**F**) APCs collect polymer nanoparticles (<50 nm, red) in the airways (lung parenchyma, blue) and migrate to the pulmonary lymph node. Scale bar, 10 μm.

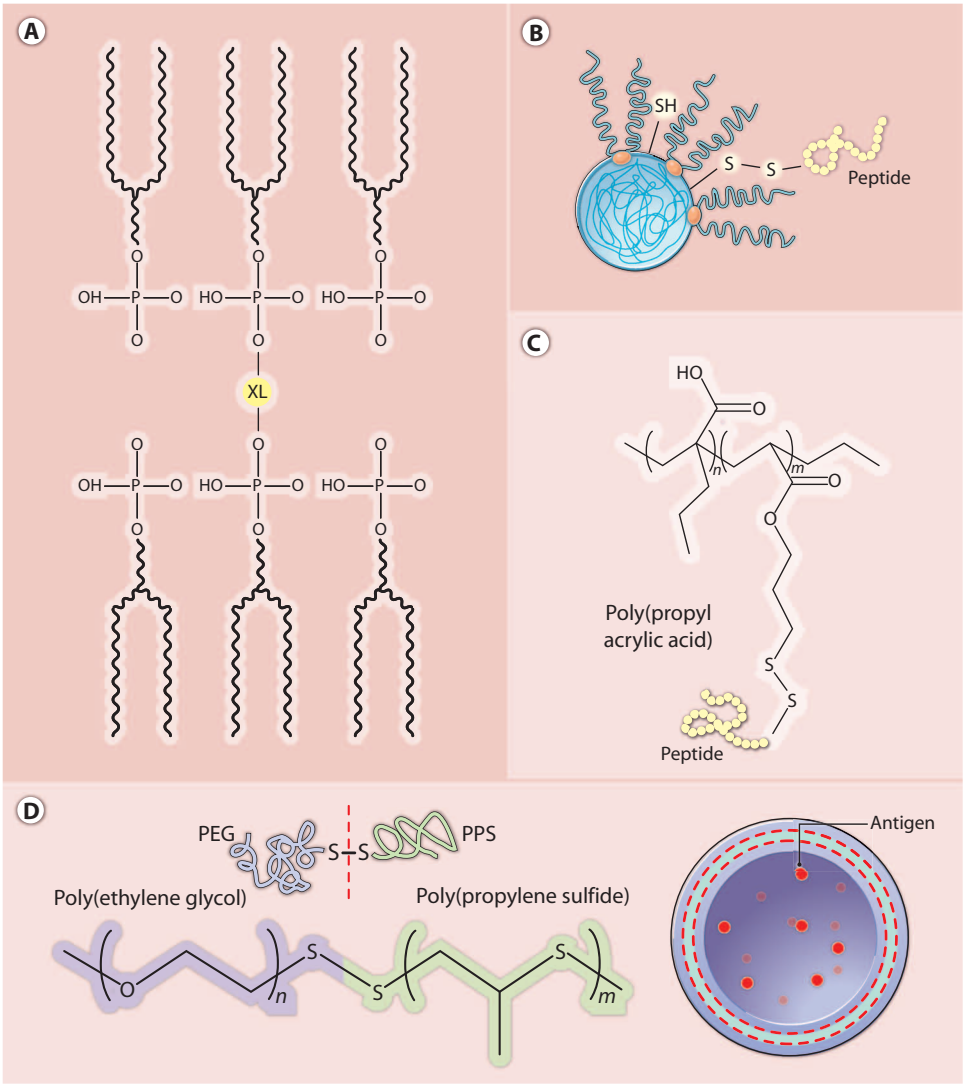

Fig. 2. Nanomaterial design strategies for intracellular targeting. (**A**) In the multilamellar lipid vesicles of Fig. 1E, a phospholipid (shown as wavy lines) with a cross-linkable (XL) domain is used to "staple" the lamellae together. Degradation of the phospholipid by intracellular lipases releases the antigen within or upon the vesicle only after endocytosis, providing antigen cross-presentation. (**B**) In the polymer nanoparticles of Fig. 1, D and F, antigen (peptide) is conjugated to the nanoparticle (blue) surface via disulfide bonds. Release by reduction occurs only after endocytosis, providing antigen cross-presentation. A steric stabilization layer of PEG (green) prevents nanoparticle aggregation. (**C**) Poly(propyl acrylic acid) (indicated by "*n*") destabilizes the endosomal membrane in a pH-dependent manner, such that acidification during endosomal maturation triggers endosomal disruption, releasing antigen directly in the cytoplasm after it is released from the polymer (indicated by "*m*") by disulfide reduction. (**D**) Block copolymers can form vesicle mesophases. When the two blocks are joined by a disulfide bond, antigen payload is released only after endocytosis, where reduction destabilizes the vesicles.

and presented on major histocompatibility complex class II (MHC II) molecules to activate $CD4^+$ T cells and to induce humoral immunity (antibody production). However, for protection and even therapy against many viral diseases and cancer, cellular immunity is also required. Intracellular antigens (from viruses or intracellular bacteria) are normally processed from the cytoplasm for presentation on MHC class I (MHC I) molecules, where they can activate $CD8^+$ T cells to proliferate and serve as cytotoxic T lymphocytes (CTLs) (*17*). Additionally, extracellular antigen can be processed from the phagosome shortly after uptake to enter the pathway for MHC I presentation (referred to as cross-presentation) (*9*, *17*).

Nanomaterials have been engineered to exploit this pathway for vaccination by enabling rapid release of antigen, only after endocytosis, into the phagosomal compartment. In one approach, multilamellar liposomal nanoparticles released their antigen owing to the enzymatic activity of phagosomal phospholipases (Fig. 2A) (*18*). Thus, these particles are relatively stable in the extracellular milieu, but are rapidly degraded to release their protein antigen payload encapsulated within the watery core of the lipid-based nanoparticle after endocytosis. In another example, peptide antigens conjugated to the nanoparticle surface by a disulfide bond were rapidly released in response to the reductive environment they encountered within the phagosome (Fig. 2B) (*19*). Both of these nanomaterial-based approaches (*13*, *18*) enhanced antigen cross-presentation and consequent generation of antigen-specific $CD8^+$ T cells compared to vaccination with soluble antigen.

Nanomaterials have also been engineered to "short circuit" the cross-presentation pathways, responding to phagosomal conditions, such as acidification with a polymer that becomes hydrophobic and thus membrane-disruptive at endosomal pH, in addition to releasing its antigen by reduction (Fig. 2C) (*20*), or reduction with a vesicle-forming block copolymer that itself is disrupted by reduction to release its contents and subsequently osmotically disrupt the endosome (Fig. 2D) (*21*). Through these approaches, the antigen payload may be delivered directly to the cytoplasm, where it can be processed as though it were of viral origin. With such responsive polymers, $CD8^+$ T cell generation was also greatly potentiated by more than an order of magnitude (*20*).

Delivering adjuvants

In subunit vaccines, recombinant antigens are formulated with adjuvant molecules that mimic pathogen-associated molecules, including agonists for the Toll-like receptors (TLRs), to which the body's innate immune system has evolved recognition. In addition to efficiently delivering antigens, nanomaterials can target adjuvant molecules to APCs. For example, the hydrophobic TLR4 agonist monophosphoryl lipid A (MPLA) was incorporated into the hydrophobic leaflets of multilamellar liposomes (*18*). MPLA, when co-incorporated into degradable poly(lactic-*co*-glycolic acid) (PLGA) particles with the TLR7

agonist R837, prolonged antibody responses and protection against H1N1 influenza in nonhuman primates compared to responses with either adjuvant molecule delivered independently (*22*). Also, sustained release of poly(inosinic:cytidylic acid), a TLR3 agonist, from PLGA micro- and nanoparticles directly injected in the lymph nodes (thus, the nano-dimension is less important) demonstrated more powerful cellular immune responses compared to those obtained with soluble poly(inosinic:cytidylic acid) when vaccinating against the model antigen ovalbumin in mice (*23*).

In addition to mimicking pathogenic signals using TLR agonists, nanomaterials have been engineered to mimic endogenous signals such as the granules that are secreted by mast cells during an inflammatory response (*21*). In this example, St. John *et al.* complexed polyanions (for example, heparin) and polycations (for example, chitosan) to form a particle encapsulating tumor necrosis factor, for DC activation, and interleukin-12 (IL-12), to bias $CD4^+$ T cell responses along a T helper 1 (T_H1) direction (*24*). When used as a vaccine adjuvant, these particles enhanced the adaptive immune response and increased survival of mice on a lethal challenge.

Lastly, some materials may themselves function as an adjuvant—in other words, generate potent immunity in the absence of additional biomolecular adjuvants. For example, self-assembling peptides have been engineered to assemble in long, unbranched β sheet nanofibrils. The peptides can be designed to display antigenic peptides on the surface of the fibrils, which have been used to generate potent humoral immunity in mouse models, even in the absence of additional biomolecular adjuvants (*25*, *26*). It is unclear if these nanofibrillar materials remain long-term in the tissue interstitium as a depot, or if they are trafficked to the draining lymph nodes independently or within APCs that phagocytose them. Next-generation approaches are needed to present larger protein domains that assume biological structure that can be recognized by B cell receptors. Such recognition will induce antibodies capable of neutralizing pathogens. Nevertheless, this initial work demonstrates the power of these nanomaterials to induce potent humoral responses.

A final example of nanoparticulate materials that do not need exogenous adjuvants is cationic nanoparticles. These positively charged particles have been developed to serve as a depot to the negatively charged mucus that coats tissue surfaces, thus creating a reservoir effect. Toward a nasal vaccine for *Clostridium botulinum*, for instance, such cationic nanoparticles have been able to release antigens and induce strong neutralizing antibody responses (*27*).

Targeting suppressors of vaccine responses

APCs are not the only cellular targets for nanomaterial-formulated adjuvants. In cancer, a myelomonocytic population, referred to as myeloid-derived suppressor cells (MDSCs), inhibits T cell activation and effector functions, suppressing antitumor immunity and thwarting cancer vaccine efficacy (*28*, *29*). Very small size proteoliposomes (VSSPs) derived from outer membrane vesicles of *Neisseria meningitidis* and containing the immune-potentiating proteolipid GM3 are in preclinical development as a cancer vaccine platform (*30*). Studies in mice have shown that these nanomaterials target MDSCs to induce their differentiation into mature APCs, rescinding their suppressive functions and thus blocking their activities in inhibiting vaccine responses (*31*). Although these VSSPs are biologically derived, it is conceivable to obtain analogous function with designed synthetic nanomaterials, which may present manufacturing advantages. Lipid nanomaterials have been developed to effectively deliver small interfering RNA to monocytes in the spleen and liver of mice (*32*), suggesting the possibility of suppressing the inhibitory activity of MDSCs resident in the spleens of tumor-bearing subjects, as well as in the tumors and draining lymph nodes.

Thus, in subunit vaccines, new developments in nanomaterials are leading to new ways to target particular cell populations, such as those resident in lymph nodes and in the mucosae, as well as to target particular subcellular compartments within those cells (to enable efficient cross-presentation or to bypass it altogether). As such, synthetic nanomaterials are promising more protective mucosal immunity and more potent cellular immune responses than antigens alone. At present, most of these exciting synthetic nanomaterials remain in preclinical development stages. The most notable synthetic nanomaterial to have entered vaccine clinical trials is based on nanoparticles composed of PLGA, because of the long history of clinical use of this polymer in drug delivery and surgical applications. With co-formulated antigen and biomolecular adjuvant, these PLGA-based nanoparticles are in clinical testing in generation of humoral responses to neutralize nicotine as an aid to smoking cessation in trials being carried out by Selecta Biosciences (ClinicalTrials.gov identifier: NCT01478893).

The burden for proof of safety of engineered nanomaterials as antigen or adjuvant carriers depends on the application. In the case of prophylactic vaccination, in which vast numbers of children would be treated, the bar for safety is extremely high. In therapeutic vaccination to treat patients with life-threatening chronic viral diseases or cancers (for which, in both fields, no examples of peptide- or protein-based subunit vaccines exist yet), the pathway for introducing new synthetic nanomaterial vaccine technologies may be clearer. Thus, many companies focus their new technology development efforts toward therapeutic vaccines, most notably in cancer.

SCAFFOLDS IN VACCINES

Rather than deliver nanomaterials to APCs, one emerging approach is to use a biomaterial scaffold to recruit the APCs to the material to create a nidus of prolonged immune activation to a delivered antigen (Table 1). A notable example from Ali and coauthors involved mimicking the biological processes of inflammation in response to infection, but in the context of therapeutic vaccines targeting cancer (*33*, *34*). The authors engineered a cancer vaccine that consisted of a macroporous PLGA scaffold loaded with three factors: the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF); the TLR9 agonist CpG, which mimics bacterial DNA; and the specific tumor lysate as antigen. The system was designed to provide for short-term presence of GM-CSF to recruit APCs to the scaffold, whereas CpG was delivered over a longer period to provide a durable APC activation signal. After APCs were activated and collected the target antigen in the scaffold, they presumably migrated into the regional lymph nodes to induce effector responses. This approach has been used to induce impressive tumor killing in mouse models of glioma (*33*) and melanoma (*34*). The prolonged presence of tumor antigen and activation signals within the scaffold may be important in stimulating clonal expansion of the rare, tumor-specific $CD8^+$ T cells critical for therapeutic vaccination in humans as well.

MOLECULAR ENGINEERING APPROACHES IN TOLERANCE

Novel molecular approaches for inducing immunological tolerance are being actively pursued by immunoengineers to treat autoimmune diseases such as multiple sclerosis and type 1 diabetes mellitus (*35*–*37*), to prevent transplant rejection (*38*), and to prevent autoimmune responses to therapeutic proteins. Natural mechanisms of establishing and maintaining tolerance are complex and depend on where, when, and in what context a potentially immunogenic antigen is encountered. During establishment of central tolerance, thymocytes are positively selected for potent antigen recognition capabilities and then negatively selected to remove most autoreactive T cells before they leave the thymus as naïve T cells. However, some self-antigen–specific T cells escape negative selection. These cells are subjected to peripheral tolerance mechanisms, which suppress or delete T cells that have escaped thymic selection and thus prevent autoimmune responses, such as after injury or inflammation.

To prevent or treat autoimmunity, new therapeutic strategies are being pursued to remove the low numbers of autoreactive cells that may escape central tolerance in genetically predisposed individuals before disease, or even after development of autoimmunity, by exploiting mechanisms of peripheral tolerance to ameliorate the deficits in central tolerance. One such approach seeks to target the antigen (to which tolerance is desired) to immature or regulatory DCs (*39*), without inducing their up-regulation of costimulatory molecules that are needed for effector T cell activation. This leads to effector T cell deletion or anergy or to activation of T_{reg} cells and apoptosis of the cognate T cells or regulatory responses (*35*, *39*, *40*). For example, antibodies that recognize the DC receptor CD205 (or DEC205) have been engineered to deliver an antigenic epitope fused to a domain on the antibody. This approach has shown promising results in the prevention of autoimmune sequelae in mouse models of multiple sclerosis (*35*) and type 1 diabetes (*36*). Nanoparticles can also present MHC-loaded antigens in the absence of costimulatory molecules, thus mimicking the function of tolerogenic DCs. Tsai and colleagues loaded nanoparticles displaying MHC I with diabetes-related antigens; when administered to mice, these nanoparticles stimulated autoregulatory $CD8^+$ T cells to prevent or even reverse type 1 diabetes. These regulatory responses, although clonally induced, were shown to have immune-suppressive capabilities that spread beyond their clonal targets (*41*).

Another approach to tolerance is motivated by the natural mechanisms induced by apoptotic cells, which often present their antigens in a tolerogenic manner (*38*, *40*). From an engineering perspective, antigen can therefore be osmotically loaded within or chemically conjugated to the surfaces of apoptotic cells and infused into the recipient so that the antigen will be cleared and processed just as though it were a natural component of the apoptotic cell. This approach has been successful in T cell adoptive transfer models (*42*) and in autoimmune mouse models of type 1 diabetes (*37*) and myocarditis (*43*), among others.

ENGINEERING IMMUNE MICROENVIRONMENTS

Two active areas of immune tissue engineering are (i) the in vivo induction of functional lymphoid-like tissues for therapeutic translation, and (ii) the development of in vitro models that can recapitulate features of microenvironments important for immune cell activation or function, as tools for basic immunology research using human immune cells (*44*).

The first area addresses a major goal of immunotherapy—to manipulate T cell education—by creating an artificial environment in vivo that encourages T cell recruitment and guides their education. To date, it has only been explored in preclinical studies and often uses cytokines such as lymphotoxins that are known to drive lymphoid tissue development both prenatally and in tertiary lymphoid structures often associated with chronic inflammation (*45*–*47*), which is likely to complicate therapeutic outcomes. The second addresses the need for a better understanding of lymph node immune physiology, and aims to develop in vitro tools in which specific hypotheses can be tested using human or mouse cells. Lymphoid tissue engineering therefore seeks to mimic and control the environments in which T cells interact with APCs. Although engineered lymphoid microenvironments have been shown to recapitulate certain key functions of natural lymphoid tissues, they lack the complex tissue architecture, stromal and chemokine organization, and flow pathways that guide the transport and distributions of antigens and lymphocytes in secondary lymphoid organs, which are critical to adaptive immune responses (*12*, *47*).

In vivo lymphoid tissue engineering

Still, some progress has been made in guiding lymphoid-like tissue formation in vivo in mice. Biodegradable polymer scaffolds seeded with disaggregated immune cells can provide structural stability and are often loaded with collagen to support cell motility, adhesion, and growth. For example, tissue-engineered neointestinal cysts were developed from cells isolated from mouse jejunum, cultured to form organoids in polymers, and then transplanted into mice (*48*). Twenty weeks after being implanted into mice, the scaffolds contained mucosal immune cell populations that were identical to those in the small intestine and that could treat experimentally induced intestinal insufficiency. Similarly, a tissue-engineered spleen was developed to address the problem of sepsis after splenectomy (*49*). In that study, biodegradable polymeric scaffolds seeded with splenocytes were implanted into mice, which protected the mice against a pneumococcal sepsis challenge after 16 weeks (*49*). A study by Suematsu and Watanabe demonstrated that lymphoid tissues could develop in mice starting with only thymic stromal cells, transfected to express lymphotoxin α (LT-α), and activated DCs embedded into a collagen scaffold (*50*). LT-α, which is important in lymphoid neogenesis (*47*), attracted lymphoid tissue inducer (LTi) cells to transform the construct into structures resembling lymph nodes. These artificial lymph nodes contained organized B cell and T cell zones, germinal centers, high endothelial venule–like vessels, and follicular stromal cell networks, and could mount both humoral and cellular immune responses to vaccination (*50*).

A major goal in oncology is to enhance antitumor immunity. To this end, therapeutic strategies to mimic lymph node functions in the tumor itself have been explored. By using lymphoid chemokines such as CCL21 or CCL19 (both CCR7 ligands) that attract LTi cells and other CCR7-expressing cells, including DCs and naïve T cells, to the tumor, the strategy aims to promote lymphoid neogenesis around the tumor and in turn stimulate the in situ education of antitumor cytotoxic T cells [reviewed in (*44*)]. On this topic, however, the literature can appear contradictory because CCR7 ligands play multiple roles in attracting different lymphocyte subsets (including T_{reg} cells) and can inhibit T cell proliferation and promote the death of activated T cells, depending on the complex inflammatory environment (*51*). Furthermore, many aggressive tumors express CCL21 endogenously. Shields *et al.* showed that melanoma expression of CCL21 (at physiological

levels similar to those expressed by resting lymph nodes) actually drove immune tolerance (*52*), presumably by promoting T cell education in the highly immune regulatory tumor microenvironment (for example, containing suppressive macrophage and monocyte subsets, and rich in cytokines such as transforming growth factor–β and IL-10). Thus, systematic studies of how dose, location, and microenvironment affect the host immune response to chemokines are needed before they can be effectively used to inform immunoengineering strategies for antitumor therapy.

Additionally, the potential for in vivo lymphoid tissue engineering to be used in any immunotherapy strategies will not be realized until we have a deeper understanding of the duality of lymphoid stromal functions in both promoting adaptive cellular immunity and maintaining peripheral tolerance to self-antigens (*51*). Tertiary lymphoid structures are often found in areas of chronic inflammation associated with autoimmunity (*53*, *54*). As mentioned above, context is everything; in addition to attracting naïve T cells and APCs, CCL21 also attracts T_{reg} cells and MDSC subsets, and its expression in the lymph node is required for acquired tolerance (*51*, *55*). Lymph node stromal cells, while supporting interactions leading to adaptive immunity, also present endogenous self-antigens on MHC molecules for peripheral tolerance maintenance (*56*) and can even cross-present exogenous tumor antigens for deletional tolerance (*57*). Indeed, tumors may hijack their tolerance-maintaining functions because tumor-draining lymph nodes are immune-privileged sites that promote tumor growth (*57*, *58*). Whereas lymphoid chemokines can attract and position immune cells to a localized site (lymph node or tumor) for education, it is the integration of other factors present—the host of cytokines, immune cell types, and other immunomodulatory molecules—that determines T cell fate, considering that the context is of utmost importance for therapeutic relevance in humans.

Therefore, future strategies to engineer lymphoid tissues that can guide desired immune responses in vivo will require a deeper understanding of how lymph nodes simultaneously maintain tolerance while guiding adaptive immunity. In turn, such understanding can be greatly facilitated by lymphoid tissue engineering, which allows specific microenvironmental conditions, such as cytokines, cells, and matrix, to be more precisely controlled and manipulated. In this way, even simple in vivo approaches and in vitro models, described below, may help instruct design criteria for future therapeutic strategies.

In vitro models

A second area of lymphoid tissue engineering focuses on developing in vitro models of immune cell activation to generate adaptive immune responses for therapeutic use or to study, predict, and screen immunomodulatory effects in human cells. For example, when primary tonsil cells were cultured on macroporous microspheres in a packed bed bioreactor, the cells reorganized into their natural organization and B cell areas could produce antibodies to delivered antigens (*59*). Cocultures of human dermal fibroblasts and keratinocytes in synthetic three-dimensional (3D) matrices have been shown to support the development of functional T cells with a diverse receptor repertoire from hematopoietic precursor cells (*60*), suggesting new therapeutic strategies for de novo generation of functional T cells ex vivo from skin biopsies. Randolph *et al.* modeled the initial steps in adaptive immunity in vitro and demonstrated that human peripheral blood mononuclear cells (PBMCs), when cocultured with human endothelial cells atop a 3D matrix, could be rapidly differentiated into functional DCs (*61*); without endothelial cells, PBMCs required culture in GM-CSF and IL-4 for 7 to 10 days to differentiate into DCs. This finding was further developed commercially by VaxDesign into a human predictive screening tool for vaccine testing, where the vaccine-activated DCs, matured in a similar in vitro system, can then be cocultured with human T cells to simulate early events in adaptive immunity (*62*).

Other examples of lymphoid tissue engineering include the development of a scaffold to support T cell motility and chemokine binding, which could be transplanted in vivo for controlled immune cell priming (*63*). A model of the lymph node paracortex, containing T cell zone fibroblastic reticular cells (FRC) within a composite scaffold, has been developed to organize and express CCL21 when exposed to interstitial fluid flow (*64*). Such in vitro models may be useful for determining therapeutic design criteria in lymphoid tissue engineering, as explained above, but are still far from realization into transplantable constructs for manipulating human immune responses. Indeed, translation is mostly limited by our still-inadequate understanding of how lymphoid tissues orchestrate immunity and tolerance, a complex topic that systems immunology (described below) aims to address.

CELL SURFACE ENGINEERING

Engineering approaches to manipulate cell surfaces with nanomaterials, specific effector proteins, or receptors are rapidly evolving to inform the design of new immunotherapeutics (*65*) (Fig. 3). These strategies to engineer immunity by modifying the cell surface have focused mainly on $CD8^+$ T cells to enable them to more potently kill tumor cells. One of the earliest approaches, and closest to clinical translation, was to augment $CD8^+$ T cell recognition of target cells by supplementing the TCR with chimeric antigen receptors (CARs) (*66*–*70*). T cells can home to tumor targets by sensing cytokine gradients and, as such, can be thought of as actively targeted cytotoxic vectors. Normally, they recognize a tumor target through ligation of the TCR to tumor antigen–loaded MHC I, but down-regulating MHC I expression is one of many mechanisms that tumors use to evade T cell immunity.

To circumvent this mechanism, CARs have been developed in which a single-chain antibody fragment (scFv) recognizing a tumor-specific or a tumor-associated antigen was fused to the ζ chain of the TCR/CD3 complex, along with other costimulatory domains (*71*) (Fig. 3A). When the scFv bound to its target, T cells were activated, including acquisition of killing capabilities. The CAR thus conferred to the T cell new target specificities without human leukocyte antigen (HLA) matching (that is, without having to use a specific MHC I depending on the genetic background of the subject) and at much higher affinity than achieved with TCR interaction with peptide-loaded MHC I. In recent clinical work, use of the CD137/4-1BB costimulatory domain with an scFv targeting CD19 (found on B cells) was shown to induce proliferation of engineered T cells transfected with the modified CARs in leukemia patients, which was associated with potent killing of cancerous as well as noncancerous B cells (*71*). As a conceptual alternative to CARs, antibody-based molecules to bridge the TCR/CD3 complex to a target on a tumor cell have been developed to enhance tumor killing; for example, antibody fragments (*6*), "BiTE" antibodies [from Micromet Inc. (*72*, *73*), currently in clinical trials (NCT00560794)], and dual-affinity re-targeting (DART) diabodies (MacroGenics) can enhance target cell

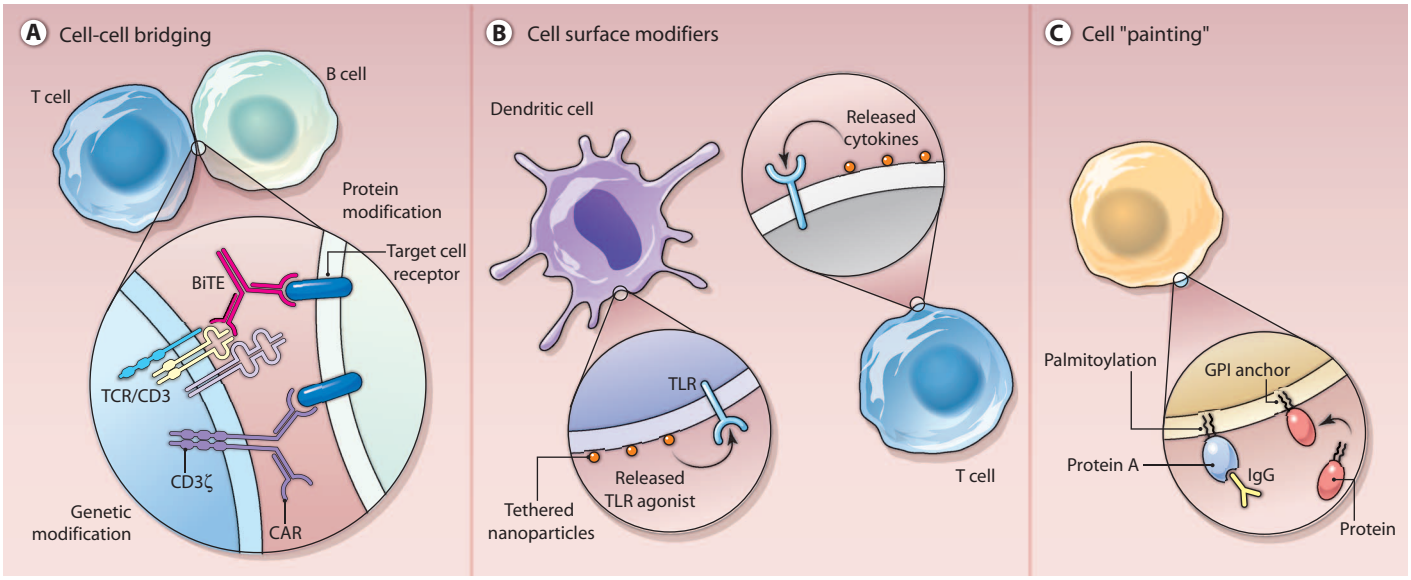

Fig. 3. Cell surface engineering for immunomodulation. (**A**) Cell-cell bridging interactions between $CD8^+$ T cells and their targets can be engineered. In an approach with CARs, an antibody fragment (scFv) is fused to the signaling domain from CD3 ζ chain along with other costimulatory domains. Binding of the scFv to its target triggers T cell activation, including proliferation and acquisition of cytotoxic function (*71*). In an alternative approach that does not require ex vivo genetic manipulation of the T cells, cell-cell bridging molecules can be applied exogenously as antibody or antibody fragments (*6*). For example, BiTE antibodies (*72*, *73*) and DART diabodies (*74*) can enhance target cell killing by engaging target cell recognition molecules with TCR/CD3 complexes on the surface of effector cytolytic T cells. (**B**) Cell surface modifiers. Nanoparticles loaded with biomolecules can be tethered to the surfaces of APCs or T cells ex vivo, so that they migrate through the body carrying their own agonists, released in a pseudo-autocrine manner (*75*). (**C**) Cell "painting." The cell surface can be modified with immunomodulatory molecules by first painting them with proteins that contain hydrophobic moieties, such as palmitoyl and GPI-based anchors (*94*). This approach can be used to immobilize cytokines on cell surfaces.

killing by engaging CD19 as a B cell lymphoma target and the TCR/CD3 complex on effector cytolytic T cells (*74*) (Fig. 3A).

Drug delivery approaches are being explored to ensure efficient survival and strong proliferation of adoptively transferred T cells during cell therapy. Unlike with other drug delivery methods, these approaches avoid genetic modification of the T cells to provide a relatively easier path to translation. T cell proliferation can be induced by addition of cytokines, such as IL-15 superagonist and IL-21; however, systemic administration of these cytokines after adoptive T cell therapy has proved ineffective (*75*). To address this, nanoparticles loaded with these cytokines have been tethered to tumor antigen–specific $CD8^+$ T cells to deliver these inducers of T cell proliferation in a pseudo-autocrine manner (Fig. 3B) over a course of several days. In a mouse melanoma model, this led to enhanced proliferation and improvements in tumor killing compared to transfer with soluble cytokines (*75*).

Other approaches to cell surface engineering are being pursued. Novel chemical schemes are being developed that may allow cell surface engineering ex vivo and even in vivo. For ex vivo modification, proteins are being developed for anchoring to the cell surface through lipidic anchors; for example, palmitoylated (*76*) or glycosylphosphatidylinositol (GPI)–anchored proteins (*77*) can be introduced exogenously to "paint" the cell surface with immunoactive molecules, such as decay-accelerating factor (Fig. 3C). Because many proteins are naturally displayed on the surface of cells using GPI anchors, this modification approach mimics the inherent biology of immunological stimulation. In vivo modification may be even more powerful, not requiring the complexity of processing cells outside the body followed by reinfusion. To this end, sialylated glycans have been metabolically labeled by the Staudinger ligation reaction (*78*) and copper-free Click chemistry (*79*) in vivo in mice to image and discriminate tumor cells from normal tissue by the differential sialylation on malignant cell surface glycans (*78*). This in vivo labeling requires two steps: first, metabolically labeling the glycans with modified sugars; second, adding the visualization reagent that has been tailored for conjugation. This same chemistry, used for conjugation of imaging agents, could be used to selectively target immunologically active biomolecules in vivo to tumor cell surfaces in this manner.

The full potential of cell surface engineering is just starting to be revealed. Many of the approaches explored thus far are complex, requiring ex vivo processing or genetic manipulation of T cells for adoptive therapy (Fig. 3). Owing to this complexity, translation to the clinic is slow; however, progress in humans is being decisively made, for example, with CARs (*66*–*70*). Simpler immunoengineering-based molecular (as opposed to cellular) approaches are being developed, including biomolecular [for example, the BiTE and diabody approaches described above (*72*–*74*)] and nanomaterial targeting [for example, cell-surface cytokine delivery described above (*75*)] of T cells without ex vivo processing, which promise to accelerate the pace of translation.

SYSTEMS ANALYSIS OF IMMUNITY

Systems-based analytical approaches to immunology could provide fundamental new insights into the signaling networks that drive and

control cellular immune responses, leading to discovery of new pathways and targets for intervention. Although not an exhaustive review of systems immunology [for which we refer the reader elsewhere (*4*, *80*–*83*)], we highlight examples of promising advances that may affect translational immunology, with a specific focus on engineering-related progress. These emerging approaches are building the necessary foundation for the science component of "immunoengineering"—that is, to develop quantitative, predictive models and rational design criteria that are needed to both improve current immunotherapeutic strategies and guide future strategies.

Immune regulation—distinguishing self from foreign or danger—depends on intercellular communication. Although many of the molecular players in immune-related signaling pathways are known, this complex web of information and response has been studied in a qualitative manner. The real system is a dynamic one, with individual cells vacillating between different stable states and smaller subpopulations, the latter of which is sometimes more important to the response than the dominant populations, for example, polyfunctional T cells being generated in response to a vaccine. Thus, immunological signaling can be informed by engineering systems analysis, using computational approaches to reveal mechanisms of interactions or dominant pathways and players (*84*, *85*), using microfluidic approaches to examine individual cells collected in large populations (*86*), using molecular read-outs to characterize signals between individual cell pairs (such as APC–T cell or T cell–T cell) (*87*), and using quantitative imaging and biophotonic approaches (*88*, *89*) to investigate on an individual-cell basis the dynamics of these immunological processes.

Whereas traditional immunological approaches have measured responses from large cell populations in culture, or have used flow cytometric methods to characterize individual cell responses at fixed times, the development of massively parallel immunoengineering approaches to measure at the molecular and cellular levels the dynamic responses of individual cells and paired cells in comparatively large populations will provide fundamental new insights into basic immunology, leading to new approaches to intervention. For example, high-throughput arrays of subnanoliter wells have been developed to characterize the HIV-specific $CD8^+$ T cell cytokine response in minute samples from blood or tissue, in a manner that allows harvesting of the T cell clones such that each clone is associated with a specific cytokine response (*90*). Collection of such information from patient samples could allow personalized characterization of cellular responses to vaccination (*87*), viral infection, and autoimmunity, for example, in tandem with personalized mouse models of human immunity (*91*).

Antigen specificities in B cell receptor and TCR repertoires arise stochastically and are selected and fine-tuned in early development as well as throughout life via a wide variety of mechanisms in the thymus, liver, and secondary lymphoid organs. Technologies have been developed to characterize humoral responses to vaccination or disease by deep sequencing of the variable regions of antibodies from bone marrow plasma cells of mice, which govern the circulating antigen-specific antibody repertoires (*92*). In mining the transcriptional data from these specific cells to determine the antibody repertoire at the genetic level, the antigen-specific antibody screening step at the protein level could be eliminated. This approach may now be extended to human samples, with the hopes of streamlining antibody discovery and production.

Quantitative systems analysis has provided insight into both how immunological repertoires evolve and how they are maintained. For example, one mechanism by which T cells maintain diversity in their TCR repertoire is through IL-7 receptor signaling. Palmer *et al.* showed that naïve T cells display variable sensitivity to IL-7 depending on their CD5 expression such that different signaling thresholds affected their survival and proliferation at steady state (*93*). In a second example, systems immunologists have uncovered new insights into how the immune system balances the determination of self in thymic selection with the detection of pathogens, thus addressing the question of why some patients with certain *HLA* alleles can control HIV infections of HIV without developing AIDS (*85*). Thus, computational systems approaches could reveal that selections in T cell repertoire during development in the thymus greatly influence the host response to pathogens and its ability to mount effective immune responses to contain chronic disease.

CONCLUSIONS

Immunoengineering is an emerging field that is rapidly being colonized by cellular engineers, systems biologists, protein engineers, biomaterials scientists, tissue engineers, and others who recognize the increasing importance of immunology in almost all areas of biomedicine, who are inspired by the translational potential of immunotherapeutics, and who find ways to apply their tools, methods, and viewpoints to address outstanding questions in immunology. Our understanding of immunology stands to benefit substantially from a more diverse array of quantitative and synthetic tools that can help dissect the extremely complex cellular and molecular interactions that govern our immune system and underlie all autoimmune, infectious, neoplastic, and inflammatory diseases. The integrative and design-oriented approaches that these researchers bring will in turn lead to new therapeutic pathways in these areas, given the time necessary for development from concept to demonstrations in mouse and potentially nonhuman primate models, and eventually human clinical testing.

Early clinical developments from translational immunologists have been encouraging thus far, as exemplified by the clinically approved cancer vaccine Provenge (Dendreon), which stimulates autologous DCs ex vivo with prostate tumor–derived antigens. However, as immunomodulatory strategies often target complex pathways involved in regulating both immunity and tolerance, the path for clinical translation may be substantially longer than for small-molecule drugs with simpler mechanisms of action. We believe that this represents the greatest translational challenge for immunoengineering. Moving forward, immunoengineers will continue to help develop more powerful and cost-effective therapeutic vaccines in cancer and chronic viral disease, as well as immunomodulatory therapies in autoimmunity. Immunoengineering seeks to accelerate the translation of immunotherapeutic strategies to the marketplace in several ways, such as by driving new synthetic and molecular alternatives to cell-based approaches, which will improve cost-effectiveness and shorten regulatory hurdles; by targeting specific cells, tissues, or lymph nodes, which will increase efficacy, spare dosage, and reduce potential toxicity; by developing systems-based frameworks that will help optimize kinetics and dosage as well as optimize antigen epitopes; and by improving immunological assays, which will help realize the potential of personalized immunotherapy.

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