HHS Public Access

Author manuscript

Adv Drug Deliv Rev. Author manuscript; available in PMC 2018 May 19.

Published in final edited form as:

Adv Drug Deliv Rev. 2017 May 15; 114: 161–174. doi:10.1016/j.addr.2017.05.013.

Combinatorial drug delivery approaches for immunomodulation

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Abstract

Immunotherapy has been widely explored for applications to both augment and suppress intrinsic host immunity. Clinical achievements have seen a number of immunotherapeutic drugs displace established strategies like chemotherapy in treating immune-associated diseases. However, single drug approaches modulating an individual arm of the immune system are often incompletely effective. Imperfect mechanistic understanding and heterogeneity within disease pathology have seen monotherapies inadequately equipped to mediate complete disease remission. Recent success in applications of combinatorial immunotherapy has suggested that targeting multiple biological pathways simultaneously may be critical in treating complex immune pathologies. Drug delivery approaches through engineered biomaterials offer the potential to augment desired immune responses while mitigating toxic side-effects by localizing immunotherapy. This review discusses recent advances in immunotherapy and highlights newly explored combinatorial drug delivery approaches. Furthermore, prospective future directions for immunomodulatory drug delivery to exploit are provided.

Keywords

Immunotherapy; Combination therapy; Drug delivery; Biomaterials; Immunomodulation; Autoimmunity Cancer

1. Introduction

The complexity of the immune system affords ample opportunities for pathologies to develop, both from aberrant immune activation and misguided immunosuppression. Recent advances delineating immunobiological mechanisms in infectious disease, cancer, and auto-immunity have helped inform novel therapeutic approaches. Immuno-therapy, an increasingly popular methodology, attempts to modulate specific arms of the immune system by interfacing with host biology to augment or suppress natural immune responses. Since Edward Jenner first developed a vaccine for smallpox in the late 18th century, immunotherapy has played a prominent role in improving human health and quality of life.

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Similarly, allergy immunotherapy has been used clinically for more than a century, yielding positive outcomes for patients with asthma, dietary, and seasonal allergies [1]. Today, immunotherapies are supplanting several well-established clinical treatment paradigms. For example, cancer immunotherapy has emerged in recent decades as an attractive alternative to chemotherapy, as administration of broadly cytotoxic drugs has dangerous and potentially fatal side effects. By contrast, controlled modulation of innate and adaptive immunity can generate robust anti-tumor responses while minimizing systemic toxicity. Recently, a number of innovative immunotherapeutic strategies have garnered attention. Enthusiasm for immunotherapies such as Sipuleucel-T dendritic cell therapy, chimeric antigen receptor (CAR) T cells, and monoclonal antibodies is buoyed by success in clinical trials. Sipuleucel-T immunotherapy, wherein isolated autologous dendritic cells are exogenously activated, loaded with tumor-specific antigen and are re-administered, was the first FDA-approved therapeutic vaccine for cancer of any kind and showed improved survival in men with metastatic prostate cancer [2]. Similarly, adoptive transfer of $CD19^+$ B cell targeting CAR T cells demonstrated sustained remission of acute lymphoblastic leukemia in children and adults [3]. Overall, immune modulating interventions to harness specific features of the immune system are becoming widespread and multipurpose.

The abundance of immunotherapy strategies being explored is in large part due to the expanding number of therapeutic tools. The increased mechanistic understanding and availability of immunomodulatory drugs including recombinant cytokines (e.g., IL-2, TGF-β, IFNγ), small molecule adjuvants (e.g., CpG, MPLA, Pam3CSK4), and monoclonal antibodies (e.g., anti-PD1, anti-CTLA-4, anti-IL-10) have facilitated development of immunotherapy approaches. In cancer immunotherapy alone, there are over 50 immunotherapy agents currently being used in the clinic or in clinical trials [4]. The targets of these single drug approaches are wide ranging, but similar in that they engage an isolated immune pathway. In one clinical trial, for example, immunotherapy with low-dose administration of IL-2 resulted in a dose-dependent increase of $FoxP3^+$ regulatory T cells (Tregs) in patients with type 1 diabetes, a cellular phenotype critically lacking in many autoimmune conditions [5]. On the other hand, recent work using monoclonal antibodies for checkpoint blockade therapy has potentially revolutionized cancer immunotherapy. Nivolumab, a monoclonal antibody that prevents T cell inhibition by impeding programmed death-1 (PD-1) signaling, dramatically increased survival in metastatic melanoma patients, outperforming a standard first-line chemotherapy regimen [6]. In other clinical trials, monoclonal antibody therapy with ipilimumab, a monoclonal antibody that inhibits cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), improved survival in patients with metastatic melanoma by over 10 months and ~20% exhibited long term survival after five years [7,8]. While clinical achievements from single drug immunotherapies cannot be understated, such approaches are limited by a number of factors. In particular, incomplete understanding of disease pathology and associated immune pathways hinders identification and application of relevant monotherapies. Additionally, heterogeneity within disease pathogenesis and among patient populations can limit the efficacy of drugs that engage individual pathways.

Combinatorial strategies to modulate multiple immune axes in coordination are seen as an attractive strategy to overcome these barriers, the growth of which is well documented [4,9–

11]. Combinatorial immunotherapy success is represented by recent clinical trials involving simultaneous administration of nivolumab and ipilimumab [12,13]. This groundbreaking work demonstrated the importance of engaging multiple immune pathways, as metastatic melanoma patients with programmed death ligand 1 (PD-L1) negative tumors displayed significantly reduced survival when administered either agent alone. Conversely, when PD-1 and CTLA-4 monoclonal antibodies were delivered in combination, PD-L1-negative tumor patients had improved survival by over 5 months. While the clinical success of combinatorial immunomodulation has fueled a dramatic increase in such approaches, concerns about toxicity associated with systemic administration have restricted their therapeutic potential. Poorly managed global immunostimulation when using activating adjuvants [14], CAR T cells [15], or monoclonal antibodies [16] can induce life-threatening cytokine storm or autoimmune disease. Similarly, chronic global immunosuppression, often employed for organ transplant and autoimmunity, leaves patients at risk for opportunistic infections and tumor development [17]. To address such problems associated with systemic delivery, controlled-release platforms are increasingly being explored for combination therapies.

Biomaterial-based drug delivery systems have been extensively investigated for immunotherapy applications. Controlled-release systems exhibit a number of advantageous chemical and physical properties that have been exploited to augment immunomodulation compared to soluble drug administration. One frequently employed characteristic, biodegradable materials can deliver sustained release of encapsulated drugs to prolong therapeutic levels, reducing high and/or frequent dose requirements. Similarly, drug degradation and clearance, two critical limitations associated with soluble drug delivery, can be minimized by biomaterial encapsulation. Drug delivery approaches can also enhance the mass and frequency of payload delivered to immune populations, which can facilitate and augment immunotherapy. For example, biomaterial vehicles can be modified to target drug delivery to key cells or tissues. Alternatively, implantable controlled-release platforms have been developed to localize drug delivery at the site of administration, adjacent to relevant immune-associated structures. Such biomaterial approaches are also particularly appealing in order to minimize undesirable systemic immunomodulation. While there is a vast array of bio-materials that have been developed for drug delivery, distinct platforms (e.g., hydrogels, micro- or nanoparticles, liposomes) each offer unique advantages for immunotherapy (Fig. 1). Particle-based systems have been extensively explored to deliver payloads to antigen-presenting cells [18]. Conversely, biomaterial scaffolds have been engineered to actively recruit immune populations in vivo [19]. The various tunable parameters of biomaterial platforms have made them a particularly useful tool for immunotherapy.

This review highlights recent work on combinatorial drug delivery strategies for immunosuppression and immunostimulation. The primary focus will be on biomaterial platforms (Table 1), although core immunotherapy principles and background results will be discussed for context. Specifically, we emphasize recent experimental work in drug delivery approaches that rely on cellular targeting modalities and controlled-release strategies to recruit immune cells *in vivo*. While significant progress has been made toward integrating immunomodulatory therapies in the clinic, we also discuss the limitations in current approaches and suggest a number of future directions that show promise.

2. Targeted drug delivery to immune cells

2.1. Targeting antigen-presenting cells

Drug delivery approaches using targeting modalities are seen as particularly appealing for immunotherapy. Targeted drug delivery to disease-relevant organs, tissues, or cells can attenuate the dangerous side effects associated with broad, systemic immunotherapy. Additionally, selective delivery to specific immune compartments can enhance downstream immune responses.

There have been broad efforts developing immunotherapeutics targeting antigen-presenting cells (APCs). Operating at the interface between innate and adaptive immunity, APCs are critical in mediating the balance between immune tolerance and activation. Dendritic cells (DCs) are the most efficient APC in the body [20]. As multifunctional regulators of immunity, various DC subsets exist with differential capacities for immunogenicity [21]. Upon exposure to inflammatory signals, such as engagement of toll-like receptors (TLRs) on DCs by pathogenic ligands (e.g., LPS, CpG), DCs become functionally mature. DC maturation results in phenotypic and functional changes that promote migration to lymphoid organs, secretion of inflammatory cytokines, and T cell activation. However, in a resting state, DCs process and present antigen in an immature fashion, maintaining homeostatic tolerance toward self-antigen [22]. In addition to immature DCs, tolerogenic DC phenotypes can be induced which can promote peripheral tolerance. Tolerogenic DCs employ a variety of modalities to preserve tolerance including induction of T cell anergy or deletion, release of anti-inflammatory cytokines (e.g., TGF-β, IL-10), and expansion of regulatory T cells [23]. Insight into TLRs, DC subpopulations, and the interface between innate and adaptive immunity have driven the continued development of tailored DC-based therapies [24–27]. The robust capacity of APCs to orchestrate immunity, particularly in antigen-specific directions, makes them a frequent target for immunotherapy.

Immunotherapy with exogenously manipulated DCs has demonstrated positive therapeutic outcomes in both preclinical and clinical investigations [28,29]. Since the first clinical trial using DC-based vaccination in 1996 [30], numerous studies have demonstrated induction of antigen-specific T and B cell responses against malignant cancers using DCs primed with tumor-associated antigens [31,32]. Not until 2010, however, was the first DC-based vaccine for cancer immunotherapy, Sipuleucel-T, approved by the FDA. Sipuleucel-T immunotherapy demonstrated success in patients with castration-resistant prostate cancer, improving mean survival time by ~4 months and increasing tumor-specific antibody titers and antigen-specific T cell proliferation [2]. While the anti-tumor effects demonstrated were modest, as tumor burden was not significantly diminished, the first-of-its-kind regulatory approval generated enthusiasm for DC-based immunotherapy. Alternatively, “negative vaccination” using DCs to induce tolerance has been investigated in models of graft survival, multiple sclerosis, and type 1 diabetes [33-36]. One approach that employed DC adoptive transfer for type 1 diabetes was recently explored in a phase I clinical trial, attempting to modulate autologously isolated DCs toward an immunosuppressive state via a combination of antisense oligonucleotides against co-stimulatory molecules CD40, CD80, and CD86 before being re-administered [37]. While the study was not powered to determine

therapeutic benefits, there was no measurable change in C-peptide levels or in regulatory T cell frequency. Notably, however, the approach demonstrated tolerable safety profiles. Similarly, combinatorial drug strategies to modulate DCs ex-vivo have also been an active area of investigation [38,39]. While DC-based immunotherapy has demonstrated clinical efficacy, administration of exogenously-treated DCs exhibits numerous barriers for widespread implementation including high cost, poor yield, and inefficient DC homing to regional lymph nodes [40,41].

A promising strategy to overcome these limitations is the targeted delivery of immunomodulatory factors to DCs *in vivo*. Nano- and micro-particles of various shapes, sizes, and compositions (e.g., lipids, polymers, metals) have been extensively studied as vehicles for *in vivo* drug delivery [42]. Particle-based approaches are valuable for drug delivery because they are often highly tunable, provide a sustained release depot for encapsulated agents, reduce systemic toxicity and dosing requirements, and simplify manufacturing, storage and shipping concerns [43,44]. Delivery to endosomes is facilitated when targeting APCs, as particles less than ~5 μm in diameter are endocytosed by APCs without the need for specific receptor-targeting strategies [45]. This strategy of “passive targeting”, relying on the intrinsic phagocytic activity of APCs for particle-based drug delivery, has drawn significant interest for immunomodulation. Recent work by the Giannoukakis laboratory established microparticles containing antisense oligonucleotides against CD40, CD80, and CD86 co-stimulatory molecule transcripts can combinatorially downregulate DC maturation and ameliorate disease in a type 1 diabetes mouse model, laying the foundation for the DC-based clinical trial mentioned above. Upon subcutaneous injection, microparticles were shown to be taken up by DCs, traffic to draining lymph nodes, augment antigen-specific regulatory T cell proliferation, and reverse new-onset diabetes [46,47].

In contrast to micron-sized particles, which exclusively rely on uptake by APCs in order to traffic to secondary lymphoid organs, nanoparticles can be fabricated small enough to migrate to draining lymph nodes through lymphatic vessels [45]. Reddy et al. demonstrated this effect in a passive targeting strategy to activate lymph node-residing DCs using ultra-small polypropylene sulfide nanoparticles (Fig. 2) [48]. Initially, the authors established the importance of nanoparticle size in lymphatic drainage, as 100 nm nanoparticles were only 10% as efficient in trafficking to draining lymph nodes compared to 25 nm particles. When 25 nm particles were surface-coated with pluronic, previously shown to activate the complement cascade [49], intradermal injection resulted in upregulation of co-stimulatory molecules CD80, CD86, and CD40 on lymph node DCs similar to levels seen by LPS activation. Furthermore, nanoparticles conjugated with ovalbumin (OVA) antigen generated significant antigen-specific humoral and cellular responses. In a follow-up manuscript, Thomas and colleagues took advantage of this lymphatic transport phenomenon using a combinatorial drug cocktail to activate DCs in tumor-draining lymph nodes [50]. Building on their original work, they hypothesized that delivery of adjuvants to tumor-draining lymph nodes, which contain high concentrations of tumor-associated antigens, would induce activated DC phenotypes and generate a potent anti-tumor response. They confirmed their hypothesis in a murine model of melanoma. Intradermal injection of 30 nm nanoparticles encapsulating CpG oligodeoxynucleotide (CpG), a TLR9 agonist, and paclitaxel, a well-

established anti-proliferative drug and TLR4 ligand [51], hindered tumor growth and skewed the $CD4^+$ T cell distribution toward a Th1 phenotype. Results also demonstrated the requirement of drainage to tumor-draining lymph nodes for effective therapy, as contralateral injections did not generate as robust antigen-specific $CD8^+$ T cell responses compared to ipsilateral injections upstream of tumor-draining lymph nodes. This work also highlights a recurring theme of controlled-release drug delivery platforms: encapsulation of the combinatorial drug cocktail produced a more efficacious immune response than soluble administration of adjuvants.

As important intermediaries between innate and adaptive immunity, DCs express high concentrations and heterogeneity of TLRs to direct diverse immune responses. Improved immunogenicity through combinatorial TLR agonist delivery is being explored. For example, mice immunized with bone marrow-derived DCs activated with TLR7 and TLR3 agonists improved cytotoxic lymphocyte responses in vivo compared to individual agonists [52]. Similarly, engagement of distinct stimulatory pathways through TLR4 and TLR8 on human DCs in vitro induced cytokine IL-12 and IL-23 levels 50–100 fold higher than those induced by single TLR agonists [53]. Biomaterial drug delivery systems have sought to take advantage of combinatorial TLR approaches. A breakthrough study in 2011 demonstrated the benefits of controlled-release vehicles to deliver two TLR agonists in vivo [54]. Mice subcutaneously injected with poly(lactide-*co*-glycolide) (PLGA) nanoparticles bearing monophosphoryl lipid A (MPLA; TLR4 agonist), imiquimod (TLR7 agonist), and OVA antigen generated persistent germinal center formation and plasma-cell responses, which were present in lymph nodes for >1.5 years following immunization (Fig. 3). Notably, they found immunization with the combinatorial formulation increased the antigen-specific humoral response compared to nanoparticles with individual TLR agonists. Results also demonstrated that TLR engagement on B cells, in addition to DCs, was critical in producing antibody responses. As cells differentially express TLRs [55], this work suggests that TLR-based vaccines can be optimally designed by rational inclusion of TLR agonists to generate tailored immune responses. Higher order TLR combinations are also being investigated. Recent work described high-throughput methods for combinatorial loading of factors in microparticles [56], as well as a high-throughput microarray to assess DC activation in response to combinations of three TLR-encapsulating microparticles [57,58]. The adjuvant-screening microarray demonstrated microparticle combinations delivering poly(inosinic:cytidylic acid) (poly(I:C)), MPLA, or CpG elicited differential expression of DC activation biomarkers CD86, MHC-II, CCR7, IL-12, and IL-10.

Classically, antigen that is endocytosed by DCs is presented by MHC class II molecules and restricted to $CD4^+$ T cell presentation [59]. Cross-presentation is the process by which antigen is released from the endosome into the cytosol and, through alternative pathways, presented on MHC class I molecules to $CD8^+$ cytotoxic T cells [60]. Cross-presentation is vital to vaccines dependent on cytotoxic T cell responses. Controlled-release particles have been shown to be advantageous in generating $CD8^+$ T cell responses. In addition to promoting prolonged antigen presentation, sustained release of antigen from biodegradable microparticles has demonstrated a roughly 1000-fold increase in cross-presentation compared to soluble protein [61]. In a similar vein, a recent nanoparticle-based approach pioneered by the Fahmy laboratory employed combinatorial TLR activation to generate

robust $CD8^+$ T cell immunity [62]. The platform utilized PLGA nanoparticles surface-conjugated with MPLA and encapsulated CpG and OVA. An important finding from their research demonstrated the significance of the mode of presentation when designing TLR-based vaccines. CpG efficacy as an immunostimulant, characterized by cytokine profiles, antibody titers, and antigen-specific T cell responses, was maximized when encapsulated in nanoparticles compared to when surface bound. As the receptor for CpG, TLR9, is localized endosomally [63], the authors suggest that physiologically relevant presentation of CpG is important in generating a robust immune response. This work additionally showed the importance of co-delivery, as single TLR agonists failed to replicate the potent $CD8^+$ T cell response generated with the combinatorial formulation.

Strategies for APC targeting have also been developed that bind surface moieties to increase retention and uptake. This approach seeks to minimize off-target drug interactions and can produce more potent immune responses due to the efficiency with which drug cargo is more selectively taken up by APCs. Particulate formulations functionalized with surface moieties including α-CD40, α-DEC205, and α-CD11c antibodies have been explored for DC targeting [64]. As noted earlier, in contrast to microparticles, nano-sized particulates injected intradermally or subcutaneously can migrate and drain through lymphatic vessels. While advantageous in certain applications, nanoparticles are prone to non-specific cellular uptake and this competition for uptake is compounded by hepatic filtration and renal clearance [65]. Thus, targeting strategies to improve DC uptake of nanoparticles is particularly valuable. Recent work characterized the impact of targeting moieties based on particulate size, demonstrating that particles surface conjugated with the human C-type lectin receptor DC-SIGN dramatically improved DC uptake for polymeric nanoparticles, but only produced a modest increase in uptake for micron-sized particles [66]. The choice of targeting moiety is often dictated by the therapeutic intention. DC maturation is undesirable when delivering immunomodulatory drugs for tolerance-inducing purposes. Along these lines, microparticles modified with antibodies against CD11c or DEC-205, an integrin and C-type lectin respectively that are highly expressed on DCs, or functionalized with peptides P-D2 or RGD, targeting intercellular adhesion molecule-4 and surface integrins respectively, were found to efficiently target DCs, both *in vitro* and *in vivo*, in a non-activating manner [67].

Conversely, targeting ligands have been studied to improve uptake by DCs and simultaneously augment immunogenicity. Rosalia and colleagues demonstrated that covalent coupling of α-CD40 to PLGA nano-particles containing antigen, Pam3CSK4 and poly(I:C), a TLR2 and TLR3 agonist respectively, improved selective DC uptake and activated DCs *in vivo* [68]. In a tumor model of melanoma, subcutaneous administration of α-CD40-functionalized adjuvant-bearing nanoparticles improved the antigen-specific $CD8^+$T cell response and prolonged survival compared to mice treated with nanoparticles conjugated with an isotype control. While the authors confirm that conjugation of α-CD40 to nanoparticles augmented nanoparticle uptake and delivery of TLR agonists to DCs, it was not investigated whether DC maturation was improved by engagement of CD40 inflammatory signaling pathways. In another approach, Kwong and colleagues used a combinatorial drug approach delivered intratumorally to generate an anti-tumor response [69]. Intratumoral injection of PEGylated liposomes surface conjugated with CpG and antibody against CD40 significantly mitigated tumor growth in a mouse model of

melanoma. Importantly, retention at the local tumor site was confirmed. Surface conjugation of α-CD40 and CpG, the receptors for which are highly expressed on APCs, aided tumor retention. Liposome uptake by $CD11c^+$ DCs and $F4/80^+$ macrophages was observed at extended time periods, 24 and 48 h post injection. Similarly, their drug delivery approach curtailed systemic inflammation, as intratumoral administration of soluble immunomodulators increased serum levels of TNF-alpha and IL-6 and resulted in weight loss compared to the lipo-some formulation.

2.2. Targeting T cells

While APC-targeting therapies attempt to modulate immunity through critical cellular mediators, drug delivery strategies have also been developed to target other immune compartments more directly. Like DC-based therapies, various strategies to modulate T cell numbers and activity *ex-vivo* for adoptive transfer therapy have been investigated (reviewed in [70]). However, limitations with adoptive cellular transfer similarly plague T cell approaches. T cell targeting strategies have been conducted to work around these limitations. In one approach, researchers developed a targeting strategy to minimize T cell loss upon adoptive transfer. Three days after adoptive transfer of allogeneic T cells bearing the distinct congenic marker Thy1.1, intravenous injection of PEGylated liposomes conjugated with targeting ligands against Thy1.1 effectively marked>95% of transferred cells [71]. As the authors suggest, this targeting approach could be universally applied for adoptive cellular transfer through introduction of distinct surface markers by genetic engineering of cells pre-transfer. In another approach for targeted drug delivery to discrete T cell populations, Vincent et al. developed a MHC-I tetramer construct to deliver payloads to antigen-specific T cells [72]. Conjugation of MHC-I tetramers loaded with islet-specific peptide antigen to saporin, a ribosomal toxin, enriched ablation of antigen-specific, autoreactive $CD8^+$ T cells in a mouse model of type 1 diabetes. Three intravenous injections of the construct reduced diabetes incidence in 8 week old non-obese diabetic mice by 30% after >50 weeks compared to controls. This approach, employing selective delivery to distinct T cell compartments, is desirable in order to minimize global immunotoxicity.

Regulatory T cells (Tregs) are critical for the maintenance of immune tolerance. Impaired Treg function and reduced numbers have been identified as important factors in cases of autoimmunity, organ transplant rejection, and allergy (reviewed in [73]). Immunotherapy strategies to augment Treg activation, proliferation, and function remain highly desirable, and have been explored through a number of diverse drug delivery platforms. In one example, microparticles delivering sustained release of the Treg chemokine CCL22 were shown to recruit Tregs locally and delay organ rejection in a murine allotransplant model [74]. In another unique strategy, the Hubbell laboratory developed an erythrocyte-targeting platform for induction of antigen-specific tolerance [75]. This system was composed of erythrocyte-binding constructs that were coupled with antigen, built around the premise that apoptotic cells, and erythrocytes are frequently recycled, are cleared through self-tolerance promoting pathways (reviewed in [76]). In a follow-up study by the Hubbell group, researchers explored the mechanisms by which the erythrocyte-targeting platform induced tolerance [77]. Results using antigen-specific T-cell-transgenic OT mouse models demonstrated that blockade of PD-1/PD-L1 signaling, but not CTLA-4/CD28, impaired

antigen-specific T cell deletion, anergy, and expression of regulatory biomarkers. Additionally, the platform significantly increased the frequency of Tregs compared to administration of soluble antigen. Depletion of Tregs through α-CD25 demonstrated the critical role Tregs played in mediating $CD4^+$ and $CD8^+$ T cell suppression. The concept of antigen coupled to apoptotic cells recently demonstrated promise in a phase I clinical trial for multiple sclerosis [78]. In the trial, patients that received apoptotic leukocytes covalently coupled with various multiple sclerosis-specific peptide antigens demonstrated favorable safety profiles. Furthermore, patients receiving higher doses of apoptotic coupled cells displayed decreases in antigen-specific T cell responses.

As numerous factors have been identified as important modulators for Treg induction, a combinatorial approach engaging multiple pathways has been suggested as an ideal strategy for robust Treg responses. In one such approach, PLGA microparticles were loaded with three known Treg inducing agents: rapamycin, TGF-β1, and IL-2 [79]. Micro-particles were separately loaded with individual factors and demonstrated sustained release of encapsulated agents over 3–4 weeks. Notably, the system was most effective in skewing murine $CD4^+$ T cells to express FoxP3 *in vitro* when all three microparticles were included. This work also showed the combinatorial microparticle formulation was also effective for induction of human Tregs *in vitro*, suggesting the translatability of their approach. Using an *in vivo* targeting scheme, McHugh and colleagues developed a similar approach by targeting delivery of TGF-β and IL-2 to T cells using PLGA nanoparticles surface-conjugated with α-CD4 antibodies [80]. Their results demonstrated that nanoparticles surface modified with α-CD4 dramatically increased binding of T cells by ~18-fold *in vitro*. Furthermore, the increased bio-availability of these pro-tolerogenic factors was shown to skew the $CD4^+$ T cell populations in vivo toward a regulatory phenotype, increasing the percentage of $CD4^+$ T cells expressing FoxP3. The authors suggest this approach can also minimize the pleiotropic effects of these cytokines when delivered solubly, as TGF-β is well known to cause severe adverse responses when administered systemically [81]. However, as they note, a potential shared drawback of particle-based systems is non-specific uptake of nanoparticles by APCs through passive phagocytic activity. As such, it may be advantageous to minimize undesirable phagocytosis when designing vehicles for drug delivery to non-APC cellular compartments. Strategies such as functionalizing peptides from membrane proteins CD47 or CD200, reported ubiquitous self-identifiers, have been employed for drug delivery applications to mitigate macrophage-mediated phagocytic clearance [82,83]. Additionally, surface charge has been explored to modulate phagocytic uptake of particulate delivery systems, albeit with varying results. It has been reported that particles exhibiting high negative or positive charges are more readily taken up by DCs and macrophages compared those displaying a neutral surface charge [84,85]. However, concerns about toxicity may limit drug delivery applications for such approaches. In 2014, for example, Getts et al. described how anionic microparticles were taken up by monocytes via the scavenger receptor, MARCO, which resulted in sequestration of monocytes in the spleen and subsequent apoptotic clearance [86]. Similarly, it has been demonstrated that cationic particles can result in the generation of reactive oxygen species or mitochondrial injury pathways [87,88]. Lastly, particle shape has been described as an influential design consideration to avoid phagocytosis. Unlike spherical particles which are phagocytosed

within minutes, rat alveolar macrophages had greatly diminished uptake capacity when incubated with ellipsoid polystyrene particles [89]. The initial point of contact between macrophages and particles was shown to be pivotal in internalization kinetics. Macrophages that attached to the spheroid end of an ellipsoid particle internalized it within minutes, however, those that encountered the flat portion of the ellipsoid did not internalize the particle for over 12 h. These approaches have been suggested to increase payload delivery to non-APC targets. Understanding the parameters that effect phagocytosis are critical in designing particle-based drug delivery systems and need to be further explored.

2.3. Alternative targeting strategies

Alternative immunotherapy targeting strategies have also been pursued. A recent innovative targeting approach modulates immunity by “hitchhiking” onto albumin protein draining to peripheral lymphoid organs [90]. Instead of targeting cell surface receptors, Liu et al. sought to localize an immunostimulatory vaccine intranodally by conjugating a lipophilic albumin-binding moiety to CpG. Subcutaneous administration of the albumin-binding construct resulted in an 8-fold increase in accumulation to peripheral lymph nodes compared to soluble CpG. Localization was further amplified as the albumin-binding vaccine showed sustained retention in lymph nodes after seven days. Notably, their approach dramatically increased anti-tumor effects in a murine model, while reducing systemic inflammation by attenuating serum IL-6 and IL-12 levels.

Localization of immunomodulatory agents to sites of concentrated immune activity has also been explored by more direct delivery approaches. In one strategy, researchers highlighted the efficacy of controlled-release particles to elicit robust immunity via intranodal delivery. By mapping the lymphatic system of mice using the tracer dye Evans blue, Jewell et al. intranodally injected PLGA microparticles containing poly(I:C) and OVA as model antigen to generate antigen-specific immunity [91]. Results demonstrated a single intranodal injection of the microparticle vaccine expanded the frequency of OVA-specific $CD8^+$ T cells by over 8-fold compared to intramuscular immunization. Additionally, encapsulation of vaccine components in controlled-release microparticles augmented poly(I:C) intranodal persistence. Fluorescently-conjugated poly(I:C) levels released from microparticles were ~2.5-fold higher measured after four days compared to an equivalent soluble intranodal dose. This work also displayed a core paradigm of controlled-release drug delivery approaches by reducing the adjuvant dose necessary to achieve similar immunogenicity. Microparticles loaded with a poly(I:C) and antigen generated a stronger OVA-specific $CD8^+$ T cell response than soluble poly(I:C) given at a 10-fold greater dose. Analogous intranodal microparticle immunotherapy has also demonstrated antigen-specific suppression in a mouse model of multiple sclerosis by delivering myelin self-antigen and the immunoregulatory drug rapamycin [92].

Drug delivery approaches have also been explored to localize immuno-therapy agents to the tumor microenvironment. In a strategy to promote T cell proliferation and simultaneously attenuate the immunosuppressive tumor microenvironment, Park and colleagues designed PEGylated liposomes to deliver IL-2 and a TGF-β inhibitor (Fig. 4) [93]. The nanoscale vehicles were composed of a lipid bilayer surrounding a degradable, polymeric core

encapsulating the hydrophilic cytokine, IL-2, and the small hydrophobic TGF- β inhibitor, SB505124 (SB). To deliver sustained release of both drugs upon hydrolysis of the internal polylactide-PEG core, SB was solubilized with β-cyclodextrans to enable encapsulation of the small hydrophobic molecule. Solubilization minimized the burst release observed compared to when SB was loaded in the absence of β-cyclodextrans and improved sustained release to over 7 days *in vitro*. The authors demonstrated the efficacy of this combination therapy as weekly intratumoral injections of nanoscale liposomes significantly improved survival and delayed tumor growth in a mouse model of melanoma, more so than liposomes bearing individual drugs. Strikingly, intratumoral numbers of activated $CD8^+$ T cells and natural killer (NK) cells more than doubled upon liposome administration. Lastly, using NK-depleted mice, they showed liposomal-mediated tumor suppression required NK cells.

Targeting of multiple immune compartments for coordinated activation can also be desirable. In one example, researchers covalently linked CpG to small interfering RNA (siRNA) to preferentially target myeloid and B cells rich in TLR9 [94]. Their combinatorial approach sought to activate the TLR9 immunostimulatory pathway and silence Stat3, an important oncogenic transcription factor that mediates immunosuppressive activity. By covalently linking CpG to Stat3 siRNA, the researchers hypothesized that TLR9 ligation, located endosomally, would facilitate cytosolic escape of the construct and improve siRNA gene silencing. The efficacy of their engineered platform was demonstrated in murine tumor models of melanoma and metastatic lung cancer. Administration of non-conjugated CpG and *Stat3* siRNA produced only minor anti-tumor effects compared to the covalently linked combination. Additionally, the frequency of $FoxP3^+$ Tregs within the $CD4^+$ T cell compartment intratumorally diminished from ~60% to 25% post CpG-Stat3 siRNA peritumoral injections. The authors found that CpG-siRNA gene silencing was dependent on TLR9 expression, though it was unclear whether engagement of TLR9 promoted endosomal release or altered intracellular processing.

3. Controlled-release approaches to recruit immune cells

While targeting systems have been investigated for in vivo drug delivery, controlled-release materials to promote cellular recruitment and localized conditioning have also gained traction for immunotherapy. Traditionally, synthetic biomaterial scaffolds have primarily been investigated in applications for tissue engineering (reviewed in [95]). Scaffolds with high porosity are compatible carriers for cellular engraftment or recruitment and also allow encapsulation of biologics to direct cell function. Many of the same attributes that makes biomaterial structures desirable for tissue engineering can be applied for immunomodulation. Biomaterials can be engrafted with immune cells in adoptive cell therapies to improve leukocyte viability and subsequent immune responses [96]. Similarly, biological signals incorporated into biomaterial scaffolds have been broadly explored for immunomodulation (reviewed in [97]). This section of the review discusses the controlled-release material approaches that have been developed to recruit and modulate immune cells *in situ*.

Inflammatory responses following biomaterial implantation coincide with recruitment of innate immune cells to the site of injection. This is advantageous for combinatorial therapies

that seek to deliver immunomodulatory drugs to APCs. However, the significance of DCs as the most effective APC can be diminished by its low frequency [21]. In response, some drug delivery approaches intend to maximize DC recruitment, differentiation, and proliferation. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine produced by a number of myeloid and supporting cells that features prominently in the pursuit of a number of DC-recruiting biologics. GM-CSF has demonstrated capacity for promoting DC proliferation, recruitment of DCs, antigen cross-presentation, and differentiation of monocytes into DCs [98–101]. Incorporation of GM-CSF has been explored both in emerging immunotherapy technologies and clinically. As has been discussed earlier, Sipuleucel-T immunotherapy utilizes GM-CSF to mature and expand DCs upon autologous isolation. GM-CSF has also been used as a supplementary therapeutic for cancer treatment to improve myeloid recovery following chemotherapy (reviewed in [102]).

Recently, a number of groups have combined GM-CSF with immunomodulatory agents in controlled-release materials to activate or suppress DCs. In 2009, researchers in the Mooney laboratory created a synthetic biomaterial scaffold to program DCs in a protective, anti-tumor fashion [19]. The authors fabricated biodegradable PLGA scaffolds that were made macroporous in order to create space for DC recruitment and antigen-presentation. Initial investigations demonstrated scaffolds loaded with GM-CSF were shown to mediate DC recruitment in a dose-dependent manner, improving DC recruitment by up to 12 times that of unloaded scaffolds. Upon the addition of the TLR9 agonist CpG, DC recruitment to the biomaterial was further improved as ~1.5 million DCs were isolated 7 days following *in vivo* implantation. Consequently, the highlight of this work was the combinatorial effect CpG and GM-CSF had in mitigating tumor burden in the mouse model of melanoma. PLGA matrices loaded with tumor lysate as antigen, CpG, and GM-CSF increased antigen-specific $CD8^+$ T cell recruitment, DC trafficking to lymph nodes, and resulted in a 90% survival rate by day 90 compared to 0% survival for unloaded scaffolds.

This combinatorial approach of using GM-CSF and CpG to modulate DC maturation has been explored in the same laboratory through other drug delivery vehicles as well. One strategy implemented an injectable material that agglomerated *in vivo* to form a macroporous scaffold, improving the safety profile by removing the need the surgical implantation [103]. Results from this work show mesoporous silica rods subcutaneously injected can deliver sustained release of GM-CSF, CpG, and OVA as a model antigen to generate a potent OVA-specific cellular and humoral response (Fig. 5). By altering the size of the microrods, they demonstrated that larger aspect-ratio material augmented cellular recruitment, which they attributed to the larger interparticle pores allowing robust cellular infiltration. Notably, they found that three repeated booster injections of the unencapsulated soluble factors were required to produce an OVA IgG_{2a} antibody response comparable to that of the single injection of the microrod technology encapsulating the same factors. This combinatorial drug delivery strategy also significantly minimized tumor growth and survival using an EG.7-OVA tumor mouse model.

Applications of GM-CSF as a DC chemokine has also been explored in tandem with other immunomodulatory drugs to moderate immune tolerance. Lewis et al. describe a dual-microparticle approach consisting of small (~1 μm), phagocytosable microparticles and

large (~30 μm), non-phagocytosable microparticles designed to recruit DCs and induce tolerance in type 1 diabetes upon subcutaneous injection [104]. Briefly, large particles encapsulating GM-CSF and the tolerogenic drug TGF-β1 served to create a microparticle depot to recruit and condition DCs, respectively, via extracellular pathways. Simultaneous delivery of small, phagocytosable particles containing insulin B:9–23 peptide, a primary autoantigen for type 1 diabetes, and an additional immunomodulatory factor, vitamin D3, targeted the intracellular delivery of factors to recruited DCs. The premise behind the system was to promote presentation of autoantigen in a tolerogenic context to reeducate aberrant autoreactivity. *In vitro* characterization demonstrated the efficacy of this system, inducing tolerogenic DC phenotypes with downregulated expression of CD86 and MHC-II molecules, suppression of allogeneic T cell proliferation, and induction of $FoxP3^+$ Tregs. Cumulatively, its effect *in vivo* resulted in a 40% prevention rate of type 1 diabetes in 4-week old non-obese diabetic mice.

In a similar approach to induce antigen-specific tolerance in a type 1 diabetes model, researchers fabricated a peptide hydrogel that delivered sustained release of GM-CSF to recruit DCs to an immunomodulatory microenvironment [105]. The hydrogel contained PLGA microparticles encapsulating denatured insulin to promote self-tolerance to recruited DCs and, notably, CpG. While CpG is more classically used as a vaccine adjuvant in an inflammatory capacity, the authors note it can elicit anti-inflammatory effects such as inducing IL-10 production [106]. This approach protected disease incidence in 40% of pre-diabetic mice. Additionally, results demonstrated that hydrogel implantation generated a resolvable granulomatous tissue at the site of administration that recruited an array of leukocyte populations and with some characteristics of a tertiary lymphoid organ.

In another DC recruiting strategy pioneered by the Roy group, the DC chemokine MIP3α, also known as CCL20, was loaded into a crosslinkable dextran vinyl sulfone and tetra-functional PEG thiol hydrogel along with PLGA microparticles encapsulating IL-10 targeting siRNA and plasmid DNA antigen [107]. The authors built off their original research which demonstrated microparticles bearing IL-10 siRNA boosted DC maturation, T cell proliferation, and skewed the *in vivo* T cell response to a Th1 pheno-type [108]. By incorporating a DC chemokine into biodegradable hydrogels, Singh et al. hypothesized that more DCs would encounter siRNA and plasmid antigen and the hydrogel might serve as an artificial DC priming center. The results demonstrated that a sustained chemokine gradient exuded in a controlled-release manner from the hydrogel was pivotal to its success, as the combinatorial system recruited ~5-fold more DCs over an extended period of time compared to an equivalent bolus dose in vitro. The recruited DCs efficiently infiltrated hydrogels and displayed notably diminished gene expression of IL-10. *In vivo* results demonstrated that intramuscular injection of the combinatorial DC-recruiting platform induced T-cell class switching toward a Th1 response, inducing $CD8^+$ CTL responses ~3-fold more robust compared to administration of soluble components alone [109]. An iteration of this work was further explored, when in 2014 Pradhan et al. incorporated the adjuvant CpG into the IL-10 siRNA and plasmid DNA antigen bearing microparticles [110]. Hydrogels formulated with the full combinatorial payload (MIP3α and microparticles containing IL-10 siRNA, CpG, and plasmid DNA) significantly improved prophylactic survival in mice immunized with A20 B-lymphoma cells compared to controls without all four agents.

Incorporation of biological factors into scaffolds can also be accomplished by surface ligation. This may be advantageous when designing systems that benefit from localized effects and to limit systemic toxicity from soluble factors. This approach has been suggested as a potential solution for allograft tolerance, wherein researchers conjugated TGF-β1 to glass beads to generate Tregs [111]. Combinatorial approaches of ligation of biologics has also been explored. Hume and colleagues functionalized IL-10 and TGF-β1 to PEG hydrogels in an effort to minimize DC maturation in vitro [112]. Immature DCs seeded in hydrogels with immobilized pro-tolerogenic cytokines maintained their immature phenotypes following incubation with LPS, displaying reduced expression of MHC-II molecules and IL-12 production. In another example, researchers demonstrated that incorporation of cell-adhesion moieties improved subsequent interaction between recruited T cells and immunomodulatory microenvironment in a hydrogel platform [113].

Specifically, the Anseth laboratory developed a PEG hydrogel functionalized with intercellular adhesion molecule-1 and α-Fas antibodies, where Fas is a well-established death receptor, to modulate localized T cell apoptosis. This platform could be valuable as a complementary immunotherapy on the surface of cell encapsulation materials to reduce allograft rejection. Results show that hydrogels coated with both ligands improve T cell apoptosis by 50% *in vitro* compared to hydrogel only containing α-Fas antibodies.

Macroporous biomaterial scaffolds contain large surface area which encourages complex biomaterial design and conveys higher order spatiotemporal control of the surrounding microenvironment. In a particularly innovative combinatorial approach, Stephan et al. developed an alginate scaffold implant to improve adoptive transfer of tumor-specific T cells [114]. In the approach, tumor-specific T cells were loaded into the alginate scaffold which was conjugated with the collagen-mimetic peptide (GFOGER) to stimulate T cell migration (Fig. 6). Additionally, the scaffold contained silica microparticles with surface-bound α-CD3, α-CD28, and α-CD137 antibodies to stimulate lymphocyte proliferation and encapsulated an IL-15 superagonist as a T cell survival signal. This multipronged approach minimized numerous steps normally required for adoptive cellular transfer (e.g., T cell expansion and viability, intratumoral delivery), as biological factors included in the alginate scaffold account support these functions *in vivo*. Notably, upon peritumoral administration of the biopolymer in a murine tumor model, adoptively transferred tumor-specific T cells were isolated from tumor beds and found to suppress tumor growth. The inclusion microparticles bearing survival and proliferative factors was shown to generate robust T cell expansion and improve the viability of encapsulated cells released from the scaffold. Additionally, GFOGER linkage onto the polymer matrix augmented T cell migration and resulted in a significantly higher number of T cells released from the scaffold.

4. Future directions

There is mounting evidence that combinatorial immunotherapy can offer significant advantages in treating complex immune-associated diseases. While we have described a number of approaches that have demonstrated potential as therapeutic strategies, the future of combinatorial drug delivery is not limited to the approaches discussed. The continually expanding body of immunotherapy research provides a strong foundation from which

scientists can draw upon. Certain drug delivery opportunities may prove particularly important in establishing successful immunotherapies in the future.

4.1. Combinatorial use of monoclonal antibodies

Checkpoint blockade therapy has buoyed the enthusiasm for monoclonal antibody immunotherapy, as it has produced some of the most successful human clinical trials for cancer in recent years (reviewed in [115]). Drug delivery strategies to exploit the expanding field of monoclonal antibody immunotherapy could be immensely beneficial. For example, controlled-release approaches can aid in mitigating antibody clearance that limits their therapeutic effects [116]. Additionally, localized release adjacent to specific immune-associated structures (e.g., peritumorally, perinodally) could augment immunotherapy and address renewed concerns of systemic toxicity. However, limitations with biomaterial incorporation to deliver sensitive biologics are well established. Functional activity can be lost when attempting to encapsulate or conjugate large proteins to drug delivery vehicles [117]. Novel drug delivery strategies to circumvent these challenges are actively being explored. Recent work, for example, developed a method to reduce antibody oxidation encapsulated in silk biomaterials via methionine functionalization [118]. Strategies that utilize monoclonal antibodies in combination with other immunotherapeutics have also demonstrated promise in pre-clinical investigations. One approach explored this past year saw a vaccine composed of four components (anti-PD-1, tumor-specific antibodies, recombinant IL-2, and albumin-binding CpG-peptide antigen constructs) build upon checkpoint inhibitor blockade therapy to mediate improved, antigen-specific survival in tumor-bearing mice [119]. Another recent approach utilized lipoprotein nanodiscs to deliver multiple tumor-associated epitopes and the adjuvant CpG while intraperitoneally administering checkpoint blockade therapy [120]. The combinatorial technology, when applied in coordination with anti-PD-1 and anti-CTLA4 antibodies, dramatically improved anti-tumoral immunity compared to nanodiscs alone. These results suggest that applications involving monoclonal antibody therapy could benefit from combinatorial and controlled-release approaches.

4.2. Combinatorial use of immunoablation and cellular reprogramming

Often accomplished through monoclonal antibodies, selective depletion of immune compartments has been investigated as an alternative therapy for systemic, cytotoxic chemotherapy. Monoclonal antibodies against $CD20^+$ B cells are frequently employed for Non-Hodgkin Lymphoma [121,122]. Similarly, depletion of $CD25^+$ Tregs has been investigated in a clinical setting for augmenting anti-tumor vaccines [123]. Approaches utilizing immunoablating techniques have also generated interest in autoimmune disease to deplete autoreactive cells in order to reprogram homeostatic immunity. Combination therapy with anti-thymocyte globulin and GM-CSF has been shown to increase Treg frequency, alter DC pheno-types, and improve survival in mouse models of type 1 diabetes [124,125]. This combinatorial approach was validated when explored in clinical trials of type 1 diabetics, improving Treg frequency and preserving β-cell function compared to placebo controls [126,127]. While careful consideration should be given to therapies that completely ablate leukocyte subpopulations for fear of promoting systemic immunosuppression, there is

growing evidence that suggests immunoablation in combination with immunomodulation can be a useful approach.

4.3. Combinatorial use with antigen-specific therapy

A recurring theme throughout the research discussed in this review has been the incorporation of disease-specific antigen in combinatorial drug delivery platforms. Antigen-specific immunotherapy is highly desirable to elicit controlled, directional immune responses. Viable antigen-specific therapies are advantageous compared to broadly cytotoxic or immunosuppressive drugs that can result in systemic toxicities. There is a wealth of research on immunotherapy systems that utilize disease-relevant antigen in coordination with immunomodulatory drugs, which indicates the value of this combination (reviewed in [9]). Controlled-release biomaterials have been extensively studied to deliver antigen, as their tunable properties can be beneficial in shaping immune responses. As discussed, sustained release of antigen from biodegradable PLGA nanoparticles has improved both prolonged antigen-presentation and the capacity for cross-presentation in bone-marrow derived DCs [61]. Mimicking long-standing clinical approaches for allergy immuno-therapy, biomaterials have also been studied to deliver self-protein to induce antigen-specific tolerance. In one approach, intravenous administration of polystyrene nanoparticles conjugated to myelin sheath epitopes ameliorated multiple sclerosis in a mouse model [128]. Vaccines that rely on immunomodulatory drugs may be improved upon by the inclusion of antigen. For example, nanoparticles containing the immuno-suppressive drug rapamycin were most effective in generating sustained humoral and cellular responses when antigen was included in the formulation [129,130]. Modulating immunity in antigen-specific immune directions will likely continue to be an important feature of forthcoming combinatorial immunotherapy strategies.

5. Conclusions

There is growing interest in combinatorial drug delivery strategies for immunomodulation. The breadth of new research highlighting the efficacy of multi-drug immunotherapy schemes is perennially expanding. Biomaterial platforms offer numerous advantages to optimize drug delivery for immunotherapy. While there are many challenges associated with successful immunotherapy, we are optimistic that combinatorial drug delivery platforms can help overcome the limitations associated with previous therapeutic interventions.

Acknowledgments

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases R01DK091658 and R01DK098589.

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Abbreviations

CAR	chimeric antigen receptor
Treg	regulatory T cell
PD-1	programmed death-1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
PD-L1	programmed death ligand 1
APC	antigen-presenting cell
DC	dendritic cell
TLR	tolllike receptor
OVA	ovalbumin
PLGA	poly(lactide-co-glycolide)
MPLA	monophosphoryl lipid A
poly(I:C)	poly(inosinic:cytidylic acid)
SB	SB505124
NK	natural killer
siRNA	small interfering RNA
GM-CSF	granulocyte-macrophage colony-stimulating factor

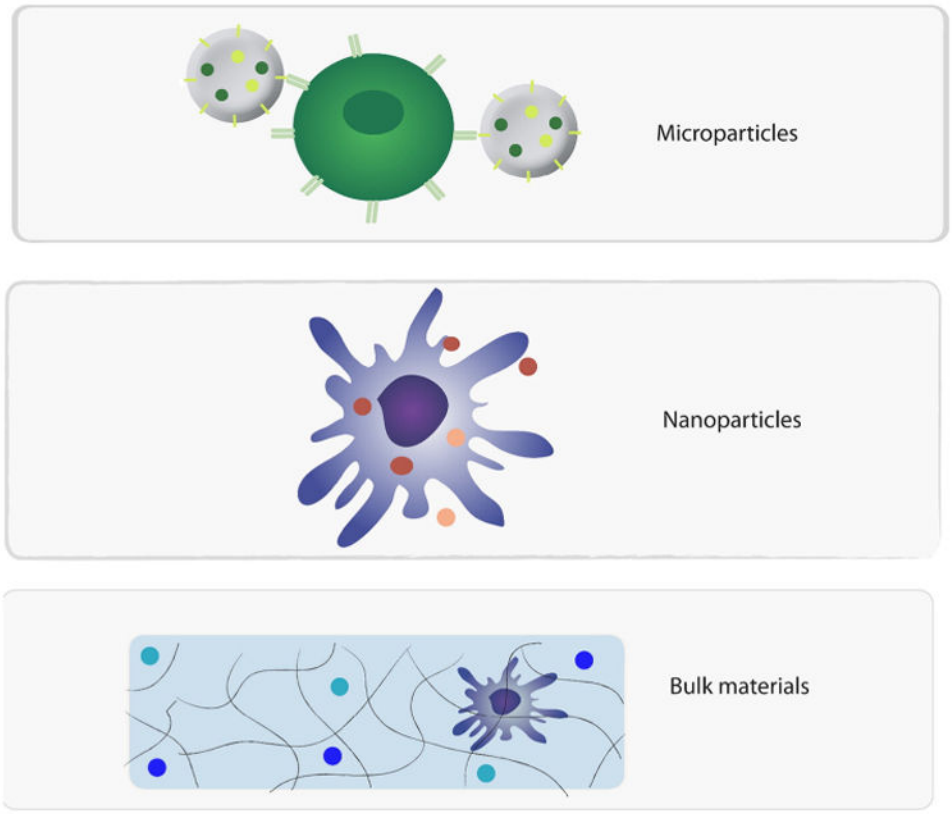

Fig. 1.

Various biomaterial platforms have been developed for combinatorial drug delivery to modulate immunity. A few of the most frequently applied systems are depicted here. (Top) Micro- and nanoparticle vehicles have been designed for specific targeting/retention to modulate subpopulations of immune cells (e.g., T cells, DCs). (Bottom) Alternatively, bulk materials have been explored to actively recruit immune populations *in vivo*.

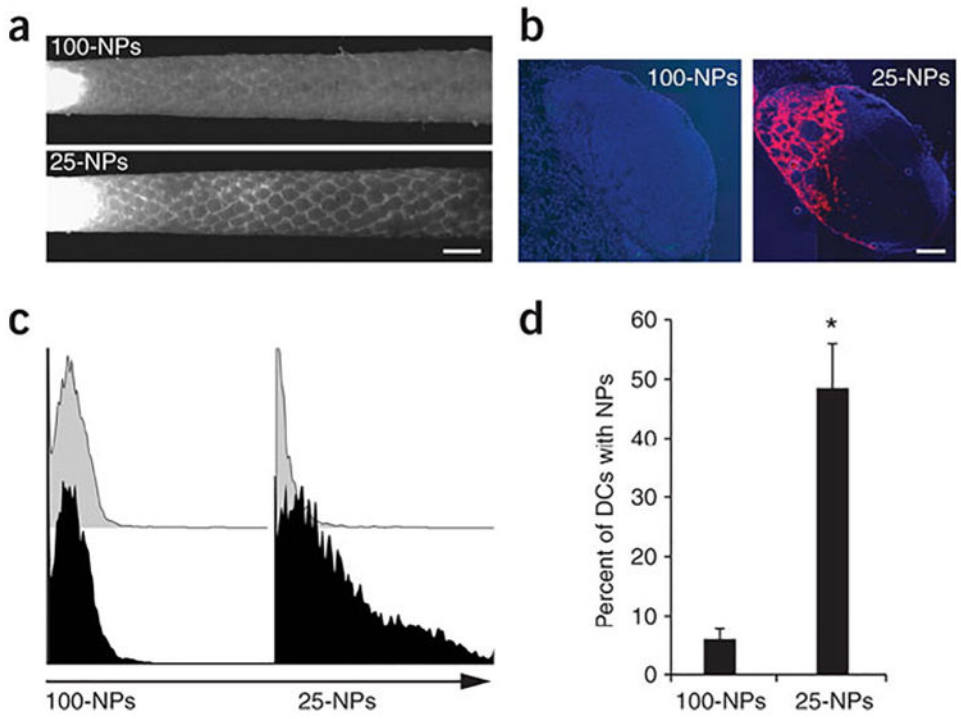

Fig. 2.

Ultra-small nanoparticles (25 nm) more efficiently drain to lymph nodes and are taken up by DCs than large nanoparticles (100 nm) upon intradermal injection. Fluorescently-labeled nanoparticles are seen draining through lymphatic capillaries (a; scale bar, 1 mm) and from isolated lymph nodes (b; scale bar, 200 μm; blue:cell nuclei, red:nanoparticles), with 25 nm particles trafficking more adeptly. Flow cytometry histograms (c) examined DC uptake of nanoparticles (black) or phosphate-buffered saline (grey) isolated from draining lymph nodes and was quantified (d). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

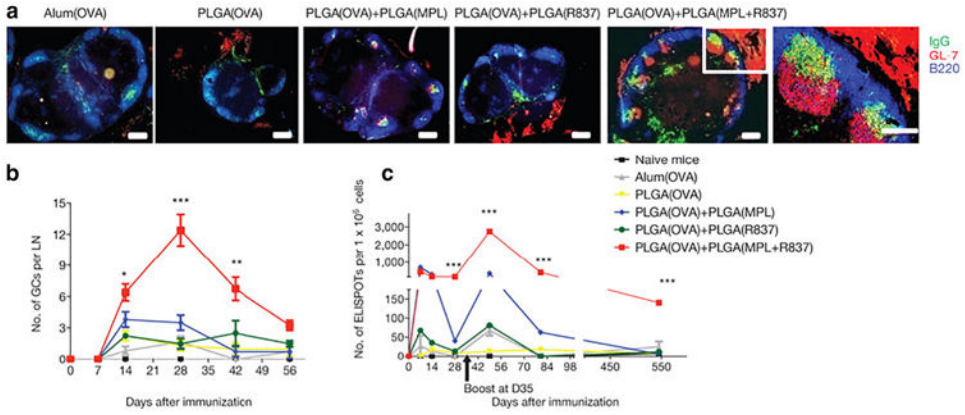

Fig. 3.

Immunization with combinatorial TLR agonists (MPLA:TLR4 and R837:TLR7) encapsulated in polymeric PLGA nanoparticles induces robust germinal center formation. (a) Draining lymph nodes were isolated four weeks post-immunization and stained for germinal center markers, with combinatorial agonist delivery producing the most persistent formation (scale bar, 200 μm). (b) The number of germinal centers in draining lymph nodes over time was stratified by treatment group. (c) Combinatorial TLR agonists promote longevity (~1.5 years) of antibody-secreting cells compared to single TLR agonists as determined by an ELISPOT assay. Reprinted by permission from Macmillan Publishers Ltd.: Nature [54], copyright 2011.

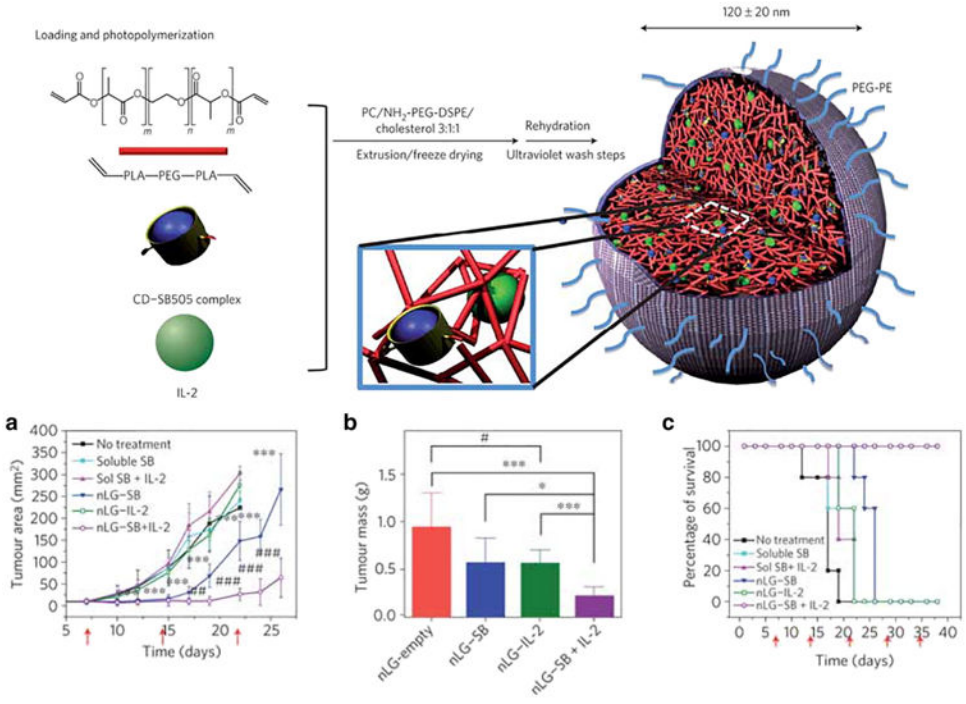

Fig. 4.

Synthesis scheme of PEGylated liposomes with polymeric cores to deliver the T cell proliferative factor, IL-2, and a TGF-β inhibitor, SB505124. Methacrylated cyclodextrans (CD) were used to solubilize the small, hydrophobic inhibitor (SB505). (Top) CD-SB505 complexes and IL-2 were loaded into a biodegradable polymer core (red) with a PEGylated liposomal exterior (grey). Photoinduced polymerization of the polymer core and the acrylated-CD induces nanoscale liposome gel (nLG) formation, capable of delivering sustained release of both drugs upon hydrolysis of the internal polymeric core. (a–c) B16 melanomas were tracked over time in response to various treatments, with combinatorial nLGs delaying tumor growth most effectively. Red arrows indicate intratumoral treatment administration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

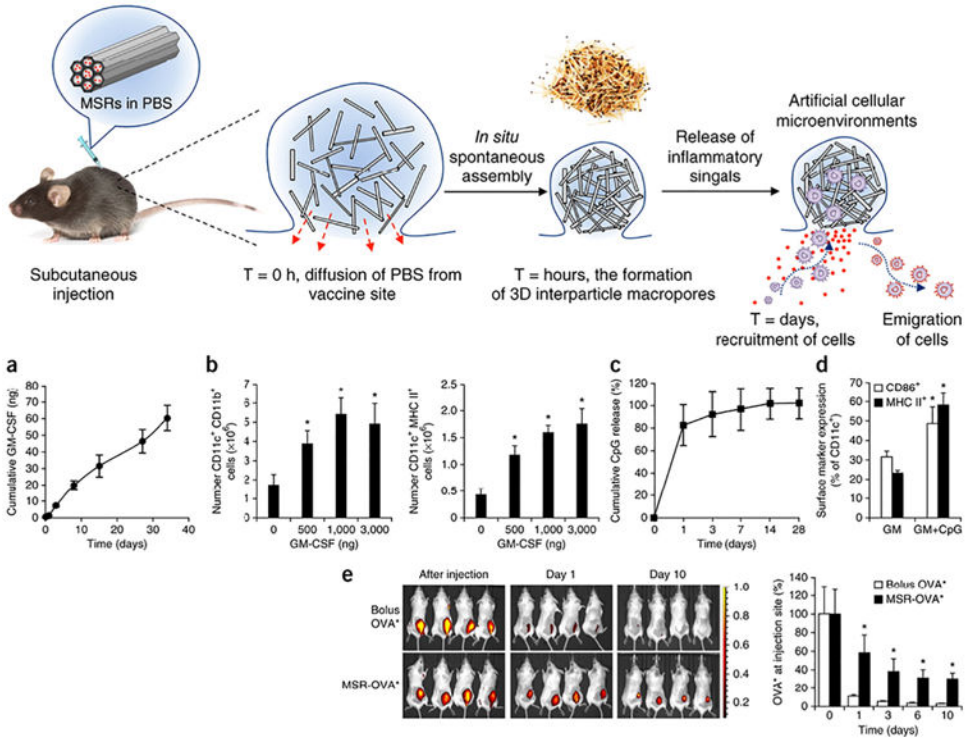

Fig. 5.

Mesoporous silica rods (MSRs) aggregate upon injection, forming macroporous spaces where host cells can be recruited and educated byencapsulated immunomodulatory drugs, GM-CSF and CpG. (a) *In vitro* cumulative release of GM-CSF from MSRs (b) $CD11c^+$ $CD11b^+$ (left) and $CD11c^+$ MHC II^+ (right) DCs were recruited to MSR scaffolds in a dose-dependent manner. (c) *In vitro* cumulative release of CpG from MSRs. (d) CpG inclusion *in* MSRs augmented DC activation, increasing surface expression of CD86 and MHC class II molecules. (e) Fluorescent in *vivo* imaging demonstrated MSRs loaded with OVA antigen deliver sustained release of protein over an extended period compared to bolus OVA injection.

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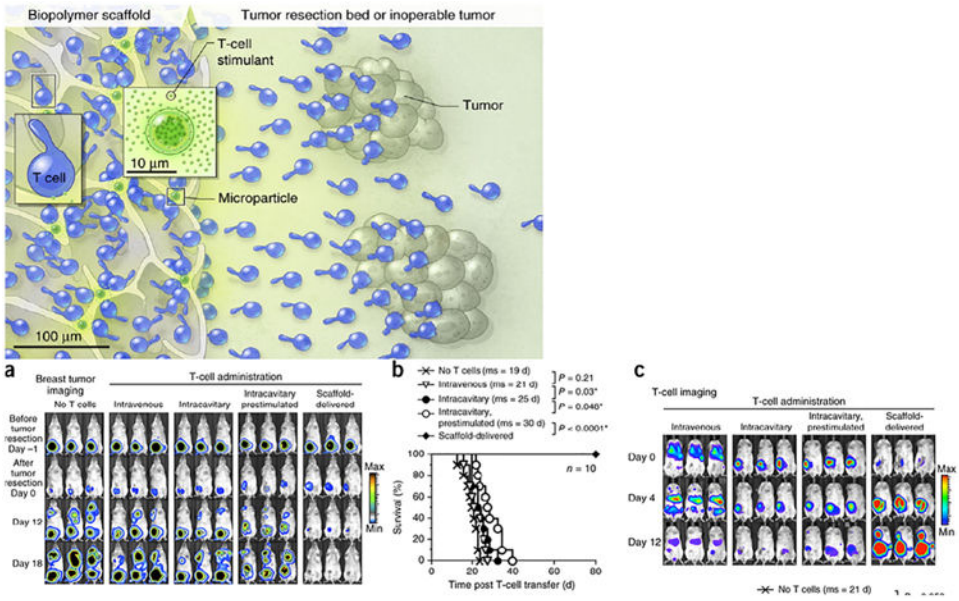

Fig. 6.

(Top) Illustration of the biomaterial system. Collagen-mimetic peptides conjugated to the polymeric scaffold encourage trafficking of loaded tumor-specific T cells into surrounding tumor beds. Additionally, microparticles in the scaffold encapsulating an IL-15 superagonist and with surface-bound anti-CD3, anti-CD28, and anti-137 stimulate lymphocyte survival and proliferation.(a) Bioluminescent imaging of 4T1 breast tumors demonstrates the potency of the biomaterial system, as minimal tumor presence is observed with the combinatorial technology compared to other treatments. (b) Kaplan-Meier survival curves for treated and control mice (ms:median survival). (c) Bioluminescent imaging of adoptively transferred T cells, showing the scaffold technology encourages robust T cell proliferation and migration. Reprinted by permission from Macmillan Publishers Ltd.: Nature Biotechnology [114], copyright 2014.

Table 1

Biomaterial platforms explored in vivo for combination immunotherapy.

Reference	Delivery system	Delivery target	Drug(s) delivered	Outcome
46, 47	PLGA/PROMAXX™ microparticles	DCs	Antisense oligonucleotides against CD40, CD80, and CD86	Reversed new-onset diabetes in non-obese diabetic (NOD) mice
50	Polypropylene sulfide nanoparticles	Tumor-draining LNs/DCs	CpG and paclitaxel	Skewed $CD4^+$ T cells to a Th1 phenotype and hindered melanoma growth
54	PLGA nanoparticles	DCs/B cells	MPLA, imiquimod, and antigen	Produced persistent (N1.5 yr) germinal center formation and plasma-cell responses
62	PLGA nanoparticles	DCs	Surface conjugated MPLA and encapsulated CpG and antigen	Robust antigen-specific T cell response was dependent on combination delivery
68	PLGA nanoparticles covalently coupled with α-CD40	DCs	Surface conjugated α-CD40 and encapsulated Pam3CSK4, poly(I:C), and antigen	α-CD40 coupling improved selective DC uptake and prolonged survival in a melanoma model
69	PEGylated liposomes covalently coupled with CpG and α-CD40	DCs	Surface conjugated α-CD40 and CpG	Surface conjugation improved nanoparticle retention at the local tumor site, minimized systemic inflammation, and mitigated melanoma tumor growth in mice
80	PLGA nanoparticles covalently coupled with α-CD4	T cells	TGF-β1 and IL-2	Increased the percentage of regulatory T cells
93	PEGylated liposomes surrounding PLA-PEG core	Tumor microenvironment/T cells	TGF-β inhibitor and IL-2	Doubled the number of tumor-infiltrating NK cells and delayed tumor growth in a melanoma model
94	TLR9-ligand construct	Myeloid/B cells	Stat3 siRNA and CpG	Minimized tumor burden in melanoma and metastatic lung cancer models. Only the covalently linked construct mediated potent anti-tumor effects
19	PLGA scaffolds	DCs	GM-CSF, CpG, and antigen	Demonstrated a dose-dependent recruitment of DCs and diminished tumor burden in a melanoma model
103	Silica micro-rods	DCs	GM-CSF, CpG, and antigen	Generated robust humoral immune responses and ameliorated tumor burden
104	PLGA microparticles	DCs	GM-CSF, TGF-β1, vitamin D3, and antigen	Prevented type 1 diabetes onset in NOD mice

Reference	Delivery system	Delivery target	Drug(s) delivered	Outcome
105	PuraMatrix™ peptide hydrogel containing PLGA microparticles	DCs	GM-CSF, CpG, and antigen	Prevented type 1 diabetes onset in NOD mice
103, 104	Dextran vinyl sulfone and tetra-functionalized PEG thiol hydrogel & PLGA microparticles	DCs	CCL20 in hydrogel and PLGA MPs encapsulating IL-10 siRNA, CpG and antigen	Induced a robust Th1 response. A later iteration with CpG improved survival in mice immunized with B-cell lymphoma
114	Alginate scaffold seeded with tumor-specific T cells and silica microparticles	Seeded T cells	GFOGER, α-CD3, α-CD28, α-CD137, and an IL-15 superagonist	Improved T cell migration, viability, and expansion, resulting in augmented tumor suppression
120	Lipoprotein nanodiscs	DCs	CpG, antigen, and soluble anti-PD-1 and anti-CTLA4	Nanodiscs alone increased the frequency of antigen-specific T cells. In combination with checkpoint inhibitor therapy, eliminated tumor burden in colon carcinoma and melanoma mouse models