Engineered Cytokines for Cancer and Autoimmune Disease Immunotherapy

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Abstract

Cytokine signaling is critical to a range of biological processes including cell development, tissue repair, aging, and immunity. In addition to acting as key signal mediators of the immune system, cytokines can also serve as potent immunotherapies with more than 20 recombinant products currently FDA-approved to treat conditions including hepatitis, multiple sclerosis, arthritis, and various cancers. Yet despite their biological importance and clinical utility, cytokine immunotherapies suffer from intrinsic challenges that limit their therapeutic potential including poor circulation, systemic toxicity, and low tissue- or cell-specificity. In the past decade in particular, we and others have devised methods with which to engineer cytokines in order to overcome such challenges and here, we review the myriad strategies that may be employed in order to improve the therapeutic potential of cytokine and chemokine immunotherapies with applications in cancer and autoimmune disease therapy, as well as tissue engineering and regenerative medicine. For clarity, we collect and present these strategies as they vary across size scales, ranging from single amino acid substitutions, to larger protein-polymer conjugates, nano/micrometer-scale particles, and macroscale implants. Together, this work aims to provide readers with a timely view of the field of cytokine engineering with an emphasis on early-stage therapeutic approaches.

Graphical Abstract

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Competing Interests
The authors declare no competing interests.
Cytokines are master regulators of the human immune system and dysregulation of cytokine signaling networks is a hallmark of cancer, autoimmunity, and a variety of other diseases. In recent years, we and others have developed methods with which to engineer potent, safe, and specific cytokine immunotherapies that, here, we review with an emphasis on early-stage and materials-based therapeutic approaches.

Keywords
immunotherapy; drug delivery; immune cell signaling; drug design

1. Introduction
Therapies that promote adaptive immunity have demonstrated great potential in the treatment of pathogens, malignancies, and other immune-dysfunction driven diseases. Cytokines, as the so-called “third signal” required for antigen-specific immune activation, thus represent an important and often indispensable component of modern approaches to immunotherapy.[1] Yet, despite their biological importance, the safe and effective administration of cytokines in the clinic presents a variety of challenges ranging from poor circulation to toxicity and pleiotropic signaling to hyperactive immune responses.[2] To address these challenges, investigators from a wide range of disciplines have come together to explore innovative approaches to better model the complexity of immunity and disease, improve the bioavailability of biologics, and mitigate the toxicity of potent biotherapeutics; for example, through enhanced 3D modeling of tissues, polymer conjugation, or targeted, hyperlocal delivery, respectively. Here, we provide an overview of the myriad approaches
which have been employed to improve the therapeutic benefit of both immune-stimulating and immunosuppressive cytokine therapy, with a particular emphasis on early-stage, engineering-based approaches. For clarity, we hierarchically categorize these approaches based on relative size, ranging from single amino acid substitutions (i.e. muteins) to protein-polymer conjugates and macroscale biomaterials implants, with the goal of providing the reader with an appreciation of both the diversity and therapeutic promise of engineered cytokine-based immunotherapies.

2. Immunostimulatory Cytokines

2.1. Applications in Cancer

Recombinant cytokines represent one of the earliest examples of cancer immunotherapies approved by the FDA. Interferon-alpha (IFN-α) for example, was first approved in 1986 for treatment of hairy cell leukemia, due to its ability to promote apoptosis and limit growth of tumor cells.[3] Shortly thereafter, interleukin-2 (IL-2) was approved for use in 1992 as a treatment for metastatic renal cancer.[4] Both appeared to be highly promising candidates, with small subsets of patients achieving complete response.[2c,5] However, systemic administration of both cytokines yielded severe adverse events in many patients, including treatment-related deaths in some instances.[6] Specifically, clinical trials of high-dose IL-2 therapy in patients with metastatic cancer resulted in high incidence rates of severe cardiotoxicity, neurotoxicity, and treatment-related deaths.[6c,7] Similarly, a recent clinical trial with recombinant IL-15 demonstrated increases in peripheral CD8+ T cell and natural killer (NK) cell populations, but was often coupled with severe hypotension, lymphopenia, or neutropenia, and did not yield any clinical responses.[8] To localize the clear benefits offered by cytokine administration and to attenuate off-target effects, many engineered cytokine designs have been created for specific trafficking into tumor microenvironments. As will be further discussed herein, these designs can incorporate small proteins or antibodies that target proteins upregulated at tumor sites, while hydrogels and other implants can be administered subcutaneously or intramuscularly to better constrain the area of cytokine activity (Figure 1). Other designs have sought to address other limitations of recombinant cytokine administration, such as short half-life in circulation.[9] A general comparison of the strengths and limitations of these design strategies can be found Table 1.

2.1.1. Mutant and Designer Cytokines—Amino acid substitution represents one of the most salient methods by which cytokine activity can be modulated. Such mutant proteins, or muteins, often exhibit enhanced or receptor-biased binding affinity that can improve the potency or specificity of associated immune activation. Due to its often pleotropic effects in vivo, the cytokine IL-2 serves as an ideal candidate for pharmacological re-engineering, whereby preferential binding with interleukin-2 receptor (IL-2R) βγc on CD8+ T cells and NK cells, rather than IL-2Rαβγc (CD25) on immunosuppressive T regulatory cells (Tregs), can dramatically improve drug-induced immune elimination of cancer.[10] Boyman and Garcia demonstrate such an approach in the development of an IL-2 superkine, an engineered variant of the protein with enhanced affinity towards IL-2Rβc.[11] Using yeast surface display, they identified IL-2 mutations (L80F/R81D/L85V/I86V/I92F) that conferred high affinity binding with IL-2Rβ and potent downstream phosphorylation of
STAT5. Treatment with IL-2 superkine in a syngeneic B16F10 melanoma model reduced tumor burden more favorably than wild-type (WT) IL-2 and to a comparable extent as IL-2/m monoclonal antibody (mAb) complexes (discussed in later sections). Importantly, therapeutically relevant doses of the protein resulted in less pulmonary edema than WT IL-2, a prominent side-effect of such therapy in patients.\textsuperscript{12} Carmenate \textit{et al.} adopt a related but complementary approach to engineer an IL-2 mutein (R38A, F42A, Y45A, and E62A) with decreased activity towards T\textsubscript{reg}, rather than increased activity towards CD8\textsuperscript{+} T cells and NK cells.\textsuperscript{13} More recently, Sun \textit{et al.} further advance this approach through two protein engineering methods detailed in later sections: fusion of an IL-2 superkine with (i) Fc protein to prolong circulation and (ii) epidermal growth factor receptor (EGFR) antibody fragments to promote tumor-targeting.\textsuperscript{14} In several syngeneic cancer models, this fusion protein (MDNA109, Medicenna Therapeutics) generated a strong and durable anti-tumor immune response, either as a monotherapy or in combination with tyrosine kinase inhibition. Together, these data demonstrate that seemingly minor but structure-guided perturbations in protein sequence can lead to dramatic improvements in the therapeutic utility of cytokines.

In addition to cell-specific immune activation, muteins can also be engineered to exclusively activate synthetic receptors present on adoptively transferred cell therapies. Ribas, Bluestone, and Garcia demonstrate the potential of this approach by developing an orthogonal IL-2/IL-2R pair which interact only with one another and not with their endogenous ligand/receptor pair (Figure 2).\textsuperscript{15} Using crystal structures of the ligand/receptor complex, they engineered a double mutant IL-2R\textsubscript{β} (H134D, Y135F) which lacked binding to the WT cytokine, then used yeast display-based evolution to mutate IL-2 in a manner that restored affinity only to the double mutant receptor. Following adoptive transfer of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells expressing orthoIL-2R\textsubscript{β} and adjuvant orthoIL-2 therapy in immunocompetent mice, they observed selective expansion of the engineered T cell population without incidence of serious toxicities. Transfer into a syngeneic B16F10 murine melanoma model demonstrated similar survival outcomes between WT and engineered IL-2 systems, further illustrating the potential for designer cytokine immunotherapies to synergize with existing treatment modalities for cancer such as chimeric antigen receptor (CAR)-T cell therapy. While reviewed in detail elsewhere,\textsuperscript{16} see Table 2 for a summary of current CAR-T cell therapies engineered (i.e. armored) to express various cytokines.

While promising, the introduction of cytokine mutations can often lead to manufacturing challenges associated with low protein stability, host immune responses that cross-react with the endogenous protein,\textsuperscript{17} and incomplete disruption of deleterious cell interactions (e.g. IL-2 binding to IL-2Rs). Baker and coworkers recently addressed this challenge through \textit{de novo} design of proteins that mimic the favorable interaction of IL-2 with IL-2R\textsubscript{βγc} but excluded those with IL-2R\textsubscript{α} (Figure 1a).\textsuperscript{18} The subsequent protein, Neo-2/15 (NL-201, Neoleukin Therapeutics), exhibited higher affinity for IL-2R\textsubscript{βγc} than human or mouse WT IL-2, and was incapable of binding IL-2R\textsubscript{α}. Additionally, in combination with anti-melanoma mAb TA99, the engineered protein demonstrated both reduced toxicity and significantly improved survival outcomes compared to native IL-2 combination treatment. While cytokine orthologs or mimics (and their engineered receptor pairs) hold broad therapeutic potential, their clinical translation remains nascent and, at present, it remains to be seen whether these approaches will continue to demonstrate impressive results in more
representative disease models or whether further iteration – computational or otherwise – will be required for clinical translation of this promising approach to immunotherapy.

2.1.2. Cytokine-Polymer Conjugates—Protein PEGylation, first developed in the 1970s, represents one of the earliest and most popular chemical modification strategies for therapeutic cytokines and other proteins. Modification of small (approx. <100 kDa) proteins with polyethylene glycol (PEG), or PEGylation, can often serve many purposes but is frequently employed to prolong drug circulation and thus enhance systemic drug exposure and associated drug activity. These polymers, characterized by their very high water solubility and conformational flexibility, are generally well-tolerated in patients with weak to no immunogenicity and are typically appended to proteins as linear polymers at N termini, as well as at lysine or cysteine residues. PEGylation can greatly increase protein hydrodynamic size (26 nm – 40 nm, for 5 kDa – 20 kDa PEG respectively), decreasing renal filtration, and can also decrease non-specific protein association that leads to opsonization or enzymatic degradation. PEGylated therapies have been developed for many disease contexts, including both solid and hematologic cancers and neutropenia, as well as autoimmune disorders such as multiple sclerosis and hemophilia A/B. Please see refs for detailed reviews of clinically approved cytokine-PEG conjugates. In contrast to the previously discussed methods which seek to bias the cell-selectivity of IL-2 through mutagenesis or protein design, bempegaldesleukin (NKTR-214, Nektar Therapeutics) achieves immune-cell selectivity through polymer modification at key ligand/receptor interfaces. The conjugate contains an average of six (20 kDa) PEG chains per molecule, each appended to lysine residues that are enriched at the interface between IL-2 and IL-2Rα, present on immunosuppressive Tregs, thus biasing the activity of bempegaldesleukin towards IL-2Rβγc. The cytokine-polymer conjugate additionally features a hydrolysable polymer linkage which sheds PEG at a controlled rate following administration, thereby prolonging IL-2 circulation, improving tumor accumulation (500-fold compared with rhIL-2), and facilitating sustained cytokine delivery to T cells both in the tumor microenvironment and in the peripheral blood. As a result, bempegaldesleukin has shown to induce efficient immune elimination and immune memory against rechallenge in syngeneic mouse models of breast and colon cancer, as well as the ability to enhance the persistence and antitumor immunity in combination with adoptive cell transfer (ACT) therapy in preclinical cancer models. The drug was well-tolerated in Phase I clinical studies, inducing tumor regression in 35% of patients and durable disease stabilization in 53.8%. Phase II/III trials of the drug in combination with nivolumab in urothelial cancer, muscle invasive bladder cancer and advanced melanoma (NCT04209114, NCT03729245, NCT03635983) are currently recruiting. Given the promising impact of ligand-receptor interface engineering demonstrated here, other cytokines with cognate heterotypic receptors (e.g. those with common βc or gp130 subunits) may benefit from related approaches.

Pegilodecakin (PEGylated IL-10; Eli Lilly) is another cytokine-polymer conjugate immunotherapeutic against a variety of solid tumors. While its precise structure is not publicly disclosed, the long-acting IL-10 receptor agonist induces oligoclonal T-cell expansion which has been shown to increase levels of CD8+ T cell tumor infiltration, as
well as associated granzyme and interferon-gamma (IFN-\(\gamma\)) levels, in preclinical models of cancer.\[^{20a-c,20e}\] In later Phase I clinical trials, responses to pegilodecakin monotherapy were relatively modest (27% overall response rate, ORR, in renal cell carcinoma);\[^{20b}\] however, subsequent trials presented substantial improvement when combined with programmed death receptor-1 (PD-1) antibodies such as nivolumab and pembrolizumab, yielding favorable treatment responses in renal cell carcinoma patients (40% ORR) and in non-small cell lung carcinoma patients (43% ORR).\[^{20a,20c,20e}\] Despite these promising findings, development of pegilodecakin was recently discontinued after Phase II and III studies failed to demonstrate significant improvements in overall survival when compared to, or combined with, FOLFOX therapy in pancreatic cancer patients (folinic acid, 5-fluorouracil and oxaliplatin)\[^{26}\] or anti-PD-1 therapy\[^{27}\] in non-small cell lung cancer patients.

As the previous examples suggest, one key challenge to the effective use of cytokines in cancer immunotherapy is the precise control – or targeting – of their activity. Inspired by the ability of a subset of cytokines (e.g. TGF-\(\beta\)1, IL-1\(\alpha\), IL-33) to activate in response to various biological stimuli,\[^{28}\] we recently hypothesized that modification of cytokines with photosensitive polymers could blunt cytokine activity when conjugated, then restore protein function upon exposure to light, allowing for enhanced control over the location, strength, and timescale of cytokine signals (i.e. “photokines,” Figure 1b, Figure 3a–d).\[^{29}\] We modified IL-2, IL-15, and IL-12 with PEG (5–20 kDa) appended to various \(\sigma\)-nitrobenzyl linkers and found that simple blue LED light exposure could selectively restore protein activity upon light exposure. Photo-labile polymer modification, alone, favorably biased IL-2 binding towards IL-2R\(\beta\gamma\)c, much like NKTR-214, and improved IL-12 plasma half-life 16-fold following intravenous injection in C57BL/6 mice. Monochromatic light exposure not only restored protein size, but also capacity for IL-2 induced T-cell proliferation, antigen-specific T-cell activation, and JAK/STAT pathway signaling. Current studies investigate the impact of light-guided cytokines on the persistence and targeting of adoptively transferred T cells, the use of photocages with high tissue penetrance, and optical logic-gated immune responses.

### 2.1.3. Cytokine-Protein Fusions

Another promising strategy for targeting cytokine activity to tumors is fusion with proteins or subdomains with affinity towards extracellular matrix (ECM) components which are enriched in the tumor microenvironment, such as collagen.\[^{30}\] Given that collagen is not a tumor cell-associated protein, but instead exists in the tumor stroma, it can serve as a “tumor-agnostic” targeting modality, potentially serving as the foundation for a broadly applicable immunotherapeutic. Wittrup and coworkers employ such a strategy in the engineering of IL-2 or IL-12 fused to lumican, a collagen type I/IV-binding protein (Figure 1c).\[^{31}\] Intratumoral administration of IL-2 fusion protein significantly improved treatment responses and tolerability in syngeneic mouse models of melanoma, as compared to recombinant IL-2 monotherapy, and further potentiated treatment response in combination with CAR-T or checkpoint blockade immunotherapy.

Hubbell and coworkers also employ a related strategy whereby the A3 domain of von Willebrand factor, previously reported to be a collagen-binding domain (CBD), was fused to both IL-2 or immune checkpoint protein blocking antibodies targeted against programmed death ligand-1 (\(\alpha\)PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (\(\alpha\)CTLA4).\[^{32}\] Following systemic therapy in syngeneic mouse models of melanoma, they observed...
complete remission in 9/13 mice receiving combined CBD-checkpoint inhibitor and CBD-IL-2 treatment, as compared to 1/13 mice achieving complete remission with unmodified combination treatment; monotherapy with CBD-IL-2 did not provide similarly promising results. In a separate study, the group also explored CBD fusion with IL-12 (Figure 1d), [33] finding that a single intravenous injection was capable of inducing complete response in 10/15 mice bearing syngeneic melanoma tumors. They also observed drug-induced immune memory responses in immunocompetent mouse models of breast cancer following tumor rechallenge. The more striking effects of CBD-IL-12 compared with CBD-IL-2 were attributed to the former cytokine’s ability to better stimulate an immune response within an immunologically cold tumor. As a tumor type-agnostic approach, microenvironment-targeting strategies hold great potential to improve the therapeutic benefit of cytokine and other protein-based immunotherapies.

In addition to fusing cytokines with proteins that alter their disposition within the body, they can also be combined with fragments of their cognate receptors in order to conditionally modulate their activity or sterically bias their affinity towards particular subsets of immune cells. One such superagonist complex, ALT-803, has demonstrated promising immunotherapeutic potential in both preclinical and clinical studies.[34] ALT-803 consists of an N72D mutant IL-15 that exhibits increased binding affinity towards IL-15Rβ, fused with a tandem IL-15Rαa fragment (sushi domain) and human IgG1 Fc protein (Figure 4). The resulting complex binds with 150-fold increased affinity to the IL-15 receptor expressed on both NK cells and T cells, and thus has greater biologic activity than IL-15 alone. This characteristic has provided for enhanced survival and tumor immunity in 5T33P and MOPC-315P multiple myeloma cancer models through a memory CD8+ T-cell mechanism, though independent from IFN-γ. Furthermore, proliferation of CD8+CD44high memory T cells was accompanied by upregulation of NKG2D without the presence of exhaustion markers such as PD-1. Together these characteristics suggest an innate-like phenotype capable of non-specific tumor killing.[35] Thus, unsurprisingly a comparison between ALT-803 and IL-15 in B16F10 and CT26 tumor models demonstrated superior anti-tumor activity by the so-called superkine; owing to its greater than 20 fold-higher in vivo half-life in addition to a dose-dependent increase in circulating immune cells. This increase in peripheral NK, CD4+, and CD8+ memory T cells also occurred after weekly dosing in cynomolgus monkeys and established the dosing regimen currently utilized in clinical studies.[36]

The first multi-center phase I trial of ALT-803 (NCT01885897) tested the therapeutic potential of ALT-803 in leukemia and lymphoma patients following relapse after hematopoietic cell transplantation. The study found no dose-limiting toxicities and greater than 96-hour serum concentrations after subcutaneous injection. Further, ALT-803 increased the expansion and function of CD8+ T cells and NK cells with no measurable effect on Tregs, which resulted in a response in 19% of patients.[34] This response rate may be increased via combination therapy. In preclinical studies evaluating the combination of anti-CD20 (rituximab) and ALT-803, short-term ALT-803 stimulation significantly increased degranulation, IFN-γ production and antibody-dependent cell cytotoxicity by human NK cells against B-cell lymphoma cell lines and primary follicular lymphoma cells. This effect significantly reduced lymphoma burden, and increased survival compared to anti-CD20
alone and enhanced the functionality of NK cells triggered by FcγRIIIa against lymphoma cells (in both Daudi lymphoma model in SCID mice and Raji lymphoma model in NSG mice).\textsuperscript{[37]} ALT-803 in combination with nivolumab was well tolerated with minimal grade 3 events in a phase I clinical trial (NCT02523469); a phase II trial of this combination is currently ongoing.\textsuperscript{[38]} Other promising approaches to cell-specific cytokine delivery include fusion with peptide-HLA domains to target IL-2 to CD8\(^+\) T cells with specificity towards HPV-associated tumors (CUE-101, Cue Biopharma; NCT03978689)\textsuperscript{[39]} and fusion with IL-2Rα (CD25) to bias IL-2 activity towards effector T cells rather than T\(_{\text{regs}}\) (ALKS 4230, Alkermes; NCT02799095, NCT03861793).\textsuperscript{[40]}

\section*{2.1.4. Antibody-Cytokine Complexes and Fusions—}

In addition to fusions with other proteins, cytokines may also be combined with IgG antibodies, or fragments thereof, to further improve tissue specificity, circulation, toxicity, and/or potency.\textsuperscript{[41]} This approach has been adapted to a range of cytokines including IL-2, IL-21, IL-12, tumor necrosis factor α (TNFα), IL-4 and IL-10, demonstrating efficacy in both murine models and clinical trials discussed further herein. These immunotherapies can incorporate whole antibodies or their smaller antigen-binding fragments (e.g. Fab, scFv, diabody, or nanobody domains) and may be linked with recombinant cytokines via affinity binding (\textit{i.e.} complexes) or via flexible peptide linkages (e.g. glycine-serine linkers) as so-called immunocytokines.

As discussed previously, cytokine affinity can be biased towards particular immune cell subsets through the introduction of site-specific mutations or chemical modifications (\textit{e.g.} polymer conjugation) that sterically disfavors binding with particular cognate receptor subunits. Analogous effects may also be achieved through complexation with antibody clones that bind cytokines in a site-specific manner.\textsuperscript{[10c]} For example, Boyman and coworkers found that IL-2-S4B6 murine anti-IL-2 mAb complexes preferentially bind with CD8\(^+\) T cells and NK cells (expressing IL-2Rβγc) while substitution of this antibody clone with JES6–1 led to almost exclusive binding with IL-2Rαβγc-expressing T\(_{\text{regs}}\).\textsuperscript{[42]} Delivery of these complexes \textit{in vivo} resulted in extended half-life and expansion of their cognate cell populations. This concept has been further enhanced by engineering antibody clones to mimic IL-2Rα binding (\textit{e.g.} the mimobody, NARA1) and thus abrogating IL-2Rα binding and T\(_{\text{reg}}\) activation \textit{in vivo} (Figure 5a–c).\textsuperscript{[43]} Treatment with IL-2-NARA1 complexes increased proliferation of CD8\(^+\) T cells with high effector function and low markers of exhaustion, resulting in tumor growth inhibition and increased survival in B16-F10 and transgenic \textit{Tyr::N-Ras}\textsuperscript{Q61K} \textit{Ink4a}−/− melanoma models. IL-2 antibody complex treatment was further enhanced by combination therapy in which treatment with both complexes and dendritic cell (DC) immunization increased antigen specific CD8\(^+\) T-cell proliferation and resulted in 100% survival of this treatment group in a B16F10 model.\textsuperscript{[44]}

Rather than biasing cell-specific activity, IL-2 may also be engineered to exert tissue-specific functions. This approach is exemplified by Hu14.18-IL-2, an immunocytokine composed of IL-2 fused to antibody clone 14.18 targeting GD2, a disialoganglioside commonly found in neuroectodermal tumors, including neuroblastoma and melanoma, at the Fc terminus. Hu14.18 was the first immunocytokine to enter clinical trials after demonstrating eradication of metastatic melanoma and neuroblastoma in immunocompetent murine models.\textsuperscript{[45]} Recent phase I/II clinical trials of Hu14.18-IL-2 indicate tolerability and efficacy that varies based...
on combination with additional therapies. In clinical trials of high risk neuroblastoma, Hu14.18-IL-2 combined with GM-CSF produced objective responses in 16.1% of patients; however, combination with induction chemotherapy produced a partial response in 76% of patients and a 2-year event free survival rate of 85.7%.\textsuperscript{[46]} Hu.14.18-IL-2 is currently in clinical trials for treating Stage IV unresectable melanoma in combination with radiation therapy, nivolumab, and ipilimumab (NCT03958383), as well as, in neuroblastoma for \textit{ex vivo} expansion of activated NK cells (NCT03209869). Results of these studies are expected in 2024 and 2021, respectively.

The fusion of cytokines with IgG antibodies targeting immune checkpoint proteins can also be used to achieve simultaneous immune stimulation and de-repression, respectively.\textsuperscript{[47]} In syngeneic Panc02 pancreatic tumor models, treatment with IL-2 proteins fused with a heavy chain-only antibody fragment (VHH) against PD-L1 led to >50% reduction of tumor burden at day 21 when compared with therapy from either αPD-L1 or control VHH-targeting cytokines.\textsuperscript{[48]} Interestingly, treatment with VHH-IFN-γ fusions increased M1 phenotypic characteristics of intratumorl macrophages resulting in reduced Panc02, KPC, and M19 (KPC organoid) orthotopic tumor burden. These results demonstrate that cytokine-checkpoint inhibitor antibody fusions may demonstrate efficacy in otherwise immunotherapy treatment-refractory tumor types.

Immunocytokines may also be engineered to target cell-surface markers on a variety of cell types. Cergutuzumab-IL-2v represents one such cell-biased cytokine which targets carcinoembryonic antigen via IgG with decreased FcγR binding but retained neonatal Fc receptor (FcRn) binding (Figure 5d–f).\textsuperscript{[49]} As a single agent, CEA-IL-2v elicited tumor reduction and substantial NK and CD8\textsuperscript{+} T-cell tumor infiltration, and when combined with PD-L1 checkpoint blockade achieved greater overall survival in A549 and LS174t xenograft murine models.

Rather than targeting cell-surface markers, immunocytokines may also target other components of the tumor microenvironment such as neovasculature or ECM components. Such antigens include the extra domain A (EDA) domain of fibronectin, the A1 domain tenascin-C, or the extra domain B (EDB) domain of fibronectin, targeted by the F8, F16, and L19 antibody clones, respectively.\textsuperscript{[50]} F8-IL-2 fusions have been shown to preferentially deliver IL-2 to the angiogenic neovasculature and to serve as an effective treatment for relapsed acute myeloid leukemia in preclinical models when combined with cytarabine. This combination achieved complete and durable responses in immunocompetent mouse models of C1498 leukemia.\textsuperscript{[51]} This effect has been further validated in other studies; F8-IL-2 therapy induced tumor eradication in 82% of mice when combined with paclitaxel in a mouse models of metastatic melanoma. Therapy with an F8-diabody-IL-2 fusion similarly induced tumor-specific immune cell infiltration and tumor growth retardation that was enhanced by paclitaxel and dacarbazine in syngeneic K1753M2 mouse models of melanoma.\textsuperscript{[52]} TNFα- and IL-2-based immunocytokines with affinity towards the same antigen were also shown to be effective in these cancer models; a single intratumoral injection of both immunocytokines resulted in dramatic eradication of neoplastic lesions.\textsuperscript{[52]} Immunotherapy with tandem fusions of both cytokines and F8 antibody has likewise been explored in combination with PD-L1 antibody therapy, demonstrating tumor eradication.
in immunocompetent CT26 and WEHI-164 tumor bearing Balb/c mice, but less striking efficacy in 129/SvEv mice bearing murine teratocarcinoma and in C57BL/6 mice bearing murine Lewes Lung Carcinoma.\textsuperscript{53} This difference may be explained by a lack of reduction of T\textsubscript{regs} in the later models and highlights the need for engineering IL-2R\beta\gamma\textsubscript{c}-biased IL-2 therapies. Pro-inflammatory cytokines IL-1\beta and IL-6 fused to F8 EDA-targeting antibodies have also been engineered, demonstrating reduced tumor inhibition in comparison to F8-TNF.\textsuperscript{54}

L19-IL-2 immunocytokines targeting the EDB domain of fibronectin are the most clinically advanced of the ECM-targeting class. In preclinical studies, L19-IL-2 immunotherapy induced CD8\textsuperscript{+} T-cell infiltration and tumor regression in both teratocarcinoma or orthotopic pancreatic cancer models,\textsuperscript{55} and also synergized with radiotherapy of EDB\textsuperscript{high} (C51) syngeneic models in a fibronectin abundance-dependent manner.\textsuperscript{56} In clinical trials, immunotherapy with L19-IL-2 induced a complete response rate in 25\% of patients with stage III metastatic melanoma, as well as disease stabilization in 83\% of patients with advanced renal cell carcinoma.\textsuperscript{57} A phase I trial (NCT02086721) of this combination demonstrated multi-year (3–4 year) disease free progression in 33\% of NSCLC patients with toxicities below grade 3 leading to the advancement of a phase II trial (ImmuNoSabr2) evaluating the efficacy of this combination in Stage IV NSCLC (NCT03705403), a study which is currently recruiting.\textsuperscript{58}

Building on the strong track-record of anti-CD20 therapy (e.g. rituximab) in treating hematological malignancies, Bhatt \textit{et al.} recently developed an anti-CD20-IgG1-IL-21 immunocytokine that extended the cytokine half-life 80-fold and both retarded tumor growth and extended survival in a murine A20-hCD20 lymphoma model.\textsuperscript{59} IL-21 has also been fused with PD-1 blocking antibodies, demonstrating treatment efficacy in humanized cancer models refractory to anti-PD-1 monotherapy.\textsuperscript{60} Here, treatment-associated tumor control and CD8\textsuperscript{+} T-cell cytolytic activity were superior to anti-PD-1 therapy alone, with comparable levels of PD-1 inhibition.

Collectively, these studies demonstrate that the combination of cytokines with IgG domains can dramatically alter cell- or tissue-specificity and the ultimate safety and efficacy of otherwise poorly tolerated or weakly effective cytokine immunotherapies. Future studies focusing on the tumor microenvironment (new immunosuppressive or metastasis-associated ECM components), new antibody structures (minibody- or camel Ig- fusions), and cytokine function in immune surveillance (muteins, \textit{de novo} design) may lead to further advances in the engineering of antibody-cytokine complexes and fusions, as well as correspondingly improved treatment outcomes for patients treated with this promising class of immunotherapy.

\textbf{2.1.5. Cytokine Delivery via Nano- and Micro-particles}

\textbf{IL-2 and IL-15:} While engineered cytokines and their derivatives may be administered systemically and with increasing safety and potency, their sustained delivery to cells or tissues for durations longer than the intrinsic half-life of soluble proteins can be attractive both alone and in combination with other cell-based therapies including islet or hematopoietic stem cell transplants and T or NK cell-based ACT. Mitogens such as

\textit{Adv Healthc Mater. Author manuscript; available in PMC 2021 December 07.}
IL-2, for example, are well-known to improve the persistence and activity of adoptively transferred T cells, including CAR-T cells (Table 2); however, frequent or high dosing of IL-2 is associated with cardiopulmonary toxicities including capillary leak syndrome and hypotension.\[^{2c,61}\] To overcome this challenge, researchers have sought to achieve both cell/tissue-specific delivery and prolonged release of cytokines through their encapsulation in nanometer- or micrometer-scale particles which are actively targeted via affinity ligands or passively targeted via size-dependent tissue accumulation. Irvine and coworkers in 2010 pioneered an approach to tether cytokine-loaded nanoparticles (NPs), so-called backpacks, onto the surfaces of adoptively transferred T cells and, more recently, extended this approach to simultaneously tether IL-2-Fc functionalized liposomes to both T cells and NK cells by way of surface-bound αCD137 (4-1BB) agonist, thus exploiting the synergistic effects of CD137 costimulatory signals and IL-2 stimulation on CD8\(^+\) T-cell activation.\[^{62}\] This nanometer-scale combination immunotherapy elicited treatment responses comparable to each of its constituent monotherapies, significantly delaying tumor growth in a syngeneic B16-F10 murine melanoma model; however, in contrast to the monotherapies, it showed no evidence of toxicity. In related work, this group sought to mitigate the toxicity of cytokine immunotherapies by exploiting the preferential expression of cell-surface thiols on T cells, B cells and hematopoietic stem cells (relative to red blood cells).\[^{62b}\] By coupling maleimide-functionalized, multilamellar liposomes encapsulating IL-15 superagonist and IL-21 to the surfaces of T cells \textit{ex vivo}, they were able to achieve sustained pseudoautocrine stimulation of these cells following ACT \textit{in vivo}. Following ACT of backpack-conjugated Pmel-1 CD8\(^+\) T cells, they observed striking curative responses in syngeneic models of melanoma.

In addition to high basal expression on T cells, B cells, and hematopoietic stem cells, cell-surface thiol groups are further upregulated on T cells after activation, a phenomenon also accompanied by increased redox activity. This novel finding has also been recently exploited in order to tether another type of backpack – nanogels – to the surfaces of adoptively transferred T cells. By crosslinking IL-15 superagonist (ALT-803) or IL-2-Fc protein using a bis-NHS crosslinker containing an internal disulfide group and stabilization using PEG-polylysine block copolymers, Tang \textit{et al.} demonstrated T cell stimulation-dependent release of cytokines from the nanogels (Figure 6d–f).\[^{63}\] ALT-803 nanogels were further functionalized with anti-CD45 antibodies for T-cell anchoring while IL-2-Fc nanogels were electrostatically coordinated to the negatively charged T-cell surface. Backpacking of adoptively transferred T cells with IL-2-Fc nanogels markedly improved T-cell expansion (80-fold compared with free IL-2-fusion), preferentially induced CD8\(^+\) memory precursor differentiation, reduced markers of T-cell exhaustion (PD-1, LAG-3), and induced no apparent toxicity or effects on tumor-infiltrating T\(_{\text{reg}}\)s in B16F10 murine melanoma models. ACT with ALT-803-CD45 nanogels expanded intratumoral T cells 16-fold \textit{in vivo} and improved overall in the same melanoma models while allowing 8-fold higher dosing without significant toxic effects.\[^{63}\] Together, these data demonstrate significant opportunities for combination immunotherapy incorporating ACT and engineered cytokines.

**IL-12:** The cytokine IL-12 has been shown to promote antitumor immunity in a variety of preclinical cancer models; however to-date, associated therapies have not advanced to phase III trials due to poor circulation and toxicity.\[^{64}\] To address this challenge, Wang \textit{et al.}
developed a microenvironment-responsive polymeric NP formulation of IL-12 (Figure 6g–i). \[65\] The poly(β-amino ester) copolymer utilized in this system, consisting of 1,6-hexanediol diacrylate, 2-(4-imidazolyl)ethylamine, and amino-terminal PEG, degrades in hypoxic tumor microenvironments, thus selectively delivering the encapsulated IL-12 cargo. This system was shown to be stable at physiological pH, substantially increase intratumoral IL-12, inhibit tumor growth, and reverse the phenotype of tumor associated macrophages without signs of toxicity in a B16-F10 xenograft murine tumor model. Another innovative approach by Hammond and coworkers utilizes layer-by-layer technology to shield IL-12-functionalized liposomes in multilayers of poly-L-Arginine, poly-L-Glutamic acid, and hyaluronic acid. \[66\] This unique architecture functions to limit the systemic toxicity of the IL-12 via polymer shielding and increases the association of the particle with cancer cells through electrostatic and receptor-specific targeting. IL-12 layer-by-layer particles demonstrated localized delivery of IL-12 to cell surfaces, generating an anti-tumor response in syngeneic mouse models of colorectal and ovarian cancer.

In a related approach, we recently developed a method to deliver IL-12 – or other cytokines – to the immunological synapse between T cells and malignant B cells via self-assembly of antibodies about magnetic iron oxide NPs (Figure 1e, Figure 6a–c).\[67\] Inspired by the clinical success of bispecific T cell engager (BiTE) immunotherapies and the challenges of IL-12 therapy in patients, we posited that incorporation of IL-12 onto multivalent BiTEs would both enhance anti-tumor activity and improve upon the low half-life of recombinant IL-12 in circulation. To examine this hypothesis, we developed a workflow to (i) generate an array of compounds with varying compositions, which we referred to as bi-specific T cell engaging cytokines (BiTEokines), and (ii) to determine ideal therapeutic candidates in a high-throughput phenotypic screen. Notably, this process required just days for hit identification and the analysis of important structure-function relationships. Using this approach, we identified CD19 x CD3 x IL-12 compounds that exhibit ex vivo lytic activity comparable to current FDA-approved immunotherapies for leukemia and correlated drug treatment with specific cell-cell contact, cytokine delivery, and leukemia cell lysis. Due to the rapid and modular nature of the approach, this delivery platform could likely be further implemented in a large number of immunotherapeutic contexts.

**TNFα:** Tumor necrosis factor α – as its name may suggest – represents one of the earliest-studied cytokines with anticancer activity; yet despite significant interest in its biology, the precise impact of TNFα on cancer cell signaling and antitumor immunity remain an active area of research.\[3\] As the potent, pro-inflammatory cytokine induces apoptosis in a variety of cell types, it is administered via isolated limb perfusion (ILP), receiving EMEA-approved for this treatment setting in soft tissue sarcoma patients in 1998. To overcome the necessity for ILP, Tamarkin and coworkers devised a method to deliver rhTNFα \textit{in vivo} via conjugation of protein to 27 nm gold NPs by way of an Au-S bond.\[68\] The particles are additionally stabilized via surface conjugation of PEG-SH. In recent Phase I clinical trials, the highest dosing of CYT-6091 (Cytimmune, Inc.) exceeded the previous maximum tolerated dose of rhTNFα by three-fold with no observed dose-limiting side effects such as hypotension. Other extensions of this approach include \(^{89}\)Zr/TNFα- and IFN-γ-conjugated colloidal gold (CYT-Z-TNF and CYT-IFNg, respectively). In an effort to...
further improve responses from this and related therapies, Curnis et al. recently examined the impact of affinity targeting using Asn-Gly-Arg (NGR), a ligand of CD13 expressed by tumor neovasculature. While NGR is specific to CD13, it rapidly deaminates into an isoform that preferentially binds to αvβ3 integrins. To address this, the authors created cyclized NGR peptides for particle antigen targeting and found tumor reduction to correlate with NGR affinity in a CD13-dependent manner in a WEHI 164 fibrosarcoma murine tumor model. Together, these data demonstrate that particle-engineered formulations may rescue the suboptimal effects of cytokine immunotherapies through improved tolerability, circulation, and tumor targeting.

**IFN-γ**: The sole member of the type II interferon class, IFN-γ is predominantly secreted by NK cells and serves as an important effector cytokine for CD8+ T cells, DCs, and pro-inflammatory M1 macrophages. In the context of cancer, IFN-γ has demonstrated utility as a predictive biomarker for response to chemotherapy and immunotherapy, but has shown little success as a therapeutic agent itself in clinical trials for a variety of cancers. Similar to other recombinant cytokines, engineered delivery platforms for IFN-γ have sought to address the limitations of systemic administration by improving localization to the tumor site before clearance. An early design by van Slooten et al. encapsulated mouse IFN-γ within liposomes, to serve as an adjuvant for a melanoma vaccine. After B16 melanoma tumor challenge, mice vaccinated with liposomal IFN-γ showed significant improvements in tumor protection as compared to vaccinated mice not treated with IFN-γ or treated with free IFN-γ, suggesting that improved localization could dramatically enhance therapeutic benefit. Later work by Mejías et al. made use of dimercaptosuccinic acid-functionalized iron oxide NPs to magnetically guide the delivery of surface-adsorbed IFN-γ. Direction of IFN-γ-carrying NPs with an external magnetic field improved tumor infiltration of CD8+ T cells and macrophages compared to non-directed NPs in a syngeneic Pan02 pancreatic cancer model, ultimately resulting in decreased tumor volume. More recently, Mitragotri and coworkers reported a strategy in which IFN-γ “backpacks” were adhered to the surface of macrophages, yielding a cell-based delivery system in which the therapeutic payload would directly interact with its carrier cell. These backpacks, consisting of a cell-adhesive layer, a polyvinyl alcohol (PVA) layer for cytokine incorporation, and two poly(lactic-co-glycolic acid) (PLGA) layers for structural support, managed to adhere to a majority of macrophages in vitro while predominantly avoiding phagocytosis over 5 days. Use of this macrophage-cytokine delivery strategy in a syngeneic 4T1 mammary tumor model promoted M1 macrophage polarization, reduced tumor volume, and improved overall survival when compared to combined macrophage and free IFN-γ treatment. In another novel cell therapy-based approach, Zeytin et al. developed a replication-deficient fowlpox virus engineered to express murine IFN-γ, finding that both in situ administration with virus and vaccination with virally transfected cancer cells was capable of inducing potent antitumor immune responses in syngeneic mouse models of colon adenocarcinoma. The latter vaccine approach was associated with the presence of antigen-specific T cell in regional lymph nodes and was protective against both primary tumor formation and rechallenge. Interestingly, antitumor immune responses were lost upon NK cell depletion, suggesting a role for these cells beyond their classical contributions to innate immunity.
**IL-10**: IL-10 is secreted by cells of the innate and adaptive immune system in order to modulate the activity of pro-inflammatory cytokines. Although typically considered an immunosuppressive cytokine, recent studies indicate that autocrine IL-10 signaling may indeed prolong the effector activity of CD8$^+$ T cells, thus providing a rationale for its use in immunotherapy for cancer – particularly in combination with immune checkpoint inhibition.[3] In addition to PEGylated IL-10 (see pegilodecakin, above), NP systems exploiting the function of IL-10 have demonstrated anti-tumor efficacy in both colon and lung carcinogenesis mediated through T-helper 17 (Th17) cell activity.[77] Oral delivery of IL-10 was achieved by polylactic acid (PLA) microspheres created through phase inversion nanoeapsulation and, following administration, resulted in suppressed tumor growth and enhanced survival in the APC$^{min/+}$/Bacteroides fragilis spontaneous colon cancer model. These remarkable therapeutic effects were attributed to a reduction of pro-tumorigenic T$_{regs}$ (CD4$^+$ Foxp3$^+$ ROR$\gamma^-$) and Th17 (CD4$^+$ ROR$\gamma^+$ IL-17$^+$) cells in combination with increased CD8$^+$ effector T cells.[77b] Reversal of Th17 axis dysregulation and attenuated tumor growth via PLA IL-10 microparticles was also seen after intratracheal administration in LSL-K-ras$^{G12D}$ (LSLKras) genetic model of lung cancer. Interestingly, this study found localized delivery was necessary for disease remediation, furthering the advantage of particle systems in cytokine delivery. This study also found the primary effect of IL-10 was on inflammatory macrophages which suppress CD4$^+$ T-cell IL-17 production.[77a]

**TRAIL**: In addition to targeting adaptive immunity, engineered cytokines are also well-suited to therapeutic applications which additionally modulate innate immune responses. One such cytokine, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is up-regulated on monocytes and macrophages after stimulation with lipopolysaccharide (LPS) and interferon-β (IFN-β), as well as on monocytes, DCs and NK cells following stimulation with IFN-γ.[78] TRAIL has been shown to induce apoptosis in a variety of cancers via binding with death receptors 4 and 5 which are preferentially upregulated on malignant cells. TRAIL immunotherapy has demonstrated efficacy in numerous preclinical cancer models and has been clinically investigated in at least six distinct types of cancer. Yet, to date TRAIL has only shown modest patient responses in part due to its poor circulation (20 kDa) and limited tumor accumulation.[79] To improve TRAIL-induced cancer immunotherapy, multiple NP formulations have been developed to-date including polymer, gold, lipid, and DNA-based structures, which we describe further herein.

TRAIL liposomes have also demonstrated the ability to selectively target tumor tissues and to achieve superior therapeutic activity when compared with the soluble protein in both in vitro systems and multiple cancer models; thus, validating the benefit of membrane-bound presentation of this protein.[80] For example, TRAIL-functionalization of liposomes has been shown to improve ligand-mediated apoptosis, increase death receptor (DR) 5 clustering, and reduce tumor volume in xenograft mouse models of colon cancer.[80a,81] More complex systems have also been designed including TRAIL and R8H3 dual-functionalized hyaluronic acid gel coated liposomes that encapsulate doxorubicin.[82] This gel-lipid nanostructure devised by Gu and coworkers was found to deliver TRAIL and doxorubicin with high specificity and accumulation in tumors (in part, owing to the high concentration of hyaluronidase within tumors), achieving both specificity and anti-tumor
potential in xenograft mouse models of breast cancer. In another approach relying on natural biomaterials, Bae et al. synthesized TRAIL- and transferrin-functionalized, human serum albumin NPs loaded with doxorubicin chemotherapeutic. These NPs efficiently accumulated in tumor sites in an HCT-116 xenograft model but were not assessed for anti-tumor activity.

To further promote tumor-specific TRAIL delivery, Sun et al. recently described a microenvironment-responsive delivery system that combined a TRAIL-loaded, self-assembled DNA nanocore encapsulated by a phospholipase A2 degradable-1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposome shell. DNA nanocores were synthesized through rolling circle amplification and modified with Ni²⁺NTA to effectively bind histidine tagged TRAIL protein and loaded into POPC liposomes via reverse phase evaporation. The system was further restricted through the need for two particles consisting of one of two complimentary DNA sequences that would hybridize to form microscale fibers after lipid degradation, thus, preventing cell internalization and enabling TRAIL presentation. This system exhibited exclusive membrane binding and enhanced in vitro cytotoxicity in the two-particle case, supporting a novel proof-of-concept mechanism for controlled delivery through DNA complementarity. King and colleagues employ another novel approach to targeted TRAIL delivery, utilizing E-selectin- and TRAIL- dual-conjugated liposomes to achieve blood leukocyte attachment and tumor cell cytotoxicity, respectively. By assembling TRAIL carriers with leukocytes in vivo under shear, the authors demonstrated enhanced killing of circulating tumor cells (CTC) and prevention of metastasis in a xenograft mouse model of prostate cancer.

Polymer-based TRAIL delivery is another active area of research which has led to significant improvements in accumulation of the cytokine within tumors and an improved understanding of associated receptor-mediated signaling. Early studies by Kim et al. demonstrated that TRAIL conjugated with a linear PEG10k-transferrin construct could retard tumor growth in a xenograft mouse model of colon cancer. Microspheres of PLGA have also been shown to electrostatically complex positively charged TRAIL and to facilitate its delivery in xenograft mouse models of cervical cancer. More recently, Langer and coworkers built upon their finding that tumor cells, but not normal cells, exhibit increased sensitivity to TRAIL-mediated apoptosis under fluid shear stress conditions. By simply conjugating PLGA NPs to the tumor cell surface via antibody-PEG linkers, amplification of fluid shear by these so-called mechanical amplifiers enhanced TRAIL-mediated tumor cell killing, both in vitro and in xenograft mouse models of colon and pancreatic cancer.

Treatment with these mechanosensitizing particles followed by TRAIL and resveratrol reduced PC-3 xenograft tumor growth by 80% in a caspase-dependent manner without significant toxic effects.

While lipid- and polymer-based delivery systems for TRAIL have demonstrated significant promise, inorganic nano- and micro-particles can in many cases offer increased or more diverse functionality that can improve the performance of associated cancer immunotherapy. For example, Gu and coworkers recently used graphene oxide (GO) nanosheets to exploit synergy between anthracycline chemotherapeutics and TRAIL immunotherapy (Figure 6j–l). In this system, doxorubicin was conjugated with GO nanosheets via π-π
stacking. TRAIL conjugation was achieved through amino-PEG-azide derivatization of carboxylated GO, followed by addition of a furin-cleavable peptide substrate via click chemistry. TRAIL was then attached to the peptide-conjugated GO by means of an amine-to-sulfhydryl crosslinker. Due to these mechanisms of attachment, doxorubicin was released under hypoxic conditions and TRAIL was released when in proximity to cell membrane containing sufficient furin. Doxorubicin sensitization in combination with cleavable TRAIL demonstrated synergistic cell toxicity *in vitro*, as well as tumor targeting and growth suppression *in vivo* in a xenograft mouse model of lung cancer. Interestingly, TRAIL release increased anti-tumor potential of this nanocarrier in comparison to a non-furin-cleavable control. Due to their unique stimuli-responsive behavior, facile surface functionalization, and utility as combined imaging contrast agents, iron oxide- and gold-based NP systems have also been extensively investigated as vectors for TRAIL-encoding gene therapies. Cho et al. for example capitalize on the magnetic properties of iron oxide particles to achieve field-induced activation of TRAIL receptors. Clustering of DR4 induced by magnetic field exposure of anti-DR4-conjugated particles mimicked TRAIL-induced signaling and enhanced caspase-mediated apoptosis *in vitro*, as well as *in vivo* in a zebrafish model. More recently, King and coworkers adopted a biohybrid approach to TRAIL delivery, coating TRAIL-conjugated silica particles with plasma membrane extracts from platelet cells in order to prolong their circulation and to target CTC. These platelet-like particles incorporated into CTC-associated lung micro-thrombi *in vivo* and decreased lung metastases in xenograft mouse models of metastatic breast cancer. Taken together, these studies illustrate the tremendous versatility and functionality afforded by nano- and micro-scale drug delivery systems for TRAIL immunotherapy. For a more in-depth review of TRAIL delivery systems, see Mitchell and coworkers.

2.1.6 Cytokine Delivery via Hydrogels and Implants—In addition to genetic-, chemical-, and particle-based approaches to protein modification, cytokines may also be embedded within macroscale matrices (i.e. implants) in order to overcome the aforementioned limitations of systemic cytokine immunotherapy. In the early 2000s, Hennink and coworkers developed non-degradable methacrylated dextran (dex-MA) hydrogels and degradable (lactate-)hydroxyethyl methacrylated dextran (dex-lactate-HEMA) hydrogels that allowed for controlled and tunable release of IL-2. Implantation of IL-2-loaded hydrogels in mouse models of metastasized lymphosarcoma yielded comparable therapeutic benefit to free IL-2 treatment and rejection of tumor cell rechallenge. More recent studies by Lv et al. leverage a related combination approach, in which IL-2, IFN-γ, and doxorubicin were loaded into poly(γ-ethyl-L-glutamate)-poly(ethylene glycol)-poly(γ-ethyl-L-glutamate) (PELG-PEG-PELG) hydrogels. Treatment with this combination-loaded hydrogel in syngeneic mouse models of B16F10 melanoma elicited almost twofold greater tumor growth inhibition when compared to the soluble protein combination. Importantly, hydrogel implantation and drug release induce no apparent toxic side-effects. A similar immunotherapy-chemotherapy combinatorial hydrogel approach was investigated by Wu et al., in which thermosensitive mPEG-b-PELG-based hydrogels were loaded with both IL-15 and cisplatin. B16-F10 xenograft-bearing C57BL/6 mice treated with IL-15/cisplatin-loaded hydrogels achieved curative responses after just 18 days. Xu et al. investigated a related approach, incorporating IFN-α into hyaluronic acid-tyramine
hydrogels of varying stiffnesses (Figure 1f), finding that IFN-α-loaded hydrogels, but not soluble protein, inhibited tumor progression in xenograft mouse models of hepatic cancer.\(^{[98]}\) Hydrogel-mediated delivery of TRAIL is also a highly active area of research. Youn and coworkers recently developed a hyaluronic acid-based hydrogel containing the cytokine,\(^{[99]}\) finding that PEG-TRAIL hyaluronic acid hydrogels dramatically reduced tumor burden when compared to TRAIL counterparts in xenograft mouse models of pancreatic carcinoma. They attributed these effects to enhanced stability of PEGylated cytokine within the hydrogel structure. This group later refined this approach through the creation of an albumin-cross-linked PEG hydrogel capable of \textit{in situ} gelling within 60 seconds, a significant improvement over the 4 hour gelation time of their previous design. Alternatively, Zhang \textit{et al.} devised a thermosensitive TRAIL-encapsulating hydrogel through use of superparamagnetic iron oxide NPs, achieving significant tumor reduction in a xenograft mouse model of glioblastoma (Figure 7).\(^{[100]}\) With regards to combinatorial TRAIL approaches, Erkoc \textit{et al.} designed a protease-sensitive hydrogel that incorporated both TRAIL and quinacrine, a chemosensitizer of TRAIL-induced apoptosis.\(^{[101]}\) This combination demonstrated significant synergy \textit{in vitro}, promoting of greater glioblastoma cell apoptosis when compared to the combination of both soluble drugs.

### 3. Immunosuppressive Cytokines

#### 3.1. Applications in Autoimmune Diseases

In contrast to immunostimulatory cytokines that are frequently used to enhance immune elimination and decrease (malignant) tissue growth, immunosuppressive cytokines can be employed to therapeutically dampen hyperactive immune responses (\textit{e.g.} cytokine release syndrome), to promote tissue regeneration, or to counteract autoimmune or inflammatory disease pathologies (\textit{e.g.} rheumatoid arthritis, systemic lupus erythematosus) (Table 3). In practice, this can be achieved by a variety of means including small molecule inhibitors and blocking antibodies; however here, we review the rich body of work surrounding novel methods to engineer and refine the therapeutic activity of immunosuppressive cytokines (Figure 8). Similar to immunostimulatory cytokines, clinical trials for recombinant, immunosuppressive cytokines have yielded mixed results. For example, as a treatment for patients with Crohn’s disease, recombinant IL-10 was shown to be well-tolerated but unable to meaningfully induce remission when compared to placebo, potentially due to low local concentrations of IL-10 in the intestine.\(^{[102]}\) Thus, many engineered, immunosuppressive cytokine designs have sought to bias activity towards specific tissues, receptors, or cell subsets in order to improve clinical benefit.

#### 3.1.1. Mutant and Designer Cytokines—

Cytokines, the subunits they are composed of, and their receptors often exhibit structural and biological redundancy which can be exploited in many cases for therapy. IL-12 family cytokines include IL-12, IL-23, IL-27, and IL-35, with each mature protein formed by a dimer of subunits.\(^{[103]}\) For example, IL-12 consists of p40+p35, IL-27 consists of Ebi3+p28, and IL-23 consists of p40+p19. While p40 can be paired with either p35 or p19 to form IL-12 or IL-23, respectively, not all IL-12 family cytokine subunits are found to be interchangeable in nature, prompting Flores \textit{et al.} to investigate the effect of combining p40 with p28, forming a new synthetic cytokine they
deemed “IL-Y”. Interestingly, although IL-12 and IL-23 generally have immunostimulatory effects, combining the common subunit p40 with p28 of the IL-27 protein resulted in anti-inflammatory effects demonstrated using pre-diabetic NOD mice.\textsuperscript{[104]} Alternatively, mutating the amino acid structure of a cytokine can alter its natural biological behavior to have a novel effect. For instance, by a single mutation from asparagine to aspartic acid (N88D) in the human IL-2 protein, receptor affinity of IL-2 to the IL-2Rβγc receptor can be lowered, resulting in a preferential binding to the high affinity IL-2Rαβγc on T_{reg}.\textsuperscript{[105]} Fused to an IgG antibody lacking Fc effector functions through point mutations, Peterson et al. crafted a T_{reg}-preferencing, longer-lasting IL-2 therapy capable of increasing the percentage of T_{reg} within the CD4\textsuperscript{+} T cell population in a humanized murine model. Amgen has also recently published work describing IL-2 muteins with T_{reg} selectivity in humanized mouse models (Figure 8a).\textsuperscript{[106]}

3.1.2. Cytokine Polymer Conjugates—Due to their small size (ca. 12–70 kDa), recombinant cytokines typically exhibit short circulation half-lives relative to larger proteins such as antibodies,\textsuperscript{[3]} thus necessitating high and/or frequent dosing in patients in order to realize therapeutic benefits. As with immunostimulatory cytokines in cancer, the PEGylation of immunosuppressive cytokines can overcome such delivery challenges in order to improve treatment outcomes for patients with a range of diseases characterized by dysfunctional immune responses (Figure 8b).\textsuperscript{[107]} In multiple sclerosis (MS), an autoimmune disorder characterized by inflammation and neurodegeneration, Avonex, a 22.5kDa recombinant IFN β–1a protein therapy, is a standard recommended intramuscular injection with an elimination half-life of 10 hours, self-administered weekly.\textsuperscript{[108]} Patients may also be treated with Rebif, another 22.5kDa recombinant IFN β–1a therapy that is administered subcutaneously with more frequent dosing at 3x per week.\textsuperscript{[109]} To extend the duration-of-action of this treatment regimen and decrease its frequency of administration, Biogen recently devised a method to PEGylate IFN β–1a at its amino-terminus with 20kDa PEG, thus enabling its sustained release and circulation.\textsuperscript{[22e]} In two Phase I trials in healthy volunteers, cytokine half-life following intramuscular injection was extended dramatically following administration of PEGylated IFN β–1a (ca. 24.3 to 45.4 hours), and extended even further with subcutaneous injection (ca. 48.4 hours). After Phase III trials demonstrating improved annualized relapse rates in MS patients receiving the PEGylated IFN β–1a versus placebo, Plegridy was FDA-approved for the treatment of MS patients via subcutaneous injection (every 14 days).\textsuperscript{[22d]}

3.1.3. Antibody-Cytokine Complexes and Fusions—Just as cytokine size and circulation time can be increased via polymer conjugation, the addition of fusion protein domains can also improve the pharmacokinetics of many immunosuppressive cytokines with therapeutic potential. As discussed previously, IgG antibodies can serve as an attractive fusion partner for such proteins due to their large size (ca. 150 kDa) and potential for FcRn recycling\textsuperscript{[110]} enabling affinity and cytokine domains to be mixed and matched to tailor activity to a specific disease setting or site. For example, EDA-fibronectin, a damage-associated molecular pattern that activates toll-like receptor 4 and is often expressed at sites of tissue remodeling and inflammation,\textsuperscript{[111]} can be affinity targeted in order to localize protein fusion partners at a wide range of inflammatory disease sites. Using a fusion of the
anti-fibronectin antibody clone F8 and IL-10, Schwager et al. demonstrated the targeting of IL-10 to arthritic lesions in collagen-induced mouse models of the disease following both intravenous and subcutaneous injection, as well as inhibited disease progression. When fused to IL-22, the same F8 clone was also capable of decreasing disease severity (by score) in a dextran sodium-sulfate induced murine model of colitis compared to a saline control. Quattrone et al. examined the anti-inflammatory effects of F8-IL-4 treatment in mouse models of endometriosis, finding that the novel therapy downregulated expression of proteins involved in tissue invasion (MMP3) and angiogenesis (VEGF), thereby decreasing the total number of lesions in syngeneic mouse models of the disease. (N.B. endometriosis is not classified as an autoimmune disease; however we include its discussion here due to structural similarities between this and previously discussed therapies, as well as the notably inflammatory nature of the disease). As of 2019, F8-IL-4 was under consideration as a clinical trial candidate for treatment of endometriosis.

As an alternative to anti-fibronectin targeting, IL-10 has also been fused with (i) anti-collagen type II antibodies to target cytokine activity to arthritic joints in an antigen-induced murine model of arthritis or (ii) anti-CD86 antibodies to target cytokine delivery to antigen presenting cells located at sites of inflammation (Figure 8c, Figure 9). In addition to cytokine-antibody fusion, direct cytokine-cytokine fusion can also prolong circulation and impart novel therapeutic effects. Eijkelkamp et al. for example, developed an IL-4/IL-10 fusion protein that completely mitigated inflammatory pain in a carrageenan-induced persistent inflammatory pain murine model (Figure 8d).

In addition to the fusion of cytokines with one another or IgG directed towards sites of disease, they may also be fused with their cognate receptor(s) or antibodies which bias their activity, with the aim of achieving novel therapeutic effects. Garcia and colleagues, for example, found that the IL-2 antibody JES6–1 could preferentially bias IL-2 activity towards T_{reg} cells, inducing their selective expansion in vivo and reducing disease severity in dextran sodium sulfate-induced mouse models of colitis. Using the same anti-IL-2 antibody, the research group also later engineered a glycine-serine linker-fused construct that maintained T_{reg}-selective activity and improved stability. Alternatively, Ward et al. produced an IL-2/IL-2Rα fusion protein with a glycine-serine linker and found a preference for T_{reg} expansion over conventional T cells. Given the broadly immunosuppressive activity of T_{regs}, such therapies hold promise for a range of diseases including rheumatoid arthritis, hepatitis C vasculitis, graft-versus-host disease, type 1 diabetes, and systemic lupus erythematosus.

3.1.4. Cytokine Delivery via Nano- and Micro-particles—One key challenge to the targeted delivery of cytokines in vivo is their high affinity to cells both in the peripheral blood and in the vicinity of disease sites, which in some cases can override targeting by the antibody itself. Cytokine delivery via nano- and micro-particles in these situations, as well as other situations which elicit acute toxic effects or suffer from poor circulation, can be highly advantageous. Because the encapsulation of cytokines in conventional lipid vesicles is typically low, encapsulation in PLGA particles is a common method to achieve sustained and/or targeted cytokine delivery. Based on their observation that leukemia inhibitory factor (LIF) supports T_{reg} maturation, Fahmy and coworkers employ this approach...
to target the delivery of LIF to CD4+ T cells via the conjugation of anti-CD4 antibodies to LIF-loaded PLGA NPs using avidin-biotin antibody conjugation (Figure 8e). Treatment with LIF-PLGA NPs in a mismatched murine heart allograft rejection model significantly prolonged graft survival and selectively expanded T\textsubscript{regs} from non-human primates \textit{ex vivo}. In related work, Shea and coworkers found that antigen-specific immune tolerance could be achieved via administration of TGF-\(\beta\)- and antigen-loaded PLGA NPs (Figure 10). They found that both subcutaneous and intravenous administration of these therapies could reduce disease severity in mouse models of multiple sclerosis (experimental autoimmune encephalomyelitis) and attributed these effects to a reduction in the inflammatory phenotype of bone marrow-derived DCs, inducing T\textsubscript{regs}. TGF-\(\beta\)-induced T\textsubscript{reg}-skewing in combination with IL-2 has also been investigated using anti-CD4- and anti-CD2/4-targeted PLGA NPs, the latter inhibiting pathogenic immune response in mice with lupus-like disease, selectively expanding CD4\textsuperscript{+} and CD8\textsuperscript{+} T\textsubscript{regs}, suppressing anti-DNA antibody production, and reducing renal disease \textit{in vivo\textsuperscript{[121-123]}}.

Beyond using NPs as a vehicle for cytokines, the properties of such materials can also be exploited to improve therapeutic activity. Meka et al. demonstrate such an approach by anchoring a lipid-conjugated targeting peptide (ART-1) to the surfaces of IL-27-loaded lipid vesicles in order to facilitate their preferential delivery to the inflamed joints in arthritic rats, thereby suppressing disease progression. Silver NPs have likewise demonstrated intrinsic biological activity in reducing inflammation at burn wound sites via a decrease in IL-6 and an increase in IL-10 at the site of injury. In later studies, silver NPs were found to significantly reduce TNF-\(\alpha\) production of LPS-stimulated murine macrophages \textit{in vitro}. These findings led to pairing the delivery of immunosuppressive cytokine IL-10 with silver NPs, with the aim to reduce inflammatory cytokine production (TNF) by murine macrophages \textit{in vitro}. Here, the combination was found capable of reducing TNF more so than free IL-10. Similarly, hyaluronic acid, a naturally occurring biopolymer, can be used to target cytokine delivery to macrophages via binding with CD44 receptors. Using hyaluronic acid NPs formed via divinyl sulfone cross-linking, Shahbazi et al. demonstrated that macrophages could be polarized towards an anti-inflammatory (e.g. M2) phenotype through delivery of encapsulated IL-4 \textit{in vitro\textsuperscript{[127]}}.

As an alternative to the use of nano- and micro- particles to deliver anti-inflammatory cytokines themselves, Fussenegger and coworkers recently explored the possibility of encapsulating engineered, cytokine-secreting cells within alginate-(poly-L-lysine)-alginate microcapsules for the treatment of psoriasis. With microsphere-encapsulated Hek293 cells that AND-gate express IL-4 and IL-10 in response to two extracellular biomarkers locally secreted during psoriatic inflammation (TNF and IL-22), the researchers demonstrated the ability to prevent the onset of psoriatic flares, to stop acute psoriasis, and to improve psoriatic skin lesions in an imiquimod-induced mouse model of the disease.

### 3.2. Other Applications

In addition to ameliorating autoimmune disease pathogenesis, anti-inflammatory cytokines can also be used to promote or control tissue growth/repair. Macrophones are a frequent target in this regard whereby a pro-inflammatory (M1-like) phenotype is characterized...
by secretion of IL-1β, IL-6, IL-12, and TNF-α,[129] and an anti-inflammatory (M2-like) phenotype is associated with secretion of IL-10 and TGF-β.[130] Because the latter cell subtype often contributes to functional tissue regeneration, Mooney and coworkers recently examined the hypothesis that sustained IL-4 signals could polarize M1-like macrophages into M2-like macrophages, thus inducing regeneration of skeletal muscle following ischemic injury in C57BL6/J mice (Figure 11).[131] They observed that intramuscular injection of 30 nm gold NPs conjugated with both PEG-SH and IL-4 (via Au-thiol bonds) resulted in significantly increased M2 phenotype expression, muscle-fiber area, and muscle contraction force. Interestingly, these effects were not observed following injection of soluble IL-4, a discrepancy attributed to M2 polarization, which they found to be highly dependent on IL-4 display valency. Kwon et al. adopted a related approach for the local delivery of IL-4 using mesoporous silica NPs bearing “extra-large” pores which they synthetically tuned to achieve sustained delivery of the protein.[132] While therapeutic effects were not studied in this work, expression of M2-like markers were significantly elevated in peritoneal macrophages following intraperitoneal injection of NP-encapsulated IL-4 – but not the soluble IL-4 – suggesting that NP-encapsulation enabled more prolonged cytokine exposure and subsequent signal transduction.

As discussed previously, immunosuppressive effects of the cytokine IL-10 are highly context-dependent but frequently result in a dampening of T- and NK-cell cytotoxicity and/or an inhibition of the antigen-presenting activity of DCs. For this reason, the protein is frequently studied in the context of graft tolerance and nerve regeneration. To this end, Hellenbrand et al. developed a sustained release system for IL-10 via incorporation into mineral-coated microparticles.[133] Intramedullary injection of these particles in a rat spinal cord injury model resulted in significant increases in axon preservation and significant improvements in motor deficits (functional scores) compared with soluble IL-10.

Given that both chronic and acute inflammation underlie foreign body response (FBR) to both medical and cosmetic implants, anti-inflammatory cytokines can also play an important role in reverting or preventing host rejection in these circumstances.[134] Hachim et al., for example, examine the hypothesis that early and sustained IL-4 delivery following implantation will inhibit the formation of foreign body giant cells that predicate fibrotic capsule formation around implants following rejection.[135] Using layer-by-layer (LbL) assembly, they coated polypropylene mesh implants with chitosan and dermatan sulfate polymer multilayers entrapping IL-4. The cytokine-loaded implant exhibited sustained release of IL-4 over 14 days under physiologic conditions, and substantially diminished fibrotic capsule formation at 90 days following subcutaneous implantation into C57BL/6 mice. Other novel approaches to delivering IL-4 include the use of heparin-functionalized supramolecular elastomers,[136] starPEG-heparin hydrogels (Figure 8f),[137] hydrogel-coated titania nanotubes,[138] and silk fibroin films (Kaplan and coworkers).[139]

4. Engineered Chemokines

Chemokines are small chemotactic cytokines (8–10 kDa) that guide leukocyte trafficking via tissue-specific concentration gradients.[140] These proteins are classified into one of four subfamilies (C, CC, CXC, CX3C) based on the primary sequence of conserved cysteine
residues. Because chemokine receptors are differentially expressed by immune cell subsets, exogenous introduction of chemokine gradients may confer pleiotropic effects including inflammatory, developmental, homeostatic, or pathogenic outcomes. Immunoengineering strategies that impart cellular- and tissue-level control over chemokine signals however, improve treatment outcomes in a variety of diseases which we discuss further herein.

4.1 Mutant and Designer Chemokines

As discussed previously, the engineering of recombinant proteins that exhibit mutations which bias their activity is one of the most conceptually simple methods of chemokine engineering, albeit one that requires tremendous skill. For example, Hanes et al. recently investigated the potential of a CXCL12 mutein as a novel cancer immunotherapy.\(^\text{[141]}\) CXCR4 and ACKR3 are both receptors that bind to CXCL12 and appear to promote the progression of cancer, inflammatory diseases, and multiple sclerosis; thus, therapies which antagonize these receptors could hold significant medical value.\(^\text{[142]}\) Efforts focused on mutations in the N-terminus of CXCL12 and subsequent engineered variants exhibited a twofold increase in CXCR4 binding affinity compared to native protein, without functional activation of the receptor. In a mouse model of experimental autoimmune encephalomyelitis, which mirrors T cell-dependent multiple sclerosis in humans, administration of their candidate CXCL12 antagonist both delayed disease onset and decreased its severity. Similarly, Getschman et al. examined a panel of CCL20 muteins as a potential treatment for psoriasis, due to the CCL20/CCR6 interaction playing a role in disease-related inflammation.\(^\text{[143]}\) By locking CCL20 into a dimer conformation via cysteine (S64C) introduction, they observed a dramatic reduction in CCR6\(^+\) cell migration in vitro upon drug treatment, as well as blocked recruitment of CCR6\(^+\) T cells to the epidermis in an IL-23-dependent mouse model of psoriasis.

While less research is currently published on chemokine structural exploitation, some noteworthy and direct protein modification strategies open the possibilities for chemotactic intervention and adjuvant immunotherapy \textit{in vivo}. Gschwandtner et al. engineered a modified CXCL12 structure to interfere with the mechanism of metastatic intravasation through blood vessels.\(^\text{[144]}\) CXCL12 cognate receptors, CXCR4 and CXCR7, are critical for metastatic intravasation through endothelium, and chemokines like CXCL12 can form solid-phase gradients by binding to glycosaminoglycans (GAGs) expressed on vascular endothelium. GAGs comprise negatively-charged polysaccharides such as heparin and heparan sulfate, which form nonspecific yet selective electrostatic patterns for chemokine binding via positively-charged amino acid patterns and site-specific hydrophobic interactions and hydrogen bonds.\(^\text{[144]}\) Gschwandtner et al. report that mutant CXCL12\(\alpha\) – with 8 leading amino acids deleted at its N-terminus in addition to L29K and V39K mutations – exhibits increased affinity for heparin and heparan sulfate GAGs, nearly abrogated receptor binding, and disabled signaling. By blocking CXCR4/CXCR7 signaling, they showed a reduced number of liver metastases in a xenograft mouse model of breast cancer treated with mutant CXCL12\(\alpha\). Taken together, these three approaches highlight the range of mutein modifications that can disrupt chemokine signaling networks that promote disease pathogenesis.
4.2 Antibody-Chemokine Fusions

In addition to mutations which alter GAG binding affinity, the surface charge of chemokines can also be modulated to tune binding affinity to GAGs, thus modifying the accumulation of immune cells at sites of disease. Ploegh and coworkers employ such an approach in the design of a protein which fuses CCL21 mutein with a PD-L1-specific VHH binding domain by way of a flexible peptide linker (Figure 12). This CCL21 mutein domain exhibits a deletion of the polycationic C-terminus, a mutation which is designed to decrease binding with GAG polyanions and improve lymphocyte infiltration of tumor sites. At the same time, the anti-PD-L1 domain was intended to increase accumulation of the fusion protein at tumor sites and to also block interactions with PD-1⁺ lymphocytes within the tumor microenvironment. As a proof-of-concept, they showed that this novel fusion protein bound murine B16 melanoma tumor spheroids in a PD-L1-dependent manner using a tumor microenvironment-on-a-chip model, and that the protein directed the chemotaxis of CCR7-expressing DCs to these tumors in vitro. Given the modular nature of this approach, we anticipate a number of future extensions of this design strategy in the future; for example, constructs targeting ECM components themselves, and those including additional recombinant proteins that synergize with inhibition of the PD-1 immune checkpoint.

4.3 Chemokine Delivery via Nano- and Micro-particles or Hydrogels

One method by which chemokine concentration gradients may be controlled is by immobilizing these proteins on or within a biomaterials scaffold. Liu et al. adopt this approach in order to examine the metastatic potential of cancer cells by adsorbing CXCL12 to poly-L-lysine/hyaluronic acid polymeric films to replicate ECM-bound chemokine gradients. When seeded with CXCR4-expressing hepatocellular carcinoma cells, they observed dose-dependent epithelial-to-mesenchymal transition-like morphological transitions in these cells that may be used in disease grading. In principle, the CXCR4-CXCL12 pair may be swapped for disease-specific study of chemokines that downregulate tumor cell motility or that recruit CD8⁺ T cells. Singh and Roy extend this approach, showing that injectable hydrogels containing immobilized chemokines can act as synthetic T-cell priming centers to convert weakly immunogenic B-cell lymphomas into responsive tumors (Figure 13). They combined DC-activating factors (microparticles containing siRNA against IL-10 and plasmid DNA coding for antigen) with immature DC chemoattractant, CCL10 (MIP3α) in a dextran vinyl-sulfone and tetra-thiolated PEG hydrogel. After quad muscle injection in syngeneic mouse models of B-cell lymphoma, they observed muscle-specific DC recruitment, a bias towards T-helper 1 (Th1) cell-specific inflammatory cytokine release, and amplified CD8⁺ cytotoxic T-cell response specific to A20 lymphoma cells. This synthetic priming center achieved protection in mice re-challenged with lymphoma injections; thus, chemokines co-delivered with immunomodulatory factors in polymeric scaffolds may synergize within synthetic priming centers to direct the immune elimination of otherwise immunologically “cold” tumors.

Kobayashi et al. adopt another extension of this approach to construct artificial tertiary lymphoid organs (artTLOs) to attract memory B and T cells and subsequently generate an antigen-specific immunity in immunocompromised patients. Using collagen sponge-containing Medgel beads, they incorporated four chemokines (CCL19, CCL21, CXCL12, CXCL13), as well as lymphotoxin-α1β2 for stimulation of tissue-resident stromal cells,
within their implants. artTLOs were generated following transplantation into the renal subcapsular space of OVA-immunized, immunocompetent mice, then transplanted again into immunodeficient (SCID) recipient mice prior to OVA immunization. Interestingly, SCID mice that were not expected to mount an immune response displayed anti-OVA-producing cells in both their implants and within their spleens, suggesting that memory B cells and CD4+ T cells from artTLOs populated the spleens of recipient mice to confer OVA immunity. While such results are exciting, we eagerly await future studies focusing on the impact of major histocompatibility complex matching and the potential for cross-priming or autoimmunity from this system. Together, these studies highlight both reductionist and complex approaches to improving immune responses to disease via chemokines.

In addition to their applications in cancer, localized chemokine delivery platforms can also be utilized in order to improve, rather than block, tissue growth and regeneration. Werner and coworkers examine this potential using hydrogels containing SDF-1α, a chemoattractant for endothelial progenitor cells. Building on prior studies involving polymer matrices, here they incorporated the chemokine into starPEG-heparin hydrogels that enable highly tunable delivery kinetics. In this work, they achieved controlled release of SDF-1α in vitro over the course of one week, and upon subcutaneous implantation of these gels in immunocompromised mice they observed a significant enrichment of endothelial cells following explantation. When administered alongside endothelial progenitor cells, SDF-1α-incorporated hydrogels demonstrated a similar ability to serve as a chemoattractant. Later studies tested the use of SDF-1α in other delivery formats and disease contexts. Gao et al. developed SDF-1α-coated platinum coils for treatment of intracranial aneurysms, while Temenoff and coworkers engineered SDF-1α microparticles to modulate M2 macrophage polarization and stem cell migration in rotator cuff injuries.

### 4.4 Chemokine Delivery via Engineered Cells

Another application of engineered chemokines stems from rapid advances in the design and use of cellular therapies over the past decade. In this manner, chemotaxis induced by these proteins may improve the persistence or homing of cellular therapies to sites of disease, thus improving treatment outcomes. In addition to chimeric antigen receptors, CAR-T cells have been engineered to secrete chemokines such as CCL19. CCR7, the cognate receptor to CCL19, is endogenously expressed on naïve T cells and DCs in order to home to the lymph nodes where activation and maturation occurs. Adachi et al. engineered CAR-T cells to express IL-7 and CCL19 (7 × 19), showing enhanced migratory potential in transwell migration assays. In combination with cyclophosphamide chemotherapy, 7 × 19 CAR-T cells completely eradicated tumor growth in syngeneic mouse models of melanoma, whereas conventional CAR-T cells were unable to control tumor growth. Similar responses were observed in syngeneic mouse models of pancreatic and lung cancers. Notably, 7 × 19 CAR-T cells increased T-cell and DC infiltration in pre-established solid tumors, thus demonstrating that ectopic chemokine secretion in engineered T-cell vectors can regulate immune responses.

In addition to the ligands themselves, CAR-T cells can also be engineered to express chemokine receptors. This strategy is unique in that it facilitates the recruitment of
engineered cells to tissues expressing cognate chemokines and, in some cases, such signaling can synergize with T-cell receptor (TCR) activation. Engineered CCR2 expression in CAR-T cells has shown enhanced tumor trafficking, along with rapid and strong control of tumor growth in a range of CCL2-expressing tumors in vivo, including lung, [153] malignant pleural mesothelioma,[154] neuroblastoma,[155] and spontaneous prostate metastases.[156] CCL2 is expressed by many other types of tumors, such as melanomas, gliomas, breast and ovarian cancers; notably, it is secreted by tumor stromal cells, T_{reg}s and tumor-associated macrophages as well.[153] As shown in these studies, the power of engineering ectopic chemokine receptor expression is that chemokine secretion profiles in non-immunogenic “cold” tumors can draw in engineered CAR-T cells that subsequently turn the tumors “hot.”

Engineered expression of other chemokine receptors on CAR-T cells has also been shown to augment tumor homing in other cancers: CCR4-CCL17/CCL22 in xenograft mouse models of Hodgkin’s lymphoma;[157] CXCR2-CXCL1/CXCL2 in xenograft[158] and syngeneic mouse models of melanoma,[159] as well as xenograft mouse models of colorectal,[159] hepatocellular carcinoma,[160] ovarian, pancreatic, and breast tumors.[161] Human NK cells have also been engineered to express CXCR2 for enhanced trafficking to CCL2-expressing renal cell carcinomas and, similarly, NK cells expressing EGFRVIII-CAR and CXCR4 demonstrated improved tumor infiltration and disease control in xenograft mouse models of glioblastoma.[162]

As mentioned above, chemokine signaling can also synergize with the effector cell functions of T cells. Moon et al. posited that CCR2 signaling augmented T-cell cytotoxicity, independent of chemotactic guidance.[154] Using CCR2-engineered CAR-T cells, they found that just a single dose of cells was necessary to achieve curative responses in mouse models of mesothelioma. Asai et al. devised a related CAR-T approach, finding that CCR2 stimulation cooperated with TCR signaling in a dose-dependent manner to improve IFN-γ production and cytotoxic granule formation in xenograft mouse models of squamous cell lung cancer.[153] In a similar demonstration of augmented endogenous effector function, Rapp et al. showed that engineered expression of CCR4 in cytotoxic T lymphocytes (CTLs) enhanced activation and subsequent tumor killing.[163] Among T-cell subsets, they showed that CCR4 expression is restricted to T_{reg}s and that its cognate ligand, CCL22, is highly expressed in pancreatic tumors and DCs. By engineering ectopic CCR4 expression in CTLs, they demonstrated increased affinity between T-cell LFA-1 and DC ICAM-1 adhesion molecules, which correlated with increased DC-CTL clustering. In a syngeneic mouse model of pancreatic cancer, CCR4-expressing CTLs induced rapid and durable tumor regression, and eradicated tumors in 40% of mice. From these examples of CCR2-TCR and CCR4-CCL22 interactions, one may conclude that transduced chemokine receptor expression in engineered T cells not only enhances tumor homing ability, but also – if the receptor is appropriately selected – cooperates with endogenous signaling to augment tumor killing.
5. Conclusions & Future Directions

In conclusion, the multitude of studies described above highlight not only the tremendous enthusiasm that exists for cytokine immunotherapies, but also the sheer diversity of approaches can be employed in order to unlock their therapeutic potential. Possibly more exciting than this is the fact that each of these techniques rely on notably cross-cutting research approaches that marry structural biologists, chemists, materials scientists, immune/cell biologists, clinicians, and protein/polymer engineers together into teams that we anticipate will continue to innovate and to grow this existing but nascent field of therapeutics. Going forward, we envision growth in a number of key areas: Given significant investments in the research and development of PEG-cytokine conjugates (e.g. bempegaldesleukin) and antibody fusions (e.g. Hu.14.18-IL-2) in the past several years, we anticipate new clinical approvals in these areas, particularly in combination with other immunotherapies which often synergize with cytokine immunotherapy such as immune checkpoint inhibition or CAR-T cell therapy in cancer. While PEG continues to be a workhorse for the field of conjugates, we anticipate extension of this approach to other materials including polyoxazolines, polysaccharides, elastin-like polypeptides, and microenvironment-responsive chemical linkages in addition to covalent modifications. As methods for de novo protein design become more widespread, we expect that new, chimeric, or cell subset-specific cytokine structures (e.g. NL-201) to emerge. Due to recent advances in the production and engineering of cellular therapies, delivery technologies for mRNA, and techniques for engineering functional gene circuits, we also expect cytokine-based approaches to play an important role in the expansion of these fields and their therapeutic potential in the coming years. Lastly, while a majority of clinically approved cytokine immunotherapies are currently used to treat malignancies, we envision that recent improvements in our understanding disease biology resulting from single-cell transcriptomic and high-dimensional cytometric techniques will lead to new opportunities for the development of precision cytokine therapies and new abilities to improve treatment outcomes for patients.

Acknowledgements

This work was supported in part by the St. Baldrick’s Foundation (Research Grant 641261), the US Department of Defense (Idea Award CA180783), the AAI Careers in Immunology Fellowship Program, the National Institutes of Health Research Training Program in Immunoengineering (T32EB021962), the American Cancer Society (IRG-17-181-05), the Donaldson Charitable Trust (Research Synergy Fund), the Winship Cancer Institute of Emory University, the Aflac Cancer and Blood Disorders Center at Children’s Healthcare of Atlanta, and the Wallace H. Coulter Department of Biomedical Engineering at Emory University and the Georgia Institute of Technology. The content here is solely the responsibility of the authors and does not necessarily represent the official views of the organizations acknowledged herein.

Biography

Adv Healthc Mater. Author manuscript; available in PMC 2021 December 07.
**Biaggio Uricoli** is a Ph.D. student in the Coulter Department of Biomedical Engineering at the Georgia Institute of Technology and Emory University. He received his B.S. in biomedical engineering from Rowan University in 2018. His research focuses on developing safer forms of cancer immunotherapy via modification with photolabile polymers, particularly in the areas of immune checkpoint blockade and cytokine therapies.

![Biaggio Uricoli](image1)

**Christopher Porter** is a pediatric hematologist-oncologist and directs a lab that studies molecular and cellular mechanisms of hematopoiesis and leukemogenesis, with the goal of developing novel therapeutic strategies to improve the care of patients with hematologic malignancies. He has a long-standing interest in cell autonomous and non-cell autonomous mechanisms of leukemogenesis and therapy resistance. In particular, he has studied how tissue fitness and micro-environmental perturbations promote carcinogenesis, and how those might be exploited therapeutically.

![Christopher Porter](image2)

**Sarwish Rafiq** is an assistant professor of hematology and medical oncology at Emory University School of Medicine. She received her B.A. degree in Biology from CUNY Hunter College, M.A. in Molecular Biology from the CUNY Graduate School, and Ph.D. from The Ohio State University in Immunology and Cancer Biology. Following her Ph.D., she completed postdoctoral training in cellular immunotherapy at Memorial Sloan Kettering Cancer Center. She leads a translational research team focused on developing novel engineered immune cell therapies for cancer.

![Sarwish Rafiq](image3)

**Erik Dreaden** holds joint faculty appointments in the Department of Biomedical Engineering and the Department of Pediatrics at Emory’s School of Medicine and the Georgia Institute of Technology. Prior to his current appointment as an assistant professor, he conducted postdoctoral training at the Koch Institute for Integrative Cancer Research at MIT. A native of Georgia, he obtained his PhD in Chemistry and Biochemistry from the Georgia Institute of Technology and his BS in Chemistry from the University of Georgia. Dr. Dreaden is a Children’s Healthcare of Atlanta Pediatric Research Scholar and holds faculty

*Adv Healthc Mater. Author manuscript; available in PMC 2021 December 07.*
memberships at the Aflac Cancer and Blood Disorders Center, the Winship Cancer Institute, and the Petit Institute for Bioengineering and Bioscience.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IFN-α</td>
<td>Interferon-alpha</td>
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<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>IL-2R</td>
<td>interleukin-2 receptor</td>
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<td>CD25</td>
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<td>mAb</td>
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<td>PEG</td>
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<td>overall response rate</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>CBD</td>
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<td>programmed death ligand-1</td>
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<td>NPs</td>
<td>nanoparticles</td>
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*Adv Healthc Mater. Author manuscript; available in PMC 2021 December 07.*
<table>
<thead>
<tr>
<th>Acronym</th>
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<tbody>
<tr>
<td>BiTE</td>
<td>bispecific T cell engager</td>
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<tr>
<td>BiTEokines</td>
<td>bi-specific T cell engaging cytokines</td>
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<td>ILP</td>
<td>isolated limb perfusion</td>
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<td>T-helper 17</td>
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<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis-inducing ligand</td>
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<td>dex-MA</td>
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<td>(lactate-)hydroxyethyl methacrylated dextran</td>
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<td>poly(γ-ethyl-L-glutamate)-poly(ethylene glycol)-poly(γ-ethyl-L-glutamate)</td>
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<td>Th1</td>
<td>T-helper 1</td>
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<tr>
<td>artTLOs</td>
<td>artificial tertiary lymphoid organs</td>
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[164]. Wong HC, Jeng EK, Rhode PR, OncoImmunology 2013, 2, e26442.


Adv Healthc Mater. Author manuscript; available in PMC 2021 December 07.


Figure 1. Strategies for engineering cytokines with immunostimulatory therapeutic activity. (a) Mutant and designer cytokines, (b) cytokine-polymer conjugates, (c) protein complexes and fusions, (d) small molecule- and peptide-conjugates, (e) nano- and micro-particle formulations, and (f) macroscale implants and hydrogels. IL-2R: interleukin-2 receptor, CBD: collagen-binding domain, MSA: mouse serum albumin, HA-Tyr: hyaluronic acid-tyramine, IFN-α2a: interferon-α2a. Figures adapted with permission from: (a) Silva et al.; [18] (b) Perdue et al.;[29] (c) Momin et al.;[31] (d) Mansurov et al.;[33] (e) Do et al.;[67] (f) Xu et al.[98] Copyright (a) 2019 Springer Nature, (b) 2020 American Chemical Society, (c) 2019 American Association for the Advancement of Science, (d) 2020 Springer Nature, (e) 2020 American Chemical Society, and (f) 2013 Elsevier B.V.
Figure 2. A biorthogonal, cytokine-receptor pair enables immune cell-exclusive stimulation.
(a) Illustration of mutually exclusive cognate receptor binding by WT and mutant- (ortho-) IL-2 and discovery via yeast display-based evolution. (b) Selective activation of WT and ortho T cells using WT or orthoIL-2 as measured by STAT5 phosphorylation. (c) Binding affinity of WT or orthoIL-2 to IL-2Rβ or orthoIL-2Rβ. (d) In vivo expansion of WT or ortho CD8+ T cells in response to administration of WT or orthoIL-2. WT: wild type, IL-2: interleukin-2, IL-2Rβ: interleukin-2 receptor β. Figures adapted with permission from Sockolosky et al. Copyright 2018 American Association for the Advancement of Science.
Figure 3. Cytokine-polymer conjugates enable optically triggered immune stimulation.
(a) Illustration demonstrating light-induced cytokine de-repression following protein modification with (b) o-nitrobenzyl-linked PEG. (c) Wavelength-selective polymer cleavage. (d) Effect of photocaging and uncaging on IL-2-induced T-cell proliferation. IL-2: interleukin-2. Figures adapted with permission from Perdue et al.\textsuperscript{[29]} Copyright 2020 American Chemical Society.
Figure 4. Cytokine-protein fusion can enhance the magnitude and duration of IL-15 activity.
(a) ALT-803, a mutant IL-15:IL-15Rα chain: IgG1 Fc complex. (b) Waterfall plot of change in tumor volume from a Phase Ib trial of ALT-803 in combination with anti-PD-1 immunotherapy. (c) Proliferative response of immune cell subsets measured after ALT-803 treatment in a single patient and (d) in comparison to other trial participants. IL-15Rα: interleukin-15 receptor α, IFNγ: interferon γ, PD-L1: programmed death ligand-1, NK cells: natural killer cells. Figures adapted with permission from: (a) Wong et al.;[164] (b-d) Wrangle et al.[38] Copyright (a) 2013 Taylor & Francis and (b-d) 2018 Elsevier Ltd.
Figure 5. Antibody-cytokine complexation or fusion favorably biases cell- and tissue-specific antitumor immune activation.
(a) Illustration of αhIL-2 antibody (NARA-1) binding with IL-2 in relation to immunosuppressive IL-2Rα (CD25) binding. (b) Decrease in tumor volume and (c) pulmonary metastases following treatment with αhIL-2:hIL-2 complexes as compared to free hIL-2 treatment. (d) Illustration of IL-2 fusion with a tumor-targeting, CEA-specific antibody. (e) Increased tumor infiltration by T cells following antibody-cytokine complex treatment. (f) Overall survival following treatment antibody-cytokine fusion therapy and/or immune checkpoint blockade therapy. IL-2: interleukin-2, CEA: carcinoembryonic antigen, PD-L1: programmed death ligand-1. Figures adapted with permission from: (a-c) Arenas-Ramirez et al.;[43] (d-f) Klein et al.[49] Copyright (a-c) 2016 American Association for the Advancement of Science and (d-f) 2017 Taylor & Francis.
Figure 6. Nanoparticle-based cytokine immunotherapies improve antitumor immunity.
(a) Illustration of bispecific T cell engaging cytokines (BiTEokines) for immune synapse-targeted cytokine delivery. (b) Transmission electron micrographs of BiTEokines and (c) CD8+ T cell-mediated lysis of leukemic B cells following BiTEokine treatment. (d) Illustration of cytokine nanogels and stimuli-responsive protein delivery. (e) Transmission electron micrographs of cytokine nanogels and (f) enhanced, tissue-specific CD8+ T cell counts relative to free IL-15Sa treatment, both following adoptive cell transfer therapy. (g) Illustration of microenvironment-responsive IL-12-loaded polymer nanoparticles. (h)
Transmission electron micrographs and dynamic light scattering data of nanoparticle size. (i) Change in tumor volume following treatment with free and polymer-encapsulated IL-12.


Figure 7. Hydrogel implants improve cancer immune elimination via heat-responsive cytokine release.
(a) Illustration of temperature-dependent gelation and release of cytokine via magnetic hyperthermia. (b) Transmission electron micrographs of TRAIL/SPION nanocomplexs and (c) TRAIL release kinetics from various hydrogels held at normal body and hyperthermic temperatures. (d) Change in tumor volume following treatment with hydrogel implants and varying cycles of hyperthermia. PPZ: poly(organophosphazene), TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand, SPIONs: superparamagnetic iron oxide nanoparticles, MHT: magnetic hyperthermia. Figures adapted with permission from Zhang et al.\textsuperscript{100} Copyright 2017 Elsevier Ltd.
Figure 8. Strategies for engineering cytokines with immunosuppressive therapeutic activity. (a) Mutant and designer cytokines, (b) cytokine-polymer conjugates, (c) protein complexes and fusions, (d) small molecule- and peptide-conjugates, (e) nano- and micro-particle formulations, and (f) macroscale implants and hydrogels. IL-4: interleukin-4, IL-10: interleukin-10, scFv: single-chain variable fragment, PLGA: poly(lactic-co-glycolic acid), starPEG: star-shaped polyethylene glycol. Figures adapted with permission from: (a) Ghelani et al.;\textsuperscript{106} (b) Xu et al.;\textsuperscript{107} (c) Zhao;\textsuperscript{108} (d) Eijkelkamp et al.;\textsuperscript{116} (e) Park et al.;\textsuperscript{121} (f) Schirmer et al.\textsuperscript{137} Copyright (a) 2020 Ghelani, Bates, Conner, Wu, Lu, Hu, Li, Chaudhry and Sohn, (b) 2018 The Royal Society of Chemistry, (c) 2020 Springer Nature, (d) 2016 Eijkelkamp, Steen-Louws, Hartgring, Willemen, Prado, Lafeber, Heijnen, Hack, van Roon, and Kavelaars, (e) 2010 American Chemical Society, and (f) 2016 WILEY-VCH Verlag GmbH & Co.
Figure 9. Antibody fragment-targeted delivery of IL-10 decreases arthritis severity.  
(a) Illustration of IL-10 fusion with the variable domain of 1–11E that targets ROS-modified collagen II. (b) Knee joints from mice with antigen-induced arthritis treated display strong Safranin O cartilage staining and (c) reduced knee swelling in response to targeted but not control (C7-) IL-10 fusion protein. (d) Reduced serum cytokine levels following treatment with targeted but not control IL-10 fusion protein. MMP: matrix metalloproteinase, IL-10: interleukin-10, IFN-γ: interferon-γ, TNF: tumor necrosis factor, HC: healthy control.  
Figures adapted with permission from Hughes et al.\textsuperscript{[115b]} Copyright 2014 Springer Nature.
Figure 10. Nanoparticle co-delivery of peptide antigen and tolerogenic cytokines induces immune tolerance.

(a) Illustration of antigen (proteolipid peptide) conjugation with poly(lactide-co-glycolide), self-assembly, and particle surface conjugation with tolerogenic TGF-β. (b) Improvement in EAE clinical score in mouse models of multiple sclerosis treated with tolerogenic NPs after disease onset. (c) Immunosuppressive T\textsubscript{reg} counts in the liver elevated in response to tolerogenic NP treatment. PLG: Poly(lactide-co-glycolide), Ag: antigen, TGF-β: transforming growth factor beta 1, EAE: experimental autoimmune encephalomyelitis, T\textsubscript{reg} cells: regulatory T cells. Figures adapted with permission from Casey \textit{et al}.\cite{122} Copyright 2018 American Chemical Society.
Figure 11. Gold nanoparticles surface-conjugated with cytokines induce anti-inflammatory macrophage polarization.
(a) Illustration gold nanoparticle PEG- and cytokine- conjugation via Au-S bond formation.
(b) Timeline of murine muscle fiber regeneration study. (c) Localization of gold nanoparticles to tibialis anterior muscle. (d) H&E staining of tibialis anterior muscles treated with control- and cytokine-loaded nanoparticles. (e-g) Percentage of tibialis anterior cross-sections with the presence of nuclear and muscle fiber staining, or lack thereof. (h-i) Maximum contraction force and velocity generated by mice after treatment with control- and cytokine-loaded nanoparticles. PEG: polyethylene glycol, IL-4: interleukin-4, IM:
intramuscular, PA4: IL-4–conjugated gold nanoparticles. Figures adapted with permission from Raimondo et al.\textsuperscript{[131]} Copyright 2018 United States National Academy of Sciences.
Figure 12. Antibody fragment-chemokine fusions direct leukocyte infiltration to tumors. (a) Illustration of various constructs fusing anti-murine PD-L1 (B3) with chemokine ligand CCL21 truncated (T) to reduce receptor-independent extracellular matrix interactions. Immunofluorescence imaging of CCL21 fusions indicate (b) decreased binding with blood and lymphatic endothelial cells and (c) increased tumor cell surface-specific interactions upon chemokine truncation in a microfluidic model of the tumor microenvironment. (d) Quantification of tumor cell-selective binding from the various fusion proteins. ECs: endothelial cells, TC: tumor cell, ECM: extracellular matrix. Figures adapted with permission from Fang et al. [145] Copyright 2019 American Chemical Society.
Figure 13. Chemokine-loaded hydrogels act as synthetic T-cell priming centers for cancer vaccination.
(a) Illustration of intramuscular injection with hydrogels containing MIP3α chemokine and microparticles with both DNA antigen and siRNA against IL-10. (b) Illustration of chemokine-mediated recruitment of immature dendritic cells, as well as subsequent antigen delivery to, and siIL-10-mediated Th1-biasing of, dendritic cells that later migrate away to present antigen to naïve Th cells (c) Image of in-situ crosslinked hydrogels extracted from mouse quadriceps muscle. (d) Change in CD11c+ dendritic cell infiltration into hydrogels with and without MIP3α chemokine and with varying rates of degradation. IL10: interleukin-10, siRNA: small interfering RNA. Figures adapted with permission from Singh et al.\cite{147} Copyright 2011 Elsevier Ltd.
Table 1.
Generalized comparison of various methods with which to engineer therapeutic cytokines.

<table>
<thead>
<tr>
<th>Delivery Strategy</th>
<th>Selected Advantages</th>
<th>Selected Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muteins</strong></td>
<td>• Scalable, recombinant production&lt;br&gt;• Smaller scale modification; less hindrance for tissue infiltration&lt;br&gt;• Easily combined with other delivery strategies (i.e. cytokine-protein fusions)</td>
<td>• Modifications limited in scope (i.e. often, truncations and point mutations)&lt;br&gt;• Potential to generate a cross-reactive immune response towards endogenous protein&lt;br&gt;• Protein stability can be reduced&lt;br&gt;• Circulation half-life can remain low</td>
</tr>
<tr>
<td><strong>Small molecule, peptide conjugates</strong></td>
<td>• Smaller scale modification; less hindrance for tissue infiltration&lt;br&gt;• Can preferentially target cells/tissues&lt;br&gt;• Circulation half-life can be extended&lt;br&gt;• Receptor selectivity can be biased</td>
<td>• Resistance possible due to downregulation of affinity target</td>
</tr>
<tr>
<td><strong>Polymer conjugates</strong></td>
<td>• Extended circulation half-life&lt;br&gt;• Receptor selectivity can be biased</td>
<td>• Separate production process for modifications&lt;br&gt;• Polymer-specific immune responses may be a concern</td>
</tr>
<tr>
<td><strong>Antibody-cytokine fusions</strong></td>
<td>• Scalable, recombinant production&lt;br&gt;• Preferential cell/tissue-localization&lt;br&gt;• Extended circulation half-life&lt;br&gt;• Receptor selectivity can be biased</td>
<td>• Resistance possible due to downregulation of affinity target&lt;br&gt;• Cytokine receptor binding can be difficult to overcome with affinity ligands</td>
</tr>
<tr>
<td><strong>Nano/microparticles</strong></td>
<td>• Can preferentially target cells/tissues&lt;br&gt;• Extended circulation half-life&lt;br&gt;• Controllable release kinetics&lt;br&gt;• Multifunctionality greater than recombinant techniques</td>
<td>• Separate production process for encapsulation/conjugation&lt;br&gt;• Regulatory considerations for both drug and carrier</td>
</tr>
<tr>
<td><strong>Hydrogels/implants</strong></td>
<td>• Controllable release kinetics&lt;br&gt;• Can act as scaffold for cell infiltration&lt;br&gt;• Multifunctionality greater than recombinant techniques</td>
<td>• Separate production process for encapsulation/conjugation&lt;br&gt;• Regulatory considerations for both drug and carrier&lt;br&gt;• Administration sites limited</td>
</tr>
</tbody>
</table>
Table 2.
Cytokines expressed by engineered (i.e. armored) chimeric antigen receptor (CAR) T cells.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Design in CAR construct</th>
<th>Activity</th>
<th>Clinical Trial</th>
</tr>
</thead>
</table>
| IL-12    | Fusion gene encoding the complete IL-12 protein with a serine-glycine repeat between the p35 and the p40 chain-coding domains | ○ Enhanced CAR T cell anti-tumor activity in vivo[165]  
○ CAR T cells resistant to Treg cell-mediated inhibition[165a]  
○ CAR T cell engraftment without preconditioning chemotherapy[165a,166]  
○ CAR T cell expansion from limited numbers of cells isolated from umbilical cord blood samples[167]  
○ Induced Th1 phenotype of CAR T cells[168]  
○ CAR T cells resistant to apoptosis and PD-L1-induced dysfunction[169]  
○ IL-12-secreting CAR T cells to treat patients with recurrent MUC16ecto+ solid tumors (NCT02498912) | IL-12-secreting CAR T cells to treat patients with recurrent MUC16ecto+ solid tumors (NCT02498912) |
|          | NFAT/IL-2 promoter-driven expression of inducible IL-12 | ○ Inducible delivery of IL-12 to the tumor microenvironment engages macrophages response to antigen-negative tumors[170]  
○ Enhanced elimination of solid tumors[171] | CD19-targeted CAR T cells with membrane-bound IL-15 to treat patients with recurrent/refractory CD19+ lymphoid malignancies (NCT03579888) |
| IL-15    | Membrane-tethered IL-15 | ○ Enhanced CAR T cell anti-tumor activity and long-term persistence[172]  
○ Induced memory-like phenotype in CAR T cells[172]  
○ Prevented disease relapse[172] |                                                                                                                    |
| IL-18    | Biologically active 18-kDa form of IL-18 | ○ Enhanced CAR T cell anti-tumor function[173]  
○ Modifies tumor microenvironment to increase proinflammatory M1-polarized macrophages and deplete anti-inflammatory M2-polarized macrophages and Treg cells[173a]  
○ Recruits endogenous tumor-specific T cells[173a] |                                                                                                                    |
| IL-1     | IL-1 Receptor agonist | ○ Prevents cytokine release syndrome while maintaining effective anti-tumor efficacy[174] |                                                                                                                    |

Nuclear factor of activated T-cells (NFAT), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-12 (IL-12), interleukin-15 (IL-15), interleukin-18 (IL-18), programmed death ligand-1 (PD-L1), regulatory T cell (Treg), T-helper 1 (Th1)
### Table 3.
Engineered cytokines in clinical development for the treatment of non-malignant diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Drug</th>
<th>Company</th>
<th>Composition</th>
<th>Target</th>
<th>Cell Type</th>
<th>Phase</th>
<th>Results</th>
<th>Dosing</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapsed multiple sclerosis</td>
<td>Plerix</td>
<td>Biogen</td>
<td>Interferon beta-1a attached to polyethylene glycol side chain</td>
<td>IFNAR</td>
<td>Antigen presenting cells</td>
<td>FDA approval granted after Phase III trials (NCT00906399)</td>
<td>○ 36% reduction in annualized relapse rate at week 48 ○ 35% reduction in sustained disability progression at week 48</td>
<td>○ 125μg every two weeks s.c. injection</td>
<td>[224]</td>
</tr>
<tr>
<td>Chronic Hepatitis C with compensated liver disease</td>
<td>Pegasys</td>
<td>Hoffmann-La Roche (Genetech)</td>
<td>Interferon alfa-2a attached to polyethylene glycol side chain</td>
<td>IFNAR</td>
<td>Antigen presenting cells</td>
<td>FDA approval granted after Phase III trials</td>
<td>275% increase in sustained viral response for PegIntron + ribavirin (54%) compared to interferon alfa-2a + ribavirin (47%)</td>
<td>○ 180μg per week (Adults) ○ 180μg/1.73 m² body surface area (Pediatrics) ○ s.c. injection</td>
<td>[175]</td>
</tr>
<tr>
<td>Chronic Hepatitis C with compensated liver disease</td>
<td>PegIntron</td>
<td>Merck &amp; Co., Inc.</td>
<td>Interferon alfa-2b attached to polyethylene glycol side chain</td>
<td>IFNAR</td>
<td>Antigen presenting cells</td>
<td>FDA approval granted after Phase III trials</td>
<td>15% increase in sustained viral response for PegIntron + ribavirin (54%) compared to interferon alfa-2b + ribavirin (47%)</td>
<td>○ 1.5μg/kg/week (Adults) ○ 60μg/kg/week (Pediatrics) ○ s.c. injection in combination with ribavirin</td>
<td>[176]</td>
</tr>
<tr>
<td>Acute radiation syndrome</td>
<td>Neulasta</td>
<td>Amgen Inc.</td>
<td>Granulocyte colony-stimulating factor attached to a polyethylene glycol side chain</td>
<td>Granulocyte colony-stimulating factor receptor</td>
<td>○ Hematopoietic stem and progenitor cells</td>
<td>FDA approval granted after preclinical studies</td>
<td>Increase in 60-day survival in irradiated non-human primates for Neulasta (91%) compared to control (48%)</td>
<td>○ 6mg, two doses s.c. injection</td>
<td>[177]</td>
</tr>
<tr>
<td>Rheumatoid Arthritis (RA)</td>
<td>Kineret</td>
<td>Swedish Orphan Biovitrum AB</td>
<td>Nonglycosylated IL-1R antagonist with addition of single methionine residue at the amino terminus</td>
<td>IL-1R</td>
<td>○ Monocytes ○ Macrophages</td>
<td>FDA approval granted after Phase III trials for RA (178) ○ 100ng/day (RA) ○ 1–2mg/kg/day (NOMID) ○ s.c. injection</td>
<td>○ 1.0-mg/kg Anakinra + melilotrate had significantly higher ACR20 response rate (46%) than placebo (19%) (178) (RA) Improvements occurred in all individual disease symptoms and serum markers of inflammation (179) (NOMID)</td>
<td>○ 100ng/day (RA) ○ 1–2mg/kg/day (NOMID) ○ s.c. injection</td>
<td>[178]</td>
</tr>
<tr>
<td>Chronic Hepatitis B (HBeAg+ patients)</td>
<td>Peginterferon lambda</td>
<td>Bristol- Myers Squibb</td>
<td>Interferon lambda-1a attached to a polyethylene glycol side chain</td>
<td>Interferon gamma receptor</td>
<td>○ Hepatocytes ○ Dendritic cells</td>
<td>Phase II Completed (NCT01204762)</td>
<td>At 24 weeks post-treatment, HBeAg seroconversion rates lower for lambda (13.8%) than peginterferon alfa-2a (30.1%)</td>
<td>○ 180μg every week for 48 weeks s.c. injection</td>
<td>[180]</td>
</tr>
<tr>
<td>Disease</td>
<td>Drug</td>
<td>Company</td>
<td>Composition</td>
<td>Target</td>
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<tr>
<td>Rheumatoid arthritis</td>
<td>Dekavil</td>
<td>Pfizer</td>
<td>IL-10 fused to anti-F8 antibody</td>
<td>○ Alternatively Spliced Extra Domain A of fibronectin</td>
<td>○ IL-10R</td>
<td>○ Macrophages</td>
<td>Phase II (NCT02270632), not yet completed</td>
<td>○ No SUSARs nor treatment-related deaths</td>
<td>○ 1x every week for 8 weeks, ○ s.c. injection with methotrexate</td>
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<tr>
<td>Ulcerative colitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.8% patients exhibited ACR responses after 8 cycles</td>
<td></td>
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</tr>
<tr>
<td>Psoriasis</td>
<td>APVO210</td>
<td>Aptevo Therapeutics</td>
<td>Monomeric IL-10 fused to anti-CD86 antibody</td>
<td>○ CD86</td>
<td>○ CD86+ monocytes</td>
<td>Phase I (NCT03768219) Estimated end date: Sept. 2020</td>
<td>No results posted</td>
<td>i.v. infusion</td>
<td>[115a]</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
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American College of Rheumatology (ACR), body surface area (BSA), interleukin-10 (IL-10), interleukin-10 receptor (IL-10R), intravenous infusion (i.v. infusion), natural killer cells (NK cells), subcutaneous injection (s.c. injection), suspected unexpected serious adverse reactions (SUSAR), type I interferon receptor (IFNAR), type I interleukin-1 receptor (IL-1R1).