nature reviews bioengineering

https://doi.org/10.1038/s44222-023-00030-y

Review article

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Engineering cytokine therapeutics

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Abstract

Cytokines have pivotal roles in immunity, making them attractive as therapeutics for a variety of immune-related disorders. However, the widespread clinical use of cytokines has been limited by their short blood half-lives and severe side effects caused by low specificity and unfavourable biodistribution. Innovations in bioengineering have aided in advancing our knowledge of cytokine biology and yielded new technologies for cytokine engineering. In this Review, we discuss how the development of bioanalytical methods, such as sequencing and high-resolution imaging combined with genetic techniques, have facilitated a better understanding of cytokine biology. We then present an overview of therapeutics arising from cytokine re-engineering, targeting and delivery, mRNA therapeutics and cell therapy. We also highlight the application of these strategies to adjust the immunological imbalance in different immune-mediated disorders, including cancer, infection and autoimmune diseases. Finally, we look ahead to the hurdles that must be overcome before cytokine therapeutics can live up to their full potential.

Sections

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Key points

- Cytokines are crucial regulators of the immune system and have important roles in health and disease.
- Recombinant cytokine therapy is hampered by the short blood half-life and severe side effects of cytokines.
- Increased knowledge of cytokine biology converges with new bioengineering approaches to develop engineered cytokine therapeutics.
- Cytokine therapeutics can be applied to modulate dysregulated immune responses in disorders such as cancer or autoimmune diseases.

Introduction

Finely tuned cooperation between diverse cell types is essential for the immune system to effectively exert its various functions. The immune system protects the host against pathogens and supports tissue repair, while safeguarding homeostasis in different tissues[1]. Communication during immune responses occurs through either direct cell-to-cell interaction or the release of biomolecules, the most important of which are small proteins known as cytokines[2–4]. Immune cells are the main source of cytokines, secreted in response to infections, inflammation or endogenous stimuli[5].

Several major cytokine classes can be distinguished according to their functions. Pro-inflammatory cytokines activate antimicrobial and immunostimulatory programmes and include tumour necrosis factor (TNF), IL-1β, IL-6 (ref. [6]), IL-17 and IL-22 (ref. [7]). Anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1RA) and transforming growth factor-β (TGFβ), resolve inflammation and promote wound healing[8,9]. Chemokines, such as IL-8 and CC-chemokine ligand 2 (CCL2), comprise another class of cytokines that direct immune cell migration[10]. Interferons are vital to antiviral immunity[11], and colony-stimulating factors, such as granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF), regulate homeostasis and immune cell progenitor proliferation[12]. Although immune cells produce most cytokines, non-immune cells and tissues can also secrete cytokines[5]. For example, endothelial and epithelial cells in the skin and the lungs express IL-33 and IL-25, which are involved in allergic inflammation[13,14]. Adipose tissue can release cytokines known as adipokines, which, among many capacities, regulate the body's metabolic state. Although leptin and resistin are adipokines, they also exert proinflammatory functions[15], illustrating the complex and diverse functions of cytokines.

Cytokines induce intracellular signalling after binding to their receptor on a target cell[16]. Cytokine receptors are as diverse as their ligands and are often multimers consisting of multiple transmembrane domains[17]. Upon binding, intracellular signalling prompts specific biological processes that, depending on the cytokine and cell type, can range from activating enzymes that regulate epigenetic modifications[18], cytokine synthesis[19] and augmented metabolism[15] to triggering cellular proliferation[20,21] or apoptosis[22]. A defining characteristic of cytokines is their ability to induce different phenotypic traits[23]. This phenomenon is known as pleiotropy, where a specific cytokine receptor can be present on various cell types, resulting in a variety of biological consequences[24] (Fig. 1a). Additionally, some cytokines can bind to multiple receptors, which in turn can produce diverse downstream effects, such as inducing cell differentiation or inactivating effector functions[25]. Different cytokines can also function as co-activators, generating different effects depending on how they synergize[26,27]. Moreover, cytokines show partial functional redundancy, where more than one cytokine can have the same biological function. For example, both IL-2 and IL-15 induce T cell proliferation[28], whereas IL-1α and TNF induce endothelial cell activation[29]. On the one hand, this ensures the immune system's robustness; on the other hand, it complicates both therapeutic immunoregulation and the design of cytokine-based immunotherapies.

Because cytokines have critical roles in many immune-mediated diseases, they have been investigated as therapeutic targets[30]. Inhibiting cytokine function by either monoclonal antibodies or receptor blockers is particularly successful in conditions characterized by exacerbated cytokine production, such as autoimmune and inflammatory diseases. Blocking TNF is an effective treatment strategy for rheumatoid arthritis and Crohn's disease[31]. Psoriasis can be treated by suppressing IL-17 or IL-23 (refs. [4,31]). IL-6 and IL-1 antagonists can treat COVID-19 (ref. [33]). Cytokines can also be therapeutically administered to direct immune responses. For example, recombinant protein technology has generated some approved cytokine drugs, such as colony-stimulating factors for haematological disorders[34] or type I interferons for viral diseases such as hepatitis[35]. However, developing cytokine-based therapeutics remains challenging. Cytokines' short blood half-lives, pleiotropism and unfavourable tissue distribution all contribute to their narrow therapeutic range[36]. In the late 1980s, a clinical trial with IFNα resulted in severe side effects such as WHO grade III flu-like symptoms in patients treated for hairy-cell leukaemia[37]. An IL-12 phase II clinical trial for treatment of renal cell carcinoma resulted in two deaths in 1995 (ref. [38]), and IL-2 clinical trials for metastatic melanoma lead to six deaths[39]. Here, we first discuss how advances in bioanalytical technologies can be deployed to deepen our understanding of cytokine biology. Then, we describe innovations in protein engineering, nanomedicine, RNA technology and cellular engineering in relation to cytokine therapeutics. We show that bioengineers can gain and harness knowledge to develop safe, effective cytokine-based therapies for a variety of immune-mediated diseases.

Understanding cytokine biology

Advances in bioengineering have greatly increased our understanding of cytokine biology over the past 40 years. Our knowledge has expanded on various levels, ranging from cytokine structure and receptor binding to cytokine pleiotropy and redundancies in signalling pathways, all of which ultimately help us to comprehend different cytokine functions. Early elucidation of protein identity was achieved after the development of polymerase chain reaction (PCR)[40] and Sanger sequencing[41]. Innovations in analytical modalities, including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for macromolecules, such as proteins, in the 1980s[42,43], helped us to study cytokine protein structure, including post-translational modifications.

Observing the 3D structure of cytokines is important to discern their function. Innovations in protein nuclear magnetic resonance (NMR)[44], X-ray crystallography[45] and cryogenic electron microscopy (cryo-EM)[46] have greatly advanced the cytokine research field. For example, structural analyses of cytokine–receptor complexes allowed

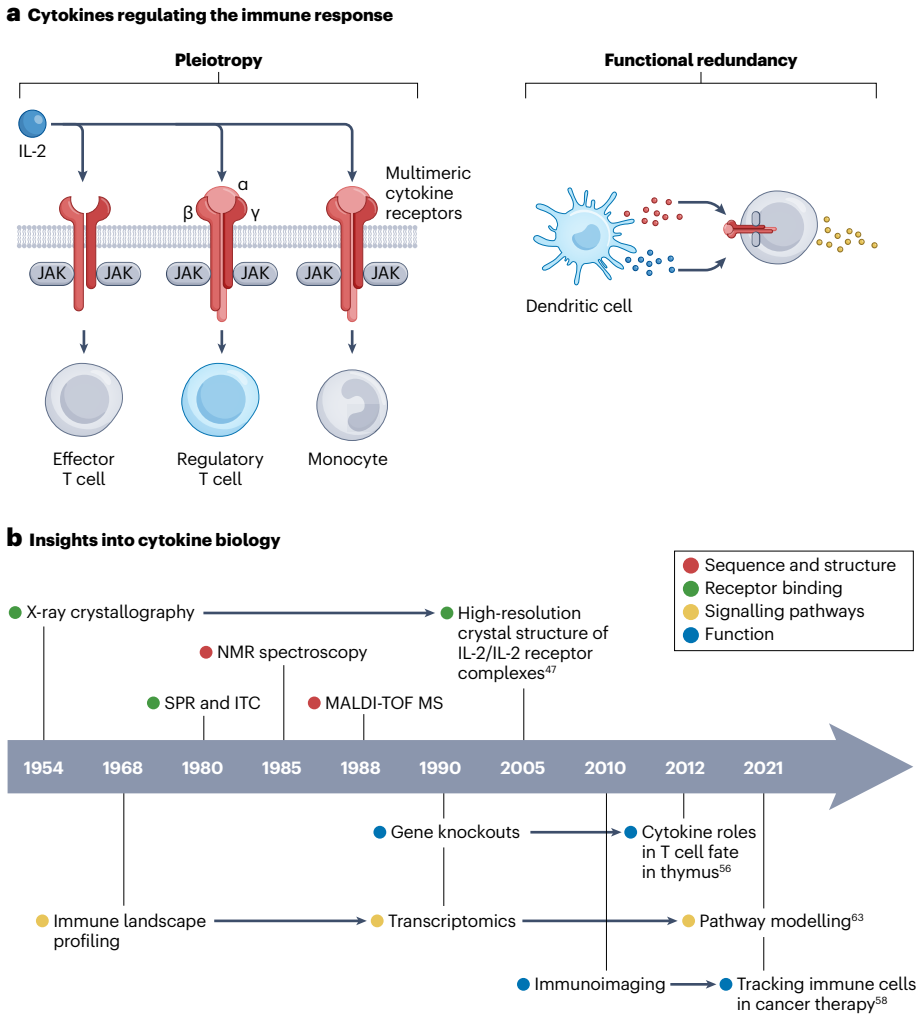

Fig. 1 | Cytokine biology and mechanisms. a, Cytokines are key regulators of the immune system and can be classified according to their function, for example, as pro-inflammatory or anti-inflammatory. Upon binding to the (multimeric) cytokine receptor on a target cell, cytokines can activate enzymes that regulate epigenetic modifications, cytokine synthesis, augmented metabolism, cellular proliferation and apoptosis. Cytokine pleiotropy refers to the ability to induce different phenotypic traits, resulting in a variety of biological consequences. The ability of cytokines to act on the same receptor indicates their redundancy. **b**, Insights into cytokine biology owing to advances in bioengineering, including greater knowledge about cytokine sequence and structure, receptor binding mechanisms, signalling pathways and function. Data are taken from refs. [47,56,58,63]. ITC, isothermal titration calorimetry; JAK, Janus kinase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; SPR, surface plasmon resonance.

us to understand the interleukin IL-2's interactions with its receptor (IL-2R) in detail. High-affinity IL-2R is composed of three subunits: IL-2Rα, IL-2Rβ and γ_c. IL-2Rβ and γ_c are sufficient for effective signal transduction through the formation of intermediate-affinity IL-2R, and IL-2Rα acts as an important affinity modulator that increases the sensitivity of cells to circulating IL-2 (refs. [47,48]). Supplementary to structural knowledge, techniques such as surface plasmon resonance (SPR)[49] and isothermal calorimetry (ITC)[50] can reveal binding kinetics and thermodynamics. Combined with insights obtained from structural biology, these bioanalytical techniques have enabled rational modifications of cytokines to regulate receptor binding and thus function. Comprehending complex cytokine signalling routes can help us to develop 'smart' cytokine therapeutics to overcome pleiotropy and thus off-target effects[51].

Ultimately, the binding of a cytokine to its receptors results in activation of the receiving cell and triggering of an intracellular signalling cascade. Various methods have been devised to study cytokines' functional effects, such as genetic techniques, imaging and single-cell technologies.

Advances in genetic techniques such as gene ablation allowed the generation of knockout mice[52]. Knockout and knock-in technologies have been crucial in understanding how cytokines and their receptors participate in host defence. For example, studies using *Il6*-knockout mice have identified IL-6's key role in resisting infections[53]. Cytokine knockout mice facilitated research into redundancies in the IL-1β pathway and helped to define distinct cytokine pathways for different fever-inducing stimuli[54]. Since the early 2010s, organ-specific conditional knockout of cytokine genes is used to investigate cytokines' role in

physiological processes, such as reproduction and T cell differentiation in the thymus[55,56].

Innovative imaging techniques also contribute to our understanding of cytokines in pathological processes. For example, intravital microscopy is used to investigate cytokines' roles in infections[57]. Nuclear imaging modalities, such as positron emission tomography with computed tomography (PET–CT), allow longitudinal tracking of labelled proteins in vivo, which can be used to study immune cells' spatial distribution[58].

Developments in single-cell technologies have considerably increased knowledge of cytokine functions in immune cells. One of the most important tools for exploring immunity is flow cytometry, which can be used to study immune cell activation and differentiation. Spectral flow cytometry allows the effects that cytokines exert on up to 40 parameters of a single cell to be probed[59]. Engineers have further expanded this with a new approach that combines flow cytometry with mass spectrometry: mass cytometry (cytometry by time of flight (CyTOF))[60]. In this modality, cell surface markers are labelled with lanthanide metals, which implies that theoretically 120 cellular markers can be detected simultaneously, although in practice the panel size is typically limited to roughly 60 parameters[61]. Single-cell RNA sequencing and transcriptomic analyses also contribute to understanding cytokine signalling and how cytokines affect immune cells. For example, proteomics combined with RNA sequencing datasets are applied to study naive and memory T cell responses to cytokines, revealing the heterogeneity of T cell reactivity in mounting a rapid cytokine response[62]. Transcriptomic profile datasets can also be used to model the interplay between cytokines. The complexity of cytokine biology means that computational modelling of cytokine signalling pathways should help to uncover new therapeutic targets, such as IL-8 in the case of severe COVID-19 (ref. [63]).

The bioanalytical innovations discussed above illustrate the sizeable leaps researchers have made in understanding cytokine biology (Fig. 1b). However, very recent discoveries such as trained immunity[64] and the existence of orphan cytokine receptors[65] indicate that we still do not completely comprehend the immune system's complexity. New analytical technologies will continue to deepen our insight into cytokine biology, which in turn will empower the development of cytokine therapeutics.

Re-engineering cytokine therapeutics

Our increased understanding of cytokine biology warrants revisiting their therapeutic exploitation. Together with advances in analytical techniques, innovative protein engineering tools have been developed to modify cytokine function, signalling and distribution. The introduction of PCR facilitated small protein modifications through point mutations[40,66]. Since then, the protein engineering field has progressed tremendously. In this section, we discuss how cytokine-based therapeutics (with an emphasis on IL-2 as an example) are designed using different protein engineering modalities.

Directed evolution

Two strategies can be employed for developing protein mutants; directed evolution and rational protein design. In directed evolution, mutations are introduced into a gene of interest and subsequently a variety of selection methods, such as yeast and phage display, are used to obtain a protein with the desired function. In contrast to rational design, directed evolution does not rely on detailed structural knowledge and protein 3D-structure prediction because it is based on functional selection of mutants. Thus, directed evolution is a popular and successful strategy for engineering proteins[67]. The method can be applied to create an IL-2 mutein with improved affinity for the IL-2Rβγ complex[51]. This dimeric form of the IL-2 receptor is mainly present on $CD4^+$ and $CD8^+$ memory T cells and natural killer (NK) cells, owing to low levels of IL-2Rα expression[68]. The toxicity of high-dose IL-2, which is a US Food and Drug Administration (FDA)-approved cancer therapy, is frequently associated with the activation of the high-affinity trimeric IL-2Rαβγ complex[68]. Moreover, the IL-2Rαβγ complex is abundantly expressed on regulatory T (T_{reg}) cells[68], and stimulating these immunosuppressive cells is counterproductive for anticancer approaches. In designing the mutein, error-prone PCR is used to create a mutagenic library of IL-2 variants that are screened for IL-2Rβ affinity. This eventually yields an IL-2 'superkine' with 200-fold higher affinity for IL-2Rβ. The superkine erases the affinity discrepancy between the dimeric IL-2Rβγ and the trimeric IL-2Rαβγ complex and results in greater antitumour responses with reduced toxicity in an in vivo mouse model. To improve its pharmacokinetic properties, this IL-2 superkine was fused to albumin (MDNA11) and is now undergoing a phase I/II clinical trial (NCT05086692) in patients with advanced solid tumours (Fig. 2).

Directed evolution has been applied to modify a variety of cytokines beyond IL-2. An IL-4 superkine has been developed to decouple its pleiotropic effects[69]. After binding to IL-4Rα, the IL-4/IL4Rα complex can engage either γ_c or IL-13Rα1 to form a type I or type II complex[70], respectively. One of the engineered IL-4 superkines (termed Super-4) displayed reduced pleiotropy owing to its 3,700-fold higher affinity for γ_c, effectively steering activity towards the type I receptor rather than towards the type II receptor. To improve IL-10's toxicity profile, an affinity-matured cytokine variant with an improved bioactivity profile at low dosages was developed[71]. In addition to improving affinity for receptors, directed evolution approaches can also improve specificity for receptors. IL-18 has potential as an anticancer drug, but its interactions with the inhibitory decoy receptor IL-18 binding protein (IL-18BP), which is frequently upregulated in tumours, has limited its clinical utility as an antitumour agent. Thus, directed evolution, using positive IL-18Rα and negative IL-18BP screening, can be applied to engineer an IL-18 mutein that evades its decoy receptor but still mediates inflammation[72].

Rational design

Instead of using directed evolution, methods have been developed to rationally redesign cytokine properties. These techniques rely heavily on detailed knowledge of cytokine structure and advanced protein modelling[73]. For example, IL-22's crystal structure bound to its receptor complex IL-22Rα/IL-10Rβ made it possible to reduce affinity for IL-10Rβ by mutating four key amino acid residues[74]. This quadruple IL-22 mutant has higher receptor and cell specificity, thereby diminishing undesirable pro-inflammatory properties and preserving its regenerative functions in vivo. Seeking to reduce pleiotropy, similar approaches yield variants of therapeutically potent cytokines, such as IL-10 (ref. [75]), IFNγ[76], IL-12 (ref. [77]) and IL-4 (ref. [69]). Interestingly, IL-2's pleiotropy can also be exploited to rebalance immunity towards tolerance. Concurrent with IL-2 re-engineering efforts for cancer immunotherapy, engineers have developed IL-2 muteins that selectively activate T_{reg} cells for treating autoimmune diseases[78–84].

PEGylation

In addition to their binding properties, the circulation half-life of cytokines determines the in vivo effects of cytokine-based therapeutics. Functionalizing proteins with polyethylene glycol (PEG) is a popular and effective way to prolong their persistence in the circulation[85].

PEGylation increases a protein's molecular weight and creates a hydrophilic corona that expands the hydrodynamic diameter, thus reducing renal clearance, and decreases interactions with plasma constituents, thereby diminishing immunogenicity[86]. PEGylation has successfully been applied to colony-stimulating factors and interferons (Table 1). However, although these changes facilitate alternate dosage regimens through a modified pharmacokinetic profile, they fail to reduce IL-2 toxicity[87,88]. PEGylation can also be applied for other purposes. IL-10 is known for its anti-inflammatory properties and is being clinically investigated as a therapy for inflammatory diseases (Table 1). Interestingly, at higher concentrations, IL-10 activates cytotoxic T cells, making it potentially useful for cancer immunotherapy[89]. Re-engineering was required to achieve elevated local IL-10 concentrations without causing severe side effects. This prompted the development of an IL-10 PEG-conjugate (pegilodecakin)[90], with an improved pharmacokinetic profile, which is now undergoing clinical trials for treating various cancers (Table 2). Although PEGylation has historically been used to improve the blood half-life of proteins, the new PEGylated cytokines are designed to additionally alter receptor-binding[91–93]. In an attempt to take advantage of IL-2's dual role in immunity, Nektar Therapeutics developed both T_{reg} cell-selective (NKTR-358)[93] and NK cell- and effector T cell-selective (BEMPEG/NKTR-214)[92] IL-2 PEG-conjugates. However, all clinical trials of BEMPEG have been terminated owing to lack of clinical benefit[94]. Besides PEGylated IL-2 therapeutics, Nektar Therapeutics also engineered an IL-15-PEG variant (NKTR-255) with natural receptor binding and improved pharmacokinetic properties, aiming to circumvent the toxicity caused by the frequent dosing required in conventional IL-15 cancer therapy[95] (Table 2).

Expanding the genetic code with unnatural amino acids allows a unique reactive group to be introduced at a specific site within the protein, granting full control over the PEG chains' position and stoichiometry[96]. Using this approach, Synthorx has designed a new PEG-IL-2 variant (THOR-707)[91], which is currently in a phase I/II clinical trial (NCT04009681) as a potential therapy against advanced or metastatic solid tumours. The PEG moieties of THOR-707 specifically block the binding site with the IL-2Rα subunit, but maintain interaction with the other receptor subunits (Fig. 2). As a result, THOR-707 improves effector T cell and NK cell expansion and activation, and displays improved pharmacokinetic and safety profiles in cancer models compared to wild-type IL-2. A possible limitation of PEGylated cytokines is that the patient may develop anti-PEG antibodies that could inactivate the cytokine or initiate an inflammatory immune response[97]. This phenomenon has not been observed in studies with PEGylated cytokines, but the widespread application of mRNA COVID-19 vaccines shows that PEG can cause anaphylaxis in rare cases[98].

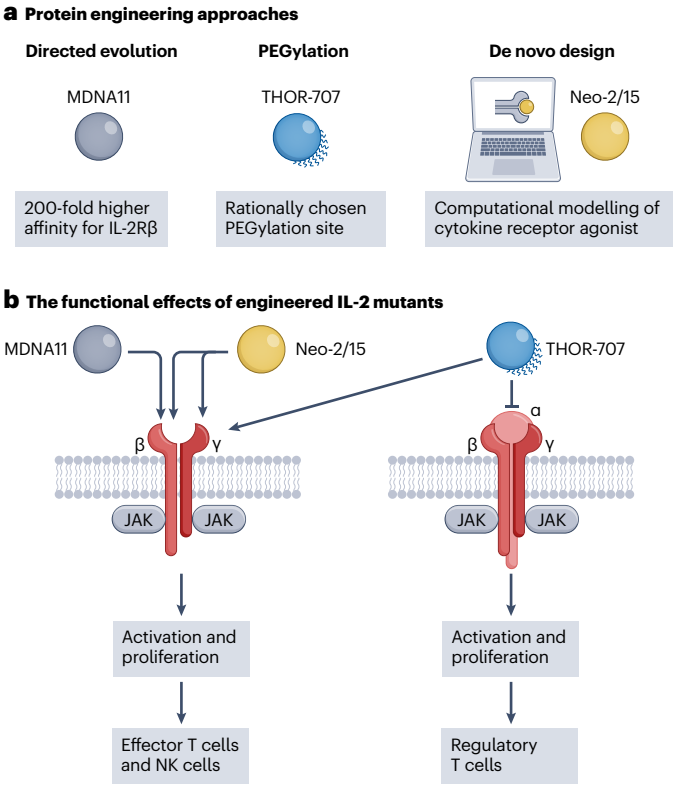

Fig. 2 | Re-engineering cytokines. a, Bioengineering methods for improving the function and pharmacokinetic properties of IL-2 include directed evolution, rational PEGylation and de novo design signalling. Taking advantage of these engineering tools, three different cytokine-based drugs (MDNA11, THOR-707 and Neo-2/15) have been developed to enhance IL-2Rβγ-mediated signalling. MDNA11 is a variant of IL-2, developed using directed evolution, with 200-fold higher binding affinity for IL-2 receptor subunit-β (IL-2Rβ) compared to IL-2. To develop THOR-707, rational PEGylation is employed to block IL-2's affinity for IL-2Rα to eliminate IL-2's innate bias towards cells that express IL-2Rα. Neo-2/15 is produced by computationally modelling peptide motifs in IL-2Rβγ without interacting with IL-2Rα, resulting in a de novo protein that selectively mediates IL-2Rβγ signalling. **b**, High specificity of cytokine-based drugs increases effector T cell and natural killer (NK) cell activation and proliferation, but prevents signalling through the trimeric form (IL-2Rαβγ), thereby minimizing undesired activation of regulatory T cells. JAK, Janus kinase; PEG, polyethylene glycol.

Computational methods

With the aid of computational and data-driven methods and recent advances in hardware and algorithmic power, proteins can be designed de novo[99–101]. Computational tools promise to help to overcome key limitations of cytokine drugs, including pleiotropy, redundancy, poor pharmacokinetics and toxicity[102]. There are multiple approaches to computer-assisted protein design, for example, relying on predicted changes in free energy[103], multiple sequence alignments[103,104], backbone redesign[105,106] or deep learning[107,108]. Moreover, computational tools initially designed for protein structure prediction, such as Rosetta[109] or AlphaFold[110], can provide valuable insights into protein design. Such computer-assisted tools could be used to customize cytokine agonists that are not necessarily related in amino acid sequence or topology to their natural equivalents. Therefore, the same function can be achieved with a completely different sequence. De novo protein design can be employed to create synthetic cytokines (synthekines) that engage unnatural receptor domain pairs, for example, IL-2Rβ/IL-10Rβ[111], IL-2Rβ/IL-4Rα or IL-4Rα/IFNαR2, and therefore activate distinct signalling pathways[112]. De novo design methods also provide a strategy to effectively decouple cytokine pleiotropy and target specific pathways, as illustrated by phase I clinical trials with Synthekine's STK-012 (ref. [113]) and Neoleukin Therapeutics's NL-201 (ref. [106]) (Table 2). NL-201 has been developed by de novo computational design, using the crystal structure of IL-2 and IL-15 bound to the IL-2Rβγ receptor subunits as a blueprint. Computational design algorithms have been subsequently employed to engineer a new protein that mimics natural cytokine interactions with IL-2Rβγ without binding to IL-2Rα. Further stability

Table 1 | Approved and failed cytokine therapies

Cytokine	Name	Route of administration	Disease	Company	Outcome
Approved					
IL-1RA	Kineret/anakinra	s.c.	Range of autoimmune disorders	Swedish Orphan Biovitrum	Approved by the FDA in 2001 and the EMA in 2002
IL-2	Proleukin/ aldesleukin	i.v. (high dose) or s.c. (low dose)	Metastatic melanoma and renal cell carcinoma	Clinigen Group	Approved by the FDA in 1992
	Ontak/denileukin diftitox/ IL2-diftitox	i.v.	T cell lymphoma	Eisai	Approved by the FDA in 2008[c]
IL-11	Neumega/ oprelvekin	s.c.	Thrombocytopaenia following myelosuppressive chemotherapy	Wyeth (now Pfizer)	Approved by the FDA in 1997[d]
TNF	Beromun/ tasonermin	Isolated-limb perfusion	Limb soft-tissue carcinoma	Belpharma	Approved by the EMA in 1999
G-CSF	Neupogen/ filgrastim	s.c.	Neutropenia	Amgen	Approved by the FDA in 1991
	Neulasta/ pegfilgrastim				Approved by the FDA and the EMA in 2002
	Lonquex/ lipegfilgrastim[a]			Teva Pharmaceuticals	Approved by the EMA in 2013
GM-CSF	Leukine/ sargramostim	i.v. or s.c. (after myelosuppressive radiotherapy)	Post radiotherapy and chemotherapy, and bone marrow and peripheral blood cell transplantation	Partner Therapeutics	Approved by the FDA in 1991
IFNα2a	Roferon A	s.c.	Chronic hepatitis C, hairy cell leukaemia and chronic myelogenous leukaemia	F. Hoffmann-La Roche	Approved by the FDA in 1995[d]
	Pegasys[a]		Chronic hepatitis B and C		Approved by the FDA in 2002
IFNα2b	Intron A	i.v., i.m. or s.c.	Chronic hepatitis B and C, hairy cell leukemia, malignant melanoma, follicular lymphoma, Kaposi's sarcoma and genital warts	Schering-Plough (now Merck)[b]	Approved by the FDA in 1995[d]
	PegIntron/ Sylatron[a]	s.c.	Chronic hepatitis C, melanoma		Approved by the FDA in 2001 and the EMA in 2000[e]
	Besremi[a]		Polycythaemia vera	PharmaEssentia	Approved by the FDA in 2021
IFN mix	Multiferon	s.c.	Malignant melanoma	Swedish Orphan Biovitrum	Approved in several European countries since 2005
IFNβ1a	Avonex/Rebif/ Cinnovex/ Soluferon	i.m. or s.c.	Multiple sclerosis	Biogen, Merck[b] and CinnaGen	Approved by the FDA in 1996/2002
	Plegridy[a]			Biogen	Approved by the FDA in 2014
IFNβ1b	Betaseron/ Extavia	s.c.	Multiple sclerosis	Bayer and Novartis	Approved by the FDA in 1993
IFNγ1b	Actimmune	s.c.	Chronic granulomatous disease	Horizon Pharma	Approved by the FDA in 1990
Epoetin alfa	Epogen/Procrit	i.v. or s.c.	Anaemia	Amgen and Johnson & Johnson	Approved by the FDA in 1989
	Aranesp/ darbepoetin alfa			Amgen	Approved by the FDA in 2001
	Mircera[a]			F. Hoffmann-La Roche	Approved by the FDA and the EMA in 2007
	Omontys/ peginesatide[a]			Affymax and Takeda Pharmaceutical	Approved by the FDA in 2012[f]
Epoetin beta	NeoRecormon	i.v. or s.c.	Anaemia	F. Hoffmann-La Roche	Approved by the EMA in 2001
Epoetin theta	Biopoin	s.c.	Anaemia	Teva Pharmaceuticals	Approved by the EMA in 2009

Table 1 (continued) | Approved and failed cytokine therapies

Cytokine	Name	Route of administration	Disease	Company	Outcome
Failed					
IL-4	NA	s.c.	Psoriasis	Schering-Plough (now Merck)[b]	Limited clinical benefit[207]
			AIDS-related Kaposi's sarcoma	NIAID	Limited clinical benefit[208]
IL-7	NA	s.c.	Metastatic melanoma, locally advanced or metastatic kidney cancer, refractory solid tumours	Revimmune and National Cancer Institute	Limited clinical benefit, formation of neutralizing antibodies[209,210]
IL-10	Tenovil	s.c.	Crohn's disease	Schering-Plough (now Merck)[b]	No clinical benefit[211,212]
	NA	i.v.	Rheumatoid arthritis	Tufts University School of Medicine	No clinical benefit and complications upon multiple dosing[213]
IL-12	NA	i.v.	Renal cell carcinoma	Wyeth (now Pfizer)	Severe toxicity issues: 2 deaths upon high dosing[38]
IL-15	NA	i.v.	Metastatic malignant melanoma or metastatic renal cell cancer	National Cancer Institute	Toxicity issues[214]
IL-21	NA	i.v.	Recurrent or metastatic melanoma	ZymoGenetics (now Bristol Myers Squibb)	No clinical benefit[215]
TRAIL	Dulanermin/AMG 951	i.v.	Colorectal cancer, non-Hodgkin's lymphoma, non-small-cell lung cancer	Amgen and Genentech (F. Hoffmann-La Roche)	No clinical benefit and adverse events[216–218]

EMA, European Medicines Agency; FDA, US Food and Drug Administration; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL-1RA, IL-1 receptor antagonist; i.m., intramuscular; i.v., intravenous; NA, not applicable; NIAID, National Institute of Allergy and Infectious Diseases; PEG, polyethylene glycol; s.c., subcutaneous; TNF, tumour-necrosis factor; TRAIL, tumour-necrosis factor-related apoptosis-inducing ligand. [a]These are PEGylated cytokines. [b]Merck Sharp and Dohme outside the USA and Canada. [c]Discontinued in 2014 following manufacturing issues. [d]Discontinued. [e]Induces severe side-effects (for example, suicidal depression). [f]Discontinued in 2013 owing to severe adverse effects.

optimizations generate the final variant Neo-2/15, which prolongs native IL-2's inactivation half-time (Fig. 2 and Box 1). Although these computational-based cytokine redesigns target the cytokine–receptor interface, new affinity-matured cytokines can also be engineered solely through computational-driven protein stabilization, as showcased in a proof-of-concept study with a new IL-2Rβ-selective variant[114]. This computational protein stabilization approach requires less experimental intervention compared to the receptor-interface-focused designs, such as STK-012 and NL-201, potentially decreasing the costs and time involved in computational cytokine engineering. De novo-designed proteins, such as NL-201, are inherently foreign and thus may present immunogenicity issues. Although not observed in preclinical studies of Neo-2/15 (ref. [106]), this remains an important consideration for the clinical translation of de novo cytokines.

Targeting and delivery

Routing cytokines with fusion proteins

Genetically fusing a cytokine to other proteins can help to reshape the cytokine's biodistribution profile. This approach can be applied to target cytokines to a specific site or cell type. Cytokine fusion constructs help to promote tumour localization and overcome poor pharmacokinetic properties and unfavourable biodistribution profiles of cytokine-based drugs.

Immunocytokines. The idea of using antibodies to improve cytokine cancer therapies was introduced at the end of the twentieth century[115,116]. Over the past 15 years, multiple antibody–cytokine fusion constructs, known as immunocytokines, with IL-2 have been engineered and are undergoing clinical trials for treating various types of cancer (Table 2). In addition to improving currently approved cytokine therapies such as IL-2, designing immunocytokines could revitalize failed cytokine therapy approaches.

Although IL-12's potency as an antitumour agent was recognized three decades ago, early clinical trials failed and resulted in two treatment-related deaths[38] (Table 1). However, genetically fusing IL-12 to tumour-targeting antibodies can increase this cytokine's therapeutic window and limit toxicity, as demonstrated by fusing IL-12 to an L19 antibody[117]. The L19 antibody is specific to fibronectin's extra-domain B (EDB), a marker overexpressed on angiogenic blood vessels, which are abundant in many cancers[118]. In another study, two IL-12 heterodimers were fused to a histone-binding antibody (NHS76)[119] to form the NHS–IL-12 immunocytokine that targets extracellular DNA in regions of tumour necrosis and apoptosis (Fig. 3a). The genetic fusion increases IL-12's blood half-life, improves its therapeutic window, improves tumour localization and augments antitumour activity in murine Lewis lung carcinoma, MC38 colon carcinoma and B16 melanoma models. Both of these IL-12-based immunocytokines are currently undergoing clinical trials (NCT01417546 and NCT04471987) as treatments against solid tumours. Although most immunocytokines aim to deliver a pro-inflammatory cytokine to the tumour, the platform's versatility can also direct anti-inflammatory cytokines to inflamed tissues. This is illustrated by the IL-22 immunocytokine (ABBV-022; out-licensed by Abbvie) which targets the gut mucosa for treatment of ulcerative colitis[120].

Table 2 | Engineered cytokine therapeutics in clinical trials

Approach	Cytokine	Aim	Administration route	Disease	Company	Clinical trials
Re-engineered cytokines						
Amino acids	BAY 50-4798/ N88R mutein	Non-β-IL-2	s.c.	HIV	Bayer	NCT00059462
	PT101/MK-6194			Ulcerative colitits	Pandion Therapeutics (acquired by Merck)[a]	NCT04924114
Glycosylation	CYT107	Prevent IL-7 antibody neutralization	i.m.	COVID-19, refractory nontuberculous mycobacterial lung disease, locally advanced, inoperable or metastatic urothelial carcinoma	Revimmune	NCT03513952, NCT04154826, NCT04379076
PEGylation	THOR-707	Non-α-IL-2	i.v.	Advanced or metastatic solid tumours	Synthorx (acquired by Sanofi)	NCT04009681
	NKTR-358	Non-β-IL-2		Systemic lupus erythematosus	Nektar Therapeutics	NCT03556007
	NKTR-255	Improve pharmacokinetics IL-15	i.v.	Colorectal cancer and head and neck squamous cell carcinoma		NCT04616196
	Pegilodecakin	Improve IL-10	s.c.	Advanced solid tumours	Eli Lilly and Company	NCT02009449
	Peginterferon lambda-1a	Improve pharmacokinetics IFNλ1a		Chronic hepatitis D, COVID-19	Eiger BioPharmaceuticals	NCT05070364, NCT04967430
Directed evolution	MDNA11/IL-2 'superkine'–albumin	non-α-IL-2	i.v.	Advanced solid tumours	Medicenna Therapeutics	NCT05086692
	MDNA55/circular permuted IL4–PE	Tumour (IL-4R) targeting of PE		Recurrent/progressive glioblastoma	Medicenna Therapeutics	NCT02858895
De novo	NL-201	Non-α-IL-2/IL-15	i.v.	Relapsed or refractory cancer	Neoleukin Therapeutics	NCT04659629
	STK-012	λ/β-selective PEGylated IL-2 mutein	s.c.	Advanced solid tumours	Synthekine	NCT05098132
Targeting and delivery						
Immunocytokines	CEA-IL2v/RG7813	Tumour (CEA) targeting + non-α-IL-2 fusion	i.v.	Advanced or metastatic solid tumours	F. Hoffmann-La Roche	NCT02350673
	Simlukafusp alfa/ RG7461/FAP–IL-2v	Tumour (FAP) targeting + non-α-IL-2 fusion				NCT03386721
	ANV419/IL-2A–IL-2	Non-α-IL-2		Advanced solid tumours and multiple myeloma	Anaveon	NCT04855929
	F16-IL2	Tumour (TNC A1) targeting		Acute myeloid leukemia	Philogen	NCT02957032
	Darleukin/L19–IL-2	Tumour (EDB fibronectin) targeting		Metastatic non-small-cell lung cancer	Maastricht University	NCT03705403
	DI-Leu16-IL2	Tumour (CD20) targeting		B cell non-Hodgkin lymphoma	Alopexx	NCT02151903
	RG6279/ RO7284755/ PD1–IL-2v	Tumour (PD1) targeting		Advanced or metastatic solid tumours	F. Hoffmann-La Roche	NCT04303858
	hu14.18-IL2	Tumour (GD2) targeting	i.t.	Advanced melanoma	Apeiron Biologics	NCT03958383
	Efavaleukin alfa/ AMG 592/Fc–IL-2v	Non-β-IL-2	s.c.	Autoimmune diseases	Amgen	NCT04987333
	XmAb564/Fc-IL-2				Xencor	NCT04857866
	Efineptakin alfa/ GX-I7/NT-I7/ IL-7–hyFc/ hyleukin-7	Improve pharmacokinetics of IL-7	i.p.	COVID-19, Kaposi sarcoma, squamous cell carcinoma	Genexine and NeoImmuneTech	NCT04730427, NCT04893018, NCT04588038
	Dekavil/F8–IL-10	Inflamed tissue (EDA fibronectin) targeting	s.c.	Rheumatoid arthritis	Philogen	NCT02270632

Table 2 (continued) | Engineered cytokine therapeutics in clinical trials

Approach	Cytokine	Aim	Administration route	Disease	Company	Clinical trials
Targeting and delivery (cont.)						
Immunocytokines (cont.)	Dodekin/ IL-12–L19L19	Tumour (EDB fibronectin) targeting	i.v.	Advanced solid tumours	Philogen	NCT04471987
	NHS–IL12	Tumour (NHS76) targeting		Solid tumours	Merck and Co[a]	NCT01417546
	Fibromun/L19TNF	Tumour (EDB fibronectin) targeting	i.v.	Metastatic soft tissue sarcoma, grade III/IV glioma, glioblastoma	Philogen	NCT03420014, NCT03779230, NCT04443010
	TAK-573/ CD38–IFNα2b	Tumour (CD38) targeting		Refractory multiple myeloma	Takeda Pharmaceutical	NCT03215030
Fusion protein: IL-2–E7–HLA	CUE-101	Activation of tumour-specific (HPV16) T cells	i.v.	Recurrent/metastatic head and neck quamous cell carcinoma	Cue Biopharma	NCT03978689
Fusion protein: cpIL-2–IL2Rα	ALKS 4230/ nemvaleukin alfa	Non-α-IL-2	i.v.	Advanced or metastatic solid tumours	Alkermes	NCT04592653
Fusion protein: IL-15–IL-15Rα (sushi) heterodimer	NIZ958/ IL-15–sIL-15Rα	IL-15 superagonist	s.c.	Metastatic cancers	Novartis	NCT02452268
	XmAb24306/ RO7310729	IL-15 superagonist–Fc complex	i.v.	Advanced or metastatic solid tumours	Genentech (F. Hoffmann-La Roche)	NCT04250155
	Anktiva/N-803/ ALT-803			Advanced solid tumours	Altor BioScience	NCT01946789
Prodrug	TransCon IL-2β/γ	Long-acting non-α-IL-2	i.v.	Advanced or metastatic solid tumours	Ascendis Pharma	NCT05081609
	XTX202	TME-selective prodrug		Advanced solid tumours	Xilio Therapeutics	NCT05052268
Nucleic acid therapeutics						
Plasmid	NNC0361-0041/ TOPPLE T1D	Antigen-specific tolerance through IL-2, preproinsulin, TGFβ1 and IL-10 expression	s.c.	Type 1 diabetes	Novo Nordisk	NCT04279613
	pIL-12 electroporation	IL-12 expression in tumour	i.t.	Metastatic melanoma	H. Lee Moffitt Cancer Center and Research Institute	NCT00323206
mRNA	mRNA-6231	IL-2–human albumin fusion protein expression	s.c.	Autoimmune diseases	ModernaTX	NCT04916431
	BNT-151	Optimized IL-2 expression	i.v.	Advanced or metastatic solid tumours	BioNTech	NCT04455620
	BNT-153	IL-2 expression				NCT04710043
	BNT-152	IL-7 expression				NCT04710043
	MEDI1191	IL-12 expression in tumour	i.t.		ModernaTX and Medimmune (now AstraZeneca)	NCT03946800
	SAR441000/ BNT-131	IL-12, IL-15Rα (sushi), IFNα and GM-CSF expression in tumour			Sanofi and BioNTech	NCT03871348
	mRNA-2752	IL-23 and IL-36γ expression in tumour			ModernaTX	NCT03739931
Cell therapy						
TCR T cell	LMBP2-specific IL-12 expressing TCR T cell	Improve TCR therapy with IL-12	i.v.	EBV-positive metastatic/ refractory nasopharyngeal carcinoma	TCRCure Biopharma	NCT04509726
CAR NK cell	IL-15 expressing CAR NK cells	Enhance in vivo expansion of CAR NK cells with IL-15	i.v.	CD19-positive lymphoid tumours	M.D. Anderson Cancer Center	NCT03056339

CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; EBV, Epstein–Barr virus; EDA, extra-domain A; EDB, extra-domain B; FAP, fibroblast activation protein; HPV16, human papillomavirus 16; IFN, interferon; i.m., intramuscular; i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous; NK cell, natural killer cell; PD1, programmed cell death 1; PE, *Pseudomonas* exotoxin; PEG, polyethylene glycol; s.c., subcutaneous; TCR, T cell receptor; TGFβ1, transforming growth factor β1; TME, tumour microenvironment; TNC, tenascin C; TNF, tumour necrosis factor. [a]Merck Sharp and Dohme outside the USA and Canada.

Box 1

Translational considerations

Protein engineering methods in academic laboratories differ vastly from those used in scaled-up good manufacturing practice (GMP) production. In research laboratories, protein therapeutics, including cytokine-based drugs, are often recombinantly expressed with purification tags in bacterial hosts, such as *Escherichia coli*. Although this approach allows swift development and downstream purification, it makes downstream processing challenging[219]. Bacterial endotoxins can be detrimental, particularly for drugs that regulate immune responses[220]. Therefore, for translational purposes, mammalian expression workflows are preferred. In addition to host organism considerations, production scale must be considered, in part because preclinical (mouse) models are much smaller than clinical (human) models. Moreover, stability studies, bioburden characterization and analytical methods often require large quantities of therapeutics. Substantial amounts of protein must also be generated to optimize the upstream manufacturing processes and the downstream processing[221] required to purify the protein and minimize potentially harmful contaminants, such as endotoxins.

Upon establishing a robust, GMP-ready manufacturing process, investigational new drug (IND)-enabling studies must include dose range finding and toxicity experiments. The choice of species here is crucial. Unlike for small-molecule drugs, differences in cytokine biology and cross-species reactivity must be carefully considered when developing cytokine-based therapeutics. If the cytokine is not cross-reactive between human and murine systems, a mouse variant of the human cytokine must be developed and then evaluated in vitro and in vivo. Using the mouse surrogate protein, the pharmacological and immunological properties of the molecule can be studied in animal models of the disease. Optimizing binding affinity and specificity is most efficiently done using in vitro assays. Although in vitro models may provide some information on potential dosing and toxicity, full-scale toxicity data must be obtained in animal models, preferably in non-human primates. Drug biodistribution and in vivo bioavailability can only be studied in animal models.

The shelf-life, storage conditions and worldwide distribution of drugs are typically not considered in the early development stages; however, these considerations are crucial as development progresses. For IND-enabling dose range finding and toxicity studies, the formulation and storage conditions must resemble those used for subsequent phase I and II studies in humans. For example, for protein-based drugs with limited stability that require storage at low temperatures, cryoprotectants must be added to the formulation. Such considerations are best established early in the development trajectory.

Despite these improvements in cytokine localization, immunocytokine interactions with off-target cells can still cause side effects. For example, IL-2 immunocytokines mainly associate with IL-2R-expressing innate immune cells instead of accumulating in the tumour microenvironment (TME)[121]. To improve receptor and cellular specificity, new engineered therapeutics combine the immunocytokine approach with re-engineered cytokine mutants (Table 2). IL-2 can be fused to the anti-IL-2 antibody JES6-1, which blocks IL-2 interactions with the IL-2Rβ and γ_c domains, but displays a unique allosteric exchange mechanism between the antibody binding site and IL-2Rα, selectively activating IL-2Rα-expressing T_{reg} cells[122]. Orionis Biosciences is developing a special class of immunocytokines, called Activity-on-Target cytokines (AcTakines), which combine a targeting antibody and an engineered cytokine mutant with reduced receptor affinity. The reduced affinity of AcTakines hampers cytokine activity until the fusion protein accumulates near the cytokine receptor on a target cell, lowering off-target effects[123,124]. Despite these advances, immunocytokines contain foreign epitopes that can induce immunogenicity, as reported in a phase I clinical trial (NCT03958383) of an IL-2 immunocytokine for treatment against melanoma and neuroblastoma[125]. Nevertheless, numerous immunocytokines are progressing to phase II clinical trials, demonstrating the promise of this therapeutic approach (Table 2).

Tumour localization. Rather than targeting protein antigens on cells, alternative fusion protein strategies can be adopted to overcome cytokine therapeutics' limitations. Collagen is the major component of the TME and is therefore an interesting target for cytokine tumour localization[126]. Two collagen-binding protein fusion constructs have been engineered with either IL-2 or IL-12, anchoring these cytokines to collagen in the TME upon intratumoral administration[127] (Fig. 3a). This approach increases cytokine retention in the tumour, consequently reducing systemic toxicity and boosting cellular antitumour immunity. In a similar strategy, IL-12 fused to a phosphorylated aluminium hydroxide (alum, the FDA-approved vaccine adjuvant) binding peptide tag shows weeks-long retention in the tumour in mouse models[128]. An alternative tumour retention strategy, that is, fusing IL-12 to the A3 collagen-binding domain of von Willebrand factor, can increase the cytokine's tumour accumulation and decrease systemic toxicity upon intravenous administration[129]. The same strategy can be applied to IL-2, highlighting this approach's adaptability for improving cytokine tumour accumulation[130].

Prodrug strategies. One approach to overcoming off-target effects is to design prodrug constructs, whereby a peptide or protein linked to the cytokine renders it inactive. The inactivating unit can be cleaved, for example, by proteases overexpressed at the disease site, to reactivate the cytokine[131]. This strategy is particularly useful in the context of cancer treatment, for which several tumour-specific proteases have been identified[132]. Cytokine prodrug designs are often combined with re-engineered cytokines. For example, ProIL2 is an inactive form of the IL-2 superkine and is activated through cleavage by tumour-associated proteases[133]. Another non-IL-2Rα prodrug with a similar mechanism[134] (Table 2 and Fig. 3a) is currently being tested in a clinical trial (NCT05052268) treating advanced solid tumours. Arrow Immune[135] and Werewolf Therapeutics[136,137] are employing this tumour protease approach to develop prodrugs of various cytokines, including IL-2, IL-12 and IFNα2b. Ascendis Pharma uses a different prodrug strategy with TransCon IL-2β/γ involving transient conjugation[138]. In addition to elevating IL-2Rβγ bias, this sustained-release prodrug is designed to prevent IL-2 spike concentrations and prolong blood half-life and has advanced to a phase I/II clinical trial (Table 2).

Routing cytokines with nanomedicine

Nanomedicine approaches have the potential to modulate the bioavailability and biodistribution profile of cytokines by routing them to specific organs or (immune) cells. However, as most cytokines act

through interactions with cell surface receptors, nanomedicine strategies should generally avoid uptake by immune cells to facilitate interactions at the cell surface (Fig. 3b). To accomplish this, two approaches have been pursued: cytokines can be presented on the nanoparticle surface to enable direct interactions with their respective receptors, or nanomedicines can be directed to non-cellular targets, such as collagen in the extracellular matrix, where cytokines are released for local interaction with their receptors. The first approach was demonstrated by surface-functionalization of PEGylated liposomes with IL-2 and anti-CD137 to route IL-2 to effector T cells[139] (Fig. 3b). Upon intravenous injection, the liposomes preferentially accumulate in the tumour, expand $CD8^+$ T cells and induce an antitumour response in mice. A non-cellular targeting approach involves a type-IV-collagen-binding polymeric nanoparticle delivering anti-inflammatory IL-10 to atherosclerotic plaques[140] (Fig. 3b). Upon collagen binding of these particles, polymer degradation slowly releases IL-10, allowing its subsequent interaction with inflammatory cells. A therapeutic regimen consisting of four injections over a four-week period prevented plaque formation in a murine model of atherosclerosis[140].

mRNA-based cytokine therapeutics

The COVID-19 pandemic has propelled the development, clinical approval and widespread deployment of mRNA-based vaccines and RNA nanotherapeutics in general[141]. As a result, there is potential for therapeutic strategies based on regulating cellular cytokine expression following intracellular mRNA delivery by nanoparticles. However,

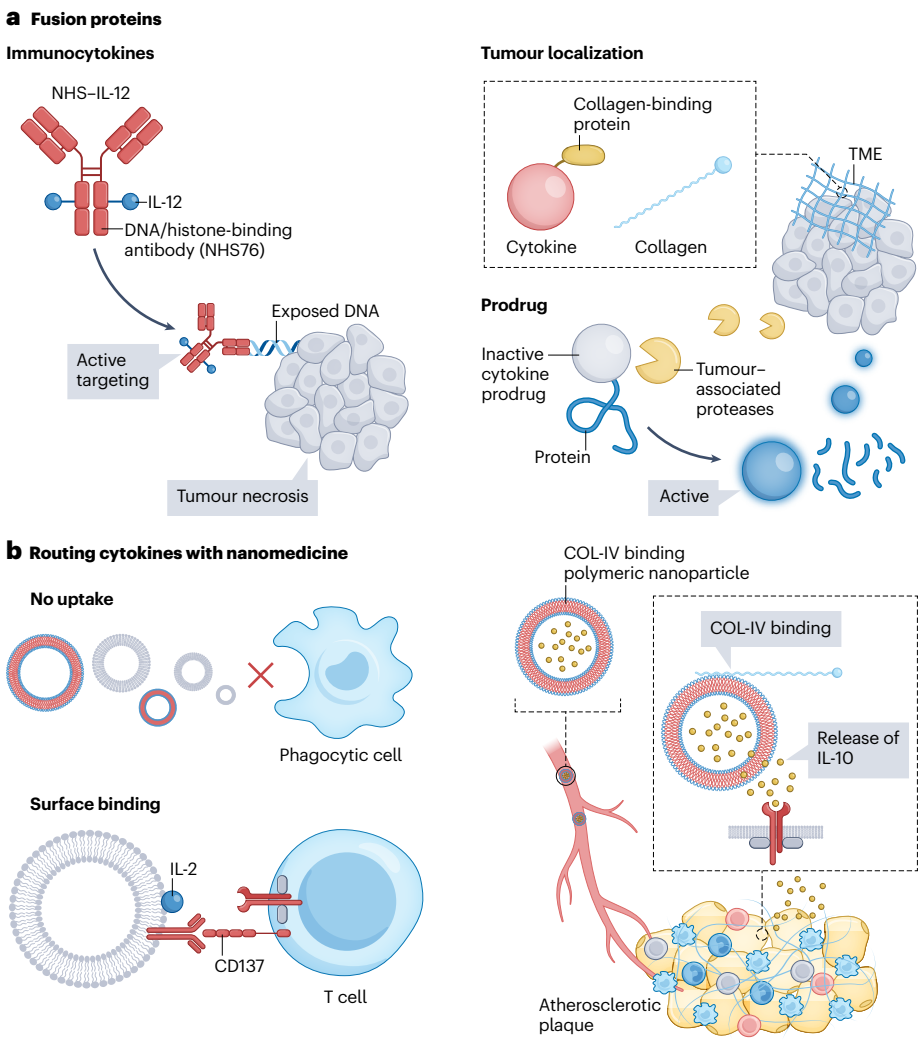

Fig. 3 | Cytokine targeting and delivery. a, Cytokine fusion proteins and their roles in redirecting cytokines to a specific site or cell type. Immunocytokines are cytokines fused to antibodies that target cytokines to distinct locations. This strategy can, for example, be used with the pro-inflammatory cytokine IL-12 to actively target exposed DNA found in necrotic areas within the tumour microenvironment (TME). Tumour localization can be achieved by directing cytokines to the tumour's extracellular components, such as collagen. Fusing a cytokine to collagen-binding proteins can anchor it in collagen and increase its retention in the tumour. Cytokine prodrugs can be engineered by fusion protein technology. These prodrugs usually exploit tumour-associated proteases that remove the inactivation domain from the cytokines through cleavage to release tumour-specific active cytokines. **b**, Routing cytokines with nanomedicine. Cytokine receptors are expressed on the cell surface and, thus, nanomedicine strategies must avoid uptake by immune cells. Nanoparticles can be designed with surface-displayed cytokines to target T cells[139]. In another approach, polymeric nanoparticles target collagen in the extracellular matrix, aiming for local sustained release of cytokines. COL-IV, type IV collagen.

systemically administering mRNA leads to its rapid degradation by endonucleases and recognition by Toll-like receptors, such as TLR7 and TLR8, resulting in innate immune activation and the concurrent secretion of pro-inflammatory cytokines, such as TNF and IL-6 (ref. [142]). To avoid TLR7 activation, and the resulting induction of cellular apoptosis, mRNA chemical modifications, such as N1-methylpseudouridine, enable efficient protein translation with reduced innate immune activation[143–146]. Additionally, mRNA must be stably delivered and transported across the cellular membrane into the cytosol. Efforts to accomplish this led to lipid nanoparticle (LNP) technology for mRNA delivery[147] (Fig. 4a). In brief, LNP–mRNA systems are composed of carrier phospholipids, PEGylated lipids, cholesterol and ionizable cationic lipids to complex negatively charged mRNA. Clinical data from the COVID-19 mRNA vaccines indicate consistent innate immune activation, which serves as a de facto adjuvant strategy[148,149]. Although advantageous in vaccine technology, innate immune activation can hamper the application of mRNA for conditions in which exacerbated immune responses need to be tempered, such as autoimmune diseases. These challenges are particularly relevant to cytokine therapy and innovations are necessary to overcome them.

Current mRNA nanotherapeutic strategies involve local and systemic administration routes (Fig. 4b). In this section, we describe how applying these two strategies to cytokine therapeutics can surmount their current limitations.

Local mRNA administration to tumours

To improve IL-12's tumour retention, an LNP–mRNA has been designed that encodes IL-12 fused to the collagen-binding protein lumican[150]. An ionizable lipid in the LNP formulation provokes an immune response and improves the fusion protein's translation. Upon intratumoral injection, the expressed IL-12 fusion protein mediates antitumour effects and systemic immune memory activation, leading to distal tumour

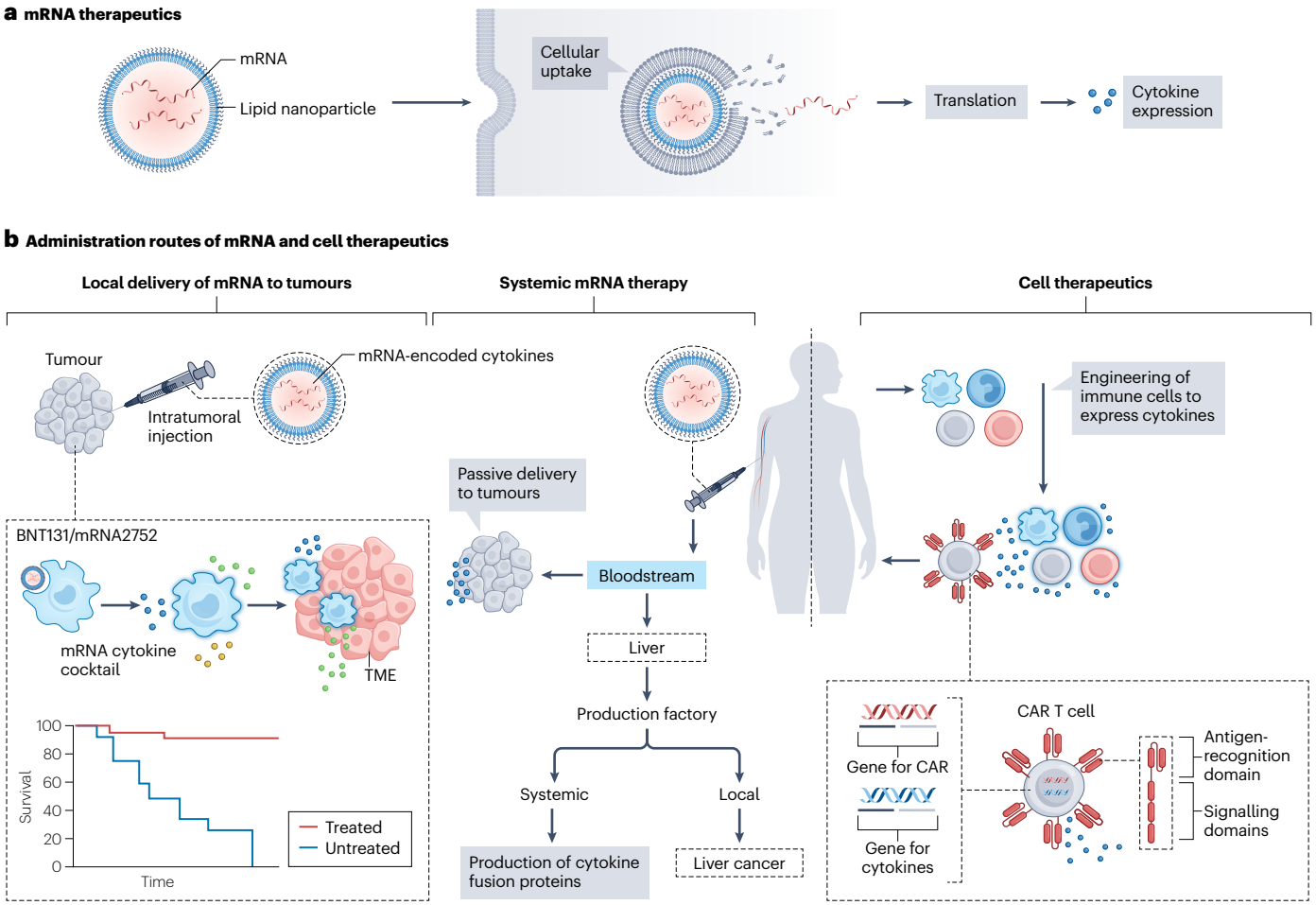

Fig. 4 | mRNA encoding cytokines and cell therapeutics. a, mRNA encoding cytokines is packaged in lipid nanoparticles and taken up by cells. The mRNA is then released into the cytosol and translated into cytokines. **b**, Local cytokine delivery to tumours using lipid nanoparticles encapsulating mRNA-encoding cytokines that locally induce inflammation against the tumour. Systemic mRNA therapy strategies include: harnessing the liver as a production factory to generate re-engineered cytokines, such as an IL-2–albumin fusion protein; treating liver cancer with mRNA encoding pro-inflammatory cytokines; and passively targeting mRNA encoding pro-inflammatory cytokines to the tumour to reshape the tumour microenvironment (TME). Chimeric antigen receptor (CAR) T cells can include a gene to express cytokines, thereby enhancing immune activation. This technique can also be used to engineer other immune cells, for example, genetically engineered myeloid cells, dendritic cells and natural killer cells. These engineered immune cells can modulate the immunosuppressive tumour environment, after systemic adminstration.

regression in a mouse melanoma model. A similar mRNA therapy encoding IL-12, IL-27 and GM-CSF suppresses tumour growth without substantial toxicity in the same melanoma model[151]. Moreover, intratumorally injectable mRNA therapies encoding a cytokine cocktail can reshape the TME[152,153]. LNP–mRNA encoding IL-23, IL-36γ and OX40L can boost T cell responses against cancer cells[152] (Table 2). This expressed cytokine mixture improves the response rate compared to treatment with mRNA coding for any of these cytokines separately. Furthermore, owing to the sustained release of cytokines encoded by mRNA, this mixture has higher antitumour efficacy than a cocktail of recombinant cytokines in a murine colon carcinoma model.

Despite their local administration, nanoparticles can still leak from the tumour into the vasculature, reach the liver and cause off-target effects[154]. Alternatively, a microRNA-122 (miR-122) binding site can be introduced into the mRNA sequence. Upon miR-122 binding, the complementary mRNA strand is cleaved, and protein expression is halted. This strategy exploits the abundance of miR-122 in the liver, preventing mRNA translation in this organ[154]. A different cytokine mRNA mixture encoding GM-CSF, IFNγ, IL-15 and IL-12 without LNP encapsulation[153] (Table 2) shows higher IFNγ response and increases $CD4^+$ and $CD8^+$ T cell proliferation compared to separate mRNA-encoded cytokines in immune cells isolated from cancer patients[153]. Several companies are currently conducting phase I clinical trials to explore the safety of these new mRNA-encoding cytokine-based immuno-oncology treatments (Table 2). Although local delivery strategies are clearly promising, this approach faces challenges for treating deep-seated tumours, which necessitate systemic mRNA therapy strategies[155].

Systemic LNP–mRNA therapy

Using systemically administered mRNA therapeutics to modulate the immune landscape can help to avoid the toxicities of recombinant cytokine therapies. One strategy for systemic mRNA therapy exploits high LNP accumulation in the liver to express cytokines and turn the liver into a cytokine production 'factory'. This approach improves cytokines' blood half-life and decreases their toxicity with lower and less frequent dosing[156]. In another example, the translation of a therapeutic mRNA encoding an IL-2–human albumin fusion protein in the liver systemically stimulates T cells to eradicate tumours[157]. This drug is currently undergoing a phase I/IIa clinical trial (NCT04455620) for treating solid tumours (Table 2). In a similar way, LNP accumulation in the liver could be harnessed to deliver mRNA encoding pro-inflammatory cytokines to treat hepatocellular cancer. LNP–mRNA encoding IL-12 can activate and recruit immune cells in the liver[158], reducing tumour growth in mice without inducing systemic toxicity.

Targeting other sites beyond the liver with mRNA is complex but holds potential for a broader range of therapeutic applications. In this context, a passive tumour-targeting approach, harnessing the enhanced permeability and retention effect, could be applied to express cytokines that can reshape the TME. For example, an LNP–mRNA encoding IL-15 can treat pulmonary metastasis tumours[159]. This mRNA therapy inhibits tumour growth without systemic toxicity following intravenous injection in a colorectal cancer model[159]. Despite promising preclinical results, mRNA therapeutics' clinical translation still faces challenges, such as immunogenicity triggered by cationic ionizable lipids[148,149,160]. Although these immunogenic effects complicate the use of mRNA in autoimmune disorders, LNP–mRNAs are being generated for treating inflammatory bowel disease. Adopting an active targeting approach, *Il10* mRNA combined with a Ly6C-antibody-anchored LNP can specifically target inflammatory monocytes[161]. The therapy induces IL-10 expression in monocytes and effectively tunes the inflammatory state in an inflammatory bowel disease mouse model.

The biodistribution profile of the LNP–mRNA remains similar when the mRNA sequence is altered, which facilitates switching between encoded therapeutic proteins[162]. However, changing the LNP composition alters the biodistribution profiles and can help to skew accumulation to specific organs[163]. This has potential in either promoting or dampening immune activation by targeting mRNA-encoded cytokines to haematopoietic organs such as the spleen and bone marrow[164].

Cell therapeutics

The translation of ex vivo engineered cells as therapeutics is progressing, as illustrated by the approval of chimeric antigen receptor (CAR) T cell therapy[165]. CAR T cells are genetically engineered to recognize tumour antigens to specifically eliminate cancer cells. Using autologous cells reduces the chance of immune-mediated host rejection but adds complexity to the manufacturing process and substantially increases costs[166]. Despite CAR T cell therapy's clinical success in haematological malignancies, it is only effective in a fraction of patients[167]. Combining cellular engineering and cytokine therapeutics will help circumvent the current difficulties facing both cell and cytokine therapies (Fig. 4b).

Improving immune cell therapy

One issue hampering CAR T cell therapy's effectiveness is the inability of engineered T cells to overcome the immunosuppressive TME[168]. Trials to reverse this suppressive state have been initiated by engineering IL-12-expressing CAR T cells, which effectively deplete tumour-associated macrophages[169,170]. The therapeutic efficacy of this approach has been demonstrated in a clinical trial but caused dose-limiting toxicities[171]. Additionally, CAR T cell therapy is often restricted by low cell expansion and survival[172] as well as poor CAR T cell accumulation in the TME[168]. IL-2 potently stimulates T cell proliferation and improves persistence of adoptively transferred T cells[173], whereas IL-7 and CCL19 are critical for maintaining a T cell zone in lymphoid organs[174]. However, directly administering these cytokines in combination with CAR T cells can induce cytokine-mediated toxicity[175]. To avoid the need to administer cytokines, CAR T cells can be engineered to express IL-2 (ref. [176]) and IL-7 with CCL19 (ref. [174]).

Cytokine re-engineering can also be integrated into cellular engineering. For example, an orthogonal IL-2 variant can be created to activate only the co-developed IL-2Rβ mutant by a lock-and-key approach[177]. The IL-2 variant can selectively target T cells expressing orthogonal hIL-2Rβ, without interfering with native T cells and natural IL-2 signalling. The orthogonal IL-2–IL-2Rβ pair improves the efficacy of adoptive T cell therapy in a mouse melanoma model, with negligible toxicity and off-target effects. The same strategy has been tested for a more challenging mouse leukaemia model, achieving anticancer effects[178]. The orthogonal IL-2–IL-2Rβ pair can also be translated to adoptive T_{reg} therapy[179]. T_{reg} therapy improves bone marrow acceptance in animal allo-engraftment models[180], but these positive results are dampened by limited cell expansion. Therefore, an orthogonal IL-2–IL-2Rβ pair was introduced into T_{reg} cells to enable selective signalling in vivo. Using orthogonal IL-2–IL-2Rβ to selectively expand adoptive T_{reg} cells improves heart allograft acceptance in a murine haematopoietic mixed chimerism model.

Protein engineering can be employed to overcome challenges arising before engineered T cells are administered. For example, a new IL-2 variant can stimulate ex vivo T cell expansion and maintain them in a more stem-cell-like state[181]. This effectively prevents T cells from

terminally differentiating before in vivo transfer, thereby improving therapeutic efficiency.

Engineered cells for cytokine delivery

Cellular engineering can also be employed for cytokine delivery. For example, GM-CSF-expressing T cells can target cytokines to central nervous system malignancies[182], possibly providing new opportunities for cytokine delivery across the blood–brain barrier. As another example, synthetic Notch receptors can be introduced into engineered T cells[183]. The synNotch receptors recognize specific cancer-related antigens, and the T cells display customizable protein expression. This approach can be exploited for the local selective expression of therapeutic cytokines, such as IL-2 and TNF-related apoptosis-inducing ligand (TRAIL), in the tumour.

Importantly, cellular engineering is not limited to T cells; for example, IL-12-expressing dendritic cells can be designed to activate T cells for tumour elimination[184,185]. Dendritic cells with combined IL-12 and IL-18 expression more effectively induce T cell-mediated antitumour immunity than dendritic cells expressing either IL-12 or IL-18 alone[186]. In addition to expressing IL-12, dendritic cells can be engineered to additionally produce and present tumour-associated antigens to activate T cells[187]. A similar strategy has been applied to a cancer vaccine by including GM-CSF expression to improve the vaccine's efficiency in inducing cytotoxic antitumour activity[188]. Dendritic cells can also be engineered to activate NK cells instead of antitumour T cells through expression of IL-15. Cancer cells lose major histocompatibility complex (MHC) class I molecules to avoid being killed by $CD8^+$ T cells as an immune-evading mechanism. As part of immune surveillance and second defence line, NK cells recognize and kill cells that downregulate MHC class I[189]. In vitro experiments show that NK cells derived from healthy donors and acute myeloid leukaemia patients acquire cytotoxic activity against tumour cells after co-culture with IL-15-transpresenting dendritic cells[190].

NK cells themselves can also be genetically modified in a more direct approach; for example, anti-CD19 CAR NK cells as treatment for CD19-positive cancers[191]. These CAR NK cells are modified to additionally express IL-15 to improve their expansion and persistence. A majority of patients in a phase I and II clinical trial (NCT03056339) of IL-15 CAR NK cells therapy showed response to the treatment, with seven out of eleven patients showing complete tumour remission, illustrating that CAR therapy can be developed with immune cell types beyond T cells. Similarly, CAR-macrophages can be engineered to express IFNγ along with the CAR construct, mediating a shift towards an M1 phenotype in the CAR-macrophages, which inhibits tumour growth[192,193].

Myeloid cells drive the inhibitory environment in pre-metastatic niches, allowing metastases to develop. IL-12-expressing genetically engineered myeloid cells can modulate the immunosuppressive TME and prevent metastasis[194]. IL-12-expressing genetically engineered myeloid cells improve survival in multiple metastatic tumour models by inducing antigen presentation and T cell activation. Alternatively, a hydrogel encapsulating IL-2-producing retinal pigmented epithelial cells can be delivered in alginate-based capsules[195], which can be implanted in the intraperitoneal cavity. Upon IL-2 release, the local concentration of IL-2 reaches therapeutically relevant levels, whereas systemic concentrations remain low enough to avoid IL-2-mediated toxicity. This approach results in decreased tumour volume and improved survival in ovarian and colorectal mouse models. Pharmacokinetic studies after intraperitoneal administration in non-human primates showed limited toxicity and an increase in $CD8^+$ T cells[195]. Engineered mesenchymal stem cells are suitable for cytokine delivery in cancer immunotherapy because of their inherent tropism and preferential homing to tumours and low immunogenicity[196,197].

Outlook

Immunotherapy has become a mainstay of treatment for numerous immune-mediated diseases, ranging from autoimmune and inflammatory disorders to cancer. New immune-based approaches such as monoclonal antibodies[198], checkpoint inhibitor drugs[199] and CAR T cells[165] have improved and extended the lives of millions of patients. Cytokines are one of the most intensively studied immunology domains, and engineered cytokine-based therapies represent a new evolution of immunotherapeutics.

By the end of the twentieth century, cytokine-based drugs were among the earliest immunotherapies being developed for advanced treatment of cancer. Unfortunately, poor pharmacokinetic properties and systemic side effects have limited the applications of cytokine drugs, with a few notable exceptions, such as interferons for chronic hepatitis[200] or multiple sclerosis[201], rIL-2 in renal carcinoma[202] and colony growth factors in neutropaenia[203]. However, the bioengineering advances described in this Review provide a developmental framework for major progress in the number and efficacy of cytokine-based therapies in the coming years. Advances in cytokine therapies are expected to emerge from two directions; first, technological innovations to re-engineer cytokines and deliver them to the site of pathological progress; and second, improved trial designs, sophisticated patient stratification and precision-medicine-based approaches. Both directions are needed to take advantage of new cytokine-based therapies in various diseases. As these research directions progress, scientists must bear in mind that protein engineering that deviates from endogenous molecules runs the risk of immunogenicity. For example, immunogenicity has been reported for an IL-2 immunocytokine[125], and modifications to proteins, such as PEGylation, can cause similar problems[204].

More approaches are expected to emerge to improve the activity, lifetime, pharmacokinetics and delivery of cytokines for therapeutic purposes. Current efforts focus mainly on developing cytokine receptor agonists; however, bioengineering can also improve therapies seeking to silence cytokine expression or dampen cytokine-receptor activation. Anakinra (recombinant IL-1RA) is a cytokine receptor antagonist that inhibits inflammation in a variety of diseases, such as rheumatoid arthritis, and hyperinflammation in severe infections, such as COVID-19 (ref. [33]). This success paves the way for similar approaches targeting cytokine receptor pathways. Protein engineering technologies can be applied to design cytokine antagonists, such as an IL-2 mutein binding IL-2Rβ with improved affinity to prevent receptor dimerization and thereby effectively impair IL-2 and IL-15 activation[205]. This approach of preventing receptor dimerization can be used as a general strategy for antagonizing cytokine activity. In addition, although monoclonal antibodies, such as anti-TNFα[31], anti-IL-6 (ref. [33]) and anti-IL-17 (ref. [32]), are established therapies for blocking cytokines, they often have poor pharmacokinetic profiles[206]. Technologies enabling the control of cytokine expression on a genetic level will probably arise as more effective cytokine therapy variants. Furthermore, emerging technologies, including single-cell analytical technologies or artificial intelligence (AI)-guided design, should continue to improve cytokine-based therapies (Box 1). These developments will lead to more rapid and more effective designs for cytokine-receptor agonists. Finally, increasing knowledge of nanoparticle structure–function relationships

will lead to developments in nanomedicine-based drugs with improved biodistribution profiles and targeting capabilities.

More clinical trials on cytokine therapeutics are expected to be conducted, based on the recent successful experimental studies on cytokine-based technologies. The success of new therapies is expected to come not only from improved formulations, but also from innovative approaches to performing clinical trials. Furthermore, cytokine-based treatments will probably be combined with established therapies, such as checkpoint inhibitors or CAR T cells, or following surgery or radiation therapy. Going forward, patient stratification and precision medicine will represent one of the most important developments in modern treatment, and cytokine-based therapies are well suited to such approaches. Moreover, the technological challenges of clinical translation of cytokine-based therapies, such as cross-species reactivity, and regulatory processes, such as GMP manufacturing, should be considered (Box 1). Accompanying diagnostics to identify the patient subgroups most likely to respond to a certain type of cytokine therapy should be a goal of all commercial entities aiming at developing cytokine-based treatments. Additionally, precision medicine will probably be integrated into future technological developments, for example, for the design of personalized mRNA therapeutics. Together, bioengineering developments, innovative approaches to clinical testing and precision medicine will help to realize the potential of cytokine-based therapies for treating various diseases.

Published online: 16 February 2023

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Author contributions

J.D., T.A., A.M.H., M.G.N. and W.J.M.M. wrote the manuscript. All authors researched data for the article, provided substantial contributions to discussion of content and reviewed and edited the manuscript before submission.

Competing interests

W.J.M.M., L.A.B.J. and M.G.N. are scientific co-founders of and have equity in Trained Therapeutix Discovery. W.J.M.M. and M.G.N. have consulting agreements with Trained Therapeutix Discovery. The remaining authors declare no competing interests.

Additional information

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Peer review information *Nature Reviews Bioengineering* thanks Yizhou Dong, Frances Balkwill, Beatrice Malacrida and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.