Engineering living therapeutics with synthetic biology

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Abstract | The steadfast advance of the synthetic biology field has enabled scientists to use genetically engineered cells, instead of small molecules or biologics, as the basis for the development of novel therapeutics. Cells endowed with synthetic gene circuits can control the localization, timing and dosage of therapeutic activities in response to specific disease biomarkers and thus represent a powerful new weapon in the fight against disease. Here, we conceptualize how synthetic biology approaches can be applied to programme living cells with therapeutic functions and discuss the advantages that they offer over conventional therapies in terms of flexibility, specificity and predictability, as well as challenges for their development. We present notable advances in the creation of engineered cells that harbour synthetic gene circuits capable of biological sensing and computation of signals derived from intracellular or extracellular biomarkers. We categorize and describe these developments based on the cell scaffold (human or microbial) and the site at which the engineered cell exerts its therapeutic function within its human host. The design of cell-based therapeutics with synthetic biology is a rapidly growing strategy in medicine that holds great promise for the development of effective treatments for a wide variety of human diseases.

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Over the past century, the development of new therapeutics has largely relied on synthetic chemistry approaches to design and optimize bioactive molecules that may have the potential to become medicines. This process typically involves cycles of lead compound functionalization and screening to iteratively enhance their efficacy, safety and pharmacokinetic characteristics[1]. Although this approach has enabled the discovery and development of important life-changing medicines, there are many unmet clinical needs across numerous human diseases that will require strategies that extend the reach of small-molecule-based and protein engineering-based therapies[2]. In recent years, the advent of synthetic biology has been changing the way life scientists and bioengineers think about designing and building therapeutic agents by establishing living cells, rather than chemical compounds, as scaffolds for the development of novel medicines. Synthetic biology approaches, akin to medicinal chemistry methodologies, enable the functionalization of a wide variety of cell types through the introduction of synthetic gene circuits to generate engineered living therapies (ELTs) (FIG. 1).

Synthetic biology is an exciting discipline that emerged at the turn of this century, combining molecular biology tools with forward-engineering principles to construct genetic systems that programme a desired cellular behaviour[3–5]. It builds upon the existence of genetic circuits of interacting genes and proteins that underpin endogenous cellular functions as well as the response of a cell to its environment[6]. Genetic circuits can be deconstructed into simpler functional units or modules with defined inputs and outputs; for instance, translation can be viewed as a two-input (an mRNA and a ribosome) and one-output (a protein) functional module. Thanks to this modularity, the overall behaviour of the system can be expressed as a composite set of interconnected operations (BOX 1). Using this framework, synthetic biologists can independently design, test and characterize novel functional modules using genetic parts that have been previously characterized. Importantly, the interaction between the parts that compose these modules can be described using well-established mathematical models, which enables the implementation of model-based design approaches for the integration of functional modules into more complex synthetic genetic circuits[7,8]. Although many of the synthetic genetic circuits created thus far are proof-of-principle designs, the field has moved towards application-based designs that promise important breakthroughs in the development of novel therapeutics.

The structure of a genetic circuit can be generally divided into three basic modules. First, an input module that detects biotic or abiotic signals and transforms them into interpretable molecular signals. For instance,

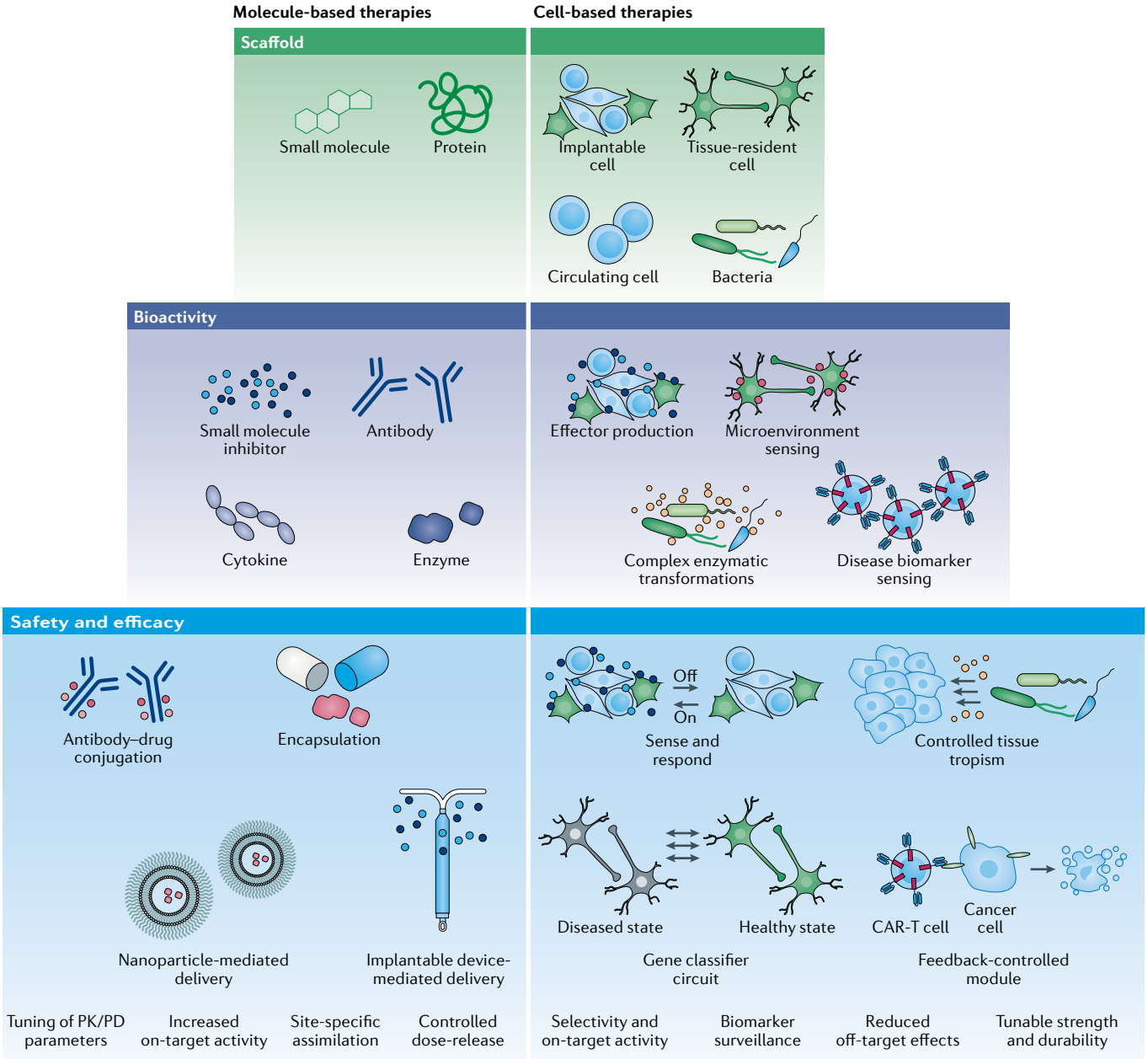


Fig. 1 | **Endowing biological scaffolds with therapeutic capabilities.** Small molecules and protein-based biologics (left) have traditionally served as scaffolds for the development of new therapeutic agents. The intrinsic bioactivities of such molecules are usually enhanced through medicinal chemistry, protein engineering and/or drug-delivery approaches to achieve therapeutic efficacy and safety. The advent of synthetic biology and its capacity for programming novel functionalities into cells present opportunities for the development of a new class of therapeutics. Like lead small molecules and proteins, living cells display natural biological activities that can be harnessed for therapeutic applications. Numerous human cell types and human-associated microorganisms (right) can be used as living scaffolds to build genetic programmes that endow them with therapeutic capabilities. PK/PD, pharmacokinetics/pharmacodynamics.

coupling of membrane-associated sensor proteins and downstream response regulators enables the transformation of a ligand-binding event into a controllable phosphorylation signalling cascade. Second, an operation module that computes the transmitted signal from the input module and determines the appropriate cellular behaviour. Most commonly, Boolean logic gates are used to process single or multiple inputs to generate the desired gene-expression outcome (BOX 1). Lastly, an output module converts the computed signal into the desired cellular response. In therapeutics applications, output modules are usually genes that encode biological effectors, such as enzymes, cytokines or cell receptors. The composition and integration of these modules enables the creation of complex synthetic systems that reprogramme the overall cellular behaviour in favour of the desired application (FIG. 2).

To develop dynamic, living therapeutics, engineered cells must sense and respond to environmental cues that encode information about their location, relevant

disease states and the timing for mounting an appropriate therapeutic response. For that, synthetic gene circuits must include sensor elements, signal processing/transduction elements and control elements to tune a desired dynamic response[9]. Environmental and disease biomarkers provide initial input to a sensing module to distinguish between disease and normal states (FIG. 2). A sensing module can be relatively simple, such as the

Box 1 | Genetic logic gates

To achieve a desired cellular response, Boolean logic gates can be designed to process multiple inputs and generate outputs according to digital logic rules. Biological logic gate circuits can be constructed using a combination of controllable input, operation and output modules that can produce desired outcomes at the DNA, RNA and protein levels. As an illustrative example (see the figure), we show how input modules that use small-molecule regulators can be combined with operation modules based on transcriptional activators or repressors to create logic gates that switch on (1) or off (0) the expression of the output module. The simplest operation is the YES gate, which makes the output identical to the input. This operation can be achieved when the presence of the input signal inhibits the expression and/or activity of a transcriptional repressor that controls the output gene. The opposite operation, which inverts the signal (NOT gate), can be obtained when the presence of the input activates the expression and/or activity of a transcriptional repressor controlling the output. The YES and NOT gates are single-input Boolean operations that have been widely used to control gene expression and can be layered to yield more complex logic gate circuits with multiple inputs. An AND gate, which requires the presence of all inputs to turn on the output signal, can be built by combining YES gates. In our example, an AND gate is achieved with the use of two transcriptional repressor-based YES gates, with operator sequences that condition the activity of a single promoter (P) in the output module. Another useful operation for the robust control of gene expression is the OR gate, which turns off the output signal only when all the inputs are absent. Two independent sets of transcriptional activators and their cognate promoters can serve as an OR gate when placed upstream of the output module. Furthermore, more complex logic gates can be created based on the logic combinations described previously, for example, a NAND gate can be built by connecting an AND gate to a NOT gate, and an NOR gate can be constructed by layering an OR gate to a NOT gate.

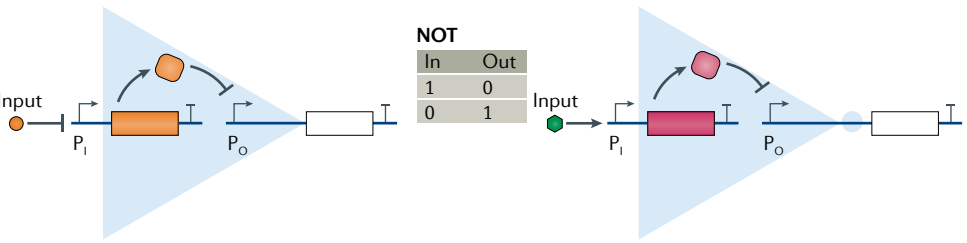
Single input logic gates

YES

In	Out
1	1
0	0

NOT

In	Out
1	0
0	1



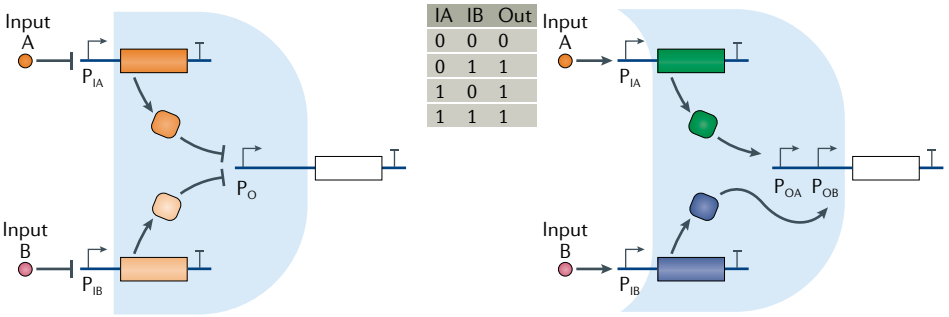
Double input logic gates

AND

IA	IB	Out
0	0	0
0	1	0
1	0	0
1	1	1

OR

IA	IB	Out
0	0	0
0	1	1
1	0	1
1	1	1



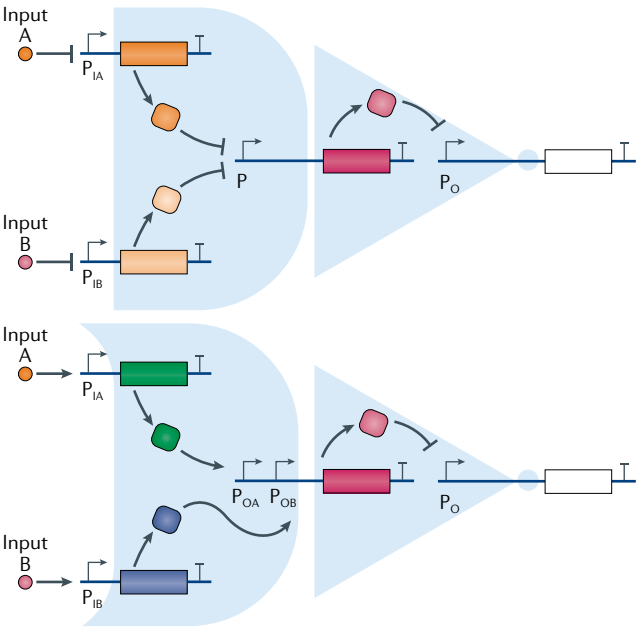
Layered, double input logic gates

NAND

IA	IB	Out
0	0	1
0	1	1
1	0	1
1	1	0

NOR

IA	IB	Out
0	0	1
0	1	0
1	0	0
1	1	0



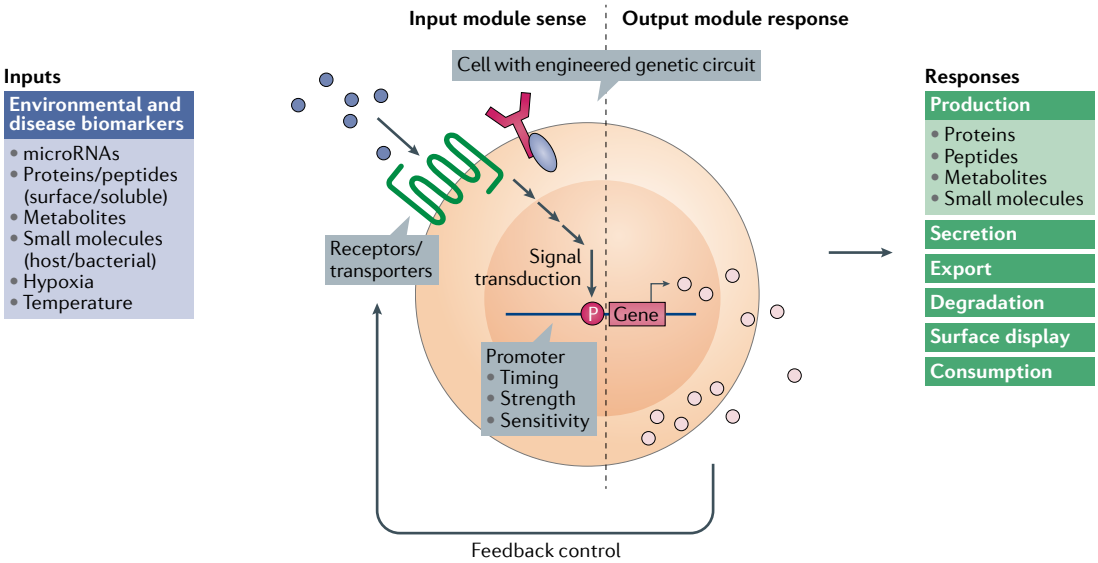


Fig. 2 | **Development of living therapeutics with engineered genetic circuits.** The components of genetic circuits introduced into a living cell are organized into functional modules with defined inputs and outputs. The initial inputs of these sensing modules may include environmental or disease biomarkers. Upon their recognition, the output module can mount a wide range of programmed responses to drive therapeutic behaviour of an engineered cell. For example, elevated blood glucose can serve as an input, which can be sensed by a glucose transporter and its signalling cascades to drive the expression of insulin[32]. Decrease in blood glucose levels following the release of insulin negatively regulates circuit activity through feedback control. The architecture of these sensing and processing modules can include various logic gates (BOX 1) to specify sensitivity, flexibility, strength and timing of a therapeutic response. P, promoter.

direct activation of a transcription factor driving a transcriptional response in the output module. However, to generate a precise response, advanced sensing modules can interface intricate signal transduction pathways with multiple control elements to fine-tune the output signal. The layering of these modules into logic gates is a powerful way of regulating timing, driving greater signal strength and improving the sensitivity of the output module (FIG. 2).

The use of synthetic gene circuits can be an important feature of cell-based therapies, and these strategies can address some of the notable limitations of conventional therapies — namely, lack of flexibility, specificity and predictability (BOX 2). The past decade has witnessed a flurry of increasingly sophisticated genetic designs to rewire cells for therapeutic applications[10,11]. These efforts have been largely directed towards mammalian cells and bacteria, which share similar fundamental principles but differ in their molecular toolkits for engineering strategies[11,12]. In this Review, we highlight noteworthy reports of engineered cell therapies that have used synthetic biology approaches to programme cellular behaviours. We focus on efforts that involve the biological sensing and computation of signals derived from intracellular or extracellular biomarkers, ultimately leading to the execution of a defined therapeutic programme. The examples presented here are categorized on the basis of cell scaffolds with physiology suitable for the creation of a therapeutic platform, namely genetically tractable human cells and human-associated bacteria.

We first describe how distinct synthetic biology approaches have been employed to functionalize human cell types that are tissue resident, implantable or capable of systemic circulation. For circulating cell types, we focus on engineered T cells that carry synthetic gene circuits capable of endowing controllability, flexibility and specificity to their therapeutic programmes. We then describe efforts towards the creation of live biotherapeutics based on microbial cells and examples of sense-and-respond systems designed to create therapeutic functions in gut- and tumour-associated bacteria. In addition to presenting synthetic biology advances, we provide insights into manufacturing and clinical development considerations related to the creation of engineered cell-based therapeutics. Finally, we analyse the future prospects for the development of engineered cells that can deliver autonomous, orthogonal and durable therapeutic programmes.

Programming therapeutic human cells

The use of human cells as chassis for engineered gene circuits represents a significant bioengineering advancement, allowing sophisticated therapeutic interventions to be imparted via genetic vectors to cells in situ or ex vivo. Implementing synthetic constructs to cells in patients can be carried out using viral or non-viral methods. Alternatively, introducing gene circuits to human tissue ex vivo allows precise manipulation in an isolated setting. In addition to being the least-foreign cellular chassis, human cells provide the native intracellular environment for exogenous therapeutic programmes, facilitating integration with physiologically relevant molecular processes (such as post-translational modifications) and biochemical pathways that are directly relevant for disease treatment (such as oncogenic signals). The synthetic biological circuits discussed below

Boolean gates
A system or device that performs a logical operation on one or more binary inputs, which results in a single binary output.

AND gate
Output is actuated only if all of the specified inputs are received.

have been developed to treat diseases such as cancer and metabolic disorders and are categorized on the basis of localization of the cellular chassis as tissue-resident, implantable or circulating therapeutic cells.

Tissue-resident engineered cells

Initial efforts to rewire cells for therapeutic use stemmed from the insertion of circuits to control biological activity in the cell of interest in response to predetermined input signals (FIG. 3). In these instances, cells are programmed to detect, integrate and respond to biological cues in the intracellular microenvironment. This concept has now been applied to the identification and discrimination of cellular states[13], such as malignant versus non-malignant behaviours. Typically, this relies on indexing and integrating groups of biomolecules of the synthetic gene circuit into a 'classifier' and, based on the output of that integration, eliciting the appropriate response in the host cell. For example, a simple 'if malignant then trigger apoptotic pathway' logic might be implemented. The distinction between non-malignant cells and cervical carcinoma (HeLa cells), represented an early proof-of-concept demonstration for the concept of cellular classifiers[14]. A set of microRNAs (miRNAs) highly expressed (HeLa-high) or absent (HeLa-low) in HeLa cells was chosen, and a double-inversion circuit was built by encoding corresponding miRNA binding sites within the transcripts of key circuit components. HeLa-low miRNAs directly degraded the output gene (apoptotic factor BAX) transcripts, and HeLa-high miRNAs degraded BAX repressor, BCL-2, to allow output expression. The resulting 'classifier' circuit elicited two discrete outcomes based on the detected cell state: the output, in this case BAX, is expressed in the presence of HeLa-high markers and repressed in their absence (FIG. 3a). Functionally, this genetic architectural design resulted in the preferential apoptosis of HeLa cells versus non-malignant HEK293 cells, when the entire circuit was introduced to the respective cells[14].

Another experimental strategy to sense intracellular processes is to regulate RNA splicing, whereby the output gene cannot be translated unless splicing occurs. Here, RNA aptamers that potentiate splicing when bound by specific input protein ligands, along with an exon encoding a stop codon, are inserted between exons of the output protein. When the protein of interest binds the aptamer, an alternatively spliced transcript for functional translation of the output protein gets generated. Initial proofs of concept have been demonstrated for inputs such as tumour necrosis factor (TNF)–nuclear factor-κB (NF-κB) and β-catenin pathways[15]. In these studies, when an aptamer was bound by its cognate factor (such as NF-κB or β-catenin), spliced transcripts led to production of the herpes simplex virus thymidine kinase, which confers sensitivity to the prodrug ganciclovir. Detection of input signals by these circuits generated non-native drug sensitivity, which could be repurposed to induce killing of target cells with a disease-specific intracellular protein.

Cancer research has been the main testing ground for the early synthetic gene circuits described above. Malignant transformations in eukaryotic cells can be characterized by genetic and epigenetic changes that result in aberrant transcriptional states leading to uncontrolled cell replication[16]. Additionally, cancer cells must escape immune surveillance processes through the subversion and evasion of several immune pathways[17]. These daunting therapeutic challenges pose profound obstacles to conventional therapeutic modalities, but synthetic biology is well placed as a complementary approach. Gene circuits can be devised that discriminate between normal and transformed cellular states, and produce therapeutic molecules to specifically destroy cancer cells by engaging combinatorial antitumour immune pathways. One such cancer immunotherapy platform has been described by Nissim and colleagues[18], and combines a 'classifier' module with an 'effector' module that produces therapeutic immunomodulatory proteins. The effective discrimination between tumour-like and normal cells is of paramount importance for the reduction of off-target toxicity (BOX 2), and as such, multiple-input Boolean gates are an attractive engineering strategy to improve circuit specificity. As an example, an AND gate can be designed in which input promoter 1 (P1) regulates an autoinhibitory mRNA that encodes the output protein (or proteins), whereas input promoter 2 (P2) regulates an RNA molecule that relieves the autoinhibition,

Box 2 | **Addressing the limitations of conventional therapies through synthetic biology**

Flexibility

A fundamental limitation of conventional therapies lies in their inflexible mechanisms of action. Whereas small molecules and biologics typically target a disease-associated cell or protein to modulate its activity or availability, synthetic gene circuits can enable engineered cells to carry out a multitude of effector mechanisms in response to various chemical, biological or environmental cues. The modular nature of biology permits the rewiring of input–output relationships in synthetic systems, offering dynamic therapeutic options that cannot be achieved by standard drugs. Modularity underlies many synthetic biology designs; synthetic biology platforms that exemplify the utility of flexible therapies include synthetic promoters coupling endogenous signalling pathways to user-defined therapeutics[32,37] and universal chimeric antigen receptor (CAR) designs with swappable antigen-binding domains[75,79].

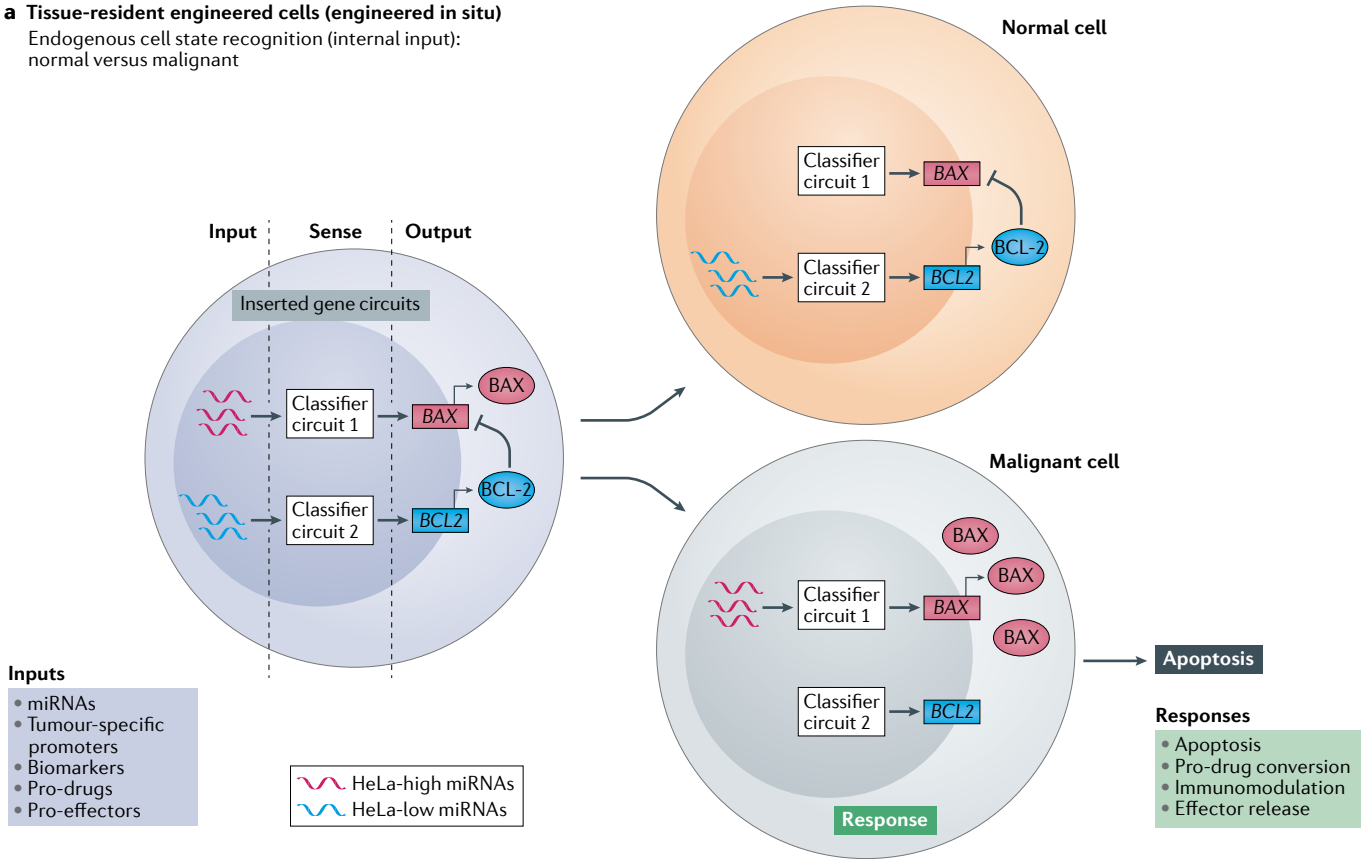
Specificity

The ability of therapeutics to distinguish between healthy and diseased states is paramount for eliciting safe and effective responses. Conventional therapies have largely relied on the availability of a single disease-associated feature to drive effector responses. However, many disease states cannot be defined or identified by just one element. Synthetic biology systems enable enhanced specificity by integrating multiple state-associated cues to deliver a therapeutic output. Designs that have capitalized on combining inputs to enhance therapeutic specificity include two-input AND gates based on disease-associated intracellular miRNAs[14], transcription factors[18] or cell surface antigens[86,88], as well as the localized production of therapeutics based on the properties of diseased tissue[40,164].

Predictability and controllability

Unpredictable outcomes in treating complex diseases represent a significant biomedical challenge. For therapies that operate within a limited therapeutic index in particular, achieving the optimal balance of efficacy and toxicity across individuals and over time is difficult when faced with inter-patient and intra-patient heterogeneity. The sense-and-respond capabilities of synthetic biology systems enable engineered live therapeutics (ELTs) to adjust to perturbations in situ and deliver tailored responses. Adaptable gene circuits have been implemented in therapeutic applications. Notable examples include feedback-controlled synthetic gene networks that regulate hormone and metabolite levels[30–32,37], inflammation-driven production of immunomodulatory cytokines[40], small-molecule controlled CAR activity[60] and self-regulated population control based on the quorum sensing of an accumulating bacteriolytic factor[169].

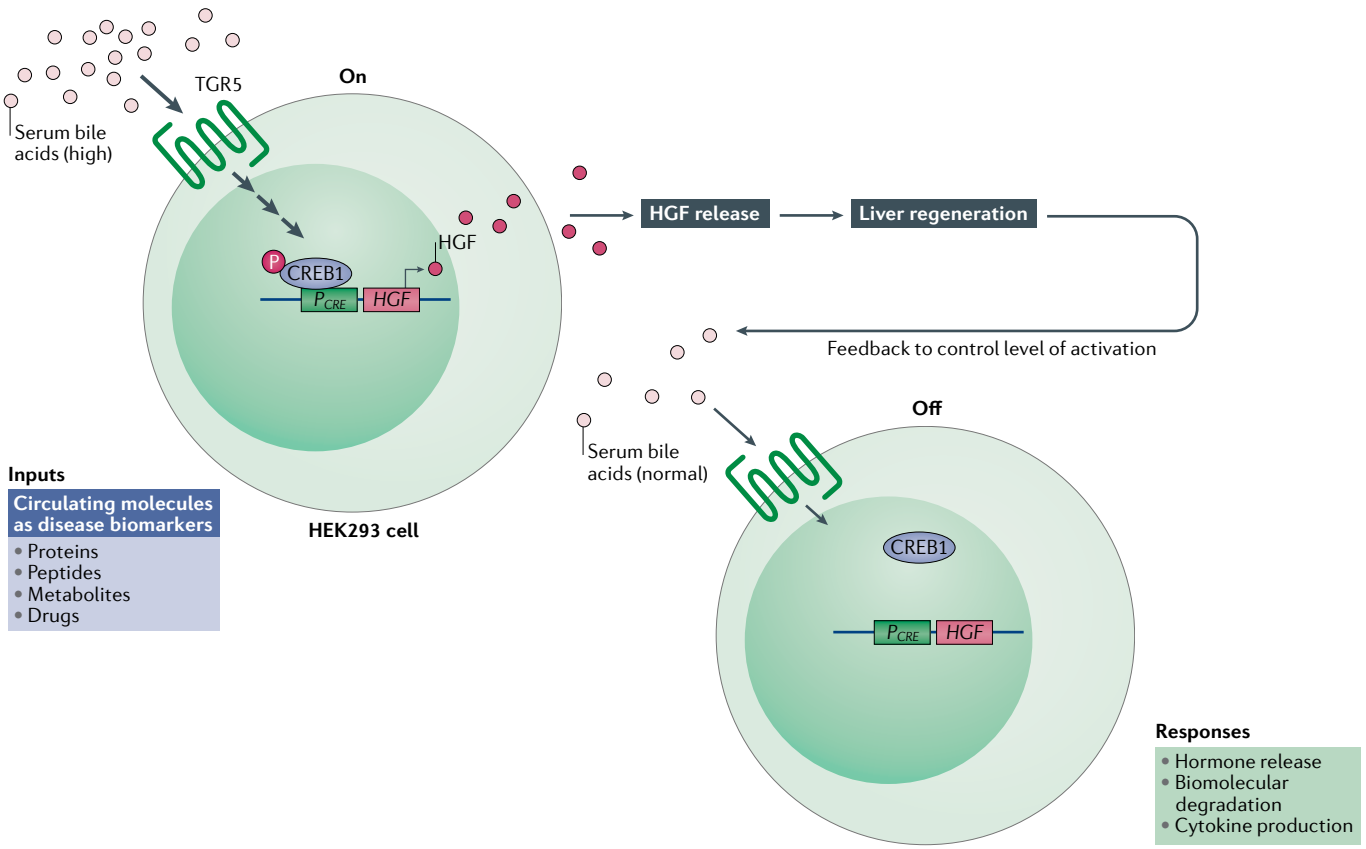
a Tissue-resident engineered cells (engineered in situ)
Endogenous cell state recognition (internal input): normal versus malignant



Inputs
- miRNAs
- Tumour-specific promoters
- Biomarkers
- Pro-drugs
- Pro-effectors

Responses
- Apoptosis
- Pro-drug conversion
- Immunomodulation
- Effector release

b Implantable engineered cells (engineered ex vivo)
Environmental state recognition (external input): normal versus disease



Inputs
Circulating molecules as disease biomarkers
- Proteins
- Peptides
- Metabolites
- Drugs

Responses
- Hormone release
- Biomolecular degradation
- Cytokine production

Fig. 3 | **Human cell therapies with engineered genetic circuits. a** | Tissue-resident cells can be engineered to recognize cell states, such as a malignant cell, by using multiple designs that include multi-input logic circuits. Multi-input sensing provides flexibility to the response, potentially leading to effective therapeutic outcomes with limited off-target toxicity. For example, sensing endogenous microRNAs (miRNAs) expressed in malignant cells (HeLa-high miRNAs) selectively triggers apoptosis through regulated expression of BAX without affecting normal cell types, in which miRNAs that are not expressed in HeLa (HeLa-low miRNAs) suppressed the expression of BAX[14]. Sensitivity to the expression levels of malignant and normal miRNAs (or other input signals) can be built into the circuit modifications. Synthetic gene circuits can be engineered to sense multiple different inputs and respond accordingly. The main challenge of this approach is gene delivery for in vivo circuit implementation. **b** | Implantable cells can be designed to react to changing environmental conditions, such as circulating metabolites or disease-related biomolecules. An excess of specific biomolecules such as uric acid, glucose or thyroid hormone leads to the activation of the response module, and the integration of self-regulating feedback loops can control and shut off the circuit activity after the system reaches normal physiological conditions. To avoid the potential toxicity of therapeutic proteins, a biomarker-sensing module with AND-gate logic can be used for tight control of the response module. In the illustrated example, high levels of serum bile acids (a biomarker of liver injury) are sensed through the bile acid membrane receptor TGR5, which leads to the activation of downstream effector CREB1 and production of hepatocyte growth factor (HGF) to stimulate liver regeneration. Because HGF not only stimulates liver regeneration but also can drive tumorigenesis, the engineered genetic circuit is designed to be activated by bile acids in a dose-dependent manner. Normalization of bile acid concentration in serum shuts off HGF production[37]. The challenge in this case is to achieve sustained activity of implanted cell and avoid rejection by the immune system.

allowing output production. P1 and P2 are activated by distinct tumour-associated transcription factors, and when expressed together are sufficient to specify a malignant cell state. In the system developed by Nissim et al., whereby P1 and P2 were driven by oncogenic transcription factors MYC and E2F1, respectively, an output set was built that included a combination of a T cell engager, a chemokine (CC-chemokine ligand 21 (CCL21)), a cytokine (IL-12) and an anti-programmed cell death 1 (PD1) single-chain variable fragment (scFv), with the potential to engage multiple antitumour immune mechanisms. Crucially, both P1 and P2 need to be concurrently activated in cells to elicit the output. As a result, any off-target activity is minimized through the combination of two tumour-associated synthetic promoters that are unlikely to be simultaneously activated in a normal cell. Efforts to discover state-specific synthetic promoters in a high-throughput fashion[19] will facilitate broader application of transcription-regulated logic gates in cell and gene therapies.

The circuit architecture developed by Nissim et al. combined multiple synthetic biology approaches and showed encouraging efficacy in mouse tumour models[18]. However, some of these systems require the delivery of large therapeutic gene circuits to cells in situ, posing a fundamental limitation in clinical applications. Improvements in viral and non-viral gene delivery technologies may help to address the challenge of in vivo circuit implementation in the future, as reviewed in detail elsewhere[20–22]. Nevertheless, it is important to design synthetic biological systems with feasibility of implementation in mind. Using the oncogenic signalling of ERBB receptors as a cancer-specific feature[23], Chung et al.[24] designed modules that bound to phosphotyrosine sites on ERBB proteins. One of the phospho-ERBB binding modules was fused to a protease and another was fused to a cleavable substrate. Hyperactive ERBB signalling promoted colocalization of both modules and release of the effector protein BH3-interacting domain death agonist (BID) to induce apoptosis. Notably, the compactness of their design allowed the delivery of the entire system by an adeno-associated virus vector. The system could be used to trigger the pro-apoptotic protein BID and had a strong preference for cancer cells with constitutively active ERBB signalling compared with normal cells, in which the pathway is only transiently activated[24]. Protease-based circuits have also been applied in other systems[25,26].

Implantable engineered cells

Unlike engineering tissue-resident cells in situ, implantable cells can be engineered ex vivo, where a wider array of applicable gene delivery methods allows for the introduction of sophisticated circuits (FIG. 3). Engineered circuits in some of the above examples are built to operate in one of a limited number of predefined cell states, and they do not dynamically adjust to changing environmental conditions. Although this design makes the system relatively simple, it poses practical limitations to its potential use in unpredictable clinical settings — a limitation shared with conventional therapies (BOX 2). Therefore, the ability to modulate circuit activity in response to an external input, such as a circulating biomolecule, drug or metabolic product, is highly attractive. One way to address this issue has been proposed in the form of prosthetic gene network devices[27], which are applicable to situations in which the target molecule in a disease state is found in circulation.

Normal physiological functions generally require key metabolic or endocrine factors to be maintained within a certain homeostatic range. Disruptions to the natural positive or negative feedback loops that control the levels of these key factors can result in disease (for instance, diabetes is a result of insufficient insulin levels to regulate blood glucose). Successful treatments for these disorders need consistent control in the bioavailability of the targeted physiological product. Thus, achieving predictable outcomes is a complicated feat for drugs that act independently of the physiological milieu after administration. As we highlight below, several synthetic gene circuits that integrate with physiological feedback loops have been successful in re-establishing normal homeostasis in preclinical disease models.

Regulating urate levels was one of the first applications for prosthetic gene network devices that sense and respond to a circulating metabolite. Urate is the end product of purine metabolism, and it accumulates to pathogenic levels in hyperuricaemia-related conditions such as gout[28], in which urate is excessively produced and/or cannot be effectively excreted. Uric acid may also protect cellular components from oxidative damage caused by free radicals[29]. Thus, a synthetic feedback module could potentially provide more dynamic and optimal control than traditional pharmacological interventions that simply reduce urate levels. In this approach[30], the sensing cassette comprises a bacterial transcriptional repressor, HucR, that binds to a DNA operator sequence motif, *hucO*, in the absence of uric

Synthetic promoters
Recombinant DNA elements that enable the binding of transcription factors and RNA polymerase enzyme to initiate transcription of RNA molecules.

Prosthetic gene network
Synthetic gene circuit that senses the bioavailability of a metabolite or hormone and autonomously corrects the physiological level of the molecule when it deviates from a targeted set point.

acid. In the presence of uric acid, HucR dissociates from DNA, which allows expression of downstream genes. This urate-sensing module is linked to a second construct encoding urate oxidase, an enzyme that degrades uric acid. Both modules, along with a human urate transporter, are transfected into HeLa cells that are then microencapsulated into alginate-(poly-L-lysine)-alginate capsules and intraperitoneally injected into urate oxidase-deficient mice. Remarkably, the naturally high levels of circulating uric acid in these mice are reduced to physiological levels for up to 7 days.

Moreover, implantable cells engineered with synthetic feedback circuits have been applied to regulate thyroid hormones[31] and blood glucose[32] with intended applications in hyperthyroidism and diabetes, respectively. Conventional methods to treat hyperthyroidism by antithyroid medication or thyroidectomy leads to hypothyroidism[33]. To address this problem, Saxena et al.[31] designed a synthetic feedback circuit using a thyroid hormone-binding nuclear receptor, which, upon ligand binding, drives the expression of a negative regulator of thyroid hormone production. Likewise, anti-diabetic medications reduce blood-glucose concentration, but can cause hypoglycaemia with varying severity[34]. Using a calcium-responsive nuclear factor of activated T cells (NFAT)-driven promoter and an ectopically expressed calcium channel, Xie et al.[32] designed a glucose-sensing circuit in HEK293 cells that interfaced with the endogenous pathway of the cell line. Elevated glucose triggers calcium influx by increasing intracellular ATP, which regulates membrane potential and voltage-gated calcium channel activation[35]. The NFAT promoter drives the expression of insulin to form a synthetic feedback loop[32]. In both cases, the engineered cells were microencapsulated and implanted in mice, and restored circulating thyroid hormone or blood glucose to physiological levels in experimentally induced pathologies[31,32].

Synthetic gene circuits with feedback can also be used to tightly regulate the expression of therapeutic proteins that carry severe toxicities. Hepatocyte growth factor (HGF) stimulates liver regeneration and can be used to treat hepatic injury. However, HGF is also a tumorigenic factor[36], thus limiting the therapeutic window of this therapy. Using a self-regulating feedback system, Bai et al.[37] coupled elevated serum bile acids to HGF production as a therapy for liver injury. Various hepatic diseases impair the ability of the liver to clear bile acids, allowing high serum bile acids to serve as an indicator of hepatic dysfunction (FIG. 3b). HEK293 cells were transfected with TGR5, a cognate G protein-coupled receptor (GPCR) for bile acids, and a module in which HGF is driven by CREB1 activation, a downstream effector of GPCRs. The circuit was activated by bile acids in a dose-dependent manner. When implanted in vivo, the system produced HGF only when hepatotoxicity was experimentally induced, and not at homeostatic levels of bile acids; this engineered cell therapy also ameliorated the induced liver damage[37].

Following a similar sense-and-respond strategy, a synthetic gene circuit was applied to treat psoriasis by linking an AND-gate-sensing module for two disease-associated cytokines: TNF and IL-22 (REFS[38,39]). The sequential AND gate was generated using an NF-κB-driven promoter, which senses TNF signalling, to express the IL-22 receptor (IL-22R). Additionally, STAT3-driven promoters, which respond to IL-22R signalling, produced immunomodulatory cytokines IL-4 and IL-10. In this system, IL-4 and IL-10 were produced only when both TNF and IL-22 were present. Mice were implanted with HEK293 cells that had been transfected with the synthetic gene circuits and microencapsulated in alginate, and then skin inflammation was induced experimentally. Under these conditions, mice carrying the implanted synthetic gene circuits showed reduced skin inflammation and diminished pro-inflammatory cytokines[40].

Successful transition from experimental settings to the clinic for these implantable systems crucially depends on the sustained activity of the implanted cells in a given body cavity or target organ, and host recognition and subsequent foreign body immune responses often compromise their efficacy. Although anti-foreign body responses and fibrosis are not fully understood, it is now clear that innate immune cells such as macrophages are involved, and that colony-stimulating factor 1 receptor (CSF1R) is a crucial signalling mediator[41]; vascular endothelial growth factor (VEGF) is another likely contributor to the response[42]. Interestingly, size and shape are also crucially important across a broad spectrum of biomaterials such as hydrogels, ceramics, metals and plastics, with spheres of ≥1.5 mm diameter preventing foreign body reactions in rodents and non-human primates[43]. Strikingly, encapsulated rat pancreatic islet cells in 1.5 mm alginate capsules were poor inducers of foreign body response and fibrosis, and were able to keep circulating glucose at normal levels in diabetic mice for up to 6 months[43]. Further optimization of implantation strategies has been explored through combinatorial chemical modifications of alginate, which might lead to more effective matrices that avoid foreign body reactions and improve the long-term performance of implanted products to enable clinical translation[44,45].

Circulating engineered cells

In the context of cancer, genetically redesigned cell therapies have provided an additional approach whereby cells can be engineered in the laboratory and transferred to the patient. Infused cells can traffic to tumours and deliver engineered payloads constitutively, in a context-dependent manner to environmental cues, or in response to exogenously delivered inputs[46–48]. Engineered chimeric antigen receptor (CAR) T cells are now an established example of this framework, and they have demonstrated profound clinical activity in haematological malignancies[49]. Although the therapeutic benefits of CAR-T cell therapy cannot be overlooked, issues such as lack of control over toxicities, lack of flexibility in adapting to evolving pathology and lack of tumour specificity limit their use in patients. As CAR-T technologies have been extensively reviewed elsewhere[50–52], we highlight here how synthetic biology is enabling the next generation of products by addressing these challenges in engineered T cells (BOX 2; TABLE 1).

Payloads
Exogenous therapeutic molecules delivered by engineered cells.

Table 1 | **Novel CAR-T cell platforms enabled by synthetic biology**

CAR platform	Limitation addressed	Mechanism of action	Refs
Inducible stabilization/ destabilization	Lack of control	CAR is covalently linked to a destabilization domain, and active degradation is induced or inhibited by a small molecule or other input	56,57,67
Inducible dimerization	Lack of control	Antigen recognition and signalling domains are separated into two proteins, each fused to a dimerization domain that is brought together by a small molecule	59,60
Inducible expression	Lack of control and specificity	CAR gene expression is driven by conditional promoters with transcriptional activity regulated by specified inputs; tumour specificity can be improved using promoters that sense microenvironment-associated signals (such as hypoxia-induced promoter)	63–66
Adaptor-based antigen recognition	Lack of flexibility	Engineered cells express a CAR that recognizes a common adaptor epitope; CAR activity is directed by antigen-specific antibodies tagged with the adaptor	74–79
OR-gated antigen recognition	Lack of flexibility	Cell product expresses multiple antigen-specific CARs, each of which can independently activate the engineered cell; a single CAR can also be designed to target multiple antigens	68–73
AND-gated antigen recognition	Lack of specificity	Simultaneous AND gate: a suboptimal primary CAR and a co-stimulatory secondary CAR that target different antigens are co-expressed	86–89,91,92
		Sequential AND gate: a primary CAR activates an orthogonal transcriptional regulator that induces expression of a fully functional secondary CAR targeting a different antigen; in both mechanisms, full activation occurs only when both antigens are presented	
NOT-gated antigen recognition	Lack of specificity	A fully functional primary CAR and a co-inhibitory secondary CAR targeting different antigens are co-expressed, such that activation occurs only when the primary but not the secondary CAR is engaged	85
Split universal programmable system (SUPRA CAR)	All of the above	A system based on a split two-module system comprising a 'zipCAR' and a 'zipFv'. The zipCAR module consists of signalling domains fused to an extracellular leucine zipper. The soluble zipFv module consists of a second, matching leucine zipper fused to an antigen-specific scFv. A functional CAR is formed by combining the two modules. Response is controlled by the availability of zipFv. Desired breadth and specificity of antigen recognition is achieved with the appropriate combinations of zipFvs and zipCARs	100

CAR, chimeric antigen receptor; scFv, single-chain variable fragment.

Controllable CAR-T cells. Currently approved CARs are synthesized constitutively, and continuously engage the target, independently of the severity of immune-mediated toxicities. Therefore, approaches that allow for the regulated expression and titration of CARs represent an important advance. Along these lines, a protein of interest can be destabilized (OFF-switch) or stabilized (ON-switch) only in the presence of an exogenously supplied pharmacological ligand[53–58]. When applied to CAR-T cells, CARs can be actively degraded upon administration (OFF-switch) or withdrawal (ON-switch) of the ligand during excessive activation to dampen the response (FIG. 4a). Alternatively, ON-switch CARs can be designed by splitting the antigen recognition and intracellular signalling domains, which are then dimerized by the addition of a small molecule[59,60]. In both stability-based and heterodimerization-based designs, cells can be circulating indefinitely and turned on and off at will, which represents a clear advantage over permanently off systems such as suicide genes[61,62]. Beyond controlling CAR activity by small molecules, alternative methods to regulate CAR expression by heat[63], ultrasound[64], light[65] and oxygen concentration[66,67] have also been developed.

Flexible CAR-T cells. Current autologous cell-based therapies are costly to manufacture, and the freedom to change their mechanism of action without the need to replace the entire cellular chassis is advantageous in the context of diseases with fluctuating antigenic landscapes. To broaden the flexibility of CAR-T cell antigen specificity, cells can be redirected to multiple targets by co-expressing multiple CARs or linking multiple scFvs in one CAR to enable OR-gate recognition[68–73]. A chimeric receptor can also be guided by a tumour-associated antigen (TAA)-specific monoclonal antibody (mAb). This is achieved using a modular design, in which a CAR targets an adaptor molecule conjugated to the tumour-targeting mAb[74–79] (FIG. 4b). These approaches can be thought of as modular and universal, as a single universal CAR can be reused for a variety of tumour targets by simply switching the guiding module (TAA-specific mAb or TAA-specific scFv).

Selective CAR-T cells. As most TAAs are not truly restricted to tumours and appear in healthy tissues as well[80,81], off-tumour on-target toxicity becomes a fundamental limitation for current engineered T cell therapies[82–84]. Improved specificity can be achieved by targeting multiple co-expressed antigens in an AND-gated or NOT-gated fashion[85–92] (FIG. 4c). For in-depth discussions of combinatorial antigen recognition, readers are directed to other reviews on this topic[48,93–95]. The careful selection of appropriate antigen combinations will be crucial for both types of logic-gated CAR-T cell strategy, and systematic analyses of candidate antigen expression patterns across healthy and malignant tissues[96–98] will play a central part in the selection of optimal combinations of target antigens. Outside of the oncological applications discussed above, cellular therapies engineered with logic-gated synthetic gene circuits can also be applied to treat autoimmune diseases[99]

OR-gate
Output is actuated if any of the specified inputs are received.

NOT-gated
Output is negated if a specific input is received.

in which a single inflammatory factor is insufficient to specify the diseased site.

Integrated controllable, flexible and selective CAR-T cells. In the examples described above, synthetic biology approaches may address different aspects of control, flexibility or specificity in isolation. A CAR system that can improve upon all of these attributes simultaneously is therefore highly desirable, and initial attempts have been made to produce a system that can be dynamically tuned, combinatorial antigens switched and cell-type responses controlled[100].

Programming therapeutic bacterial cells

Immediately after birth, humans are colonized by a multitude of microbes that establish specific human tissues as their primary niche[101–103]. Throughout life, the human body is in constant interaction with microbes that are present in the diet and the environment, and therefore, human-associated microorganisms can be used as a platform to interface with host physiological functions for therapeutic purposes. Similar to the strategies used for implantable human cells, microbial cells can be manipulated through synthetic biology methodologies and delivered to the human body to prevent or

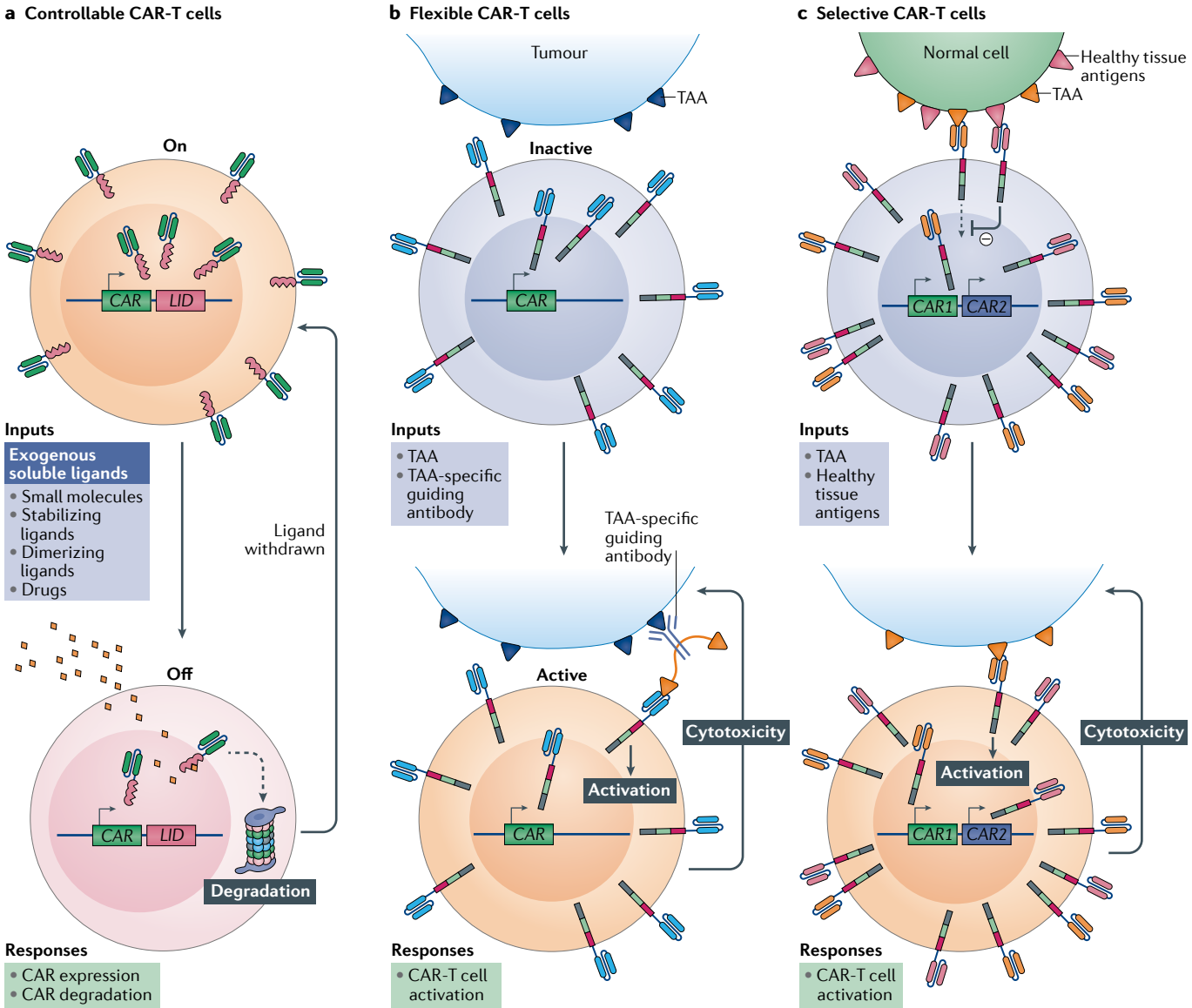


Fig. 4 | **Human circulating CAR-T cell therapies with engineered genetic circuits.** Circulating, engineered chimeric antigen receptor (CAR)-T cells can be designed for control, flexibility and specificity of cellular therapies. **a** | Controllable CAR-T cells. An exogenous soluble ligand can be used to switch ON and OFF CAR-T cell activity by stimulating CAR expression or CAR dimerization. In this manner, CAR activity is better controlled via two inputs: an antigen and a small-molecule ligand. A CAR fused with a ligand-induced degradation (LID) domain sequence is one example for improving control. **b** | Flexible CAR-T cells. To broaden the flexibility of CAR-T cells to recognize multiple tumour-associated antigens (TAAs), chimeric receptors can be designed to target an adaptor molecule conjugated to a tumour-targeting monoclonal antibody. **c** | Selective CAR-T cells. To improve the specificity of CAR-T cells and minimize their off-target effects on healthy tissue, different logic gates with multiple inputs can be engineered to drive CAR-T cell activity. This approach includes AND gates (simultaneous or sequential), NOT gates and combinatorial modules. In the illustrated example, dual CAR-T cells with multi-antigen recognition NOT gate express CAR1 for TAA (orange) and CAR2 for normal tissue antigen (pink). The CAR-T cell selectively kills tumour target cells expressing TAA, while healthy off-target cells co-expressing normal tissue antigen inhibit CAR-T cell activation and cytotoxicity.

Toggle switch
A synthetic, two-gene regulatory network in which either of two gene products represses the expression of the other gene, resulting in bistable equilibrium states.

Repressilator
Regulatory cycle of multiple genes whereby each gene represses its successor in the cycle, which is used to build an oscillating biological network.

treat diseases[104]. Microbial systems have been integral to the development of synthetic biology owing to their relative ease of genetic manipulation, metabolic simplicity and robustness[3]. The first generation of synthetic gene circuits, such as the toggle switch and repressilator, were designed, built and tested in bacteria[4,5], paving the way for more complex systems[105]. Notably, the vast diversity of microbial life serves as a seemingly inexhaustible source of genetic parts with novel functionalities for the creation of synthetic gene circuits[106–108]. These features position microorganism-based engineered therapeutics as a promising field for addressing unmet clinical needs.

The selection of bacterial strains for the development of therapeutic platforms generally depends on two factors: the safety profile of the strain and its amenability to genetic manipulation. Owing to their long-term safe usage in human nutrition, lactic acid bacteria (LAB) have been recognized as innocuous vehicles for the delivery of therapeutic payloads to human tissues[109]. In particular, *Lactococcus lactis*, which is frequently consumed as part of fermented dairy products, has been extensively developed as a host for genetic engineering efforts with industrial and clinical applications[110,111]. Similarly, various strains of *Escherichia coli*, a human gut commensal turned workhorse of molecular biology, have been extensively used for living therapeutics applications. Of note, the probiotic strain *E. coli* Nissle 1917 (*Ec*N)[112] has been safely used for nearly 100 years as the active pharmaceutical ingredient in multiple licensed medicinal products and the anti-inflammatory and antimicrobial activities of which have been described[113–116]. Alternatively, attenuated pathogens that retain their native tissue tropism can be used to deliver therapeutic or stimulatory molecules to malignant tissues. For instance, strains of *Listeria monocytogenes* that lack virulence factors such as the actin assembly-inducing protein or internalin B display reduced growth outside tumours but are capable of eliciting antitumour responses through intracellular growth within circulating immune cells[117–119]. Also, strains of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (hereafter *S.* Typhimurium), which are defective in the transport of lipids or in the production of secondary messenger metabolites, are incapable of bloodstream dissemination, but are able to target the hypoxic tumour environment when administered systemically[120,121].

The in vivo delivery of therapeutic payloads and the execution of metabolic conversions have been at the centre of most synthetic biology approaches to establish microorganisms as living therapeutic agents. Recent advances in the understanding of the interplay between host cells and microbes in the context of the intestinal[122] and tumour[16] environments have pointed to key pathways in which engineered microbes can intervene to restore cellular homeostasis. Below, we highlight key advances in developing engineered bacterial therapeutics that operate within the gut and tumour environments.

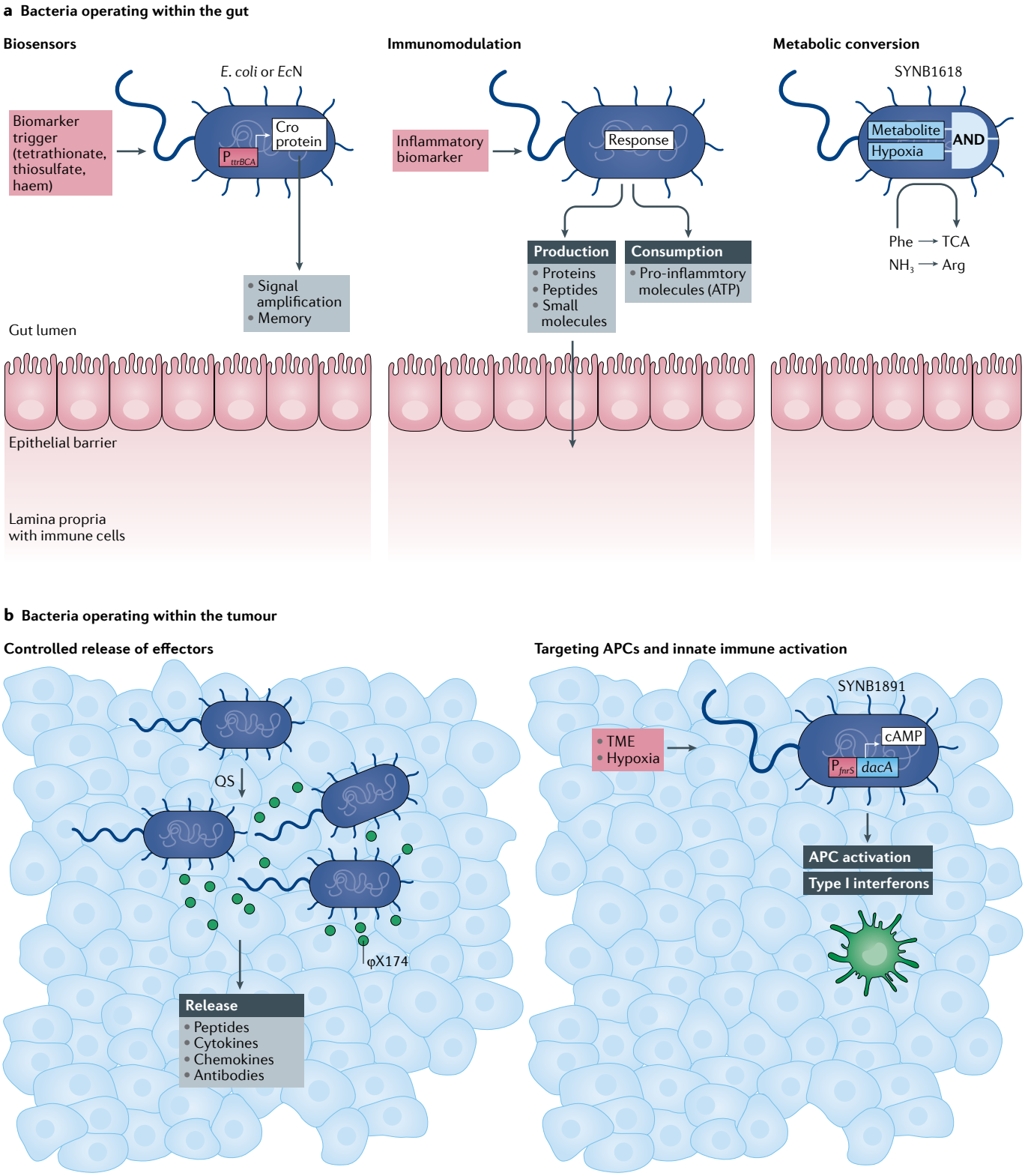
Gut-associated engineered bacteria

The ability of commensal and diet-derived bacteria to interact with mucosal surfaces of the gastrointestinal tract provides a platform for the development of living therapeutics operating within the gut. Oral administration of bacteria carrying basic production and secretion modules has proved to be a simple and effective strategy to deliver effector molecules to the gut environment. However, in recent years the combination of robust biological components and circuit design principles has enabled the creation of bacterial systems that can detect a stimulus and produce a designated response. This step-up in complexity of the synthetic gene circuits enables therapeutic microbes to achieve a more precisely localized and timed effect on the host. Below, we describe some notable examples in which engineered bacteria have been used to deliver diverse types of immunomodulatory or anti-infective effector molecules. We then discuss more complex cases of engineered bacteria that incorporate higher-level synthetic biology principles to respond to gut biomarkers for diagnostic applications or that have spatiotemporal sensing capabilities for the treatment of metabolic conditions in the gut.

Engineered bacteria as delivery vectors of effector molecules. One of the earliest applications of bacteria-based engineered therapeutics was their use as live vaccine vectors for the delivery of antigens into the human mucosa to elicit local and systemic immune responses. For instance, a recombinant strain of *Bifidobacterium* displaying a *Salmonella* antigen on its cell surface protected mice from lethal challenge of *S.* Typhimurium[123]. Similarly, *L. lactis* expressing *Helicobacter pylori* Lpp20 antigen[124] or nontoxic recombinant fragments derived from C-terminal receptor binding domains of cytotoxins TcdA and TcdB from *Clostridioides difficile*[125] yielded effective adaptive immune responses that prevented pathogenic colonization of the mouse gut. In another example, tetanus toxin fragment C was expressed by multiple native strains of lactobacilli eliciting serum pathogen-specific IgG and enhanced lymphoid responses[126]. Living vaccine vectors against viruses such as HIV have also been developed using engineered lactic acid bacteria. Chamcha et al.[127] expressed an HIV Gag antigen fused to the pilus proteins of group B *Streptococcus* on the surface of a *L. lactis* and showed that it was effective at eliciting mucosal humoral and cellular responses after oral immunization in mice, highlighting its potential as a vaccine platform against HIV.

Besides eliciting selective immune responses through mucosal delivery of antigens, engineered bacterial cells have been programmed to express other genetically encodable factors to hinder pathogen colonization or resolve inflammatory conditions in the gut. Inspired by natural microbial competition, bacterial strains have been engineered, for instance, to express antimicrobial compounds that target invading pathogenic bacteria. Along these lines, Forkus et al.[128] engineered *Ec*N to express and secrete the antimicrobial peptide microcin J25 and showed that administration of the engineered bacteria reduced the burden of intestinal *Salmonella* in turkeys.

Bacteria can also be engineered to target the virulence rather than the viability of pathogens. Some bacteria, such as the cholera pathogen *Vibrio cholerae*, can detect — or 'sense' — their own population density through a



quorum-sensing system based on levels of signalling factors, such as cholera autoinducer 1 (CAI-1), secreted by individual bacteria. Thus, when the concentration of these factors reaches a certain threshold as the bacterial population grows, it triggers a signalling pathway that results in suppression of virulence factor production. To prevent intestinal expansion of *V. cholerae*, *Ec*N was engineered to express high levels of CAI-1, and pretreatment with this engineered strain resulted in up to 92% survival of mice that ingested *V. cholerae*[129].

The luminal delivery of therapeutic proteins via the oral dosing of engineered bacteria is a particularly attractive notion in the context of inflammatory bowel disease (IBD), as this approach has the potential to deliver high

Fig. 5 | **Bacterial cell therapies with engineered genetic circuits. a** | Existing designs of live bacteria operating from the gut can be divided into three broad categories: diagnostic biosensors programmed to detect intestinal inflammation-associated biomarkers that appear in the gut lumen during disease states and that effectively amplify an output signal; bacteria engineered for the production of immunomodulatory molecules (such as trefoil factors or anti-inflammatory compounds) or for the consumption of pro-inflammatory molecules such as ATP within the gut lumen; these responses can be controlled by the presence of inflammatory biomarkers and can restore epithelial barrier function and reduce inflammation. In the third category metabolic conversions of toxic molecules in the context of metabolic disorders (conversion of toxic phenylalanine into *trans*-cinnamate (TCA) in the case of phenylketonuria, or ammonia into arginine in the case of hyperammonaemia) can be controlled by certain characteristics of the microenvironment found in the gastrointestinal tract (such as low oxygen tension). **b** | Existing designs of live bacteria operating from tumours to support antitumour immune response and tumour elimination. These designs include genetic oscillators such as quorum-sensing (QS) circuits to control the local delivery of effector molecules (such as immunomodulatory proteins, pro-apoptotic peptides, antibodies to immune checkpoint inhibitors). Other designs trigger the synthesis of immunostimulatory molecules in response to the hypoxic tumour microenvironment (TME) after engulfment of engineered bacteria by tumour-resident antigen-presenting cells (APCs).

local concentrations of a therapeutic agent at the site of inflammation (intestinal compartment), while minimizing its systemic exposure and therefore potential toxicities. The dysregulation of immunomodulatory cytokines is thought to have a crucial role in the pathophysiology of IBD, and as such the restoration of homeostatic levels of cytokine networks is an area of intense investigation[130]. Initial attempts to deliver engineered bacteria to the gut for the control of inflammation in experimental IBD settings were based on the expression of murine anti-inflammatory cytokines or nanobodies against pro-inflammatory cytokines[131]. Further steps were made to engineer *Ec*N to create inducible curli nanofibres displaying trefoil factors (TFFs), and ultimately to promote gut epithelial integrity and restore epithelial barrier function during colonic inflammation[132]. *Ec*N was engineered with a plasmid encoding a synthetic curli operon producing chimeric CsgA protein, the main component of the *E. coli* biofilm matrix. The CsgA protein was genetically fused to a therapeutic domain TFF3, a small cytokine promoting epithelial healing[133]. A synthetic curli operon was placed under the control of the arabinose-inducible promoter (P_{BAD}), which can be activated externally through the addition of arabinose to the drinking water of the mice.

Although most of these studies were not framed within a synthetic biology context of 'sense and respond', they demonstrated how simple expression modules can be incorporated in a variety of bacterial hosts to achieve the effective delivery of therapeutics. In addition, these studies suggest that simple synthetic gene circuits are functional in the context of animal studies, and several of these have moved on into further clinical development, in which they have proved safe in phase I human clinical trials[134–139].

Bacterial living biosensors of gut biomarkers. An ideal biosensor works in complex environments, has a high-resolution detection capacity, a high signal-to-noise output range, the ability to store information and can effectively amplify the output signal. These requirements can be addressed through synthetic biological principles[140]. The generalized architecture of a biosensor consists of several modules: a detection module for multiplexed pathological biomolecules (inputs) at predetermined thresholds; a Boolean module that incorporates a group of logic gates informed by user-defined clinically relevant biomarkers and/or rules, and an amplification and/or output module that produces an immediate user-interpretable signal. Alternatively, a memory module can be used to stably store information in the DNA of the biosensor cell for retrieval later. These concepts can be applied, in principle, to any biological compartment, and, as a proof of concept, Courbet and colleagues[140] incorporated amplifying genetic switches and logic gates in alginate polymer-encapsulated *E. coli* for the detection and recording of pathological levels of glucose in blood and urine in patients with diabetes[140]. Although the ex vivo detection of pathological biomarkers in fluids is attractive, the ultimate goal of synthetic biosensors is constant in vivo monitoring for real-time diagnostics. The engineering of gut commensals represents an attractive opportunity for the exploration of intestinal inflammation. A trigger–memory concept has been realized in *E. coli* with a genetic design that includes an anhydrotetracycline (aTC)-inducible promoter (P_{tet}) that controls the expression of the bacteriophage λ repressor protein Cro, and a memory element based on the λ cI/Cro region[141]. In vivo, in mice dosed with the engineered bacteria and aTC, the system was able to sense aTC and to 'remember' it after its withdrawal. This memory can be read by the user by interrogating the fraction of bacteria in the Cro-ON state, measured by a β-galactosidase reporter gene fused to Cro. This proof of concept for the trigger–memory design of a small-molecule sensor can be adapted to sense biological biomarkers upregulated in disease states, such as intestinal inflammation.

E. coli has been used to generate biosensors for tetrathionate, a metabolite produced during inflammation[142] (FIG. 5). The detection–trigger module detects tetrathionate by the *ttrR/S* genes from *S.* Typhimurium to drive expression of the Cro protein by the P_{ttrBCA} promoter. The tetrathionate-induced production of Cro results in activation of the β-galactosidase-based memory module that, once triggered, remains active in the absence of tetrathionate, which allows for subsequent signal retrieval through non-invasive faecal testing. Riglar and colleagues[142] validated this proof of concept in several mouse models of intestinal inflammation. The system was integrated into the chromosome of *E. coli* for enhanced stability, and was shown to continuously work in vivo for up to 6 months[142]. Along similar conceptual lines, a biosensor for thiosulfate (another intestinal inflammation-associated biomolecule) has been generated in *Ec*N[143] and demonstrated the ability to effectively sense, remember and report on gut inflammation in mice.

Synthetic biology approaches have been applied to probiotic bacteria to serve as in vivo diagnostics of gastrointestinal infections[144]. Mao et al.[144] engineered the lactic acid bacterium *L. lactis* into a living diagnostic that provides a point-of-need readout of cholera infection. They leveraged the transient colocalization of ingested *L. lactis* and *V. cholerae* to detect the pathogen at an early

Curli nanofibres
The amyloid fibre component of *Escherichia coli* biofilms.

Trefoil factors
(TFFs). Disulfide-rich mucosal peptides that promote epithelium protection by stimulating cell migration and increasing the viscoelasticity of the mucosa.

Curli operon
Gene cluster that encodes and regulates curli proteins.

stage of the infection. For this, they constructed a hybrid cell-surface receptor that uses components from the *V. cholerae* quorum-sensing system to detect the pathogen's cognate small-molecule autoinducers. Upon activation of the sensing module, the engineered probiotic computed the signal from the synthetic phosphorylation cascade to result in the expression and secretion of an enzymatic reporter, which is easily detected in faecal samples with a colorimetric assay. The demonstration of the functionality of the cholera-sensing *L. lactis* strain in an animal model of infection provided fundamental evidence for the use of engineered microbes for the diagnosis and surveillance of gastrointestinal infections.

A recent application of engineered *E. coli* as a biosensor used living bacteria as a component within an electronic, miniaturized ingestible device that travels through the gut and wirelessly communicates with the user[145]. This ingestible microbioelectronic device (IMBED) contained chambers in which engineered *Ec*N have access to the intestinal contents and report an output that can be electronically transformed and transmitted to the investigator. As a proof of concept, Mimee and colleagues[145] designed a gastrointestinal bleeding-sensing module based on a synthetic promoter regulated by the haem-responsive transcriptional repressor, HrtR, and an output module based on the *luxCDABE* cassette, which produces bioluminescence. The light output was detected by phototransistors, converted into a digital code by a luminometer chip, and transmitted wirelessly outside the body. IMBED was able to successfully detect and report intestinal bleeding in vivo in mice and pigs[145].

Engineered bacterial therapeutics for metabolic disorders. An important step in the generation of synthetic biology-based products for translation into human therapeutic tools is the development of cellular systems with drug-like properties. These properties include predictive pharmacokinetics, dose–response relationships, safety, manufacturability and scalability. Recently, two reports have introduced these concepts for orally delivered bacterial cell therapies in the context of two metabolic conditions, phenylketonuria (PKU) and hyperammonaemia, highlighting the role of synthetic biology in the generation of specific, flexible and controllable therapeutic entities (BOX 2). PKU, an inborn error of metabolism, is characterized by a deficiency of the enzyme phenylalanine hydroxylase (PAH), and as a consequence the food-derived amino acid phenylalanine (Phe) cannot be metabolized, accumulating in the blood to levels that cause neurological damage[146]. Isabella and colleagues[147] reported the generation of a strain of *Ec*N that was engineered with three gene circuit modules: a module based on a gene encoding the Phe transporter, *pheP*, under the control of the hypoxia-inducible promoter P_{fnrS}; a module based on a gene encoding phenylalanine ammonia lyase (PAL), *stlA*, also under the control of P_{fnrS}; and a module based on a gene encoding L-amino acid deaminase (LAAD), *pma*, under the control of the arabinose-inducible promoter P_{araC}. In this engineered *Ec*N strain (SYNB1618), LAAD is induced during fermentation and biomass production by supplementation of the culture medium with arabinose, whereas PheP and PAL are produced in vivo when the bacteria encounter the hypoxic environment of the mammalian gastrointestinal tract. Functionally, SYNB1618 is controlled by the combinatorial inputs of Phe and hypoxia, which results in the conversion of Phe into *trans*-cinnamate (TCA) (FIG. 5a). Importantly, TCA is converted by the liver into hippurate (HA) and excreted in the urine, acting as a biomarker for strain activity. This so-called 'synthetic biotic' was shown to effectively reduce systemic levels of Phe in mouse models acting from the gastrointestinal tract. Importantly, in humans, oral dosing of SYNB1618 in healthy volunteers results in a dose-dependent accumulation of the biomarker HA in the urine (NCT03516487).

Ammonia is a prevalent by-product of metabolism, and as such, the gastrointestinal tract represents a major source. In healthy individuals, gut ammonia is metabolized in the liver via the urea cycle into urea, which is then excreted in the urine. In individuals with the rare metabolic disease urea cycle disorder (UCD)[148], or with liver injury (such as cirrhosis)[149], ammonia builds up in the blood and results in hyperammonaemia and neurological damage. Therefore, capturing ammonia from the gastrointestinal tract and preventing its escape into the bloodstream is an important application for which orally delivered engineered bacteria might represent a viable therapeutic tool. In this context, a synthetic biotic was recently described, in which *Ec*N was rewired to efficiently channel gut ammonia into the arginine biosynthetic pathway, which results in the production of L-arginine. This natural amino acid is often given as a dietary supplement with no reported toxicity[150]. The engineering strategy was directed towards the effective funnelling of ammonia into the arginine pathway by deleting a negative feedback regulator of the pathway (*argR*) and creating a circuit with an arginine-resistant mutant version of the other negative regulator, *argA*, under the control of the hypoxia-induced promoter P_{fnrS}. This design results in the constant conversion of toxic ammonia into arginine when the bacteria encounter the hypoxic environment of the intestine. This strain (SYNB1020) was shown to reduce systemic levels of ammonia in animal models of liver injury in a dose-dependent manner and has completed phase I clinical trials in healthy volunteers (NCT03179878).

Most synthetic biology applications in microorganisms have been centred around *E. coli*, for which extensive genetic and biochemical knowledge has been amassed for the past half-century. However, the mammalian intestinal microbiota is dominated by other bacterial taxa, with members of the *Bacteroides* representing one of the most prevalent groups. For this reason, *Bacteroides*, and especially the commensal species *Bacteroides thetaiotaomicron*, are receiving increasing attention as additional chassis for synthetic biology applications. The development of circuit parts — most importantly, tunable promoters — as well as ribosome-binding sequence libraries and other genetic components are quickly being developed[151–154] to build next-generation engineered intestinal microbes for diagnostic and therapeutic purposes.

Tumour-associated engineered bacteria

Ever since the pioneering studies of William Coley in the late 1800s[155], bacteria have been recognized as potential anticancer agents. Coley first observed the eradication of a cervical sarcoma after an acute streptococcal infection of the tumour area, inspiring him to devote his career to the activation of the immune system with bacteria as a cancer treatment[155]. His work is credited as the first immunotherapy programme in the history of medicine. These very early observations captured two key notions: bacteria can live in tumours, and they can trigger antitumour immune responses. These insights form the basis upon which synthetic biology strategies can be harnessed to create controlled, safe and rationally designed antitumour biotherapeutics using bacteria. Below, we present notable examples in which engineered bacteria were used for the localized delivery of antitumour compounds as well as immune system modulators.

Bacteria of a wide range of taxonomic groups preferentially accumulate and proliferate in tumours, and in fact, many animal and human studies with pathogenic bacteria have demonstrated antitumour activity[156]. Unfortunately, these studies also unveiled unacceptable toxicities that prevented the development of these agents as therapeutic tools. Further attempts to attenuate pathogenicity resulted in safe but ineffective therapies, highlighting the apparent trade-off between toxicity and efficacy in these approaches[156]. Currently, most bacterial antitumour strategies are based on the use of a non-pathogenic chassis (such as *Ec*N) that is rationally engineered with synthetic biology tools to engage the immune system in the tumour microenvironment (TME) for robust and durable antitumour activity. The combination of synthetic biology approaches with our rapidly advancing understanding of tumour immunology represents an attractive opportunity for the development of novel bacterial cell therapies for the treatment of cancer (FIG. 5b).

One way to improve bacteria-based cancer therapies is to further augment their intrinsic tumour tropism[157,158]. The natural affinity of some bacteria for tumours can be enhanced by engineering synthetic adhesins targeted to bind predetermined cancer-expressed molecules such as neoantigens or other molecules that are enriched in cancer cells. Synthetic adhesins are modular membrane-anchored proteins with extracellular immunoglobulin domains that can be generated and optimized by library screens. As a proof of concept, Piñero-Lambea et al.[159] built a constitutive circuit in *E. coli* with a synthetic adhesin targeting green fluorescent protein (GFP) and demonstrated efficient in vitro and in vivo binding of the engineered bacteria to GFP-expressing HeLa cells. Crucially, the in vivo intravenous delivery of this engineered strain resulted in effective tumour colonization at a dose 100 times lower than that needed for an endogenous strain, or for a strain carrying an irrelevant adhesin, suggesting that similarly manipulated bacteria can be used to deliver therapeutic payloads to tumours at very low doses with minimal potential systemic toxicities[159]. The ability of bacteria to selectively home to tumours has also been explored for diagnostic purposes[160]. Danino et al.[160] developed a bioluminescent *Ec*N strain that also produces an enzymatic reporter. Orally delivered bacteria enabled the detection of murine liver tumour and metastases by imaging and measuring reporter enzyme substrate in the urine. These studies demonstrated that bacteria remain metabolically active in tumours, at least to an extent that allows the operation of engineered gene circuits.

Given that bacteria preferentially colonize malignant sites and naturally activate innate immune cells such as dendritic cells and macrophages, their use can provide a baseline level of immune activation in the TME. The use of immune checkpoint inhibitors such as anti-PD1 and anti-PDL1 antibodies has revolutionized cancer treatment[161]. However, only a fraction of patients can benefit from this therapeutic approach. Although the reasons for this lack of response in most patients are only partially understood, it is clear that some level of T cell infiltration at baseline is necessary for checkpoint inhibitors to work, and in fact these so-called 'hot tumours' have consistently demonstrated good prognosis. By contrast, 'cold tumours' that lack T cells show poor prognosis and therefore represent an important unmet medical need for cancer immunotherapy[162].

Synthetic biology can accordingly be applied to engineer bacteria for the controllable expression of metabolites, cytokines, scFvs or any other molecule capable of engaging T cell immune responses. This engineered function, in combination with the natural immune-engaging components present in the bacterial cell, has the potential to drive durable antitumour activity, even in cold tumours. This concept has recently been demonstrated through the engineering of a stimulator of interferon genes (STING) agonist in *Ec*N (SYNB1891) (FIG. 5b). The intracellular activation of STING by cyclic dinucleotides results in the production of type I interferons that activate innate immune cells as well as promote priming of cytotoxic T cells[163]. Leventhal et al.[164] designed a circuit in which *dacA* from *L. monocytogenes* (encoding an enzyme that synthesizes cAMP, a STING agonist) was placed under the control of the hypoxia-induced promoter, P_{fnrS}. SYNB1891 was also designed to include two auxotrophies (*thyA* and *dapA*) that result in its inability to survive outside the TME and in its inability to replicate within the TME, respectively. This gene circuit design endows the system with significant specificity (only activated in the hypoxic TME) and controllability (eliminated outside of the TME and unable to multiply within the TME) (BOX 2). In mouse tumours poorly infiltrated by T cells, SYNB1891 drove robust tumour eradication and induced long-term immunological memory rendering cured mice resistant to tumour relapses[164]. These effects were STING and $CD8^+$ T cell dependent, and further highlight the potential of bacterial synthetic biology for cancer therapy. SYNB1891 is currently being evaluated in phase I clinical studies in various types of cancer (NCT04167137).

Beyond enabling bacterial systems to deliver and overexpress payloads of interest within the TME, synthetic biology approaches allow for the tuning of the relative strength and duration of expression of the intended output. One way to control timing and duration of expression is by building a tunable gene oscillator[165].

Gene oscillators represent one of the earliest examples of the application of engineering principles to bacterial systems and can be designed by introducing competing activating and repressing factors. Stricker and colleagues[165] built a circuit in *E. coli* in which three genes — *araC*, *lacI* and *yemGFP* (reporter) — are controlled by a hybrid promoter, $P_{lac/ara1}$. This synthetic regulatory element is composed of the activation site from the *araBAD* promoter and the repression operator sites from the *lacZYA* promoter. Copies of $P_{lac/ara1}$ were then placed both upstream and downstream of transcription start sites controlling each of the three genes in the circuit. The system is activated by the AraC protein in the presence of arabinose, and repressed by the LacI protein in the absence of isopropyl thiogalactopyranoside (IPTG), and its oscillation can be tracked by fluorescence (GFP). Importantly, the period (duration) and amplitude (magnitude) of the oscillation could be accurately controlled by the relative concentrations of arabinose and IPTG added to the culture medium. The architecture of this synthetic gene network can be translated into therapeutic circuits that enable periodic delivery of payloads.

A successful strategy to build genetic oscillators in anticancer bacteria has been inspired by quorum sensing, in which the build-up of a crucial diffusible factor triggers a synchronous response in the population[166–168] (FIG. 5b). When the predefined cellular response to critical density is cell lysis, this approach enables the programmed release within the TME of engineered oncolytic factors, as well as immunomodulatory proteins that, in combination, resulted in cancer cell lysis and tumour growth inhibition in vivo[169]. A genetic circuit designed by Din et al.[169] includes a common promoter (P_{luxI}) that drives the expression of its own activator (acyl-homoserine lactone), as well as a bacteriophage-derived lysis factor (ϕX174 protein E). When the circuit is ON, ϕX174 E is produced, diffuses to neighbouring cells and triggers lysis upon reaching the critical threshold. A few surviving cells proliferate again and produce ϕX174 E until it reaches the critical threshold and triggers a new round of bacterial lysis, completing a new cycle. This pulsatile, synchronized lysis system was connected to an output of up to three cargoes — a pro-apoptotic peptide, a chemokine and haemolysin — resulting in the simultaneous release of all three in the TME each time that quorum lysis took place. This approach showed encouraging efficacy in syngeneic mouse tumour models[169].

There are other notable examples that demonstrate the utility of the coupling of programmed cell lysis with synthetic quorum-sensing systems for the release of therapeutic payloads into tumours. Chowdhury and colleagues[170] engineered non-pathogenic *E. coli* with a synchronized lysis circuit to lyse within the TME and release an encoded nanobody against the anti-phagocytic receptor CD47, which is commonly over-expressed in several human cancer types. Gurbatri and colleagues[171] built a probiotic bacteria *Ec*N with a similar lysis circuit integrated into the ϕ80 site of the *Ec*N-lux genome, where *plux* drives the transcription of *luxI* and ϕX174 E genes under a single promoter. Circuit activation led to the controlled production and intratumoural release of nanobodies targeting PDL1 and cytotoxic T lymphocyte antigen 4 (CTLA4), as well as the release of granulocyte–macrophage colony-stimulating factor (GM–CSF). In both examples, treatment with engineered bacteria led to robust tumour regression and systemic antitumour immunity.

Prospects for engineered cell-based therapeutics

As the field of engineered cell therapeutics matures and expands, it will face unprecedented challenges in terms of manufacturing (BOX 3) and clinical development (BOX 4), owing to the fact that cells have not been widely used as the fundamental unit in the in vivo production of a therapeutic. Synthetic biology has demonstrated conceptual improvements in therapeutic strategies for tackling currently intractable diseases. In silico-guided gene circuit construction could further accelerate the design–build–test cycles of engineering therapeutic platforms[172–175]. Exciting developments are underway in this field, and below we highlight some additional areas that will augment the transformative power of synthetic biology in ELTs.

Autonomy

As discussed earlier, negative feedback-controlled gene circuits can autonomously correct disease-driven perturbations to healthy physiology in complex diseases such as diabetes and hyperthyroidism[31,32]. A closed-loop sense-and-respond therapeutic system self-regulates its activity according to a predefined set point. Once administered, these systems would no longer require additional instructions. Applications that could benefit from such design principles include efforts to mitigate the toxicity of cellular therapies (such as cytokine release syndrome from CAR-T cells).

Non-autonomous platforms like those requiring exogenous control by the user with small molecules

Box 3 | Creating cell-based therapies: manufacturing considerations

The clinical-grade manufacturing of engineered live therapeutics (ELTs) uses production technologies similar to those for other cell products that are already in clinical use (such as clinical-grade probiotics or autologous lymphocytes), and is subject to the same rules and regulations. An additional challenge, however, is the inclusion of engineered, heterologous biological circuits that can impose burdens on the culture during scale-up, resulting in circuit and strain instability. Importantly, the use of synthetic biology approaches in the manufacturing process itself can offer effective solutions, whereby synthetic gene circuits can be introduced to ensure a desired process control as well as engineered pathway retention. For example, the therapeutic circuits in a given cell can be efficiently shut down during the manufacturing process so that reproducible and tightly controlled production of biomass can be ensured. For facultative anaerobic bacteria, this can be achieved by engineering hypoxia-controlled promoters and introducing defined oxygen concentrations into the microbial fermentation runs[147]. In some instances, however, activation of the synthetic gene circuits during manufacturing may be required to ensure that the engineered pathway is effective at the time of therapeutic administration.

Although the ELT field is still in its infancy, lessons from the more established industrial fermentation field provide helpful insights and potential solutions for manufacturing pitfalls. Relevant technical challenges include the accumulation of mutations during manufacturing with the consequent emergence of underproductive strain variants as a consequence of production load and metabolic burden that may make consistent production of approvable batches challenging[186,187].

Box 4 | **Creating cell-based therapies: clinical development considerations**

In the United States, the FDA regulates all aspects of the clinical development of engineered cell therapies through the Center for Biological Evaluation and Research (CBER). As there is some societal hesitancy about genetically modified organisms as a general concept, using the tools of synthetic biology to address crucial areas of unmet medical need argues for a more balanced view for the benefit to patients. In this regard, crucial areas of regulatory concern have focused on chemistry, manufacturing and control issues, which in many ways are similar to those of drug candidates from other therapeutic classes, including small molecules, monoclonal antibodies and vaccines. A complete description of the manufacturing guidelines is available elsewhere[188].

An important objective of the engineered live therapeutic (ELT) field is the development of pharmacokinetic and pharmacodynamic tools to guide the candidate selection and drug development process, applying the collective knowledge of decades of clinical development of other therapeutic modalities. ELTs present new scenarios for the construction of these relationships — for example, an orally administered agent may have a pharmacokinetic that is essentially the transit time of the organism through the gastrointestinal tract. Similarly, the pharmacokinetics for a human cell therapy delivered intravenously (such as CAR-T cells) can be determined by blood counts of the administered ELT over time after administration. However, local concentrations within the gastrointestinal tract are challenging if not completely impractical to measure, and stool samples are frequently used to generate data on the time to clearance of a specific dose. Bioengineered resources, such as artificial in vitro gastrointestinal systems, will offer important new technologies that allow drug developers to extrapolate human dosing regimens from a collection of preclinical data, informing dose selection for clinical trial design[189,190]. A similar challenge is posed by CAR-T cell engraftment, which is indicative of clinical activity but represents an additional level of complexity when characterizing in vivo behaviour of ELTs. Undoubtedly, and appropriately so, regulators and physicians will expect reassurance that a given dose of an ELT with a defined potency will provide an expected therapeutic benefit. There is limited experience to date with the development of ELTs as human therapeutics, and the approved and in-development CAR-T cell therapeutics (human cells) as well SYNB1618 and SYNB1891 programmes (bacterial cells) offer early examples of the development of tools and strategies to support candidate selection and full development.

are not ideal, as they also require a clinically useful biomarker for practical implementation and constant monitoring of patients. In the case of CAR-T cells, a synthetic gene circuit capable of dampening responses by sensing local inflammation could potentially serve as an autonomous safety switch. Tuning the sensitivity of the sensors and dosage of the output[105,176] will be crucial for engineering self-regulated ELTs that do not require additional user intervention.

Orthogonality

One of the major aims of synthetic biology is to programme novel cellular functions, often using building blocks that are derived from natural biological systems. This leads to problems with crosstalk between the synthetic and endogenous pathways that can compromise the fidelity of the intended function[177]. In the examples discussed above, CREB1 and NFAT activity downstream of bile acid and blood glucose detection modules[32,37], respectively, are common terminal transcription mediators shared by many pathways[178,179]. Therefore, the output of these circuits could be induced by other nonspecific signals. Co-opting orthogonal modules from evolutionarily distinct organisms into human cells (such as bacterial two-component systems)[180] is one way to ameliorate crosstalk with endogenous processes. Engineering artificial protein effectors[181,182] and novel signal transduction mechanisms[183,184] is also a promising area of investigation for expanding the toolkit of synthetic biology and avoiding untoward interactions with cellular host machinery.

Durability

The successful application of many ELTs will rely on the persistence of the introduced cells in vivo. The microbiota represents a niche in which a synthetically engineered commensal chassis could be coaxed to engraft and persist indefinitely, with the built-in ability to respond and activate an effector circuit only upon the sensing of a predetermined pathological cue or combination of cues. As discussed above, the rapid expansion of synthetic biology tools for prominent members of the human microbiota, such as *Bacteroides* or *Clostridium*, is paving the way for such durable approaches[151–153]. Robust, long-term functioning of the engineered gene circuits is also an important consideration for durability. For example, repetitive DNA elements in synthetic constructs can lead to genetic instability and loss of intended activity. Efforts to design nonrepetitive components[185] will be useful in constructing circuits of increasing complexity.

Concluding remarks

The ELT field is at an inflexion point. Significant biomedical challenges that once remained intractable to conventional pharmaceuticals are now being tackled by a new frontier of biological engineering approaches. Human and microbial cells can be genetically modified to correct intrinsic defects or to unlock their full therapeutic potential. In this regard, synthetic biology principles, such as molecular logic gates and feedback control, have made significant contributions to building the next generation of ELTs. Efforts to translate the innovative gene circuitry described here are currently underway. As with previous shifts in therapeutic paradigms, multiple hurdles in the research and development of next-generation ELTs still need to be addressed before clinical benefits are fully realized. Nevertheless, the intersection of synthetic biology with cell and gene therapy holds great promise for advancing medicine by expanding our ability to create specific, flexible and controllable treatments for complex diseases.

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Author contributions
A.C.-R. and T.G. wrote and edited sections of the manuscript, generated artwork and researched references. A.C.-R. and T.G. are equal contributors. A.S. edited the manuscript, generated artwork and researched references. J.J.C., T.K.L. and P.F.M. contributed to the writing and editing of the manuscript. J.M.L. conceived and coordinated the project, wrote several sections, edited the manuscript and researched references.

Competing interests
A.S. is an employee at Synlogic. J.J.C. is a co-founder and adviser of Synlogic and Senti Bio. T.K.L is an employee at Senti Bio, and co-founder and adviser of Synlogic and Senti Bio. P.M is a member of the advisory board of Synlogic. J.M.L. is a former employee at Synlogic. T.G. and A.C.-R. declare no competing interests.

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