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# Engineering strategies and therapeutic applications of synthetic Notch (synNotch) receptors in cancer therapeutics

KEYNOTE (GREEN)

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Synthetic Notch (synNotch) receptors have been developed as a novel approach to cell-based immunotherapy and will provide programmable, specific cancer treatments. Unlike existing fixed chimeric antigen receptor (CAR)-T cell therapies, synNotch receptors are a combination of modular domains, such as extracellular, transmembrane, and intracellular domains, for influencing gene expression depending on the present ligand. Through Boolean logic-based cellular decisions, synNotch receptors allow for a higher degree of precision and a lower degree of off-target effects. Logic-regulated circuits use pairs of ligands and receptors to detect indicators of the tumour microenvironment in a highly specific manner. The potential of synNotch in solid tumours such as glioma and pancreatic cancer has been demonstrated in preclinical models using CAR expression control and the ability to regulate cytokines, a method that could break through the obstacles of immunosuppressive niches and antigen escape. Continuous developments are resolving engineering chal-



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## allenges, and the fusion of synNotch with technologies such as CAR-T or induced pluripotent stem cells could be a transformative approach in cancer therapy.

**Keywords:** synNotch receptors; logic-gated CAR-T; programmable immunotherapy; synthetic biology; cancer therapeutics



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### Introduction

The synthetic Notch (synNotch) receptor system was designed to address the limitations of existing receptor-based cancer immunotherapies by retaining the modular structure and activation logic of Notch, but without the requirement for native signalling components.<sup>(p1)</sup> It is based on the endogenous Notch signalling pathway, which is an evolutionarily conserved system that controls cell fate choices through juxtacrine signalling pathways. The structural components of synNotch receptors consist of three parts: an intracellular domain (ICD) that gets released on activation to induce user-defined transcriptional outputs; a transmembrane domain (TMD) that facilitates signal transduction via proteolytic cleavage; and an extracellular domain (ECD) to target antigen recognition.<sup>(p2)</sup> Through synthetic modularity, researchers can precisely manage the spatial and temporal behaviour of cells in response to specific tumour-associated inputs through the customization of the receptors to a variety of antigenic inputs and transcriptional outputs.<sup>(p3)</sup>

The origin of the synNotch concept can be traced to the reconstitution of the canonical signalling mechanism of the Notch receptor. As a result of ligand-induced conformational changes, Notch is cleaved by ADAM (a disintegrin and metalloproteinase) and secretase-dependent proteases, thus liberating the ICD. SynNotch designs using replaceable adaptable transcription factors can perform customized genetic programming and provide a decoupled signal output, independent of endogenous pathways.<sup>(p4)</sup> This flexibility aids the application of synNotch in next-generation immunotherapies, in which cells can be instructed to perform complex tasks such as the conditional expression of checkpoint inhibitors, cytotoxic effectors, or additional synthetic receptors when specific antigens are present or absent.<sup>(p5)</sup>

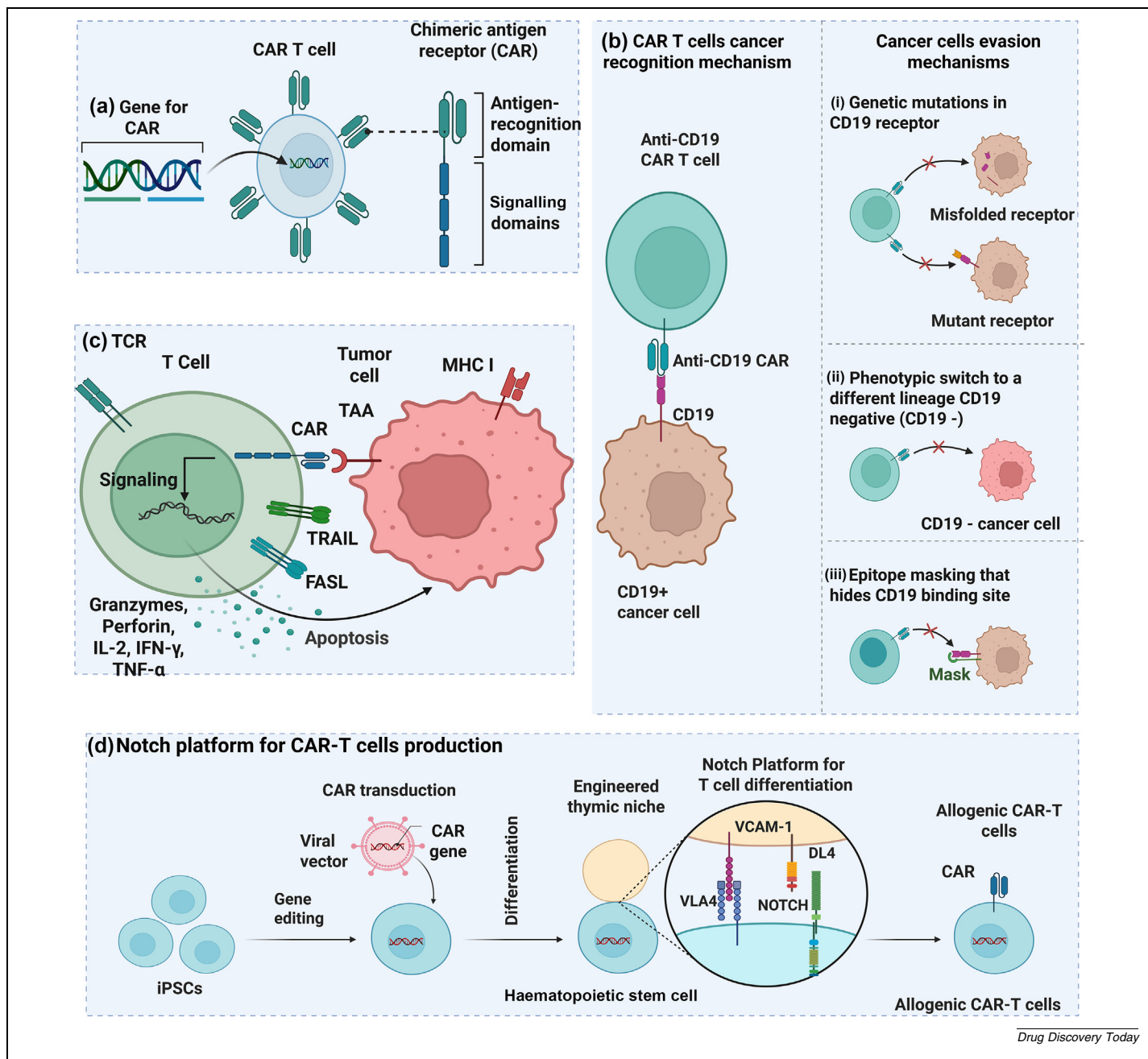
Compared with methods based on chimeric antigen receptor (CAR) and T cell receptor (TCR), synNotch receptors offer significant improvements in selectivity and functional complexity in the setting of cancer immunotherapy, as presented in [Figure 1](#). Poor efficiency in immunosuppressive tumour microenvironments (TMEs), antigen escape, and off-target damage are typical in conventional CAR-T cells.<sup>(p6)</sup> By contrast, synNotch systems allow the design of Boolean logic gates (including AND, OR, and NOT), which require combinatorial antigen recognition to become activated. This makes therapy more precise and reduces the possibility of cell death. This is of particular concern with solid tumours, because the heterogeneity of antigens and their

spatial variability present a significant challenge to successful treatment.<sup>(p7)</sup>

Morsut *et al.* conducted pioneering work in building synNotch receptors and implementing them in cancer therapies, developing synthetic receptors that could induce ligand-dependent transcriptional control using the modular Notch pathway design – a major conceptual breakthrough in the field of immune cell engineering. This proof-of-concept was able to prove that synNotch receptors could be modified to probe various different antigens and activate tailored genetic programs distinct from existing signalling.<sup>(p8)</sup> Subsequent landmark studies used synNotch receptors for tumour-specificity targeting; for example, Roybal *et al.* developed combinatorial antigen-sensing circuits to increase tumour-specificity and lower off-target toxicity in CAR-T cells. These pioneering works paved the way towards demonstrating the feasibility and clinical benefits of synNotch in the context of therapy, and offered the intellectual and experimental framework for subsequent studies in the area of Boolean logic-gated CAR expression and localized delivery of cytokines, as well as TME remodelling.<sup>(p9)</sup>

Recent preclinical studies have found synNotch-engineered T cells helpful in targeting complex tumour environments, including glioblastoma and pancreatic carcinoma, where other immunotherapies often fail. The translational potential of the synNotch platform is augmented by the fact that it can be applied across a range of cellular chassis, such as induced pluripotent stem cells (iPSCs), natural killer (NK) cells, and macrophages. Still, problems remain in optimizing ligand presentation, sustained signalling, and overcoming immunosuppressive checkpoints.<sup>(p10)</sup>

The synNotch receptor framework offers a highly flexible, modular, and programmable platform by which cell-based therapies can be made to recognize, interpret, and respond to complex tumour-derived signals, thus providing a highly flexible, modular, and programmable precision immunotherapy platform. As we advance, it is possible to speculate that combining synNotch with more physiologically relevant disease models, including tumouroids, and the most recent gene editing tools, including CRISPR, could accelerate the development of safer and more effective cancer therapies. SynNotch will have a central role in the broader enterprise of synthetic biology-based medicine, and could become a therapeutic intervention tool with its present momentum.<sup>(p11)</sup>



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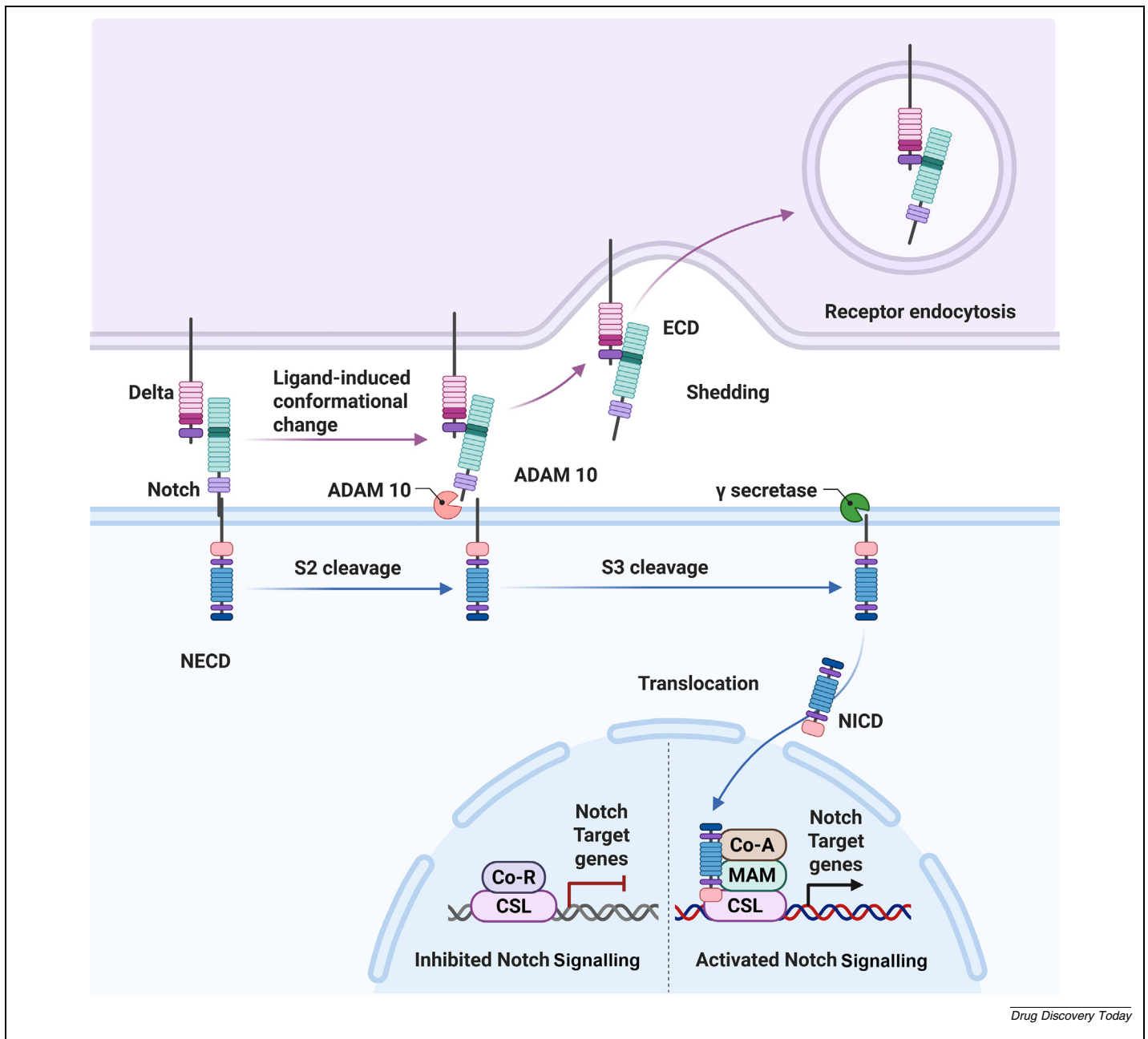
**FIGURE 1**

Overview of CAR-T cell design, tumour recognition, evasion mechanisms, and Notch-based production platforms. **(a)** The CAR structure comprises an extracellular antigen-recognition domain (e.g., scFv) fused with intracellular signalling domains, introduced via gene transduction into T cells. **(b)** Anti-CD19 CAR-T cells specifically recognize CD19<sup>+</sup> cancer cells; however, tumour cells can evade recognition through (i) genetic mutations causing CD19 receptor misfolding, (ii) lineage switch resulting in CD19-negative phenotype, or (iii) epitope masking that hides the CAR binding site. **(c)** CAR-T cells engage tumour-associated antigens (TAAs) on cancer cells, triggering intracellular signalling cascades, release of cytotoxic molecules (granzymes, perforin, TRAIL, and FASL), and proinflammatory cytokines (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ), resulting in tumour cell apoptosis. **(d)** Allogeneic CAR-T cells can be derived from gene-edited induced pluripotent stem cells (iPSCs). The Notch signalling platform, involving VCAM-1, VLA-4, DL4, and NOTCH receptors in an engineered thymic niche, promotes the differentiation of haematopoietic stem cells into functional, off-the-shelf CAR-T cells. (Created by using [BioRender.com](https://www.biorender.com).)

### Molecular architecture of synNotch

The molecular layout of synNotch receptors is a logically programmed framework that provides orthogonal transcriptional regulation and receptor personalization, while exploiting the structural and mechanistic properties of the endogenous Notch signalling pathway. Notch signalling is illustrated in [Figure 2](#).<sup>(p12)</sup>

This modular architecture consists of three distinct, functionally related domains: the ECD, the TMD, and the ICD. Recognition signal processing and gene regulation activities can be independently adjusted, because each module is synthetically interchangeable. The design ensures that in pathophysiologically relevant microenvironments (e.g., solid tumours), ligand

**FIGURE 2**

Schematic representation of the Notch signalling cascade. An allosteric repose (such as Delta) causes change in conformational shape of the Notch receptor, allowing S2 cleavage and shedding of the extracellular domain (ECD/NECD) by ADAM10. This complex of ligand–NECD is endocytosed in the signalling cell, and the rest of the membrane tethered fragment of Notch is further cleaved by  $\gamma$ -secretase, which cleaves the S3 products in the transmembrane domain (TMD). This liberates the intracellular domain of Notch (NICD), which translocates into the nucleus. Without NICD, the CSL transcription factor does not dissociate with the co-repressors (Co-R), which keep the target genes in the repressed state. When NICD is introduced, CSL converts to an active complex through the recruitment of co-activators (Co-A and MAM), resulting in the Notch target genes being transcriptionally activated. The figure shows extracellular shedding, progressive cleavages, NICD nuclear translocation, and transcriptional change between inactive and active Notch signal. (Created with BioRender.com.)

engagement on the cell surface results in tightly regulated transcriptional activation, dissociated from native pathways and poised to operate in response to non-native ligands.<sup>(P13)</sup>

#### Core components

The specificity of synNotch receptors is determined by their ECD, which generally consists of custom ligand-recognition motifs, including single-chain variable fragments (scFvs), nanobodies,

or protein scaffolds (e.g., DARPins). They facilitate the specific binding of membrane-bound antigens on neighbouring cells, and are bound at the N-terminus of the receptor. Notably, the architecture of the ECD lacks background signalling, because it is not dependent on endogenous Delta-like or Jagged ligands.<sup>(P14)</sup>

The linker region between the ECD and TMD can include spacers or protease-resistant sequences to modify the mechanical stress and receptor accessibility required to activate the receptor.

The TMD, crucial to the mechanical signals propagated on a ligand's engagement, is derived from Notch1. It contains both the S3 cleavage site, which is  $\gamma$ -secretase-dependent, and the S2 cleavage site, which is sensitive to ADAM10/17.<sup>(p15)</sup> The TMD architecture is structurally restrictive in preserving the receptor's membrane conformation and exposing the cleavage sites in a proper biophysical environment. The discrimination of activation threshold can be enhanced, and ligand-independent cleavage (leakage), a significant issue *in vivo*, can be reduced by engineering the TMD to alter membrane residence time or protease access.<sup>(p16)</sup>

The ICD comprises synthetic transcriptional activators such as Gal4-VP64, tetracycline-controlled transactivator protein (tTA), or synthetic transcriptional repressors and activators fused to nuclear localization sequences (NLSs), which replace the native RAM/ANK/PEST domains of Notch1. On proteolytic cleavage and release into the cytosol, the ICD translocates to the nucleus, where it binds to promoters engineered to contain cognate DNA-binding motifs.<sup>(p17)</sup> This enables the ICD to stimulate or inhibit the transcription of specific downstream effectors, including CARs, cytokines, and apoptosis-inducible proteins. To achieve a balance between response amplitude and timing, optimization of the design of ICDs with regulated nuclear import kinetics and low immunogenicity is in progress.<sup>(p18)</sup>

### Mechanism of action

Mechanistic activation of the synNotch signalling is triggered by a ligand-mediated force propagation across the ECD–TMD axis. ECD-membrane-anchored antigen binding on a neighbouring cell generates mechanical tension that allows ADAM-family metalloproteases to cleave the S2 site in the TMD.<sup>(p19)</sup> The ECD should ectopically dissolve so that the ICD can be liberated from the lipid bilayer through intramembrane proteolysis at the S3 site, mediated by  $\gamma$ -secretase. The juxtaposition of membranes, the anchoring of ligands, and the spatial orientation of the receptor–ligand complex – factors all affected by ECD length, glycosylation, and the mechanical rigidity of the TME – are key elements of the sequential proteolytic cascade.<sup>(p20)</sup>

The released ICD binds to minimal synthetic promoters upstream of response regions within its NLS, thus initiating transcription. Due to their inherent orthogonality, this technology can combine many synNotch receptors in parallel or with other synthetic modules, such as synthetic promoters or recombinases, to implement Boolean logic circuits (AND, OR, NOT gates). To distinguish between cancerous cells and healthy tissue with common markers, for example, one synNotch receptor can be used to control the expression of a second CAR or synNotch receptor, enabling layered transcriptional programs that respond to combinations of antigens or sequential presentation of antigens.<sup>(p21),(p22)</sup>

SynNotch receptor activation depends on the endocytosis-mediated mechanical unfolding of the negative regulatory region (NRR), exposing proteolytic cleavage sites. Ligand binding on the ECD generates pulling force (mediated by clathrin-dependent endocytosis in the ligand presenting cell) that unfolds the NRR to allow ADAM10 cleavage at the S2 site, followed by  $\gamma$ -secretase cleavage at the S3 site.<sup>(p23)</sup> This proteolytic cascade is responsible for the release of the ICD, leading to the initiation

of the transcriptional responses. This mechanotransduction is similar to endogenous Notch activation and is necessary for the ligand-specific and tightly controlled signal transduction by synNotch, as supported by several reports describing the conformational changes and (force-dependent) cleavage necessary for activation without the requirement of internalization of the ligand in the receiver cell itself.<sup>(p24),(p25)</sup>

Advanced versions of synNotch have tuneable degrons and post-translational modifications to regulate ICD stability and transcriptional half-life, as well as stacked structures to activate multiple ICDs with a single ligand input. To improve proteolytic activity or to avoid signal bleed, recent work has also introduced chimera TMDs or canonical cleavage motifs, improving signal fidelity in more complex biological settings, such as immunosuppressive tumour stroma.<sup>(p26)</sup> The synNotch receptors can display programmed ligand recognition, modular transcriptional activation, and controlled membrane cleavage owing to their flexible and orthogonally functional design. SynNotch is essential for next-generation cellular therapies that aim to target TME heterogeneity and oncogenic complexity, because its synthetic plasticity enables highly discriminative therapeutic programming in engineered immune cells.<sup>(p27)</sup>

### Engineering strategies

The ability to program the expression of genes in response to specific extracellular signals has transformed cellular immunotherapy. SynNotch is a modular platform that combines immunoengineering and synthetic biology to program intelligent therapeutic circuits that are capable of making complex decisions in the TME.<sup>(p28)</sup> Engineering techniques such as modular stacking of signalling elements, precise ligand–receptor pairing, and the design of flexible transcriptional circuits and threshold control mechanisms to regulate receptor sensitivity are relevant to synNotch function. Collectively, these approaches can make therapeutic cells exhibit conditional, temporal, and geographic specificity of their anti-tumour effects.<sup>(p29)</sup>

#### Pairing of ligands and receptors

The design of synNotch receptors requires customization of the ligand–receptor interactions that determine the specificity of downstream signalling events. SynNotch receptors use synthetic ECDs to bind specific non-natural or disease-specific antigens, but endogenous Notch systems use conserved Delta/Jagged ligands. These special ECDs can recognize tumour-associated surface proteins selectively and are often constructed with scFvs, nanobodies, or synthetic ligands.<sup>(p17)</sup> SynNotch orthogonal receptor–ligand interactions reduce cross-reactivity by decoupling receptor activation and endogenous ligands, achieving specificity in complex tissue environments. The ligand–receptor pairing can be designed to enable multi-input recognition programs, whereby a synNotch receptor minimizes on-target/off-tumour effects by only activating transcription in the presence of a specific ligand that is only expressed by tumour cells.<sup>(p30)</sup>

#### Transcriptional circuits

SynNotch receptors contain a modular ICD that can be altered to secrete any transcriptional effector on ligand binding. Due to its design, synNotch can be installed as a plug-and-play system to

initiate user-programmed gene expression experiments. A significant advance in this area has included transcriptional circuits governed by Boolean logic, enabling therapeutic cells to implement logical functions such as AND, OR, and NOT gates.<sup>(p31)</sup> Dual synNotch systems can be configured to enhance tumour specificity so that production of a lethal gene (such as CAR) requires the sequential detection of two independent antigens (AND gate). As an alternative, selectivity can be enhanced by suppressing activation in the presence of standard tissue signals using NOT gates. Such transcriptional circuits allow real-time, complicated control over cell behaviour by modulating various outputs, including cytokine secretion, immunological checkpoint inhibition, or even synthesizing additional receptors.<sup>(p32)</sup>

### Modularity and stacking

Among the most attractive aspects of synNotch engineering is its inherent modularity, which allows the generation of ECDs, TMDs, and ICDs separately and in combination. This property can be used to encode multiple synNotch receptors in the same chassis cell, each wired to distinct transcriptional outputs: a stacking technique. This stacking enables the therapeutic cells to be responsive in layers to a multiplicity of microenvironmental stimuli by enabling the hierarchical or combinatorial control of multiple genes.<sup>(p33)</sup> An orderly sequence of immunological events can be initiated, including homing, recognition, and killing, because pathways are more likely to be triggered sequentially by this architecture. Stacking also enables hybrid signalling strategies through the incorporation of synNotch with other synthetic platforms, such as CARs or CRISPR-based switches. This approach opens up the possibility of multifactorial control systems in complex disease settings.<sup>(p34)</sup>

### Tuning sensitivity

A key factor in achieving a balance of safety and therapeutic efficacy is optimizing the sensitivity of synNotch receptors. This could be achieved by modifying the proteolytic cleavage efficiency of TMD, the affinity of ECD binding, or the expression level of receptor subunits. To distinguish between malignant cells and normal cells that express the same surface markers, the synNotch systems must be capable of discriminating between cells with high and low expression of a target antigen. This is enabled through the development of adjustable thresholds, using inducible promoters or transcriptional repressors in the output circuit.<sup>(p35)</sup> These thresholding protocols can provide more reliable and stable therapeutic outcomes by averting undesired interactions or unintentional activation because of ligand spillover.

Advances in the quantitative modelling of synNotch dynamics have aided the rational design of receptors with a response curve that is optimized to specific tumour properties.<sup>(p36)</sup>

The combination of ligand specificity, transcriptional logic, modular design, and threshold calibration give synNotch receptor systems a solid basis as a next-generation programmable immunotherapy, and recent advances in computational design and synthetic circuit integration promise to make these systems even more precise and therapeutically powerful.<sup>(p37)</sup>

### Circuit dynamics: Latency, leak, and transcriptional control

SynNotch receptor circuits have their own latency factor, which in most cases is 20–24 h between ligand engagement and a maximal transcriptional response, making rapid therapeutic responses challenging. This latency is due to sequential proteolytic cleavage, intracellular trafficking, and promoter-dependent gene expression kinetics.<sup>(p25)</sup> Leakage, defined here as basal receptor activation in the absence of ligand, differs among ICD transcription factor choices (e.g., Gal4-VP64 usually generates higher activation, but higher leakage levels than tTA or TetR-based regulators). Promoter strength has an important effect on the modulation of output amplitude and noise level, because constitutive versus inducible promoters will affect signal-to-noise ratios and precision in therapeutic windows. Synthetic circuits can be optimized towards low leakage and strong activation by tuning the ICD nuclear localization signals and degrons. These parameters are crucially important to determine how synNotch functions in the context of solid tumours, and knowledge of the variants of transcription factors and promoter architectures can be used to customize the latency and specificity of synNotch systems.<sup>(p8),(p38)</sup>

### Advantages over traditional therapies

The programmability, modularity, and specificity of synNotch receptors offer distinct benefits over earlier CAR-T and TCR-based therapies. The key features of synNotch systems include a strongly adjustable ligand–receptor interaction to enable context-dependent activation of downstream effectors, along with ultra-specific recognition of tumour-associated antigens (TAAs).<sup>(p39)</sup> Because single-antigen recognition is often insufficient to differentiate between malignant and intact tissues with shared epitopes, this layout significantly reduces the prospect of off-target harm, a significant concern in conventional CAR-T treatment. SynNotch platforms could exert spatiotemporal regulation over gene expression through the transcriptionally insulated synthetic circuits. This allows engineered cells to react at particular occasions when they are exposed to specific microenvironmental inputs.<sup>(p40)</sup>

SynNotch can also be programmed, making it possible to design logic-gated therapeutic systems to respond to Boolean logic such as AND, OR, and NOT gates, ensuring therapeutic induction only occurs in the presence of specific antigenic combinations. This is most useful in the case of solid tumours, which often foil single-input therapies owing to stromal masking effect and antigen heterogeneity. SynNotch circuits provide a programmable platform on which multistage intervention strategies can be built by wiring diverse inputs to trigger sequential or combinatorial gene expression induction, including immune checkpoint blockade, cytokine release, or conditional CAR engagement.<sup>(p41)</sup> Strategies for modulating Notch signalling in cancer include virotherapy, nanoparticle delivery, immune checkpoint blockade, and synNotch-based CAR platforms. These approaches reprogram tumour-associated macrophages, enhance CD8<sup>+</sup> T cell responses, and enable multi-antigen precision targeting, offering synergistic and tumour-specific immune activation across varied therapeutic contexts, as presented in [Figure 3](#).<sup>(p42)</sup>

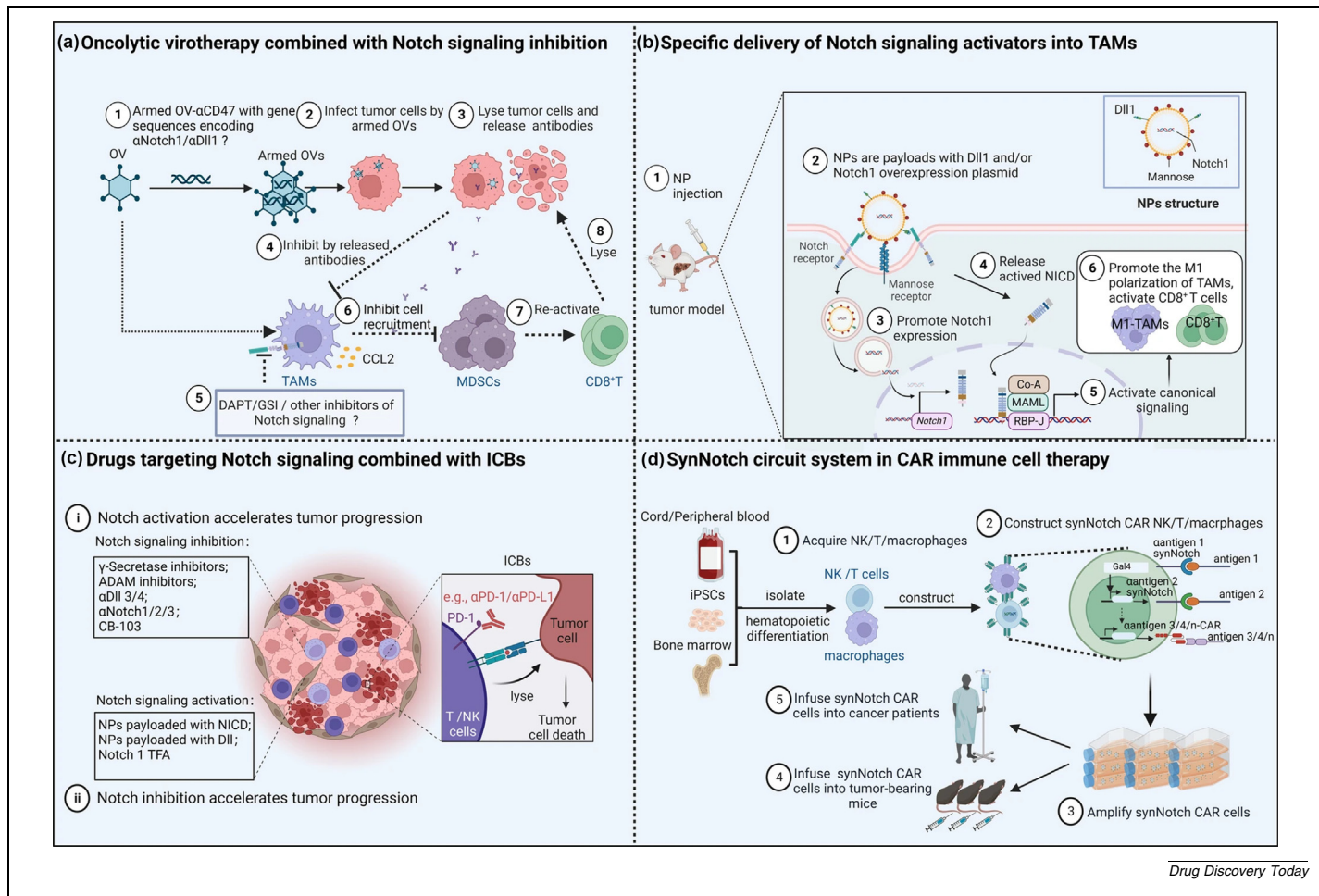
**FIGURE 3**

Illustration of innovative strategies integrating Notch signalling modulation in cancer immunotherapy. **(a)** Oncolytic virotherapy armed with anti-Notch components (e.g.,  $\alpha$ Notch1/ $\Delta$ DII1) facilitates tumour lysis, antibody release, and inhibition of tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) via Notch pathway suppression, enhancing CD8<sup>+</sup> T cell reactivation. **(b)** Nanoparticles (NPs) engineered to carry Notch1/DII1 payloads target mannose receptors on TAMs, inducing Notch1 expression and NICD release, activating canonical signalling to polarize TAMs to an M1 phenotype and boost CD8<sup>+</sup> T cell function. **(c)** Therapeutic modulation of Notch signalling combined with immune checkpoint blockade (ICBs): Notch inhibition (via  $\gamma$ -secretase, ADAM, or  $\alpha$ Notch antibodies) or activation (via NICD/DII1-loaded NPs) shows tumour-context-dependent effects and might synergize with ICBs such as anti-PD-1/PD-L1. **(d)** synNotch-based CAR platforms: immune cells (T/NK/macrophages) engineered with multi-antigen-sensing synNotch-CAR circuits are expanded into tumour-bearing models or patients, offering precise and controlled anti-tumour immunity tailored to tumour-specific antigen landscapes. Reproduced from Li X *et al.* (2023).<sup>(p42)</sup>

The synNotch architecture allows ICDs, TMDs, or ECDs to be replaced or layered without a complete redesign. These plug-and-play features augment the adaptability and scalability of designed cellular therapies and allow the customization of receptor sensitivity, signal length, and payload type. Furthermore, synNotch constructs are not limited to T lymphocytes, because synNotch can be transferred across various other immune cells, including NK cells and stem cells.

Owing to these advantages, synNotch systems are especially amenable to addressing the complex challenges posed by solid tumours, including localized immunosuppression, intratumoural heterogeneity, and immune evasion. SynNotch-based platforms are expected to have a significant impact on the future of precision immunotherapy and personalized oncology, with efforts under way to improve their receptor fidelity, signal amplification, and delivery technology.<sup>(p43)</sup>

## Therapeutic applications

SynNotch systems have the potential to enable precise responses in complicated and heterogeneous TMEs.<sup>(p44)</sup> Recent preclinical advances have demonstrated the usefulness of synNotch in the creation of multifunctional therapeutic programs that comprise logic-gated CAR-T cells, localized cytokine release, TME remodelling, and real-time intratumoural diagnostics.<sup>(p45)</sup>

### Logic-gated CAR-T cells

A canonical application of synNotch technology is the generation of Boolean logic-gated CAR-T cells, which operate on two or more antigen inputs to produce tuned activation. The synNotch-CAR cascade involves AND, OR, and NOT logic circuits and allows temporal distinction between antigen detection (via synNotch) and cytolytic involvement (via CAR).<sup>(p46)</sup>

In this case, the T cells will be initially activated in the presence of antigen A using an AND gate-based architecture that will trigger the expression of a synNotch receptor and produce CARs against antigen B. This decreases off-tumour toxicity that can occur when the single antigen is produced in normal tissues, because it requires the presence of both antigens to induce effector functions.<sup>(p47)</sup> The synNotch identification of epidermal growth factor receptor variant III (EGFR<sup>vIII</sup>) could produce interleukin-13 receptor subunit  $\alpha 2$  (IL-13R $\alpha 2$ )-targeting CARs, leading to selective lysis of tumour cells and improved survival *in vivo*. Another application of combinatorial targeting has been in pancreatic ductal adenocarcinoma (PDAC), where T cells have been engineered to initially detect a fibroblast-specific signal, enhancing CAR expression only in the tumour core and evading the immunosuppressive desmoplastic stroma. In addition to enhancing infiltration and durable response, these synNotch-CAR systems were found to be resistant to antigen escape, a key limitation of monovalent CAR designs.<sup>(p48)</sup>

#### Conditional cytokine secretion

Confinement of immunostimulatory activity to the TME makes synNotch-mediated cytokine release efficacious while avoiding systemic homeostasis.<sup>(p49)</sup> One study used synNotch to regulate IL-12 production in melanoma models, recruiting tumour-infiltrating lymphocytes (TILs) and mobilizing local dendritic cells, turning immunologically cold tumours hot. The same approach has been applied to engineer the delivery of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, and IL-15 to favour the recruitment of NK cells and the preferential expansion of T cells. Furthermore, orthogonal synNotch control of cytokine payloads allows multiplexing with pro-apoptotic factors or checkpoint blockade elements to generate multilayer treatment regimens tuned to respond to distinct tumour antigens. These multimodal therapies achieve long-term tumour destruction with minimal disruption to the immune system.<sup>(p50)</sup>

#### Tumour microenvironment modulation

Adoptive cell therapy is seriously hampered by the immunosuppressive properties of solid tumours, which are characterized by thick extracellular matrix (ECM), regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and the expression of checkpoint ligands. SynNotch receptors allow strict manipulation of these inhibitory systems through inducible payload release. For example, synNotch T cells can be designed to express matrix metalloproteinases (MMPs) or heparanase in response to tumour antigens; these proteins can then remodel the ECM and enhance T cell infiltration in stroma-rich tumours such as PDAC.<sup>(p51)</sup> Innovative strategies that integrate Notch signalling modulation in cancer immunotherapy are presented in Figure 4.<sup>(p42)</sup>

Recently, it was shown that local delivery of anti-programmed cell death protein 1 (anti-PD-1) or anti-cytotoxic T-lymphocyte associated protein 4 (anti-CTLA-4) minibodies through synNotch could rescue T cell effector function without the systemic administration of checkpoint inhibitors. Models have also used synNotch to reprogram metabolic checkpoints, such as IDO and arginase pathways, to recondition the metabolic state of the TME towards a state favouring an immune activation phenotype.

These strategies demonstrate how synNotch systems can dynamically interact with tumour biology and modify it on a real-time basis.<sup>(p52)</sup>

#### Intratumoural diagnostics and biosensing

A rather unusual, but already relevant application case is biosensing with SynNotch. Synthetic cells can be utilized as sentinels, whereby transcriptionally driven reporters, such as luciferase, GFP, or secreted enzymes, are used to report the absence or presence of specific tumour biomarkers. SynNotch biosensors have been developed to detect EGFR<sup>vIII</sup> in glioma models and activate a fluorescent reporter, allowing mapping of the spatial distribution of antigen within the tumour bed.<sup>(p53)</sup> This enables therapeutic cell activation and localization to be monitored in real time and encourages adaptive treatment modalities. Moreover, such diagnostics could be incorporated into closed-loop therapeutic layouts, where payload expression is under the control of biosensor activation, enabling self-regulating and autonomous therapy cycles. Future research will aim to broaden the diagnostic toolbox of tumour-specific features beyond antigen expression by incorporating biosensors of hypoxia, pH, and metabolic stress.<sup>(p54)</sup>

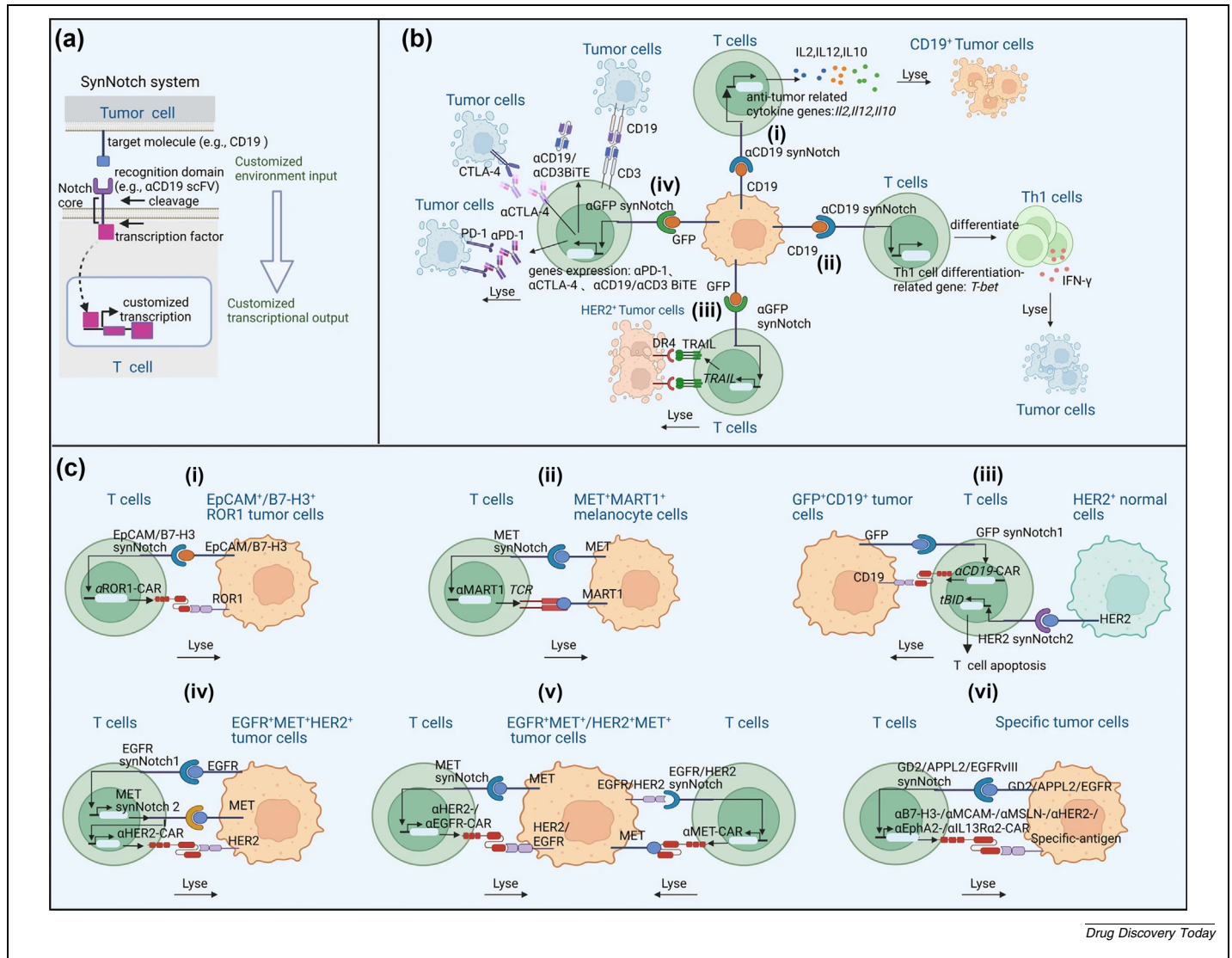
The therapeutic potential of synNotch platforms is being expanded through combination with organoid/tumouroid systems to create bespoke preclinical validation, immune cell engineering using iPSCs to enable scalable production, and CRISPR/Cas9 to enable precise genome editing. Programming immune cells *in vivo*, without *ex vivo* manipulation, requires optimizing the presentation of synthetic ligands by biomaterial scaffolds, modifying exosomes, or using virus-like particles.<sup>(p55)</sup> Furthermore, future developments in RNA-based circuit elements and synthetic promoters are likely to improve the dynamic range and sensitivity of synNotch-based responses.

SynNotch technology, in combination with real-time diagnostics, intelligent delivery strategies, and personalized immunogenomics, is bringing a new era of versatile, programmable cancer immunotherapies, although challenges remain in terms of heterogeneity, immune suppression, and safety.<sup>(p56)</sup> Table 1 provides a summary of engineering strategies and therapeutic applications of synNotch receptors in cancer therapeutics.

#### Preclinical studies

Modified T cells, NK cells, and stem cell-derived immunocytes are the primary reagents in proof-of-concept experiments to verify synNotch-mediated response against various solid tumours.<sup>(p57)</sup> Initial pioneering studies demonstrated that synNotch T cells could execute antigen-specific transcriptional programs in response to tumour-associated ligands, delivering therapeutic cargoes such as pro-inflammatory cytokines, cytotoxic effectors, or secondary CARs in a spatially restricted fashion, enhancing tumour specificity while preserving the architecture of non-malignant tissue.<sup>(p58)</sup>

Recently, this platform was improved by producing AND-gated T cells via orthogonal synNotch-CAR circuits. To cytotoxicity target only dual-antigen-positive scenarios, T cells expressing an EGFR<sup>vIII</sup>-specific synNotch receptor were designed to make an IL-13R $\alpha 2$ -targeted CAR only after ligand engagement

**FIGURE 4**

Overview of synNotch receptor-based engineering strategies and applications in cancer immunotherapy. **(a)** synNotch architecture: T cells are engineered with receptors containing an extracellular tumour antigen recognition domain (e.g.,  $\alpha$ CD19 scFV), a Notch core for proteolytic cleavage, and a transcription factor for programmable gene expression upon activation. **(b)** Functional applications of synNotch circuits:  $\alpha$ CD19 synNotch (i) induces expression of proinflammatory cytokines (IL-2, IL-12, and IL-10); (ii) drives Tbet expression for Th1 differentiation and IFN- $\gamma$  secretion; (iii) activates TRAIL in HER2<sup>+</sup> tumours to induce apoptosis; and (iv) triggers expression of  $\alpha$ CD19/ $\alpha$ CD3 BiTEs and immune checkpoint inhibitors ( $\alpha$ PD-1,  $\alpha$ CTLA-4) to enhance T-cell cytotoxicity. **(c)** Combinatorial synNotch logic gates in tumour targeting: (i, ii) AND gates for dual-antigen recognition (EpCAM/B7-H3, MET/MART1); (iii) avoidance of HER2<sup>+</sup> off-targets via layered recognition; (iv, v) multi-antigen gating for EGFR<sup>+</sup>/MET<sup>+</sup>/HER2<sup>+</sup> tumours; (vi) multiplex targeting of tumour-specific antigens (e.g., GD2, APPL2, EphA2) to enhance tumour specificity and safety of engineered T cells. Reproduced from Li X *et al.* (2023).<sup>(p42)</sup>

in glioblastoma models. This circuit improved a significant limitation of first-generation CAR therapies by successfully reducing off-target damage to healthy brain tissue.<sup>(p59)</sup> Similarly, the local precision of this approach was evidenced by high tumour shrinkage and minimal systemic toxicity in hepatocellular carcinoma xenografts expressing  $\alpha$ GPC3 synNotch-driven CAR.

SynNotch systems have been used to deliver cytokines in a localized manner. The ability of synNotch to modify the TME was dissected in melanoma murine models using T cells producing synNotch-regulated IL-12, which exhibited enhanced intratumoural T cell infiltration, increased the expression of MHC-I in tumour cells, and reversed local immunosuppression. Within the tumour core of PDAC, which is a recognized immune-

resistant tumour type, co-stimulatory ligand production through synNotch-mediated activation led to the reactivation of exhausted T cells and reduced the progression of the disease.<sup>(p60)</sup>

SynNotch designs were recently shown to be compatible with a non-T cell chassis. NK-92 cells engineered with synNotch circuits showed *in vitro* and *in vivo* ligand-inducible cytolytic responses, and the platform has applications beyond adaptive immunity. Moreover, synNotch-expressing stem cells have generated lineage-controlled immune effectors with therapeutic effects. These can be renewed to produce immune cells that can maintain anti-tumour activity.<sup>(p61)</sup>

These preclinical models show the potential of the synNotch system to enable the precise, logic-based coordination of

immune therapies. But despite these promising results, there are challenges involved in translating these findings into human environments, such as how to sustain expression, avoid immune-mediated clearance, and achieve uniform ligand presentation across various tumour types. However, synNotch-based therapies will soon commence early-phase clinical trials, marking a significant step towards the development of next-generation precision immunotherapies, as the field exhibits increasing maturity in terms of receptor design, circuit layering, and chassis optimization.<sup>(p62)</sup>

## Challenges and limitations

SynNotch receptor systems promise to transform cancer immunotherapy, yet several significant challenges remain before their universal clinical use can be realized. Such limitations include *in vivo* performance over time, delivery performance, functional fidelity, and molecular design. To achieve the maximal therapeutic effect of synNotch-based cellular engineering in solid tumours and diverse cancer microenvironments, a focus on these shortcomings is needed.<sup>(p63)</sup>

### Ligand presentation

A fundamental issue in synNotch activation is presenting cognate ligands in a context-specific and reliable fashion. Effective synNotch signalling requires expression of the membrane-bound ligands and, to achieve the best selectivity, this expression needs to be spatially confined to diseased tissues. Expression of ligand in heterotypic, transient, and non-malignant cells in the complex TME can result in inadequate activation or an unexpected response. Unlike the soluble ligands used in the traditional receptor–ligand interactions, synNotch activation requires physical cell–cell contact. This limits its applicability and requires delicate engineering of the tumour-targeting interface. Recent strategies to address this involve synthetic ligands conjugated to biomaterials, tumour-limiting antigens, or even dual-recognition logic circuits, which are only activated in multi-antigenic conditions.<sup>(p64)</sup>

The inability of synNotch receptors to be activated by soluble ligands is at the core of their mechanism of activation. SynNotch receptors are based on the conserved (i.e., endogenous) Notch receptor pathway, which requires mechanical forces through binding of these receptors to ligand on the ECD of neighbouring cells; conformational changes and proteolytic cleavage of membrane prodomain in the TMD increase the rigidity of the receptor. The resulting force-mediated cleavage liberates the ICD, which moves to the nucleus to trigger transcriptional responses. Soluble ligands do not have the membrane anchorage required to provide this mechanical tension, and so are unable to cause the conformational changes that lead to proteolytic cleavage and consequent receptor activation. Thus, the activation of synNotch is intrinsically limited to juxtacrine signalling, limiting the applicability of the receptor where targets are provided as soluble factors.

This constraint also strengthens the concept of spatial specificity, through a need for direct cell–cell interactions, which in turn reduces off-target activation, but at the same time creates difficulties for achieving a wider accessibility of the ligand in

the TME. Despite attempts at engineering ligand presentation via the use of biomaterial scaffolds or synthetic linkage of the ligand, overcoming this requirement for membrane-bound ligands remains a great obstacle. Addressing this limitation in synNotch requires innovative receptor or ligand design to translate soluble signals into mechanical inputs or different modalities of activation, thus increasing the therapy possibilities in cancers that are characterized by soluble biomarkers. Future engineering approaches could potentially increase synNotch receptor utility in immunotherapy applications.<sup>(p23),(p25)</sup>

### Delivery constraints

Other crucial limitations of the practical application of synNotch-engineered cells are associated with delivery. If the modified T or NK cells are delivered systemically, they can accumulate outside tumours, show poor infiltration into immunosuppressive tumour cores, or be lost or exhausted before interacting with the target. Other impediments to synNotch cell trafficking and activity include stromal density, hypoxic gradients, and immunosuppressive signalling networks. Furthermore, it is difficult to ensure that engineered cells can express synNotch constructs over the long term without epigenetic decay or silencing. Strategies to enhance *in situ* activation and therapeutic specificity are being investigated, including chemokine-mediated cell navigation, stromal-targeting co-receptors, and more-effective administration routes (such as intratumoural injection).<sup>(p19),(p49)</sup>

### Signal leak

Signal leak, where synNotch is activated without ligand binding, is a significant concern. This could be caused by spontaneous proteolytic cleavage of the Notch TMD, the basal activity of the transcriptional payload, or an unstable receptor conformation.<sup>(p65)</sup> Signal leaks alter the safety and controllability of engineered biological responses through spurious or premature gene expression. To overcome this, ligand recognition and proteolytic cleavage thresholds are being tightly coupled to optimize receptor scaffolds. Recent innovations such as split receptors, orthogonal ICDs, and more spatially isolated synthetic transcriptional components could ensure lower basal activity while preserving logic gate integrity.<sup>(p66)</sup>

Evidence of ligand-independent activation of Notch receptors, mainly as a consequence of endosomal trafficking dysregulation or conformational instability in the NRR, is a double-edged sword for synNotch engineering in that it could be strategically exploited to increase rather than decrease therapeutic specificity. Endogenous Notch is basally activated upon ESCRT-mediated sorting dysfunction, resulting in trapping of the receptors in endosomes, where aberrant S2/3 cleavages simulate ligand-dependent force that can release the Notch ICD (NICD) without juxtacrine cues. SynNotch receptors, although designed to limit these leaks through their optimized TMDs and ECD spacers, can still display spurious activation of their ICD and unintended transcription *in vivo*.<sup>(p67)</sup>

However, rather than being a liability, this phenomenon could be put to use: with the help of tuneable NRR stabilizers or ligands with mimicked functions as *cis*-inhibitors, the specificity of synNotch could be improved using dual-threshold gating, where weak-level noise of ligand-independent mechanical

TABLE 1

**Engineering strategies and therapeutic applications of synNotch receptors in cancer therapeutics.**

Engineering strategy	Key component/module	Description	Design goals	Representative examples	Implications in cancer therapy	Therapeutic applications	Refs
Modular receptor architecture	Extracellular domain (ECD)	Tumour-specific ligand binding via scFvs	Antigen specificity	Anti-HER2, anti-EGFR, anti-MUC1	Restricts activation to tumour sites	Tumour antigen sensing; input for logic circuits	(p90)
	Transmembrane domain (TMD)	Mediates signal transduction	Optimization of cleavage and activation	Notch1, Notch2 TMD	Signal fidelity and stability	Core activation machinery	(p38)
	Intracellular domain (ICD)	Releases TF to drive output gene	Modular transcriptional control	Gal4-VP64, tTA	Triggers desired gene expression	Controls CAR, cytokines, or suicide genes	(p64)
Ligand recognition engineering	ScFv optimization	Engineered for stability and humanization	Reduction of immunogenicity	Humanized CD19, CEA scFv	Enhances selectivity	Tumour-specific detection	(p91)
	Dual-ligand detection	Integrates two antigens via AND/OR logic	Safety enhancement	CD19 + HER2	Avoids off-tumour effects	Multi-antigen logic therapy	(p92)
Transcriptional control modules	Synthetic promoters	Controlled by orthogonal TFs	Minimization of endogenous interference	UAS, TRE, SynZiF	Tight gene expression regulation	Drives inducible gene therapy payloads	(p93)
	Logic gate circuits	Implements AND, OR, NOT gating	Enhancement of tumour-specific responses	synNotch → CAR + CAR systems	Context-specific activation	Precision immunotherapy	(p94)
Temporal and spatial regulation	Time-delay circuits	Cascading activation steps	Control of kinetics	synNotch → TF → CAR	Minimizes basal activation	Safer activation kinetics	(p92)
	Local response restriction	TME-restricted activation	Reduction of systemic toxicity	TME-sensing cytokine circuits	On-target cytokine expression	Solid tumour immune remodelling	(p94)
Payload programming	CAR expression	Inducible CAR based on antigen contact	On-demand cytotoxicity	HER2-synNotch → CD19-CAR	Spatial and conditional killing	Tumour-specific cytotoxic T cell activation	(p92)
	Cytokine secretion	Induced pro-inflammatory cytokines	Activation of immune microenvironment	IL-2, IL-12, IFN- $\gamma$ secretion	Remodels immunosuppressive niche	TME activation, T cell support	(p38)
	Suicide gene activation	Inducible safety switches	Controlled cell death	iCasp9, HSV-TK	Emergency deactivation	Safety switch in engineered cell therapy	(p64)
Structural optimization	Cleavage site engineering	Tunes S2/S3 $\gamma$ -secretase site	Optimization of receptor activation	Modified Notch1 cleavage sites	Tuning sensitivity and response time	Lower activation thresholds in dense tumour tissue	(p1)
	Spacer and linker design	Optimizes interdomain folding	Reduction of sterics, improvement in performance	GS-rich linkers	Structural refinement	Improved stability, responsiveness	(p1)
Signal amplification strategies	Cascade amplifiers	Transcriptional cascade for strong output	Overcoming weak input signals	synNotch → TF → CAR amplifier	Sustained functional activity	Low-antigen tumour killing	(p92)
	Positive feedback loops	Autocrine or gene loop circuits	Prolonged activation	synNotch → IL-2 → autocrine loop	Enhances cell survival and function	Enhances memory phenotype T cells	(p92)

(continued on next page)

TABLE 1 (CONTINUED)

Engineering strategy	Key component/module	Description	Design goals	Representative examples	Implications in cancer therapy	Therapeutic applications	Refs
Delivery and expression systems	Viral vector systems Non-viral delivery CRISPR knock-in	Stable gene delivery Transient, fast expression Precision genome integration	Long-term expression Reduction of insertional mutagenesis Expression under endogenous control	Lentivirus, AAV Electroporation, mRNA TRAC locus, AAVS1	Durable and robust expression Short-term expression trials Reduces insertional mutagenesis	Universal synNotch T cell platform Safer for early-phase testing Streamlined regulatory approval	(p14) (p14) (p14)
Multicellular system design	Division of labour circuits Distributed activation networks	Split sensing and effector modules SynNotch cells as communication hubs	Functional compartmentalization Inter-cell signalling	Sensor → effector synNotch cells SynNotch → ligand → activate neighbour cell	Multi-layer therapeutic programming Decentralized control	Complex tumour targeting Modular and scalable cell therapies	(p64) (p64)

pull could be suppressed until the combinatorial *trans*-ligands exert enough mechanical pull for robust activation. Such designs would mimic the physiological cis–trans balance, restricting organ responses to tumour microstructures that contain multiple membrane antigens clustered together, and switching off the basal response in the rest of the organ. This approach far surpasses existing orthogonal attempts at improvement using endogenous autoinhibitory logic, and could result in potentially greater improvements exceeding 15-fold for the refined synNotch variants. Integrating approach-independent mechanisms, therefore, could lead to more precise synNotch systems.<sup>(p23),(p68)</sup>

### Long-term efficacy

A separate challenge is sustaining synNotch activity over long periods. SynNotch signal transduction can be perturbed by chronic TME stresses, metabolic fatigue, and repeated ligand exposure, all of which limit the duration of therapy and induce the possibility of treatment relapse. The phenotypic stability of engineered cells and the acquisition of long-term immunological memory are also currently being investigated. Long-term response can be interfered with by epigenetic drift, receptor desensitization, and markers of T cell fatigue (PD-1, LAG-3). Epigenetic reprogramming strategies, inducible self-renewal programs and combination with immune checkpoint regulation are being studied to boost durability without compromising safety.<sup>(p69),(p70)</sup>

To approach these problems in a translational perspective, an approach that encompasses the systems level is required in the form of biomaterial science, synthetic circuit design, immunoen지니어링, and clinical manufacturing methods. Translation to the clinical setting would need scalable vector delivery (both viral and non-viral platforms), stable ligand expression in patient tumours, and strong preclinical validation in physiologically relevant models (e.g., patient-derived organoids or tumouroids).<sup>(p71)</sup>

Immunogenicity presents a serious problem to synNotch receptors, where heterologous ECDs (e.g., scFvs, nanobodies) and non-native ICD transcription factors (e.g., Gal4-VP64) cause host anti-transgene immunity, and induce clearance of engineered T cells, and reduce therapeutic persistence, respectively. In contrast to autologous CAR-T, orthogonal components of synNotch induce neutralizing antibodies and CD8<sup>+</sup> T cells to become exhausted, which suppresses logic-gated activities in solid tumours. The humanization of affinity domains or the use of hypoiimmunogenic scaffolds can mitigate this, but requires strict validation. Furthermore, circumventing immunogenicity will surely lead to clinical failure, hence there is a need for a stealth-engineered synNotch to provide long lasting anti-tumour control.<sup>(p72),(p73),(p74)</sup>

Biosafety, regulatory protocols, and manufacturing uniformity should be well evaluated, especially in autologous or customized therapy. The potential for broader use is growing as a result of the development of universal, off-the-shelf synNotch platforms that include modular payloads and safety switches. These innovations are being shaped and made more effective by progress in synthetic biology, systems immunology, and precision oncology.<sup>(p25),(p75)</sup>

## Clinical translation: Progress towards clinical use and regulatory challenges

A significant trend in programmable immunotherapy is the movement of synNotch receptor systems out of preclinical validation and into the clinic. What defines the clinical translatability of synNotch platforms is their ability to activate transcriptional programs in engineered immune cells, specifically T lymphocytes and effectors differentiated from iPSCs, with high specificity, ligand dependence, and absence of basal leakiness or unanticipated immunological reactions.<sup>(p76)</sup> Recently, chimeric ECDs have been constructed to reduce cross-reactivity with normal tissue epitopes and bind tumour-restricted antigens with high affinity and specificity. Advances are needed to minimize the risk of off-tumour/on-target toxicity, an established limitation of traditional CAR-T systems. Adding Boolean logic-gated circuits (e.g., AND, NOT, and OR) to synNotch constructions enables multi-input sensing. This improves the ability to discern between malignant and non-malignant tissue based on combinatorial antigen expression profiles that are unique to TMEs.<sup>(p77)</sup>

Clinically translatable synNotch circuits have performed well in orthotopic murine xenograft models of glioblastoma multiforme, pancreatic adenocarcinoma, and triple-negative breast cancer. SynNotch-primed CAR modules allowed T cell effectors to accomplish localized killing and conditional activation. Tight regulation of downstream genes such as cytolytic effectors (e.g., granzyme B and perforin), immune checkpoint inhibitors (e.g., PD-1 and scFv), or immunomodulatory cytokines (e.g., IL-12 and IL-18) has been achieved in such systems with inducible transcriptional units. Gal4-VP64, TetR-VP64, or artificial transcriptional repressor cleavage by synNotch controls these units. These designs can enhance the therapeutic effect in immunosuppressive TMEs and reduce the systemic burden of constant cytokine exposure associated with neurotoxicity in conventional immunotherapies and cytokine release syndrome (CRS).<sup>(p26),(p78)</sup>

Although synNotch-based therapies have exciting preclinical data, a difficult road awaits them before getting regulatory approval. Regulatory agencies such as the US FDA and EMA require comprehensive data on the pharmacodynamic behaviour, transgene expression kinetics, dose–response, and long-term persistence of the modified cells. The non-native ligands and transcriptional modules used as synNotch components are synthetic, which evokes concerns regarding immunogenicity that must be carefully considered in both autologous and allogeneic settings. Also, being a regulated protease (e.g., 7-secretase), activity in different tissues could affect the uniformity and fidelity of synNotch activation, necessitating that circuit responsiveness is normalized across patients.<sup>(p13)</sup>

The scalability and repeatability of synNotch T-cell production under GMP guidelines needs to be determined. This involves confirming the absence of replication-competent viral vectors, confirming the clonal homogeneity of the modified cells, and ensuring the integrity of lentiviral or non-viral gene delivery systems.<sup>(p79)</sup> Specifically, real-time control of synNotch activation presents clinical trial design with new challenges, namely the timing of antigen exposure relative to circuit activation windows, with adaptive dosing informed by the geographical heterogeneity of antigens.<sup>(p80)</sup>

The next important issue is the regulatory status of the synNotch platforms. Synthetic biologics, advanced therapy medical products (ATMPs), and gene-modified cellular products just scratch the surface of the regulatory spaces that synNotch-based medicines will enter owing to their synthetic, programmable nature.<sup>(p81)</sup> Hence, regulatory frameworks are urgently needed to evaluate dynamic, logic-driven treatment systems (particularly those incorporating multiplexed antigen sensing and independent decision-making). This might involve generating novel potency assays, quantifying circuit leakiness, and predicting *in vivo* behaviour through computer modelling.<sup>(p82)</sup>

Translational bioengineering in collaboration with quantitative pharmacology and systems immunology will assist the clinical translation of synNotch systems in the future. Synthetic safety switches (e.g., small molecule-controlled dimerization domains or inducible suicide genes) will increase safety and accuracy, as will non-invasive molecular imaging to monitor circuit activity and CRISPR-based genomic targeting. It is expected that refractory solid tumours whose antigenic pattern is well characterized and whose treatment requires unmet needs will represent the primary subjects of early-phase clinical trials. Incorporation of synNotch circuits into adaptive clinical trial designs, including basket or umbrella trials, can additionally speed regulatory approval pathways and enable sorting patients by immunogenomic subsets as these circuits mature.<sup>(p83)</sup>

The most important outcome of moving the synthesis of synNotch receptor systems to a broader and more comprehensive selection of biomarkers under the banner of integrative bioinformatics is to ensure their increased clinical relevance by incorporating preclinical and early clinical research outcomes. There are also emerging data showing that synNotch-engineered cells, such as iPSC-derived NK cells deployed against glioblastoma using a dual checkpoint blockade, provide localized immunomodulation with minimal off-tumour toxicity: this is an important feature of therapeutic precision and the safety profile. Nevertheless, there is little research on the pharmacologic interference of inhibitors in patients, risks associated with artificial transcription factor immunogenicity, and potential toxicities of viral vectors used for delivery. A combination of the emerging clinical trial results, such as Phase I synNotch CAR-T safety testing, and high-throughput bioinformatics of transcriptional and proteomic immune evidence can provide standards of translation preparedness. This information could be used as a practical translational manual to ensure synthetic receptor designs meet clinical regulatory requirements, streamlining the process of adopting synNotch in personalized oncology ([ClinicalTrials.gov](https://ClinicalTrials.gov) identifier: NCT06186401).<sup>(p49),(p64)</sup>

Although the regulatory and technical hurdles involved in clinical translation are yet to be resolved, synNotch receptor systems have already been successfully discovered, developed, validated, and integrated into therapeutic cell platforms, providing a strong foundation and compelling rationale for advancing towards clinical trials.<sup>(p64)</sup> To achieve the therapeutic potential of synNotch-based precision immunotherapies, challenges related to immunogenicity, the standardization of GMP-compatible production routes, and regulatory assessment tools will need to be addressed.<sup>(p84)</sup>

## Future directions

The next phase in the development of synNotch receptor technology is convergence with new platforms such as CRISPR-based genome engineering, iPSCs, tumouroid systems, and customized immunotherapeutic strategies. This combination will significantly increase the specificity, flexibility, and translational readiness of synNotch-based cellular therapies in the oncology setting.<sup>(p85)</sup> CRISPR-Cas techniques provide a highly tuneable set of tools in immune effector cells to perform precise genome editing, with the opportunity to conditionally activate synNotch circuits and enable multiplex regulation or stable integration.<sup>(p86)</sup> Combining synNotch modules with CRISPR-based logic algorithms allows the building of multi-layered genetic circuits that can respond to tumour-specific stimuli and achieve a well-defined therapeutic output with spatiotemporal precision. This co-engineering lowers systemic toxicity and tumour clearance by dynamically controlling payloads such as cytokines, cytotoxic medicines, or checkpoint inhibitors in the presence of combinatorial tumour antigens.<sup>(p87)</sup>

An additional therapeutic universe is realized through using iPSCs as a cellular chassis; it is now feasible to generate renewable patient-specific immune cells (e.g., T cells, NK cells, and macrophages) that can be edited using synNotch constructs. The iPSC-derived immune cells can be used to develop autologous or off-the-shelf immunotherapies with improved durability and reduced immunogenicity. Also, the pluripotency of iPSCs allows preclinical testing and repetitive optimization of synNotch functionality across different immune lineages before translation to the clinic.<sup>(p88)</sup> When used together with 3D patient-derived tumouroid models, synNotch technology provides a biologically relevant system for preclinical validation. Tumouroids preserve heterogeneous antigen expression, stromal interactions, and other molecular and architectural aspects of the original tumour. Within an engineered TME, tumouroids offer high-throughput circuit functionality screening, functional longevity testing, and off-target effects upon co-culture with synNotch-engineered immune cells. The approach is also valuable in the establishment of patient-selective treatment thresholds and in the optimization of ligand-receptor designs.<sup>(p89)</sup>

Personalized immunotherapy is the last therapeutic frontier, and synNotch circuits have the modularity required to tailor cellular therapies to the specific qualities of each tumour. SynNotch receptors can be custom-made to recognize specific neoantigen profiles, dynamic TME signatures, or immunosuppressive checkpoints via the use of genomic, transcriptomic, and proteomic data.<sup>(p64)</sup> Such flexibility is required to overcome tumour evolution, immune evasion, and interpatient heterogeneity. Also, using machine learning to augment the synNotch logic circuit via *in silico* modelling can accelerate the design process, and therapeutic outcome predictions can be more accurate. Combining synNotch therapies with genome editing technologies, renewable cell sources, advanced tumour modelling, and systems-level precision medicine is a prospect for the future. The combination strategies can potentially transform the field of personalized cancer therapy by creating next-generation immunotherapies with an unimaginable degree of control, efficacy, and safety.<sup>(p1),(p44)</sup>

## Conclusion

The design of synNotch receptors has now emerged as a game-changer in cancer immunotherapy, offering a level of cellular programmability and specificity that surpasses that of the established methods, such as CAR and TCR platforms. SynNotch systems enable a context-dependent therapeutic response in engineered immune cells by allowing spatially and temporally regulated gene expression, mediated by ligand-induced proteolytic cleavage and transcriptional activation. This modularity, in combination with the capability to construct Boolean logic-gated circuits (AND, OR, and NOT), would allow synthetic biology frameworks to distinguish between cancerous cells and healthy tissue with unprecedented specificity, minimizing off-target effects and increasing safety profiles, particularly in solid TMEs, which are defined by immunological evasion and heterogeneity.

This review has discussed the molecular structure of synNotch receptors and has focused on how these receptors utilize the shrewd assembly of TMD custom intracellular effectors and specialized extracellular sensing modules to generate highly tuneable signalling outputs. Orthogonal input-output designs, transcriptional circuit stacking, ligand-receptor affinity tuning, and sensitivity modulation are engineering advances that have broadened the space of functions of synNotch systems. These developments have enabled applications in TME reprogramming, intratumoural diagnostics, conditional cytokine release, and logic-gated CAR-T cells, and all have demonstrated high efficacy in preclinical cancer models.

Despite these developments, several translational obstacles remain. Such potential problems as the limited lifetime of response, *in vivo* ligand presentation, possible leakage of signalling, and the challenge of presenting the modified receptors in therapeutically relevant formats need to be addressed with care. Furthermore, regulatory validation, scalable manufacturing, and integration of the synNotch structure into clinically approved cell therapy platforms require further optimization. These issues have been solved promisingly recently with *ex vivo* gene editing, nanoparticle-mediated delivery, and synthetic promoter engineering.

It is anticipated that synNotch technology, in combination with advanced tools such as patient-derived tumouroid models, iPSCs, and CRISPR-based genome editing, will enable the development of next-generation, patient-specific immunotherapies with higher specificity and flexibility. Interdisciplinary synergies between synthetic biology and immunoengineering, materials science, and nanomedicine will be needed to overcome the current challenges and translate synNotch circuits from the bench to the bedside. SynNotch receptors could transform the field of programmable cell treatments, and their ability to execute complex decision-making processes within engineered immune cells offers an attractive platform for neutralizing the traditional limitations of immunotherapies. The future of synNotch-based systems is auspicious; as the field matures, we could see the development of more personalized, safer, and more effective cancer therapies that can adapt to the complicated and ever-changing nature of solid tumours.

**CRedit authorship contribution statement**

**Amol D. Gholap:** Writing – original draft, Software, Conceptualization. **Jai R. Vengurlekar:** Writing – original draft, Software. **Navnath T. Hatvate:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Nanasaheb D.**

**Thorat:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation, Conceptualization.

**Data availability**

No data was used for the research described in the article.

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