Rethinking cancer targeting strategies in the era of smart cell therapeutics

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Abstract | In the past several decades, the development of cancer therapeutics has largely focused on precision targeting of single cancer-associated molecules. Despite great advances, such targeted therapies still show incomplete precision and the eventual development of resistance due to target heterogeneity or mutation. However, the recent development of cell-based therapies such as chimeric antigen receptor (CAR) T cells presents a revolutionary opportunity to reframe strategies for targeting cancers. Immune cells equipped with synthetic circuits are essentially living computers that can be programmed to recognize tumours based on multiple signals, including both tumour cell-intrinsic and microenvironmental. Moreover, cells can be programmed to launch broad but highly localized therapeutic responses that can limit the potential for escape while still maintaining high precision. Although these emerging smart cell engineering capabilities have yet to be fully implemented in the clinic, we argue here that they will become much more powerful when combined with machine learning analysis of genomic data, which can guide the design of therapeutic recognition programs that are the most discriminatory and actionable. The merging of cancer analytics and synthetic biology could lead to nuanced paradigms of tumour recognition, more akin to facial recognition, that have the ability to more effectively address the complex challenges of treating cancer.

Drug development in oncology has long been influenced by Paul Ehrlich's hundred-year-old concept of a therapeutic magic bullet that can precisely target a unique and critical feature of a tumour. The era of cancer genomics has identified a host of mutant or overexpressed oncogenes, the protein products of which are potential molecular targets for such magic bullet drugs. Remarkable advances have been made in targeting these proteins with small molecules[1], antibodies[2] and even retargeted immune cells[3], leading to significant improvements in the quality of life of many patients with cancer. Nonetheless, safe and truly durable cures remain highly elusive, revealing the inherent limitations of a magic bullet strategy. First, it is increasingly clear that for most cancer types there is no single magic bullet. Cancers are derived from self, and thus any targeted therapy has an inherent risk of cross-reactive toxicity with normal tissues[4,5]. Many oncogenic target proteins are also found in diverse normal tissues and thus, alone, may not provide sufficient discrimination. Second, because cancers are highly heterogeneous and constantly mutating, the emergence of resistance to nearly all targeted therapies via antigenic loss or escape mutations is a near universal reality. For example, targeted kinase inhibitors can lead to active site resistance mutations[6], whereas antibody-directed therapies including chimeric antigen receptor (CAR) T cells can lead to loss or decreased expression of target antigens[7]. Thus, we are faced with a conundrum: cancer therapies need to be more specific, yet at the same time if they are too specific the likelihood of escape and development of resistance increases. How might we navigate this inherent dilemma to develop more effective, safe and durable therapies?

In this Perspective, we argue that engineered cell therapies offer a revolutionary new way to navigate the inherent complexities of cancer targeting. These emerging new therapies can be programmed to execute more sophisticated sensing and response actions: they can recognize multifaceted features of cancer cells and the tumour microenvironment, and they can be programmed to launch more complex, multi-tiered therapeutic actions. Thus, perhaps a better inspiration for a new cancer targeting strategy is that of facial recognition, where sophisticated algorithms combined with machine learning approaches can lead to remarkably precise identification (FIG. 1). Below we envision how emerging platforms in cell engineering and synthetic biology, combined with state-of-the-art computational analysis of genomic data, might lead to far more effective cancer targeting algorithms that strategically navigate the inherent conundrums outlined above. We focus here on the platform of engineered immune cells, but also discuss how some of these new capabilities are possible with newer, more sophisticated molecular platforms, such as antibody-based bispecific and multi-specific engagers.

Facial recognition algorithms

Facial recognition, as well as other computer vision tasks, plays an increasingly important role in our day-to-day lives. Here, we argue that facial recognition offers a new updated analogy that is very different from how we currently think about cancer recognition (FIG. 1). Moreover, engineered cell therapies are uniquely poised to utilize this kind of new approach. Key features of facial recognition algorithms that provide a conceptual framework for cancer recognition are described below.

- Multifaceted pattern recognition. Rather than focus on any single standout feature, facial recognition takes advantage of multiple features and their relationships. Identification is the result of an algorithm that recognizes informative patterns of data. By analogy, cancer targeting could, in principle, be significantly improved by recognizing patterns of molecular

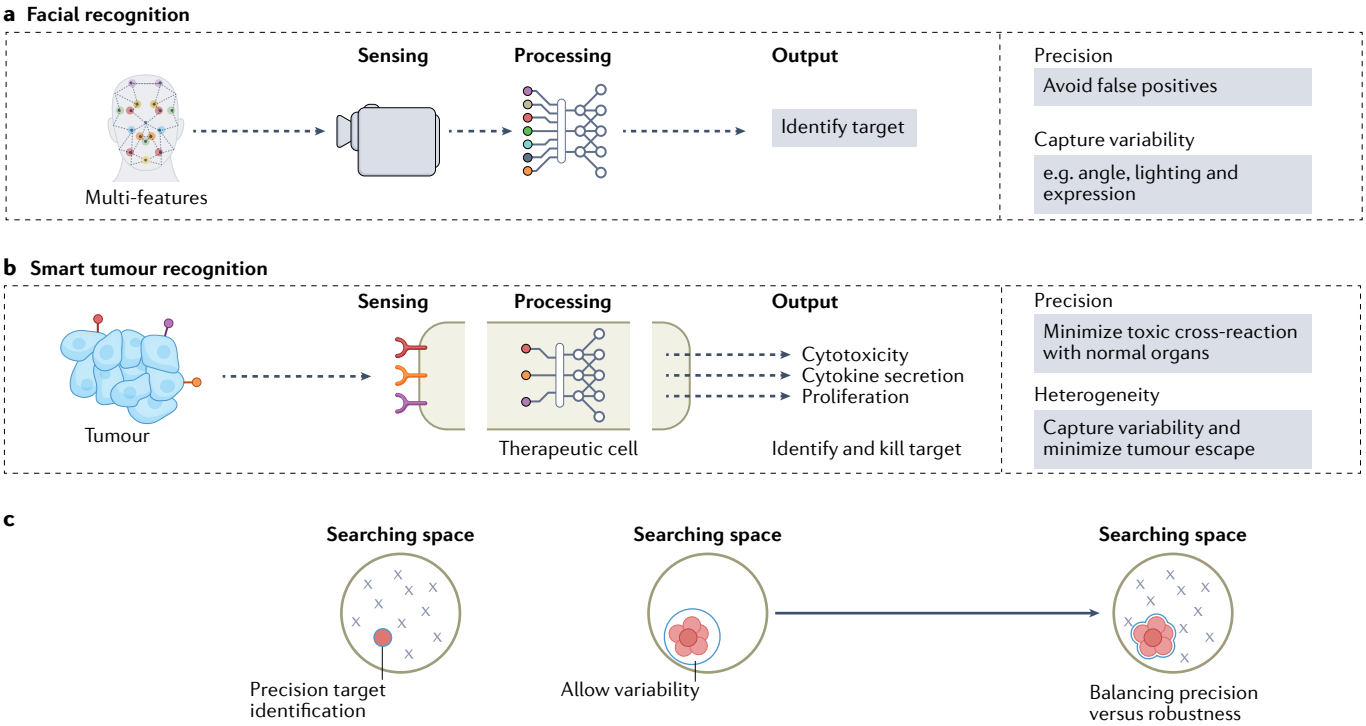

Fig. 1 | **Facial recognition as inspiration for smarter tumour recognition.** **a** | Facial recognition algorithms utilize the relationship between multiple parameters to identify a targeted individual. Machine learning allows training of algorithms to best discriminate against the population pool, and to have the flexibility to recognize an individual from different viewpoints or with different lighting or facial expressions. This paradigm of a multi-input recognition program that must balance precision and heterogeneity offers a road map for the future of cell-engineered recognition programs. **b** | Next-generation cell therapy designs can be built to incorporate this same type of information processing to balance the precise recognition of tumour cells while overcoming potential heterogeneity in target antigens. **c** | Balancing specificity with flexibility and robustness in any type of complex recognition problem.

features present in tumours, rather than any single molecule. Ideally, pattern recognition could encompass key sets of antigens present in the tumour, as well as other microenvironmental signals. Whereas most chemical drugs can only recognize one molecular species, the increasingly sophisticated toolbox of cell engineering, with its novel receptors and multi-input gating circuits, provides one of the only platforms with the potential to achieve multifaceted tumour recognition.

- Maximizing discrimination from the relevant population. Effective facial recognition algorithms are also trained using machine learning to identify particular features or profiles that make an individual stand out relative to known control population databases. By analogy, strategies for targeting cancer could be trained and evaluated on cancer and normal tissue databases, in order to identify those patterns that best discriminate the tumours from the relevant normal tissues.
- Robustness to variability. Facial recognition algorithms would be useless if they were unable to identify a target individual because of variations in the angle of view, lighting or facial expression. The algorithms must therefore be designed to be robust to these common types of variation. By the same token, ideal cancer targeting approaches should take into account the most likely mechanisms of tumour variability and escape, in order to design strategies that cast a therapeutic net that is wide enough to capture this variability. Cell therapies, unlike small molecules, can break down disease sensing and the execution of therapeutic action into distinct steps. Thus, they present the unique opportunity to use high-specificity circuits to determine whether the cell is in the tumour, and then use these factors to trigger the highly localized launch of a broader (less specific) killing response.

Below we describe in more detail how therapeutic cells could be designed to accomplish these three critical behaviours.

Improving precision

To achieve a more holistic recognition program for cancer, newer more sophisticated therapies that can incorporate multiple data points must be created. Fortuitously, the fields of synthetic biology, protein design and cell engineering have recently enabled the development of new smarter therapeutic platforms that can be designed to recognize combinatorial features of tumour microenvironments and cancer cells.

Cell therapies with multi-antigen recognition programs. Engineered cell therapies, in which immune cells are genetically modified before adoptive transfer into patients, have recently come of age with US Food and Drug Administration (FDA) approval of CD19-targeted[3] and B cell maturation antigen (BCMA)-targeted[8] CAR T cells. CARs are synthetic receptors that combine the cytotoxic activity of the T cell receptor with the targeting capability of a monoclonal antibody to recognize specific tumour-associated cell surface antigens[9]. The current approved CAR T cells all target a single antigen. Although they have been successfully employed in the treatment of B cell and plasma cell malignancies, their widespread adoption in broader cancer types, particularly solid tumours, has been significantly constrained by the limited capabilities of single antigens to accurately discriminate most tumours from normal

tissue. Even the approved CD19 CAR T cells are poor at discrimination: they kill both cancerous and normal B cells, a cross-reaction that is tolerable only because patients can survive with B cell aplasia[10].

We propose that current single-antigen CAR T cell designs represent only the beginnings of a far greater range of engineered cell therapies. Cells are a living drug capable of advanced information processing tasks that allow the construction of complex target recognition programs that were previously impossible with conventional drugs, small molecules and antibody-based therapies. Recently developed state-of-the-art recognition programs can incorporate information from multiple antigens using combinations of AND or NOT gates to precisely identify target cells and avoid normal tissue (FIG. 2).

The earliest multi-antigen combinatorial designs were based on splitting the CAR with the cytotoxic CD3ζ receptor under the control of one antigen and additional co-stimulatory or cytokine signals under the control of a second or third antigen[11–13]. These designs would only engage the full effects of CAR T cell activation, cytotoxicity and proliferation in the presence of all antigens. A key limitation in these split designs is that the CD3ζ receptor on its own can induce target cell killing, although with weaker in vivo activity. Thus, they did not effectively address the issue of avoiding toxic cross-reaction with normal tissues.

More recently, the development of synthetic Notch receptors (synNotch)[14]

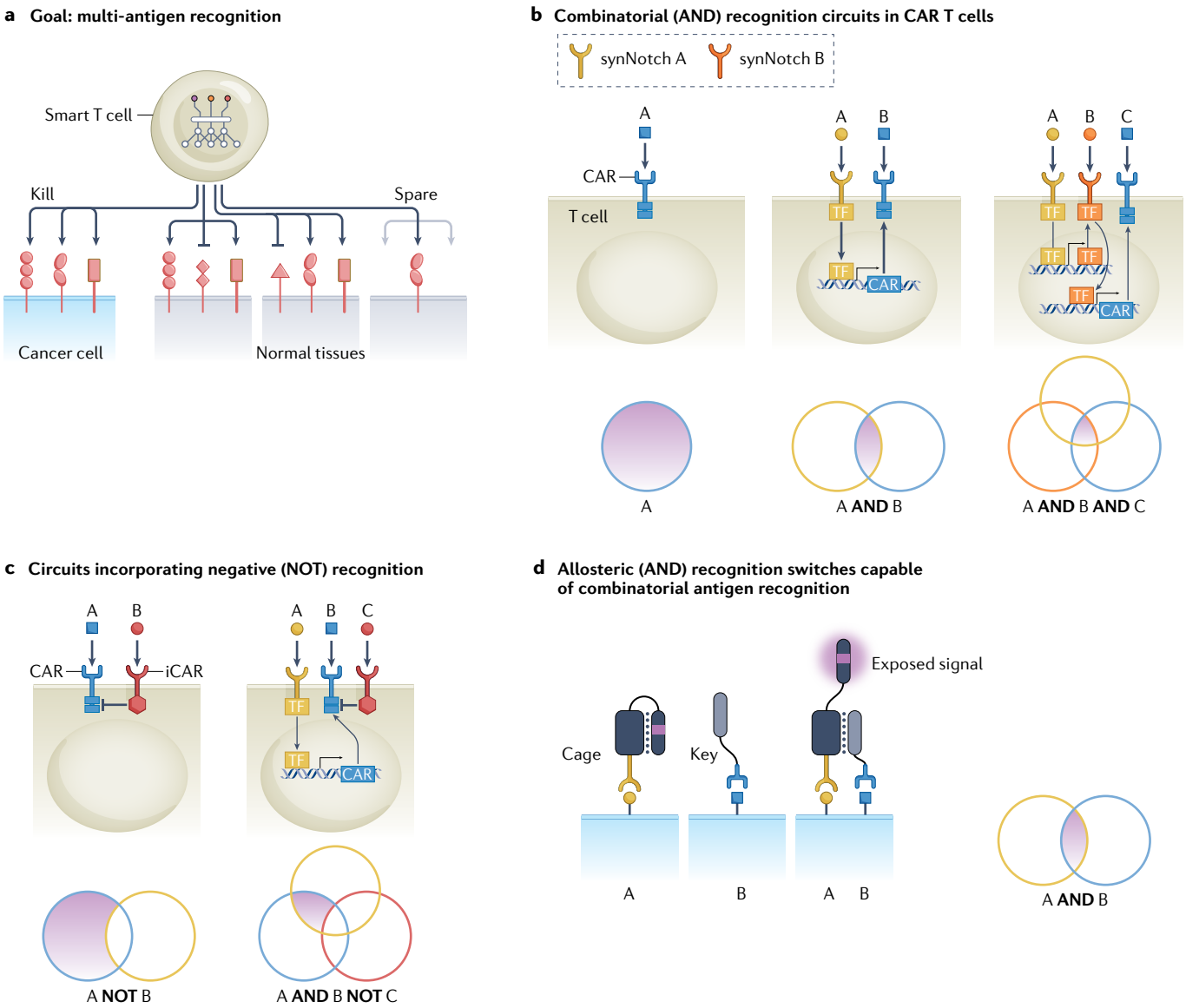

Fig. 2 | **Emerging capabilities of engineering multi-antigen recognition circuits in CAR T cells. a** | Multi-antigen recognition programs could lead to much more discriminatory chimeric antigen receptor (CAR) T cells. **b** | Examples of AND gate circuits that can be engineered by linking synthetic Notch receptors (synNotch) and CAR receptors. SynNotch-controlled expression of a CAR (synNotch → CAR) requires the presence of two different antigens (A and B) to initiate killing. Three input AND gates can be created by a cascade circuit in which synNotch A induces synNotch B, which induces CAR C. Here, killing requires three antigens (A and B and C) to be present. **c** | NOT gates in CAR T cells can be created using antigen-driven recruitment of a suppressor of T cell activation either opposing a CAR (A not B) or opposing a synNotch-driven CAR circuit (A and B not C). **d** | Next-generation macromolecule therapies have also been developed that can accomplish precision AND gating using targeted recruitment of Cage and Key proteins to the membrane to expose a CAR T cell antigen target. Only when Cage and Key moieties are adjacent on the same cell will the killing signal be exposed. iCAR, inhibitory CAR; TF, transcription factor.

to control CAR expression has enabled a new class of combinatorial targeted cell therapies (known as synNotch → CAR circuits). synNotch receptors are a novel cell-sensing receptor platform that allow a T cell to be programmed to sense an antigen of interest, and, in response, to express a genetically encoded payload of choice. Based on the Notch receptor, synNotch receptors have an extracellular recognition domain (for example, a single-chain variable fragment (scFv)) and an intracellular transcription factor (TF) domain. The transmembrane region of Notch undergoes proteolytic cleavage upon engagement of a ligand, leading to release of the synthetic intracellular transcriptional domain, whereupon it can enter the nucleus and drive expression of a payload from a responsive promoter.

In the context of a therapeutic T cell, a synNotch receptor becomes very powerful when used to control expression of a CAR: in this case, synNotch acts as an if–then operator where a priming antigen must first trigger synNotch-mediated expression of a CAR, which targets a second killing antigen[15]. Thus, T cells with a synNotch → CAR circuit must simultaneously encounter both priming and killing antigens in order to launch a cytotoxic response. Dual antigen AND gated control of CAR T cell activity with synNotch → CAR circuits has been shown to avoid potential on-target but off-tumour toxicity seen with conventional anti-ROR1 CAR T cells in mouse models of breast cancer[16].

Critically, the flexible and modular nature of synNotch-based transcriptional circuits allows complex antigen recognition programs to be designed by daisy-chaining together multiple synNotch components in series[17]. For example, three input AND gated cells can be engineered by having synNotch A induce expression of synNotch B, which in turn induces CAR expression (synNotch A → synNotch B → CAR C). Theoretically, the only limitations to recognition program size arise from the kinetics of transcriptional circuitry and the ability to introduce multi-receptor payloads into primary immune cells. Nonetheless, two to three component circuits are within the limits of current lentiviral transfection payloads.

Similarly, CAR circuits have been designed that use NOT logic — in these cases, CAR killing of a cell can be aborted by recognition of a negative antigen (for example, an antigen that is NOT expressed in cancer but is expressed in a potentially cross-reactive normal tissue). One of the first NOT gate designs, known as the inhibitory CAR (iCAR) receptor, regulates the recruitment of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) to the CAR immune synapse by the NOT antigen[18–20]. Although these iCAR designs have been successfully used in several preclinical models, they require careful balancing of the expression levels of the NOT receptor and CAR receptor as well as relatively high expression levels of the NOT antigen to fully inhibit CAR T cell killing. More recent work has also shown that synNotch induction of cell death or immune inhibitory proteins can also potentially act as a modular NOT gate that can be incorporated into multi-antigen recognition circuits that integrate both positive and negative selection[17]. Here, antigen-triggered production of truncated BH3-interacting domain death agonist (tBID) can block T cell proliferation and cytotoxicity when exposed to an off-target tissue.

Overall, the still-growing set of synthetic sensors that control CAR T cell activity can be multiplexed to provide the synthetic biologist with a complete toolbox to build diverse multi-antigen targeted CAR T cells with the potential for future clinical translation (FIG. 2b,c).

Protein therapies with multi-antigen recognition activity. Similar to the first generation of CAR T cells, initial monoclonal antibody therapeutics were designed to target a single antigen only. Although lacking the information processing potential of cellular therapies, advances in protein engineering have allowed for somewhat more complex control mechanisms. These include bispecific or trispecific antibodies with engineered target selectivity[21,22], which have shown clinical promise with favourable efficacy and reduced toxicity[23,24]. Alternative antibody engineering strategies include controlled access to antibody binding domains through enzyme-gated antibodies[25].

More recently, modular AND and NOT logic gating at the protein level has also been engineered using co-localization-induced protein switches (known as Colocalization-dependent Latching Orthogonal Cage/Key pRoteins (Co-LOCKR))[26] (FIG. 2d). Here, one surface antigen can be targeted by an antibody fused to a caged tag (which itself could be the target of a CAR or antibody). Because the tag is hidden, recognition of a cell bearing this single antigen has no impact. However, a second recognition molecule can then be added, which bears an antibody for a second antigen, fused to a key domain that displaces the caged tag on the first recognition molecule. Thus, CAR T cells targeting this tag domain were only active against tumour cells bearing both the first and second antigens. Conversely, NOT logic could be created using decoy cages targeted against the NOT antigen. The in vivo efficacy of these elegant protein switches remains to be tested, especially the robustness to varying concentrations of cage, key and decoy molecules. Nonetheless, these approaches illustrate that there are multiple levels at which multi-input recognition behaviours can be engineered.

Optimal recognition programs

Emerging data sets for cancer recognition. How do we utilize the recognition circuit engineering capabilities outlined above? These emerging therapeutic cell recognition circuits will only be useful when combined with computational analysis of cancer profiling data that identifies the multi-antigen signatures that best discriminate cancer from normal tissues. With the rapid progress in high-throughput tools to analyse genomic and proteomic data from primary patient specimens, a much clearer picture of how to best discriminate tumour from normal tissue is beginning to emerge. This rich data set includes both mutational profiling to identify neoantigens and expression profiling to identify tumour-associated antigens (potential therapeutic target proteins that are overexpressed in cancer). Efforts to build therapies to recognize neoantigens presented by major histocompatibility complex (MHC) molecules must overcome enormous technical challenges in peptide–MHC prediction algorithms[27,28], and the development of peptide–MHC-based recognition tools[29,30], with the 'private' nature of most mutational profiles requiring an almost impossibly bespoke therapy for each patient.

By contrast, tumour-associated antigens, particularly those found on the cell surface, are readily actionable 'public' antigens. These antigens are the targets of monoclonal antibodies, bispecific antibodies and CAR T cell therapies used in current clinical practice. But these public antigens come with the major cost of increased risk for toxic cross-reactivity with normal tissue. Early in the development of these therapies, target antigens were often selected based on limited data incorporating expert opinion rather than an unbiased examination of tumour and normal tissue expression. To help fill in this data gap a tremendous amount of

high-throughput RNA sequencing analysis of gene expression in tumours has been performed. The most prominent of these data sources is The Cancer Genome Atlas (TCGA), which provides a publicly available data set from 20,000 samples across 33 different cancer types[31]. Complementing expression profiling, high-throughput proteomics analysis of tumour tissue is now available including immunohistochemistry analysis[32] and mass spectrometry analysis of the surfaceome (proteins found on the plasma membrane surface)[33].

Most of the existing resources for antigen expression are from tumour specimens but, importantly, several efforts have been focused on normal human tissue antigen expression. Notably, this includes the Genotype-Tissue Expression (GTEx) project[34], which sampled 1,000 human individuals at 54 non-diseased tissue sites. The GTEx data set is also complemented by the Human Protein Atlas (HPA), which contains expression profiling from 95 human individuals representing 27 different tissues[35]. There are also ongoing projects to use single-cell transcriptomics to profile both normal mouse[36] and human tissue[37,38]. As we expand on below, improving the quality and comprehensiveness of normal tissue profiling data is an area of critical need.

Computationally identifying discriminatory recognition signatures. Systematic analysis of any potential single antigen target for a CAR or antibody-based therapy quickly highlights the poor overall discrimination that many of these tumour-associated antigens offer. For example, prostate-specific membrane antigen (PSMA; also known as FOLH1) has been under active investigation as a target of bispecific T cell engagers (BiTEs)[39] and CAR T cells[40] in prostate cancer. PSMA is highly overexpressed in prostate cancers, and has been a long-time target for drug design. However, significant expression of PSMA is clearly seen in several normal tissues by both immunohistochemistry staining[32] (FIG. 3a) and RNA expression profiling[41], albeit at lower levels than in prostate cancer. As expected, early-phase trials of CAR T cells targeting solid tumours with single antigen specificity have been limited by this type of on-target but off-tumour toxicity[4,42]. Fortunately, with our new ability to engineer multi-antigen recognition programs into T cells, we can now try to harness bioinformatic analysis of tumour and normal tissue to guide therapeutic program design choices.

Several groups have begun this process, leveraging the large bioinformatic data sets on tumour and normal tissue antigen expression to predict potentially useful antigen combinations in the recognition of specific cancer types. As an example, when targeting acute myeloid leukaemia (AML) there has been a significant challenge in discriminating AML cells from normal myeloid haematopoietic stem or progenitor cells. A direct comparison of transcriptomic and proteomic data between leukaemia stem cells and $CD45^{+}CD38^{-}$ haematopoietic cells[43] did not find a single antigen that clearly distinguishes these healthy and malignant cell types. However, several antigen pairs were predicted to more efficiently distinguish the two tissues. In 2021 a more comprehensive pan-cancer surfaceome atlas was generated[44], which integrated multiple bioinformatic resources to predict the identity of membrane proteins and then used RNA expression profiling from TCGA and GTEx to predict potentially useful AND and NOT gates. In this work, AND gates were defined as having mutually exclusive patterns of expression in normal tissue, with a relatively small number of antigen pairs identified.

A more complete examination of the potential two-antigen and three-antigen combinatorial space was performed by Dannenfelser et al.[45]. Here, RNA sequencing data from GTEx and TCGA were analysed using a spatial clustering algorithm to identify synergistic combinations of antigens using either AND or NOT logic (FIG. 3b). For each potential surface antigen pair or triplet across each cancer type in TCGA, a clustering-based score was calculated based on the average distance between tumour and normal tissue expression space and the overall distribution of samples in the two-dimensional or three-dimensional antigen space (FIG. 3c). High-scoring antigen pairs or triplets were then analysed using machine learning decision tree classifiers to calculate the precision (potential for off-target toxicity) and recall (fraction of tumour samples detected) of the antigen pair. A summary of the ability of an antigen, an antigen pair or a set of three antigens connected by AND or NOT operators to discriminate tumour from normal tissue was encapsulated in the F1 score (harmonic mean of precision and recall). This work found that as the number of antigens used in detection was increased, the ability to discriminate tumour from normal tissue also increased, with discrimination approaching optimal with three antigen classifiers (FIG. 3d). In fact, for all cancers within TCGA, combinatorial antigen detection dramatically increased the precision and recall of cancer discrimination from normal tissue when compared with the best possible single antigen target.

In summary, these computational analyses indicate that there is remarkable promise in harnessing multi-antigen recognition of cancer. For all cancer types, two-antigen or three-antigen recognition signatures appear to be sufficient to lead to near ideal discrimination from normal tissues. Moreover, for each cancer type, there are also many potential options — tens to hundreds of different two-antigen or three-antigen signatures are predicted to potentially show effective discrimination. Nonetheless, the applicability of these computational analyses is limited by the quality and extensiveness of the current tumour and tissue profiling data, which, as will be discussed later, could be significantly improved.

Engineering balanced recognition

Although great progress is being made in designing therapeutic cells that show much higher specificity, these still face the inherent dilemma of retaining sufficient flexibility to overcome tumour escape via mutation or reduced expression of the targeted antigen. As analysis of tumours at single-cell resolution improves, it has become readily apparent that a tumour is not a homogeneous tissue[46]. This heterogeneity in target antigen expression is a major driver of resistance to small-molecule kinase inhibitors[47], monoclonal antibodies[48] and CAR T cell therapies[49,50]. Below we describe innovative ways in which smart cell circuits can be designed to balance specificity and robustness to variation, an arena in which cell-based therapeutics has the potential to vastly outperform other therapeutic platforms.

Development of more flexible OR gate CAR T cells. Several advances in protein and cell design have been developed to help overcome target antigen heterogeneity and create therapies with a more flexible and/or broader recognition strategy. The most straightforward of these is the implementation of OR gates where therapies are able to trigger a cytotoxic response against two or more target antigens. At the level of protein engineering, there has been an explosive growth in the development of polyspecific monoclonal antibodies[22]. Although the majority of these molecules have been designed as immune cell engagers (to trigger T cell-mediated or natural killer cell-mediated killing), combinatorial OR gate detection mechanisms have been developed that, for instance, target human epidermal growth factor receptor 2 (HER2; also known

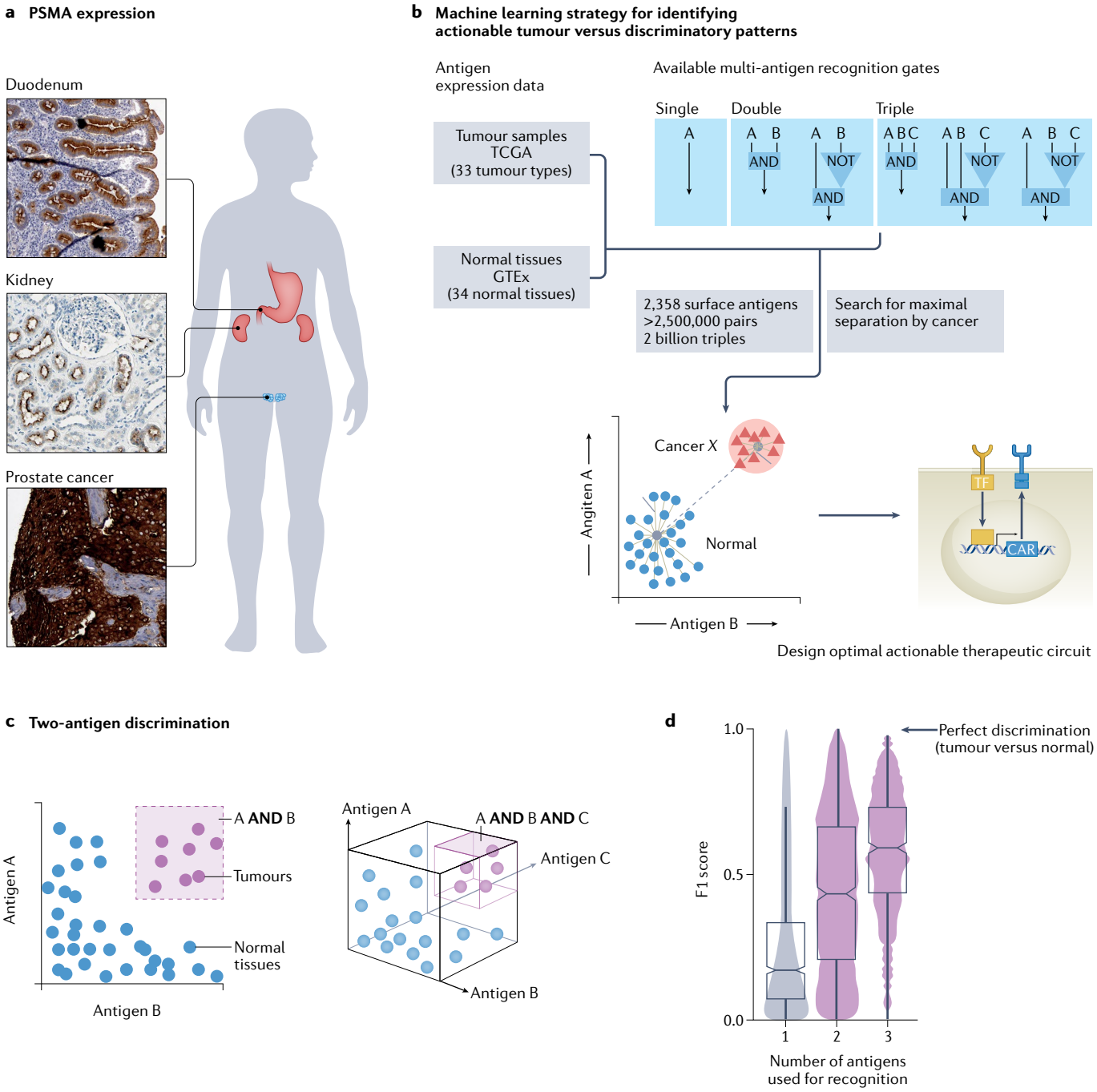

Fig. 3 | **Computational analysis of tumour profiling data to identify optimal tumour versus normal tissue discrimination circuits. a** | Prostate-specific membrane antigen (PSMA) is a canonical antigen targeted by chimeric antigen receptor (CAR) T cells and bispecific T cell engagers (BiTEs). However, it has clear evidence of expression in normal tissue (images available from v21 of the Human Protein Atlas[41]). **b** | Machine learning strategies can be employed to profile the combinatorial antigen space to identify optimal two-antigen or three-antigen gates that best separate specific tumour types from normal tissue[45]. **c** | Expanding recognition into two-dimensional or three-dimensional antigen spaces increases the ability to find ways to separate tumour versus normal cells. **d** | Analysis of all high-performing potential single-antigen, double-antigen or triple-antigen gates across each individual cancer type shows that as increasing numbers of antigens are used to identify tumour cells, the ability to discriminate tumour from normal tissue starts to approach complete precision. Here, F1 score is the harmonic mean of precision and recall of cancer versus normal tissue discrimination. Overall, two to three antigens are predicted to lead to dramatic improvement in recognition over single antigen therapies. GTEx, Genotype-Tissue Expression; TCGA, The Cancer Genome Atlas; TF, transcription factor. PMSA staining in duodenum. Image credit: Human Protein Atlas, www.proteinatlas.org[32]. Image available at the following URL: https://www.proteinatlas.org/ENSG00000086205-FOLH1/tissue/duodenum#. PMSA staining in kidney. Image credit: Human Protein Atlas, www.proteinatlas.org[32]. Image available at the following URL: https://www.proteinatlas.org/ENSG00000086205-FOLH1/tissue/kidney#. PMSA staining in prostate cancer. Image credit: Human Protein Atlas, www.proteinatlas.org[32]. Image available at the following URL: https://www.proteinatlas.org/ENSG00000086205-FOLH1/pathology/prostate+cancer#. Parts **b**, **c** and **d** adapted with permission from REF.[45], Elsevier.

as ERBB2) OR HER3 (REF.[51]). In the field of cell therapy there has been progress in the construction of multicistronic[52,53] (expression of multiple CARs from a single viral vector) and multivalent or tandem CARs (one CAR with multiple antigen recognition motifs)[54–56] (FIG. 4a). Tandem CAR designs have been favoured as they are more compact and do not require alternative codon usage to avoid recombination, although they often require optimization of linkers and binders[55,57] to avoid misfolding and tonic signalling[54,58]. These tandem designs have had some success in clinical trials, such as tandem CAR T cells targeting CD19 OR CD20, which were found to reduce the risk of antigen-negative recurrence in non-Hodgkin lymphoma[59]; although tandem designs for CD19 OR CD22 have been more challenging[57,60]. Another potential tool for implementation of OR gated cell therapies is the use of a fixed CAR design with the administration of a flexible set of adapter recognition molecules[61]. This split, universal and programmable (SUPRA)-CAR design features a single CAR with a leucine zipper extracellular domain that can bind to multiple different adapters (scFvs fused to a cognate leucine zipper domain).

Two-step prime and kill strategies. Although OR gating systems are proven tools to overcome antigen heterogeneity, they all sacrifice specificity in the name of improved sensitivity. For example, if an OR gate is made involving two different target antigens, then the potential off-target cross-reactivity will be the sum of the two individual antigens. In short, OR gates alone will in many cases dramatically increase the potential for toxicity. Fortunately, innovative new concepts in recognition are emerging that utilize the unique information processing power of engineered therapies in multiple ways.

One general emerging solution is to design therapies that execute their actions in two independently tunable steps — the first can focus on maximizing specificity of recognition, whereas the second can focus on completeness of killing (FIG. 4).

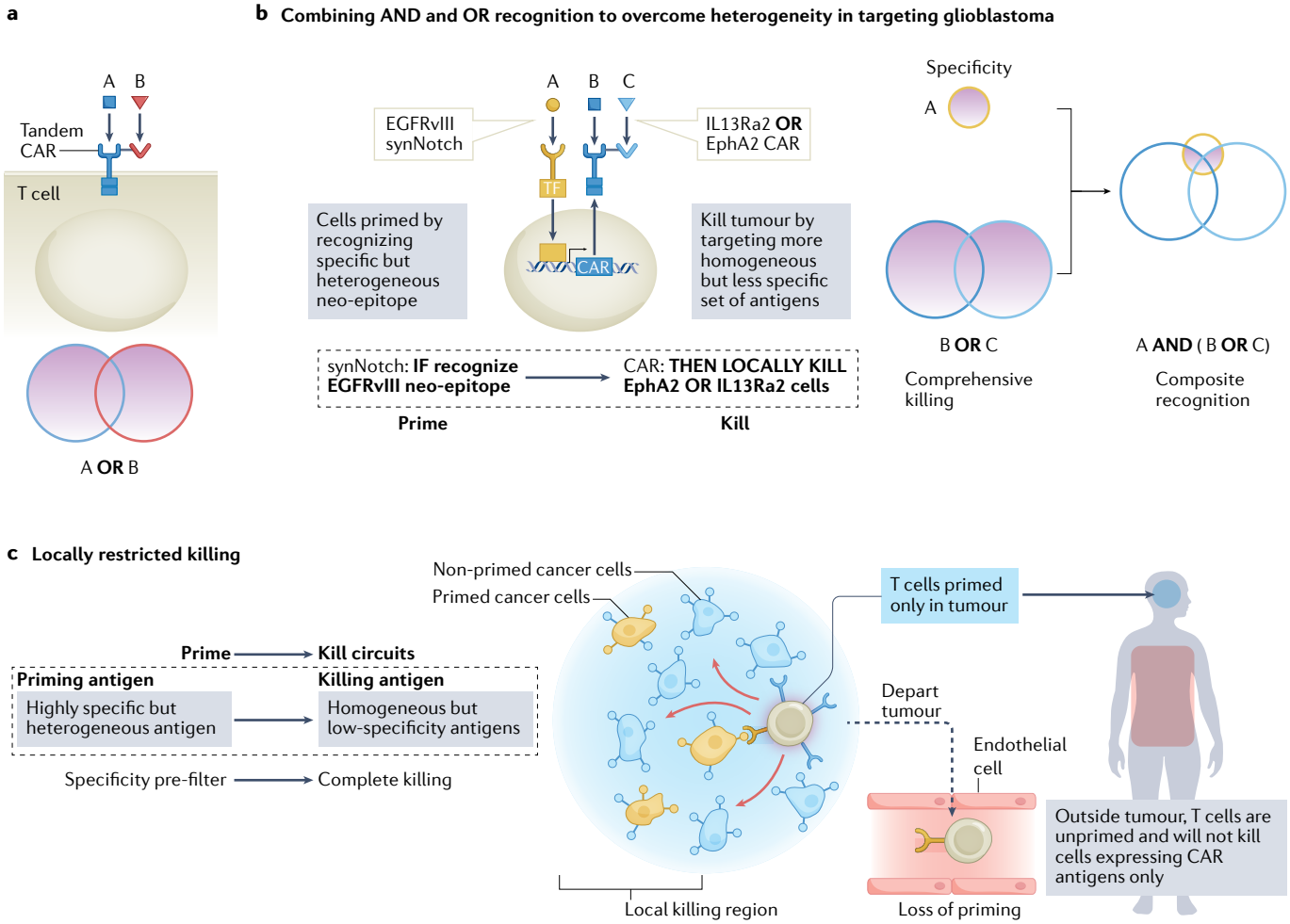

Fig. 4 | **Navigating precision and robustness to escape using nuanced combinatorial recognition circuits. a** | OR gating strategies to overcome target antigen heterogeneity allow triggering of an antibody or a chimeric antigen receptor (CAR) using any of two or more potential binding moieties. OR gates can decrease the potential for tumour escape, but can also increase off-tumour toxicity. **b** | AND gates can be combined with OR gates using synthetic Notch receptors (synNotch) to drive expression of a tandem CAR. In the case of an anti-glioblastoma therapy, the circuit was designed to be primed by synNotch recognition of a tumour-specific but heterogeneous neoepitope, epidermal growth factor receptor vIII (EGFRvIII), but to then launch killing using a tandem CAR that recognizes the more homogeneous set of antigens, ephrin A2 (EphA2) and interleukin-13 receptor α2 (IL-13Rα2). These killing antigens lack perfect specificity (they are expressed outside the brain in normal tissues), but as a composite circuit they are locally constrained by the specificity of EGFRvIII. **c** | This general strategy of locally priming therapeutic activity within the region of a tumour can be used to balance precise recognition programs with local antigen heterogeneity. TF, transcription factor. Reprinted with permission of AAAS from Choe et al., *Sci. Transl Med.* **13**, eabe7378 (2021).

This principle, for example, is harnessed at the protein therapeutics level by antibody–drug conjugates (ADCs). With ADCs, monoclonal antibodies are conjugated to membrane-permeable toxins wherein the antibodies are cleaved after intracellular uptake. For instance, trastuzumab–MMAF (monomethyl auristatin F) is a fusion of a microtubule inhibitor to a HER2 monoclonal antibody with a linker that is designed to be cleaved after intracellular uptake. Importantly, the HER2 antibody ideally targets the agent to the tumour, whereas the released toxin is then able to kill a broader range of neighbouring bystander cells (including HER2-negative cells) producing enhanced cytotoxicity against trastuzumab-resistant tumours or those with non-uniform overexpression of HER2 (REF.[62]).

Cellular therapies, with their ability to execute user-defined decision-making functions, are particularly suited to this type of multistep approach of using heterogeneous markers to identify tumour tissue and deploy a more homogeneous effector activity (FIG. 4b). Glioblastoma offers a particularly informative test case. A subset of patients with glioblastoma have a highly tumour-specific truncated isoform of epidermal growth factor receptor (EGFR), known as EGFRvIII. Although the EGFRvIII epitope might seem an ideal CAR antigen, it is highly heterogeneous (expressed in 35–85% of tumour cells)[63]. Consistent with this high heterogeneity, a phase I clinical trial showed that CAR T cells targeting EGFRvIII were able to traffic to the tumour, induce an interferon response and reduce EGFRvIII expression but had negligible clinical activity because of the escape and outgrowth of EGFRvIII-negative tumour cells[64]. Several groups have developed alternative strategies that attempt to harness the high tumour specificity of EGFRvIII to then deliver a more potent but less specific (and potentially more toxic) payload. Choi et al.[65] supplemented anti-EGFRvIII CAR T cells with the expression of a BiTE targeting wild-type EGFR. Wild-type EGFR is homogeneously overexpressed in glioblastoma, but also present on other normal tissues outside the brain. Intracranial administration of anti-EGFRvIII CAR T cells secreting an EGFR BiTE was able to overcome EGFRvIII antigen heterogeneity leading to tumour clearance, whereas intravenous administration of these T cells had minimal effect on a normal EGFRvIII-negative human skin tissue graft. The local activation and expansion of the CAR T cells in the targeted glioblastoma tumour is hypothesized to be sufficient to confine EGFR BiTE delivery to the tumour. Nonetheless, constitutive BiTE secretion carries a risk for toxicities from T cells that are still in circulation and have not yet infiltrated into the tumour.

An alternative cell engineering strategy was developed by Choe et al.[66] using synNotch-mediated recognition of tumour-specific but heterogeneous EGFRvIII to prime expression of an anti-interleukin-13 receptor α2 (IL-13Rα2)–ephrin A2 (EphA2) CAR, which subsequently executes killing (FIG. 4b). This therapy has several mechanisms to balance specificity and completeness of killing. First, it relies on EGFRvIII only as a mechanism to locate and identify the tumour, and does not rely on this heterogeneous antigen for completeness of killing. Instead, for killing, the circuit harnesses an OR gate tandem CAR that targets EphA2 and IL-13Rα2, two antigens that are far more homogeneous in glioblastoma, especially when recognized using a broader OR gate mechanism. Importantly, although these two killing antigens are expressed in some normal tissues, they are not normally expressed in the brain. In short, the circuit is designed to be very precise through recognition of the EGFRvIII neoepitope, but then very broad in terms of induced killing.

When these EGFRvIII synNotch → EphA2–IL-13Rα2 CAR T cells were intravenously administered, they were able to autonomously traffic to the brain, locally prime CAR expression only in the tumour and clear glioblastoma xenografts with heterogeneous EGFRvIII expression (50%). This therapy did not disrupt growth of a matching EGFRvIII-negative tumour implanted in the flank of the same animals, highlighting the high local activity of the circuit. Importantly, CAR T cells primed by EGFRvIII-expressing tumour cells were shown to be able to kill neighbouring glioblastoma tumour cells lacking EGFRvIII expression.

In essence, these systems integrate multiple pieces of information, using different antigens for different purposes. Certain sensors focus on highly tumour-specific antigens to mark a region for a cytotoxic response, allowing a high degree of specificity without sacrificing sensitivity. These sensors, in turn, trigger a broader killing response, that is still locally constrained by the upstream specificity sensors. By separating tumour recognition from killing, we can now incorporate features such as geographical targeting: T cell therapies that first recognize signals that tell them they are in the tumour, triggering them to induce CAR or BiTE expression — creating a localized 'killing radius' in and around the tumour (FIG. 4c). Current in vitro studies indicate that CAR activity is limited to a radius of ~100 µm around the priming signal, in the case of synNotch → CAR circuits[67]. Analysis of the synNotch → CAR circuits in preclinical models indicates that once these leave a tumour (the site of the priming antigen) they lose CAR expression within hours[15], thereby minimizing the chance of a strong off-tumour cytotoxic response, which can take days to weeks. Preclinical models have also shown that local priming of CAR expression in one tumour leaves a second distant, priming antigen-negative tumour unaffected[66], although this prime then kill approach is limited in settings of a high burden of circulating tumour cells[16]. These approaches yield strategies that are far more resistant to target antigen heterogeneity, and clearly illustrate how cell therapies are capable of far more nuanced and tunable recognition than most molecular therapeutics. To effectively utilize these strategies, we clearly require information not only about tumour versus normal specificity but also about the heterogeneity and spatial distribution of antigens. Such information will allow us to judiciously pick antigens to target for priming, and antigens to target for killing.

The path forward

The results and concepts summarized here show the promise of smart cell therapeutics to achieve a revolutionary new level of precision recognition of cancer. General principles have emerged about how to strategically design cellular circuits that recognize a particular cancer type with high specificity but with sufficient completeness to avoid escape. Excitingly, some therapies utilizing these approaches are moving forward to clinical trials, where their advantages and shortcomings can be empirically evaluated. Nonetheless, even at this point, numerous challenges remain and we can identify needs in the field to make the design of multi-targeted therapies more reliable and predictable, and these are described below.

Improvement in tumour and normal profiling databases. Although there has been substantial improvement in the data sets available to characterize tumour and normal tissue antigen expression, additional data are still required to help guide design decisions. Likely the most acute need is in

additional profiling of normal tissue gene expression. Overall, there is a significant gap in the amount of data from normal tissue compared with tumour profiling, especially normal tissue that accurately reflects the patient populations of interest. These normal tissue data are critical for deploying machine learning approaches to optimize tumour versus normal discrimination.

The availability of additional profiling data for characterizing tumour expression is also greatly needed. Tools to assess heterogeneity (single-cell RNA sequencing (scRNA-seq) or spatial sequencing) or measurements of protein expression on the surface of cells (surfaceome profiling) remain quite sparsely implemented. To highlight the importance of single-cell sequencing and measurements of heterogeneity, only 30% of overexpressed surface antigens detected by bulk RNA sequencing were found in more than half of individual cells measured by scRNA-seq[44]. In the end, the utility of computationally predicted multi-antigen signatures of cancer, especially those to use for priming versus killing, will only be as useful as the data used to generate these predictions. In addition, more profiling of metastatic tumours will be increasingly important, as many of the samples in TCGA come from earlier stage tumours still in their native environment[68].

Tissue localization signatures. The concepts guiding multi-antigen discrimination of tumour versus normal tissue can also be applied to recognition of specific organs or tissues within a body. In the treatment of cancer this can be used to limit CAR T cell activity to an effected organ in loco-regional disease, for example the brain in the treatment of glioblastoma[66]. Alternatively, it could be applied to turn off CAR T cells in specific tissue compartments where off-target CAR T cell toxicity is most problematic, for example, the lung in the treatment of HER2$^+$ malignancies[4]. Currently, there is no existing bioinformatic guide for multi-antigen circuits to recognize particular tissues with a high level of precision.

Improved cellular gating designs. The current generation of logic gates available in the engineering of immune cells is remarkably robust after what is essentially only a decade of work. However, there remain several cell engineering hurdles to overcome. Robust NOT gating is a particularly challenging design for CAR T cells, as CAR activation drives proliferation selecting for 'cheaters' that bypass CAR inhibition. Effective NOT gating will likely require local paracrine-acting suppressive factors to be effective. In addition, measuring and fine-tuning the amplitude of split receptor designs or the kinetics of synNotch inducible designs to best accommodate clinically relevant tumour antigen burdens and spatial organization remain largely unexplored.

Expanding our tools for genetic modification of primary cells. Although the general toolbox to engineer combinatorial detection programs, particularly for engineered immune cells, has largely been laid out, implementation in clinically actionable designs remains a key hurdle. In general, our ability to rationally design therapies has far outstripped our ability to make these genetic changes in primary immune cells. The primary tools for viral-based genome integration (gammaretrovirus or lentivirus)[69] are limited to delivery of payloads of around 5,000 bp, and the use of multiple viral vectors is limited by FDA limits on copy number integration and efficiency of viral integration. Non-viral transposon vectors such as piggyBac[70] or Sleeping Beauty[71] have larger payload capacities but suffer from poor efficiency, often requiring selectable markers[72]. Random integration and the potential for silencing of gene expression present further cell engineering challenges, which can largely be solved with targeted integration of genetic payloads. Adeno-associated virus (AAV)[73] and DNA template[74,75] delivery mechanisms coupled with CRISPR–Cas9 integration have all been used successfully to integrate CARs; however, current payload limitations with these techniques are even more stringent than with retroviral or lentiviral vectors. Without a significant advance in our ability to make targeted, large-scale integrations into a native genome we will continue to be limited in our ability to clinically implement highly specific combinatorial antigen recognition programs into immune cells. Nonetheless, we remain optimistic that major progress can be made now, given that two-component or three-component cell circuits can be made and the predictions that two-antigen or three-antigen recognition CAR T cells will provide a major improvement over single-antigen CAR T cells.

Conclusions

Progress in unbiased analysis of tumour and normal tissue coupled with advances in protein and cell engineering has paved the way for a new paradigm in cancer recognition. Here, aided by machine learning analysis of expression profiles, engineered therapies can be designed to selectively target the precise combination of antigens that best identify a tumour. By using synthetic circuits that localize CAR T cell killing to specific areas, this enhanced specificity does not necessarily come at the cost of loss of sensitivity.

In order to move this field forward we propose a road map for future work.

- The development of improved bioinformatic data sets that are collected from normal tissues, and not just focused on cancer.
- Increased use of single-cell and spatial analyses that better describe target antigen heterogeneity and allow us to understand the spatial geography of cell types and antigens within tumour and normal tissue.
- Continued growth in the tools and implementation of more advanced cellular and protein designs to engage and recognize tumour ecosystem features in a spatio-temporally precise manner.

We believe that thinking about cancer recognition in terms of a holistic multifaceted program will be a key to tackling the many therapeutic challenges presented by malignancies.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

W.A.L holds equity in Gilead and Intellia, is an adviser for Allogene Therapeutics and has filed patents related to this work. G.M.A. declares no competing interests.

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