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Synthetic biology approaches to engineer T cells

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There is rapidly growing interest in learning how to engineer immune cells, such as T lymphocytes, because of the potential of these engineered cells to be used for therapeutic applications such as the recognition and killing of cancer cells. At the same time, our knowhow and capability to logically engineer cellular behavior is growing rapidly with the development of synthetic biology. Here we describe how synthetic biology approaches are being used to rationally alter the behavior of T cells to optimize them for therapeutic functions. We also describe future developments that will be important in order to construct safe and precise T cell therapeutics.

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Introduction: synthetic biology meets immunology

Cells are capable of remarkably sophisticated behavior. In particular, immune cells exhibit a wide range of characteristics that are well suited for therapeutic applications. Research in cell biology and immunology has focused on dissecting the molecular mechanisms underlying these complex behaviors. However, there is now growing interest in understanding how to engineer immune cells to carry out controlled and redirected natural behavior and new, non-natural behaviors. This shift comes from the convergence of two exciting emerging areas of research. First is the establishment that engineered immune cells can be used as therapeutics to treat cancer or autoimmunity. Second is the development of synthetic biology — a field in which our understanding of molecular regulatory systems has been combined with our increasing ability to genetically modify and edit cellular systems. Thus this is a particularly exciting time: our ability to rationally engineer cells is exponentially growing, as are the potential therapeutic applications of engineered immune cells.

Synthetic biologists seek to understand the design principles of biological systems by dissecting, rebuilding and repurposing natural and synthetic components [1–6]. The biomedical relevance of engineered T cells demonstrated in recent clinical trials is one reason why T cells are emerging as an important model system for synthetic biologists. In adoptive immunotherapy, T cells are isolated from blood, processed *ex vivo*, and re-infused into patients [7••,8,9••] (Figure 1a). Although best known for cancer therapy, the application of engineered T cells includes and is not limited to treating intracellular pathogens and auto-immunity [10,11] (Figure 1b). Remarkably, engineered T cells can safely persist for years *in vivo* [12,13••]. Progress towards allogeneic, universal donor T cells is underway, and so are methods of differentiating induced pluripotent stem cells into T cells [14,15,49]. Both technologies are envisioned to significantly increase the availability of therapeutic T cells.

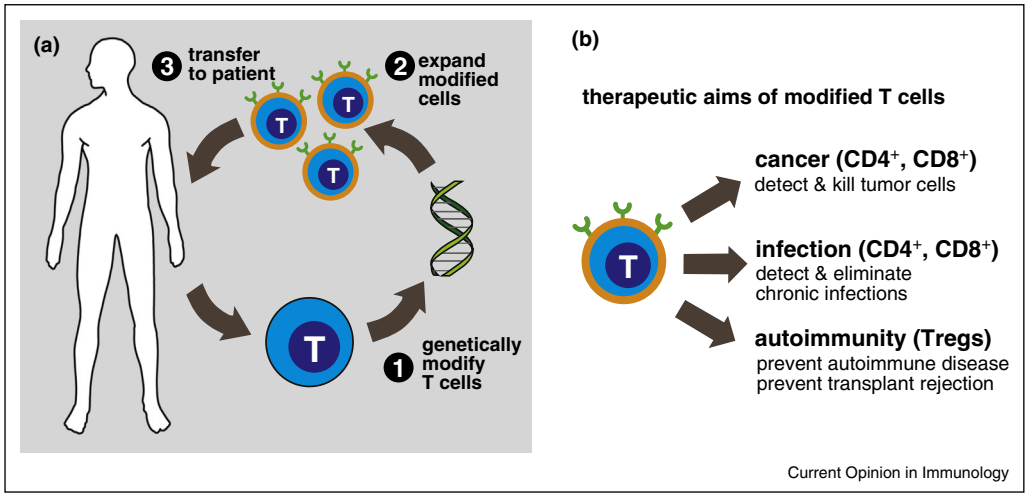
T lymphocytes and their signaling systems are an ideal test bed for synthetic engineering, thanks to decades of rigorous basic research that has generated extensive knowledge on T cell biology. The proliferative capacity of T cells also makes it relatively simple to obtain large numbers of cells for experimental and treatment purposes. Transient or stable expression of synthetic molecules in T cells can be achieved using multiple methods (Box 1) [16–20,50–52]; genome engineering via CRISPR or ZFN approaches carries immense potential for construction of complex circuits involving re-wiring, modifying, or disabling endogenous pathways. Finally, T cells provide a rich context for intercellular interactions that is amenable to engineering and can be used to explore key parameters in cell–cell communication and dynamic population behaviors [21,22].

Thus the field of T cell engineering (synthetic immunology) is rapidly growing. This review will discuss selected examples T cell engineering and how this field might expand in the future to enhance precision control over therapeutic T cells.

Progress in rewiring T cells

Detection of disease signals through synthetic T cell receptors. T cells normally use their T cell receptor (TCR) to detect

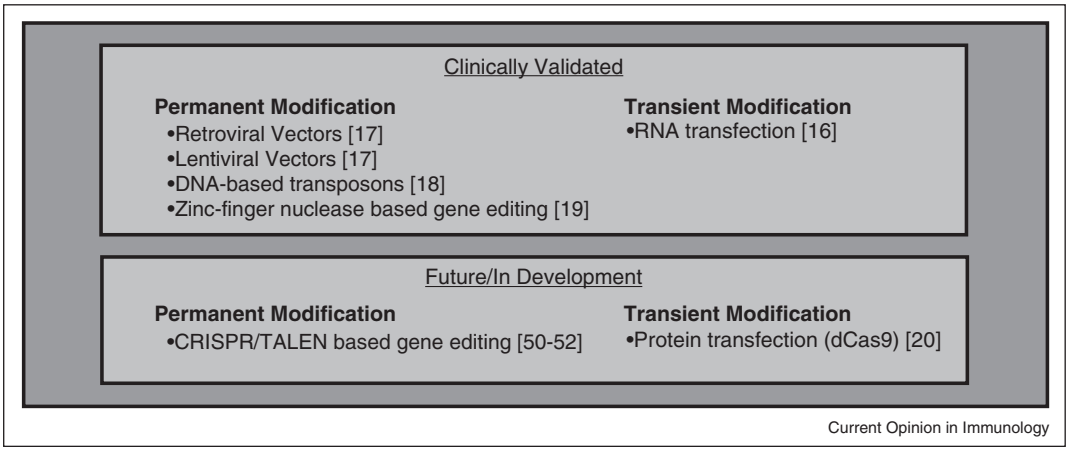
Figure 1



Engineering T cells for diverse clinical needs. **(a)** Overview of adoptive immunotherapy using genetically modified T cells. **(b)** Current and future applications for engineered T cells.

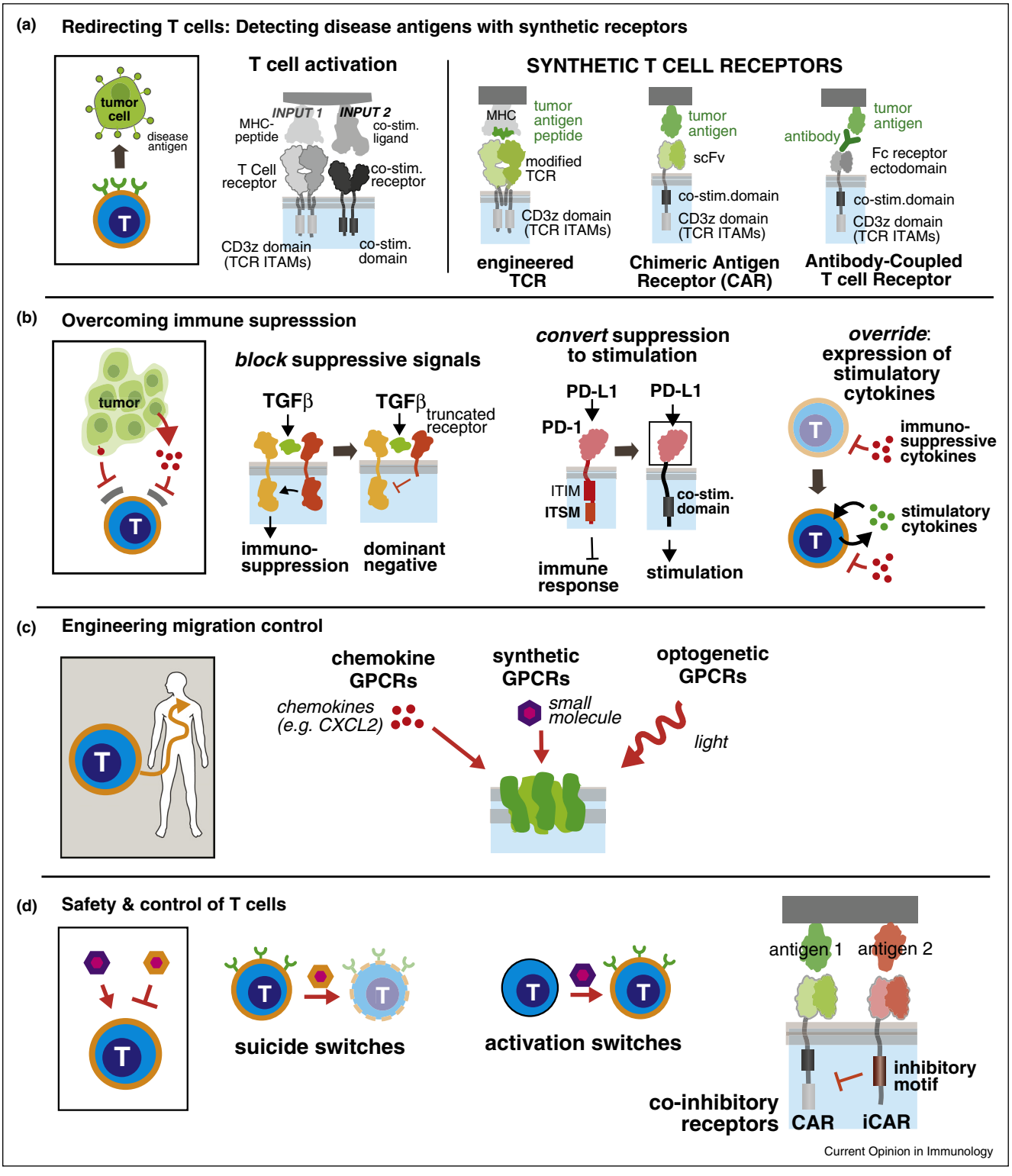
peptides presented by MHC molecules. To harness T cells in treating disease, it is critical to be able to alter T cells such that they recognize specific, selected disease signals (e.g. a tumor antigen). A streamlined way to modulate a T cell's specificity for input signals is to employ synthetic receptors, which are typically chimeras of motifs and domains of natural or synthetic origin. Synthetic TCRs, chimeric antigen receptors (CARs) and antibody-coupled T cell receptors re-direct cells to recognize disease associated ligands or antigens on target cells [7••,9••,23••,24•] (Figure 2a). The first generation of these synthetic receptors was developed nearly 20 years ago and generally only contained signaling modules from the TCR. The current generation of CARs and antibody-coupled T cell receptors typically combine intracellular signaling modules from both the TCR and co-stimulatory receptors. In this regard antigen-dependent activation of T cells equipped with such receptors is independent of MHC and costimulatory interactions. The single antigen input activates two intracellular responses — both signal 1 (antigenic stimulation) and signal 2 (co-stimulation) of T cell activation to produce desired outcomes such as cytokine production, proliferation, and survival. With the advent of current generation CARs containing costimulatory motifs, CAR T cells have shown strong *in vivo* proliferation and durability required for therapeutic efficacy. A tremendously exciting example of current CAR therapy is the CD19 CAR T cell trials for treatment of B cell cancers, which highlight the potential of cell-based therapeutics to transform cancer therapy [7••].

Box 1



Methods to engineer T cells.

Figure 2



Diverse synthetic modules have been developed to reprogram therapeutic T cells. **(a)** Synthetic receptor designs for targeting disease antigens. ITAM: immunoreceptor tyrosine-based activation motif, scFv: single chain variable fragment. **(b)** Methods for engineering T cells resistant to immunosuppressive microenvironments. ITIM: immunoreceptor tyrosine-based inhibitory motif, ITSM: immunoreceptor tyrosine-based switch motif. **(c)** Approaches to engineer migration/trafficking control. **(d)** Engineering safety switches and gated activation in gene modified T cells.

Overcoming immune suppression. The immunosuppressive conditions in tumor microenvironments remain a major challenge in cancer immunotherapy. High levels of inhibitory signals in tumor microenvironments in the form of cytokines (e.g. TGF-β and IL-4) and cell surface co-inhibitory ligands (e.g. PD-L1) can globally suppress effector T cell functions (Figure 2b). Additional synthetic receptors have been developed to overcome such immunosuppressive mechanisms. For example, a dominant negative allele of the TGF-β receptor can abrogate TGF-β mediated inhibition, rescue the survival defects of T cells, and enhance their anti-tumor activities in distinct mouse models [25,26]. In another example, a chimeric receptor consisting of the IL-4Rα extracellular domain and IL-7Rα transmembrane + intracellular domains induces Th1 polarization in the presence of IL-4, which normally suppresses Th1 effector functions. T cells expressing this chimeric receptor showed enhanced immunity against IL-4-producing tumors, likely due to the ability to continuously utilize tumor-derived IL-4 to proliferate and sustain Th1 activities [27].

The concept of converting inhibitory signals into activating signals has also been explored to mitigate PD-L1 mediated immunosuppression. A chimeric receptor consisting of the PD-1 extracellular domain and CD28 intracellular segment converts co-inhibitory signaling of the PD-1 pathway to co-stimulatory signaling of the CD28 pathway [28]. Primary human $CD8^+$ T cells expressing this chimeric receptor produced more effector cytokines when stimulated with $PD\text{-}L1^+$ target cells. The T cells also retained granzyme B expression more effectively after prolonged exposure to the PD-L1 ligand *in vitro*, suggesting sustained anti-tumor functions.

Tumor-targeting T cells can also be engineered to conditionally secrete cytokines that promote anti-tumor functions and T cell survival in tumor microenvironments. In such a controlled manner, toxicities that would otherwise result from systemic administration of the cytokine might be minimized. One example is antigen-dependent production of IL-12 through an NFAT-responsive promoter that is activated upon tumor antigen recognition by a CAR or TCR, although a recent clinical study reveals the necessity for greater stringency to avoid severe toxicities [29,30]. Increased stringency of IL-12 production could be achieved through administering a synthetic small molecule to specifically regulate the onset, duration, and magnitude of IL-12 transcription [31]. As summarized in earlier work, IL-12 exerts multiple advantageous immunomodulatory effects in tumor microenvironments through engaging both the innate and the adaptive immune components as well as altering the extracellular matrix [32]. One major effect of IL-12 is promoting neo-antigen recognition by the immune system so that tumor cells that have evaded T cell recognition by loss of MHC or target antigen expression can be eliminated. Controlled production of other cytokines (e.g. IL-2, IL-15) to promote T cell proliferation and survival can be achieved with ribozyme-based switches responsive to small molecules [33]. As co-stimulatory signals and cytokines act in concert to regulate T cell functions, the potential synergy among the synthetic devices discussed in this section would be exciting to explore in adoptive immunotherapy.

Re-directed migration of T cells. Migration of T cells can be controlled using natural or synthetic G-protein coupled receptors (GPCRs) recognizing soluble ligands of interest to increase the T cells' therapeutic potential (Figure 2c). For example, preclinical tumor models showed enhanced T cell migration to tumor sites and improved tumor regression when CAR T cells expressed a natural GPCR for the tumor-derived chemokine CCL2 or CXCR2 [34,35]. GPCRs engineered to recognize orthogonal ligands, known as receptors activated solely by a synthetic ligand (RASSLs), have been developed using directed molecular evolution [36]. Migration of human primary T cells expressing one such RASSL was exogenously regulated using the small molecule clozapine-N-oxide, an inert metabolite of the FDA-approved drug clozapine. Both *in vitro* and *in vivo*, RASSL-expressing T cells migrated up concentration gradients towards the sources of ligand [37•]. Similarly, Xu *et al*. designed a light-responsive rhodopsin-CXCR4 chimeric GPCR for controlled phototaxis of engineered T cells *in vitro* and *in vivo*. In an OVA tumor model, light stimulation induced significant intratumoral infiltration of antigen-reactive cytotoxic T cells, along with enhanced T cell proliferation and tumor regression [38•]. In summary, synthetic motility control systems using orthogonal small molecules or light could promote infiltration and retention of engineered T cells in tissues that lack T cell presence, such as certain solid tumors and immune privileged sites.

It is worth noting the exciting development of synthetic molecules specifically designed to trigger responses in unmodified immune cells, such as engineered cytokines and their mimics [39] as well as bi-specific antibodies [40]. Although most of these molecules are designed to interact with natural immunoreceptors, further engineering could be applied to yield highly specific, orthogonal pairs of synthetic ligands and receptors, which in principle would afford more precise exogenous control over engineered immune cells [41].

Safety devices. A critical design aspect moving forward will be incorporation of robust safety mechanisms to prevent unrestrained activity of engineered cells. There have been serious adverse effects associated with many therapeutic T cell clinical trials, including severe cytokine release syndrome, and in some cases death due to cross reaction with healthy tissues [7••,9••,23••]. A number of approaches have been described to make engineered

T cells safer. Arguably the simplest strategy is the 'kill switch' or 'suicide gene' that results in death of engineered cells upon addition of an artificial stimulus. Principal amongst these approaches is ectopic expression of the herpes simplex virus (HSV) thymidine kinase that causes DNA replication defects in engineered cells after addition of the FDA-approved antiviral ganciclovir, and is currently under investigation in a Phase I CAR T cell trial. An alternative is ectopic expression of an engineered protein designed to mediate inducible apoptosis. The leading example is an inducible Caspase 9 system that has also been tested in a Phase I trial. This FKBP-Caspase9 (iCasp9) fusion homo-dimerizes and stimulates the cell intrinsic apoptotic pathway upon small molecule addition [42]. Finally, cells may be engineered to express surface receptors or epitope tags such that addition of antibodies leads to depletion of therapeutic cells [43].

Each of these strategies has its associated challenges. HSV thymidine kinase has documented immunogenicity [44], which could allow rejection of therapeutic cells in immunocompetent patients; a similar concern holds for synthetic epitope tags employed for antibody depletion of target cells. Alternatively, expression of native molecules (such as EGFR) for antibody depletion strategies could have unintended consequences in the cells of interest, and may require significant engineering to render them biologically inert.

The most severe limitation of 'suicide gene' strategies in settings such as CAR T cell therapy, where the CAR is constitutively 'on' in the presence of antigen, is the requirement for 100% efficacy of the switch to avoid toxicity issues. Even small numbers of cells that inactivate the kill switch or evade deletion could expand and cause significant toxicity. Indeed, in a phase I trial of iCasp9 expressing cells, treatment with the small molecule dimerizer led to only ~95% depletion of gene modified cells [42].

An alternative approach would be to design cells that are 'off' in the basal state and exhibit controlled gating of activation ('activation switches', Figure 2d). Here cells could be rendered therapeutically inert until addition of an activating signal such as a small molecule, or detection of specific environmental antigens such as a defined tissue localization (Wu *et al.*, in press).

Alternatively, improved safety could be engineered with negative regulatory systems that mitigate activity or enhance specificity. For example, Fedorov *et al.* have reported the construction of inhibitory CARs (iCARs) that contain domains from the co-inhibitory receptors CTLA-4 or PD-1 [45•]. In T cells expressing a conventional active CAR targeting Antigen A and an iCAR targeting Antigen B, ligation of the iCAR 'overrides' the active CAR and allows discrimination between cells expressing Antigen A alone versus both Antigens A and B. Extending this approach to more complex synthetic circuits could allow construction of T cells with exquisitely specific behaviors and feedback control that allow defined periods of activation, multi-antigen gating, among others. Analogously, other groups have begun constructing CARs with tandem extracellular recognition domains [46]. These and other combinatorial recognition CARs might exhibit more specific antigen recognition, or could be used to reduce the chance of tumor escape via target antigen loss or mutation.

Future needs: the promise of synthetic T cell circuits

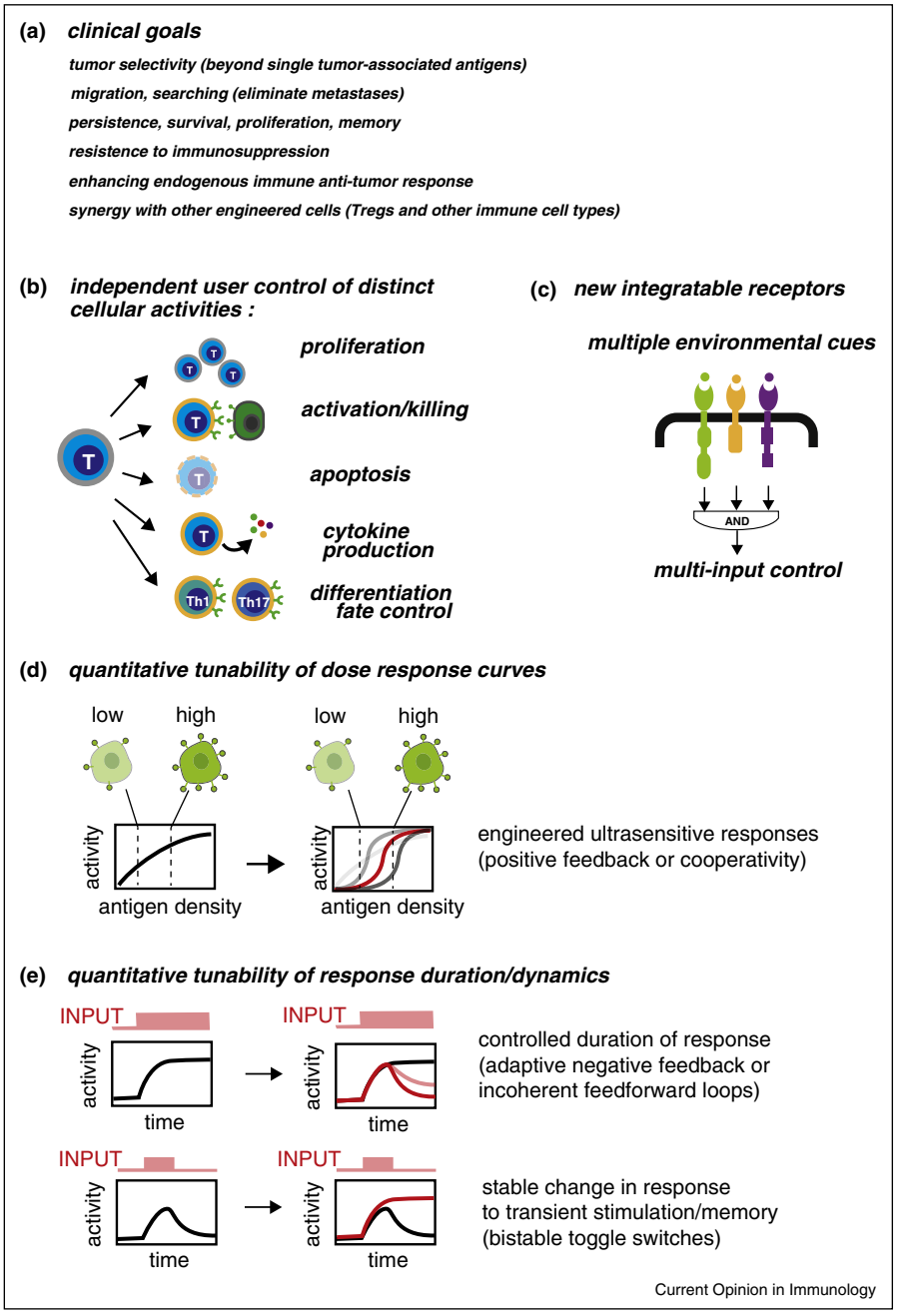
Numerous clinically desirable behaviors would be useful in next generation therapeutic T cells (Figure 3a). Desired properties include, but are not limited to, increased tumor recognition specificity, directed migration/trafficking, enhanced persistence/survival/differentiation, resistance to immunosuppressive microenvironments, and recruitment of endogenous anti-tumor response. To date current approaches to engineering T cells have been relatively simplistic, involving single transgenes or relatively static approaches that address a single challenge detailed above. We propose that going forward it will be vital to develop a diverse toolbox of dynamically regulated engineered behaviors and functionalities that can be rapidly integrated to generate customized cell therapies for diverse applications.

A critical challenge in cell based therapies is either insulating engineered circuits from the endogenous response, or understanding native behavior sufficiently to integrate the desired functionality. This is particularly relevant for T cell engineering because of developmental plasticity and the immense impact that environmental factors play in determining T cell fate, function, and localization [47,48]. Ultimately, we must develop tools for independent control of multiple T cell functions, including but not limited to survival, proliferation, trafficking, targeted cytolysis or cytokine production, differentiation, and maintenance of cell fate (Figure 3b). Designing artificial circuits for these capacities will require careful and systematic dissection of native systems, particularly given the interconnectedness of many of the parameters described above [47,48].

Another significant need is development of multi-input control that allows for complex Boolean logic gating, analogous to the way cells integrate diverse stimuli to encode downstream outputs (Figure 3c). Such combinatorial sensing could aid in discriminating healthy tissue from cancer, or limit production of secondary outputs (such as cytokines) to specific tissues to avoid side-effects of systemic production.

Along these lines, it will be critical to move beyond constitutive transgenes and/or reliance upon endogenous

Figure 3



Looking forward: design principles for next generation therapeutic T cells. **(a)** Desirable overall clinical properties for next generation therapeutic T cells. **(b)** Cellular activities to be independently controlled by user for enhanced therapeutic cell safety and precision. **(c)** Multi-input systems required for complex Boolean logic and sophisticated decision making. **(d)** Enhanced ligand/antigen density discrimination to distinguish normal versus disease target cells via tuning dose responses in engineered T cells. **(e)** Engineered control of T cell response duration and state-switching.

pathways to regulate duration and intensity of programmed behaviors. Incorporation of classical synthetic biology motifs such as positive feedback for amplification, negative feedback for limiting duration, or bistable switches to promote mixed population states (Figure 3d,e) should allow for rational control of therapeutic behaviors, both temporally and spatially [1–6].

A primary technical limitation to application of complex circuits lies in the ability to deliver large genetic payloads (3–5+ transgenes and promoters) reproducibly at high efficiency. Transposon-based approaches may prove superior to viral vectors in this regard due to larger payload delivery; alternatively, CRISPR-based homologous recombination could overcome this hurdle. Barring

advancement of this technology, it may also be possible to deploy CRISPR to selectively rewire endogenous signaling pathways by tweaking promoter strength/responsiveness, introducing destabilizing mutations, or creating dominant negative alleles in engineered cells.

In summary, T cells are one of the most exciting platforms for cellular engineering and development of mammalian synthetic biology tools. Advancing clinical efficacy of next-generation cell therapies will necessitate the development of a diverse toolkit that allows independent user control of multiple cellular behaviors and functionalities. Building upon foundational knowledge of the native behavior of T cells, this may ultimately allow rational design of customized therapeutic cells for a diverse range of unmet clinical needs.

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