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T Cell Reprogramming Against Cancer

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Abstract

Advances in academic and clinical studies during the last several years have resulted in practical outcomes in adoptive immune therapy of cancer. Immune cells can be programmed with molecular modules that increase their therapeutic potency and specificity. It has become obvious that successful immunotherapy must take into account the full complexity of the immune system and, when possible, include the use of multifactor cell reprogramming that allows fast adjustment during the treatment. Today, practically all immune cells can be stably or transiently reprogrammed against cancer. Here, we review works related to T cell reprogramming, as the most developed field in immunotherapy. We discuss factors that determine the specific roles of αβ and γδ T cells in the immune system and the structure and function of T cell receptors in relation to other structures involved in T cell target recognition and immune response. We also discuss the aspects of T cell engineering, specifically the construction of synthetic T cell receptors (synTCRs) and chimeric antigen receptors (CARs) and the use of engineered T cells in integrative multifactor therapy of cancer.

Keywords

T cell; T cell receptor (TCR); Chimeric antigen receptor (CAR); alpha beta T cells; gamma delta T cells; Memory T cells; Immune synapse; Reprogramming; Adoptive cell therapy; Signal transduction; TCR clustering

1 Introduction

Progress in immunotherapy has reached a critical point where available funding and efforts can provide practical improved clinical outcomes for patients. These advances are based on findings in academic and clinical studies in immunology, adoptive immunotherapy, gene editing, and stem cell modulation, among other fields. Despite our rapidly increasing understanding of tumor–immune system interactions, there are profound limits to our knowledge. Nonetheless, the urgent need for therapeutic improvements facilitates the development of new drugs and modified cells in parallel with new methods of their clinical evaluation. Particularly important is the opportunity to exploit combinatorial multifactor treatment protocols based on protein and cell engineering.

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Immune cells can be programmed with molecular modules that increase their therapeutic potency and specificity. Although in its infancy, modern immunotherapy strives to provide personalized therapy that is modifiable during the course of treatment based on the patient's baseline characteristics and ongoing accurate evaluation of the course of the disease. The development of immune modulation by cell reprogramming already has been translated into patient cures. This highlights both a fascinating discovery and our relative ignorance about how to prevent high morbidity, off-target effects, and other complications.

Fortunately, some of the gaps in our knowledge are being addressed rapidly. It has become obvious that successful immunotherapy must take into account the full complexity of the immune system and when possible include the use of different types of immunocytes and multifactor cell reprogramming, and apply flexible methods that allow fast adjustment during the treatment depending on the patient's conditions and needs. Today, practically all immune cells can be stably or transiently reprogrammed against cancer. The most developed field is T cell reprogramming, although quite promising results have been achieved with natural killer (NK) cells, macrophages, and others [1–5]. Several chapters in this volume exemplify these different sources of cells that can be reprogrammed (e.g., Chapters 6–9 for NK cells, Chapter 11 for dendritic cells (DCs), Chapter 14 for macrophages).

Challenges in cell engineering appear at many levels. At the subcellular level, the design of novel proteins or other molecules that can be expressed and function in accord with endogenous cell systems is nontrivial. At the cellular level, the complexity of cell-to-cell interaction dictates accurate construct adjustment and modification. In addition, there may be the need to introduce additional molecules of different classes that optimize the cognate cell function. At the organismal level, there is a need to evaluate multiple reactions by an integral combinatorial approach where cell engineering synergistically couples with other therapies.

During last 20 years, adoptive cell therapy (ACT) was developed based on two main premises: (1) Cytotoxic T cells eliminate diseased cells, and (2) artificial modular protein constructs can be designed to recognize specific antigens on the surface of target cells and trigger T cell target killing. Since 2011, the number of patents related to chimeric antigen receptor (CAR)-mediated immunotherapy has grown exponentially [6]. Today, ACT is best demonstrated in the treatment of blood B cell tumors with chimeric antigen receptor T cell (CAR-T) therapy products: Kymriah (Novartis) and Yescarta (Kite Pharma/Gilead), which are approved by the US Food and Drug Administration [7] and the European Medicines Agency [8]. Other hematopoietic cancers and solid cancers have been more challenging to target, because T cell function is impeded by the absence of specific tumor antigens, multiple barriers of tumor accessibility, and immunosuppressive conditions. Increased knowledge of the processes that take place in the tumor microenvironment, metastasis development, and immune tuning on both systemic and local levels will be necessary to improve cell engineering and resolve both the fundamental and technical problems. In this review, we briefly address the main areas in T cell reprogramming relevant to ACT of cancer and describe some obvious underdeveloped areas important for building better integrative personalized therapies.

2 T Cells

2.1 T Cell Diversity

Functional diversity of T cell populations and their T cell receptor (TCR) repertoire are important factors that determine immune health. In cancer research, attention is often focused on the rather narrow task of finding a T cell population, among a weakened immune system, that is robust enough to yield a sufficient amount and be reprogrammed to kill cancer targets and then maintained in patients long enough to achieve efficacy. However, cross-communication among the subsets of T cells, dendritic cells (DCs), and other immunocytes is also an important part of immune response. That explains growing attention to various subsets of immune cells.

In humans, it is estimated that ~7 billion T cells are present in the peripheral blood, 25 billion in the bone marrow, 30 billion in the spleen, and 150 billion in the lymph nodes. Taken together, these three organs contain $>200 \times 10^9$ T cells, which constitutes the majority of total T cells [9].

T cells consist of many subtypes, the largest of which are the “conventional” αβ T cells with “classic” major histocompatibility complex (MHC) restriction. These T cells are part of the sophisticated adoptive immune system with a relatively slow response. Some other T cells are part of the innate immune system. They are characterized by a limited TCR diversity, are either “non-classic MHC” restricted or MHC independent, and exhibit a fast immune response. They include γδ T cells, natural killer T (NKT) cells, CD1- and MHC class Ib-restricted T cells, MR-1-restricted mucosal-associated invariant T cells (MAIT), and intraepithelial lymphocytes (IELs) [10–12]. Although the subtypes of T cells are functionally different, this difference is not inflexible. For example, human peripheral γδ T cells can be transdifferentiated ex vivo into αβ T cells [13]. γδ T cells are a minor subset of peripheral lymphocytes in humans (<5%) [14] but are enriched in epithelial and mucosal tissues [15].

2.2 T Cell Development

T cells originate in the bone marrow, and most of them develop in the thymus. These cells arise from immature CD4 and CD8 double-negative thymocytes and express either the αβ or γδ TCR [14, 16]. αβ T cells undergo positive and negative selection through recognition of self-peptide–MHC (p–MHC). Most αβ T cells with high affinity to self-peptides undergo negative selection and die. However, some of them survive and after positive selection form certain subtypes, such as IELs, NKTs, and Foxp3+ regulatory T cells (Tregs) [17]. αβ T cells with low affinity to self-p–MHC develop into “conventional” CD8+ cytotoxic lymphocytes (CTL) or CD4+ helper T cells that recognize foreign or “diseased” peptide antigens presented by MHC class I and class II molecules, respectively. This difference is not rigid, as a substantial part of CD4+ T cells can be cytotoxic similar to CD8+ T cells [18–22].

After selection in the thymus, αβ T cells proceed to the periphery where they circulate as naïve CD4+ and CD8+ T cells between blood and secondary lymphoid organs. Positive

selection of naïve T cells continues as cyclical “tonic” activation as they circulate between the blood and secondary lymphoid organs where they encounter self-p–MHC [23–25].

γδ T cells recognize antigens in a “classic MHC”-independent manner. They recognize a range of structurally different moieties, such as non-classic MHC molecules, proteins, peptides, and phospholipids [26]. Studies in mice have demonstrated that γδ T cells undergo negative selection in thymus, but the role of positive selection in their development may not be obligatory [27].

2.3 Naïve, Effector, and Memory αβ T Cells

Naïve T cells are maintained in a state of quiescence, which is characterized by low cell volume, low metabolic rate, and low homeostatic proliferation. Gradual naïve T cell propagation without differentiation is upheld by IL7 signaling and tonic, low-level activity of the TCR that monitors “normal” self-p–MHC presented by surrounding cells [28, 29]. Conventional activation of naïve T cells by antigen-presenting cells (APCs) requires the simultaneous engagement of three receptors on the T cells: TCR ligation with agonist p–MHC, CD28 or another costimulatory receptor ligation with cognate ligand, and cytokine receptor ligation with IL2 or another homeostatic cytokine [30–32].

Sometimes, naïve T cells can be activated without TCR ligation. For example, in vivo T cells become activated when their population is severely depleted [33], whereas in vitro T cells can be activated in the presence of high concentrations of interleukins IL2 or IL7 [34].

Upon APC-mediated activation, naïve T cells undergo clonal expansion and differentiation. They activate mTORC1 [35], shift from catabolic to anabolic metabolism, increase glucose and amino acid (AA) uptake, remodel their mitochondrial function, and increase their cell volume and their rate of proliferation [29]. In the initial expansion phase, naïve T cell doubling time decreases from 500–1000 days [36, 37] to a few hours [38, 39]. This process may occur in different tissues and is highly dependent on receptor–ligand affinity and density, as well as environmental factors, such as pH, redox potential, and the availability of nutrients. The activation leads to clonal expansion by sequential asymmetric cell division. In this process, cell divisions convert activated naïve T cells into terminally differentiated effector T cells (T_{EFF}) and self-renewing memory lineages (T_{MEM}) [40]. Some of the progeny cells become more activated and directed toward T_{EFF}, while the others become less metabolically active T_{MEM} [41]. Thus, T_{MEM} cells are metabolically more active than naïve T cells, with higher mitochondrial mass and respiratory capacity [42], but less active than T_{EFF} cells and maintain predominately catabolic metabolism [43]. This contributes to the prolonged persistence of T_{MEM} cells, such that their longevity is between naïve and effector T cells.

Activated T_{EFF} cells include both CD8+ CTLs and CD4+ helper T cells that provide acute protection from immune challenges [44]. Activated CTLs recognize an appropriate target, bind to it, and secrete killing molecules toward the target, before detaching from the dying target [45]. In humans, the main mechanism of T cell cytotoxicity is the granzyme–perforin pathway [46–49]. Other killing systems, such as Fas/FasL, TNF-α/TNF receptor 1, and TRAIL/DR4/DR5, are usually employed in T cell activation-induced cell death (AICD),

which is an essential part of T cell homeostasis regulation [50]. However, these systems can also cause target killing [45, 51, 52].

After the immediate immune response and foreign antigen is cleared, the T cell population enters a contraction phase, and most of the T_{EFF} subsequently die by apoptosis [53].

Long-term immune protection is provided by subsets of T_{MEM} cells that include central memory (T_{CM}) and effector memory (T_{EM}) cells. T_{CM} are circulating cells that are prevalent in lymph nodes and have enhanced longevity and proliferative potential. Although T_{CM} lack effector functions themselves, they generate T_{EFF} and T_{EM} cells. T_{EM} cells are circulating cells that are more prevalent in nonlymphoid tissues [54–57]. They possess immediate effector functions; they rapidly migrate toward targets and provide antigen elimination [58]. Differentiation from naïve T cells, to T_{CM}, to T_{EM} is associated with decreasing expression of the Wnt/β catenin transcription factors, LEF-1, and TCF-1 [59–61]. Clonal development is a flexible process, and to some degree, it can be reversed. For example, in mice, long-lived memory CD8+ T_{CM} cells can develop from effector T_{EFF} cells through a process of dedifferentiation [62].

T_{MEM} population is diversified, and their discovery and classification are especially difficult if they are not circulating. Recently, such tissue-resident memory (T_{RM}) cells were located in the lungs, salivary glands, female reproductive tract, skin, and liver, where they orchestrate the response to different pathogens [63, 64].

An important subset within the T_{CM} population generated during the primary immune response has stem cell-like characteristics, defined as T_{SCM}. These cells exhibit a high capacity for homeostatic proliferation and can give rise to other memory subsets. In normal homeostasis, the pool of T_{SCM} cells in humans is believed to comprise 2–3% of all circulating T lymphocytes [61, 65].

Some CD8+ T_{MEM} cells are virtual memory cells that originate from naïve T cells under strong IL15 signaling but without agonist antigen stimulation [66–68]. The role of these cells is debatable, but one theory is that they provide innate-like protection during the earliest stages of bacterial or viral infection [68, 69].

Once created, conventional T_{MEM} cells also become independent of further antigenic stimulation, which allows their maintenance after resolving an acute infection. T_{MEM} homeostasis depends on paracrine and autocrine IL15 signaling, and T_{MEM} cells can proliferate in response to IL15 in a TCR-independent fashion [70, 71]. Because the overall number of T_{MEM} cells remains constant over long periods of time, the observed continual proliferation of T_{MEM} cells must be accompanied by a nearly equal death rate, probably as result of asymmetric division, where half of the offspring becomes apoptotic [72].

2.4 Persistence of Circulating αβ T Cells

The accurate evaluation of T cell subpopulations is difficult and varies in different studies. Vrisekoop et al. estimated that human naïve CD4 and CD8 T cells have half-lives of 4.2 and 6.5 years, respectively, whereas memory CD4 and CD8 T cells have half-lives of 0.4 and 0.7 years [73]. However, these measurements were made on circulating cells, whereas T_{MEM} in

nonlymphoid tissues and bone marrow can have much longer life spans that allow long clonal survival. In fact, some memory T cells persist for more than 10 years [74, 75].

The age-related decline in T cell population is caused by thymic involution, impaired peripheral T cell maintenance, repeated antigen exposure, and persistent inflammation. Although healthy aging individuals can maintain a sufficient T cell content over time, naïve CD4+ and CD8+ T cell numbers and repertoire gradually decline [76], and the peripheral T cell pool becomes dominated by memory T cells [77, 78].

3 The T Cell Receptor (TCR)

3.1 Formation of the αβ TCR

Present on more than 90% of the T cells, the αβ TCR comprises the predominant TCR complex on the surface of human T cells [16]. Interestingly, it is also present on some neutrophils [79, 80] and macrophages [81, 82].

To date, no intrinsic enzymatic activity has been found for any of the TCR proteins, but rather, it is provided by the set of cytosolic, transmembrane (TM), and membrane-bound (e.g., myristoylated) enzymes that associate with the TCR. These proteins are organized in a highly cooperative system where the inter-protein signaling is quite often regulated by phosphorylation–dephosphorylation. Other interactions include a variety of enzymatic reactions and cytoskeleton and membrane rearrangements, which result in the attraction of scaffold proteins and adaptors, and signal amplification through multiple downstream metabolic pathways.

The conventional αβ TCR is located in membrane lipid raft subdomains. It contains two antigen recognition proteins, α chain and β chain, and four “signal transduction” CD3 proteins, δ, γ, ε, and ζ. The α and β subunits, bound together by a disulfide bridge, are sequence-variable proteins, with very short cytoplasmic tails, that can recognize agonist peptide in context of the MHC complex. The αβ heterodimer is flanked by the CD3 proteins as non-covalently associated heterodimers of εγ, εδ, and a disulfide-linked homodimer of ζ- [83–85]. The CD3 proteins are invariant, with relatively longer cytosolic domains that contain binding sites for cytosolic adaptors and enzymes, and maintain an invariant path for information of TCR/p–MHC recognition through the cell membrane down to the cytoplasm, to achieve an adequate cellular response [86].

Subunit cooperativity in the TCR is substantially determined by coordination of ionizable residues in their transmembrane (TM) domains, which form a specific hydrophobic/ionic interface between αβ, δε, γε, and ζζ [87]. Each of the ζ, ε, and δ molecules possesses an ionizable aspartic acid in their TM regions, while γ possesses a glutamic acid residue. Together, this gives six acidic residues in the TM region of the TCR complex. The α TM domain possesses two basic residues (arginine and lysine), while the β TM domain possesses one basic residue (lysine) [88]. Therefore, an αβγεδεζζ complex has three extra negative charges in the TM region leading to a “charge imbalance.” This led to a search for an alternative bivalent TCR structure, γεαβζζαβδε, with a neutral TM region [89, 90] or even structures with higher valency [91–93]. Purification of the TCR with different

detergents or without detergents has led to various deduced TCR structures [16, 90, 92]. In fact, it is possible that the actual TCR structure may “resonate” between mono-, di-, and higher variants of valency.

TCR proteins emerge on the plasma membrane as a fully built complex. ζ is the last subunit to be associated with TCR complex in the Golgi [16, 88]. In resting human T cells, a portion of ζ associates with the actin cytoskeleton. This interaction, mediated by a sequence in the C-terminus of ζ, might be involved in the localization of the TCR into lipid raft structures as well as play a role in TCR recycling [94]. In the cytosol, ζ is present in excess, compared to the other TCR proteins, and can participate in reactions not related to TCR activity. For example, ζ interacts with the transferrin receptor (TfR), and a TfR-ζ complex is expressed on the cell surface independently from TCR. ζ is also expressed in cells other than T cells, like NK cells and neurons. In NK cells, ζ is associated with NK FcgRIII (CD16) and may participate in cell surface expression of this receptor complex [95]. ζ is also associated with NKp46 and NKp30 receptors on NK cells, and its phosphorylation is required for transmission of activating signals upon antigen binding to these receptors. ζ expression in retinal ganglion cells and brain neurons regulates neuronal development by reducing the size of the dendritic arbor [96].

3.2 TCR/p–MHC Ligation

Upon TCR/p–MHC ligation, the shift in αβ conformation determines transmission of antigen-binding energy on the cell surface down into the CD3 intracellular tails [97]. A plausible interpretation of the available data is that the signaling domains of CD3 in the inactive TCR are submersed in the inner leaflet of the cell membrane which screens them from cytosolic adaptors [16, 83, 98, 99]. The mechanical force applied during TCR/p–MHC ligation weakens intra-subunit associations, changes membrane composition, and leads to subunit rearrangement, moving the CD3 intracellular domains out of the membrane and exposing them to cytosolic signaling adaptors [83, 98, 100–102].

CD3 proteins bind cytosolic adaptors by specific docking motifs. One such motif, tyrosine-based activation motifs (ITAM), is present as a single copy in γ, δ, and ε and as three copies in ζ, for a total of ten ITAMs in the CD3 complex [16]. This distinguishes the TCR from other cell receptors with ITAMs, which contain only one or two. A plausible view on ITAMs’ composition is that ITAM tyrosines in CD3 are not redundant and might have specific roles [103]. However, the exact role of each ITAM in the CD3 subunits, including the detailed dynamics of ITAM phosphorylation, remains to be elucidated.

ITAM multiplicity determines the correct signaling for T cells developing in the thymus [104]. Decreasing the number of CD3 ITAMs to less than seven in mice impedes T cell development. These animals developed autoimmune disease probably due to inefficient signaling under negative selection in the thymus [104]. In these animals, studied prior to the onset of autoimmunity, CD3s with slightly decreased numbers of ITAMs still activated signaling cascades including cytokine production and T cell proliferation. Further decrease of CD3 ITAM multiplicity to two to four ITAMs per CD3 resulted in only a limited response with cytokine production, but not T cell proliferation [105].

For developed T cells, ITAM multiplicity appears to have minimal influence on signal amplitude [106]. Instead, it might help with the coordinated activation of all the subunits in the CD3 complex, such that there is a switch-like “all or nothing” response upon TCR binding to p–MHC [107]. Therefore, ITAM multiplicity may determine potency for synchronous activation of the T cell population [106, 107].

The ITAM motif (YXXL/I$X_{6–8}$YXXL/I) contains two tyrosines, which are usually phosphorylated by the key TCR activator, lymphocyte-specific protein tyrosine kinase (Lck). Lck associates with CD3 proteins as well as with the coreceptors CD4 and CD8 [108]. Lck exists in open (active) and closed (inactive) forms, which is determined by the state of phosphorylation. The transmembrane phosphatase CD45 activates or inhibits Lck, depending on location of dephosphorylation. Lck can also be inhibited by the cytosolic kinase Csk or by the cytosolic phosphatase SHP-1. Also, Lck can be activated through serine/threonine phosphorylation by extracellular signal-regulated kinase (ERK), and this may interfere with SHP-1 recruitment to the TCR complex [109].

Phosphorylation of both tyrosines in an ITAM by Lck attracts the cytosolic adaptor ZAP-70. After TCR/p–MHC ligation, ζ phosphorylation proceeds from the most membrane-distal ITAM toward the membrane [110], and their affinity to Zap-70 increases in the same direction [111]. When bound to the CD3 ITAM, Zap-70 is not activated, but released from its autoinhibited conformation [112]. This opens Zap-70 to be activated through phosphorylation by Lck or by autophosphorylation [106]. Zap-70 phosphorylation can occur by Lck or Zap-70 molecules that are bound to the same protein or by those associated with neighboring CD3 subunits [106], thereby enhancing CD3 cooperativity.

Two CD3 proteins, ε and ζ, deserve special attention. Both are non-glycosylated proteins and present in the TCR as two copies: ε as heterodimers with δ and γ and ζ as a homodimer with the possibility to undergo covalent S–S binding. ε and ζ subunits contain other docking sites besides ITAMs for cytosolic adaptors and can be involved in regulation beyond the ITAM–Zap-70 reaction [113–115]. For example, ε contains a proline-rich motif that can bind the adaptor protein Nck [116]. Nck participates in actin reorganization, cell adhesion, and movement. Nck binds to a partially phosphorylated ε ITAM, which contains a non-phosphorylated Y39 and a phosphorylated Y50. The Nck–ε interaction peaks in the beginning of TCR/p–MHC ligation and then decreases after 10 min as the phosphorylation of ε increases [103]. The ζ subunit has the longest cytosolic tail protein among the CD3 proteins and the highest involvement in multiplex signaling. It can bind adaptor proteins Shc and Grb2, the p85 subunit of PI3K [86], SLP-76, Vav, and negative regulators such as SLAP [117], SLAP-2 [118, 119], TRIM, CTLA4, and Unc119, as well as actin [113]. These proteins as well as GADS, phospholipase PLCγ1, Nck, p38 MAPK, and ADAP are recruited into a complex with linker for the activation of T cells (LAT) upon its phosphorylation by ZAP-70 [16, 120, 121].

The ensuing reactions may lead to signal amplification or abruption, depending on multiple factors involved in the regulation of T cell activity. When the TCR senses self-p–MHC, it enters a state of “tonic activity” with only partial phosphorylation of CD3 ITAMs and the associated cytosolic adaptors. The weak reaction on p–MHC may trigger a negative

feedback, through recruitment of Csk, CD45, and SHP-1, which inactivate Lck and lead to receptor desensitization. In contrast, a stronger reaction may turn on a positive feedback, involving further activation and phosphorylation by Lck and prevention of SHP-1 and CD45 recruitment [109]. The activation triggers further signaling in local and distant protein networks and recruitment of the TCR coreceptors, resulting in a full cellular response [122–124].

3.3 TCR Coreceptors

Upon binding agonist p–MHC on target cell, the αβ TCR attracts transmembrane TCR coreceptors CD8 or CD4 to the p–MHC and checkpoint receptors that recognize additional non-MHC proteins on the target cell membrane. These additional interactions can either augment or undermine TCR signaling outcome.

Coreceptors CD4 or CD8 bind to the p–MHC cooperatively with the TCR, which may increase the overall binding force. However, the most important role of CD4 and CD8 proteins is to deliver activated Lck, associated with coreceptors, into the area of the TCR/p–MHC interaction so that CD3 cytoplasmic tails move out of membrane, become exposed to cytosol, and can be phosphorylated, to attract Zap-70 [100, 125]. Whereas the CD4 receptor is a single protein that spans the cell membrane and binds Lck [126], the CD8 receptor contains two proteins: CD8α and CD8β. While both CD8α and CD8β span the cell membrane, only CD8α binds Lck. Thus, it is likely that CD8α is involved in both ligand recognition and signaling, and CD8β participates only in the recognition. The CD8αβ heterodimer is expressed on CD8 T cells [127]; the CD8αα homodimer is expressed on some γδ T cells, NK cells, and IELs; and the CD8ββ homodimer has not been found on lymphocytes [100]. The binding affinity of CD8 to MHC is independent of TCR specificity or affinity; therefore, the impact of coreceptors on p–MHC binding and signaling decreases with increasing TCR affinity [128].

Coreceptor involvement in TCR/p-MHC ligation is followed by Zap-70-mediated phosphorylation of the scaffold protein LAT, which in turn nucleates multiple downstream pathways. Phosphorylated LAT binds additional adaptors GADS, GRB2, and phospholipase PLCγ1(NF-kB). This LAT complex subsequently recruits other adapters and enzymes, including SLP76, VAV1, Nck, p38 MAPK, and ADAP [16, 120, 121]. Phosphorylated ZAP-70 also has a noncatalytic function as a scaffold phosphoprotein that facilitates the high-affinity state of the integrin LFA-1, which in turn increases T cell adhesion by binding ICAM-1 on antigen-presenting cells [129].

Further T cell activation turns on a cascade of reactions, including structural changes with actin rearrangement [130], attraction of the centrosome, increasing of endocytosis, and further accumulation of TCR receptors in the synapse by lateral diffusion or exocytosis (*see* also Subheading 3.6). In addition, there is inositol phospholipid hydrolysis and mobilization of Ca^{2+} through activation of phospholipase C-gamma 1 and serine/threonine kinases [120]. Finally, distant signaling pathways are induced including PI3K/Akt/mTOR, Myc [44, 105, 131–133], NFAT [134], NF-κB, and AP-1 [135]. Overall, the signal cooperativity of CD3 proteins with the coreceptors may include cross-phosphorylation among ITAMs, synergism in adaptors' binding, and cross-activation among CD3 complexes in TCR clusters.

3.4 The Immune Synapse

The structure and specific activity of immune synapses are determined by the type of T cells (cytotoxic, helper, Treg, NKT), TCR (αβ TCR and γδ TCR), coreceptors (CD4 or CD8), and the set of checkpoint receptors that bind to various ligands outside the p–MHC and add either positive or negative cooperativity. For example, the synapse between a helper CD4+ T cell and B cell exists longer and leads to different outcomes than the synapse between a cytotoxic CD8+ T cell and B cell [136]. As a second example, the synapse with DCs primes naïve CD8+ T cells to proliferate and differentiate into CTLs over the course of several days, whereas it primes CTLs to kill diseased cells by secretion of cytolytic granules at the point of TCR signaling [137].

Target cells also determine synapse structure and function. Potential target cells include “professional” APCs, such as a dendritic cell (DC), macrophage, or B cell [138]; “atypical” APCs, such as a granulocyte [139], lymphatic epithelial cell [140, 141], basophil, mast cell, or eosinophil [138]; or “true target” diseased cells that should be eliminated. Synapses between T cells and different APCs have different organizations [142, 143]. CTLs attached to dendritic cells are less toxic toward their target than CTLs attached to B cells [144, 145].

Wild-type TCRs usually have low affinity for their p–MHC targets with a dissociation equilibrium constant (K_D) of 1–100 μM [136, 146, 147]. The precise number of p–MHC target antigens per cell required for optimal αβ T cell activation can vary, but in principle, T cells can be activated in response to only a very few p–MHC antigens [148–151]. In fact, the αβ TCR accurately recognizes relatively infrequent agonist peptides among “self”-peptides in the MHC class I, with a ratio as low as ~1×10^4 self-peptides: one agonist peptide [152]. Technically, it occurs by constant p–MHC monitoring that provides low-level T cell tonic stimulation. It has been proposed that naïve T cells have the ability to adjust their activation threshold by a mechanism dependent not only on specific TCR affinity to p–MHC [153], but includes dynamic tuning with participation of coreceptors CD8 and CD4 and proteins with TCR inhibitory activity, like CD5, CD6, and CD45 [153, 154]. Intrinsic TCR affinity for self-p–MHC ligands in mice correlates with expression of CD5 [154, 155] and inversely correlates with expression of CD8 [156].

Serial activation of TCRs by p–MHC ligation may elevate reactivity to low-affinity antigens and also help to discriminate between different high-affinity ligands. For example, it is important to discriminate between an acute infection that usually results in expression of high-affinity antigens at high density and a normal high-affinity antigen that escaped thymus presentation, which is often expressed at low density. Comparison of TCRs with different affinities showed that a TCR with an affinity greater than the physiologic range mediated stronger and faster responses than wild-type TCR. Paradoxically, this leads to an inability to recognize such an antigen presented in low density on target cells [157]. Ligation of high-affinity p–MHC with TCR can be relatively stable and impede ligation of this p–MHC with other TCRs. In this case, p–MHC in high density still may provide strong serial triggering. However, serial triggering should be blocked if p–MHC is presented in low density. In this fashion, TCR affinity and clustering can be considered part of peripheral immune tolerance.

The initial TCR/p–MHC binding is sterically limited because the exodomains of the TCR and p–MHC are short (~7 nm) requiring a relatively small intracellular cleft of ~15 nm to make contact [151]. This interaction is difficult because in the cell membrane the TCR neighbors highly abundant large surface proteins like CD45, whose extracellular segment ranges from 20 to 50 nm depending on the isoform [158]. Individual TCR/p–MHC interactions are short-lived (seconds), but for T cell activation, binding should continue for minutes to hours [151]. Therefore, a close contact between the T cell and target cell within a cleft of ~15 nm must be built and persist by exclusion of large surface moieties.

T cells use microvilli to create a close contact with the interrogated antigen-presenting cells (APCs) and target cells [24, 159]. These ~150 nm-diameter cell membrane protrusions contain adhesive receptors and form contact with target membranes with a short ~15 nm cleft, which sterically excludes CD45 and other bulky membrane proteins [151]. Microvilli usually last for seconds, but if a TCR complex presented in it binds agonist p–MHC, this can lead to cytoskeleton rearrangement that stabilizes the microvilli for a longer period of time [160]. The effective binding involves redistribution of adhesion receptors CD2 and LFA-1 that bind CD58 and ICAM-1 target proteins, respectively. The relatively long LFA-1/ICAM-1 interaction (~40 nm) moves to the periphery of the contact zone, while the shorter CD2/CD58 interaction (~15 nm) is placed in close proximity to the TCR/p–MHC binding [161].

The synapse is a dynamic structure that may contain various numbers of TCRs. Often it undergoes a transition from nanoclusters (~20) of TCR to microclusters (~300). These can proceed further to increased concentric membrane aggregates that in some conditions comprise up to 20% of the cell surface. Such complexes contain three concentric supramolecular activation clusters (SMACs): a central TCR/p–MHC cluster (cSMAC) with a narrow ~15 nm cleft [137], a peripheral ring of LFA-1/ICAM-1 (pSMAC) with a ~40 nm cleft, and a distal ring that includes CD45 and F-actin (dSMAC) in a bigger cleft [162]. This structure has been observed in helper, cytotoxic, and regulatory T cells [163].

The SMAC complex is a dynamic structure that can persist over a period of hours and provides signaling that can change in a timely fashion [16]. While cSMAC was initially considered only an activation domain, the recent identification of nearby late endosomal compartments suggests it can also function as a domain of TCR downregulation [161].

After completion of the immune response, T cell activation caused by TCR ligation should be downregulated. This can occur simply by exhaustion of available antigens, by negative feedback with synapse-associated cytosolic enzymes, or by specific checkpoint inhibitory receptors, such as PD-1, CTLA-4, B7-H3, DGK-α, LAG-3, and Tim-3, that are activated in parallel with the TCR and can work in the synapse by binding cognate target cell ligands [164]. PD-1 has two ligands, PD-L1 and PD-L2, which are members of the B7 family [165]. PD-1 is located in the immune synapse interface and recruits cytosol phosphatase SHP-2 to dephosphorylate CD28 [166]. PD-1 may also dephosphorylate phosphotyrosines in other TCR-associated proteins, such as Zap-70 and CD3 [165]. CTLA-4 shares two ligands, CD80 and CD86, with the stimulatory receptor, CD28, and can downregulate CD28 activity by binding its ligands [167, 168].

3.5 Signal Transduction Downstream of the p–MHC

Not only T cells but also target cells (“true targets” and APCs) can sense the immune synapse and react accordingly. For professional APCs, TCR/self-p–MHC contact during routine immune monitoring may cause signaling on the APC side to prevent its killing. After T cell attachment, DCs activate signaling mechanisms that facilitate cell–cell communication and actin and membrane remodeling [24, 169].

T cells can activate APCs by depositing their membrane fragments that contain TCRs, costimulatory and adhesion molecules, and cytokines on cognate antigen-bearing APCs. CD4+ T cells transfer to DCs membrane proteins by trogocytosis and the budding of T cell microvilli particles (TMP). The TMPs contain CD2, CD28, CD4, CD25, and activating cytokines and, upon uptake, initiate DC activation, including a calcium response and expression of costimulatory proteins such as CD40, CD80, and CD86 [24]. Memory CD8+ T cells release the DC-activating factor TNF-α, which induces the expression of an endogenous granzyme B inhibitor, PI-9, that protects DCs from killing by CD8+ effector T cells [170].

The reactions developed by cancer cells in response to T cell binding may include various pathways, such as induction of inhibitory ligands and cytokines [171, 172], abnormal tumor angiogenesis [173], downregulation of MHC expression [174], secretion of inhibitory exosomes [175], and complex modulation of the tumor microenvironment [176].

3.6 αβ TCR Clustering

Clustering and spatial cooperation of proteins in membranes are observed in many signaling pathways. Although TCR clustering may exist without antigen activation [100], such activation leads to a spatial reorganization of TCRs into signaling-competent clusters. In turn, initial TCR clustering may cooperatively facilitate further cluster development attracting additional resources from both membrane and cytosolic compartments [177].

The efficiency of the T cell reaction can be achieved either if the TCR has a relatively high affinity to p–MHC or if the affinity is relatively low, by cooperative involvement of neighboring TCRs. TCRs on the T cell membrane are usually localized in lipid rafts as 2D nanocluster aggregates, and the reaction on a single TCR may be amplified by lateral (horizontal) activation extension in the cluster. Therefore, structural rearrangement of activated TCR adaptors in the underlying cytosol might facilitate similar processes in its vicinity and promote cooperative lateral activation without additional p–MHC ligation. A strong positive cooperativity between individual TCRs has been detected in nanoclusters containing up to 20 TCRs [90, 178, 179]. In these experiments, binding of only two p–MHC (a p–MHC dimer) could stabilize 20 TCRs in the signaling-competent state [180]. Such allosteric reactions between TCRs can be an important factor of TCR selectivity that allows the detection of a relatively rare and weak “signal” (foreign p–MHC l) in the presence of abundant “noise” (self-p–MHC) [181]. Clustering of TCR complexes can be mediated by extracellular domain oligomerization, intracellular domain interactions, and attached cytoplasmic scaffold proteins [100].

In contrast to naïve T cells, T_{EFF} and T_{MEM} cells have a lower threshold of activation and response and also an elevated level of TCR clustering [178]. Unlike B cells, T cells lack the capacity to undergo “affinity maturation” after antigen engagement. However, “functional avidity maturation” can be achieved by TCR clustering [178, 182].

3.7 Structure and Signaling of the γδ TCR

The structure and function of the TCR in γδ T cells appear to be fairly different from αβ T cells. While the γδ TCR sometimes contains the same CD3 complex as the αβ TCR, for the recognition of homodimer, it uses TCR-γ and TCR-δ chains instead of TCR-α and TCR-β chains. In addition, Hayes et al. report that some γδ TCRs lack the CD3 δ chain and instead have the stoichiometry: γδ, γε, γε, and ζζ [183–186]. Similar to αβ TCRs, γδ TCRs are contained in lipid rafts and can form clusters on the cell surface [187]. Distinct from αβ T cells, γδ T cells do not require “classic MHC” molecules to recognize antigens, and they do not require CD4 or CD8 coreceptors.

Given the underlying differences, it is not surprising that signaling mechanisms in γδ T cells are distinct from αβ T cells. The γδ TCR has a stronger signaling capacity, which may be due to the fact that they constitutively express approximately twofold more of the TCR/CD3 complex than αβ T cells [188–190]. Also, the γδ TCR may provide signal transduction without a conformation shift of the CD3 complex. This quite unexpected divergence from the αβ TCR may be caused by differences in the TCR-γ and TCR-δ amino acid content, their glycosylation and orientation in the membrane, the pattern of TCR clustering, or the complement of associated kinases [191, 192]. For example, B lymphoid kinase (Blk), an Src family kinase expressed primarily in B cells, is expressed in γδ T cells but not in αβ T cells [193]. In addition, a subpopulation of γδ T cells, but not αβ T cells, has been detected in Lck-deficient and Zap-70-deficient mice, suggesting that Lck and Zap-70 are necessary for αβ T cell viability but not for γδ T cells [192]. In primary murine γδ T cells, TCRs contain ζζ homodimers. However, following ex vivo activation and expansion, one or both ζ subunits are replaced with FcεR1γ proteins [183].

4 Synthetic Receptors

4.1 Engineered TCRs

Synthetic TCRs (synTCRs) can potentially recognize all peptides processed and presented in the context of MHC molecules, thus allowing TCRs to target both surface and intracellular antigens. On the other hand, TCRs only recognize peptides in the context of the MHC complex. Therefore, this approach is hindered by some factors, including the need for MHC matching, MHC downregulation by cancer cells, suppressive tumor environment, and off-target/off-tumor killing [194]. In addition, the activity of TCR-transduced cells may be affected by the formation of mixed dimers between exogenous and endogenous α and β proteins which may decrease activity or lead to nonspecific reactivity [195, 196]. To prevent such dimerization, the selective binding between α and β proteins in synTCRs can be achieved by rearranging specifically interacting amino acid sequences in α and β constant domain interface [197] or by adding a second disulfide bond [198]. Another approach is to construct synTCRs with a murine constant region in place of the human constant region.

This resulted in preferential pairing of the murine constant domains in α and β subunits and higher expression of the engineered TCR on the surface of the human lymphocytes [199]. One theoretical drawback in the use of mouse TCR domains might be the development of human anti-mouse TCR immune responses; however, this was not observed in clinical trials [200]. Expression of cancer-reactive γδ TCRs in αβ T cells prevents formation of mixed dimers [147]. This reprogramming accompanied by CRISPR-mediated elimination of the endogenous αβ TCR led to increased γδ TCR expression and efficiency irrespective of patient MHC type [201].

Besides optimization of amino acid content, several other factors should be considered in constructing a synthetic TCR. Adequate expression of exogenous αβ heterodimer depends on the configuration of the vector and expression cassettes. It also depends on availability of CD3 proteins (ε, ζ, δ, γ) for correct assembly in the Golgi [195]. Co-transfer of CD3 and αβ genes into primary murine T cells enhanced TCR expression and antigen-specific T cell function in vitro and in vivo [202]. TCRs with high affinity, not available in normal T cells because of negative selection in the thymus, may enhance the ability to kill target cells in the cancer environment [203]. Engineered T cells containing “high-affinity” TCRs showed efficiency in treating myeloma and synovial cell sarcomas [204, 205]. Also, generation of synthetic TCRs able to recognize specific cancer neo-antigens or known cancer-specific antigen/MHC combinations can be advantageous for developing individualized anticancer therapy [206, 207].

Another approach is to create synTCRs with antibody recognition domains. In these receptors, exodomains of α and β subunits of the TCR are modified by replacing their variable domains with antibody domains that can recognize cancer-associated antigens. It can be just variable domains, V_H and V_L [208], or Fab fragments with V_H–C_H domains fused over the TCR-α constant domain and V_L–C_L domains fused over the TCR-β constant domain [209]. The resulting chimeric TCR is expressed on the surface of cytotoxic T lymphocytes, recognizes antigen in a non-MHC-restricted manner, and transmits the signal through the CD3 complex for T cell activation [208]. The absence of p–MHC in the synapse excludes CD8 co-signaling; however, the affinity of Fab can be increased to a level that sufficiently compensates for the absence of CD8 [128].

4.2 Chimeric Antigen Receptors (CARs)

Basic chimeric antigen receptors (CARs) contains three elements: (1) a recognition domain that is a surface ligand-binding domain; (2) a transmembrane (TM) domain that is a structure ~20 amino acid long, enriched with hydrophobic amino acids (AAs) and forming an alpha helix in the cell membrane; and (3) an intracellular effector part that can contain various signaling domains needed for sustained effector cell ability to kill and propagate [208, 210].

Upon ligation to cognate antigens on the target cell, it is thought that the CAR dimerizes at the site of recognition and undergoes a conformational shift in its cytoplasmic domains, which leads to their phosphorylation, binding, and activation of Zap-70 with sequential activation of multiple signaling cascades [211]. In support of dimerization occurring, a mutation in a CAR’s ectodomain that facilitates spontaneous dimerization (even without

antigen recognition) increased its functionality [212], whereas mutations in a CAR transmembrane domain that resulted in disruption of dimerization led to decreased CAR-T cell activation and cytolytic activity [213].

First-generation CARs contain a single-strand antibody (ScFv), TM, and CD3-ζ signaling domain [210, 214]. Second-generation CARs include a coactivator cytoplasmic domain in *cis* to provide additional T cell co-stimulation. The most widespread is a CD28 or 4–1BB signaling domain inserted between the TM and ζ domains. CD28 signals through activation of LCK, PI3K-Akt [215], Grb2, and Gads [216] and induces Bcl-X_L [217] and IL2 [216]. 4–1BB signaling upon aggregation (trimerization) of 4–1BB ligand attracts TNF receptor-associated factors and forms a "signalosome" that activates T cell proliferation and survival [218]. This leads to phosphorylation of CD3 proteins ε and ζ, Lck, and LAT [219]. Other costimulatory domains, like ICOS, OX40, and CD27, can also function in CARs between the TM and ζ domains [220–222]. Third-generation CARs include two costimulatory domains, like CD28 and 4–1BB inserted between TM and ζ [223]. This additional co-stimulation apparently increases the basal activity of CARs and can be counterproductive due to baseline activation and auto-toxicity [224, 225].

CARs can apparently function in many different cytotoxic immunocytes [1, 226]. For human CD8+ T cells, the granzyme–perforin pathway seems to be the most common activated by the CAR, as this is the predominant cytotoxic mechanism in human T cells [46, 47, 227]. However, other pathways are also used as Hong et al. demonstrated Fas-mediated killing by CD30 CAR-T cells [228]. Because some CD4+ T cells possess cytotoxic activity, they also can be reprogrammed for CAR-mediated killing [227, 229]. Beyond conventional αβ T cells, CAR-mediated killing has also been shown in NK cells [230, 231], γδ T cells [232, 233], NKT cells [234, 235], and neutrophils [236]. While the mechanisms of killing by other effector cells reprogrammed with CARs might be more diverse, it is assumed that upon target recognition, CARs can activate the natural cytotoxic signaling pathways present in a host cell. Interestingly, for macrophages, a CAR that contains the cytosolic domains of Fc receptor instead of the ζ-signaling domain leads to phagocytosis upon target recognition instead of cytotoxicity [5].

In T cells, analysis of CAR-mediated targeting showed that affinity to cognate antigen in the interval of 10 μM to 1 mM allows for both effective recognition and dissociation when the T cell action is completed [237, 238]. However, lower affinity might be preferable to prevent off-tumor killing [239]. Steric hindrance both inside and outside the cell should be taken into consideration when designing a CAR. The length of the extracellular segment should be comparable with the optimal TCR/p–MHC distance at ~15 nm (*see* Subheading 3.4) [212, 240]. Likewise, steric limitations should be applicable to the cytosolic part of the CAR, because the signaling adaptor proteins should act in a certain distance from the cell membrane. Of course, the sophisticated TCR architecture makes it difficult to easily deduce the ideal CAR sequence. That is why during CAR construction, combinations of ecto-, TM, and endodomain amino acid contents have to be tested in parallel to determine variations in target affinity and signaling. It is important to note that even small differences in amino acid content can dramatically change the tertiary structure of the CAR, with obvious consequences for protein stability and function [241].

4.3 Comparing CARs with αβ TCRs

In engineering T cells, the simplicity of a CAR compared to a synTCR leads to both advantages and disadvantages. One advantage is that CARs recognize and bind targets independently of MHC and coreceptors like CD4 or CD8. In addition, CARs can recognize a wide spectrum of ligands on the cell surface, including proteins, carbohydrates [242], glycolipids, and other moieties [243], that are usually not recognized by TCRs. The CAR recognition motif structure appears flexible as scFvs, ligands (e.g., CD70 that binds CD27 receptor) [244], and single-strand avidin (that binds biotinilated targets) [245, 246] are all functional. Compared to TCRs, CARs provide a faster killing dynamic [149] and can be used in the presence of other CARs and TCRs in the host, both independently of them and in cooperation [247].

The main disadvantages of CARs are that they cannot target intracellular antigens and they do not communicate through the balanced system of CD3 proteins and coreceptors leading to less regulation in cytosolic signaling. That can undermine some important functions such as antigen recognition proofreading and adequate dynamics of the cell response. For example, first-generation CARs with a sole ζ chain as a signaling domain and without a coactivator signal were unable to maintain robust T cell viability in the presence of cognate antigen. In contrast to the TCR, the conventional CAR is not involved in cell monitoring and combinatorial antigen evaluation. Rather, it works as a binary operator that turns on the response as soon as it recognizes the target. Compared to conventional CTLs, CAR-T cells are less sensitive to p–MHC density. Whereas a CTL's response may need only a few agonist p–MHCs per target cell [148–151], a CAR-T cell response may need about ~200 antigens per cell [248, 249].

Another major flaw of CARs compared to TCRs is that they usually produce a high basal signal, which can be deleterious for T cell viability [250]. Whereas the TCR emerges from the cell membrane as a tightly cooperative complex with accurate regulation of its subunits' conformation, the CAR emerges as a single protein prone to specific and nonspecific reactions with surrounding molecules. Thus, while TCR tonic signal is caused by ligation with self-p–MHC and is a part of T cell homeostasis, the basal signal activity of the CAR is independent of antigen presentation and may disrupt T cell homeostasis. This basal activity is correlated with the density of CAR proteins on the cell membrane [250, 251]. In fact, CAR proteins can spontaneously aggregate in the cell membrane independent of external ligands, potentially because of thermodynamic driving factors and variation in physicochemical properties of CARs and surrounding proteins [252]. Multiple factors may determine CAR aggregation and toxicity including the spacer connecting the CAR's recognition and TM domains [253], the configuration of the CAR's active ITAMs [254], and the activity of T cell death signaling pathways, Fas and DR5 [255].

The comparison of CD19 CARs containing 4–1BB-ζ and CD28-ζ cytoplasmic tails showed that CD28-ζ CAR had higher spatial aggregation and more "basal" ζ phosphorylation and was more toxic for T cells [250]. However, basal CAR toxicity also has been shown for 4–1BB-ζ CAR [251].

4.4 Complications in the Clinical Use of CAR-T Therapy

Although CAR-T cells have been very successful in some clinical trials, this therapy is associated with serious complications. Among other factors, problems associated with CAR-T cells include uncontrollable activation, expansion, and persistence, as well as on-target/off-tumor and off-target/off-tumor killing. Upon introduction in patients, CART-19 cells can achieve rapid proliferation (up to 10^4-fold expansion), which may result in tumor lysis syndrome (TLS), cytokine release syndrome (CRS), and neurotoxicity [256–259]. In addition, CART-19 therapy kills all CD19+ cells leading to B cell aplasia [256]. On the other hand, B cell tumors with a mutated CD19 can escape CART-19 killing [260–262].

5 Next-Generation Strategies for T Cell Engineering

This quickly developing field has extended in many directions in an attempt to improve upon the original CAR and TCR approaches. Designs to improve both safety and efficacy include modifications of the CAR itself, combining multiple CARs, and adding multiple factors in addition to the CAR.

5.1 Construction of CARs with New Domains

Since the first description of a CAR, an impressive number of structural modifications with a wide number of variations have been introduced. Considerable attention has been focused on the discovery of novel scFv recognition domains to target different antigens. However, any structure able to bind a cancer cell is a theoretically viable alternative.

Several “universal” CAR systems have been constructed. In one strategy, the antigen-recognizing domain is replaced with a monomeric avidin moiety that binds biotin. Biotinylated tumor-specific molecules, such as a monoclonal antibody, can then direct the CAR-T cell to different target cells. Simply changing the biotinylated antibody redirects the CAR-T cell to recognize and trigger killing of cancer cells that are “stained” with the biotinylated monoclonal antibody [245, 246]. In a second strategy, Cho et al. replaced the scFv with a leucine zipper domain, such that the CAR could bind to a second chimeric protein composed of the cognate leucine interaction domain fused to an scFv. Introduction of such second chimeric proteins can continuously redirect the CAR-T cell activity [263]. A third strategy fuses a tumor antigen-specific Fab with a peptide that binds to a CAR. Peptide-associated Fabs that can have different specificities can be systemically delivered in vivo to connect the CAR-T with cognate antigen on the target cells. In this system, the antigenic diversity of Fabs and their dose determine CAR-T antigen specificity and the level of activation [212].

In addition to universal systems, another approach attempts to develop a CAR that can emulate the recognition ability of a TCR for agonist p–MHC. Here, a two-gene system is used. The first gene encodes a CAR where the scFv is replaced with a TCR-α chain that is truncated at the TM region and contains a cysteine [264]. The second gene encodes a TCR-β chain that is also truncated at the TM region and contains a cysteine to mediate disulfide bridging. When expressed, the TCR-β chain binds to the TCR-α chain of CAR, creating an αβ TCR/CAR hybrid. This approach increases the spectrum of antigen recognition, by

inclusion of intracellular antigens, although in the context of MHC. It can be especially important in the treatment of cancer cells with neo-antigen markers as an alternative to TCR-mediated therapy.

Whereas the previous approach attempts to bring together TCR binding with CAR signaling, others have tried to create CARs with scFv binding that directly engage with TCR signaling. One approach to directly utilize TCR signaling is a “T cell antigen coupler” (TAC) [265]. In this design, the CAR contains an anticancer scFv recognition domain attached to the CD4 TM and signaling domains. In addition, a CD3-ε-binding domain is inserted in the CAR between the scFv and the TM, which results in CAR attachment to the CD3-ε subunit of the TCR. When the CAR’s scFv binds cognate antigen, the signal transduction goes through the CD4–TCR complex. In mouse models, this approach yielded increased antitumor efficacy with reduced toxicity. A second approach, called T cell receptor fusion constructs (TRuCs), was developed by sequentially attaching scFv domains to each subunit of the TCR [266]. In TRuC-T cells, the scFv is incorporated into the TCR and binding an antigen in an MHC-independent manner engages the signaling capacity of the entire TCR. Among the different TRuCs tested, fusing the scFv to the ε subunit showed the highest level of functionality. This is potentially due to its stoichiometric advantage as well as its specific cytoplasmic docking sites for Nck [116], GRK2 [267], CAST [268], and phospholipid-binding motif [269].

A separate tactic to increase CAR-T cell functionality is to insert in the CAR’s cytoplasmic tail domains that can emulate the signaling by homeostatic interleukins. Kagoya et al. introduced binding motifs for STAT5 and STAT3 in CD19 CAR and showed that the new CAR strengthened the activation of JAK kinase and STAT3 and STAT5 signals, elevated in vivo persistence of CAR-T cells, and increased their antitumor activity [270].

Finally, significant improvements can be made even without modifying any of the functional domains but only taking into account the tertiary structure of CAR protein and the distances between recognition domains and signaling domains that could be crucial for signaling. Using CD19 CAR, Ying et al. modified nonenzymatic “scaffold segments” of the CAR by increasing the length of the hinge and the distance between TM and signaling domains. By computer modeling, they showed that such variations can dramatically change tertiary structure of the protein. Then, by adding additional amino acids to the hinge and two amino acids between the TM and CD28-ζ, they were able to decrease the level of CAR signaling and the rate of CAR-T propagation in the presence of targets; and by that means virtually eliminate the development of chemokine shock in a mouse model and a clinical trial [241].

5.2 Combining More Than One CAR in a Cell

Expression of multiple constructs in T cells potentially can provide recognition of multiple antigens on the targets to discriminate between cancer cells and normal cells, and obtain adequate T cell signaling by switching both activation and inhibition pathways [221, 271–273].

This approach may increase CAR-T cell efficiency in situations where tumors downregulate the expression of cognate antigens. For example, failure of the CD19 CAR against B cell tumors is sometimes caused by internal deletions in CD19, which remove the recognized

epitope [274]. In such a situation, the expression of an additional CAR that recognizes other B cell markers can prevent tumor escape [275]. This has been accomplished both by supplying two separate CARs and by supplying one CAR that contains two tandem scFvs, each for a different antigen [276]. In the latter case, because different antigens may have different length, the CAR should have appropriate sterical configuration of its recognition domains that not only allows simultaneous ligation with antigens but also maintains an appropriate distance to the target membrane for synapse formation.

A two-CAR system also may increase CAR-T cell selectivity. For example, consider the scenario where a normal cell has only one of two surface antigens present on a cancer cell. A T cell expressing two CARs, one that recognizes each antigen with sufficiently low affinity, may proceed with killing only when both CARs are simultaneously ligated with the target [273, 277]. In another scenario, a cancer cell may have only one of two surface antigens present on a normal cell. In this case, one CAR can recognize the shared antigen promoting killing, while a second CAR, known as an inhibitory CAR (iCAR), can recognize only normal cells and contain a signal inhibitory domain instead of the activation ζ domain. Fedorov et al. have shown that an iCAR with a segment of the inhibitory PD-1 cytoplasmic tail can prevent the stimulation of the other CARs when the T cell interacts with a cell containing both antigens [278].

Playing off the theme of converting positive to negative signals, switch receptors were designed to convert a negative signal to a positive one. Here, the extracellular domain of PD-1 is combined with the cytoplasmic domain of CD28. When the switch CAR binds the inhibitory ligand PD-L1, it activates T cells through the CD28-mediated pathway. In solid tumor models, T cells reprogrammed with both CAR and the switch receptor showed augmented efficacy compared to CAR-T cells [279–282].

5.3 Using Additional Genes to Reprogram the T Cells

The fourth generation of CARs, armored CARs, has been made by combining two expression cassettes: one coding a second-generation CAR and another coding an additional metabolically active protein, such as a cytokine, antibody, or another ligand [283]. Since CAR-T cells accumulate in the tumor, the active proteins are delivered locally to the site of disease, minimizing the toxicities often associated with active proteins delivered systemically. T cells loaded with a CAR construct armored with the proinflammatory cytokine IL12 showed elevated antitumor efficacy [284, 285]. CAR-T cells armored with IL18 increased CAR-T cell survival and enhanced immune response by modulating tumor microenvironment [286]. A CD20 CAR-T cell armored with IL7 and the chemokine ligand CCL19 improved immune cell infiltration and CAR-T cell survival in the tumor [287]. CAR-T cell persistence and efficiency can also be enhanced when cells are reprogrammed with constitutively active homeostatic receptors, such as IL2, IL7, or IL15 receptors, or with chimeric cytokine receptors that switch a negative signal produced by inhibitory cytokines, such as IL4, to a positive signal [288]. CD19 CAR-T cells, armored with the immune activator protein CD40L, exhibited increased cytotoxicity against CD40+ tumors and extended the survival of tumor-bearing mice in a xenotransplant model of CD19+ systemic lymphoma [289]. CAR-T cells loaded with a PD-1-blocking scFv enhanced the survival of

PD-L1+ tumor-bearing mice in syngeneic and xenogeneic mouse models through both autocrine and paracrine mechanisms [290].

Suicide switch constructs can eliminate CAR-T cells upon systemic delivery of a signaling molecule. For example, this construct may contain caspase-9 protein fused with a protein that can be dimerized by a drug. In the presence of the drug, the caspase-9 dimerizes and activates the intrinsic apoptotic pathway [291]. A second strategy introduces a truncated epidermal growth factor receptor (EGFRt) that is recognized by the antibody cetuximab, which mediates antibody-dependent cellular cytotoxicity (ADCC) against the CAR-T cells [82]. However, kill switches do not allow control of the rate of T cell activation and expansion. Rather, they are turned on after the recognition of a problem and once activated cannot be reversed. In addition, leaky expression of an inducible suicide gene can undermine efficacy, while incomplete activation or targeting can undermine the purpose of the suicide switch.

Lim et al. improved the functionality of CAR-T cells by adding a synthetic Notch (synNotch) receptor that contains an antigen recognition domain (scFv), fused to the Notch regulatory core domain and a transcription activator domain. Binding of the cognate antigen stimulates cleavage of the receptor and releases the transcriptional activator, which can enter the nucleus and drive ectopic expression of genes inserted in the T cell DNA under an activator-specific promoter. One synNotch receptor, upon antigen binding, can activate multiple genes regulated by the same promoter [271, 292].

6 Challenges and Potential Future Solutions

A combinatorial approach for cancer treatment is important to emulate the complexity of the immune system in fighting tumors. Here, we will consider ways to (1) better emulate the TCR, (2) use other cells instead of αβ T cells, and (3) use other cells in combination with αβ T cells.

6.1 Better Approximation of the TCR

Conceptually, CAR-mediated activity may emulate TCR function with similar intercellular synapses and signaling pathways. Further improvement of CAR functionality might lay in better understanding the relationship between CARs and TCRs. CAR-mediated synapses are structurally and functionally different from TCR synapses, being smaller, less structured, and shorter-lived [149]. The CAR-T synapse, created without a TCR, is obviously deficient in coreceptors (CD8 or CD4) and in underlying processes related to coreceptor binding to MHC such as delivering additional Lck activator for Zap-70 (Subheading 3.3). In addition, they do not bind to MHC on target cells, so the target cell membrane state also can be different.

An important goal is to attain CARs that build a cooperative complex with CD3 (*see* Subheading 5.1). If the cytoplasmic tail of the CAR is not included in a tight TCR complex, it may constantly be in a “loose” conformation in the cytosol, prone to some degree of nonspecific aggregation or phosphorylation by Lck and basal signaling.

Optimizing the spatial and functional relationship of the CAR to the TCR is predicted to boost activity. Current experimental data suggests that the CAR–TCR relation can be quite flexible. T cells with defective TCR expression are still able to provide CAR-mediated killing [263, 293]. On the other hand, cells with an active TCR may contain TCRs as part of the CAR synapse [266]. A more integrated relationship between CARs and TCRs may be advantageous for CAR-T cell efficiency.

6.2 Expanding Beyond αβ T Cells

Another direction comes out of the fact that TCRs and CARs can work not only in αβ T cells but also in other immune cells involved in the innate response. In such cells, ectopic TCR and CAR activity can be combined with innate modalities to create a more complex immune response. As already mentioned, expression of endogenous TCRs has been shown in various myeloid cells, including eosinophils [294], neutrophils [79], monocytes, and macrophages [81, 294–296]. Ectopic TCRs and CARs that redirect immune cells against cognate targets can be expressed in NK cells [297, 298], NKT cells [299], γδ T cells, and cytokine-induced killer (CIK) cells [2]. In contrast to αβ T cells, certain subsets of innate immune cells have natural attraction to tumors through the recognition of stress-related tumor antigens.

γδ T cells serve as a particularly illustrative example. One reason they are attractive for ACT is that they have natural tropism for several types of cancer by monitoring stress-induced and inflammatory markers, such as lipopeptides, pyrophosphates, microorganism-derived proteins, and self-proteins through their γδ TCR, Toll-like, NK, and CD16 receptors [191, 192, 300]. In addition, they detect alterations in cell surface molecules, such as the MHC class I chain-related ligands A and B (MICA and MICB), and cell-associated antibodies [301, 302]. Their antitumor activity involves cytokine and chemokine secretion [303] and cytotoxicity. In contrast to αβ T cells, γδ T cells do not have strong lineage separation between helper and cytotoxic subsets, but do include T_{EFF}, T_{CM}, and T_{EM} cells [303–307]. Cytotoxicity is accomplished through perforin–granzyme, TRAIL, FasL, and ADCC [308]. In addition to cytotoxicity, γδ T cells are able to phagocytose and present tumor antigens to CD8+ αβ T cells, as well as induce DC maturation by TNF-α secretion [309]. Finally, as an added advantage, γδ T cells are mainly not alloreactive and do not induce GVHD [310].

Both ectopic TCRs and CARs can be used to reprogram γδ T cells for adoptive therapy [301]. Transfer of ectopic αβ TCRs into γδ T cells can be provided without TCR mispairing and formation of mixed TCR heterodimers. γδ T cells engineered to express human αβ TCRs exhibited high levels of antitumor cytotoxic activity and cytokine release [311, 312]. Both GD2 and CD19 CARs have been used in peripheral blood-derived γδ T cells and shown to exhibit target-specific IFN-γ secretion and cytotoxicity [313, 314]. In the case of a GD2–CD28–CD3-ζ CAR, the reprogrammed γδ T cells showed enhanced GD2-specific killing beyond the γδ T cells without the CAR, which also recognized the tumor cells with endogenous “stress receptors.” Expanded CAR-T cells retained the ability to take up tumor antigens and cross present the processed peptide to responder αβ T cells [233]. To refine the specificity of γδ T cell cytotoxicity, Fisher et al. created a GD2 CAR in which the ζ domain

had been replaced with innate NKG2D signaling molecule DAP10 [315]. These γδ CAR-T cells killed GD2+ glioblastoma cells, but not GD2+ control cells, by working with the endogenous γδ TCR targeting glioblastoma "stress receptors." One way to augment the expression of tumor stress-related antigens can be by less specific treatments, like chemo- and radiotherapy [316].

Two obstacles to overcome in using γδ T cells are relatively low cell numbers, and they can also promote cancer progression by inhibiting antitumor responses, enhancing cancer angiogenesis, and increasing the population of myeloid-derived suppressor cells (MDSCs) [14, 317]. Thus, accurate amplification, evaluation, and selection of γδ T cell subsets will be an important part of their optimization for therapy. To some extent, the number and distinct activity of γδ T cells can be modulated with cytokine stimulation [14].

6.3 Total Immune System Engagement

The human immune system is very complex with various types of cells, proteins, and subcellular particles working synergistically against various diseases, including infection, cancer, and aging. The ability of pathological agents for fast propagation, modification, and population plasticity dictates the general immune system works as a multifactor combinatorial defense. Although today immune cell reprogramming is still limited to relatively simple combinations tested in clinical studies, it is clear that in the future, multi-cell, multi-ligand treatment will need to be developed for an efficient and flexible therapeutic approach. This can be especially important for the treatment of solid tumors that are often resistant to conventional CAR-T therapy [2] because of poor tumor recognition, penetration, and inhibition by the tumor microenvironment.

In addition to using T cells expressing multiple CARs and other chimeric receptors, B cells, NK cells, and other combinations of reprogrammed immune cells could be used. Most of the anticancer therapeutic designs are not mutually exclusive and can be applied in combinations to maximize the outcome. The improvement of therapy employing CARs and ectopic TCRs probably will depend on their cross talk with tumor-resident immunocytes. For example, the treatment may include co-introduction of αβ and γδ T cells reprogrammed with CARs and TCRs, DCs loaded ex vivo with tumor-specific antigens, cytokines, and checkpoint inhibitor ligands delivered locally to the tumors by "armored" T or NK cells. The versatile treatment will integrate the most efficacious combination of the helper, effector, and memory cells with their modulators that allow to maximize the therapeutic specificity and safety.

As a second example, tumor-infiltrating lymphocytes (TILs) are cells that can recognize and penetrate tumors. These cells can be extracted from the tumor, activated and propagated ex vivo, and then used for ACT. However, some solid tumors contain very low numbers of infiltrating T cells. So, combining therapies that increase T cell recruitment with subsequent TIL treatment might be beneficial. Although T cell recruitment can depend on chemokine gradients created by tumor-resident dendritic cells, the recruitment of DC in tumors is a process that is not fully understood. It may depend on multiple factors, including both activation and inhibition influenced by other tumor-resident cells such as macrophages and NK cells [318]. Here again, understanding better the mechanisms of intratumoral immune

cell signaling might lead to approaches to more fully engage and reprogram multiple arms of the immune system.

7 Conclusions

T cell reprogramming has demonstrated that methods of genetic and cell engineering can be broadly used to great clinical benefit by augmenting the body's own immune defense. Simple designs combining a few domains are starting to give way to more complicated approaches that either more closely emulate actual TCR signaling or integrate secondary signaling pathways. Similar approaches have been successfully used in reprogramming other immunocytes and have rather universal applicability in cell biology for other cell types and other diseases. This is especially encouraging for the development of combination therapies that employ multiple reprogramming factors and reprogrammed immune cells, with accurate monitoring of clinical outcome and fast adjustments of the treatment. The accumulation of knowledge from numerous academic, biopharmaceutical, and clinical results is rapidly translating our thought processes and ability to conquer cancer.

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