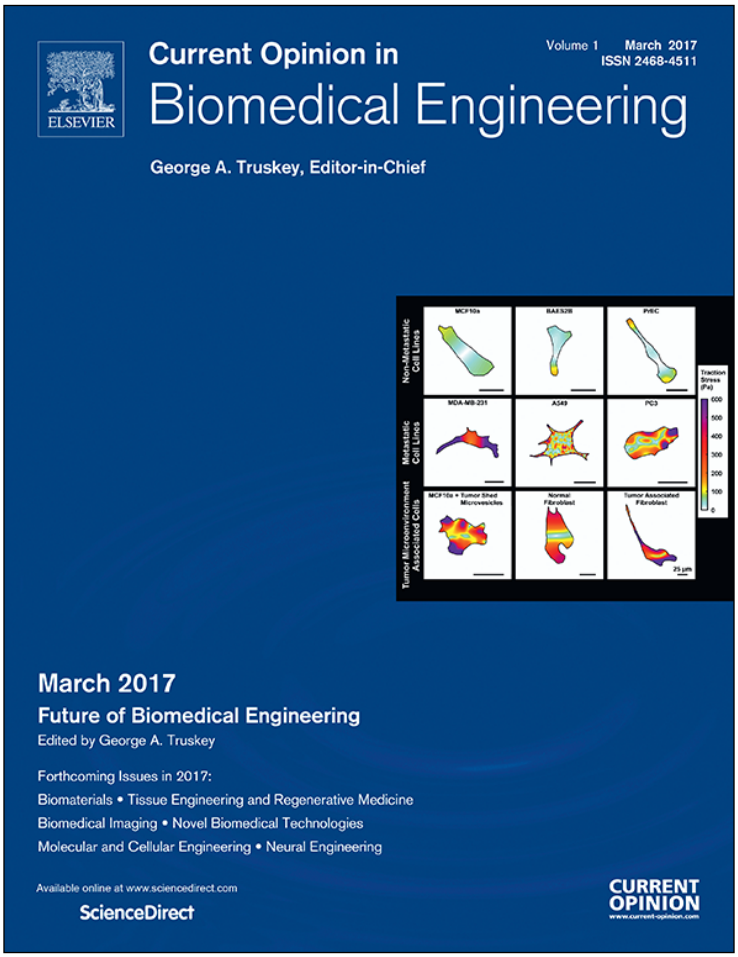

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Immune engineering: From systems immunology to engineering immunity

Ning Jiang

The smallpox vaccine represents the earliest attempt in engineering immunity. The recent success of chimeric antigen receptor T cells (CAR-T cells) in cancer once again demonstrates the clinical potential of immune engineering. Inspired by this success, diverse approaches have been used to boost various aspects of immunity: engineering dendritic cells (DCs), natural killer (NK) cells, T cells, antibodies, cytokines, small peptides, and others. With recent development of various high-throughput technologies (of which engineers, especially biomedical engineers/bioengineers contributed significantly), such as immune repertoire sequencing, and analytical methods, a systems level of understanding immunity (or the lack of it) beyond model animals has provided critical insights into the human immune system. This review focuses on recent progressed made in systems biology and the engineering of adaptive immunity.

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Introduction

Engineering and medicine have gone hand in hand in human history; after the problem is identified, it is intuitive to try to fix it and this is what engineers do best. Recent advances in high-resolution imaging modalities, mass cytometry, and next generation sequencing have provided the requisite tools for advancing biomedical research and health care. Immunology is one research area that has benefitted from this series of technology developments. This combination of technology and quantitative analysis marks the beginning of a new research area: systems immunology. The complexity of the immune system renders it a superb candidate to be tackled by a systems biology approach that combines computational and experimental techniques. Various discoveries made in systems immunology will provide new targets for immune engineering and the potential for personalized diagnosis. With expertise in quantitative analysis and engineering design principle, biomedical engineers are poised to be at the front of engineering immunity.

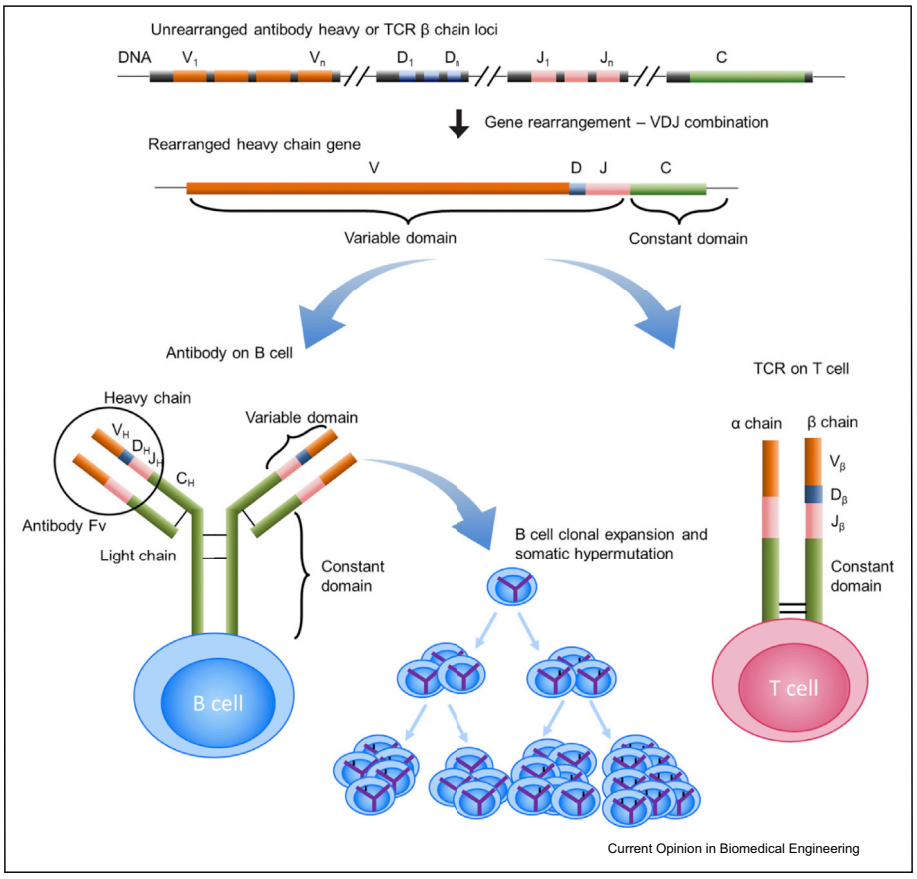
Mirroring the complexity of the immune system, immune engineering targets many cellular and molecular processes: boosting the antigen presentation, reinvigorating exhausted T cells, engineering immune effector molecules and cells, optimizing drug delivery to target specific immune cells or tissues, and engineering new materials for immunotherapy. In this review, I will focus on some of the recent advances in the area of engineering of the adaptive immunity, which includes antibodies and T cells.

From antibody repertoire profiling to engineering

The human immune system generates an enormous repertoire of B cells, each bearing a new antibody gene that is the product of the recombination of a set of canonic gene segments (Figure 1). Upon antigen stimulation, responding B cells go through an activation and proliferation process that is accompanied by introducing mutations to their antibody genes [1,2]. B cells that bear favorable antigen-binding mutations will have a competitive edge to proliferate and survive over those bearing mutations that disfavor antigen binding [3,4]. Thus, the composition of the antibody repertoire changes after each infection and vaccination [2,5]. Tracking these changes offers insights to how a protective immune response is generated, and ultimately informs engineering immunity.

Immune repertoire sequencing leverages the capacity of next-generation sequencing technologies to sequence millions of genes encoding either antibodies or T cell antigen receptors (TCRs). This technology development has made it possible to provide a comprehensive view of the expressed antibodies or TCRs in a sample [6–9], which has been a black box for decades. Coupled with statistic analysis and information theory, this technology has provided in depth analysis of the antibody repertoire in response to vaccinations [10–12],

Figure 1



Antibody and T cell receptor repertoire diversity generation. Genes encode the variable domains of the antibody heavy and light chains and the T cell receptor (TCR) α and β chains go through a recombination process each time a new naïve B or T cell is generated. One gene segment from each of the three groups of gene segments (V, D, and J) are randomly recombined to form a new antibody or TCR sequences. There are also random nucleotides introduced at the junction of V and D, and D and J. In this way, a set of canonical gene segments are able to generate an enormous repertoire of antigen receptor sequences. Unique to antibody or B cell receptor, its gene sequences can also change itself by introducing random mutations when the B cell expressing a particular antibody is stimulated by antigen and clonally expands. Thus, progeny B cells become a mixture of sub-species, each expresses a different antibody sequence and is represented by different number of cells. This mutational process has a higher rate compared to somatic mutation, thus was coined the term 'somatic hypermutation'. On the other hand, T cells do not have somatic hypermutation, thus the TCR sequences expressed by the parental cell pass on to all its progenies.

infections [12], autoimmune diseases [13], and cancer therapy [14]. These analyses shed light on fundamental questions that were not possible to answer in immunology before. One such study was designed to examine the conflicting finding that there was an apparent stereotypy in antibody VDJ gene segment usage in young animals, but this stereotypy breaks when animals grow up. A large number of antibody sequences from individual organisms, from fish [15] to mammals [10], helped to demonstrate that stochastic somatic hypermutations and associated clonal expansion in some B cells when they encounter antigens mask the stereotyped VDJ usage that naïve B cells have. Another study showed that elderly are more likely to clonally expand existing B cells rather than improving the quality of antibody sequences that B cells express by introducing somatic hypermutations [10]. This level of detailed analysis has helped tremendously the search for broad neutralization antibodies against HIV [16]. The ability to obtain a large amount of sequences makes it possible to re-construct the evolutionary path a particular antibody sequence went through, which provides insights on critical mutations that help the development of broad neutralization antibodies against HIV. This helped the rational design of stepwise changing immunogenes that are capable of inducing these types of broad neutralization antibodies in animal models [17–21]. More recently, increased sequencing length, pairing of the heavy and light chains, and other molecular methods made it possible to cover a longer piece of the constant region of the antibody sequences to differentiate antibody sub-isotypes [22–24]. This facilitates Fc receptor based immune engineering aiming to modulate downstream of antibody-antigen binding. At the same time, technology development in mass spectrometry-based antibody repertoire profiling helps to illuminate the

antibody repertoire that executes protection in the serum [25,26].

Engineering T cells

By the same token, T cells rearrange their T cell antigen receptor (TCR) genes during T lymphogenesis (Figure 1). Contrary to somatic hypermutation in B cells, TCRs do not introduce mutation, and therefore the TCR sequences can be used as markers to track T cells as they develop into different functional phenotypes after activation [27]. Depending on which co-receptor is expressed, T cells can be separated into $CD4^+$ (also called helper) and $CD8^+$ (also called cytotoxic) T cells. Combinatorial expression of a set of transcriptional factors, cytokines, cytokine and chemokine receptors, adhesion molecules, and other surface and/or intracellular proteins can further classify $CD4^+$ T cells into subtypes, each with a distinct function. The role of many of these subtypes of $CD4^+$ T cells can be considered beneficial in some diseases but detrimental in others. For example, one subset of $CD4^+$ T cells, regulatory T cells (Treg), function to dampen or eliminate autoimmune response in healthy individuals with the breakdown of Treg functions being implicated in many autoimmune diseases [28]. However, this beneficial function of Treg is considered one of the major contributors to the immune-suppressive cancer microenvironment. Although $CD8^+$ T cells are not as versatile as their $CD4^+$ T cell counterparts in terms of functions, they are vicious killers once activated. A single $CD8^+$ T cell is able to kill multiple target cells in a day [29,30]. However, even these vicious killers can get exhausted (below) in persistent viral infection and cancer, which impacts their ability to properly form memory cells [31]. For both $CD4^+$ and $CD8^+$ T cells, forming memory cells at the end of a natural infection is how immune memory continues, which is the goal of vaccination and immunotherapy.

Adoptive cell transfer therapy in cancer and persistent viral infections

Researchers have spent a tremendous amount of effort harnessing the specific killing capacity of $CD8^+$ T cells for cancer immunotherapy [32] and persistent viral infection. Early effort using in vitro expanded tumor infiltrating lymphocytes (TILs) demonstrated durable results in certain cancers, such as melanoma [33] and human papilloma virus–associated cancers [34]. However, relying on tumor derived TILs has limited the wide use of the adoptive cell transfer (ACT) therapy. The advancement of genetic engineering has made it possible to engineer any T cell to express the TCR of choice, thus alleviating the problem of relying on surgically removed tumor materials to isolate TILs. However, the challenge now is to determine which TCRs to genetically engineer into T cells for ACT. One choice is to express TCRs that recognize cancer differentiation antigens (CDAs), such as NY-ESO-1. These antigens are abundantly expressed in cancerous cells but minimally expressed in healthy tissues. A recent clinical trial that used an NY-ESO-1-reactive TCR on patients bearing metastatic synovial cell sarcoma or metastatic melanoma demonstrated that on average 58% of the patients in both groups showed objective clinical responses and over 30% of the patients achieved 3 year survival [35]. It is worth noting that many patients in this trial had failed prior PD-1 blockade (below) treatment, one of the latest FDA approved cancer immunotherapies, before enrolling to this trial, demonstrating the clinical value of ACT using CDA specific T cells.

One bottle neck for using this approach is the difficulty of identifying high-affinity TCRs to these CDAs. High affinity, self-antigen (including CDAs) specific T cells are rare. This is, in part, because many of these high affinity self-antigen specific T cells have been deleted in the thymus before they are released into the peripheral blood and tissues to avoid auto-immunity [36]. In addition, the standard method to measure TCR affinity, surface plasmon resonance, requires labor-intensive cloning and protein purification and thus is a hurdle to screening a large number of antigen-specific T cells to look for those bearing high-affinity TCRs. Alternatively, protein engineering or yeast display screening can generate high-affinity TCRs. However, it has been shown that this kind of TCR can cause detrimental side effects by cross-reacting with other self-proteins expressed in healthy tissue, possibly due to the lack of thymic negative selection on engineered TCRs [37]. Recently, we have developed an adhesion based *in situ* TCR affinity and sequence test (iTAST) [38] that measures TCR affinity and sequences from primary single $CD8^+$ T cells directly purified from human samples with a throughput of approximately 75 cells per day. Furthermore, some of the hepatitis C virus specific T cells we obtained have an affinity higher than commonly recognized high-affinity mouse $CD8^+$ T cells, such as OT-I. In addition, these cells have passed thymic negative selection, therefore are not likely to induce auto-immunity and therefore, iTAST is a great tool for identifying high-affinity TCRs for ACT in persistent viral infection and cancer.

Recently discovered neo-antigens are another promising class of antigens for cancer immunotherapy. Neo-antigens are somatically mutated proteins expressed by cancer cells. Because of the mutations, neo-antigens are considered as foreign antigens. Unlike T cells recognizing CDAs, T cells recognizing these neo-antigen are not subject to the limitation of thymic negative selection that deletes most of the high-affinity T cells recognizing self-antigens including CDAs. Lennerz et al., pioneered the characterization of several of these neo-antigens and neo-antigen recognizing T cells found in a melanoma patient before the era of next generation

genomic sequencing [39]. With tens of thousands of cancer genomes sequenced and many more single cell cancer genomes, it is clear that somatic mutations are widely spread in cancers, ranging from 0.001 to several hundred per megabase [40]. At the same time, it was shown that cancer mutation load correlates with immune check point blockade therapy efficacy (see below) [41]. The obvious explanation lies again in the thymic selection: somatic mutations cause amino acid sequence changes which make these mutation-bearing peptides foreign to the immune systems. Therefore high-affinity TCRs recognizing these mutant peptides are exempted from thymic negative selection and can be recruited to fight cancer.

Since then, a series of high-profile papers have been published describing various approaches that take advantage of neo-antigens. Neo-antigen based peptide or mRNA vaccines induced antitumor immunity in MHC-humanized animal model of gliomas [42] and murine tumor models [43]. Shortly after, Steven Rosenberg and colleagues demonstrated the feasibility of identifying $CD4^+$ and $CD8^+$ T cells that recognize neo-epitopes in human gastrointestinal cancers, which are known to have fewer somatic mutations [44]. Although there were no shared neo-epitopes detected in nine patients, one patient did harbor a $KRAS^{G12D}$ hotspot driver mutation found in many human cancers [44]. Recently, the same group demonstrated the therapeutic potential of adoptively transferring a TCR recognizing this $KRAS^{G12D}$ mutation in a metastatic colorectal cancer patient who expressed mutant $KRAS^{G12D}$ and its binding MHC, HLA-C*08:02 [45]. An earlier study showed that patient unique neo-antigens recognized by T cells can be lost from the tumor population and new neo-antigens can emerge [46]. These results suggest that targeting public hotspot driver mutations might be a better strategy in treating cancer than looking for patient private neo-antigens.

Applying ACT to persistent viral infections, such as cytomegalovirus (CMV), was demonstrated as a safe and long lasting treatment in humans almost 25 years ago [47]. With new generations of viral constructs and delivering methods, ACT has been applied to several persistent viral infections, such as Epstein-Barr virus (EBV), adenovirus (ADV), influenza viruses, hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV). In fact, there are more than 20 registered clinical trials at ClinicalTrials.gov alone. T cells recognizing viral epitopes are exempt from the thymic negative selection due to their foreign origin and, thus, high-affinity viral-specific TCRs should be retained. However, our recent study showed that in healthy individuals, the affinity distribution of polyclonal $CD8^+$ T cells recognizing a single epitope ranges three orders of magnitude. Complicating this finding, we also demonstrated that some donors either lacked or had significantly fewer high-affinity T cells compared to other donors. Therefore, iTAST could be an ideal tool to generate high-affinity viral epitope specific T cells for ACT in persistent viral infection [38].

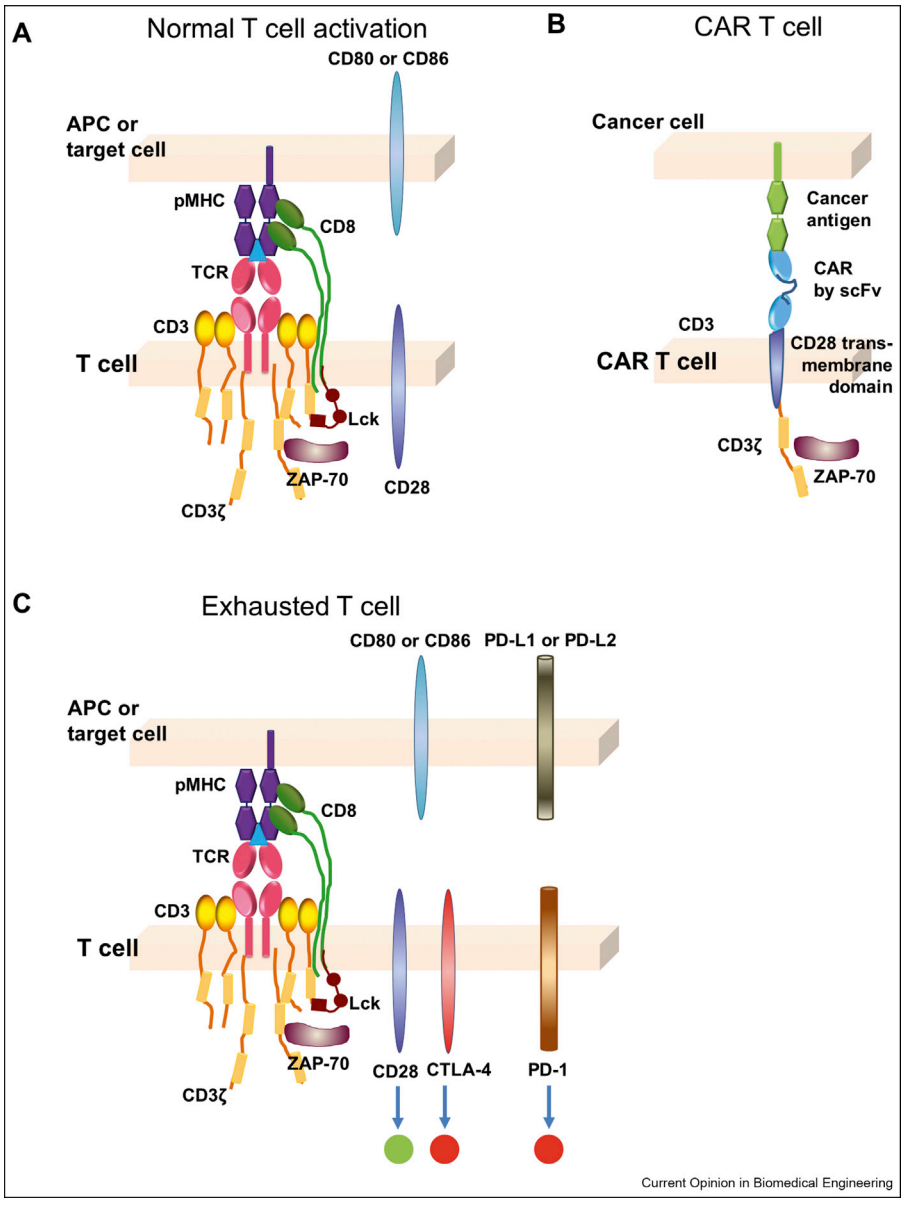
CAR T cells for immunotherapy in cancer and autoimmune diseases

Chimeric antigen receptor (CAR) T cells link the specificity of antibody-cancer antigen interactions directly to the killing ability of $CD8^+$ T cells by using an engineered chimeric receptor (Figure 2). This combination eliminates the constraint that T cells only kill targets with matched MHC molecules and processed peptide antigens. Since the implementation of this concept was first published 25 years ago [48,49], numerous efforts have been focused on its clinical effect, applications to other cancers, and controlling its activity and fate in the host [50–52]. The first generation of CARs was simple by current measures: it was encoded by a single-chain variable fragment from a monoclonal antibody recognizing a tumor antigen with an essential intracellular signaling element [53]. Progressively, more elements were added to the CAR, such as costimulatory signaling domain [54,55]. With a detailed understanding of signaling transduction in T cell activation and diverse tools available in synthetic biology, more safety switches and specificity controls have been added to CARs, such as 'suicide' genes [56–59], logic-gated CARs [60], inhibitory CARs [61], and 'ON-switch' CARs [62].

Most current CARs are focusing on hematologic cancers because the 'off-target' effect of killing non-cancerous blood cells by the CARs can be rescued by normal hematopoiesis. However, in solid tumors, identifying cancer specific antigens with sufficient expression level for engineered CARs to target remains a bottleneck. Recently, the study from Carl June's group broke this bottleneck and showed that a novel CAR based on an antibody to a Tn-MUC1 glycopeptide epitope widely expressed by adenocarcinomas eliminated Tn-MUC1-expressing tumors in mouse models of leukemia and pancreatic cancer [63]. Further clinical trials will truly test the non-specific killing induced complications that have previously been shown in CARs to solid cancers [64]. We should expect more effort along this line of work in the near future, particularly in combination with synthetic biology approach to engineer 'smart' CARs.

In addition to cancer, it has been recently shown that CARs can be used to treat autoimmune disease [65]. The autoimmune disease, pemphigus vulgaris, is caused by autoantibodies to the keratinocyte adhesion protein desmoglein [66]. Instead of fusing antibody to the signaling modules on T cells as the traditional design for all CARs so far, these authors intelligently reversed the receptor-ligand pair and put desmoglein,

Figure 2



Schematics of TCR-ligand interaction during a normal T cell activation, CAR T cell activation, and exhausted T cell activation. TCR along with CD3 complex, co-receptor (CD8 in this case), and co-stimulatory molecule (CD28) translate the binding of a cognate ligand to initiate intracellular signaling cascade (**A**). Replacing the TCR variable region with single chain variable region (scFv) of a cancer-antigen recognizing antibody is the essential design of a CAR T cell. In addition, this extracellular receptor region is fused with the intracellular signaling part of various T cell signaling molecules, which transduce ligand binding signal mediated by the CAR (**B**). In addition to normal T cell signaling complex formed upon T cell ligand recognition, exhausted T cells in persistent viral infection or tumor also express inhibitory molecules, such as CTLA-4 or PD-1, that either compete with co-stimulatory molecule for ligand binding (in the case of CTLA-4) or bind to its own ligand (in the case of PD-1). These bindings generate inhibitory signals that limit the activation signals generated by TCR ligand binding, thus inhibit T cell activation (**C**).

the autoantigen, as the CAR and name it CAAR for chimeric autoantibody receptor. In human skin xenograft model, these CAAR T cells exhibited specific cytotoxicity toward desmoglein-specific B cells and without off-target toxicity in vivo. Although this approach needs further evaluation in clinical trials, it expands the design of CARs to potentially treat autoimmune diseases.

Immune checkpoint blockade for cancer therapy

Prolonged activation of T cells and/or the cancer microenvironment induce T cells to express inhibitory molecules that dampen T cell activation. Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) [67–69] and programmed cell death protein 1 (PD-1) [70] were the first two inhibitory molecules discovered. In

2011, the FDA approved the anti-CTLA-4 monoclonal antibody ipilimumab for the treatment of metastatic melanoma. Ipilimumab is the first drug of any kind shown to extend survival in metastatic melanoma essentially reviving interest in cancer immunotherapy. In 2013, cancer immunotherapy topped the Science list of breakthroughs of the year. Now, there are four FDA approved antibodies targeting these two immune checkpoint inhibitors, CTLA-4 and PD-1 (Figure 2), in treating five different cancers: melanoma, non-small cell lung cancer, renal cell carcinoma, Hodgkin lymphoma, and bladder cancer. Many more immune checkpoints are in the pipeline, such as those targeting LAG-3 [71], TIM-3 [72], and VISTA [73], as well as co-stimulatory molecules include ICOS [74], OX40 [75], and 4-1BB [76].

One of the biggest challenges in immune checkpoint blockade is that even with CTLA-4 and PD-1 combination therapy, a significant portion of the patients do not respond [77]. Thus, figuring out the difference between responders and non-responders is essential to inform future therapies and the development of new therapeutics. Systems immunology that leverages the power of high-throughput technologies and computational analysis is critical in dissecting the molecular mechanism of the immune checkpoint blockade resistance directly in humans. It can inform therapy regiment design and guide the development of new therapeutics. Three papers published recently shed light on the usefulness of this approach. Zaretsky et al. found that mutational defects in the pathways associated with interferon-receptor signaling and in antigen presentation were associated with acquired resistance to PD-1 blockade therapy [78]. Using an interferon and receptor PCR array, Gao et al. showed that CTLA-4 non-responders have significantly higher incidents of genomic aberrations (copy-number alterations (CNAs) and single-nucleotide variants (SNVs)) in their melanoma cancer cells compared to CTLA-4 responders [79]. In another study, Benci et al. discovered that prolonged interferon signaling promotes epigenomic and transcriptomic features of CTLA-4 blockade resistant tumors in mice and that inhibiting interferon signaling in tumor cells can reverse their resistance to CTLA-4 blockade treatment. In addition, they showed that high expression level of interferon-stimulated genes correlates with progression after anti-PD-1 treatment in humans [80]. This study corroborates two other recently published studies on the epigenetic effects of T cell exhaustion [81,82] where it was demonstrated that the epigenetic stability of exhausted T cells may limit current immune checkpoint blockade therapy. Therefore, genomic editing of the PD-1 gene in CAR T cells is an appealing way to prevent exhaustion [83]. It is important to note, however, that this needs to be carefully evaluated in primary T cells because PD-1 has been shown to regulate memory T cell generation [84] and targeting PD-1 regulatory element might be a better strategy in controlling T cell differentiation states and exhaustion [81,82]. These constraints make the study by Benci et al. valuable, as their study offered a plausible direct treatment regimen in CTLA-4 blockade resistant cancer where immune checkpoint blockade could be combined with small molecule inhibitors that specifically target interferon stimulating gene induced immune checkpoint blockade resistance in cancer cells [80].

From systems immunology to engineering immunity

With various immune modulation based therapies emerging for the treatment of cancer, personalized therapy may not require manufacturing personalized medications, but rather could be achieved with personalized combinations of off-the-shelf therapies given knowledge of the status of patient's immune system. In the complex tumor micro-environment, suppressive innate immune cells (such as macrophages, dendritic cells, and neutrophils) along with regulatory T cells coach $CD8^+$ T cells into an exhausted state [31] by expressing suppressive cytokines (such as TGF-β and IL-10) and inhibitory molecules (such as PD-L1 and PD-L2) [85]. Exhausted T cells are characterized by the loss of robust effector functions, expression of multiple inhibitory receptors, and an altered transcriptional program [31]. High-throughput single cell analyses, such as single cell RNA sequencing (sequencing the entire transcriptome of single cells) [86,87] and mass cytometry (flow cytometry using isotope labeled antibodies) [88,89], offer an opportunity to study this complexity. This kind of analysis also yields new insight [90] into the interplays among cancer, immune, stromal, and endothelial cells [90] that can guide future therapeutics.

Knowing the interplays of the immune systems offers rational design of engineering approaches to treating cancer. Recently, Moynihan et al. demonstrated a combination therapy that harnesses both innate and adaptive immunity to eliminate large established tumors in mice, tumors that were very difficult to treat while relying on purely endogenous immunity [91]. Applying the synthetic biology approach, Wendell Lim's group recently demonstrated versatile functionalities and activation states that engineered T cells have by taking advantage of specific transcriptional responses induced by customized synthetic Notch receptor/ligand interactions [92]. Additionally, efforts in engineering immunity via engineering the microenvironment using scaffolds [93,94] or organoid culture [95,96] have paved the way of applying in vitro engineered immune cells for therapeutics. With continued development of high-throughput technology and synthetic biology tools, immune engineering will be a critical link between basic

immunology discoveries and their application. It will no doubt impact how cancer, autoimmune diseases, and infections are treated in the near future.

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Conflict of interest

The author is a scientific advisor of ImmuDX, LLC.

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