ANNUAL REVIEWS

Annual Review of Immunology

CRISPR-Based Tools in Immunity

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Annu. Rev. Immunol. 2019. 37:571–97

First published as a Review in Advance on January 30, 2019

The *Annual Review of Immunology* is online at immunol.annualreviews.org

https://doi.org/10.1146/annurev-immunol-042718-041522

Keywords

CRISPR, genome engineering, immunity, autoimmunity, cancer immunotherapy, genetic circuits, cell therapy

Abstract

CRISPR technology has opened a new era of genome interrogation and genome engineering. Discovered in bacteria, where it protects against bacteriophage by cleaving foreign nucleic acid sequences, the CRISPR system has been repurposed as an adaptable tool for genome editing and multiple other applications. CRISPR's ease of use, precision, and versatility have led to its widespread adoption, accelerating biomedical research and discovery in human cells and model organisms. Here we review CRISPR-based tools and discuss how they are being applied to decode the genetic circuits that control immune function in health and disease. Genetic variation in immune cells can affect autoimmune disease risk, infectious disease pathogenesis, and cancer immunotherapies. CRISPR provides unprecedented opportunities for functional mechanistic studies of coding and noncoding genome sequence function in immunity. Finally, we discuss the potential of CRISPR technology to engineer synthetic cellular immunotherapies for a wide range of human diseases.

FUNCTIONAL GENETIC STUDIES OF IMMUNITY

The completion of the human genome reference sequence in the early 2000s marked a turning point for immunological research. The Human Genome Project identified the ~3.2 billion bases of our DNA, but we lacked understanding of their functions. Immune cells—which are critical for human health and can be studied ex vivo and in vivo in established animal models—have been a major focus of genomic exploration. Over the past two decades, diverse immune cells have been subjected to chromatin state and transcriptional profiling to map DNA elements and the genetic circuitry underlying immune cell types, states, and functions. However, key questions surrounding DNA and immunity can only be answered through genetic perturbation. What are the functional sequences in our DNA and what is their biological importance? What is the genetic circuitry—encoded in genes, noncoding sequences, and *trans*-regulators—that wires specific cellular pathways and specialized functions in immune cells? How does variation in critical coding and noncoding sequences alter cellular function and contribute to risk of immune-mediated disease? Can we utilize our understanding of natural immune cell genetic circuits well enough to reprogram them for the next generation of engineered cellular therapies? The answers depend on new technologies to rewrite genomes in immune cells. CRISPR is one such technology, and with it immunologists are beginning to manipulate immune cell genomes to reveal the genetic underpinnings of immunity.

Here we review the tool set that has emerged rapidly for CRISPR-based genome engineering. CRISPR is a flexible system for targeted genome modifications. It has been used to knock out gene function or knock-in new genetic sequences in cell lines, primary human cells, and animal models. Beyond this, CRISPR has been adapted as a modular system to recruit diverse effector functions to specific sites in the genome in a programmable manner. CRISPR-based tools for transcriptional modulation, epigenetic modification, chromatin imaging and biochemistry, and targeted base-editing are introduced. Finally, we discuss how CRISPR targeting and large-scale CRISPR-based forward genetic screens are being deployed to reveal how immune cells are wired, how their circuits fail in disease states, and how they might be reprogrammed for new treatments.

CRISPR TOOL KIT

Gene Editing

The ability to induce double-stranded DNA breaks at specific sites in the genome of a cell can enable targeted genome modifications. Pioneering work showed that exogenous DNA sequences could be incorporated into a cell's genome through a process called homologous recombination (1). However, the spontaneous efficiency of this process was low. Induction of a double-stranded break (DSB) in genomic DNA could catalyze site-specific repair mechanisms and promote homology-directed repair (HDR) at the target site (2, 3). These findings sparked the beginnings of genome engineering, which began with restriction enzymes in yeast and moved to nucleases with longer recognition specificities, like meganucleases (4, 5), and engineered DNA-specificities including zinc fingers (6, 7), and transcription activator–like effector nucleases (TALENs) (4–6, 8). Due to the complexity of having to reengineer nuclease protein sequences to target different sites in the genome, the use of these tools remained largely restricted to labs and companies with specialized expertise. CRISPR has overcome this limitation by utilizing a highly predictable, RNA-programmable system. The ease of use and versatility of CRISPR has transformed genome engineering into a widely accessible and adaptable laboratory tool.

The term CRISPR, or clustered regularly interspersed short palindromic repeats, originates in observations as far back as the 1980s that some bacteria harbored short repetitive DNA sequences in their genomes that surrounded short spacer sequences resembling viral DNA

(9–14). Decades later, we now understand that CRISPR evolved in some bacterial species as a DNA targeting system that cleaves foreign genomes (15–19). Advances in our understanding of the basic mechanisms of this bacterial system enabled its widespread adoption for genome engineering. Different CRISPR systems continue to be identified today, but the best known and most widely used is the type II CRISPR system, in large part due to its simplicity. Whereas other CRISPR systems have multisubunit effector complexes that mediate nuclease activity, the type II CRISPR system uses a single DNA nuclease. Cas9 is the most widely known type II CRISPR nuclease and is the major focus of this review (18). Cas9 is targeted to DNA sequences by a guide RNA (gRNA), which is made up of a *trans*-activating RNA (tracrRNA) and a CRISPR RNA (crRNA) in bacteria (**Figure 1*a***). The Cas9:gRNA complex scans DNA for sequences complementary to the crRNA that are appropriately spaced from a required protospacer adjacent motif (PAM) (20–23) (**Figure 1*a***). Upon recognition, Cas9 cleaves the DNA to create a DSB between the third and fourth nucleotides upstream of the PAM site (18) (**Figure 1*a***). By linking the crRNA and tracrRNA into a single guide RNA (sgRNA), Jinek et al. (23) reduced CRISPR into a two-component technology for DNA targeting. By varying RNA sequences in the crRNA region of the sgRNA, Cas9 could be reprogrammed to cut distinct DNA sequences (23).

This ability to introduce targeted DSBs at specific DNA sequences is fundamental for precise and efficient genome editing. Eukaryotic cells have evolved multiple mechanisms to repair DSBs, the most prominent being nonhomologous end joining (NHEJ) and HDR, which are differentially utilized and ultimately lead to different repair outcomes (24). NHEJ is an error-prone mechanism that rejoins the two ends of a DSB with frequent small nucleotide insertions or deletions (indels). These errors in NHEJ repair can be exploited for gene ablation (knockout can be achieved by frameshift mutations and deletions) and sequence perturbation studies (25–27) (**Figure 1*b***). In contrast, HDR relies on homologous DNA sequences to template repair of DSBs, which can be exploited to promote specific nucleotide sequence replacement. By adding exogenous DNA templates one can co-opt the cell's HDR pathway to deliver sequences at the site of a DSB (28) (**Figure 1*b***). In 2013, the first applications of CRISPR for mammalian cell DNA editing were reported (25–27). The ease with which the Cas9 nuclease could be reprogrammed to cut at different genomic sites by altering the gRNA made the system flexible to rapidly target sites throughout the genome. This also allowed for multiplexed editing by using multiple gRNAs to simultaneously target Cas9 to different parts of the genome (25). For the first time biologists had a genome-engineering tool that could be deployed quickly and efficiently to edit diverse sequences in the genetic code.

DNA editing with the CRISPR-Cas9 system is limited to sequences adjacent to PAM sites. The NGG PAM requirement for *Streptococcus pyogenes* Cas9 is not particularly stringent, which has made this nuclease useful for most DNA-editing applications. However, for applications like therapeutic gene editing that require targeting of specific sequences the PAM requirement can be limiting. In addition, increasing the number of targetable sites can be useful for functional studies on noncoding sequences (29). One approach to expanding the genome editing space of CRISPR has been to identify CRISPR systems from new microbial species that may have different PAM requirements (30–32). An alternative approach has been to engineer Cas9 PAM specificities by structure-guided mutations (33–36) and directed evolution (37–39). These efforts are expanding the CRISPR tool kit toward flexible targeting across the genome.

Genetic Screens with CRISPR

Unbiased genetic screens have the potential to reveal unappreciated biological pathways and to identify new genetic circuits. CRISPR has facilitated large-scale genetic screens due to the ease, and relatively low cost, with which Cas9 can be reprogrammed to target different genomic sites

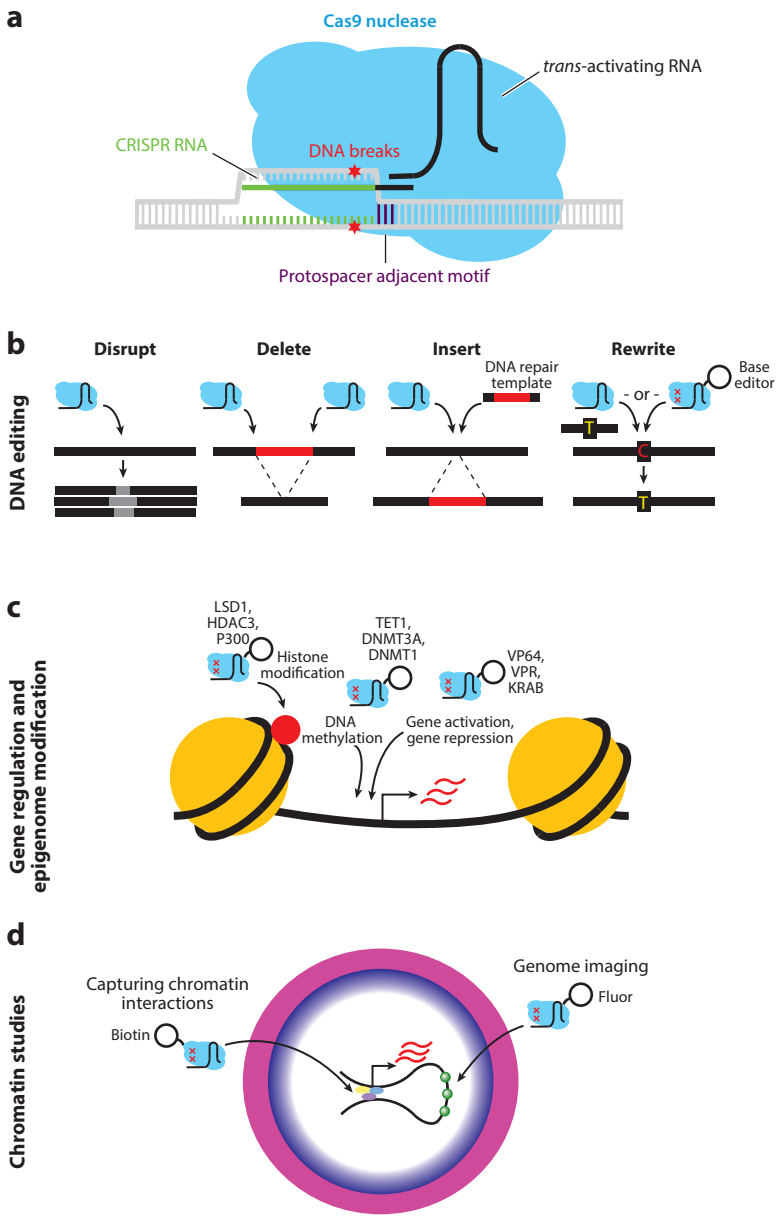

Figure 1

The CRISPR toolbox. (*a*) Schematic of CRISPR-Cas9 gene editing showing Cas9:gRNA complex at on-target genomic site inducing a double-stranded DNA break, (*b*) CRISPR-based tools for gene editing, (*c*) gene regulation and epigenome modification, and (*d*) chromatin studies.

simply by coupling the nuclease to varying gRNAs. CRISPR screens can be carried out in an arrayed or pooled fashion.

Libraries of gRNAs can be used to generate pools of cells with CRISPR perturbations (40–42) (**Figure 2*a***). Pooled screens to study the immune system have been performed in cell lines

engineered to stably express Cas9 (43, 44) and primary immune cells derived from Cas9-expressing transgenic mice (45). More recently, genome-scale pooled screens have also been performed in primary human cells (46, 47). Viral transduction of gRNA libraries with low multiplicity of infection ensures that the majority of transduced cells receive one gRNA, and therefore harbor a single genetic perturbation. In a large population of perturbed cells, cells with a phenotype of interest can be selected, and the causative perturbations can be mapped by sequencing the gRNAs in the selected population (**Figure 2*a***). In this format, genomic integration of the gRNAs links the phenotype to the perturbation caused by a particular gRNA. Pooled screening methods have enabled forward genetic screens at a genome-wide level. However, pooled screens generally are restricted to individual selectable phenotypes including cell survival/proliferation (40–42) or selectable protein markers (44).

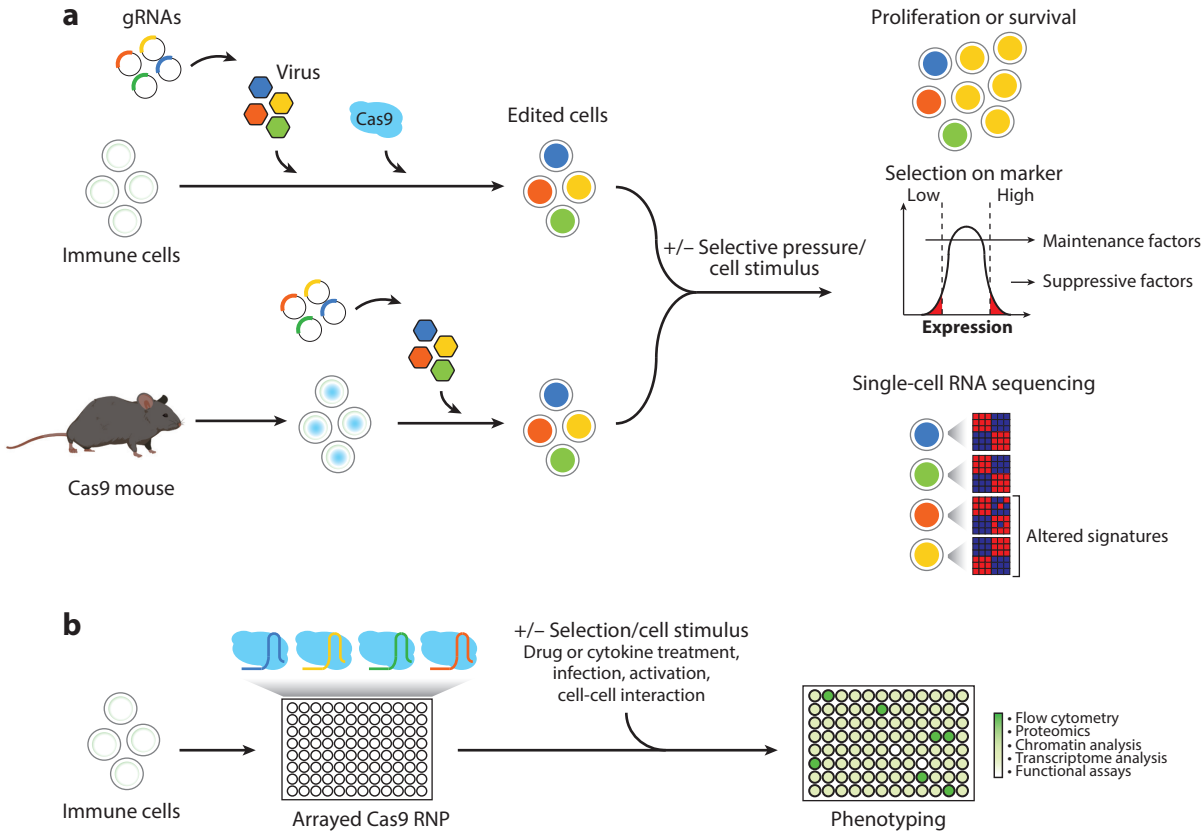

Figure 2

CRISPR genetic screens. (*a*) Pooled CRISPR screen workflow for immune cells. Generally, libraries of gRNAs are transduced into cells such that transduced cells on average receive a single gRNA that mediates a single genetic perturbation. Cas9 can be transduced or electroporated as protein to generate genetic perturbations. Genetic perturbations are made in a pool, and their effects on cellular proliferation and survival, protein expression, or other cell phenotypes can be assessed by deep sequencing gRNAs from cells with and without selection. These types of screens can be used to rapidly test large numbers of genetic perturbations. (*b*) Arrayed CRISPR screen workflow for immune cells. Single genetic perturbations are introduced to cells. Phenotypic effects can be measured for each genetic perturbation in edited cells. These screens are lower in throughput but allow for rich phenotypic readouts. Abbreviations: gRNA, guide RNA; RNP, ribonucleoprotein.

In an arrayed format, different populations of cells are targeted with unique genetic perturbations (48) (**Figure 2*b***). Arrayed screens generally are lower throughput than pooled approaches, but they allow for complex phenotypic readouts due to the homogeneity of the targeted population (48) (**Figure 2*b***). Additionally, arrayed screens also are uniquely suited to study cell-cell interactions and assess cell nonautonomous effects of a genetic perturbation (48) (**Figure 2*b***). Pooled and arrayed CRISPR screens serve complementary roles for functional genetic studies.

Specificity of Genome Editing

CRISPR-Cas9 gene editing can have unintended consequences in the genome. In screening approaches, off-target effects of individual guides can be handled by including multiple guides targeting each gene of interest. However, unintended edits are a significant concern for CRISPR-based generation of animal models and human therapeutic applications. Early on it was recognized that Cas9 can cleave DNA sequences homologous to the on-target site despite complementarity mismatches (49). The nucleotides immediately adjacent to the PAM, the seed sequence, are critical for targeting and generally do not tolerate mismatches. However, mismatches in the remainder of the gRNA can be tolerated and lead to DNA cleavage. Bioinformatic tools have been developed to minimize off-target effects and maximize on-target editing efficiency (49, 50). The algorithms serve as a general guide for gRNA design but do not account for all factors that govern success of CRISPR editing, including the local chromatin environment (51–53). In addition, on-target DNA cleavage can cause unintended mutations in neighboring sequences due to DNA repair (54–56). This has sparked efforts to engineer more specificity into the DNA-editing machinery and to develop tools to capture unintended mutations (56–61).

Several approaches have been developed to limit CRISPR-Cas9 off-target cleavage. Early efforts focused on limiting Cas9 nuclease activity and engineering Cas9 to require two neighboring targeting events to introduce a DSB (25, 62). Structure-guided studies have also had success in reducing off-target effects by mutating Cas9 residues that are not necessary for DNA binding or cleavage but interact with the DNA phosphate backbone (34–36). A directed-evolution approach was successful in improving targeting specificity of Cas9 (39). Modifications to the gRNA length and sequence composition can also attenuate Cas9 off-target activity (63, 64). Finally, limiting Cas9 activity in targeted cells is emerging as an important factor. Several strategies have been used to do this, including delivery of Cas9 protein (63) or mRNA (65, 66), either of which is degraded relatively rapidly; self-limiting circuits (67); tunable systems (68–72); and CRISPR inhibitors (73).

Targeted Genome Sequence Replacement

Targeted sequence replacement at endogenous genomic sites is a critical goal of genome engineering. Cas9:gRNA complexes are sufficient to disrupt genome sequences based on indels introduced during imperfect NHEJ repair or by excising DNA sequences when they are introduced in pairs (**Figure 1*b***). However, cut-and-paste functionality for genome editing requires co-introduction of a DNA repair template (**Figure 1*b***). DNA repair templates have been introduced into cell lines on plasmids that allow for long homology arms to the target sequence, which is important for HDR efficiency and specificity (6, 74). However, generating plasmids can be laborious, and delivery to primary cells can be inefficient and toxic. To overcome these technical barriers, short single-stranded DNA oligos (~200 bases) have been employed by multiple groups (75–77). However, the short oligos limit the length of sequence replacement that is possible, especially when accounting for homology arms (78).

Many research and therapeutic goals depend on technologies to replace or insert larger sequences at endogenous sites. There have been concerted efforts to increase the size of the sequence payload that can be delivered while maintaining HDR efficiency and cell viability. One approach that has become widespread is combining Cas9, or other targeted nucleases, with adeno-associated virus (AAV) strains engineered to encode homology arms and the sequence to be introduced at the target site. The viral genome therefore serves as an HDR template and has been used to efficiently rewrite target sequences in human $CD34^+$ hematopoietic stem cells (79, 80), T cells (81–83), and B cells (84). These methods are powerful, but they rely on viral production and transduction, which can be a bottleneck for both research and clinical applications. Recent efforts have revealed that Cas9 ribonucleoproteins (RNPs) can be coupled with long (>1 kilobase) DNA templates that are either double stranded (dsDNA) or single stranded (ssDNA) for HDR (85–87). Efficient nonviral genome targeting can be achieved in primary human T cells by optimizing cell culture conditions, Cas9 RNP concentrations, DNA template concentrations, and electroporation parameters (86). This method is likely to be adaptable for other immune cell types as well. However, some cells—especially nondividing cells—may not be competent to undergo efficient HDR. Several groups have developed homology-independent targeted integration (HITI) (88) or microhomology-dependent precision integration into target chromosomes (PITCh) (89), which could help to expand the set of cells where targeted integrations can be achieved. Collectively, these technologies to knock-in large sequences will allow us to tag genes in their endogenous loci for biochemical and imaging studies. Furthermore, they will enable efforts to rewrite coding and noncoding sequences at specific sites in the genome to correct pathogenic mutations and reprogram immune cell functions.

Engineering Mouse Models

Mouse models are indispensable for functional genetic studies of the immune system. Gene targeting of embryonic stem cells by homologous recombination had been the method of choice for genome modifications (90). However, this methodology is time consuming, expensive, and limited to certain genetic backgrounds for which established embryonic stem cell lines are available (91). CRISPR-Cas9 overcomes these limitations and is now used routinely to engineer knockout and knock-in mice in a few months (92, 93). CRISPR modification of zygotes can be especially powerful for multiplex editing of multiple targets (93) or adding new modifications to existing mouse models that already carry multiple transgenic alleles. Gene targeting with CRISPR-Cas9 is possible on diverse murine backgrounds as long as gRNAs are properly designed based on the targeted genome. Recent work successfully introduced genetic modifications on the autoimmune-prone nonobese diabetic genetic background, which is commonly used for human cell transfers and studies of autoimmune type 1 diabetes (94). Cas9 nuclease can be microinjected into single-cell zygotes as DNA, RNA, or protein along with appropriate gRNAs. Exogenous DNA including plasmids or short, single-stranded oligonucleotides can be co-delivered for knock-ins by HDR at the Cas9 cut site (92, 93). Microinjection of the CRISPR machinery along with long ssDNA templates has proven useful for larger targeted modifications, including introduction of floxed alleles (95). Electroporation of mouse zygotes with Cas9 RNP (CRISPR-EZ) eliminates the need for laborious zygote microinjections (96, 97). This method is efficient and simple and facilitates higher-throughput mouse generation. Although gene editing with CRISPR-EZ is highly efficient, further work is needed to enable HDR with larger repair templates. Taken together, these new tools are accelerating the production of engineered murine models.

CRISPR can also be used to engineer somatic murine cells as an alternative to germline editing. To facilitate CRISPR targeting of somatic cells and murine models for CRISPR screening,

transgenic mice have been generated that express Cas9 constitutively (45), conditionally (98), or inducibly (99). This facilitates genome editing in primary immune cells, where Cas9 delivery can be challenging. In Cas9 transgenic mice, cells can be modified simply by transducing sgRNA sequences individually or in pools. This has enabled studies of individual gene knockouts in somatic cells in addition to ex vivo and in vivo primary cell screens (45). Finally, recent efforts have successfully used Cas9 RNP electroporation to directly edit primary immune cells isolated from mice (100). These tools accelerate assessments of phenotypes arising from target perturbations in mature cells of the immune system.

Controlling Gene Expression

Beyond genome editing, CRISPR-Cas9 offers tremendous utility as a programmable scaffold to target effector molecules to DNA sequences. To transform Cas9 into a DNA-targeting scaffold, the nuclease domains were mutated to create a nuclease-deficient dead Cas9 (dCas9) that no longer cuts DNA but could still target and bind to DNA sequences in a gRNA-programmable manner (101). Targeting dCas9 to gene bodies could reduce gene expression through direct transcriptional interference with the RNA polymerase, without altering the genome sequence (101). The utility of dCas9 was expanded further with the recognition that different effector molecules could be tethered to the inactivated enzyme to control gene expression. dCas9 tethered to a transcriptionally repressive domain (e.g., dCas9-KRAB) silenced gene expression when the molecule was targeted to gene promoters (102). Alternatively, tethering a transcriptional activator (e.g., VP64) could increase gene expression (103–106). CRISPR inhibition (CRISPRi) and activation (CRISPRa), as these systems are now known, have made it possible to toggle target gene expression in a controlled manner.

The effects of dCas9-VP64 on gene activation were often modest (104, 105, 107, 108), but CRISPR-based control of transcriptional regulation continues to improve. Potent gene activation has been achieved by tiling multiple dCas9-VP64 molecules at a gene promoter (103, 105) or by increasing the number of activation domains a single dCas9 can recruit (109). Alternatively, tethering additional activation molecules to dCas9 could synergistically boost the effect of VP64, leading to more robust gene activation (110). The gRNA sequence can also be engineered to recruit effector molecules (111). Likewise, CRISPRi continues to be improved (112). Improved ability to tune gene expression positively and negatively offers opportunities to test the effects of transcript levels on cell function and to perform large-scale complementary loss-of-function and gain-of-function screens (113). Furthermore, the controlled expression of groups of genes by multiplexing gRNAs can be used for directing cellular differentiation and other genetic programs (114, 115).

Epigenome Reprogramming

Epigenetic regulation of gene expression involves chromatin modifications that can be stably passed on to future generations of cells. Our understanding of epigenomic modifications has increased with the advent of next-generation sequencing technologies that have profiled histone modifications and transcription factor binding by chromatin immunoprecipitation. A major undertaking in the field has been cataloging of epigenomic signatures across cell types and under different cell conditions, to understand the interplay among chromatin modifications, gene expression, and cell function. However, these data are correlative. A remaining fundamental question is which chromatin modifications have causal roles in gene expression and epigenetic memory. Engineered CRISPR systems are providing tools to recruit chromatin-modifying enzymes to specific genome sites and directly test the effects of epigenomic modifications.

RNA-programmable CRISPR systems have been to used recruit enzymes that modify histones or control DNA methylation to specific genomic loci. Fusing dCas9 to the catalytic domain of the DNA methyltransferase DMNT3A alone (116) or in combination with other factors (117) can specifically and efficiently methylate endogenous loci. Fusing dCas9 to TET1 can remove DNA methylation from silent loci, inducing expression of genes that were otherwise transcriptionally inactive (116, 118–120). These tools have been used to assess mechanistic consequences of site-specific DNA methylation. They are also potential therapeutic tools for diseases characterized by inappropriate methylation. Histone modifiers have also been engineered to allow precise control of locus-specific histone epigenetic marks. These include dCas9 fusions with histone demethylases (LSD1) (121) and methyltransferases (SMYD3, PRDM9 and DOT1L) (122, 123), as well as deacetylases (HDAC3) (124) and acetyl transferases (p300) (125, 126). Chromatin-modifying versions of CRISPR are being used in pooled formats to assess the functions of site-specific chromatin marks that have been previously mapped (125, 126).

One potential advantage of epigenome reprogramming over CRISPRi or CRISPRa approaches discussed above is that the consequences on cell function can be more stable. This has raised interest in hit-and-run epigenetic reprogramming with CRISPR (127). With this method, CRISPR is used to recruit multiple modifications to a target locus to induce stable gene silencing. This has potential for therapeutic manipulation of immune cells. Gene targets could be disabled with a transient treatment that does not alter any genetic sequences. Epigenome reprogramming of immune cells holds notable potential for adoptive cellular therapies.

Biochemistry and Imaging Studies of Chromatin

Gene regulation programs depend on physical interactions between transcriptional regulators and *cis*-regulatory elements, and complex three-dimensional interactions among chromatin sites. CRISPR tools are being developed to image chromatin sites and facilitate proteomic and genomic studies of chromatin interactions. Biotinylated dCas9 can be used to pull down endogenous genomic sequences and identify bound transcription factors in an unbiased manner by mass spectrometry and local 3D chromatin interactions by sequencing (128, 129). Fluorophore fusions to dCas9 have been used to visualize genomic loci in living cells in real time (130). Using the SunTag system to recruit additional fluorophores to a single dCas9 molecule or similar methodologies to improve the signal may allow imaging of single genomic sites to address questions of nuclear organization and chromatin remodeling (109). With the ability to write large exogenous sequences into the genome it is possible to begin to tag endogenous transcription factors (86, 131). High-affinity tags can be fused to transcription factors to enable pulldown studies to map DNA binding sites and interacting partners (129, 131, 132). Additionally, nuclear factors can be fused to fluorescent proteins or tags to study their localization in the cell and their dynamic interactions with DNA (86). These emerging tools collectively should shed light on the physical interactions that contribute to immune cell circuitry.

Base Editing

Engineered effectors coupled to nuclease-deficient CRISPR systems now offer genetic reprogramming in addition to epigenetic reprogramming. Base editing is a new approach to rewriting genetic sequences. Base editors use cytidine deaminases to introduce nucleotide changes at the targeted genomic site (133). This approach to genome editing has potential advantages over nuclease-dependent strategies, as DSBs are not introduced and desired editing outcomes may be achieved more homogeneously. Several iterations of this technology have been published that

have improved the editing efficiency and flexibility of base editors (134–136). Preclinical evidence suggests that this technology may be useful for therapeutic purposes to correct single-nucleotide mutations or introduce disease-causing mutations in cells to model disease. The mutagenic potential of cytidine deaminases does raise concern for off-target effects. Further modifications to base editor systems promise to deliver precise single-nucleotide edits at the on-target site. Base editing technology has also proven useful for targeted mutagenesis screens. Recent platforms were engineered to have mutagenic activity over larger DNA sequences (~100 bp), introducing distributed transition and transversion nucleotide changes giving rise to allelic diversity (137, 138). This functional diversification of DNA sequences at a target site, which could include loss-of-function and gain-of-function mutations, cannot be achieved readily with Cas9 cutting, which mostly introduces indels. Saturation mutagenesis screens using base editing across noncoding and coding DNA sequences promise insights into the grammar of our DNA.

Additional CRISPR Systems

New CRISPR systems continue to be discovered in bacteria and mined for new functions. For example, although CRISPR systems have largely been used to target DNA sequences, some have been discovered now that target RNA sequences (139). Ongoing discovery of microbial systems is likely to provide an expanded CRISPR toolbox for genetic engineering of immune cells.

GENETIC VARIATION AND IMMUNE REGULATION

Mapping Genetic Circuits of Immunity

Immune homeostasis depends on complex coordination of cellular programs. Diverse cell types must traffic to appropriate sites, recognize antigenic targets, and respond effectively to threats without causing autoimmunity. These specialized, cell type–specific, and stimulus-responsive programs are governed by molecular circuits comprising signaling pathways, *trans*-regulators (e.g., transcription factors and epigenetic regulators) and networks of *cis*-regulatory elements and target genes. Correlative cellular measurements like transcriptome and chromatin analyses have been used extensively to infer elements of this circuitry. However, true understanding of circuit function depends on the ability to perturb the putative elements and observe the effects. CRISPR technology is well suited for systematic perturbation studies, and we can now directly test causal relationships between genome sequences and cellular phenotypes. CRISPR enables rapid high-throughput functional studies of immune cells to comprehensively map genetic regulators of cellular phenotypes.

Pooled CRISPR screens are emerging as a powerful approach to identify genes that control immune functions. A genome-wide CRISPR screen dissected the innate immune response of bone marrow–derived dendritic cells (BMDCs) (44). BMDCs isolated from Cas9-expressing mice were transduced with gRNAs to introduce different genetic perturbations across the population. gRNA-transduced BMDCs were stimulated with LPS and then sorted based on the resulting levels of Tnf induction to identify the gRNAs that targeted key regulators. This unbiased screen identified known and unknown regulators of Tnf induction. Hits could be grouped into common biological pathways and known protein complexes illustrating the power of high-throughput CRISPR screens. This flow-based approach can be adapted to study diverse biological pathways or cellular phenotypes that can be selectively enriched by FACS.

Deeper measurements of the molecular phenotypes arising from each CRISPR perturbation would provide richer insights into gene circuity. Combining single-cell RNA sequencing

(scRNA-seq) with pooled CRISPR libraries has allowed investigators to observe the effects of individual genetic perturbations on a cell's transcriptome. Three studies published simultaneously at the end of 2016 established this pooled CRISPR screening approach (140–142). The power of this approach linked genetic perturbations to divergent cellular responses, developmental pathways, and gene regulatory circuits. This required technical advances to obtain single-cell transcriptome data and simultaneously capture the gRNA found in each cell, as the gRNAs could not be directly captured by scRNA-seq due to their short length and absence of 3′ poly A tails. Instead these studies sequenced the lentiviral constructs to pair individual gRNAs with a unique bar code in the 3′ untranslated region of a fluorescent protein transcript. A variant of this protocol termed CROP-seq developed a vector in which gRNA sequences are captured directly by scRNA-seq (143). The current costs associated with scRNA-seq can be prohibitive for large high-throughput studies. However, rapid advances in the technology, lower costs, and computational methods are beginning to make these studies feasible, which greatly accelerates our ability to map genetic circuits of immunity.

Noncoding Elements in Immune Circuitry and Disease Risk

CRISPR is also a powerful tool to probe noncoding elements in the genome. Deciphering the genetic underpinnings of common autoimmune diseases and other complex diseases of the immune system requires improved understanding of how noncoding sequence variation regulates gene expression and immune cell function. Most common autoimmune diseases are thought to have a complex genetic etiology that stems from the combined effects of common variants and environmental factors. Although individual autoimmunity variants have relatively small effects on disease risk, they mark important regions of our genome that are critical for normal immune function. Over the last two decades, genome-wide association studies (GWAS) have linked common variation in hundreds of loci across the genome to risk of autoimmunity. Roughly 90% of the genetic variants implicated in autoimmune disease risk do not alter protein-coding sequences but rather fall in noncoding regions of the genome that remain relatively poorly understood (144). Mapping functional noncoding sequences that harbor autoimmunity variants and identifying the biological programs they regulate will be critical in understanding how common variants predispose to autoimmunity.

Noncoding sequences harbor hundreds of thousands of putative enhancers—transcription factor docking sites that shape transcriptional programs in response to specific cellular signals. Consortia like ENCODE and the Roadmap Epigenomics Project have profiled transcription factor binding and epigenomic marks across diverse cell types and cell states to map putative enhancers in noncoding sequences (145, 146). These maps revealed that a majority of autoimmunity variants reside in sequences with features of immune enhancers (77, 86, 147). Taken together these data suggested that the dysregulation of transcriptional circuits plays a causal role in autoimmunity. Our ability to delete, paste, and rewrite the genetic code with CRISPR is now transforming our understanding of noncoding sequences and has enabled rapid functional testing of autoimmunity variants.

Sequence perturbation is the gold standard for identifying functional noncoding sequences. Given how little is known about the molecular grammar of sequences outside of amino acid–coding regions, CRISPR-based saturation mutagenesis screens of noncoding stretches of the genome have proven useful. These relatively unbiased functional screens use thousands of gRNAs to tile across entire loci, saturating them with Cas9-induced indels. Edited cells are then binned and sorted on target gene expression or downstream phenotype and sequenced to quantify the enrichment or depletion of gRNAs, a measure of the regulatory effects of the targeted

genomic site. Several Cas9 cutting screens have been published showing the utility of this approach in identifying functional noncoding sequences (148, 149). Similarly, CRISPRi recruitment of the transcriptional repressor dCas9-KRAB can also be used to map functional enhancers (150). These loss-of-function strategies identify elements that are required for gene regulation in the particular context in which the screen is performed. However, many enhancers only contribute to gene regulation in the context of specific extracellular cues. We adapted CRISPRa to map functional enhancers where the activation domain was sufficient to induce a specific target gene. By recruiting dCas9-VP64 via CRISPRa to thousands of genomic sites in pooled experiments, immune enhancers were mapped across two key autoimmunity risk loci, *CD69* and *IL2RA* (151). In these experiments, CRISPRa was able to identify stimulus-responsive enhancer elements even in unstimulated cells. Taken together, CRISPR-based approaches are revealing functional noncoding elements and linking them to their target genes.

CRISPR is also being used to fine-map critical sequences within individual enhancer elements. In enhancer bashing studies, Cas9 perturbations are targeted to every gRNA site within an enhancer. By sorting enhancer-edited cells on target gene expression or a downstream phenotype and correlating enhancer edits with gene expression, it is possible to footprint nucleotides required for optimal enhancer function (152). This approach improves the resolution with which we can study noncoding sequences and variation within them. Ultimately, developing methods to efficiently introduce autoimmunity variants by HDR or base-editing on an isogenic background will allow for direct assessment of variant function. This fine-resolution functional mapping moves us toward an understanding of how single-nucleotide variants can tune gene programs.

The next frontier will be the mapping of functional enhancers across the genome and capturing their effects on specific transcriptional programs. Further resolution of functional sequences can be achieved by tiling regions with nucleases that have different PAM specificities. Screening with other dCas9-coupled effector molecules could discriminate classes of enhancers in different functional chromatin states—for example, poised versus active enhancers. In addition, a recent study screened combinations of noncoding CRISPR perturbations with scRNA-seq to understand the regulatory logic of superenhancer clusters (153). Although technical and analytic challenges remain, these approaches are linking noncoding elements to downstream gene programs, providing biological insights into how noncoding variants can alter immune cell circuitry and contribute to disease risk.

Reverse Genetics of Pathogenic Sequence Variants

GWAS and genome sequencing of patients with monogenic immune dysregulation are identifying a growing number of candidate causal pathogenic sequences affecting the immune system. Genome editing is critical for testing which of these are truly causing disease phenotypes and for determining the underlying mechanism of pathology. One challenge is that natural variants are often inherited in combinations, making it very difficult to differentiate the ones that cause disease risk from neutral variants in the same individuals. CRISPR provides a tool to create isogenic cells that differ only at a single targeted genomic site and to assess the in vitro and in vivo cellular effects of genetic variants.

Modeling conserved human variants provides an opportunity to pinpoint functional consequences of disease variants in an intact immune system. For example, CRISPR mouse engineering enabled functional assessment of a common coding single-nucleotide polymorphism (SNP) in *PTPN22* implicated in risk of type 1 diabetes and other autoimmune diseases. As predicted from human population studies, nonobese diabetic (NOD) mice CRISPR-engineered with the human risk allele had increased incidence of autoimmune diabetes. CRISPR-engineered mice can also

test the functional consequences of noncoding variants implicated in immune disease. Noncoding variants may only affect target gene regulation in particular cell types or in response to particular stimuli. We recently used CRISPR mice to study a noncoding autoimmunity SNP found in an *Il2ra* intron (151). The fine-mapped SNP explains the risk for Crohn disease at the *IL2RA* locus (154). Interestingly, this same SNP is protective for type 1 diabetes, suggesting it might have context-restricted effects with divergent outcomes on disease (155). We engineered SNP knock-in mice that differed in only the SNP nucleotide. We examined different T cell subsets under different states to map the context within which *Il2ra* might be dysregulated. We found that the SNP resides within a conserved stimulation-responsive *Il2ra* enhancer and delays the kinetics of *Il2ra* induction on naive T cells as they respond to stimulation (151). Looking forward, CRISPR-generated animal models of human disease variants provide opportunities to localize disease risk to specific cellular compartments, test epistasis of multiple risk variants, and assess candidate pharmacological interventions.

Many genetic variants implicated in human disease are not conserved in mouse. Thus, genome editing in human cell lines and primary immune cells often is required to assess causal effects. Although Cas9 delivery to human cells was initially challenging, CRISPR can now be efficiently deployed for genome modification in primary human cell types. Purified recombinant Cas9 protein can be mixed in vitro with gRNAs to make Cas9 RNPs (63, 156, 157). Cas9 RNPs can be electroporated in primary human immune cells to generate knockout and knock-in modifications (77, 86, 147). This approach has been used to engineer DNA sequences in hematopoietic stem cells (HSCs), T cells, and B cells (77, 100, 147, 158). Genetic modification using Cas9 RNPs is likely to be successful in other primary immune cell types as well. Electroporation of Cas9 mRNA has also been employed for gene modifications in immune cells (147, 159).

CRISPR now can be used to rapidly test variants, including variants in linkage disequilibrium with each other, to functionally fine-map genetic associations. For example, a recent study found a noncoding SNP in the *CEBPA* locus associated with altered basophil counts (160). CEBPA is a hematopoietic transcription factor, but it had not been previously linked to basophil abundance. In situ perturbation of the SNP site in human stem and progenitor cells revealed it resided within a *CEBPA* enhancer that influences basophil differentiation (160). Remarkably, pursuing this natural genetic association revealed new biology about a *cis*-regulatory code underpinning basophil differentiation. Performing these studies in different cell types and states will be critical to assigning function to immune disease variants.

Therapeutic Correction of Monogenic Mutations in Immune Cells

CRISPR not only provides a means to confirm and characterize pathogenic variants but could also provide an avenue to correct the genetic causes of immune cell dysfunction. Therapeutic correction of causal mutations in the affected cell types or their progenitors can be curative for patients with immune-related disorders (**Figure 3*a***). Recent work has focused on improving CRISPR-Cas9 editing efficiency in primary human immune cells to rewrite monogenic-disease-causing variants (86, 161–163). Diseases where the standard of care is currently allogeneic stem cell transplantation may eventually be treated with ex vivo stem cell correction and autologous transplantation. There are potential risks that must be explored, including unintended genome modifications (49, 55, 56, 164–166) and perhaps altered cell programs that result from the CRISPR machinery (167, 168), electroporation, or DSBs (169, 170). On the other hand, the risk of graft-versus-host disease would be reduced and treatments could become available for patients without donor matches. For example, severe combined immunodeficiency (SCID) is a rare genetic disorder that can be caused by diverse mutations. CRISPR with HDR-mediated correction could provide

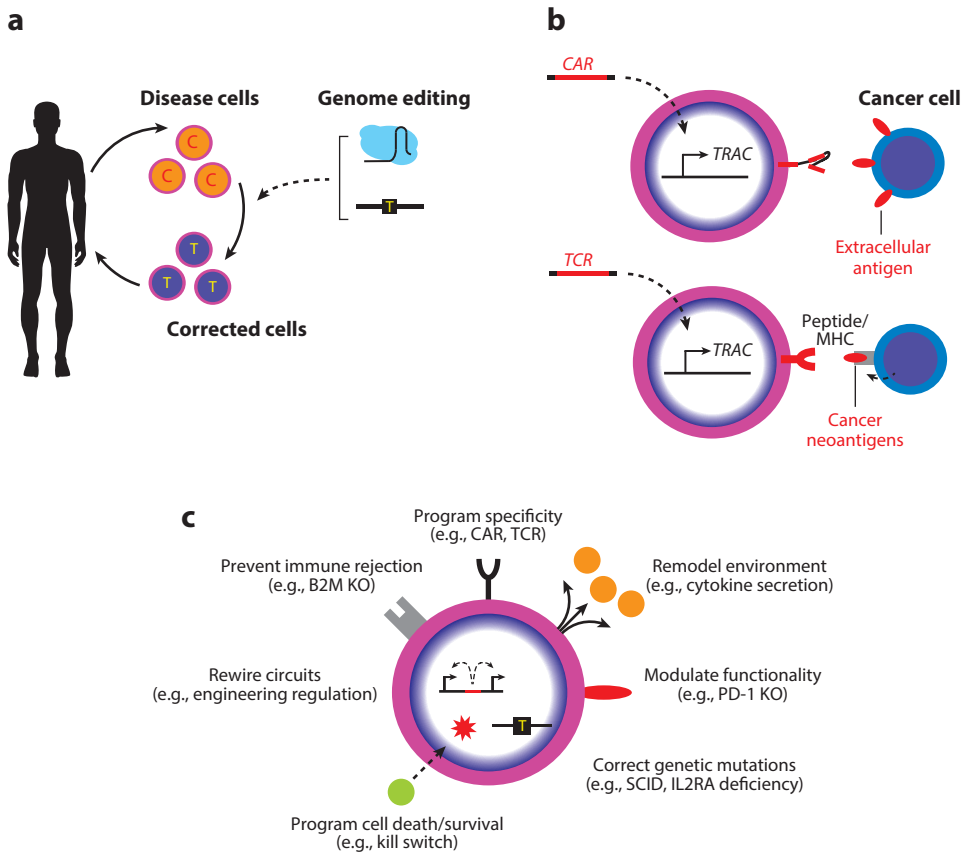

Figure 3

Using CRISPR to engineer immune cell therapies. (*a*) CRISPR-Cas9 gene editing of immune cells for correction of immune-related genetic disorders. (*b*) Engineering immune cell specificity through CRISPR targeting of CARs or TCRs at endogenous loci. (*c*) Desired genetic modifications for cellular immunotherapies. Abbreviations: CAR, chimeric antigen receptor; SCID, severe combined immunodeficiency; TCR, T cell receptors.

a flexible system for therapeutic correction of mutations in HSCs to restore gene function needed for healthy T cell generation. The base editor system is an alternative approach to therapeutic correction of T-to-C mutations, which does not require DSB formation or exogenous DNA for repair. As protocols improve for differentiation of pluripotent stem cells, induced pluripotent stem cells could provide renewable resources of patient cells for experimental optimization and perhaps therapeutic gene correction (161).

Mutation correction in differentiated immune compartments is also being explored as a therapeutic strategy. For example, siblings in a family with varying autoimmune manifestations caused by recessive mutations in *IL2RA* have FOXP3$^+$ regulatory T cell (Treg)-like cells that do not express appropriate levels of IL2RA and are dysfunctional. We demonstrated that nonviral CRISPR-based genome targeting could correct a pathogenic *IL2RA* mutation and partially rescue IL2RA expression in T cells from these patients (86). Correction could be achieved in FOXP3$^+$ cells, raising the possibility of autologous transfer of gene-corrected Treg therapy for the affected children. Tregs can enforce dominant tolerance, suggesting that a relatively small number of

corrected Tregs might restore immune homeostasis to affected children. In the future, there may be more opportunities to tailor gene surgery approaches to specific cell populations that are impaired by a particular patient mutation. Ex vivo CRISPR gene correction is advancing rapidly toward the clinic. As delivery strategies for CRISPR continue to advance, there may eventually be opportunities for targeted in vivo editing of somatic cells to treat monogenic disease. These fields continue to evolve and much validation remains to be done, but there are concerted efforts to achieve safe and effective targeted genome surgery in immune cells.

MICROBIAL IMMUNITY

A primary function of the immune system is to recognize and eradicate pathogens. Understanding the genetic factors that regulate how immune cells interact with pathogens could reveal critical pathways co-opted by pathogens and open new therapeutic avenues. Almost 40 million people around the world are infected with human immunodeficiency virus (HIV). This virus selectively infects $CD4^+$ T cells and causes life-threatening immunodeficiency. The virus is well known to depend on host factors in human T cells at multiple stages of its life cycle. Several groups used RNA interference (RNAi) knockdown approaches to search for these host dependency factors in cell lines (171–173), but results were inconsistent. CRISPR, which tends to have fewer off-target effects than RNAi and can generate complete knockout cells, has renewed hopes for systematic identification of host factors that influence HIV infection.

Both pooled and arrayed CRISPR screens have been performed to identify host factors that influence HIV infection. A genome-wide CRISPR screen was performed in a human T cell line to identify genome modification that confers strong resistance to HIV (174). This unbiased approach identified a remarkably restricted set of factors, including known entry receptors, that could be deleted to confer cell survival and resistance to infection upon challenge with HIV in vitro. The function of a novel gene pathway that posttranslationally modifies the CCR5 coreceptor was validated by CRISPR modification of primary human T cells. Targeted arrayed screens have also been piloted in primary human T cells. Several candidate host factors implicated by protein-protein interaction studies (175) and the HIV literature were individually deleted with CRISPR. Knockout primary human $CD4^+$ T cells for each gene were generated using three different Cas9 RNPs and tested for HIV infection (176). This arrayed platform quantified rates of HIV infection in each cell population with high-throughput flow cytometry and identified both known and unknown host factors important for HIV infection. More broadly, this work established an arrayed CRISPR platform to test effects of genetic perturbations in primary human immune cells for studies of infectious diseases. Taken together, pooled and arrayed CRISPR screens are providing clues to critical functional interactions between immune cells and pathogens that infect them.

Various groups are considering how to translate CRISPR insights into HIV pathogenesis into new strategies for an HIV cure. Deleting host factors in human $CD4^+$ T cells or HSCs to limit HIV infection is one gene therapy approach for HIV therapy. Ablation of the HIV coreceptors CCR5 and CXCR4 can generate $CD4^+$ T cells that are resistant to infection (177–179). As above, CRISPR screens are identifying additional host factors that may also be modified to ensure viral resistance. Direct CRISPR targeting of the HIV genome has also been explored, although therapeutic delivery would be challenging. Recent work used gRNAs in the long terminal repeats of HIV that flank the viral genome to excise the virus from human T cells (180, 181). In a similar approach, conserved sequences of HIV were targeted by CRISPR-Cas9 to functionally ablate the virus (182). In another approach pioneered with TALENs and meganucleases rather than Cas9, genome engineering was performed to knock-in an anti-HIV chimeric antigen receptor (CAR)

sequence into the *CCR5* locus (81). Similar approaches could be extended to other pathogens to understand the genetics of their interactions with the immune system in addition to designing genome-engineering approaches for therapy (183–186).

CANCER IMMUNITY

Immunotherapy is offering new hope for previously untreatable cancers. Checkpoint inhibitors are reversing T cell dysfunction and causing productive anticancer immune responses in some patients (187). In addition, adoptive transfer of tumor-infiltrating lymphocytes and genetically engineered T cells have demonstrated the potential for cellular therapies as a new drug class. Despite these advances in immunotherapy, a large fraction of malignancies remain incurable. CRISPR is being used to understand cancer immunity through unbiased genetic perturbation studies in immune cells and cancer cells. Unbiased CRISPR screens are rapidly revealing the genetic underpinnings of T cell responses and pointing toward new targets for pharmacological checkpoint blockade or genetic engineering in cell therapies.

CRISPR-Cas9 pooled screens can be used to rapidly map gene circuits that regulate cancer immunity. T cell exhaustion through activation of the PD-1/PD-L1 pathway is a major mechanism by which cancer cells evade the immune system. Given the remarkable clinical success of drugging this pathway, there has been great interest in understanding the regulation of these molecules. Genome-wide CRISPR screens using a FACS-based sorting strategy for PD-1 in T cells (174) or PD-L1 in cancer cells (188) identified factors that were critical for their expression. These studies identified novel *trans*-factors and implicated specific cellular pathways in the regulation of PD-1/PD-L1 expression. CRISPR has also helped to decode *cis*-regulatory circuitry of the PD-1/PD-L1 pathway. Sen et al. (189) mapped chromatin accessible sites in acute and chronically activated murine $CD8^+$ T cells to identify putative exhaustion enhancers in the PD-1 locus. A CRISPR-Cas9 pooled screening approach was used to saturate accessible sites with perturbations confirming required *cis*-regulatory sequences for eight of these putative enhancers (189). Taken together, CRISPR screens are a powerful platform to map coding and noncoding sequences that regulate pathways for cancer immunotherapy.

Productive immune clearance of malignancies depends on genetic programs in cancer cells and immune cells. In vitro CRISPR experiments with coculture systems have been used to identify mutations in cancer cells that affect their survival in the presence of antigen-specific $CD8^+$ T cells (190). This work identified genes in antigen processing and presentation critical for T cell killing (190, 191). In vivo CRISPR screens have also been used to study the interaction between the immune system and transplantable tumors (192). Due to the limit in numbers of cells that can be assayed in in vivo screens, this study focused on genes that represent key functional pathways. Pools of edited cancer cells were transplanted into immunosufficient mice that were then given immunotherapies to identify genes that were important for resistance or susceptibility to these treatments. Immunodeficient animals were used to control for cancer cell–autonomous effects of gene knockouts. TNF activation/NF-κB signaling, antigen processing and presentation, inhibition of kinase signaling, and ubiquitin proteasome pathway were all found to increase the efficacy of immunotherapy. In addition, PTPN2 knockout was found to sensitize cancer cells and promote immunotherapy by increasing antigen presentation and IFN-γ signaling. CRISPR-based functional studies in T cells can prioritize novel targets for immunotherapy drug development and improve the design of genetically reprogrammed adoptive cellular immunotherapies. We recently overcame challenges of genome-wide CRISPR screens in primary human immune cells to identify regulators of T cell stimulation and immunosuppression responses (46). Together these

approaches are revealing the complex network of genetic factors that mediate immune responses to cancer immunotherapy.

ENGINEERING CELLULAR THERAPIES

Cellular therapies are an emerging treatment class for human diseases. The US Food and Drug Administration has now approved two genetically modified T cell therapies for cancer immunotherapy. These products rely on nontargeted viral integrations to insert CARs into T cell genomes, programming the immune cells to recognize an antigen found on malignant cells (**Figure 3*b***). As cell therapies gain momentum, CRISPR provides countless opportunities to modify endogenous immune cells' genome sequences to enhance therapeutic properties. For example, efforts are underway to engineer T cells to overcome the immunosuppressive tumor microenvironment. For example, several groups have demonstrated PD-1 can be ablated in engineered antigen-specific T cells (193). The first CRISPR-Cas9 clinical trials are now enrolling patients for treatment with engineered TCR specificity (NY-ESO-1 TCR viral transduction with CRISPR-mediated *TRAC* and *TRBC* gene deletion) plus CRISPR *PD-1* deletion. Numerous academic and commercial groups are pursuing additional gene targets that can be deleted to enhance anticancer efficacy of immune cells (194) or to make off-the-shelf allogeneic CAR T cells that escape immune rejection (159).

The delivery of therapeutic transgenes to endogenous loci helps to prevent collateral damage to other genes and preserves endogenous regulation of the transgene, which can be important for engineered cellular function. For example, recent work with CRISPR editing plus AAV templates suggested that targeted site-specific integration of CARs can produce products with more homogenous CAR expression and improved efficacy in preclinical models (83). We recently developed a strategy for nonviral integration of specific TCRα and TCRβ pairs into the endogenous TCRα locus of polyclonal T cell populations to generate functional cells with a desired antigen specificity (86) (**Figure 3*b***). Thus, without the need for time-consuming virus-production steps, newly identified TCRs can be rapidly engineered into T cells for immunotherapy. More broadly, nonviral genome-targeting technology will enable us to delete, insert, or rewrite genetic sequences to replace genes, tune regulatory programs, and rewire immune cells to obtain desired functions.

CRISPR has been widely adopted to decode the fundamental circuitry of the immune system, as we have reviewed here. Beyond decoding, CRISPR also offers the opportunity to program new biology into immune cells (**Figure 3*c***). As discussed above, immune cell specificity can already be written. Genome surgery will be attempted to correct pathogenic mutations that cause primary immune dysregulation syndromes. Likewise, CRISPR editing has the potential to strengthen Treg cell programs to suppress autoimmunity, graft-versus-host disease, and transplant rejection. In time, we are likely to have new tools to rewrite how cells sense extracellular signals and how they traffic, proliferate, and survive in the body, and to modify the effector programs they activate in specific settings (195).

We have discussed how CRISPR is providing insight into coding and noncoding gene programs that shape how immune cells contribute to autoimmunity, interact with pathogens, and participate in cancer immunotherapy. These CRISPR-generated functional maps will point us to the genomic sites that can be modified to alter these codes. With improving technology to rewrite nucleotides at those sites, synthetic biology approaches could be harnessed to confer complex functional logic into cells and tune their therapeutic properties. As we think about the next generation of engineered cellular therapies, it is important to consider how to manufacture these cells safely and ensure their beneficial effects in the human body.

CONCLUSIONS AND FUTURE DIRECTIONS

The past decades have seen a revolution in reading genome sequences. An ever increasing number of patient genomes are being sequenced and analyzed. Common variants that modulate the risk of immune dysregulation have been mapped, along with rare mutations that cause Mendelian forms of immune dysregulation. However, major challenges remain to determine causal mutations, relevant genes, and affected cellular pathways. Moreover, the critical challenge remains to translate new genetic knowledge into new clinical interventions. These challenges require the ability to alter genome sequences and not merely the ability to read them. CRISPR has now brought the next revolution in writing genome sequences. In the post-CRISPR era, immunologists can query which genome sequences control specific immune functions. As adoptive immune cell therapies continue to advance, there are mounting opportunities to employ CRISPR-engineered cells for new targeted treatments. This could transform how genetic diseases of the immune system are treated in the future. CRISPR will help to validate causal mutations in affected cell types. As we develop tools needed for safe and effective genome surgery, impaired immune cells or hematopoietic stem cells could be corrected ex vivo and adoptively transferred into patients. Looking forward, decoding of immune cell programs with CRISPR will also enable more complex reprogramming of immune cells to make them more flexible and effective cellular drugs for a wide range of human diseases.

DISCLOSURE STATEMENT

A.M. is a cofounder of Spotlight Therapeutics, Sonoma Biotherapeutics, and Arsenal Biosciences, serves as a scientific advisory board member to PACT Pharma, and was previously an advisor to Juno Therapeutics. The Marson laboratory has received sponsored research support from Juno Therapeutics, Epinomics, and Sanofi and a gift from Gilead. The Marson laboratory has received funding from the Parker Institute for Cancer Immunotherapy, CZ Biohub, and the Innovative Genomics Institute. The Marson laboratory has received reagents from Illumina. A.M. has had paid or unpaid speaking and/or consulting engagements with Thermo Fisher, Bernstein, Merck, Abbvie, Genentech, Illumina, Arcus, Jackson Laboratories, Nanostring Technologies, GLG, Lonza, and Health Advances. The Marson laboratory at UCSF has filed patent applications related to CRISPR-based modifications of immune cells. New companies may result from some of the work described in this review. D.R.S. is a co-founder of Beeline Therapeutics.

ACKNOWLEDGMENTS

We thank Patrick Hsu, Jeffrey Bluestone, Abul Abbas, Chun Jimmie Ye, Andrew Levine and members of the Marson laboratory for input and discussion. A.M. received a career award from the Burroughs Wellcome Fund, funding from the Parker Institute for Cancer Immunotherapy (PICI), gifts from Galen Hoskin and Jake Aronov, and funding from the Innovative Genome Institute (IGI) and is a Chan Zuckerberg Biohub investigator.

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