intra-tumoral *CD8A* expression was associated with increased amounts of transcripts encoding for proteins associated with T cell exhaustion, including the inhibitory receptors *PD1*, *LAG3*, *HAVCR 2*, *CD244*, *CD160*, and the transcription factors *PRDM1* and *EOMES*. These findings suggest that at least in a subset of patients, immune evasion and T cell exhaustion coevolve with the acquisition of MAPKi resistance (Figure 1).

The study from Hugo *et al*. along with several recent preclinical and clinical studies highlights the importance of integrating studies of intratumoral immune dynamics into our understanding of the signaling pathways that drive tumor development and the impact of different therapies on the cancer cells and the tumor microenvironment. For example, $BRAF^{V600E}$ melanoma cells express immunosuppressive cytokines (IL-6, IL-10, VEGF), which promote the recruitment of myeloid derived suppressor cells and regulatory T cells [6]. In a mouse model of melanoma, constitutively active β-catenin signaling was shown to result in T cell exclusion and resistance to checkpoint blockade through defective dendritic cell recruitment and T cell priming [7]. The findings of Hugo *et al*. suggest that for patients afflicted with melanoma tumors that are resistant to MAPKi and exhibit decreased mRNA levels of antigen presentation genes and *CD8A*, subsequent salvage therapy with immune checkpoint blockade may not be efficacious. This raises the question of whether underlying characteristics of the baseline tumor prior to MAPKi treatment can be used to predict which tumors will develop an immunosuppressive microenvironment with few infiltrating CD8 T cells along with MAPKi resistance.

These findings also emphasize the need to understand cancer evolution along multiple dimensions: genomic, non-genomic, and environmental (Figure 1). While genomic, epigenomic, and transcriptional analyses can be performed on small amounts of fixed tumor samples, direct analysis of the immune cell populations in these samples (e.g., flow cytometry) is challenging. The approach taken by Hugo *et al.* of extracting information regarding immune cell infiltration and function from bulk tumor transcriptional data presents a valuable alternative. These analyses will be aided by bioinformatics tools developed to deconvolute immune cell composition and functional states from complex tumor and tissue transcriptomic datasets [8]. Furthermore, two recent studies demonstrate that metabolic alterations in tumors also drive immune suppression and cancer progression [9,10]. Cancer cells and T cells compete for metabolites in the tumor microenvironment, and nutrient deprivation may not only diminish T cell responses, but also increase cancer cell resistance to therapy.

Cancer development, therapy-induced remission, and drug resistance/relapse result from the complex interplay of coevolutionary genetic, transcriptional, epigenetic, immune and metabolic events (Figure 1). A surprising finding of Hugo *et al.* is that the genomic and non-genomic alterations found in MAPKi-resistant melanomas was quite diverse; thus there are likely many pathways leading to resistance. Whether this diversity is due to intrinsic properties of the tumors, patient characteristics, environmental factors, or all three, is a critical question for future studies. While genome sequencing and other 'omics' technologies rapidly advance, we need to understand the interplay of these multidimensional factors determining cancer evolution to develop effective truly personalized therapies.

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[1]Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

*Correspondence: schietia@mskcc.org (A. Schietinger).

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References

1. Holzel, M. *et al.* (2013) Plasticity of tumour and immune cells: a source of heterogeneity and a cause for therapy resistance? *Nat. Rev. Cancer* 13, 365–376
2. Postow, M.A. *et al.* (2015) Immune checkpoint blockade in cancer therapy. *J. Clin. Oncol.* 33, 1974–1982
3. Ilieva, K.M. *et al.* (2014) Effects of BRAF mutations and BRAF inhibition on immune responses to melanoma. *Mol. Cancer Ther.* 13, 2769–2783
4. Hugo, W. *et al.* (2015) Non-genomic and immune evolution of melanoma acquiring MAPKi resistance. *Cell* 162, 1271–1285
5. Paley, M.A. *et al.* (2012) Progenitor and terminal subsets of $CD8^+$ T cells cooperate to contain chronic viral infection. *Science* 338, 1220–1225
6. Sumimoto, H. *et al.* (2006) The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J. Exp. Med.* 203, 1651–1656
7. Spranger, S. *et al.* (2015) Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature* 523, 231–235
8. Gentles, A.J. *et al.* (2015) The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat. Med.* 21, 938–945
9. Chang, H.Y. *et al.* (2015) Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell* 162, 1229–1241
10. Ho, P.C.A. *et al.* (2015) Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses. *Cell* 162, 1217–1228

Spotlight

Efficient Gene Editing in Primary Human T Cells

Yvonne Y. Chen[1,*]

Recent advances in T-cell therapy for cancer, viral infections, and autoimmune diseases highlight the broad therapeutic potential of T-cell engineering. However, site-specific genetic manipulation in primary human T cells remains challenging. Two recent studies describe efficient genome editing in T cells using CRISPR and TALEN approaches.

Immunotherapy has generated tremendous excitement in recent years by demonstrating the potential to transform medical practices in diverse disciplines

[1]. In particular, the manipulation of T cells as either therapeutic effectors or treatment targets has emerged as a central focus [2]. The adoptive transfer of autologous T cells expressing chimeric antigen receptors (CARs) targeting CD19 has achieved 90% remission rate among heavily pretreated patients with B-cell malignancies [3]. Furthermore, checkpoint inhibitor therapies that booster T-cell effector functions by blocking CTLA-4 and PD-1 signaling have shown marked efficacy in the treatment of advanced melanoma [4]. Beyond cancer therapy, zinc-finger nuclease-based editing of CCR5 and CXCR4 expression has been shown to confer HIV resistance upon edited $CD4^+$ T cells [5], and the engineering of regulatory T (Treg) cells holds promise as a treatment option for autoimmune diseases [6]. As these T-cell applications mature, an increasing number of genetic manipulations, for example, introduction of suicide genes or elimination of endogenous inhibitory receptors, have been proposed to further enhance the safety and/or efficacy of T-cell therapeutics [7]. However, implementation of such systems requires efficient and precise methods to genetically modify primary human T cells (Figure 1), a challenge that is further complicated by the requirement for rapid cell manufacturing in clinical applications such as adoptive T-cell therapy.

Lenti- and retroviruses as well as Sleeping Beauty transposon systems have been used to stably integrate transgenic constructs into primary T cells [2], but these methods cannot site-specifically insert or disrupt genetic elements. In a recent article published in the *Proceedings of the National Academy of Sciences*, Schumann *et al*. reported on the site-specific genome editing of primary human T cells using Cas9 ribonucleoproteins (RNPs) [8]. Cas9 RNPs are recombinant Cas9 proteins complexed with *in vitro*-transcribed single-guide RNAs (sgRNAs), and Cas9 RNPs delivered via electroporation were shown to significantly reduce or eliminate CXCR4 and PD-1 expression in $CD4^+$ T cells by introducing insertions and deletions (indels) in the targeted genes. Furthermore, the inclusion of a homology-directed region (HDR) template

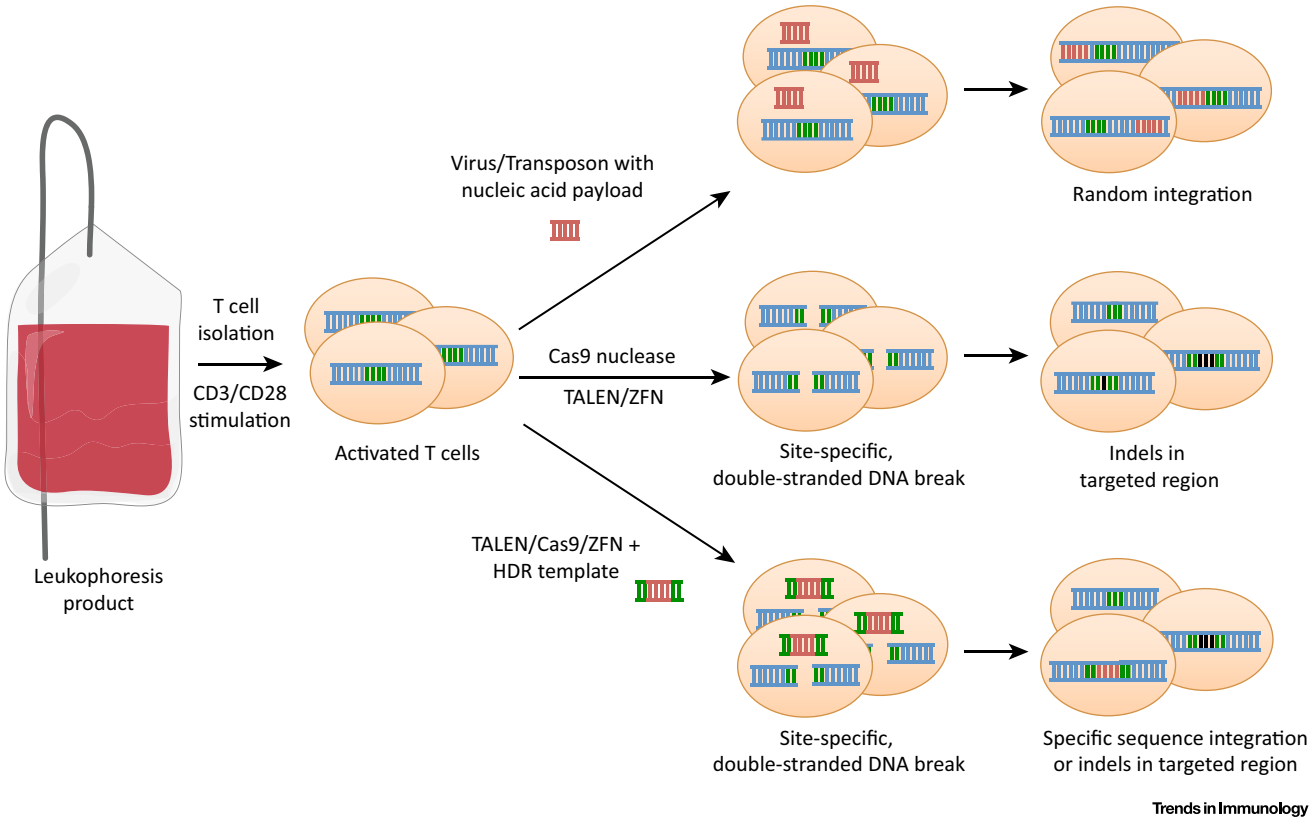

Figure 1. Primary Human T Cells Can be Genetically Modified to Introduce Exogenous Sequences or Disrupt Endogenous Genes. T cells isolated from donor blood are activated through CD3/CD28 stimulation. Activated T cells can be transduced with lenti- or retroviruses or electroporated with Sleeping Beauty transposon system components to achieve non-site-specific integration of transgenic constructs. Alternatively, activated T cells can be electroporated with DNA, mRNA, or proteins encoding Cas9 nuclease, transcription effector nuclease (TALEN), or zinc-finger nuclease (ZFN) to achieve site-specific gene disruption. Finally, Cas9, TALEN, and ZFN can be co-delivered with homology-directed repair (HDR) templates to site-specifically integrate transgenic constructs. None of the editing methods achieves 100% efficiency, thus some cells will remain unedited. In the case of genome editing with HDR templates, a fraction of the cells will integrate the desired exogenous sequences while others will experience insertions/deletions (indels) in the targeted region without integration of the desired sequence.

successfully introduced exogenous DNA into the genome at the Cas9 cleavage site [8]. Although the exact editing efficiency was donor dependent and varied with the quantification method used, deep sequencing results indicated that up to 55% of treated cells contained indels in the targeted region, with ~20% of cells incorporating the exogenous DNA sequence introduced through the HDR template [8]. The electroporation method described by Schumann *et al*. can be accomplished using commercially available reagents and equipment, making it an accessible and readily implementable technique for T-cell genome editing.

The work by Schumann *et al*. is joined by other recent reports focused on primary human T-cell engineering using site-specific nucleases. Hendel *et al*. reported the disruption of CCR5 expression in T cells by codelivering chemically modified sgRNAs with Cas9 mRNA or protein via electroporation, achieving up to 49% indel frequency in activated primary human T cells based on tracking of indels by decomposition (TIDE) analysis [9]. These Cas9-based editing techniques will enable the deletion of a wide variety of target genes, including the checkpoint receptors CTLA-4 or PD-1 in tumor-targeting T cells or the virally targeted chemokine receptors CCR5 and CXCR4 in T cells of HIV patients. The insertion of exogenous DNA sequences using HDR templates demonstrated by Schumann *et al*. further opens the possibility of site-specifically integrating transgenic constructs [8], thereby lowering the risk of gene insertion in oncogenic sites. However, current efficiency of gene integration by nuclease-mediated homologous recombination is low compared to standard viral transduction, thus the latter is likely to remain a dominant method for stable transgene integrations that do not require site specificity. Consequently, an important next step will be to develop protocols that allow combinatorial application of multiple gene-editing techniques in primary T cells.

A major challenge in primary T-cell engineering lies in the limited time frame in which genetic manipulation can be achieved with high efficiency. Unstimulated primary T cells are significantly less receptive to exogenous nucleic acid or protein uptake compared to activated T cells [9]. On the other hand, repeated stimulation will lead to T-cell exhaustion and similarly decrease genome-editing efficiency. Therefore, protocol optimization will be required to enable the effective application of multiple genetic manipulation techniques on one T-cell product. To this end, Poirot *et al*. recently reported in *Cancer Research* a method to both disrupt endogenous genes and introduce CARs into primary human T cells on a schedule that is compatible with typical clinical T-cell manufacturing processes [10]. There, the authors activated freshly isolated human T cells via CD3 stimulation, lentivirally transduced cells at 72 h postactivation to stably integrate a CAR transgene, and electroporated the transduced cells at 120 h with mRNA encoding transcription activator-like effector nucleases (TALENs) to simultaneously disrupt the expression of CD52 and the T-cell receptor α constant region [10]. Editing efficiency using this combined protocol was also donor dependent, with results indicating >70% viral integration efficiency and >40% TALEN-mediated double-knockout efficiency in most production runs [9]. This manufacturing procedure yielded CAR-T cells that were specific to $CD19^+$ targets, resistant to the chemotherapeutic agent alemtuzumab, and incapable of triggering graft-versus-host disease (GVHD), highlighting the ability to generate multi-functional T cells with efficient genome-editing techniques.

Many T-cell engineering applications thus far do not require 100% efficiency in gene modification. For example, adoptive cell therapy trials regularly use unsorted CAR–T-cell products that contain a fraction of unmodified T cells [3]. However, applications such as suicide-gene integration or the production of 'off-the-shelf' T cells lacking antigens responsible for GVHD will require complete editing of the T-cell population to achieve their desired purpose. Further advancements in methods to edit primary T cells, and subsequently isolate and expand edited cells, will greatly increase the safety and efficacy of engineered T cells for diverse applications.

[1]Department of Chemical and Biomolecular Engineering, University of California—Los Angeles, Los Angeles, CA 90095, USA

*Correspondence: yvchen@ucla.edu (Y.Y. Chen).

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References

1. Couzin-Frankel, J. (2013) Breakthrough of the year 2013. Cancer immunotherapy. *Science* 342, 1432–1433
2. Corrigan-Curay, J. *et al.* (2014) T-cell immunotherapy: looking forward. *Mol. Ther.* 342, 1564–1574
3. Maude, S.L. *et al.* (2014) Chimeric antigen receptor T cells for sustained remissions in leukemia. *N. Engl. J. Med.* 371, 1507–1517
4. Topalian, S.L. *et al.* (2015) Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* 27, 450–461
5. Didigu, C.A. *et al.* (2014) Simultaneous zinc-finger nuclease editing of the HIV coreceptors ccr5 and cxcr4 protects $CD4^+$ T cells from HIV-1 infection. *Blood* 123, 61–69
6. Barrett, D.M. *et al.* (2015) Chimeric antigen receptor- and TCR-modified T cells enter Main street and Wall street. *J. Immunol.* 195, 755–761
7. Jensen, M.C. and Riddell, S.R. (2015) Designing chimeric antigen receptors to effectively and safely target tumors. *Curr. Opin. Immunol.* 33, 9–15
8. Schumann, K. *et al.* (2015) Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10437–10442
9. Hendel, A. *et al.* (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol.* 33, 985–989
10. Poirot, L. *et al.* (2015) Multiplex genome edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. *Cancer Res.* 75, 3853–3863