will tell us if these therapeutic approaches may find a different indication for the CLL with unmutated *IGHV* (U-CLL) or mutated *IGHV* (M-CLL). U-CLL and M-CLL carry distinctive cellular origin, biology, epigenetics and genetics, and clinical behavior.[6] Therefore, prognostic algorithms may need to be generated separately in these 2 types of CLL. Only extended observation time and a better understanding of how to combine new targeted drugs will make it possible to identify the best prognostic factors of OS. In the interim, when combined with the outcome data in Langerbeins et al, the CLL-IPI remains a tool that assists prognostication for patients with CLL in the era of chemotherapy-free targeted regimens.

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

Comment on Kath et al, page 2599

Putting together the pieces: *CAR* into *CD3ζ* locus

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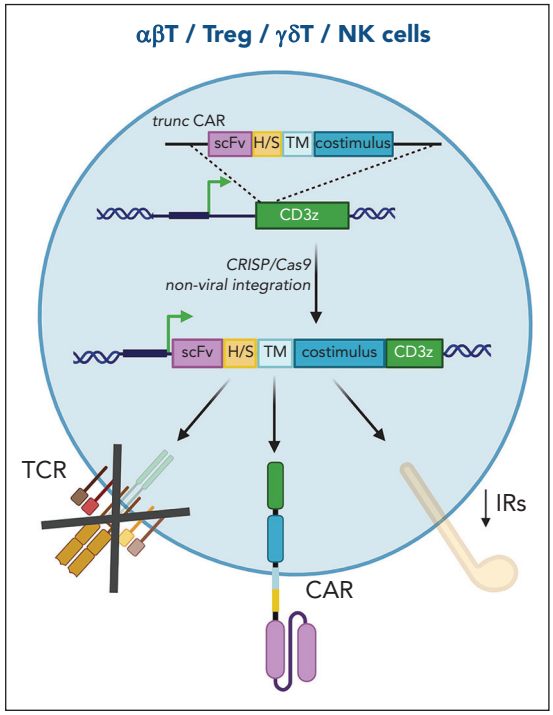
In this issue of *Blood*, Kath et al[1] report a novel virus-free strategy to redirect cell specificity via in-frame integration of CD3ζ-deficient chimeric antigen receptors (CARs) within the *CD3ζ* gene, thereby producing functional CAR fusion genes. This knock-in approach (1) results in T-cell receptor (TCR) ablation; (2) allows physiological CAR regulation by the *CD3z* promoter; and (3) can be applied to different cell types, that is, TCR-αβ and TCR-γδ effector T cells, regulatory T cells, and natural killer (NK) cells (see figure).

Most current approaches to generate CAR T cells rely on viral vector–mediated delivery of the CAR transgene into T cells, which results in semirandom integration into the genome and drives CAR expression through strong exogeneous promoters. This approach carries the intrinsic risk of insertional mutagenesis that may promote malignant transformation. Although T cells are considered relatively resistant to genotoxicity, sporadic cases of CAR^+ T-cell malignancies have been recently reported using viral vectors and transposons.[2,3] Existing data from follow-up studies suggest very low risk with CAR T-cell therapies compared with other cancer treatments, but longer monitoring periods of treated patients and development of safer strategies for CAR redirection are desirable, especially for application in nonlethal diseases such as autoimmune conditions. Kath et al developed a virus-free gene transfer strategy to precisely integrate the *CAR* gene into the *CD3ζ* locus, thus reducing the risk of insertional mutagenesis. This approach exploits cell transfection with precomplexed CRISPR-Cas9 ribonucleoproteins and double-stranded DNA, which creates a targeted double-strand break in the *CD3ζ* locus and serves as a template for homology-directed DNA repair. The resulting fusion gene is composed of the truncated *CAR* linked to the endogenous *CD3ζ*, which serves as the activation domain, and is placed under the transcriptional control of the *CD3ζ* promoter for physiological CAR regulation. The authors report encouraging data regarding off-target assessment, suggesting high-precision *CD3ζ* targeting. However, caution is called for, because in silico methods can fail to predict experimentally determined off-target sites.

Kath et al show that *CD3ζ* editing shares some advantages with CAR insertion into the *T-cell receptor (TCR) α-chain constant region (TRAC)* locus, which is considered the gold standard for gene-edited CAR T cells.[4] Importantly, both strategies restrict transgene expression to specific cell lineages, preventing unwanted CAR expression in B-cell leukemic blasts that may cause relapse in the autologous setting.[5] Moreover, in both cases, TCR/CD3 complex assembly and expression on the cell surface is impeded, reducing the risk of graft-versus-host disease in the allogeneic setting but still requiring association with strategies to avoid CAR T-cell rejection. Finally, both *TRAC* and *CD3ζ* integration allow physiological TCR-like CAR down-regulation after antigen engagement, which may prevent T-cell exhaustion and



CD3ζ-editing strategy. A CAR missing the activation domain (*trunc*CAR) is integrated in the *CD3ζ* locus through CRISPR/Cas-9 nonviral technology to obtain a fully functional CAR construct. The proposed strategy brings the CAR under the transcriptional regulation of the *CD3ζ* promoter, reducing the expression of inhibitory receptors, and ablates the expression of the endogenous TCR. This approach can be applied to all T-cell subsets and NK cells. αβT, T-cell receptor-αβ T lymphocytes; CAR, chimeric antigen receptor; H/S, hinge/spacer sequences; scFv, single-chain fragment variable; TCR, T-cell receptor; TM, transmembrane domain; Treg, regulatory T lymphocytes; γδT, TCRγδ T lymphocytes; NK, natural killer.

terminal differentiation. The enrichment of less differentiated and poorly exhausted T-cell subsets in the infusion product has been associated with improved antitumor activity and reduced inflammation-related toxicities in preclinical models and patients.[6,7] Although excessive signal strength and duration induced by constitutively active promoters may compromise CAR T-cell fitness, calibrated activation and/or transient rest have been reported to prevent terminal differentiation and exhaustion.[4,8,9] Despite CAR downmodulation observed with both *TRAC* and *CD3ζ* editing upon antigen engagement, the authors reported lower basal expression levels when the CAR is expressed in the *CD3ζ* with respect to the *TCR* locus. Further studies are required to identify whether these differences are promoter-related, construct-related (full CAR vs truncated CAR), or procedure-related (more efficient access to one site over the other). Interestingly, the authors show that CAR expression in the *CD3ζ* locus can be increased by inserting a GSG linker before the 2A self-cleavage peptide. Functionally speaking, although *CD3ζ*-edited CAR T cells performed similarly overall to their *TRAC*-edited counterpart, *CD3ζ*-edited CD19 CAR T cells carrying the GSG linker showed small but significant superiority in vivo. Further investigation of this design using other CAR targets, such as human epidermal growth factor 2, and the relative impact of the selected costimulatory domain over others will be instrumental to highlight specific advantages.

As opposed to *TRAC*, *CD3ζ* editing allows a reduction in required transgene size and permits targeted integration in all T-cell subsets and NK cells, which have shown great promise for CAR-based therapy of cancer.[10] Kath et al show that *CD3ζ* disruption in primary human NK cells did not impair canonical NK functions, such as antibody-dependent cellular cytotoxicity and degranulation, and report for the first time nonviral knock-in to redirect NK specificity with CARs. However, these results are still in the proof-of-concept stage and strategies to increase the efficiency of reprogramming are required before testing in suitable in vivo models. Also, the functional edge of having regulated CAR expression in NK cells remain to be proved, because CAR-NK functionality seems more closely linked to immediate cytotoxic activity than to functional persistence.

CD3ζ editing has great potential for use in the allogeneic setting, using either T cells or NK cells. From this perspective, because electroporation can negatively affect cell survival, expansion, and phenotype, a careful setup of the manufacturing procedure is warranted to generate adequate numbers of high-quality CD3ζ-edited CAR products. Allogeneic platforms carry the great advantage of using healthy donors for starting material, overcoming functional defects imprinted in patients by the underlying disease or previous treatments. Moreover, these strategies would make the treatment available to patients with lymphopenia and speed up drug administration for rapidly progressing diseases. Overall, this would result in simpler manufacturing procedures and reduced costs, allowing for full exploitation of CAR-based approaches.

Kath and colleagues' strategy provides important improvements for engineered T-cell therapies. Regulated expression through the *CD3ζ* promoter may improve antitumor efficacy by reducing excessive chronic activation. Nonviral integration of the *CAR* gene into a specific locus can reduce the risk of insertional mutagenesis. The expression of the *CD3ζ* gene in different cell types permits editing of all T-cell subsets and NK cells. Finally, their approach is suitable for application in the allogeneic setting, expanding the use of CAR-based therapies to a wider cohort of patients.

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LYMPHOID NEOPLASIA

Comment on Gulla et al, page 2612

Immunogenic therapy: new actors in myeloma

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In this issue of *Blood*, Gulla et al identify GABA type A receptor-associated protein, GABARAP, as a key player in mediating resistance to immunogenic chemotherapy in multiple myeloma (MM), by halting calreticulin translocation to the external leaflet of the cell membrane. By shedding light on immunogenic cell death (ICD), the authors provide a framework for potential novel combination treatments including ICD inducers to restore anti-MM immunity.[1]

The recognition and removal of stressed, ageing, and dying cells by phagocytes is vital for maintaining health. These homeostatic processes also play a crucial role in the immune response against cancer. During ICD, deteriorating tumor cells shed specific signals conducive to phagocytosis, including the exposure of calreticulin, an endoplasmic reticulum protein, on the cell surface. This presentation of an "eat me" signal facilitates the engulfment of cancer cell by antigen-presenting cells (APCs) such as dendritic cells and macrophages. These APCs subsequently process and present tumor antigens to T cells, thereby initiating an adaptive antitumor immune response.[2,3] Cancer cells can exploit various pathways to overcome the induction of ICD. Thus, a deeper understanding of the biologic mechanisms involved by these cancer cells and devising strategies to counteract them is an important strategy to improving cancer treatment.[4]

Gulla et al reported in 2021 that bortezomib induces calreticulin-dependent ICD in MM cells.[5] The induction of ICD is associated with a strong antitumor immune response in immunocompetent mice, with the simultaneous induction of immunological memory. Thus, a low dose of bortezomib produces tumor regression in mice bearing syngeneic tumors.[5]

Here, Gulla et al investigated the mechanisms involved in resistance to the immunogenic effects of bortezomib in MM. They analyzed a public data set to identify the prognostic role of GABARAP, a well-described vesicular trafficking and autophagy regulator.[6] Next, by using MM cell lines, animal models and single-cell RNA sequencing, the authors identified GABARAP as a mediator of bortezomib resistance, by modulating phagocytosis, T cell–mediated cell lysis, and autophagy. Furthermore, the authors found that GABARAP impacts the fitness of organelles such as the Golgi apparatus and endoplasmic reticulum, suggesting its involvement in cellular homeostasis and immune evasion.

Conventionally, cytotoxic sensitivity loss has been linked to extrinsic and intrinsic mechanisms.[7] Gulla et al figured out a novel mechanism of drug resistance via GABARAP-calreticulin-dependent ICD. In vitro, a lower level of GABARAP impaired calreticulin exposure and T-cell responses. This finding reveals a mechanism of primary resistance to proteasome inhibition due to a lack of both spontaneous and ICD-mediated anti-MM immunity. Based on the proposed model, a multimodal approach to quantify the effects beyond direct cytotoxicity is needed to fully characterize the clinical impact of these findings.[5]

Gulla et al also found that rapamycin restored signaling lost with GABARAP knockout during bortezomib treatment, identifying autophagy modulation as a potential clinical strategy. However, ICD is also triggered by other anti-MM therapeutics.[7] Therefore, boosting immunogenicity deserves broader exploration, such as the impact on BCMA-, CD38-, SLAMF7-, GPRC5D-, and FcRH5-directed therapies.[7] Thus, the GABARAP level might impact other clinical scenarios besides bortezomib-induced ICD. A deeper understanding of the immune response and its impact on drug resistance could pave the way to revive antitumor immunity with combination approaches.

Gulla et al's research also was based on the hypothesis that MM immune evasion in present in some patients with high-risk disease, as the GABARAP locus is on the short arm of chromosome 17 that is deleted in this subgroup of patients. Indeed, GABARAP expression is broadly downregulated in MM plasma cells compared with normal plasma cells from