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blood commentary

IMMUNOBIOLOGY AND IMMUNOTHERAPY

Comment on Selli et al, *page 3153*

Signatures of dysfunctional CAR T cells

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In this issue of *Blood*, Selli et al[1] have discovered that whereas CD28-bearing chimeric antigen receptor (CAR) T cells become dysfunctional and exhausted via the classical events occurring with chronic stimulation from tumors or chronic infections, 4-1BB–bearing CAR T cells become dysfunctional in a different way that is driven by the transcription factor FOXO3.

A little over a decade ago, CAR T cells were shown to have remarkable efficacy in B-cell malignancies.[2-4] All of them targeted the B-cell molecule CD19 and bore the intracellular signaling domain of 1 of 2 costimulatory molecules, either CD28 or 4-1BB. The incorporation of a costimulatory domain into the CAR was hypothesized to improve persistence and function and, therefore, antitumor effects. Owing to the remarkable initial success of these initial products, all 6 constructs that are approved by the US Food and Drug Administration, including those targeting the B-cell maturation antigen expressed by myeloma plasma cells, and most other CARs in clinical development across a variety of indications, incorporate 1 of these 2 signaling domains.

Because CARs recognize antigen with high affinity and are expressed by constitutive promoters, investigators noted early on that continuous stimulation with antigen, and sometimes even just high-level CAR expression, could drive T-cell dysfunction. These dysfunctional T cells had reduced cytotoxicity, cytokine production, and proliferation after antigen stimulation; this could be overcome by intermitting rest[5] from signal transduction. In patients, high tumor burden has been correlated with reduced response and increased T-cell dysfunction.[6] Until now, this dysfunction was thought to be essentially the same phenomenon as T-cell "exhaustion," which has been extensively described in the setting of chronic viral infection and cancers treated with checkpoint blockade.[7]

Here, Selli et al modeled chronic activation by repetitive in vitro stimulation of CD19-directed CAR T cells bearing either CD28 or 4-1BB intracellular signaling domains to generate dysfunction, and then performed a comprehensive analysis of the transcriptional, epigenetic, and phenotypic programs. The CAR T cells were stimulated with new tumor cells at a low effector-to-target ratio every 48 hours. After 2 weeks, the CAR T cells lost their ability to kill antigen-positive targets, could no longer make cytokines, and could no longer proliferate. Next, they interrogated these dysfunctional CAR T cells phenotypically, using multiparameter cytometry by time-of-flight. They found that CAR T cells bearing CD28-based CARs bore the hallmarks of classic T-cell exhaustion, including resurgent expression of PD-1, TIGIT, LAG3, TIM3, and CTLA4 (see figure). In contrast, dysfunctional T cells bearing 4-1BB CARs occupied different t-distributed stochastic neighbor embedding space by nearest neighbor analysis and expressed higher CD62L and CD25 on their surface. Interrogation of the transcriptional programs of these dysfunctional CAR T cells using RNA sequencing revealed that CD28-based CAR T cells were highly enriched for exhaustion-associated genes, whereas 4-1BB–based dysfunctional CAR T cells did not have the classic exhaustion signatures. Recent studies of T-cell exhaustion have emphasized that the state is defined by specific epigenetic alterations.[8] The investigators also confirmed these differences using assay for transposase-accessible chromatin with sequencing (ATAC-seq), particularly of the chromatin accessibility of the gene encoding PD1 (*PDCD1*), which looked like classic exhaustion in the CD28-based CAR T cells but not the 4-1BB–based CAR T cells. The authors then used longitudinal single-cell RNA sequencing and identified that most of the dysfunctional 4-1BB CAR T cells were defined by a distinct set of genes involved in cytotoxicity (*GNLY, CCL5, PRF1, GZMA, GZMK, CTSW*), natural killer cell identity (*KLRK1, KLRC2*), and T-cell differentiation (*ID2*), which they termed the T_{BBD} signature (see figure). Intriguingly, their T_{BBD} signature was confirmed in a single patient who had received tisagenlecleucel (an anti-CD19/4-1BB CAR construct) and whose lymphoma had progressed despite persisting CAR T cells in their blood.

To understand the origin of this novel molecular program of dysfunction, they interrogated transcription factor binding accessibility over time. As expected, CD28-CAR dysfunction had increased accessibility for Jun:Fos (AP1) binding, but in contrast, 4-1BB-CAR dysfunction opened homeobox (HOX) and forkhead box (FOX) sites, and the small conditional RNA-sequencing data revealed high activity of *FOXO3*. Furthermore, in mechanistic studies, disruption of FOXO3 by CRISPR/Cas resulted in resistance to dysfunction after repetitive antigen stimulation, and, conversely,

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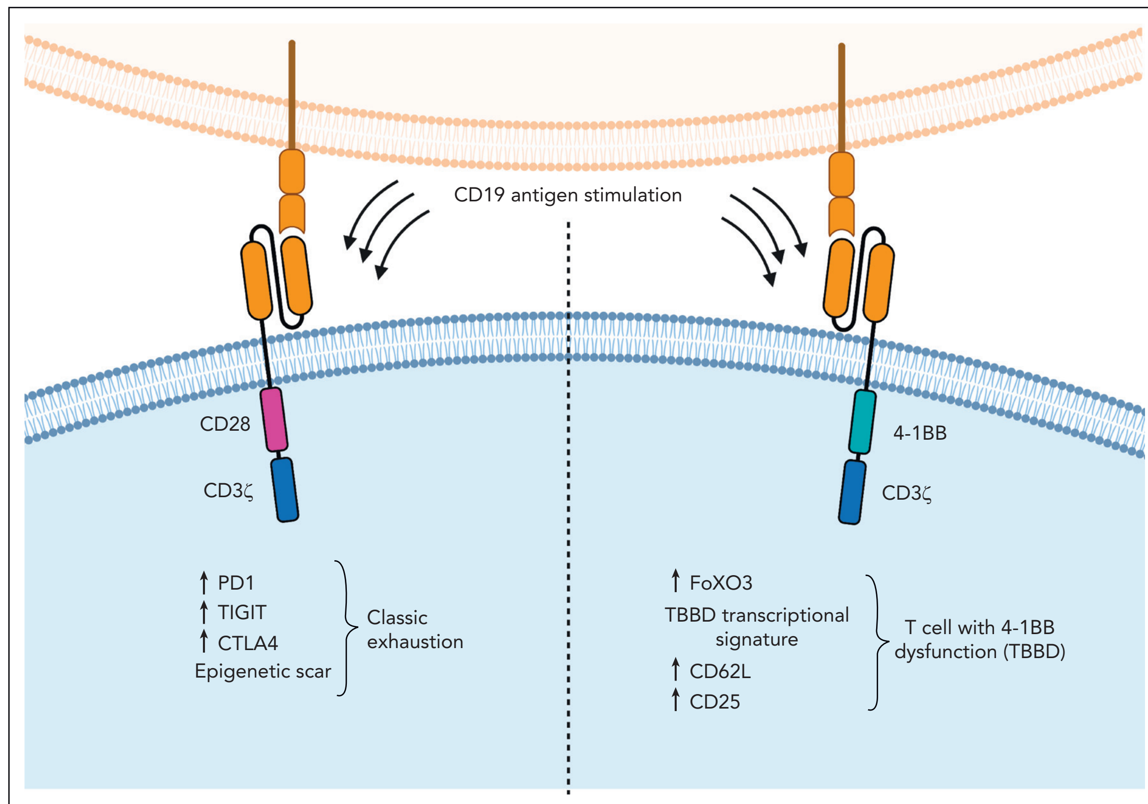

CAR T cells bearing different costimulation domains exhibit different transcriptional, epigenetic, and phenotypic signatures when they become dysfunctional after chronic stimulation. CAR T cells bearing a 4-1BB costimulation domain reactivate FOXO3, which drives a novel program of dysfunction that is distinct from classic T-cell exhaustion.

FOXO3 overexpression dramatically reduced the expansion of 4-1BB (but not CD28) CAR T cells in the setting of repetitive antigen stimulation. In murine xenograft stress models, in which very low numbers of CAR T cells are injected to treat higher tumor burdens, FOXO3 knockout CAR T cells bearing a 4-1BB intracellular signaling domain improved survival.

Remarkably, the authors discovered a new molecular mechanism by deep technological interrogation, spanning from high-dimensional cytometry to longitudinal single-cell RNA sequencing, and while still using a relatively simple in vitro model (repetitive antigen stimulation). The molecular program itself, and reactivation of FOXO3, is interesting and perhaps not typically found in nature. In natural biology, 4-1BB stimulation is temporally dissociated from T-cell receptor (TCR) stimulation, and TCR expression itself cycles with antigen exposure, thus reducing the possibility of tonic signaling. Future studies could include further validation of the T_{BBD} transcriptional signature and FOXO3 activity in more patients who received tisagenlecleucel and did not respond. Likewise, a deeper understanding of whether tisagenlecleucel gets exhausted in patients based on chronic exposure to nascent $CD19^+$ B cells, regardless of disease status, should be explored. Interestingly, chronic exposure to nascent B cells was thought to have a role in the decade-long persistence of tisagenlecleucel in the first 2 patients treated, both of whom had prolonged remissions.[9] In addition, it will be interesting for the field to see whether 4-1BB–bearing CAR T cells that are specific to other antigens, such as both of the B-cell maturation antigen (BCMA)–targeted CAR T cells that are approved for multiple myeloma, also exhibit the T_{BBD} signature and FOXO3 reactivation when patients with BCMA-positive disease recur despite persistent BCMA-directed CAR T cells. Finally, at the mechanistic level, it will be interesting to see whether FOXO3 knockout or c-Jun overexpression[10] is more potent in reducing CAR T cell exhaustion.

Conflict-of-interest disclosure: M.V.M. is an inventor on patents related to adoptive cell therapies, held by Massachusetts General Hospital (some licensed to Promab) and University of Pennsylvania (some licensed to Novartis); receives grant/research support from Kite Pharma; has served as a consultant for multiple companies involved in cell therapies; holds equity in 2Seventy-Bio, Century Therapeutics, Neximmune, Oncternal, and TCR2; and serves on the board of directors of 2Seventy Bio.

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https://doi.org/10.1182/blood.2023020935

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LYMPHOID NEOPLASIA

Comment on Largeot et al, *page 3166*

Targeting mRNA translation in CLL

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In this issue of *Blood*, Largeot et al[1] show that flavagline FL3-mediated inhibition of enhanced messenger RNA (mRNA) translation in chronic lymphocytic leukemia (CLL) cells has multiple effects, including blocking proliferation, rewiring MYC-driven metabolism, and controlling CLL growth in a murine model.

CLL is the most frequent leukemia in older people in the Western world. The disease now may often be controlled for years or sometimes may not even require treatment for extended periods. However, CLL still can not be cured, and aggressive forms, such as a transformation into a diffuse large B-cell lymphoma (Richter transformation) or the immunodeficiency caused by CLL, can be rapidly life threatening. Thus, there is a continuing search for cellular vulnerabilities that can be therapeutically exploited. Largeot et al focused on the prior observation that CLL cells have an enhanced protein translation rate compared with normal B cells[2] and on indications that inhibiting mRNA translation may be toxic for CLL cells.[3] The authors first validated an increased translation rate in human CLL cells and then showed that this is also a feature of murine CLL cells in the TCL1-driven murine CLL model. Triggering B-cell receptor (BCR) or Toll-like receptor pathways further increased translation in CLL cells. A known translation inhibitor and modifier, the synthetic flavagline compound FL3, inhibited translation in CLL cells. FL3 was active at a low concentration, at which most other immune cells were not affected. Thus, CLL cells seem particularly sensitive to translation inhibition, opening a promising therapeutic window. Treatment of activated CLL cells with FL3 modified several pathways, including a reduction of MYC activity. Importantly, FL3 treatment also decreased proliferation of CLL cells, rewired multiple metabolic pathways, and induced apoptosis in these cells. MYC seems to play a major role in the metabolic rewiring, as direct MYC inhibition showed similar effects as FL3 treatment. This may be linked to a positive feedback loop, as strong translation initiation promotes MYC activity, and MYC itself promotes translation.

The striking effects of FL3 on CLL cells prompted the authors to investigate its mechanism of action in more detail. An unexpected finding from these studies was that the primary effect of FL3 on its prohibitin targets was not the known impairment of the RAS/RAF pathway. Rather, in CLL cells prohibitins bind directly to the eukaryotic translation initiation factor complex involving eIF4A, eIF4G, and eIF4F, and this binding is inhibited by FL3 (see figure). Thus, a novel mechanism of FL3-mediated inhibition of translation initiation was identified for CLL cells. The important role of prohibitins for translation initiation in CLL cells was confirmed by showing that silencing of prohibitin expression had similar inhibitory effects on translation as FL3 treatment. Finally, in the CLL mouse model, the authors showed that FL3 treatment of transplanted CLL cells slowed CLL growth and prolonged survival of the mice. Notably, FL3 treatment in this model not only inhibited translation in CLL cells (while not affecting normal B cells) but also in regulatory T cells, which also have a relatively high translation rate. As regulatory T cells promote CLL development,[4] the additional effect of FL3 on these cells may be of added value for FL3 treatment of CLL.

The obvious question is whether these in vitro findings are relevant in vivo. Indeed, most CLL proliferation takes place in proliferation centers in lymph nodes, where CLL cells are in contact with T helper cells and presumably receive autoantigen triggering of their often autoreactive BCRs. In these proliferation centers, a fraction of the CLL cells shows MYC expression.[5] Hence, FL3 may specifically target these proliferating and metabolically active tumor cells, thereby attacking the root of CLL tumor clone expansion.

Several aspects deserve further considerations and study. First, CLL is presumably derived from mature $CD5^+$ B cells, and MYC expression and activity were reported for a subset of these normal $CD5^+$ B cells.[6] Hence, a moderate propensity for increased translation may already be an inherent property of the normal counterparts of CLL cells. Second, CLL is usually preceded by the premalignant condition monoclonal B-cell lymphocytosis, in which expanding B-cell clones already carry genetic lesions typical for CLL cells. It would be interesting to know whether these premalignant cells also already show increased translation initiation and metabolic rewiring. If this is the case, would treatment with a translation initiation inhibitor such as FL3 (assuming it

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