Title
Killer fatigue: Transition to NK-cell-like phenotype is a signature of CAR-T cell exhaustion

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Understanding CAR-T cell exhaustion to improve anti-tumor function

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Abstract

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Main text

Chimeric antigen receptor (CAR)-T cells have shown remarkable response rates in treating refractory B-cell malignancies and became the first genetically modified cell-based therapy to receive FDA approval (Westin et al., 2021). However, CAR-T cell therapy has shown limited efficacy against solid tumors, in part due to the abundance of immunosuppressive factors and cell types in the solid tumor microenvironment (TME) that render solid malignancies especially intractable (Hong et al., 2020). The unfavorable TME contributes to CAR-T–cell exhaustion—a state of hypofunctionality characterized by distinct epigenetic, metabolic, and phenotypic signatures—and presents a major roadblock for the effective application of CAR-T cells against solid tumors (Hong et al., 2020; Hou et al., 2021). Many efforts to mitigate CAR-T–cell exhaustion have been reported in recent years, including antibody-mediated blockade of inhibitory receptors such as PD-1 and CTLA-4, depletion of immunosuppressive cell types like myeloid-derived suppressor cells (MDSCs), pharmacological downregulation of CAR expression to enforce T-cell rest, and supplementation of metabolites to delay T-cell differentiation to terminal exhaustion (Tang et al., 2021; Weber et al., 2021). In addition, with the advent of gene-editing technologies such as CRISPR/Cas9, the genetic disruption of relevant genes—such as inhibitory receptors like PD-1, CTLA-4, TIM-3, and LAG-3; TGF-β receptor; and the TOX, TOX2, or NR4A family transcription factors—presents additional approaches to prevent T-cell dysfunction (Tang et al., 2021; Weber et al., 2021). Nonetheless, a more comprehensive understanding of the molecular mechanisms of T-cell exhaustion during antigen stimulation can provide useful insight for novel T-cell engineering strategies to prevent dysfunction in CAR-T cells. In this issue of *Cell*, Good et al. tackle this challenge by developing an *in vitro* model that employs prolonged continuous antigen exposure (CAE) to drive CAR-T cell

exhaustion, and observed through transcriptional, epigenetic, and protein-level analyses that exhausted CD8+ T cells undergo a transition to a natural killer (NK)-like phenotype (Good et al., 2021). The authors further identified ID3 and SOX4 as two transcription factors whose modulation may help prevent or delay CAR-T–cell exhaustion upon prolonged antigen exposure.

To recapitulate characteristics of CAR-T cell exhaustion efficiently and easily, Good et al. developed an *in vitro* model, in which mesothelin-targeting CAR (M5CAR)-T cells were driven to a dysfunctional state through continuous antigen exposure (CAE) by mesothelin-expressing tumor cells (Figure 1A). CD8+ M5CAR-T cells after CAE displayed hallmark signs of T-cell exhaustion, including reduced proliferation, decreased cytokine production, increased expression of inhibitory receptors, and downregulation of surface CAR expression. Bulk RNA sequencing (RNA-seq) of M5CAR-T cells after CAE revealed an enrichment of genes associated with T-cell exhaustion (e.g., *CTLA4, TOX, TIGIT, NR4A2, NR4A3, HAVCR2*), and ATAC-seq (Assay for Transposase-Accessible Chromatin with high-throughput sequencing) results showed CAE leads to chromatin remodeling to resemble that of exhausted human tumor-infiltrating lymphocytes (TILs). Interestingly, several genes associated with natural killer (NK) cells (e.g., *KLRC1, KLRC2, KLRC3, KLRB1, KLRD1, KIR2DL4*) were also upregulated in the post-CAE CAR-T cells. Single-cell RNA sequencing (scRNA-seq) of M5CAR-T cells after CAE enabled the curation of an unbiased dysfunction gene signature comprising the top 30 genes upregulated in CAE-driven dysfunctional clusters, including several genes with previously unknown links to T-cell exhaustion (e.g., RGS16, SRGAP3, DUSP4, NDFIP2, CD9). The upregulation of exhaustion markers and NK-cell receptors was also observed in scRNA-seq data, and a number of transcription factors—such as *ID3* and *SOX4*—were uniquely upregulated in dysfunctional clusters. The transition of CD8+ T cells to NK-like T cells and its correlation with T-cell dysfunction were further bolstered with protein-level assessment using flow cytometry and CyTOF (cytometry by time of flight), in which high levels of checkpoint receptors and NK-associated receptors were observed on M5CAR-T cells after CAE. Of note, the NK-like signature was also observed in M5CAR-T cells recovered from mice with recurrent pancreatic tumors, as well as in CD19-targeting CAR-T cells recovered from lymphoma patients who did not respond to therapy.

Despite their nomenclature, NK-associated inhibitory receptors are not restricted to NK cells, and CD8+ cytotoxic T lymphocytes (CTLs) have been reported to express NK-associated inhibitory receptors such as the killer-cell immunoglobulin-like receptor (KIR) family in humans, or the lectin-like Ly49 family of receptors in mice (McMahon and Raulet, 2001; Vivier and Anfossi, 2004). The expression of NK-associated inhibitory receptors is implicated in increasing the threshold for T-cell activation and concordant dampening of T-cell effector functions (McMahon and Raulet, 2001; Vivier and Anfossi, 2004). However, the functional role of NK-associated receptors in CD8+ T cells and whether their expression is a cause, consequence, or simply correlation of T-cell dysfunction remain uncertain. Nevertheless, Good et al. shed light on the regulatory players in the expansion of dysfunctional, NK-like CD8+ CAR-T cells after CAE by identifying a correlation with the enrichment of transcription factors such as ID3 and SOX4, as well as increased chromatin accessibility at SOX4 sites.

The authors demonstrated that *ID3* knockout (KO) or *SOX4* KO CAR-T cells displayed improved *in vitro* cytotoxicity, as well as reduced expression of T-cell–dysfunction signature genes and NK-associated receptors. Interestingly, *ID3* KO resulted in the abrogation of SOX4 expression, and *SOX4* KO resulted in a partial loss of *ID3* expression, suggesting more complex regulatory networks involving these transcriptional factors may be at play. ID3 is a non–DNA-binding transcription factor that inhibits other transcription factors from binding DNA (Good et al., 2021), and repression of its expression has been found to suppress CD8+ memory T-cell formation (Ji et al., 2011). SOX4 is a high-mobility group (HMB) transcription factor that regulates various developmental processes, and its knockdown has been shown to inhibit the recall proliferation of memory CD8+ T cells (Hu and Chen, 2013). The pleiotropic roles of ID3 and SOX4 and the suggested interaction between them may be further elucidated by in-depth studies of *ID3* KO and *SOX4* KO CAR-T cell phenotype and function in the context of the TME *in vivo*. Looking toward clinical translation, the role of NK-associated inhibitory receptors in maintaining NK-cell self-tolerance and their potential connection to T-cell tolerance would call for safety assessments on the effects of downregulating their expression through *ID3* or *SOX4* KO (Vivier and Anfossi, 2004).

In conclusion, Good et al. address a major hurdle in actualizing CAR-T cell therapy for solid tumors—i.e., the emergence of terminally exhausted, dysfunctional T cells with reduced effector function that thwart successful clinical responses. By identifying gene signatures of dysregulation using an *in vitro* model of T-cell exhaustion, a T-cell engineering strategy was identified to ablate the expression of genes that positively regulate T-cell dysfunction, resulting in more potent cytotoxicity and favorable T-cell fitness. Insights from this study can guide the engineering of more robust, efficacious CAR-T cells for therapeutic applications *in vivo.*

Figure legend

Figure 1. An *in vitro* model of CAR-T cell exhaustion driven by sustained antigen exposure facilitates the identification of gene signatures associated with diminished CAR-T–cell function.
(A) Mesothelin CAR (M5CAR)-T cells are repeatedly stimulated with AsPC-1 pancreatic tumor cells that express mesothelin (MSLN) on the surface. After the continuous antigen exposure (CAE) regimen, M5CAR-T cells are sorted and analyzed using epigenetic, transcriptional, and protein-level analysis technologies to study their phenotypes. RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA sequencing; ATAC-seq, Assay for Transposase-Accessible Chromatin with high-throughput sequencing; CyTOF, cytometry by time of flight.
(B) CRISPR/Cas9-mediated knockout (KO) of transcription factors ID3 or SOX4 can rescue CAR-T cells from terminal exhaustion by downregulating the expression of NK-associated genes and dysfunction signature genes.

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