RNA polymerase II pausing factor NELF in CD8+ T cells promotes antitumor immunity

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T cell factor 1 (TCF1) is required for memory and stem-like CD8+ T cell functions. How TCF1 partners with other transcription factors to regulate transcription remains unclear. Here we show that negative elongation factor (NELF), an RNA polymerase II (Pol II) pausing factor, cooperates with TCF1 in T cell responses to cancer. Deletion of mouse Nelfb, which encodes the NELFB subunit, in mature T lymphocytes impairs immune responses to both primary tumor challenge and tumor antigen-mediated vaccination. Nelfb deletion causes more exhausted and reduced memory T cell populations, whereas its ectopic expression boosts antitumor immunity and efficacy of chimeric antigen receptor T-cell immunotherapy. Mechanistically, NELF is associated with TCF1 and recruited preferentially to the enhancers and promoters of TCF1 target genes. Nelfb ablation reduces Pol II pausing and chromatin accessibility at these TCF1-associated loci. Our findings thus suggest an important and rate-limiting function of NELF in anti-tumor immunity.
T cells undergo rapid proliferation and acquire effector function after encountering antigens, co-stimulation signals, and inflammatory cytokines\textsuperscript{4,5}. Short-lived effector cells often undergo apoptosis after the clearance of foreign antigens. A small subset of effector cells develops into long-lived memory T cells\textsuperscript{5}. Both effector and memory T cells play critical roles in controlling virus infection and tumor outgrowth. However, unlike acute virus infection, tumor antigens often persist in an excessive amount and thus induce an exhaustion phenotype of tumor-reactive T cells\textsuperscript{6}. Exhausted T cells, which exhibit upregulation of multiple inhibitory receptors and loss of polyfunctionality, have been the major target for reinvigorating T cell function in anticancer immunotherapy\textsuperscript{7}. Despite clinical success in adoptive cell therapy and immune checkpoint blockade-based therapeutics\textsuperscript{8,9}, most patients still cannot benefit from these immunotherapies, partly due to the lack of persistent tumor-reactive memory T cells.

Recent studies reported a number of key transcription factors that control memory T cell differentiation, including TCF1\textsuperscript{10}, FOXO1\textsuperscript{11}, MYB\textsuperscript{12}, BACH2\textsuperscript{13}, and BATF3\textsuperscript{14}. In particular, TCF1, protein, encoded by the gene TCF7, plays a critical role in the regulation of T cell development\textsuperscript{12}, differentiation\textsuperscript{13}, and effector function preservation during exhaustion\textsuperscript{14,15}. TCF1 promotes chromatin accessibility that favors memory T cell differentiation\textsuperscript{16}. Meanwhile, TCF1 deletion exacerbates, and enforced TCF1 expression ameliorates T cell exhaustion\textsuperscript{15,16}. TCF1 also promotes long-term T cell survival by promoting the anti-apoptotic factor BCL2\textsuperscript{15} while suppressing the pro-apoptotic factor BIM\textsuperscript{16}. A TCF1\textsuperscript{+} T cell population is critical in response to immune checkpoint blockade immunotherapy\textsuperscript{17}. Although the role of TCF1 in the determination of T cell fate is well established, little is known about the partners that facilitate its function in T cell differentiation-related transcriptional programming.

Pol II pausing plays a pivotal role in regulating metazoan gene expression\textsuperscript{18–20}. At the molecular level, promoter-proximal Pol II pausing prevents nucleosome assembly and thus maintains a permissive chromatin structure for future rounds of transcriptional activation in response to environmental stimuli\textsuperscript{21}. Recent studies also showed that Pol II pausing and release play a role in transcriptional enhancers\textsuperscript{22}. NELFB is one of the four subunits of the NELF complex that controls Pol II pausing\textsuperscript{23}. Mouse genetic studies demonstrate that NELFB is indispensable for early embryogenesis\textsuperscript{24} and mammary gland development\textsuperscript{25}. In addition, NELF is involved in various aspects of adult tissue homeostasis including energy metabolism in the myocardium\textsuperscript{26}, inflammatory responses in macrophages\textsuperscript{27}, myofiber repair after injury\textsuperscript{28}, and maintenance of functional integrity\textsuperscript{29}. However, the functional significance of NELF-dependent Pol II pausing in the context of T cell function remains unclear.

Here, we define a CD8\textsuperscript{+} T cell-intrinsic role for NELF during antitumor immune response. Using tissue-specific mouse genetic models, we demonstrate that NELF in CD8\textsuperscript{+} T lymphocytes is important for antitumor immunity. Mechanistically, NELF is associated with TCF1 and facilitates chromatin accessibility at TCF1-bound transcriptional enhancers and promoters. We further establish that ectopic NELF expression boosts host antitumor immunity in mouse models and the efficacy of CAR-T immunotherapy.

**Results**

**Nelfb deletion impairs memory T cell recall response.** Memory T cell response is critical for mediating the protective role of vaccination\textsuperscript{33,34}. To determine the impact of *Nelfb* KO on memory function during tumor antigen-initiated vaccination, we chose two aggressive, ovalbumin (OVA)-expressing tumor models: lymphoma E.G7-OVA and melanoma B16-OVA (Fig. 2a and Supplementary Fig. 2a). In both tumor models, non-vaccinated (non-vac), tumor-bearing KO mice tended to have worse survival than their *Nelfb\textsuperscript{+/−}* counterparts, though the difference was not statistically significant (Fig. 2b and Supplementary Fig. 2b). This is likely because the robust tumor growth outstripped host antitumor immunity. When mice were vaccinated with OVA protein and then challenged with tumor cells, vaccination significantly lengthened survival in both *Nelfb\textsuperscript{+/−}* and KO hosts. However, vaccination exhibited substantially greater protection against tumors in *Nelfb\textsuperscript{+/−}* than KO mice (p = 0.002 in Fig. 2b, p = 0.01 in Supplementary Fig. 2b). In addition, vaccination markedly increased CD8\textsuperscript{+} T cell abundance in *Nelfb\textsuperscript{+/−}*, but not KO, hosts (Fig. 2c), suggesting that *Nelfb* KO impairs memory T cell response.

To ascertain the role of NELF in memory T cell function, we next used heat-inactivated B16 tumor cells as the vaccine based on an established protocol\textsuperscript{35}. Consistent with the findings from OVA-mediated vaccination, B16 tumor growth was comparable...
**Fig. 1** Nelfb is required for CD8+ T cell-intrinsic antitumor function. **a, b** Growth curves of E0771 (**a**) and AT-3 (**b**) tumors in Nelfb+/− and KO mice. **c, d** E0771 tumor weight (**c**) and image (**d**) upon harvest in Nelfb+/− and KO mice. **e-h** TIL analysis for E0771 tumors in Nelfb+/− and KO mice: **e** CD45+ (% of all live cells), **f** CD8+ (% of CD45+), **g** effector memory CD44+CD62L− (% of CD8+), **h** Ki67+ (% of CD8+), n = 5/group. **i, j** E0771 tumor growth curve (**i**) and weight (**j**) in Rag1−/− mice receiving Nelfb+/− or KO CD8+ T cells. **k, l** TIL analysis for E0771 tumors in Rag1−/− mice receiving Nelfb+/− or KO CD8+ T cells. **k** CD8+ (% of CD45+), **l** effector memory CD44+CD62L− (% of CD8+); n = 5/group. Data were presented as mean ± SEM; Student’s t-test (for two groups) or one-way-ANOVA (for three or more groups). Tumor curves were compared using two-way ANOVA followed by multiple comparisons. Two-sided tests were used. Source data are provided as a Source Data file.
Nelfb deletion in T cells impairs memory response to tumor antigen vaccination. a Scheme of vaccination procedure using OVA protein followed by E.G7-OVA tumor challenge. b Survival curve for E.G7-OVA tumor-bearing Nelfb/f and KO mice with or without vaccination. c CD8+ percentages among E.G7-OVA tumor-infiltrating CD45+ cells; n = 4/group. d B16 tumor growth curve in Nelfb/f and KO mice with or without vaccination; Nelfb/f non-vac (n = 4), Nelfb/f vac (n = 7), KO non-vac (n = 6), KO vac (n = 9); one-way-ANOVA for comparing mean differences. Tumor curves were compared using two-way ANOVA followed by multiple comparisons. Source data are provided as a Source Data file.

Nelfb KO causes functional defects in CD8+ T cells. To further characterize CD8+ T cell defects in Nelfb KO mice, we sought to define cellular functions affected by Nelfb loss during T cell receptor (TCR) activation. Carboxyfluorescin succinimidyl ester (CFSE) labeling showed that KO CD8+ T cells had relatively normal proliferation rates shortly after in vitro TCR activation (days 1–4), but exhibited significant proliferation defects on day 5 (Fig. 3a and Supplementary Fig. 3a). This indicates that Nelfb deletion unlikely directly impaired TCR-activated cell cycle entry. On the other hand, significantly more KO CD8+ T cells underwent apoptosis compared to their Nelfb/f counterparts as early as 24 hr after TCR activation (Fig. 3b and Supplementary Fig. 3b).

Interleukin 2 (IL2) is critical for the expansion of activated T cells but also promotes an exhaustion phenotype36. As expected, under prolonged in vitro incubation with IL2, both Nelfb/f and KO CD8+ T cells gradually increased expression of the immune inhibitory receptors PD1 and TIM3 (Fig. 3c–f). Notably, the magnitude of increase in either PD1/TIM3 single or double-positive exhausted populations was significantly higher in KO versus Nelfb/f cells (Fig. 3c–f). Loss of polyfunctionality is a hallmark of reduced memory stem cell population and increased T cell exhaustion37. Extended in vitro proliferation also resulted in a substantially lower abundance of interferon γ (IFNγ) and tumor necrosis factor α (TNFa) double-positive, polyfunctional KO T cells versus Nelfb/f (Fig. 3g, h and Supplementary Fig. 3c, d). In aggregate, our data strongly indicate that Nelfb ablation exacerbates CD8+ T cell exhaustion during ex vivo expansion.

To confirm the impact of Nelfb deletion on CD8+ T cell functionality, we performed single-cell RNA-sequencing (scRNA-seq) using total CD8+ T cells isolated from Nelfb/f and KO mice. Based on canonical marker gene expression patterns, we identified four major subsets—naïve, memory, exhausted, and senescent populations (Fig. 4a and Supplementary Fig. 4a). Compared to Nelfb/f CD8+ T cells, KO samples had smaller naïve and memory, but larger exhausted and senescent, cell populations (Fig. 4b and Supplementary Fig. 4b–d). Using an established algorithm for trajectory analysis38, we found two differentiation trajectories initiated from the naïve stage; one leads to the exhausted stage and the other to the senescent stage (Fig. 4c).

Pseudotime is a parameter that describes the positioning of individual cells from the differentiation starting point along the specific trajectory, therefore representing the degree of the differentiation from the naive to terminally differentiated stage39. Pseudotime analysis of the differentiation trajectory clearly showed that for both exhausted and senescent lineages, KO cells had significantly higher pseudotimes versus Nelfb/f (Fig. 4d). This indicates that KO cells are further downstream from their differentiation origin and therefore are more terminally differentiated than Nelfb/f cells. Together, our data support the notion that NELF prevents precocious terminal differentiation of CD8+ T cells.

Preferential NELF-dependent Pol II pausing at TCF1 targets. To elucidate the molecular mechanism by which NELF regulates T cell functionality, we performed NELFB ChIP-seq in primary mouse CD8+ T cells. An unbiased analysis of transcription factor binding motif enrichment showed that NELFB chromatin binding overlapped most significantly with that of TCF1 (Fig. 5a)40. Of note, NELFB ChIP-seq signals were substantially higher at both enhancers and transcription start sites (TSS) of TCF1
targets versus non-TCF1 targets (Fig. 5b). Next, we conducted Pol II ChIP-seq in primary Nelfb/+ and KO CD8+ T cells. We used Pol II pausing index to assess the degree of promoter-proximal enrichment of Pol II, and value D to denote the maximum difference in vertical distance between two cumulative distributions (Fig. 5c). In Nelfb/+ CD8+ T cells, TCF1 targets had a significantly higher Pol II pausing index versus non-TCF1 targets (Fig. 5c, D = 0.3137, p < 2.2e-16). Nelfb KO decreased the Pol II pausing index to a greater extent at TCF1 targets (D = 0.13387, p < 2.2e-16, Fig. 5c, Supplementary Fig. 5a). When enhancers and TSS were analyzed separately, the reduction in Pol II binding upon Nelfb KO was more pronounced at TCF1 targets than at TCF1 non-bound targets (Fig. 5d and Supplementary Fig. 5b).

Together, our data indicate that NELF-dependent Pol II binding in primary CD8+ T cells is preferentially associated with TCF1-bound regulatory regions.

NELF regulates chromatin accessibility of TCF1 targets. Pol II accumulation at promoters and enhancers have been implicated in chromatin accessibility. We, therefore, used an assay for transposase-accessible chromatin sequencing (ATAC-seq) to assess global chromatin openness in primary naive Nelfb/+ and Nelfb KO T cells cultured in vitro, n = 3/group. Following anti-CD3/CD28 activation, percentages of apoptotic cells among Nelfb/+ and KO CD8+ T cells were assessed by annexin V-APC/PI for flow cytometric analyses, n = 3/group. Nelfb deletion promotes apoptosis and exhaustion of CD8+ T cells. a Time course of CFSElow population as a percentage of Nelfb/+ and KO CD8+ T cells cultured in vitro, n = 3/group. b Percentages of apoptotic cells among Nelfb/+ and KO CD8+ T cells after 24 h of anti-CD3/CD28 activation, assessed by annexin V-APC/PI for flow cytometric analyses, n = 3/group. Data were presented as mean ± SD; mean differences were compared using Student’s t-test. Time-dependent curves were compared using multiple comparisons. Two-sided tests were used. Source data are provided as a Source Data file.
KO CD8<sup>+</sup> T cells. Principal component analysis showed that Nelfb<sup>−/−</sup> and KO had distinct chromatin opening states (Supplementary Fig. 5c). Three-quarters of the differentially accessible regions exhibited reduced chromatin openness upon Nelfb deletion (Supplementary Fig. 5d), suggesting a role of NELF in promoting chromatin accessibility. Reminiscent of its enrichment in NELFB chromatin binding regions (Fig. 5a), TCF1 binding was the most significantly enriched motif in the chromatin regions with KO-impaired accessibility (Fig. 6a). Nelfb KO-triggered reduction in chromatin openness occurred more at TCF1 targets than non-TCF1 targets, and furthermore, the KO effect was more pronounced at enhancers than TSS of TCF1 targets (Fig. 6b and Supplementary Figs. 5e, 6a, b). These data support the notion that NELF in naïve CD8<sup>+</sup> T cells preferentially facilitates chromatin accessibility at TSS and to a greater extent, enhancers of TCF1 targets.

The propensity of NELF chromatin binding and its action for TCF1 targets prompted us to discern a physical relationship between these two transcription factors. TCF1 protein levels were comparable in naïve Nelfb<sup>+/−</sup> and KO CD8<sup>+</sup> T cells (Supplementary Fig. 6c), suggesting that NELF unlikely impacts TCF1 targets by regulating its expression. Using proximity labeling, we found that NELF was in close proximity with TCF1 (Fig. 6c). As a positive control, similar physical proximity was detected between NELFB and NELFE, another NELF subunit (Fig. 6c). This finding is consistent with the idea that NELF and TCF1 work at a common set of targets to regulate chromatin accessibility in naïve CD8<sup>+</sup> T cells.

Chromatin accessibility at transcriptionally regulatory regions often precedes transcription of associated genes. We, therefore, conducted deep RNA-sequencing using Nelfb<sup>−/−</sup> and KO CD8<sup>+</sup> T cells before and after in vitro TCR activation by anti-CD3/CD28 plus IL2. While baseline transcriptomes were similar between Nelfb<sup>−/−</sup> and KO cells, TCR-activated transcriptomes of WT and KO cells were quite distinct (Fig. 6d, Supplementary Fig. 6d, and Supplementary Data 1). Of note, gene set enrichment analysis (GSEA) showed that Nelfb deletion was associated with enriched gene signatures for TCF1 deficiency, T cell exhaustion, and aging signature (Fig. 6e). Furthermore, KO cells exhibited reduced gene signatures for memory T cells and fatty acid metabolism, a hallmark of memory T cells (Fig. 6e). In keeping with our findings, GSEA analysis of published scRNA-seq data from human melanoma showed that high NELFB expression in tumor-infiltrating CD8<sup>+</sup> cells significantly correlated with gene signatures for memory T cell and fatty acid metabolism.

NELFB overexpression boosts antitumor immunity. To determine whether NELFB overexpression could enhance adaptive immunity, we established a T cell-specific transgenic mouse model (referred to as Tg hereafter, Supplementary Fig. 7a). In addition to elevated NELFB levels (Fig. 7a), the expression of NELFA and NELFC, two other NELF subunits, were also increased in CD8<sup>+</sup> T cells of Tg mice (Supplementary Fig. 7b), likely through stabilization of the entire NELF complex. In an in vivo competitive assay, KO or Tg CD45.2<sup>+</sup>CD8<sup>+</sup> T cells were mixed at a 1:1 ratio with their corresponding control CD8<sup>+</sup> T cells carrying a congenic marker CD45.1<sup>+</sup>, which were subsequently transferred into B16 tumor-bearing recipient mice. Tumor-infiltrating CD8<sup>+</sup> cells were analyzed 2–3 weeks post-transfer (Fig. 7b). As expected, KO cells were significantly
outcompeted by control cells (Fig. 7c, d). In contrast, Tg cells comprised most of the tumor-infiltrating CD8\(^+\) cell population (Fig. 7e, f), indicating that NELFB-overexpressing T cells are superior to their WT counterparts. When purified WT and Tg CD8\(^+\) cells were transferred separately into Rag1\(^{-/-}\) immunodeficient hosts followed by E0771 tumor challenge, Tg CD8\(^+\) T cells again exhibited more potent antitumor activity than their WT counterparts (Fig. 7g–i). Furthermore, mice receiving Tg CD8\(^+\) cells had significantly more total leukocytes (Fig. 7j) and CD8\(^+\) TILs (Fig. 7k). NELFB overexpression increased effector memory (Fig. 7l) and proliferative CD8\(^+\) T cell populations (Fig. 7m) while reducing the exhaustion marker PD1 expression (Fig. 7n). In addition, NELFB overexpression increased both single and double IFN\(\gamma\)/TNF\(\alpha\) CD8\(^+\) cells (Fig. 7o–q). Collectively, our data suggest that NELF is a rate-limiting factor in boosting CD8\(^+\) T cell antitumor activity, which likely occurs through reducing T cell exhaustion and increasing memory and polyfunctionality.

T cell exhaustion and the lack of sustained persistence are the major barriers to successful CAR-T therapy\(^50\). We, therefore, sought to determine whether human NELFB (hNELFB) can boost T cell functionality in a more clinically relevant setting. We engineered a bi-cistronic CD19-specific CAR vector based on an established CAR construct, anti-CD19-28z\(^31\) (anti-CD19-28z-
P2A-hNELFB). Lentivirus-mediated transduction of primary human T cells resulted in overexpression of hNELFB (Supplementary Fig. 8a) without boosting CAR expression or altering the CD4:CD8 ratio during in vitro expansion (Supplementary Fig. 8b–d). In vitro expanded human T cells with hNELFB overexpression displayed an increased percentage of CD62L+CD8+ populations (Fig. 8a, b). In a Raji lymphoma model using NSG immunodeficient mice, T cells carrying the parental anti-CD19-CAR-28z vector significantly prolonged the survival of tumor-bearing mice when compared with mice receiving PBS or mock-infected T cells (Fig. 8c). Of note, T cells with anti-CD19-CAR-28z-hNELFB conferred markedly superior host survival benefits over those with the parental CAR-T vector (Fig. 8c). In a solid tumor model, in which CD19 antigen was engineered in human breast cancer cell line MDA-231, host mice receiving hNELFB-expressing CAR-T cells exhibited smaller tumor growth than those with parental CAR-T cells (Supplementary Fig. 8e). Furthermore, compared to parental CAR-T, hNELFB-expressing CAR-T conferred more tumor infiltration of CD8+ and CD4+ T cells (Supplementary Fig. 8f, g), and higher memory marker CD127 expression and fewer cells with the exhaustion markers TIM3+CD39+ in both CD8+ and CD4+ populations (Supplementary Fig. 8h–k). Our data, therefore, provide the proof of principle that hNELFB overexpression could boost CAR-T anticancer immunotherapy.

**Discussion**

In the current study, we defined a CD8+ T cell-intrinsic function of the Pol II pausing factor NELF in regulating adaptive immunity. Upon genetic ablation of Nelfb, CD8+ T cells exhibit...
accelerated apoptosis, impaired proliferation, loss of poly-functionality, and reduced memory cell populations, all of which likely contribute to defective antitumor immune responses. The effect of \textit{Nelfb} KO on memory T cell response can be attributed to a direct role of NELF in the memory cell population or an indirect consequence of NELF functions in effector T cells, analogous to the findings from the genetic studies of other T cell transcription factors such as \textit{Batf} and \textit{Irf4}.

Combining scRNA-seq and differentiation trajectory analyses, we found that T cell-specific deletion of \textit{Nelfb} led to more terminally differentiated populations while diminishing naive and memory progenitor pools. These findings are consistent with a recent study using a muscle stem cell (MuSC)-specific \textit{Nelfb} KO model, in which \textit{Nelfb} deletion impaired MuSC replenishment in response to repetitive muscle injury due to premature terminal differentiation of myogenic progenitors. We point out that NELFB binding to repetitive muscle injury due to premature terminal differentiation of myogenic progenitors.

**Fig. 7 NELFB overexpression promotes the tumor-killing capacity of CD8\(^+\) T cells.** a Representative Western blots for NELFB and ACTIN in WT/Tg splenic CD8\(^+\) T cells. The experiments were independently repeated three times with similar results. b Scheme of competitive co-transfer assay. c Representative flow cytometry plot of TILs in the mice that received WT/KO CD8\(^+\) T cells. d Percentage of total TILs CD8\(^+\) cells in mice receiving WT/KO CD8\(^+\) T cells, \(n=6/\)group. e Representative flow cytometry plot of TILs in mice receiving WT/Tg CD8\(^+\) T cells. f Percentage of total TILs CD8\(^+\) cells in mice receiving WT/Tg CD8\(^+\) T cells, \(n=10/\)group. g-i E0771 tumor growth curve (g), weight (h), and image (i) in Rag1\(^{-/-}\) mice receiving WT or Tg CD8\(^+\) T cells; WT (\(n=6\)), Tg (\(n=5\)). j-q TIL analysis for E0771 tumors in Rag1\(^{-/-}\) mice receiving WT or Tg CD8\(^+\) T cells, \(n=6/\)group. j CD8\(^+\) (% of total live cells), k CD8\(^+\) (% of CD45\(^+\)), l effector memory CD44\(^+\)CD62L\(^-\) (% of CD8\(^+\)), m Ki67\(^+\) (% of CD8\(^+\)), n Median fluorescence intensity (MFI) of PD1, o IFNg\(^+\) (% of CD8\(^+\)), p TNF\(^+\) (% of CD8\(^+\)), q polyfunctional IFN\(^+\)TNF\(^+\) (% of CD8\(^+\)). Data were presented as mean \(\pm\) SEM; mean differences were compared using Student’s t-test. Tumor curves were compared using two-way ANOVA followed by multiple comparisons. Two-sided tests were used. Source data are provided as a Source Data file.
were compared using Student’s t-test. Log-rank (Mantel-Cox) tests were used for survival analyses. Source data are provided as a Source Data file.

tissue-specific Nelfb KO mouse models. It is conceivable that both the protein amount of individual NELF subunits and the stability of the NELF complex can affect T cell lifespan and functionality. The robust phenotypes of our KO and transgenic mouse models provide compelling evidence for an important and rate-limiting role of NELF in sustaining CD8+ cell functions in antitumor immunity.

NELF-dependent Pol II pausing at promoter-proximal regions is known to facilitate transcription by maintaining an accessible chromatin architecture and stabilizing the transcription initiation complex. In contrast, relatively little is known about the functional consequence of Pol II accumulation at transcriptional enhancers. Our genome-wide survey of primary CD8+ T cells supports the notion that NELF-dependent Pol II accumulation has even stronger effects on chromatin accessibility in enhancers versus promoters. While the exact mechanism by which enhancer-associated Pol II regulates transcription remains to be determined, we envision at least two plausible scenarios: (1) enhancer-associated Pol II may facilitate enhancer–promoter looping/interaction (Fig. 8d); and (2) Pol II-transcribed enhancer RNA could serve as the anchor for additional transcription factors/cofactors. Histone-based epigenetic programming has been implicated in various stages of T cell development and differentiation. For example, terminally exhausted and stem-like memory T cells have distinctive histone modification and chromatin accessibility profiles. In addition, histone methyltransferase Suv39h1 has been shown to restrain the transcriptional program of memory T cell differentiation by enabling H3K9me3 deposition at memory-related genes. We propose that NELF-mediated Pol II pausing may be part of the epigenetic programming that dictates the differentiation and function of memory T cells.

Our work suggests a strategy to improve outcomes of CD8+ T cell-based adoptive cell therapy. NELFB overexpression in both human and mouse T cells significantly enhances antitumor immunity and prolongs the survival of tumor-bearing hosts. Consistent with our findings, TCF1 overexpression has been shown to boost antitumor immunity and thus proposed to enhance CAR-T efficacy. We propose that NELF belongs to an expanding list of transcription factors whose overexpression bolsters CAR-T efficiency. It will be of interest to determine whether these
transcription factors regulate the same or distinct transcriptional programs in T cell-based immunotherapy. The anti-CD19-28z CAR construct we used is more exhaustion-prone than newer generations of anti-CD19 CAR constructs carrying the 4-1BB or ICOS signaling domain. To strengthen the translational potential of NELF overexpression in boosting adoptive cell therapy, future experiments are needed to test NELF overexpression in other CAR constructs with tonic signaling and exhaustion-prone phenotypes, such as GD2.8z CAR-T. Because CD8+ T cell exhaustion was observed as a pan-cancer phenotype, NELF function in mitigating immune exhaustion could be applied to multiple cancer types.

In summary, our current study shows that NELF in CD8+ T cells works at both enhancers and promoters of TCF1 target genes to potentiate chromatin accessibility and transcriptional activation. We propose that the functional cooperation between NELF-dependent Pol II pausing and TCF1 target genes is a mechanism that controls conversion between memory T cells and differentiated effector cells, ultimately dictating antitumor immunity and efficacy of cell-based immunotherapy against cancer.

Methods

Mice. All animal protocols were approved by the Institutional Animal Care and Use Committee at George Washington University. Nelfbfl/fl and distal Lck-cre (dLck-cre) mice were generated as previously described. Nelfbfl/fl mice were generated by CRISPR-based gene editing (Oxygen Biosciences). Briefly, the gRNA to ROSA26, a donor vector containing CAG promoter-loxP-stop-loxP mouse Nelfb cDNA-polyA, and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted knockin offspring. F0 founder animals, identified by PCR followed by sequence analysis, with brood at WT mice and assessed for germline transmission and Fl mouse generation. T cell-specific Nelfb KO or transgenic mice were generated by crossing dLck-cre with Nelfbfl/fl or Nelfbfl/fl, respectively. The Cre and flox strain behaved the same as WT B6 mice; we, therefore, used the floxed mice as WT. Rag1fl/fl (stock no. 002216), NSG (stock no. 005557), and C57BL/6 CD45.1 congenic mice (stock no. 002018) were purchased from The Jackson Laboratory. Mice that are 10-week-old were considered adults and were used for tumor studies.

In vivo tumor challenge and assessment. For direct tumor challenge, syngeneic mammary tumor E0771 cells (5 × 105 cells) (CH3 Biosystems, 940001) were inoculated subcutaneously into the backanks of mice and tumor growth was measured. For single-cell RNA-seq, total CD8+ T cells were isolated from mouse spleenocytes (STEMCELL Technologies, 19853). Cells were then activated by anti-CD3/CD28 (2 μg/mL each) per million cells with a target of 3000 cells per sample. Single-cell RNA-seq libraries were processed further as follows. RNA samples were processed further as follows. Briefly, about 500 ng total RNA was used for library preparation following the protocol for Illumina TruSeq stranded mRNA-seq. PolyA-containing mRNA were enriched and converted into first-strand cDNA by random primers and reverse transcriptase. Second-strand cDNA were then synthesized and final RNA-seq libraries were generated by PCR. An Illumina HiSeq 3000 platform was used to carry out 50 bp single-read sequencing.

Sequencing data were demultiplexed and processed by Cell Ranger (version 3.1.0). STAR was used to map reads to the mouse reference genome (reddata-cell-rmm-10.3.0). The outputs of individual samples were loaded into the Seurat R package (version 3.1.5). High-quality cells were filtered based on the number of detected genes and the percentage of cleaned cells (top 10000) and the presence of specific molecular identity markers (UMIs) mapped to mitochondrial genes (<12%). Individual samples were integrated, and principal components were calculated. The first ten principal
components were used for cell clustering and tSNE visualization. Data QC analysis was done as previously described. Tagmented DNA was included in Supplementary Fig. 9e. For genomic analyses, active enhancers were quantified using a MinElute PCR purification kit (Qiagen, 28004), amplification and sequenced with 50 bp single-read sequencing using an Illumina HiSeq 3000 platform. After sequencing, fastq files were generated with Bcl2fastq2. ChIP-seq data quality was checked by Filt-ER software. For ATAC-seq assays, freshly isolated naïve CD8+ T cells from mouse spleen (STEMCELL Technologies, 200-0046) or frozen (STEMCELL Technologies, 21024) were quantified by Qubit and Agencourt, and then pooled for cbot amplification and sequenced with 50 bp single-read sequencing using an Illumina HiSeq 3000 platform. After sequencing, fastq files were generated with Bcl2fastq2. ChIP-seq data quality was checked by MultiQC (v1.9). For ATAC-seq assays, freshly isolated naïve CD8+ T cells from mouse spleen (STEMCELL Technologies, 19858) were washed and centrifuged at 500 × g for 5 min. Cell pellets were then resuspended in a cryopreservation solution containing FBS and 10% DMSO. Approximately 100,000 frozen cells of each sample were used by the Active Motif to perform ATAC-seq and analyses. Briefly, cells were thawed at 37 °C. Cell nuclei were first isolated to perform Tn5 tagmentation and make libraries of open chromatin, as previously described. Tagmented DNA was then purified using a MiniElute PCR purification kit (Qiagen, 28004), amplification and sequenced with 10 cycles of PCR, and purified using Agencourt AMPure SPRI beads (Beckman Coulter). Afterward, pooled-end (PE)24 sequencing with a depth of 30 million reads (a total of 60 million reads) was performed using NextSeq 500 (Illumina). FRIP scores and peak counting were used as quality controls. Reads were aligned to the mouse genome (mm9) using the BWA algorithm. Duplicate reads were removed, and only uniquely mapped reads (mapping quality ≥ 21) and reads mapping as matched pairs were used for further analysis. Peaks were identified using the MACS 2.1.0 algorithm. Peak reads numbers correlation were included in Supplementary Fig. 9e. For genomic analyses, active enhancers were defined by H3K27ac binding peaks at non-TSS regions in CD8+ T cells, and active promoters/TSS were defined as H3K4me3 peaks that overlap with TSS in CD8+ T cells. TCF1 targets were defined using previously published CD8+ TCF1 ChIP-seq data.

Co-transfer experiment: Splenic CD8+ T cells were isolated from congenic marked WT C57BL/6 (carrying CD45.1) or dLck-cre-Nelbf/f mice (carrying CD45.2), mixed at a ratio of 50:50, and intravenously transferred into B16 melanoma tumor-bearing Rag1−/− immunodeficient mice. When comparing WT versus Nelbf-overexpressing cells, WT (CD45.1) or dLck-cre-Nelbf/f (CD45.2) were co- transferred. A total of 2 × 106 cells were transferred in 100 μl PBS injected via the tail vein of CD45.1 (BioLegend, 110706) and CD45.2 (Bio- Legend, 109984) in tumor-infiltrating CD8+ cells were assessed by flow cytometry 2–3 weeks post-transfer.

Proximity labeling and western blotting: APEX2-mediated proximity biotinylation was done as previously described. Briefly, APEX2-encoding DNA sequences were fused to the mouse Nelbf gene using standard molecular cloning techniques, and the overexpression plasmid was packaged with helper plasmids in 293 T cells to generate lentivirus stocks. Afterward, Jurkat cell lines (ATCC, TIB-152) were infected with the lentivirus and a stable cell line was selected using neomycin. Biotin-labeling was conducted with 30 min incubation in 500 μM biotin-photon. Cells were then exposed to 1 mM H2O2 at room temperature for 1 min. The reactions were then stopped with ice-cold Dulbecco’s phosphate-buffered saline with quenches (10 mM sodium azide, 10 mM sodium ascorbate, 5 mM Trololo, 10 mM sodium azide, and 1 mM PMSF), then pelleted by centrifugation and lysed by RIPA lysis buffer containing 1 mM PMSF, 1 mM Trololo, 10 mM sodium azide, and 1 mM sodium ascorbate. A slurry of streptavidin magnetic beads (NEB, SI4205) was incubated with cell lysate and rotated at room temperature for 1 h. The beads were subsequently washed and boiled to elute biotinylated proteins. Cells with negative controls. The resultant protein lysates were analyzed using standard Western blotting techniques. Primary antibodies included anti-TCF1 (CST, 2023 S), anti-NELF (Proteintech, 10705-1-AP), and anti-GAPDH (Bio-Rad, 1204167). Other antibodies used for Western blotting included anti-NELF (Cell Signaling Technology, 14894 S), anti-NELFA (Proteintech, 10456-1-AP), and anti-NELFC (Cell Signaling Technology, 12265 S). All antibodies were used at 1:1000 dilution.

CAR-T generation and characterization. A lentiviral vector pELPs-CAR19-28z was a generous gift from Dr. Carl H. June’s lab at the University of Pennsylvania. The human NELFB gene coding sequence was inserted downstream of the CAR19- 28z-encoding sequence, with the P2A cleavage sequence in between (Gene Universal, Inc.). Ultra-purified high-titer viruses for both unmodified and modified lentiviral vectors were packaged and generated by VectorBuilder Inc.

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Statistical analysis. Mean differences between the two groups were tested using Student’s t-test. Mean differences between three or more groups were tested using one-way ANOVA. Tumor curves were compared using two-way ANOVA followed by multiple comparisons. Two-sided tests were used. Survival analyses were done by Log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests. GSEA was done using GSEA software. To analyze human melanoma tumor-infiltrating lymphocytes (TIL), we downloaded the single-cell RNA-seq dataset from GSE72056. Activated CD8+ TILs (CD8a +25 and CD4a −22) were used for GSEA as previously described. Population distributions in scRNA-seq data analyses were examined using Chi-square tests. Wilcoxon rank-sum test with continuity correction was used for genomic analysis. Statistics were performed using GraphPad Prism software. p < 0.05 was considered significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The sequencing data that support the findings of this study have been deposited to NCBI Gene Expression Omnibus under accession codes GSE182862. Human melanoma tumor-infiltrating lymphocytes (TIL) data were downloaded from GSE72056. The remaining data were available within the Article, Supplementary Information, or Source Data file. Source data are provided with this paper.

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Author contributions

R.L. managed and oversaw the overall project. R.L. and B.W. designed the experiments and wrote the manuscript. B.W., X.Z., H.C.C., H.P., B.Y., P.M., L.Q., and H.S. carried out the experiments. B.W., X.Z., C.N.Y., E.Y., Y.H., N.Z., and R.L. analyzed the data.

Competing interests

R.L. and B.W. are co-inventors of a pending patent (application number 62/982,514) filed by The George Washington University on the therapeutic utility of NELFB in CAR-T therapy. The remaining authors declare no competing interests.

Additional information

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