PNAS RESEARCH ARTICLE | IMMUNOLOGY AND INFLAMMATION OPEN ACCESS

The NAE1-mediated neddylation operates as an essential post-translational modification checkpoint for effector CD8$^+$ T cells

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Edited by Marc Jenkins, University of Minnesota Medical School, Minneapolis, MN; received November 27, 2024; accepted January 2, 2025

Optimal activation of CD8+ T cells is crucial for immunity-mediated destruction of cancer, requiring a substantial amount of proteins involved in metabolism, proliferation, and effector function. Despite extensive studies emphasizing the role of transcriptional regulation in this process, paired transcriptomic and proteomic analyses reveal that the RNA profile is poorly correlated with protein levels. This discrepancy underscores the importance of post-translational modifications (PTMs) in controlling protein abundance during activation. However, the impact of PTMs on the CD8+ T cell protein dynamic remains underexplored. We identify that neddylation, a recently discovered PTM, is activated in response to T cell receptor (TCR) stimulation and enriched in effector CD8+ T cells from colon cancer patients. Mechanistically, we found the rate-limiting enzyme of neddylation, neural precursor cell expressed developmentally down-regulated protein 8 activating enzyme E1 (NAE1), is induced by the NFATc1, a critical transcription factor downstream of TCR signaling. Our observation revealed that genetic ablation of NAE1 significantly disturbed the proteomic landscape related to activation and mitochondrial function. As a result, CD8+ T cells lacking NAE1 exhibited severely compromised activation, proliferation, and survival, which was accompanied by impaired mitochondrial function. Consistently, deletion of NAE1 in CD8+ T cells abolished their antitumor function and promoted tumor progression. By contrast, the overexpression of NAE1 significantly improved the function of tumor-infiltrating CD8+ T cells. Overall, we uncovered neddylation, a previously underappreciated PTM, as a proteomic checkpoint for CD8+ T cell activation. Enforced expression of NAE1 offers promising therapeutic potential for boosting the antitumor CD8+ T cell responses.

immunotherapy | T cells | post-translational modifications

Significance

Our study illuminates the pivotal role of neddylation, a previously overlooked post-translational modification, in orchestrating the activation of CD8 T cells is crucial for immune responses against cancer. By focusing on NAE1, a key enzyme in the neddylation pathway, we found that its upregulation, governed by the T cell receptor/nuclear factor of activated T cells 1 (TCR/NFATc1) axis, enhances mitochondrial fitness and protein dynamics essential for CD8 T cell activation. Importantly, our findings reveal that NAE1 deficiency impairs antitumor immunity while augmenting NAE1 expression enhances the efficacy of adoptive cell transfer therapies targeting tumors. This research not only expands our understanding of post-translational mechanisms in T cell biology but also suggests avenues for immunotherapeutic interventions in cancer treatment.

The activated cluster of differentiation 8 (CD8$^+$) T cells are critical for eradicating tumors through their specific lytic function in immunotherapy. Upon activation, CD8$^+$ T cells undergo significant cellular reprogramming to generate a substantial amount of proteins to support metabolism, clonal expansion, and effector T cell differentiation (1–4). Most current studies focus on epigenetic and transcriptional regulation and provide an instrumental understanding of this process (5, 6). However, emerging evidence challenges the correlative relationship between the transcripts and proteins during T cell activation (7–11). Several recent studies performed paired RNA sequencing and proteomics analyses and demonstrated significant discrepancies between messenger RNA (mRNA) and protein abundance (12–14). This discordance has been revealed in effector molecules such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and mitochondrial ribosomal proteins (12, 14). These studies have demonstrated the importance of regulatory mechanisms after transcription that regulate the proteomic landscape. Consistently, it is becoming increasingly apparent that post-translational modifications (PTMs) are crucial regulators of T cell activation by altering the activity, stability, and localization of proteins involved in TCR signaling, effector function, and metabolism (15–18). However, our knowledge of the impact of PTMs on protein dynamics during T cell activation remains limited. Thus, identifying novel PTMs that remodel the proteomic landscape is required to better understand the mechanism of T cell activation. This knowledge will provide the critical foundation for developing novel interventions that therapeutically target PTMs to boost the T cell immunity against cancer and eliminate the tumor.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2424061122/-/DCSupplemental.

Published March 3, 2025.

Through bioinformatic analysis, we identified that key enzymes initiating neddylation are significantly induced upon activation in $CD8^+$ T cells. Additional analysis of published clinical studies suggests a significant association between neddylation and effector $CD8^+$ T cells, supporting the premise that neddylation is required for T cell activation in colon cancer (19). Neddylation is a ubiquitylation-like enzymatic cascade process that covalently tags the neural precursor cell expressed developmentally down-regulated protein 8 (NEDD8) onto the target proteins (20, 21). This modification starts with NEDD8 activation by the E1 NEDD8 activating enzyme (NAE), a heterodimer of NEDD8 activating enzyme E1 (NAE1) and UBA3, which is followed by transferring the activated NEDD8 to the target protein by E2 conjugating enzyme and E3 NEDD8 ligase (21). NEDD8 was initially discovered in the mouse brain as a critical regulator of the development and specialization of the central nervous system (22). Recent studies have unveiled its role in other settings, such as hepatic lipogenesis, adipogenesis, cardiac disease, tumorigenesis, and immune regulation (20, 23–27). Despite reports indicating that the neddylation deficiency leads to impaired $CD4^+$ T cell differentiation and function in conditions like airway inflammation (28), malaria infection (29), graft-versus-host disease (30), and chronic lymphocytic leukemia (31), knowledge regarding the role of neddylation in $CD8^+$ T cell differentiation and antitumor immunity cell remains obscure.

Distinct from ubiquitylation, neddylated proteins are not subjected to proteasome-mediated degradation. Instead, neddylation usually increases stability and modulates the function and subcellular localization of targeted proteins (20, 32). The neddylation target proteins play a vital role in various biological pathways, including mitochondrial morphology and function. Recent studies also identified that the mitochondrial proteins can be directly neddylated to enhance its stability by preventing ubiquitylation-mediated degradation in hepatocytes (33). Consistent with these observations, evidence obtained through pharmacologically targeting NAE1 confirms that neddylation is involved in the mitochondrial regulation in hepatocytes and cancer cells (33–35); however, such a concept has never been explored in immune cells. A growing number of evidence highlights the impact of maintaining mitochondrial fitness in effector $CD8^+$ T cells (36). Upon TCR stimulation, $CD8^+$ T cells must rewire their mitochondria for enhanced respiratory function to support effector T cell differentiation (36, 37). Despite current studies emphasizing the importance of transcriptional regulation in this process (38, 39), the stability of mitochondrial proteins can also be regulated by PTMs (40, 41). Therefore, it is plausible that neddylation remodels the mitochondrial proteome to endorse their function, in turn, supporting T cell activation.

In this study, we demonstrated the vital role of neddylation in T cell responses. We showed that genetic ablation of NAE1 leads to diminished activation, proliferation, and survival in activated $CD8^+$ T cells. Mechanistically, the proteomic analysis revealed a significant reduction in mitochondrial proteins in the absence of NAE1. This correlates with reduced mitochondrial respiration and an accumulation of depolarized mitochondria in NAE1 null $CD8^+$ T cells. These findings suggest that NAE1 is required for the neddylation of mitochondrial proteins to sustain mitochondrial fitness and support T cell activation. Using a murine MC38 colon cancer model, we found that mice with $CD8^+$ T cell–specific deletion of NAE1 exhibited impaired antitumor immunity and accelerated tumor growth. Furthermore, the function of tumor-reactive effector T cells can be boosted by overexpressing NAE1. These findings highlight a previously unappreciated role of neddylation as a PTM checkpoint in regulating $CD8^+$ T cell response in tumors.

Results

NAE1-Mediated Neddylation Is Required for $CD8^+$ T Cell Activation In Vitro. To determine whether the neddylation pathway is upregulated upon activation, we reanalyzed recent proteomic datasets from T cells following TCR stimulation (4). The key enzymes involved in initiating neddylation were upregulated during T cell activation (Fig. 1*A*). To confirm this observation, our immunoblotting data revealed that the protein level of NAE1, the key enzyme in neddylation, was negligible at the naïve stage but gradually increased upon T cell activation (Fig. 1*B*). To gain insight into the critical transcription factor (TF) involved in the upregulation of NAE1, we interrogated published ATAC-seq data from activated $CD8^+$ T cells (42). We performed TF binding motif enrichment analysis focusing on the locus of *Nae1* (*SI Appendix*, Fig. S1*A*). This analysis identified several TFs whose motifs were enriched in the promoter region of *Nae1*, including nuclear factor of activated T cells 1 (NFATc1) (43), a key TF downstream of TCR signaling and involved in T cell activation (Fig. 1*C*). Thus, we next tested whether NFATc1 induces the expression of *Nae1*. The CRISPR/Cas9-mediated approach was employed to delete NFATc1 in CD8+ T cells followed by TCR activation. We found that the mRNA level of *Nae1* was significantly abolished in cells that received gRNA targeting *Nfatc1* (Fig. 1*D*). Ablation of other TFs such as IRF1, NRF1, and ZNF281 has no impact on the expression of Nae1 (*SI Appendix*, Fig. S1*B*). Thus, it is possible that the TCR-endorsed expression of *Nae1* is under the NFATc1-mediated transcriptional regulation.

To determine the intrinsic role of NAE1 in $CD8^+$ T cells, we have conditionally deleted *Nae1* in the T cell lineage by breeding the $Nae1^{flox/flox}$ mice with transgenic $dLck^{Cre}$ mice, which express Cre recombinase under the control of the distal T cell *Lck* promoter. The deletion efficiency of *Nae1* in $CD8^+$ T cells was confirmed by immunoblotting (*SI Appendix*, Fig. S1*C*). At homeostasis, this new strain $Nae1^{flox/flox}$ $dLck^{Cre+}$ ($Nae1^{T\text{-}CKO}$) exhibited comparable T cell profiles to littermate controls $Nae1^{flox/flox}$ ($Nae1^{T\text{-}WT}$) in the spleen, thymus, and bone marrow (*SI Appendix*, Fig. S1*D*), suggesting that the lack of NAE1 has no impact on T cell development. Upon activation of $CD8^+$ T cells through anti-CD3/CD28 stimulation, we observed a significant decrease in the proportion of granzyme B $(GzmB)^+$ $CD44^+$ effector cells as well as levels of other activation markers (CD69 and CD25) in $Nae1^{T\text{-}WT}$ $CD8^+$ T cells (Fig. 1*E* and *SI Appendix*, Fig. S1*E*). Consistently, this compromised activation is accompanied by a substantial reduction in cell proliferation and survival (Fig. 1 *F* and *G* and *SI Appendix*, Fig. S1*F*). These phenotypic defects are similar to previous observations in $Nfatc1^{-/-}$ $CD8^+$ T cells (43), which is consistent with our above data suggesting NAE1 is under the regulation of NFATc1. Overall, our data indicate that NFATc1 transcriptionally upregulates NAE1, which plays a crucial role in the activation, proliferation, and survival of $CD8^+$ T cells.

Neddylation Regulates the Proteomic Landscape and Mitochondrial Function in $CD8^+$ Cells. To gain a more in-depth understanding of how NAE1 regulates the T cell activation, we performed RNAseq to profile the transcriptome. In-depth analysis demonstrated that only 21 genes are significantly downregulated in $Nae1^{T\text{-}CKO}$, and none are involved in T cell function, survival, and proliferation (Fig. 2*A*) (44). Surprisingly, the transcripts encoding *Cd44*, *Cd69*, and *GzmB* were comparable between $Nae1^{T\text{-}WT}$ and $Nae1^{T\text{-}CKO}$, which does not correlate with their protein abundance measured by flow cytometry (Fig. 2 *B* and *C*). These observations prompted us to characterize the proteomics profile impacted by *Nae1* ablation. Compared to WT, 1,632 proteins were reduced,

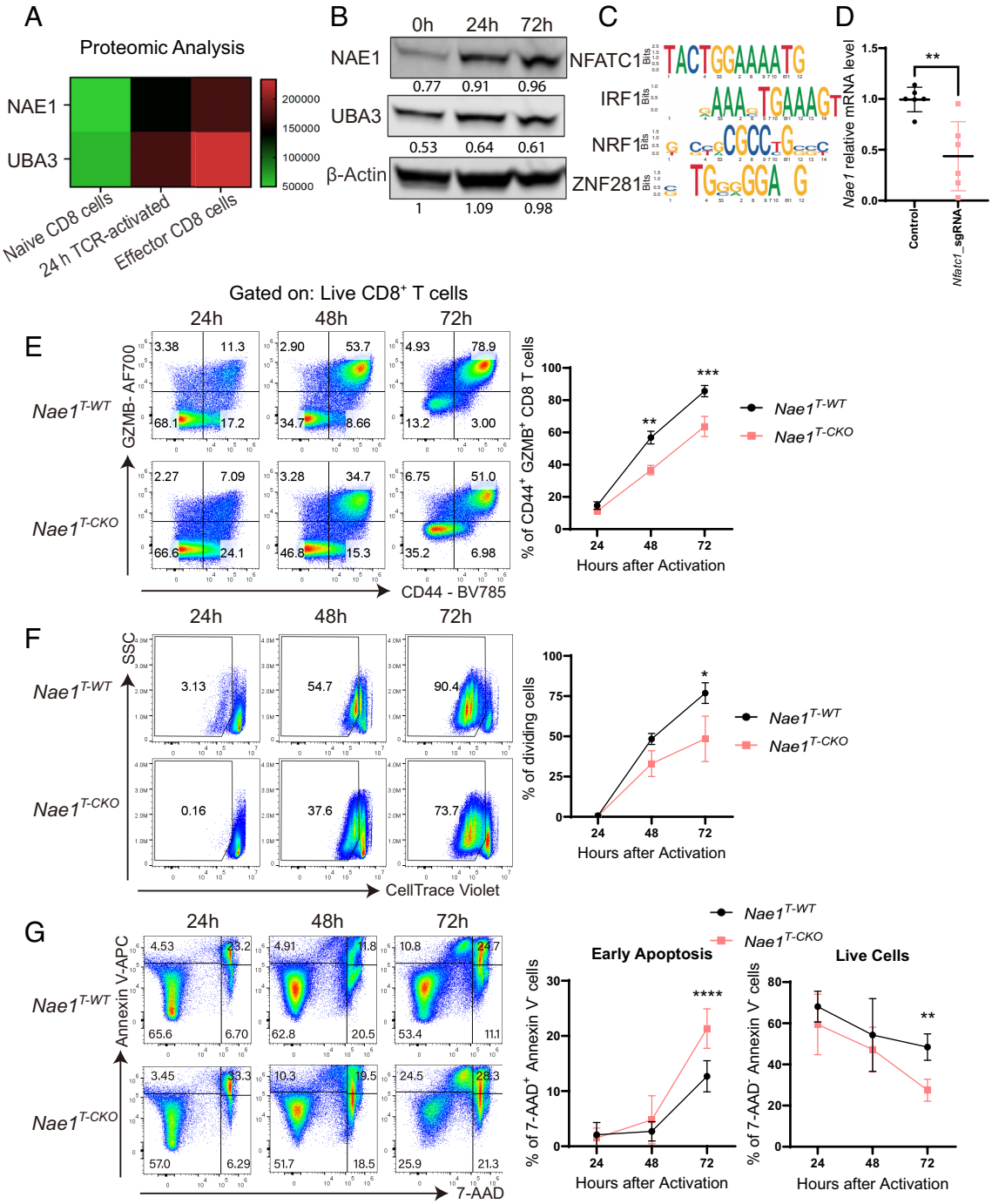

Fig. 1. NAE1-mediated neddylation is required for CD8$^+$ T cell activation in vitro. (*A*) The proteome dataset from naïve, 24 h TCR activated, and effector CD8$^+$ T cells was reanalyzed. The protein levels of NAE1 and UBA3 were shown in heatmap. (*B*) The CD8$^+$ T cells from C57BL/6 mice were activated by anti-CD3/28 mAb with 10 unit/mL IL-2. The protein levels of NAE1 and UBA3 were examined by western blot at 0, 24, or 72 h after activation. (*C*) The motif analysis was performed on published ATAC-seq data from 48 h activated CD8$^+$ T cells. The consensus motif was enriched within the regulatory region of *Nae1* loci. (*D*) The CD8$^+$ T cells were isolated from C57BL/6 mice and subjected to CRISPR/Cas9-mediated gene knockout approach. These cells received nontargeting control Cas9 RNP (sgNTC) or gRNA targeting Nfatc1 (sg*Nfatc1*). The expression of *Nae1* was detected by RT-qPCR 72 h after in vitro activation. Data are expressed as mean ± SD and n = 6. (*E*) The CD8$^+$ T cells from *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ were activated as described above. The expression of GzmB and CD44 were examined by flow cytometry and shown in representative flow plots and summary data. (*F*) These CD8$^+$ T cells were labeled with CellTrace Violet (CTV) followed by stimulation. The proliferation of CTV labeled CD8$^+$ T cells was analyzed by flow cytometry at 24, 48, and 72 h post labeling. (*G*) Representative dot plots (*Left*) are shown for the staining of Annexin-V and 7-AAD on *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells at 24, 48, and 72 h after activation. The frequencies of early apoptotic cells (Annexin-V$^+$, 7-AAD$^-$) and live cells (Annexin-V$^-$, 7-AAD$^-$) are shown in the bar graphs (*Right*). Data are expressed as mean ± SD and n = 8. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

whereas 1,434 proteins were increased in *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells (*SI Appendix*, Fig. S2*A*). The levels of T cell activation proteins, such as CD44, GzmB, CD27, IFNγ, TNFα, and perforin (Prf1), were substantially reduced in the absence of NAE1 (Fig. 2*D*). Consistently, IL-2 and mTOR signaling pathways and molecular signatures involved in effector CD8$^+$ T cells were significantly reduced in *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells (Fig. 2*E*). Conversely, the apoptosis pathway was significantly enriched in *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells (Fig. 2*E*), correlating with the accumulation of apoptotic cells (Fig. 1*G*). Next, we explored the mechanism by which neddylation reprograms the proteomic profile. Since the transcription of the key activation markers is not affected by lack of NAE1, we sought

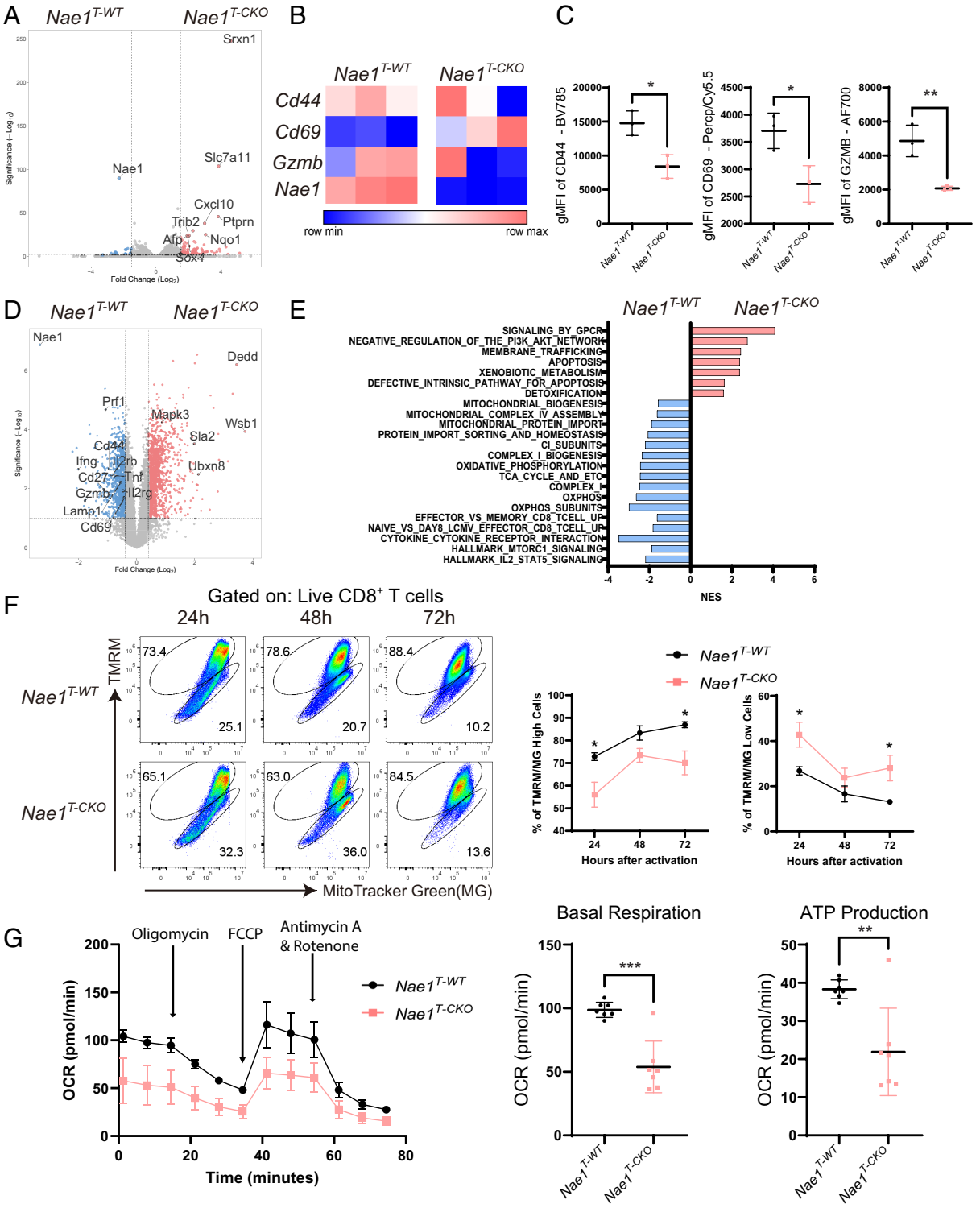

Fig. 2. NAE1 deficiency disrupts proteome and mitochondrial fitness during T cell activation. (*A*) The *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells were activated for 48 h and subjected for bulk RNA-seq. The volcano plot demonstrates the genes are differentially expressed between *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ cells. (*B*) Heatmap shows the transcript level of *Cd44*, *Cd69*, *GzmB,* and *Nae1* between The *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ cells. (*C*) The summary data displays the geometric Mean Fluorescent Intensity (gMFI) of CD44, CD69, and GzmB from 48 h activated cells. (*D*) The proteomic analysis of 48 h activated *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells was shown in volcano plot. (*E*) GSEA plot demonstrates that pathways related to T cell–activating signaling, function, and mitochondrial fitness were negatively enriched, whereas the apoptosis pathway was positively enriched in *Nae1*$^{T\text{-}CKO}$. (*F*) Mitochondrial mass and membrane potential of *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells from 24, 48, and 72 h after in vitro activation were examined by MitoTracker Green (MG) and TMRM, respectively. Data are expressed as mean ± SD and n = 8. (*G*) Line graphs depicting the OCR of *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ activated CD8$^+$ T cells in response to the Mito Stress assay. Bar graphs quantifying basal respiratory capacity and ATP production in *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ CD8$^+$ T cells. Data represent cumulative results from three independent experiments with a total n = 8. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

to identify which process after transcription will be impacted by NAE1. We directly evaluated translation rate by measuring the incorporation of L-homopropargylglycine (HPG), a methionine analog, into newly synthesized proteins and revealed comparable protein synthesis rate between *Nae1*$^{T\text{-}WT}$ and *Nae1*$^{T\text{-}CKO}$ (*SI Appendix*, Fig. S2*B*). These mild impacts on transcriptional

and translational regulation uncovered an essential role of NAE1 in regulating the protein stability at the posttranslation level in activated $CD8^+$ T cells.

Next, we explored how neddylation regulates $CD8^+$ T cell activation. Our proteomic analysis suggested that NAE1 deficiency abolishes mitochondrial biogenesis, TCA cycle, mitochondrial complex I, and oxidative phosphorylation (Fig. 2*E*). This indicates that neddylation is required to regulate mitochondrial function to support T cell activation. Consistently, the mitochondrial proteome including enzymes in the TCA cycle (SDHA), mitochondrial ribosomal protein (MRPL48), mitochondrial translocase of the outer mitochondrial membrane (TOMM40), translocase of the inner mitochondrial membrane (TIM23), and NADH dehydrogenase subunit 6 (NDUFB6) were profoundly reduced at the protein level in the absence of NAE1 (*SI Appendix*, Fig. S2*C*). Indeed, we did observe that the NAE1-null activated $CD8^+$ T cells accumulated more depolarized mitochondria ($TMRM^{lo}/MG^{high}$) as defined in a previous publication (Fig. 2*F*) (37). Furthermore, real-time metabolic seahorse analysis demonstrated that *Nae1* deficiency markedly attenuated basal mitochondrial respiration, and ATP production without significant impact on basal glycolysis (Fig. 2*G* and *SI Appendix*, Fig. S2*D*). It has been reported that these dysfunctional mitochondria can impair effector $CD8^+$ T cell activation and function (37). Collectively, these data suggest that neddylation reprograms protein dynamics to maintain mitochondria fitness and support T cell activation.

NAE1 Deficiency in $CD8^+$ T Cells Impairs Effector Function and Antitumor Immunity. To determine the functional importance of NAE1 in antitumor immunity, we generated $CD8^+$ T cell–specific NAE1 knockout mice ($Nae1^{CD8\text{-}CKO}$) by crossing $Nae1^{flox/flox}$ mice with the $E8I^{Cre}$ mouse. We have confirmed the deletion of NAE1 in activated $CD8^+$ T cells from $Nae1^{CD8\text{-}CKO}$ mice (*SI Appendix*, Fig. S3*A*). Upon TCR stimulation, the $Nae1^{CD8\text{-}CKO}$ $CD8^+$ T cells exhibited similar defects in activation and function as the $Nae1^{T\text{-}CKO}$ cells (*SI Appendix*, Fig. S3*B*). To assess the impact of NAE1 deficiency on $CD8^+$ T cells in tumors, we challenged $Nae1^{CD8\text{-}WT}$ and $Nae1^{CD8\text{-}CKO}$ mice with MC38 colon carcinoma. Intriguingly, the tumor growth was significantly accelerated in $Nae1^{CD8\text{-}CKO}$ mice compared to littermate controls (Fig. 3*A*). To better understand the underlying mechanism, we profiled the $CD8^+$ T cells from tumors 10 d after tumor inoculation, before the tumor growth bifurcated. The proportion of total tumor-infiltrating $CD8^+$ T cells was profoundly decreased in $Nae1^{CD8\text{-}CKO}$ mice (Fig. 3*B*). More importantly, we also observed significantly reduced activated total $CD44^{high}$ $CD8^+$ T cells and abolished CD44 protein abundance in $Nae1^{CD8\text{-}CKO}$ mice (Fig. 3 *C* and *D*). This impaired activation was accompanied by substantially reduced levels of PD1 and TIM3 (Fig. 3 *E* and *F*). Consequently, the function of $Nae1^{CD8\text{-}CKO}$ $CD8^+$ T cells, such as the IFNγ production, was also decreased (Fig. 3*G*). To ensure that the phenotypic and functional defects were specific to tumor-infiltrating T cells, we also examined the $CD8^+$ T cells from the spleens and tumor-draining lymph nodes. The frequency and phenotype of $CD8^+$ T cells in these lymphoid organs were comparable between $Nae1^{CD8\text{-}WT}$ and $Nae1^{CD8\text{-}CKO}$ (*SI Appendix*, Fig. S3 *C* and *D*). Motivated by these results, we further evaluated the role of NAE1 on antitumor CD8 T cells using an MB49 bladder tumor model and obtained similar observations (*SI Appendix*, Fig. S3*E*). Our analysis collectively implies that NAE1 is important for the effector function of antitumor $CD8^+$ T cells.

Neddylation Overexpression Enhances the CD8+ T Cell Immunity against Cancer. To further define the role of neddylation in regulating antitumor T cell responses, we interrogated recent scRNA-seq datasets of immune cells from colon tumors (19). The expression of *NAE1* is preferentially upregulated in the effector and progenitor $CD8^+$ T cell subsets, whereas it is barely detectable in native and exhausted $CD8^+$ T cell subsets from colon tumors (Fig. 4*A*). Further analysis confirmed the significant positive association between NAE1 and genes encoding effector function (*SI Appendix*, Fig. S4 *A* and *B*). In accordance with this enriched expression of NAE1 in functional $CD8^+$ T cells, our TCGA-based analysis revealed that colon cancer patients with elevated levels of neddylation activating enzyme (NAE1 and UBA3) correlated significantly with better survival (Fig. 4*B*). Based on the vital role of NAE1 in antitumor immunity, we reasoned that selectively activating NAE1 expression could boost the function of tumor-reactive T cells. To explore whether NAE1 was sufficient to augment effector function, we used the MSCV-IRES-Thy1.1 (MIT) retroviral vector (RV) to overexpress NAE1 in $CD8^+$ T cells, with transduced cells identified by Thy1.1 expression. To test this idea in an antigen-specific setting, we employed glycoprotein (GP) expressing MC38 tumor cells (MC38-HELLO) (45) and $CD8^+$ T cells from P14 T cell receptor (LCMV GP33-specific) transgenic mice as donor cells (Fig. 4*C*). The activated P14 cells were retrovirally transduced with MIT-empty or MIT-NAE1 vector and transferred into mice that were inoculated with MC38-HELLO tumor 7 d prior (Fig. 4*C*). To determine whether NAE1 endorses early activation, we examined the immune phenotypes of P14 cells 4 d after transfer. We demonstrated that the P14 cells transduced with MIT-empty or MIT-NAE1 contained a comparable proportion of $CD44^+$ $CD8^+$ T cells in the tumors (Fig. 4*D*). However, NAE1 overexpression increased the protein level of GzmB in tumor-infiltrating P14 cells compared to MIT-empty controls (Fig. 4*E*). Consistently, the frequency of polyfunctional $IFN\gamma^+$ $TNF\alpha^+$ $CD8^+$ T cells and the expression of IFNγ was also enhanced by enforced expression of NAE1 (Fig. 4 *F* and *G*). Compared to mice receiving P14 cells with MIT-empty vector, the enforced expression of NAE1 improved antitumor efficacy resulting in significantly reduced tumor progression (Fig. 4*H*). These findings suggest that activation of NAE1 can augment effector function of antitumor $CD8^+$ T cells, which ensures a durable and effective response in treating solid tumors.

Discussion

Our findings highlight an essential role of NAE1 in reprogramming proteomic landscapes to support $CD8^+$ T cell activation. While previous studies suggested a predominant role of epigenetic and transcriptional regulation, more recent work has increasingly recognized the contribution of proteomic reprogramming to the effector T cell differentiation. However, we still have an incomplete molecular understanding of how the protein dynamic is regulated during T cell activation. To address this gap, our bioinformatic analysis has suggested that neddylation, an underappreciated PTM, is strongly associated with the functional $CD8^+$ T cells in colon tumor. Supporting this notion, our work identified neddylation as a PTM regulator of the proteome and mitochondrial fitness in effector $CD8^+$ T cells. Our evidence supports a model in which the TCR/NFATc1 axis upregulates NAE1, the key enzyme in neddylation, which in turn modulates effector molecules and mitochondria proteins to support the T cell activation. Consequently, loss of NAE1 impaired the activation, proliferation, and survival accompanied by an accumulation of dysfunctional mitochondria in $CD8^+$ T cells in vitro. More importantly, genetic

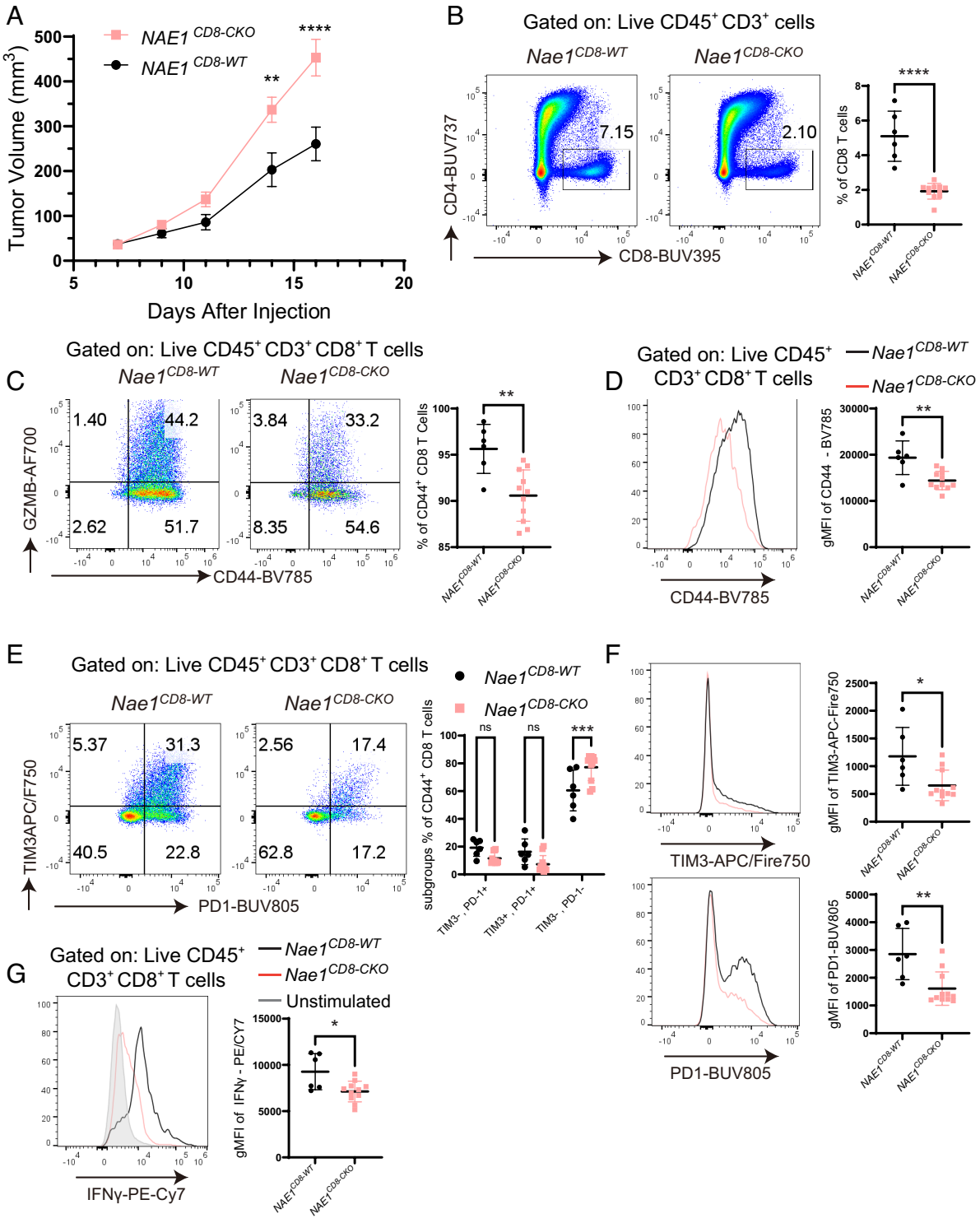

Fig. 3. NAE1 is required for antitumor effector CD8$^+$ T cell response in vivo. (*A*) *Nae1*$^{CD8\text{-}KO}$ (n = 6) and littermate control *Nae1*$^{CD8\text{-}WT}$ (n = 10) mice were inoculated with MC38, and tumor growth was monitored and compared by two-way ANOVA. (*B*) 10 d after inoculation, both groups of mice were harvested for examining immune cells from tumor. The proportion of CD8$^+$ T cells in total CD45$^+$ immune cells from tumor were measured by flow cytometry. (*C*) The representative flow plots (*Left*) and quantification (*Right*) of CD44$^+$ CD8$^+$ T cells from tumors. (*D*) The protein level of CD44 was measured by flow cytometry and shown in histogram and summary dot plots. (*E*) The percentage of TIM3 and PD1 on tumor-infiltrating CD8$^+$ cells are shown in representative flow plots and quantitative scatter plots respectively. (*F*) The protein abundance of TIM3 and PD1 were shown in representative histogram and dot plots. (*G*) Ex vivo stimulation of tumor-infiltrating T cells to measure the IFNγ producing CD8$^+$ T cells between *Nae1*$^{CD8\text{-}WT}$ and *Nae1*$^{CD8\text{-}CKO}$. Data for all panels represent cumulative results from two independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

ablation of *Nae1* in CD8$^+$ T cells abolished their antitumor activity, leading to enhanced tumor progression. We further demonstrated that forced expression of NAE1 in CD8$^+$ T cells improves their effector function upon adoptive transfer into the tumor-bearing host. Our findings unraveled an underexplored mechanism by which neddylation sustains the protein dynamics required for effector T cell differentiation and is critical for improving the efficacy of adoptive cell therapy (ACT).

Although the molecular mechanism underlying T cell activation has been investigated for several decades, most studies have focused

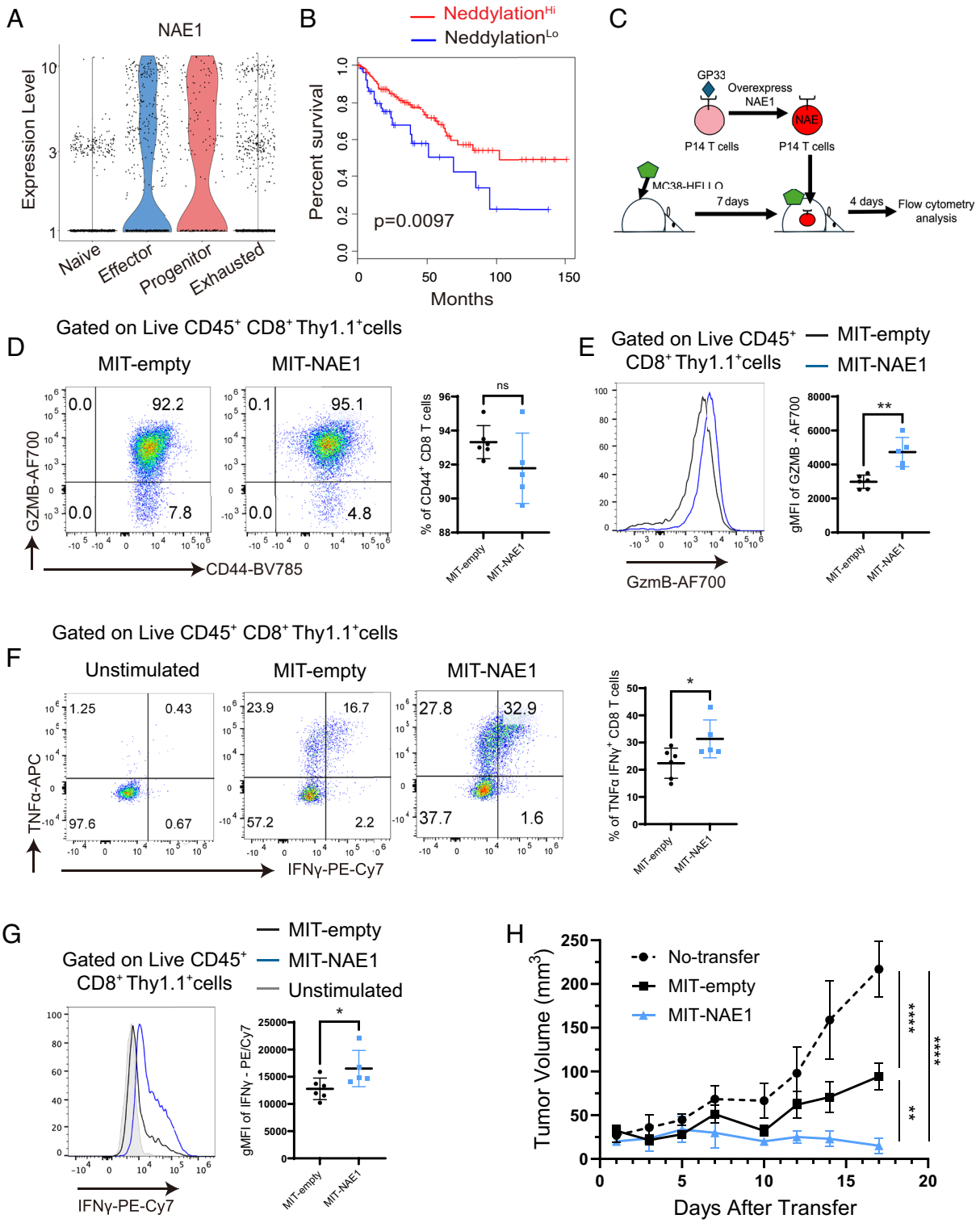

Fig. 4. Overexpressing NAE1 improves the effector function of CD8$^+$ T cells in tumor. (*A*) The scRNA-seq dataset from colon tumor tissues was reanalyzed. The expression of NAE1 in various subsets of CD8$^+$ T cells was shown in violin plot. (*B*) Kaplan–Meier survival curve of colon cancer patients with high (n = 216) or low (n = 54) neddylation activation signature gene, NAE1, and UAB3. (*C*) The design of adoptive transfer experiments. (*D*) Two groups of C57BL/6 mice were inoculated with MC38-HELLO tumor cells and received P14 cells transduced with either MIT-empty (n = 6) or MIT-NAE1 (n = 5) RV. At day 4 after transfer, the intratumoral Thy1.1$^+$ RV-transduced P14 cells were examined for the frequency of CD44$^+$ CD8$^+$ T cells and shown by representative flow plots (*Left*) and summary data (*Right*). (*E*) The expression of GzmB was detected on these cells and shown in representative histograms and dot plots. (*F*) Dot plots show the proportion of Thy1.1$^+$ cells among the IFNγ and TNFα double-positive populations following ex vivo TCR stimulation. (*G*) Histograms show the expression of IFNγ in the intratumoral Thy1.1$^+$ RV-transduced P14 cells. (*H*) C57BL/6 mice were inoculated with MC38-HELLO tumor cells and followed by transfer of P14 cells transduced with either MIT-empty (n = 5) or MIT-NAE1 (n = 5) RV or mock (n = 5). Tumor growth was monitored and compared by two-way ANOVA, (*D–G*) represent cumulative results from two independent experiments. *P < 0.05, **P < 0.01.

on transcriptional regulation. With the recent advancement in proteomics technologies, several studies have comprehensively characterized the critical role of PTMs in regulating the proteome dynamics during T cell activation (1, 3, 17). As one of the newly discovered PTMs, the role of neddylation has yet to be adequately explored in effector T cell differentiation. Previous studies have

revealed that neddylation is required for Th1/Th2 differentiation in $CD4^+$ T cells (28). Mechanistically, the neddylation of Shc might enhance the formation of a ZAP70–Shc–Grb2 signaling complex, affecting downstream ERK activation and influencing $CD4^+$ T cell activation (28). Several additional studies reported that pharmacological inhibition or genetic ablation of critical enzymes in neddylation abolished $CD4^+$ T cell response during allergic inflammation, viral malaria infection, and graft-versus-host disease (28–30). Despite these studies, the role of neddylation in regulating $CD8^+$ T cell activation has not been explored. Our study demonstrates that NAE1-mediated neddylation is a positive PTM regulator of $CD8^+$ T cell activation. Supporting this notion, we have shown that the NAE1-null $CD8^+$ T cells exhibited severe defects in activation and mitochondrial function. Meanwhile, our proteomic analysis suggested that the neddylation sustains the proteomic landscape associated with effector molecule production, proliferation, survival, and mitochondrial fitness. Consequently, we observed a subverted antitumor $CD8^+$ T cell response and accelerated tumor progression in NAE1 knockout mice. Overall, our findings reveal a previously underappreciated role of neddylation in regulating the differentiation and function of $CD8^+$ T cells.

While emerging evidence highlights the role of neddylation in the immune system, mechanisms of how neddylation is induced, especially in T cells, remains limited. Our study is a prospective report to support a model in which TCR stimulation induces NFATc1 to upregulate *Nae1* in $CD8^+$ T cells. Similar to previous observations (29), we also showed the *Nae1* expression was low at the native stage but dramatically increased in response to TCR stimulation. By analyzing the open regulatory region within the *Nae1* gene loci, we have identified NFATc1 as a key TF that highly likely controls *Nae1* expression. Supporting this notion, CRISPR-Cas9-mediated deletion of NFATc1 significantly reduces the *Nae1* transcript levels in activated T cells. This is consistent with NFATc1 as a key TF downstream of TCR stimulation and regulating numerous genes involved in T cell effector function (46). The family of NFAT transcription factors consists of five members that share a common DNA-binding domain of approximately 300 amino acid residues, and most of their activity is induced by the TCR-triggered calcium release (47). Several studies reported that the ablation of NFATc1 attenuates T cell activation, cytokine production, and cytotoxic effector functions (43, 48, 49), similar to our observations in *Nae1* null $CD8^+$ T cells. Taken together, this evidence supports a model that TCR signaling induces NFATc1 to transcriptionally upregulate *Nae1* for supporting effector T cell differentiation.

Our study provides several lines of evidence indicating that neddylation remodels the mitochondrial proteome to control their function, which is essential for T cell activation. First, impaired neddylation led to a disturbed protein profile involving OXPHOS complex I subunits. Second, neddylation deficiency also reduces proteins related to the outer mitochondrial membrane (TOM40) and the inner mitochondrial membrane (TIM23) (50, 51). Extensive studies confirmed that the disruption in the mitochondrial membrane leads to impaired ion and metabolite exchange, which is detrimental to cells (40, 41). Thus, it is possible that neddylation may regulate mitochondria fitness by maintaining the mitochondrial membrane integrity. Third, while several reports revealed the association between neddylation inhibition and mitochondrial dysfunction in cancer cell lines (32), we demonstrated that the lack of *Nae1* in activated $CD8^+$ T cells promoted the accumulation of depolarized mitochondria and obstructed respiratory activity. This finding parallels the observation using liver cancer cell lines and hepatocytes, which showed that the pharmacological inhibition of neddylation reduces mitochondrial function (33, 35). We conclude from these studies that neddylation controls essential mitochondrial proteins indispensable for sustaining mitochondrial biogenesis during T cell activation. However, the possibility that neddylation may regulate mitochondria fitness by governing mitophagy, a process that removes damaged mitochondria, cannot be excluded because neddylation deficiency also reduced key mitophagy promoter HUWE1, which regulates alternative mitophagy pathways and acts independently of the PINK1–Parkin pathway (52). A recent study suggests that HUWE1 can be recruited to damaged mitochondria and interact with AMBRA1 to induce the clearance of dysfunctional mitochondria (52). Future studies are warranted to investigate the role of neddylation in HUWE1-mediated mitophagy.

In summary, our results unveil the broad function of an underappreciated PTM, neddylation, in regulating various aspects of $CD8^+$ T cell activation, which is critical for effective antitumor immunity. The key enzyme of neddylation, NAE1, is enhanced by the TCR/NFATc1 axis to sustain mitochondrial fitness and support T cell activation. Consistently, we uncovered that ablation of NAE1 profoundly obliterates the effector $CD8^+$ T cell differentiation in colon tumors. Ultimately, we highlight the translation value of inducing neddylation in ACT by demonstrating that enforced expression of NAE1 profoundly improved $CD8^+$ T cell effector function. This proof-of-principle study has clearly demonstrated the feasibility of targeting NAE1 to boost the therapeutic efficacy of ACT. Our work reveals the fundamental principles of the emerging connections between PTM and metabolic regulation during T cell activation. Also, this study provides crucial knowledge to developing a promising ACT for colon cancer by manipulating the neddylation pathway in T cells.

Methods

Mice. C57BL/6, C57BL/6 CD45.1, Lck^{Cre}, $E8I^{Cre}$ mice and P14 mice were purchased from Jackson Laboratory. $Nae1^{floxed/floxed}$ mice were acquired from Nuo Sun from the College of Medicine, Ohio State University (Columbus, OH). $Nae1^{T\text{-}CKO}$ mouse line was generated by crossing the Lck^{Cre} mice with $Nae1^{floxed/floxed}$ mice, and $Nae1^{CD8\text{-}CKO}$ mouse line was generated by crossing the $E8I^{Cre}$ mice with $Nae1^{floxed/floxed}$ mice. All in vivo experiments used 8- to 12-wk-old gender-matched mice, and all mouse handling conformed to the requirements of the Institutional Animal Care and Use Guidelines of Ohio State University.

CD8 T Cell Isolation and Activation. Polyclonal $CD8^+$ T cells were isolated from the mice spleens with the EasySep™ Mouse $CD8^+$ T Cell Isolation Kit (STEMCELL Technologies). The spleens harvested from mice were placed in cold RPMI-1640 medium supplemented with 1% FBS (Gibco), 100 U/mL HyClonepenicillin/streptomycin (Cytiva), and 1% GlutaMAX™(Gibco) before being mashed through 70 μm cell strainers to get a single cell splenocyte suspension. Without ACK lysis, the splenocytes were resuspended in EasySep™ Buffer at 1×10^8 cell/mL and proceeded to CD8 T cell isolation per the manufacturer's instruction.

Polyclonal activation was done by culturing isolated $CD8^+$ T cells in 24-well plates coated with 3 μg/mL anti-CD3ε antibody overnight at 4 °C (145-2C11, BioLegend), in T cell culture medium with additional 1 μg/mL anti-CD28 antibody (37.51, BioLegend) and 10 U/mL IL-2 (Peprotech). The T cell medium is prepared by adding 10% FBS (Gibco), 100 U/mL Hyclone penicillin/streptomycin (Cytiva), 1% GlutaMAX™(Gibco), 55 μM 2-Mercaptoethanol (Gibco), 1 mM Sodium Pyruvate (Gibco), 1% MEM Non-Essential Amino Acids Solution (100X, Gibco), and 10 mM HEPES (VWR international) in RPMI-1640 medium.

P14 $CD8^+$ T cells were prepared from splenocytes isolated as previously stated, followed by red blood cell lysis with ACK lysis buffer (Quality Biological Inc). Then the splenocytes were cultured in T cell medium in the presence of 1 μg/mL GP33 peptide (Genscript) and IL-2 (Peprotech) for 3 d.

Tumor Inoculation. MB49 and MC38 cell lines were obtained from ATCC. MC38-HELLO cells were prepared by transducing MC38 cells with the HELLO Lentiviral vector (LV). The LV backbone for HELLO has been previously described (45, Cell). HELLO-LV contains HELLO driven by the MCV promoter. HELLO was created as a protein fusion of codon-optimized hen egg lysozyme (HEL), LCMVgp33-43 (KAVYNFATCGI), LCMVgp61-80 (GLNGPDIYKGVYQFKSVEFD), and

2A peptide–linked RFP, with PuroR (45). The transduced MC38 cells were sorted by FACS, resulting in a pure RFP population of MC38-HELLO cells. Subsequently, the cells were diluted to half-cell per well and subjected to puromycin selection at varying doses to isolate single clones. All tumor cells were cultured in DMEM (Gibco) Supplemented with 10% FBS (Gibco), 1% GlutaMAX™(Gibco), HyClone penicillin/streptomycin (100 U/mL; Cytiva). For in vivo inoculation, only the tumor cells within their 8th passage were used. 1×10^6 tumor cells were subcutaneously injected in the flank of C57BL/6 mice in 100 μL of sterile PBS.

Immune-Cell Isolation. Lymphocytes were isolated from the spleen and other organs by mashing through 70 μm cell strainers followed by red blood cell lysis in ACK lysis buffer (Quality Biological, Inc.) to form single cell suspension for subsequent staining and analysis. The tumors were dissected from mice 10 d after tumor inoculation and were minced into small pieces in RPMI-1640 medium supplemented with 1% FBS (Gibco), 2 mg/mL Collagenase Type I (Worthington Biochemical Corporation) and Collagenase Type IV (Worthington Biochemical Corporation), and incubated for 30 min at 37 °C. The tumor tissues were then disassociated with gentleMACS™ Tissue Dissociator (Miltenyi Biotec). The cell suspension was subsequently enriched for lymphocytes with Lymphocyte Separation Medium (Corning™) per the manufacturer's instruction.

Flow Cytometry. Single-cell suspension isolated from the spleen, other organs, and tumors were stained and analyzed by flow cytometry. For cytokine production assay the lymphocytes isolated from tumors underwent polyclonal activation as previously described in presence of brefeldin A (Biolegend) for 6 h before subsequent staining and analysis. Dead cells were excluded with LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Invitrogen™) and all antibodies were purchased from eBioscience and BD Biosciences except TCF1/7 (Cell Signaling Technology, Inc.). Cell apoptosis assay was done with APC Annexin V Apoptosis Detection Kit with 7-Amino-actinomycin D (7-AAD) (BioLegend) per the manufacturer's instruction. The flow cytometry data acquisition was done on Cytek Aurora (Cytek® Biosciences), which was subsequently spectro-unmixed with SpectroFlo® software and analyzed by FlowJo V.10.10.0 (BD Biosciences).

Immunoblotting Assays. CD8 T cells were activated as described above. After 3 d, the cells were lysed in RIPA buffer (Thermo Scientific, 89901) supplemented with 1× Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, 78440) and 1 mM PMSF Protease Inhibitor (Thermo Scientific, 36978). SDS–polyacrylamide gel electrophoresis separated the resulting total cell lysates and transferred to PVDF membrane. The membranes were blocked with 5% nonfat milk (VWR) in TBST and then immunoblotted with the indicated antibodies diluted in 3% BSA (Sigma-Aldrich) in TBST at 4 °C overnight. Specifically, the anti-NAE1 antibody (Cell Signaling, #14321) and anti-GAPDH antibody (Cell Signaling, #97166) was used at a 1:1,000 dilution. The immunoblots were then washed with TBST and incubated with secondary antibody in 5% BSA (Sigma-Aldrich) in TBST. Finally, the membranes were developed with Pierce™ ECL Western Blotting Substrate (Thermo Scientific, 32106) on Chemidoc MP system (Bio-rad).

Retrovirus-Mediated NAE1 Overexpression and Cell Transfer. P14 CD8 T cells used in retrovirus-mediated overexpression were activated as previously stated before transfection. MIT RV was obtained from Weiguo Cui. Murine NAE1 was subcloned into MIT to generate the MIT-NAE1 vector. Empty or NAE1-containing vectors were transfected into 293T cells. After 48 h, culture supernatant containing retrovirus was collected and added to day 2 activated P14 cells for the spin transfection. Then, the P14 cells were cultured for another 2 d and adoptively transferred into the recipient CD45.1 mice.

RNA Extraction and RT-qPCR. RNA was extracted from cells using TRIzol reagent (Invitrogen) and Direct-zol RNA Microprep Kits (ZYMO Research). First strand cDNA was synthesized using LunaScript® RT SuperMix Kit per user manual. The SYBR green method was used for RT-qPCR with primers from Bio-rad or IDT on QuantStudio 3 Real-Time PCR Systems. Relative expression was normalized by β-actin in each sample. The following primer sequences were used: Nae1 (qMmuCED0047217, Bio-rad), β-actin (forward,5′-GTGACGTTGACATCCGTAAAGA-3′; reverse, 5′-GCCGGACTCATCGTACTCC-3′).

Transcriptomics Analysis. The CD8 T cells isolated from *Nae1*$^{T\text{-}CKO}$ and *Nae1*$^{T\text{-}WT}$ mice were activated as previously described for 2 d before transcriptomics assay. 3×10^6 activated CD8 T cells were flash frozen in ethanol/Dry ice slush and shipped to core facilities at Novogene, where library construction and sequencing was done per standard protocol. The resulting raw reads were trimmed by Trim Galore v0.6.10 and aligned to reference genome mm10 with HISAT2 V2.2.1 in combination with Samtools V1.2. The DEG assay was done by DESeq2 V 3.19. GSEA was done on GSEA desktop software v4.3.3 on classic preranked mode. Volcano plots were generated by VolcaNoseR, and heatmaps were generated with Morpheus (https://software.broadinstitute.org/morpheus) and pheatmap V 1.0.12.

Proteomics Analysis. The CD8 T cells isolated from *Nae1*$^{T\text{-}CKO}$ and *Nae1*$^{T\text{-}WT}$ mice were activated as previously described for 2 d before proteomics assay. 3×10^6 activated CD8 T cells were rinsed in cold PBS and lysed with pure trifluoroacetic acid (Sigma-Aldrich) and processed with E3filter as described previously (PMID: 38866007). The resulting desalted peptides were dried and stored under −80 °C before further analysis. The peptides were later analyzed with LC-MS on an Ultimate 3000 RSLCnano system in tandem with an Orbitrap Eclipse mass spectrometer and FAIMS Pro Interface (Thermo Scientific) as described in previous publication (PMID: 37729920) and processed with DIA-NN software V 1.8.1 (PMID: 31768060). Bioinformatics analyses including *t* test, correlation, clustering analyses were performed using Perseus software V 1.6.2.3. GSEA, heatmaps, and volcano plots were done with the same procedures as transcriptomics analysis.

CRISPR-Cas9-Mediated Gene Deletion. CRISPR–Cas9 system was used to knock out gene encoding transcription factor NFATC1 in $CD8^+$ T cells. Two predesigned sgRNAs (5′-CGGCCAGTTTTTCCACGACG-3′ and 3′TCCTCGGGCAAGCATCACGG′5) targeting *Nfatc1*, CRISPR/Cas9 ribonucleo-protein complex, and electroporation enhancer was acquired from IDT. Electroporation was performed following the protocol provided by IDT CRISPR genome editing (www.idtdna.com) using Lonza 4D Nucleofector System.

Seahorse Assay. The Seahorse XF Cell Mito Stress Test Kit (Seahorse Bioscience) was utilized to assess the mitochondrial function of CD8 T cells by measuring the oxygen consumption rate (OCR) with an XF96 analyzer (Seahorse Bioscience). Approximately 5×10^5 cells were seeded per well in an XF96 cell culture microplate. During the assay, compounds were injected at specific final concentrations, and OCR was analyzed.

Statistical Analysis. Two-tailed Student's *t* tests were performed on all the data using the GraphPad Prism 10.2.1*(395) software unless otherwise stated. For 3-d activation time courses, tumor growth curves, and thymus baseline analysis two-way AVONA was used. ns $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ in all data shown.

Data, Materials, and Software Availability. The RNAseq data is deposited at GEO (GSE289216) (44), and proteomics data is available at https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp with ID-MSV000094179 (53). All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. This work was supported by 1R01CA269984 (G.X.) from the NIH, CCR231013713 from Susan G. Komen (G.X.) and RSG-23-1036499-01 (G.X.) from the American Cancer Society.

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