

Peer Review File

N-acetyltransferase 10 is implicated in the pathogenesis of cycling T cell-mediated autoimmune and inflammatory disorders



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The study emphasizes the importance of the NAT10-RACK1 axis in regulating T-cell proliferation to generate and maintain immune responses. Alterations at this control point can trigger autoimmune processes. The title indicates "cycling T cells," Figure 1 refers to this, but the term is not further defined throughout the article. The role of B lymphocytes and neutrophils in the pathogenesis of autoimmune diseases is relevant, and they were discarded.

Introduction

In this section, it is suggested that the authors review the vocabulary for some concepts to make it more relevant to the topic. A very long paragraph related to treatment (lines 59 to 88) could be reduced for the article's focus. It will be essential to include references indicating that NAT10 is important in T lymphocytes.

Materials and Methods

The sample size (n=6) for the single-cell work is small (supplementary figure legends line 5). There are missing parameters for reproducible analyses (supplementary figure legends line 314).

The methodology is solid and extensive; however, information is missing to make the experiment reproducible (supplementary figure legends, line 314).

It is necessary to include the R version being used (supplementary figure legends, line 310), the type of fixation and permeabilization used (supplementary figure legends, line 165), and the fluorochromes, clones, brand, and catalog used for labeling (supplementary figure legends, line 149).

Regarding t-SNE analysis, specific parameters used to run the t-SNE algorithm are missing (supplementary figure legends line 314).

Regarding cell culture, the culture time is not indicated (supplementary figure legends line 130). Regarding flow cytometry, it does not indicate how fixation permeabilization was performed or with which reagents or kit (supplementary figure legends line 149).

Regarding RT-PCR, the primer sequences for the NAT10 and Beta-actin genes are not indicated (supplementary figure legends, lines 291-300). Regarding sequencing, the R version is not indicated (supplementary figure legends, lines 305 and 310).

Results

Figure 1:

The description of the text in Figure 1C does not match. What does "patient CD" indicate? Lines 152, 153, and 159. In Figure 1C, it is not clear where the data come from.

What genes define cycling T cells? This is because, in Figure 1B and Supplementary Figure 1B, it is indicated that this is "consistently" (line 163).

The supplementary Figure 1C is not included in the description of the results. In Figures 1E and 1F supplementary, what gate are they referring to? Is cycling T cells of MS patients (supplementary Figure 1A) or in cells of IBD patients (supplementary Figure 1A)?

In line 194, "different from previous observations," what does it refer to? In Figure 1J, what do CE

and NE indicate? The expression of NAT10 at the protein level is shown with nuclear extracts of CD4+ T cells (Figure 1J). Were the same results obtained for CD8+ T cells? What are the implications if NAT10 is expressed only when cells are stimulated for more than 6 hours? On the other hand, what is the possible explanation for finding NAT10 in the cytoplasm when it had been reported as a nuclear protein (line 196)? It is necessary to clarify if these activated T cells and NAT10 expression evaluated at the RNA and protein levels are from the spleen or inguinal lymph nodes.

Figure 2:

Do the WT and NAT10KO mice show similar B lymphocyte levels and decreased T lymphocyte levels (Figure 2A)? Was NAT10's participation in B lymphocytes explored further, since these cells are important in the pathogenesis of autoimmune diseases such as rheumatoid arthritis and lupus erythematosus?

Why are the levels of CD3 in the spleen and iLN so disproportionate? They are low percentages (22.9 and 10.8, respectively, and 61.6 and 34.7 in iNL).

In supplementary Figure 2C, B lymphocytes are increased in NAT10KO mice; what explanation exists for this point?

The compensatory mechanism in NAT10KO mice (lines 210 and 213) is decreased T lymphocytes and increased CD69 and CD44 molecules. What do N, M, CM, and EM refer to in supplementary Figure 2D?

If no changes were observed at the thymus level (line 215), why is it said that NAT10 impacts T lymphocyte homeostasis (line 221)? There are fewer T lymphocytes in NAT10KO mice compared to WT, but they still produce higher levels in CD4+IL-17+, CD4+IFN γ +, and CD8+IFN γ (Figure 2D, line 222).

Figure 3:

In line 238, what does it mean that NAT10 plays an important role in T lymphocyte proliferation and its effector function? What is this effector function being modulated by NAT10? In the mouse model of IBD, it is demonstrated that NAT10 would participate in developing the disease. If it is demonstrated that CD4IFN γ + T lymphocytes are decreased in the spleen and iNL of 10KO mice compared to WT, it would be expected that they synthesize fewer cytokines, although it contrasts with Figure 2D.

What is DSS (line 252), and in supplementary Figure 3A, DSS treatment?

What other cells are related to colitis (line 260)? In the EAE model, similar to the IBD model, there is no disease in NAT10KO mice. In Figure 3G, what type of cells infiltrate the central nervous system in NAT10KO mice? Was immunohistochemistry performed? CD11b cells show increased levels in NAT10KO mice compared to WT. What is the explanation? Figure 3H.

Figure 4:

T cells were isolated from WT and NAT10KO mice. What percentage of lymphocytes is obtained? If low levels of T lymphocytes are shown and apoptosis is present, what implications would these alterations have on T lymphocyte function?

Based on the results shown, highlighting genes related to cell cycle arrest, it seems that NAT10 is important in the late stages of activation since the expression of CD69 (early activation antigen) is not modified (line 313).

Figure 6:

The effect of remodelin showed a slight reduction in proliferation (Figure 6A) and cell cycle (Figure 6B), although there is a significant difference. Is it possible to include semi-quantitative data in Figure 6C? What stimulus was used to activate the cells in Figures 6B and 6C and supplementary Figures 6B and 6C? The interaction between NAT10 and RACK1, Figure 6G, and 6H was significant in its demonstration.

The abstract should be more specific regarding the results, lines 457 to 459.

Discussion:

Paragraphs from lines 462 to 504 can be revised; they are very unspecific regarding the topic addressed in the article. Although it involves NAT10 participating in migration (lines 542), the data shown do not evaluate this point.

Minor:

Line 184 phenptype, phenotype

Figure 3H Brian, brain

Figure 6D = DMSO? R = Remodelin?

What does the histogram of blue color represent? The one in red?

In Figure 7G, the letters are overlapping.

Figure 7 indicates which variable was evaluated on the ordinate.

Reviewer #2 (Remarks to the Author):

This study by Li et al. investigates the role of NAT10, an acetyltransferase, in T cell development, activation and proliferation. It is suggested that deletion of NAT10 results in normal thymic development and T cell activation. However, proliferation is reduced in NAT10^{-/-} T cells. The authors then go on to suggest that this proliferation defect is due to a new function of NAT10 in protecting RACK1 from ubiquitin-mediated degradation. It is suggested that the K185 on RACK1 is a critical ubiquitination site that is acetylated by NAT10, thereby preventing ubiquitin-mediated degradation of RACK1.

I have expertise in the molecular mechanisms of proliferation control. I have focused on the results shown in Fig. 6 and Fig. 7 of the study, as these are the most relevant to my technical expertise.

I have the following major concerns over the data shown.

1. Can the authors explain why the NAT10 inhibitor Remodelin does not phenocopy NAT10 deletion?
2. In Fig. 7 and throughout the manuscript, I have concerns over the quality and the robustness of the immunoblot data shown. There must be quantitation for each immunoblot where the relevant bands are normalized to a loading control.
3. The English vocabulary and grammar used in the Introduction section is too flowery and uses archaic words whose meaning in scientific writing is unclear.

I do not believe that the study is publishable in its current form as the data in Fig. 7 do not substantiate the claim that NAT10 acetylation of RACK1 protects it from ubiquitin-mediated degradation. Furthermore, as far as I can tell, the only evidence shown that the mechanism of the proliferation defect observed in NAT10^{-/-} T cells is mediated via RACK1 is in Fig. 7L. Are the expression levels of WT and K185Q RACK1 comparable?

Comments on Fig. 7

Fig. 7A-B, quantitation and triplicate analysis

Fig. 7A band is very light – transfer issue? There is also a decrease in tubulin signal

Fig. 7B bands look ‘burnt’ from excessive peroxidase activity

Fig. 7C – middle lane suggests that FLAG-Ub was not transfected into cells, but the immunoblot shows FLAG-Ub signal. There must be a typo!

Fig. 7C – it looks like ubiquitination is globally reduced, not just potentially RACK1

Fig. 7D – again there needs to be quantitation. Most ubiquitin variants appear affected by NAT10 overexpression, not just K48.

Fig. 7E – K174 is written on the Figure, but K175 is written in the text (line 439). I also do not understand what the -NAT10 or +NAT10 means in the schematic diagram for RACK1. Please explain. Does this mean with or without NAT10 overexpression?

Fig. 7G – again, quantitation. It looks like HA signal is decreased comparing lanes 2 and lanes 2 and 7, so the differences in Myc signal could easily be explained by loading

Fig. 7H – comparing lanes 2 and 5, you would expect to see a decrease in ubiquitin signal if K185 is a major site for ubiquitination. In contrast, the signal is as far as I can tell equal. Second, there is much less NAT10 overexpressed in the presence of the K185Q mutant (see FLAG signal comparing lanes 3 and 6). Therefore, the difference in ubiquitination might be due to differences in NAT10 expression.

Reviewer #3 (Remarks to the Author):

NAT10, a N-acetyltransferase, has been recognized for its role in the acetylation of RNAs and proteins, with multiple mRNA and protein targets reported. This acetylation is vital for mRNA stability and translation, thus playing a critical role in cell proliferation and self-renewal. It is noteworthy that NAT10's necessary role in promoting cell cycle arrest, specifically in the context of p53, suggests its function in cell survival and differentiation may be contextually variable. . In addition, NAT10 seem to be broadly expressed in various cell type. Thus far, the specific effect in T cells have not been examined.

The strength and significance of the manuscript is its thorough investigation of NAT10's roles in T cell proliferation and activation, utilizing both in vitro and in vivo models to provide substantial evidence. In simple term, NAT10 is essential for T cell proliferation. The data presented is robust, comprehensive, and well-articulated. An exciting mechanism the authors described is that NAT10 acetylates a few key lysine residues of RACK1 through interaction and these acetylation prevents ubiquitination and thus increase RACK1 stability. There remains some reservation regarding the specific mechanisms by which NAT10 influences T cells. This is surprising given the promiscuous nature of protein acetylation and essential roles of NAT10 in other contexts. It may not be essential to delineate an exact molecular mechanism within this

study due to the broad influence of the factor under investigation, but strengthen supporting data would be necessary to make a compelling argument for the proposed mechanisms. Detailed comments are as follows:

1. The manuscript would benefit from a more streamlined introduction and discussion. Some sections diverge into topics not directly related to NAT10, which could be refined to enhance clarity and logical progression. While expressive language can aid the narrative, it is crucial that it remains well-organized and delivers clear and precise scientific information.

2. The representation of gene expression in Figure 1F using dot plots could potentially be misleading for representing each single-cell population. It highlights the cells expressing NAT10. Since only ~5% T cells exhibit the gene expression in question, alternative visualization techniques such as violin plots could offer a more holistic and less biased presentation of the expression variations across single-cell populations.

3. In several places in the manuscript, there appears to be discrepancies between the text and figures. This warrants a careful review to ensure that the figure and text are in concordance. Figure 2D is the opposite from “we have also observed a significant suppression of T cell homeostasis, evidenced by a diminished capacity to produce cytokines under steady-state conditions”

Figure 7C. The samples may be mis-labeled.

Figure 7D. the label is a bit hard to understand. What are the K48, K63, K6, K11, K27, K29 and K33 in “FLAG-Ub”. How are they being identified here? I cannot find description in text or methods about this.

4. Figure 6, particularly 6C, presents data that may not show a significant difference. Comparisons with figure S6 suggest the acetylated proteins in NAT10 TKO that could correspond to the activation-induced acetylation observed (~40KD) – stronger in activated T cells and TKO.

5. The interaction between NAT10 and RACK1 is highlighted as a significant finding in the paper and is used to explain functions of NAT10 in T cells. However, the supporting data are not conclusive. Figure 6D do not specifically address whether these overlap acetylated protein candidates are dependent on T cell activation, or NAT10 at all. It would be necessary for the authors to demonstrate the differences in acetylated proteins between WT and NAT10 KO T cells (particularly RACK1) and acetylated proteins between naïve and activated T cells (particularly RACK1), to substantiate the implied association between NAT10 and RACK1 acetylation.

6. With respect to the co-immunoprecipitation experiments involving NAT10 and RACK1, the possibility of an indirect interaction through a mutual binding partner such as RNA should be considered. It would be informative to assess whether the interactions observed are sensitive to RNase treatment.

7. Figure 7D, In any comparisons, the difference between WT and KO are not obvious. The authors should add quantification among biological replicates to support their statement in the text that any of these ubiquitinations are or are not different.

8. The authors propose that NAT10 acetylates RACK1 at various lysine residues, thereby preventing ubiquitination. To bolster this claim, it would be necessary to show direct evidence of such NAT10-dependent acetylations and its impact on RACK1 stability.

9. In Figure 7K, the observed differences in RACK1 between WT and K185Q mutants may arise from factors other than protein stability. At least, the inclusion of M132 inhibitors in experimental assays should be considered.

10. The authors postulates RACK1 acetylation as a key effector of NAT10 in T cell homeostasis. It would be compelling to determine if the RACK1 K185Q mutation can rectify the deficiencies in NAT10-deficient T cell functions, including metabolic activity (Figure 5E, F) and cytokine production (Figure 4C), thereby strengthening the manuscript's conclusions.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The study emphasizes the importance of the NAT10-RACK1 axis in regulating T-cell proliferation to generate and maintain immune responses. Alterations at this control point can trigger autoimmune processes. The title indicates "cycling T cells," Figure 1 refers to this, but the term is not further defined throughout the article. The role of B lymphocytes and neutrophils in the pathogenesis of autoimmune diseases is relevant, and they were discarded.

Introduction

1. In this section, it is suggested that the authors review the vocabulary for some concepts to make it more relevant to the topic. A very long paragraph related to treatment (lines 59 to 88) could be reduced for the article's focus. It will be essential to include references indicating that NAT10 is important in T lymphocytes.

Response: Following the reviewer's suggestion, we removed the content of immunosuppressive treatment in autoimmune disease. However, current reports on the function of NAT10 in lymphocytes are lacking, which further illustrates the innovation of our work.

Materials and Methods

2. The sample size (n=6) for the single-cell work is small (supplementary figure legends line 5).

Response: This is a good question. The cerebrospinal fluid single-cell sequencing data of multiple sclerosis patients were obtained from the GEO database (accession code: GSE138266). This dataset included single-cell sequencing data of cerebrospinal fluid from only 6 healthy controls and 6 multiple sclerosis patients. Due to the strict inclusion and exclusion criteria of the study, we believed that the final sample size is acceptable (PMID: 31937773); at the time of submission, we did not find larger-scale single-cell sequencing data of cerebrospinal fluid from multiple sclerosis patients published.

3. There are missing parameters for reproducible analyses (supplementary figure legends line 314). The methodology is solid and extensive; however, information is missing to make the experiment reproducible (supplementary figure legends, line 314).

Response: We are sorry for this missing information. We added them back as reviewer's suggestion.

4. It is necessary to include the R version being used (supplementary figure legends, line 310), the type of fixation and permeabilization used (supplementary figure legends, line 165), and the fluorochromes, clones, brand, and catalog used for labeling (supplementary figure legends, line 149).

Response: As suggestion, we included the R version in line 310. We also added comprehensive details pertaining to the antibodies used, including the antibody clone, catalog number, and the respective supplier's information.

5. Regarding cell culture, the culture time is not indicated (supplementary figure legends line 130).

Response: We added culture time and experimental time in the "Cell culture" section.

6. Regarding flow cytometry, it does not indicate how fixation permeabilization was performed or with which reagents or kit (supplementary figure legends line 149).

Response: We expressed our gratitude to the reviewer for the timely reminder. In response to this valuable input, we incorporated additional details regarding the experimental methodology and the utilization of kits within the Materials and Methods section.

7. Regarding RT-PCR, the primer sequences for the NAT10 and Beta-actin genes are not indicated (supplementary figure legends, lines 291-300). Regarding sequencing, the R version is not indicated (supplementary figure legends, lines 305 and 310).

Response: We are sorry for not describing the detail of RT-PCR clearly. We provided the sequence of primers for qPCR were shown in Table S3. We also added R version in M&M section.

Results

Figure 1:

The description of the text in Figure 1C does not match. What does "patient CD" indicate? Lines 152, 153, and 159. In Figure 1C, it is not clear where the data come from.

Response: We apologized for not clearly describing our research here, but "CD" patient actually describes "patient with CD". We have revised the above statement. Meanwhile, the data in Figure 1C is a further classification and analysis of the public data shown in **Figure 1A-B**.

What genes define cycling T cells? This is because, in Figure 1B and Supplementary Figure 1B, it is indicated that this is "consistently" (line 163).

Response: I would like to express my gratitude to the reviewer for the insightful question. Indeed, as depicted in **Figure 1B** and **Supplementary Figure 1B**, we have demonstrated the expression of MKI67 and TOP2A, respectively. Additionally, CENPF and STMN1 have been employed as markers for identifying cycling T cells. The term "consistently" in this context denotes the recurring observation of the presence of cycling T cells, which have been consistently found to be significantly elevated in patients with MS.

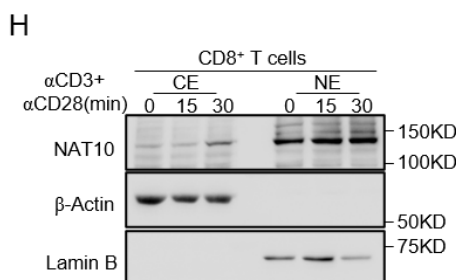
The supplementary Figure 1C is not included in the description of the results. In Figures 1E and 1F supplementary, what gate are they referring to? Is cycling T cells of MS patients (supplementary Figure 1A) or in cells of IBD patients (supplementary Figure 1A)?

Response: Thanks for the reviewer's nice reminding, we gave a description for **Supplementary Figure 1C**., as following "The energy utilized by T cells during clonal expansion is primarily allocated to support genome synthesis, thereby leading to a concomitant decrease in effector function during the proliferation process. Likewise, DEGs in cycling T cells also indicated an anticipated reduction in rRNA, mRNA, and miRNA metabolic processes, all of which were intricately linked to translation processes (**Supplementary Figure 1C**)". We also indicated that **Figure 1E-F** mainly analyzed cycling T cells from CD patients.

In line 194, "different from previous observations," what does it refer to? In Figure 1J, what do CE and NE indicate? The expression of NAT10 at the protein level is shown with nuclear extracts of CD4+ T cells (Figure 1J). Were the same results obtained for CD8+ T cells? What are the

implications if NAT10 is expressed only when cells are stimulated for more than 6 hours?

Response: To address the reviewer's query, we conducted a comparable assessment for CD8⁺ T cells. The findings indicated that in naïve CD8⁺ T cells, NAT10 predominantly resided in the nucleus. Upon stimulation with anti-CD3 and anti-CD28, a portion of NAT10 translocated from the nucleus to the cytoplasm (**Supplementary Figure 1H**), mirroring the behavior observed in CD4⁺ T cells. There was a basal expression of NAT10 in both CD4⁺ and CD8⁺ T cells. Short-term stimulation induced the relocalization of NAT10 from the nucleus to the cytoplasm, while long-term stimulation led to an upregulation of NAT10 transcription (**Supplementary Figure 1H**). These results implied that NAT10 played a role in regulating the physiological functions of T cells at various stages.



Supplementary Figure 1 (H) WT naïve CD8⁺ T cells were activated for indicated time by TCR crosslinking, the cytosolic and nuclear extracts were subjected to immunoblotting with antibody against NAT10.

On the other hand, what is the possible explanation for finding NAT10 in the cytoplasm when it had been reported as a nuclear protein (line 196)? It is necessary to clarify if these activated T cells and NAT10 expression evaluated at the RNA and protein levels are from the spleen or inguinal lymph nodes.

Response: We extend our appreciation to the reviewer for their valuable suggestion, as well as for the comprehensive elaboration and discussion provided in the results section. These findings indicated that in activated T cells, NAT10 exerted its function not only within the nucleus but also through potential interactions with proteins or RNA in the cytoplasm, thereby regulating the functionality of these molecules. Concurrently, the naïve T cells were isolated from the spleens of WT mice.

Figure 2:

Do the WT and NAT10KO mice show similar B lymphocyte levels and decreased T lymphocyte levels (Figure 2A)? Was NAT10's participation in B lymphocytes explored further, since these cells are important in the pathogenesis of autoimmune diseases such as rheumatoid arthritis and lupus erythematosus?

Response: The reviewer has raised a pivotal inquiry. Upon comparing the B cell specificity knockout mice NAT10 B cell development situation, we observed that, in contrast to T cells, the absence of NAT10 did not appear to impact the development of B cells in the spleen and bone marrow. These findings suggested that the involvement of NAT10 in the pathogenesis of autoimmune diseases primarily hinged on its functionality in T cells, rather than its direct regulation of B cells.

Response: Thank you for your insightful question. Indeed, numerous early studies have demonstrated that upon arrest of T cell development, T cells exhibited an autoactivation phenotype characterized by an increase in effector memory (EM) and central memory (CM) cells, as well as upregulation of molecules such as CD69 and CD44 (*Journal of Autoimmunity*, 21 Sep 2018, 96:74-85). This phenomenon represented a common compensatory response in T cell development. In the figure legend, we provided detailed descriptions and explanations of the abbreviations N, M, CM, and EM.

If no changes were observed at the thymus level (line 215), why is it said that NAT10 impacts T lymphocyte homeostasis (line 221)? There are fewer T lymphocytes in NAT10KO mice compared to WT, but they still produce higher levels in CD4+IL-17+, CD4+IFNγ+, and CD8+IFNγ+ (Figure 2D, line 222).

Response: The response to this inquiry parallels the aforementioned discussion. The thymus, serving as the central organ of immunity, plays a pivotal role in the development and maturation of T cells. In peripheral immune tissues, T cells primarily function in self-renewal and activation. It was imperative to emphasize the significance of maintaining homeostasis in peripheral T cells. Analogous to the aforementioned issue, aberrant T cell development precipitated T cell autoactivation, thereby accounting for the heightened expression of IFN-γ and IL-17 in the residual peripheral NAT10-null T cells. Notably, despite the marked decline in total peripheral CD4⁺ T cells, there was a concurrent elevation in T cell cytokine levels. This discrepancy suggested that while there was no overall augmentation in immune activation, it appeared to be a heightened T cell-mediated immune response phenotype.

Figure 3:

In line 238, what does it mean that NAT10 plays an important role in T lymphocyte proliferation and its effector function? What is this effector function being modulated by NAT10? In the mouse model of IBD, it is demonstrated that NAT10 would participate in developing the disease. If it is demonstrated that CD4IFNγ+ T lymphocytes are decreased in the spleen and iNL of NAT10 KO mice compared to WT, it would be expected that they synthesize fewer cytokines, although it contrasts with Figure 2D.

Response: We regret to inform that this section pertains to preliminary research findings. In this segment of the text, we addressed the paradoxical observations regarding the disease model of CD4⁺ T lymphocyte IFN-γ reduction, juxtaposed with the concomitant increase in steady-state CD4⁺ T lymphocyte IFN-γ levels. We posited that under diseased conditions, T cell activation, coupled with clonal amplification, remained unaffected by the absence of NAT10. However, a notable inhibitory effect on T cell proliferation was observed. Consequently, the proportion of CD4⁺ IFN-γ-positive effector T lymphocytes in the NAT10-null group surpassed that of the WT group. In the steady-state scenario, although the intrinsic activation capacity of T lymphocytes was compensatorily heightened, the substantial decline in total T cell count also signified a state of immune deficiency. These seemingly contradictory phenomena were not mutually exclusive.

What is DSS (line 252), and in supplementary Figure 3A, DSS treatment?

Response: We apologize for not using the full name of “DSS” in the first appearance. Dextran sulfate sodium (DSS) is a chemical compound commonly used to induce colitis in murine models

for research purposes. When administered orally, DSS disrupts the intestinal epithelial barrier, leading to increased intestinal permeability and inflammation. This disruption results in the infiltration of immune cells into the intestinal mucosa, leading to the development of colitis in mice. DSS-induced colitis is a widely utilized model for studying the pathogenesis of inflammatory bowel disease (IBD) and for evaluating potential therapeutic interventions for IBD.

What other cells are related to colitis (line 260)? In the EAE model, similar to the IBD model, there is no disease in NAT10KO mice. In Figure 3G, what type of cells infiltrate the central nervous system in NAT10KO mice? Was immunohistochemistry performed? CD11b cells show increased levels in NAT10KO mice compared to WT. What is the explanation? Figure 3H.

Response: We firstly thank the reviewer's important question. The T cell adoptive transfer-induced colitis model is a widely used experimental model for studying colitis. After moving to the colon, the effector T cells produced various cytokines and chemokines that further recruited dendritic cells and macrophages, and contribute to the pathogenesis of colitis in this model.

In the EAE model, NAT10-deficient T cells exhibited reduced infiltration in the central nervous system due to developmental defects. Our FACS results indicated an increased presence of CD45^{mid}CD11b⁺ microglia and CD45^{hi}CD11b⁺ macrophages/monocytes in the central nervous system of EAE model mice with NAT10 T cell-specific deletion. While it appeared, as suggested by the reviewer, that the proportion of CD11b⁺ cells had significantly increased, we attributed this change to the decline in effector T cell proportion and the subsequent increase in the proportion of residual cells. To address the reviewer's concern, we included the total cell count in this section. The findings revealed that the quantity of microglia in the central nervous system of mice with specific loss of WT and NAT10 T cells did not exhibit significant changes. However, the number of macrophages/monocytes in the CD45^{hi}CD11b⁺ population was notably reduced, as was the number of T cells (**Figure 3H**). Given that mice with specific loss of NAT10 in T cells did not manifest significant disease, we postulated that these locally enriched myeloid immune cells might not fulfill the same critical functions as pathogenic T cells, and further detailed analysis was not performed.

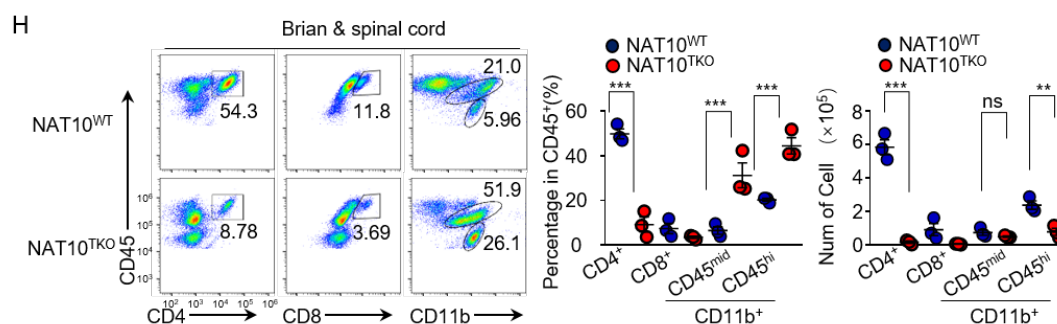


Figure 3 (H) On the 14th day after immunization, the brains and spinal cords of the immunized mice were collected and dissociated, and immunocytes infiltration was analyzed by FACS. The bar graphs were presented the percentage and absolute cell number of distinct immune cells subsets.

Figure 4:

T cells were isolated from WT and NAT10KO mice. What percentage of lymphocytes is obtained? If low levels of T lymphocytes are shown and apoptosis is present, what implications would these alterations have on T lymphocyte function?

Response: We appreciate the reviewer's inquiry. In our study, FACS was employed to isolate Naive CD4⁺ T cells for use in the T cell proliferation and differentiation assay, and to determine the proportion of purified naive CD4⁺ T cells. Our findings indicated that both NAT10^{WT} and NAT10^{TKO} mice-derived naive CD4⁺ T cells achieved a purity exceeding 98% following purification (**Figure only shown to the reviewers**). These data demonstrated that the Naive CD4⁺ T cells obtained are well-suited for the subsequent experimental purification processes.

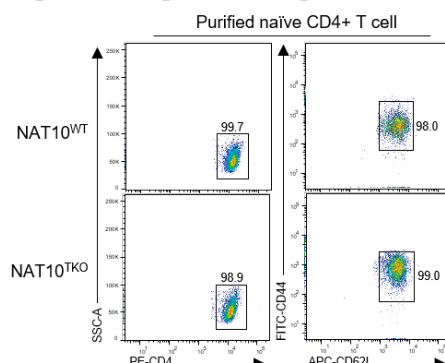


Figure only shown to the reviewers Naive CD4⁺ T cells were isolated from the spleen of NAT10^{WT} and NAT10^{TKO} mice using FACS Aria II. The purity of naive CD4⁺ T cells was verified by FACS.

Regarding the reviewer's inquiry about apoptosis in naive T cells, we conducted an analysis of apoptosis in naive CD4⁺ T cells isolated from NAT10^{WT} and NAT10^{TKO} mice. Our results indicated that, in comparison to the WT control, the absence of NAT10 in naive CD4⁺ T cells did not lead to a significant increase in apoptosis (**Figure only shown to the reviewers**). This observation might be attributed to the low expression levels of NAT10 in T cells. Thus, the apoptosis caused by NAT10 deficiency might not be involve in the activation of T cells at early stage.

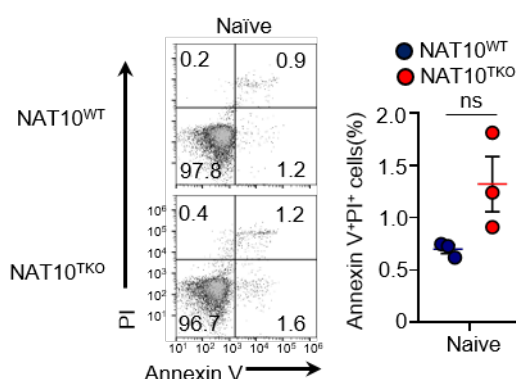


Figure only shown to the reviewers The apoptosis (Annexin V⁺PI⁺) of WT and NAT10-deficient naive CD4⁺ T cell was measured by FACS.

Based on the results shown, highlighting genes related to cell cycle arrest, it seems that NAT10 is important in the late stages of activation since the expression of CD69 (early activation antigen) is not modified (line 313).

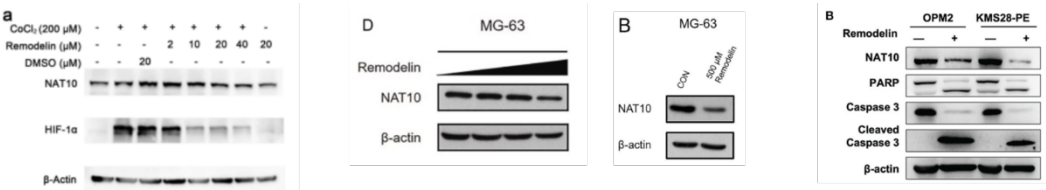
Response: We agree with the reviewer's conclusion that both the TCR-mediated pathway and the expression of CD69 indicate that NAT10 has not played its function in the early stage of T cell activation. However, in the analysis of the cell cycle, we found that the loss of NAT10 caused a significant decrease in the number of T cells entering the S phase during activation, indicating that

NAT10 played an important role in the early stage of the T cell cycle. Therefore, in this part, we defined the early stages as "early stages of cell cycle".

Figure 6:

The effect of remodelin showed a slight reduction in proliferation (Figure 6A) and cell cycle (Figure 6B), although there is a significant difference. Is it possible to include semi-quantitative data in Figure 6C? What stimulus was used to activate the cells in Figures 6B and 6C and supplementary Figures 6B and 6C? The interaction between NAT10 and RACK1, Figure 6G, and 6H was significant in its demonstration.

Response: This issue is of considerable importance. We concured with the reviewer's concerns that the effect of Remodelin was significantly less pronounced than the phenotype caused by NAT10 loss. Current research has revealed that the inhibitory effects of Remodelin depend on the functional activity of NAT10 during DNA replication (*Transl. Oncol.* 2020;13:100783). Furthermore, Remodelin has been shown to regulate the protein levels of NAT10. However, multiple studies have demonstrated that Remodelin only partially reduced the protein levels of NAT10, with an efficiency markedly lower than that observed with NAT10 deficiency as following. Consequently, we believed that the significant differences observed between these two groups were attributable to the relatively modest impact of Remodelin on NAT10.

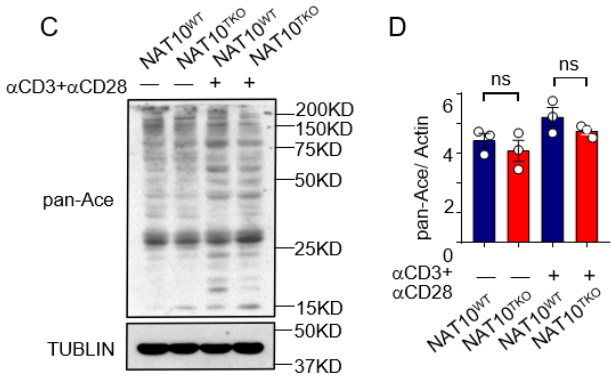


Mol Cell Biochem. 2020 Sep;472(1-2):19-31.

Heliyon. 2023 Jun 8;9(6):e17103

Front Oncol. 2022 Aug 1;12:967811.

We also included semi-quantitative data in **previous Figure 6C** as reviewer's suggestion. Furthermore, as recommended by the reviewer, we have clarified the stimulator (α CD3+ α CD28) in **Previous Figure 6**. The findings indicated that the whole acetylation level of the protein did not exhibit a significant difference in activated T cells between WT and NAT10 deficiency. Since NAT10 only participated in the acetylation of a small part of the protein, the change of total protein acetylation was not significant after the absence of NAT10. Consequently, we have decided to omit this portion of the results as it inadequately represented the relationship between NAT10 and its substrates. We hoped that the reviewer could understand our choice.



Previous Figure 6 (C-D) The pan-acetylation level of proteins in WT and NAT10-deficient CD4⁺ T cells were measured by IB (C). The statistical results of semi-quantitative pan-Ace to Actin were shown as bar graph (D).

The abstract should be more specific regarding the results, lines 457 to 459.

Response: As reviewer's suggestion, we modified the summary of the results in **Figure 7** and provided more specific conclusion.

Discussion:

Paragraphs from lines 462 to 504 can be revised; they are very unspecific regarding the topic addressed in the article. Although it involves NAT10 participating in migration (lines 542), the data shown do not evaluate this point.

Response: In accordance with the reviewer's recommendations, we have revised the discussion section and excised content that was deemed extraneous to the central focus of this manuscript.

Minor:

Line 184 phenptype, phenotype

Figure 3H Brian, brain

Figure 6D = DMSO? R = Remodelin?

What does the histogram of blue color represent? The one in red?

In Figure 7G, the letters are overlapping.

Figure 7 indicates which variable was evaluated on the ordinate.

Response: We thank the reviewer for his careful reading, and we corrected these typos as suggestion.

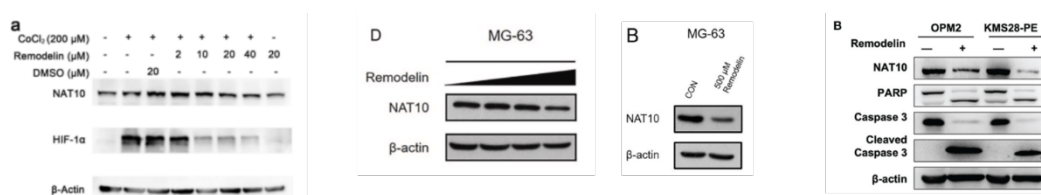
Reviewer #2 (Remarks to the Author):

This study by Li et al. investigates the role of NAT10, an acetyltransferase, in T cell development, activation and proliferation. It is suggested that deletion of NAT10 results in normal thymic development and T cell activation. However, proliferation is reduced in NAT10^{-/-} T cells. The authors then go on to suggest that this proliferation defect is due to a new function of NAT10 in protecting RACK1 from ubiquitin-mediated degradation. It is suggested that the K185 on RACK1 is a critical ubiquitination site that is acetylated by NAT10, thereby preventing ubiquitin-mediated degradation of RACK1. I have expertise in the molecular mechanisms of proliferation control. I have focused on the results shown in Fig. 6 and Fig. 7 of the study, as these are the most relevant to my technical expertise. I have the following major concerns over the data shown.

1. Can the authors explain why the NAT10 inhibitor Remodelin does not phenocopy NAT10 deletion?

Response: This issue is of considerable importance. We concurred with the reviewer's concerns that the effect of Remodelin was significantly less pronounced than the phenotype caused by NAT10 loss. Current research has revealed that the inhibitory effects of Remodelin depend on the functional activity of NAT10 during DNA replication (*Transl. Oncol.* 2020;13:100783). Furthermore, Remodelin has been shown to regulate the protein levels of NAT10. However, multiple studies have demonstrated that Remodelin only partially reduced the protein levels of NAT10, with an efficiency markedly lower than that observed with NAT10 deficiency as following. Consequently, we believed that the significant differences observed between these two groups were attributable to the relatively

modest impact of Remodelin on NAT10.



Mol Cell Biochem. 2020 Sep;472(1-2):19-31.

Heliyon. 2023 Jun 8;9(6):e17103

Front Oncol. 2022 Aug 1;12:967811.

2. In Fig. 7 and throughout the manuscript, I have concerns over the quality and the robustness of the immunoblot data shown. There must be quantitation for each immunoblot where the relevant bands are normalized to a loading control.

Response: We fully acknowledge the reviewer's concern regarding the IB data in **Figure 7**. In response, we replaced portions of the low-quality data and performed separate semi-quantitative and statistical analyses for each panel. The results of these analyses were provided in the supplementary materials corresponding to **Figure 7**.

3. The English vocabulary and grammar used in the Introduction section is too flowery and uses archaic words whose meaning in scientific writing is unclear.

Response: We revised the introduction section to enhance readability and omitted content that was not directly relevant to the focus of this study.

3. I do not believe that the study is publishable in its current form as the data in Fig. 7 do not substantiate the claim that NAT10 acetylation of RACK1 protects it from ubiquitin-mediated degradation. Furthermore, as far as I can tell, the only evidence shown that the mechanism of the proliferation defect observed in NAT10^{-/-} T cells is mediated via RACK1 is in Fig. 7L. Are the expression levels of WT and K185Q RACK1 comparable?

Response: We extend our gratitude to the reviewer for their insightful comments regarding the data presented in Figure 7. We concurred that the previously established link between NAT10 and RACK1 stability was tenuous and did not demonstrate that the acetylation of RACK1 by NAT10 protected it from ubiquitin-mediated degradation. To address this concern, we have supplemented this section with additional data, including:

1. **Identification of K185 as a critical ubiquitination site:** Utilizing various RACK1 mutant, we identified K185 as an important ubiquitination site on RACK1 (**Figure 7H** and **Supplementary Figure 7I**).
2. **Analysis of RACK1 acetylation in WT CD4⁺ T Cells:** We compared the acetylation status of RACK1 in WT CD4⁺ T cells before and after activation (**Table S3**). The results indicated the presence of multiple acetylation sites in both resting and activated T cells, with varying trends for each site. Notably, K185 was not acetylated in resting T cells but showed significant acetylation following 15 minutes of anti-CD3 and anti-CD28 stimulation (**Figure 7I**).
3. **In Vitro acetylation assay:** An in vitro acetylation assay confirmed that NAT10 could directly acetylate the K185 site on RACK1 (**Figure 7J** and **Supplementary Figure 7J**).
4. **Stability of acetylation mimic mutant RACK1 K185Q:** The acetylation mimic mutant RACK1 K185Q was found to be more stable than the WT control (**Figure 7K** and

Supplementary Figure 7K).

- Effect of NAT10 acetylase dead mutant:** We constructed a catalytically inactive mutant of NAT10 G641E and found that it was unable to induce RACK1 acetylation or inhibit its ubiquitination (**Figure 7L-M**).
- Functional analysis in RACK1 mutant-transfected NAT10-Deficient T Cells:** NAT10-deficient T cells overexpressing the K185Q RACK1 mutant exhibited enhanced proliferation, energy metabolism, and cytokine production (**Figure 7N-S**).

Collectively, these new data provided compelling evidence that NAT10 directly acetylated RACK1 at lysine 185, thereby inhibiting its ubiquitination and enhancing its stability. Furthermore, our findings highlighted the significance of RACK1 as a critical substrate of NAT10 in T cell activation.

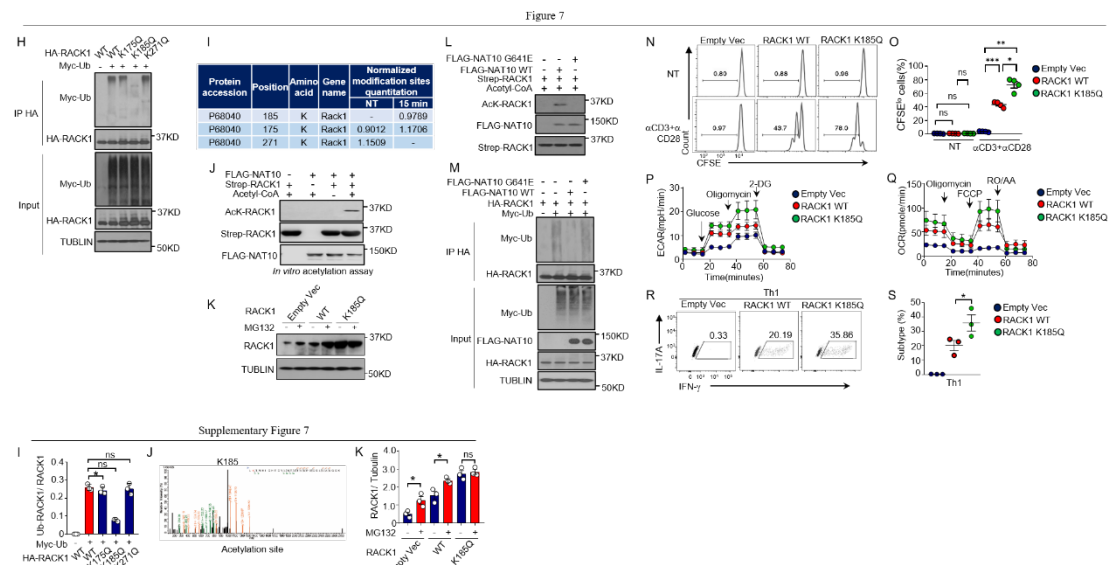


Figure 7 (H) HEK293T cells were transfected with Myc-tagged ubiquitin, indicated lysine-mutant RACK1 and NAT10 expression vectors, and subjected to ubiquitination assay 36h later. **(I)** K175, K185 and K271 ubiquitination of RACK1 in activated T cells identified by mass spectrometry. **(J)** The acetylation of Strep-RACK1 mediated by recombinant FLAG-NAT10 was measured by IB. **(K)** mCherry⁺ WT and RACK1 K185Q mutant-transfected NAT10-deficient CD4⁺ T cells isolated by FACS, and treated with 20 μ M MG132 for 2h. The protein level of WT and RACK1 K185Q mutant were detected by IB. **(L)** The acetylation of Strep-RACK1 mediated by recombinant WT FLAG-NAT10 and NAT10 G641E mutant was measured by IB. **(M)** HEK293T cells were transfected with Myc-tagged ubiquitin, HA-tagged RACK1 and FLAG-NAT10 G641 mutant and its WT control, and subjected to ubiquitination assay 36h later. **(N-O)** mCherry⁺ WT and RACK1 K185Q mutant-transfected naïve NAT10-deficient CD4⁺ T cells were labeled with CFSE, activated with anti-CD3/anti-CD28. The proliferation of these cells was measured with CFSE dilution by FACS and after 48h and presented as bar graph. **(P-Q)** ECAR and OCR of mCherry⁺ WT and RACK1 K185Q mutant-transfected naïve NAT10-deficient CD4⁺ T cells stimulated with anti-CD3 plus anti-CD28 for 18h were measured by Agilent Seahorse XF Analyzers. **(R-S)** mCherry⁺ WT and RACK1 K185Q mutant-transfected naïve NAT10-deficient CD4⁺ T cells were polarized under Th1 differentiation condition. The expression of IFN- γ were analyzed by FACS after 96h.

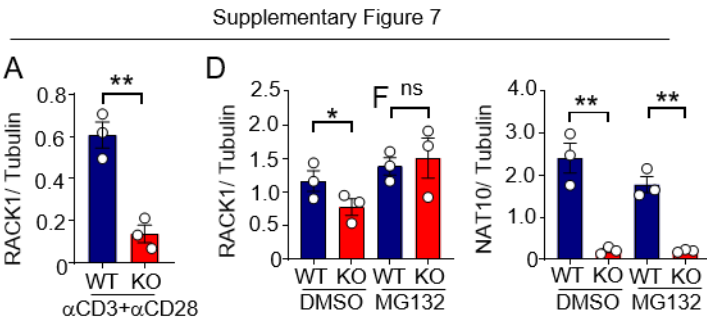
Supplementary Figure 7 (I) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7H. **(J)** NAT10-mediated K185 acetylation of RACK1 *in vitro* was

identified by mass spectrometry. (K) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7K.

Comments on Fig. 7

4. Fig. 7A-B, quantitation and triplicate analysis.

Response: In accordance with the reviewer's recommendations, we conducted a quantitative analysis of the IB data presented in **Figure 7A-B**. The statistical results of this analysis were provided in **Supplementary Figure 7A** and **Supplementary Figure 7D**.



Supplementary Figure 7 (A) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7A. **(D)** The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7B.

5. Fig. 7A band is very light – transfer issue? There is also a decrease in tubulin signal

Response: To address the reviewer's concerns, we presented new repeatability data. We aimed to ensure that the quality of the data meets the reviewer's requirements. Both semi-quantitative analysis and statistical data were provided as follows.

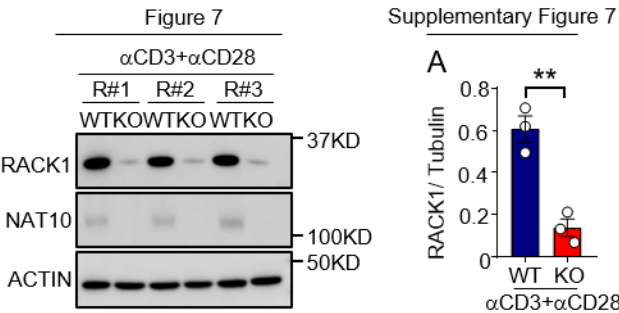


Figure 7 (A) Naïve CD4⁺ T cells isolated from NAT10^{WT} and NAT10^{TKO} mice were activated with αCD3 plus αCD28 for 24h. The protein abundance of RACK1 was measured by IB.

Supplementary Figure 7 (A) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7A.

6. Fig. 7B bands look 'burnt' from excessive peroxidase activity

Response: The reviewer's observation is accurate. In some bands, the signal intensity was excessively strong, resulting in a burnt appearance. We addressed this issue by selecting parallel films with different exposure intensities, thereby improving the quality of the new films compared to the initial results. Additionally, following the reviewer's suggestion, we conducted a semi-

quantitative analysis of the data in **Figure 7B**, which has rendered the conclusions more robust and credible.

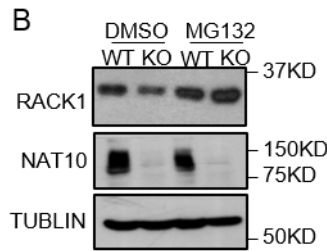


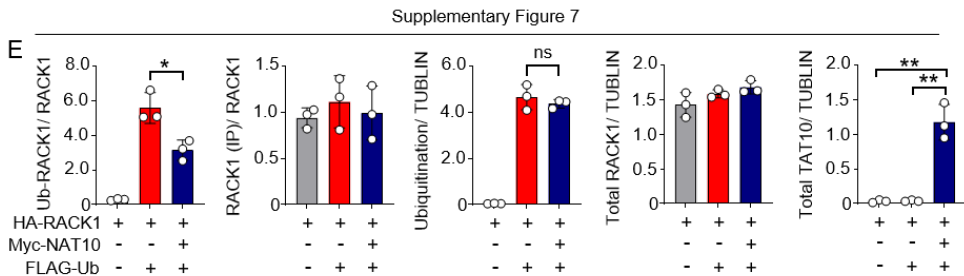
Figure 7 (B) WT and NAT10-deficient naïve CD4⁺ T cells were stimulated with anti-CD3/anti-CD28 for 22h, and treated with 20 μM MG132 for another 2h. The cell lysates were subjected to IB to measure protein abundance of RACK1.

7. Fig. 7C – middle lane suggests that FLAG-Ub was not transfected into cells, but the immunoblot shows FLAG-Ub signal. There must be a typo!

Response: We appreciate the reviewer's reminder. We corrected the labeling error of FLAG-Ub and Myc-NAT10 in the revised data.

8. Fig. 7C – it looks like ubiquitination is globally reduced, not just potentially RACK1.

Response: To address the reviewer's concerns, we repeated the experiments and generated new data, providing semi-quantitative analysis and statistical results from three replicate experiments. The results indicated that the decreased ubiquitination levels observed are specifically due to reduced ubiquitination of NAT10 and RACK1, rather than a general decline in overall ubiquitination levels.



Supplementary Figure 7 (E) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7C.

9. Fig. 7D – again there needs to be quantitation. Most ubiquitin variants appear affected by NAT10 overexpression, not just K48.

Response: We accepted the reviewer's suggestion and conducted a semi-quantitative analysis for **Figure 7D**. As noted by the reviewer, NAT10 significantly suppressed RACK1 polyubiquitination, encompassing K48-, K63-, K6-, K27-, and K29-linked subtypes (**Supplementary Figure 7F**). These findings suggested that the inhibition of RACK1 polyubiquitination by NAT10 was not specific to certain ubiquitin linkage types. To further elucidate the inhibitory effect of NAT10 on RACK1 K48-linked ubiquitination, naïve CD4⁺ T cells isolated from the spleens of WT and NAT10^{TKO} mice were activated with anti-CD3 and anti-CD28 antibodies and treated with MG132 before IB analysis. The levels of K48-linked polyubiquitination in immunoprecipitated RACK1 were then

measured. The results indicated that, in activated CD4⁺ T cells, the absence of NAT10 led to a significant increase in K48-linked polyubiquitination of RACK1 (**Figure 7E** and **Supplementary Figure 7G**). In light of these new results, we have also revised our conclusions.

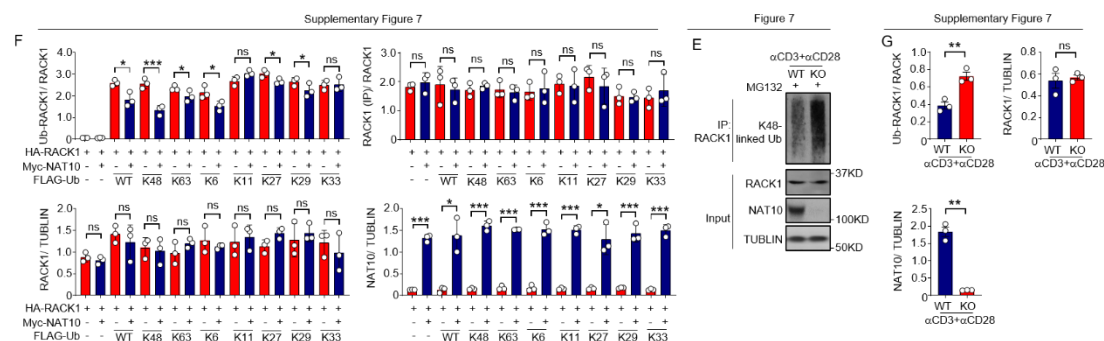


Figure 7 (E) IB analyses of α CD3+ α CD28-triggered K48-linked ubiquitination of RACK1 in WT and NAT10-deficient CD4⁺ T cells. RACK1 was isolated by IP and followed by IB detection of ubiquitin.

Supplementary Figure 7 (F) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7A. **(G)** The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7E.

10. Fig. 7E – K174 is written on the Figure, but K175 is written in the text (line 439). I also do not understand what the -NAT10 or +NAT10 means in the schematic diagram for RACK1. Please explain. Does this mean with or without NAT10 overexpression?

Response: We apologize for the error in Figure 7e. Specifically, the labeling of K175 was incorrect, and the correct representation should indeed be K175. We rectified this mistake. Regarding the reviewer's comment on the labeling as "-NAT10" or "+NAT10", the reviewer's interpretation was accurate; these labels indicated the absence or presence of NAT10 overexpression, respectively. We added the corresponding explanations in the figure legend for clarity.

11. Fig. 7G – again, quantitation. It looks like HA signal is decreased comparing lanes 2 and lanes 2 and 7, so the differences in Myc signal could easily be explained by loading.

Response: As noted by the reviewer, assessing the ubiquitination levels of RACK1 mutants has been challenging due to variations in their expression. We incorporated the reviewer's suggestion and performed semi-quantitative and statistical analyses on the data presented as following (**Figure only shown to reviewer**). Our findings revealed that ubiquitination levels were significantly reduced in RACK1 mutants K185R and K271R compared to the wild type, although the difference in K271R did not reach statistical significance (**Previous Figure 7H**). In addressing additional reviewer comments, we identified glutamine as a more suitable amino acid mutation. Consequently, we mutated each of the three lysine residues to glutamine. The new results showed that K185Q mutant was better than K185Q mutant in significantly reducing the ubiquitination level of RACK1 (**Figure 7H** and **Supplementary Figure 7I**). We replaced these new data to previous Figure 7H.

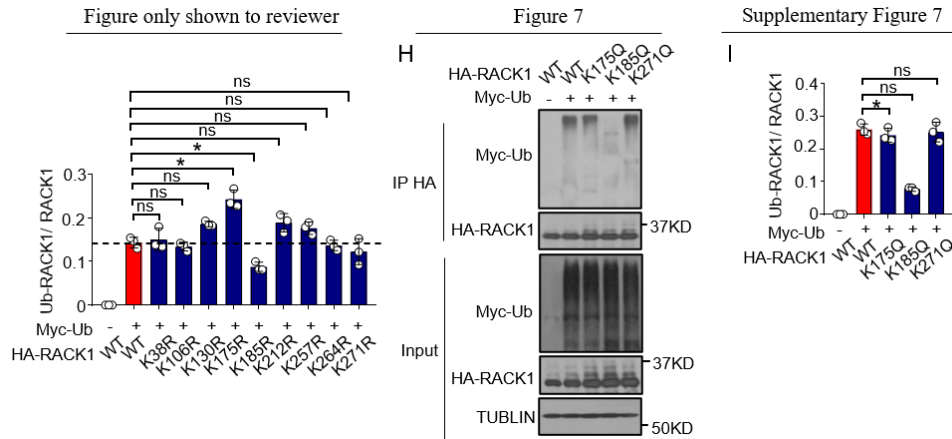


Figure only shown to reviewer The bar graph presented the semi-quantitative analysis results of the IB in the data of Previous Figure 7H.

Figure 7 (H) HEK293T cells were transfected with Myc-tagged ubiquitin, indicated lysine-mutant RACK1 and NAT10 expression vectors, and subjected to ubiquitination assay 36h later.

Supplementary Figure 7 (I) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7H.

12. *Fig. 7H – comparing lanes 2 and 5, you would expect to see a decrease in ubiquitin signal if K185 is a major site for ubiquitination. In contrast, the signal is as far as I can tell equal. Second, there is much less NAT10 overexpressed in the presence of the K185Q mutant (see FLAG signal comparing lanes 3 and 6). Therefore, the difference in ubiquitination might be due to differences in NAT10 expression.*

Response: We concur with the reviewer's perspective and acknowledge that the initial findings may not adequately capture the impact of the K185Q mutant on ubiquitination levels. To address these concerns comprehensively, we conducted a comparative analysis of ubiquitination levels among three RACK1 mutants, considering both acetylation and ubiquitination sites. Our findings revealed a significant reduction in ubiquitination specifically with the K185Q mutant (**Figure 7H** and **Supplementary Figure 7I**). This observation was consistently replicated in three independent experiments, supported by quantitative statistical analyses. Furthermore, we investigated the protein stability of the K185Q mutant, and found it to be markedly higher than that of the WT control, and was unaffected by proteasome inhibitors (**Figure 7K** and **Supplementary Figure 7K**). Additionally, we explored the impact of a NAT10 acetylase-activated dead mutant, G641E, on RACK1 ubiquitination. Our data indicated that NAT10 G641E failed to inhibit RACK1 ubiquitination to the extent observed with the WT NAT10 (**Figure 7M** and **Supplementary Figure 7N**). Together, these results demonstrated NAT10-mediated acetylation at K185 of RACK1 as a pivotal mechanism inhibiting ubiquitination at this site.

H

	HA-RACK1	WT	WT	K175Q	K185Q	K271Q
Myc-Ub	-	+	+	+	+	+
IP HA						
Input						

M

	FLAG-NAT10 G641E	-	-	-	+
FLAG-NAT10 WT	-	-	-	+	+
HA-RACK1	+	+	+	+	+
Myc-Ub	-	+	+	+	+
IP HA					
Input					

I

K

	Empty Vec	WT	K185Q
MG132	-	+	+
RACK1			
Empty Vec			
WT			
K185Q			

N

	FLAG-NAT10 G641E	-	-	+	+
FLAG-NAT10 WT	-	-	-	+	+
HA-RACK1	+	+	+	+	+
Myc-Ub	-	+	+	+	+

Supplementary Figure 7 (I) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7H. **(K)** The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7K. **(N)** The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7M.

NAT10, a N-acetyltransferase, has been recognized for its role in the acetylation of RNAs and proteins, with multiple mRNA and protein targets reported. This acetylation is vital for mRNA stability and translation, thus playing a critical role in cell proliferation and self-renewal. It is noteworthy that NAT10's necessary role in promoting cell cycle arrest, specifically in the context of p53, suggests its function in cell survival and differentiation may be contextually variable. . In addition, NAT10 seem to be broadly expressed in various cell type. Thus far, the specific effect in T cells have not been examined.

The strength and significance of the manuscript is its thorough investigation of NAT10's roles in T cell proliferation and activation, utilizing both in vitro and in vivo models to provide substantial evidence. In simple term, NAT10 is essential for T cell proliferation. The data presented is robust,

comprehensive, and well-articulated. An exciting mechanism the authors described is that NAT10 acetylates a few key lysine residues of RACK1 through interaction and these acetylation prevents ubiquitination and thus increase RACK1 stability. There remains some reservation regarding the specific mechanisms by which NAT10 influences T cells. This is surprising given the promiscuous nature of protein acetylation and essential roles of NAT10 in other contexts. It may not be essential to delineate an exact molecular mechanism within this study due to the broad influence of the factor under investigation, but strengthen supporting data would be necessary to make a compelling argument for the proposed mechanisms.

Detailed comments are as follows:

1. The manuscript would benefit from a more streamlined introduction and discussion. Some sections diverge into topics not directly related to NAT10, which could be refined to enhance clarity and logical progression. While expressive language can aid the narrative, it is crucial that it remains well-organized and delivers clear and precise scientific information.

Response: In accordance with the reviewer's recommendations, we revised the introduction section to enhance readability and omitted content that was not directly relevant to the focus of this study.

2. The representation of gene expression in Figure 1F using dot plots could potentially be misleading for representing each single-cell population. It highlights the cells expressing NAT10. Since only ~5% T cells exhibit the gene expression in question, alternative visualization techniques such as violin plots could offer a more holistic and less biased presentation of the expression variations across single-cell populations.

Response: We fully agreed with the reviewer's suggestion and have employed violin plots to illustrate NAT10 expression across different cell subsets. The results demonstrated that NAT10 was predominantly expressed in cycling T cells, albeit in a relatively small proportion, as noted by the reviewer. We believed this small proportion of NAT10⁺ cycling T cells was related to the heterogeneity of the cell cycle phases within the cycling T cell population.

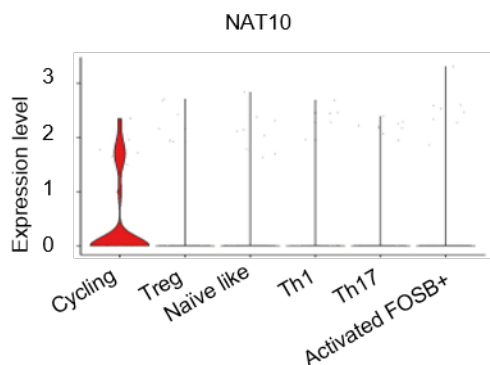


Figure 1 (F) Expression features of *NAT10* gene in different clusters were showed as violin plots.

3. In several places in the manuscript, there appears to be discrepancies between the text and figures. This warrants a careful review to ensure that the figure and text are in concordance. Figure 2D is the opposite from “we have also observed a significant suppression of T cell homeostasis, evidenced by a diminished capacity to produce cytokines under steady-state conditions”

Response: We apologize for any inaccuracies in our initial data presentation. Specifically, our

intention was to demonstrate that NAT10-deficient T cells exhibited an autoactivated phenotype while maintaining an overall immunosuppressive state due to a significant reduction in cell number. To enhance the clarity and comprehensibility of our findings for both the reviewer and readers, we revised the relevant section as follows. “Although NAT10 impacted the development of T cells, we observed a significant autoactivation of peripheral T cell homeostasis, evidenced by an upregulated capacity to produce cytokines under steady-state conditions (Figure 2D). Indeed, numerous early studies have demonstrated that upon arrest of T cell development, T cells exhibited an autoactivation phenotype characterized by an increase in effector memory (EM) and central memory (CM) cells, as well as upregulation of molecules such as CD69 and CD44.”

4. Figure 7C. The samples may be mis-labeled.

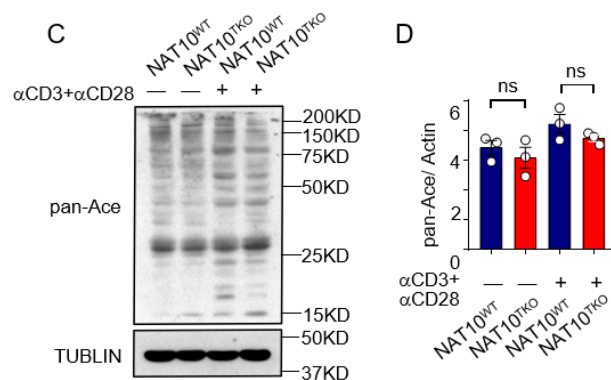
Response: We appreciate the reviewer's reminder. We corrected the labeling error of FLAG-Ub and Myc-NAT10 in the revised data

5. Figure 7D. the label is a bit hard to understand. What are the K48, K63, K6, K11, K27, K29 and K33 in “FLAG-Ub”. How are they being identified here? I cannot find description in text or methods about this.

Response: First and all, we would like to express our gratitude to the reviewer for their insightful comments. We apologized for the initial lack of clarity in the description of our experimental results. To facilitate a better understanding of our findings by both readers and reviewers, we added the following statement to the main text. “Polyubiquitination refers to the attachment of multiple ubiquitin molecules to a substrate protein, forming a ubiquitin chain. There are several types of polyubiquitination, determined by which lysine residue of the ubiquitin molecule is used for chain formation. As the most well-known type, K48-linked polyubiquitination targets proteins for degradation by the proteasome. K63-linked polyubiquitination is involved in non-degradative processes such as DNA repair, signal transduction, and endocytosis. K6-, K27-, and K29-linked polyubiquitination are less well-characterized but are known to be involved in various cellular processes, including DNA repair, mitochondrial maintenance, and regulation of signaling pathways. K11-linked polyubiquitination is associated with cell cycle regulation and protein degradation through the endoplasmic reticulum-associated degradation (ERAD) pathway.”

6. Figure 6, particularly 6C, presents data that may not show a significant difference. Comparisons with figure S6 suggest the acetylated proteins in NAT10 TKO that could correspond to the activation-induced acetylation observed (~40KD) – stronger in activated T cells and TKO.

Response: This is an excellent question. To address the reviewer's concern, we included semi-quantitative data in **Previous Figure 6C**. The findings indicated that the whole acetylation level of the protein did not exhibit a significant difference in activated T cells between WT and NAT10 deficiency. Since NAT10 only participated in the acetylation of a small part of the protein, the change of total protein acetylation was not significant after the absence of NAT10. Consequently, we decided to omit this portion of the results as it inadequately represented the relationship between NAT10 and its substrates. We hoped that the reviewer could understand our choice.



Previous Figure 6 (C-D) The pan-acetylation level of proteins in WT and NAT10-deficient CD4⁺ T cells were measured by IB (C). The statistical results of semi-quantitative pan-Ace to Actin are shown as bar graph (D).

7. The interaction between NAT10 and RACK1 is highlighted as a significant finding in the paper and is used to explain functions of NAT10 in T cells. However, the supporting data are not conclusive. Figure 6D do not specifically address whether these overlap acetylated protein candidates are dependent on T cell activation, or NAT10 at all. It would be necessary for the authors to demonstrate the differences in acetylated proteins between WT and NAT10 KO T cells (particularly RACK1) and acetylated proteins between naïve and activated T cells (particularly RACK1), to substantiate the implied association between NAT10 and RACK1 acetylation.

Response: We fully concur with the reviewer's opinion. Indeed, our previous data did not entirely support the hypothesis that NAT10 directly acetylates RACK1. Initially, our research focused on identifying proteins bound to NAT10 that were acetylated during T cell activation as potential substrates of NAT10. The reviewer suggested comparing the protein acetylation profiles between WT and NAT10 KO T cells. However, due to the significant reduction of peripheral T cells in NAT10 TKO mice, we were unable to obtain sufficient cell numbers for acetylomics analysis. Despite this limitation, we analyzed the potential acetylation changes at each lysine site of RACK1 in WT CD4⁺ T cells before and after activation (Table S3). The results indicated that RACK1 displayed a series of acetylation sites in both resting and activated T cells, although the acetylation patterns varied. Specifically, K185 was acetylated in activated T cells but was not detected in resting T cells (Figure 7I). To further investigate whether NAT10 could directly acetylate RACK1 at K185, we conducted in vitro acetylation assays. In these assays, which included only NAT10 and RACK1 recombinant proteins, we observed acetylation of RACK1, indicating that NAT10 directly acetylated RACK1 (Figure 7J). Mass spectrometry analysis confirmed that this acetylation occurred at position K185 (Supplementary Figure 7J). These new data supported our previous conclusion.

Figure 7

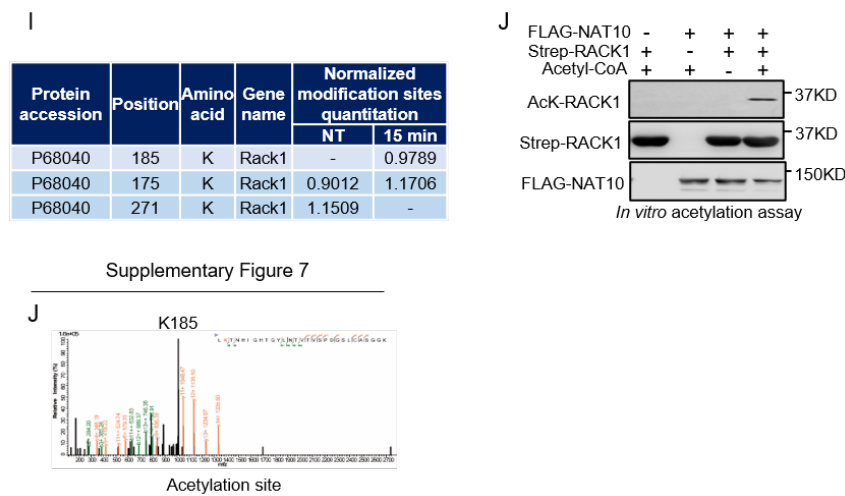
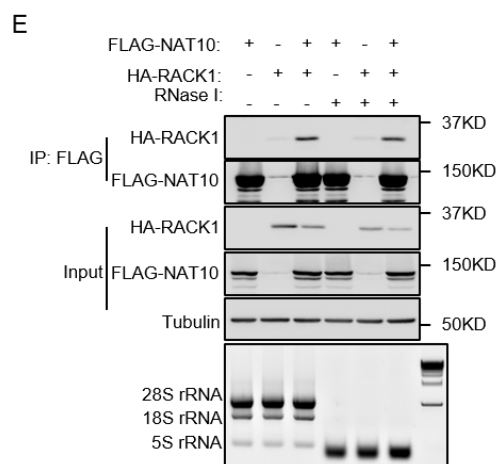


Figure 7 (I) K175, K185 and K271 ubiquitination of RACK1 in activated T cells identified by mass spectrometry. **(J)** The acetylation of Strep-RACK1 mediated by recombinant FLAG-NAT10 was measured by IB.

Supplementary Figure 7 (J) NAT10-mediated K185 acetylation of RACK1 in vitro was identified by mass spectrometry.

8. *With respect to the co-immunoprecipitation experiments involving NAT10 and RACK1, the possibility of an indirect interaction through a mutual binding partner such as RNA should be considered. It would be informative to assess whether the interactions observed are sensitive to RNase treatment.*

Response: This is an excellent question. As an enzyme that possesses both RNA acetyltransferase, it is possible that NAT10 binding to RACK1 is dependent of its RNA-binding activity. We therefore pretreated these samples with RNase for co-immunoprecipitation experiments. The results indicated that, although the levels of 28S, 18S, and 5S rRNA were reduced, the interaction between NAT10 and RACK1 remained unaffected. This finding suggested that the association between NAT10 and RACK1 is independent of the association of NAT10 with RNA (**Supplementary Figure 6E**).



Supplementary Figure 6 (E) HA-RACK1 and FLAG-NAT10 were co-expressed in HEK293T cells, followed by immunoprecipitation of HA-RACK1. The presence of immunoprecipitated

NAT10 was analyzed using an anti-FLAG antibody. To assess the potential role of RNA in the interaction between RACK1 and NAT10, 20 units of RNase I were added to the cell lysate during immunoprecipitation. The efficiency of RNA digestion was confirmed by agarose gel electrophoresis (bottom panel).

9. Figure 7D, In any comparisons, the difference between WT and KO are not obvious. The authors should add quantification among biological replicates to support their statement in the text that any of these ubiquitinations are or are not different.

Response: We concur with the reviewer's concerns. It is important to note that the comparison here focuses on the effect of NAT10 on various ubiquitination isoforms of RACK1 in HEK293T cells under conditions of overexpression versus non-overexpression. Given that HEK293T cell proliferation naturally results in high expression of NAT10, we believed that the observed slight decrease in RACK1 polyubiquitin modification upon NAT10 transfection was due to adequate expression of NAT10 non-transfected condition. Additionally, we incorporated the reviewer's suggestion and performed a semi-quantitative comparison and statistical analysis of the immunoblotting data presented in **Figure 7D**. Based on statistical results, NAT10 significantly suppressed RACK1 polyubiquitination, encompassing K48-, K63-, K6-, K27-, and K29-linked subtypes (**Supplementary Figure 7F**). These findings suggested that the inhibition of RACK1 polyubiquitination by NAT10 was not specific to certain ubiquitin linkage types.

To further elucidate the inhibitory effect of NAT10 on RACK1 K48-linked ubiquitination, naive CD4⁺ T cells isolated from the spleens of WT and NAT10^{TKO} mice were activated with anti-CD3 and anti-CD28 antibodies and treated with MG132 before IB analysis. The levels of K48-linked polyubiquitination in immunoprecipitated RACK1 were then measured. Different from the mild change under overexpression condition in HEK293T, the absence of NAT10 led to a significant increase in K48-linked polyubiquitination of RACK1 (**Figure 7E** and **Supplementary Figure 7G**). Based on these new results, we have also revised our conclusions.

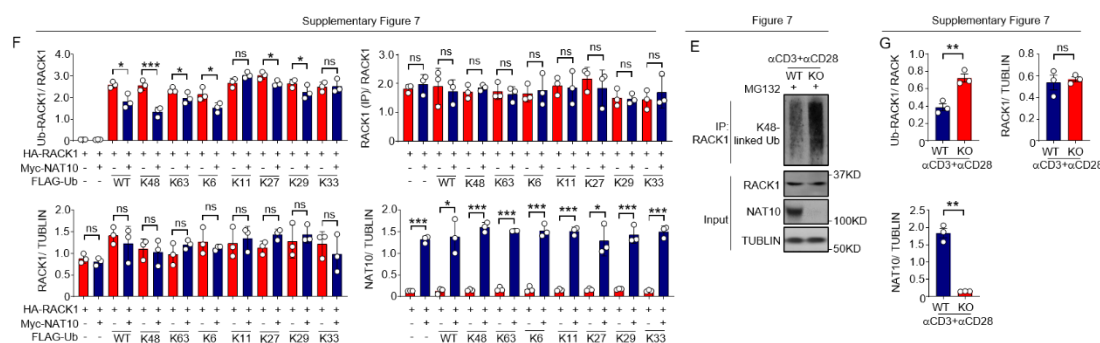


Figure 7 (E) IB analyses of α CD3+ α CD28-triggered K48-linked ubiquitination of RACK1 in WT and NAT10-deficient CD4⁺ T cells. RACK1 was isolated by IP and followed by IB detection of ubiquitin.

Supplementary Figure 7 (F) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7A. **(G)** The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7E.

10. The authors propose that NAT10 acetylates RACK1 at various lysine residues, thereby preventing ubiquitination. To bolster this claim, it would be necessary to show direct evidence of

such NAT10-dependent acetylations and its impact on RACK1 stability.

Response: We thank the reviewer's nice question. In order to address this question, we first demonstrated that NAT10 could directly acetylate RACK1 at lysine 185 (**Figure 7H** and **Supplementary Figure 7I**). An *in vitro* acetylation assay confirmed that NAT10 directly acetylated the K185 site on RACK1 (**Figure 7J** and **Supplementary Figure 7J**). The acetylation-mimic mutant RACK1 K185Q exhibited enhanced protein stability compared to its WT counterpart, which was regulated by proteasome (**Figure 7K** and **Supplementary Figure 7K**). We further constructed a catalytically inactive mutant of NAT10 G641E and found that it was unable to induce RACK1 acetylation or inhibit its ubiquitination (**Figure 7L-M**). Collectively, these new data provided compelling evidence that NAT10 directly acetylated RACK1 at lysine 185, thereby inhibiting its ubiquitination and enhancing its stability.

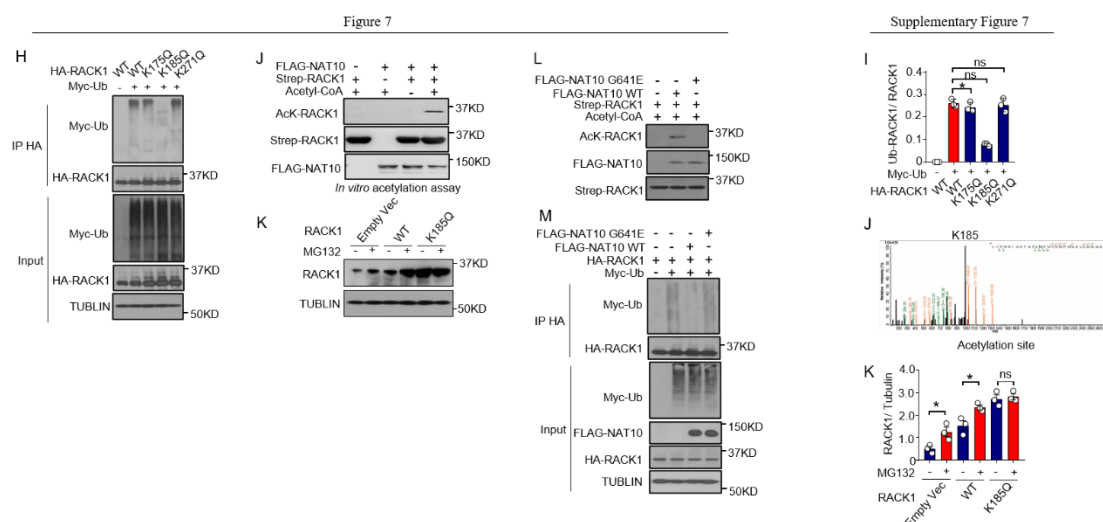


Figure 7 (H) HEK293T cells were transfected with Myc-tagged ubiquitin, indicated lysine-mutant RACK1 and NAT10 expression vectors, and subjected to ubiquitination assay 36h later. **(J)** The acetylation of Strep-RACK1 mediated by recombinant FLAG-NAT10 was measured by IB. **(K)** mCherry⁺ WT and RACK1 K185Q mutant-transfected NAT10-deficient CD4⁺ T cells isolated by FACS, and treated with 20 μ M MG132 for 2h. The protein level of WT and RACK1 K185Q mutant were detected by IB. **(L)** The acetylation of Strep-RACK1 mediated by recombinant WT FLAG-NAT10 and NAT10 G641E mutant was measured by IB. **(M)** HEK293T cells were transfected with Myc-tagged ubiquitin, HA-tagged RACK1 and FLAG-NAT10 G641 mutant and its WT control, and subjected to ubiquitination assay 36h later.

Supplementary Figure 7 (I) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7H. **(J)** NAT10-mediated K185 acetylation of RACK1 *in vitro* was identified by mass spectrometry. **(K)** The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7K.

11. In Figure 7K, the observed differences in RACK1 between WT and K185Q mutants may arise from factors other than protein stability. At least, the inclusion of M132 inhibitors in experimental assays should be considered.

Response: We appreciate the Reviewer for proposing an important suggestion. In response to this concern, we compared the protein levels of different RACK1 mutants in NAT10-deficient CD4⁺ T cells before and after MG132 treatment. The results showed that under the condition of constant

transfection efficiency and mRNA level (**Supplementary Figure 7L-M**), the protein of WT RACK1 was significantly restored after inhibiting proteasome activity, while the protein level of RACK1 K185Q mutant was significantly increased in activated T cells compared with that of the WT control (**Figure 7K**). Furthermore, upon MG132 treatment, the protein levels of the RACK1 K185Q mutant were not elevated anymore (**Figure 7K**), indicating that the K185Q mutation obstructed the degradation pathway of RACK1 in activated T cells.

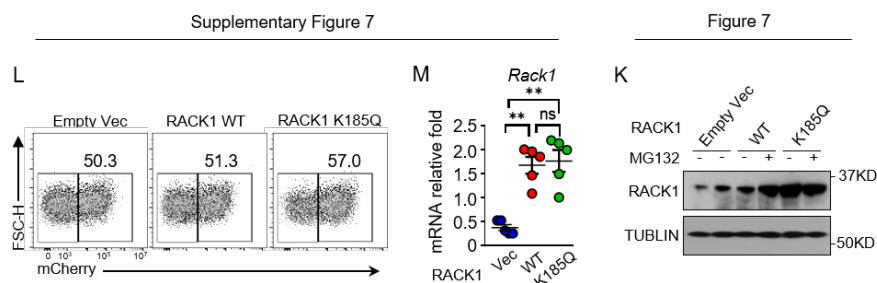


Figure 7 (K) mCherry⁺ WT and RACK1 K185Q mutant-transfected NAT10-deficient CD4⁺ T cells isolated by FACS, and treated with 20 μ M MG132 for 2h. The protein level of WT and RACK1 K185Q mutant were detected by IB.

Supplementary Figure 7 (L) Naïve NAT10-deficient CD4⁺ T cells were infected with retrovirus carrying WT or K185Q mutant RACK1-mCherry. The percentage of infected cells was detected and isolated based on mCherry by FACS.

(M) The mRNA level of RACK1 in mCherry⁺ CD4⁺ T cells isolated as above were detected by RT-qPCR. These qPCR data are presented as fold change relative to the *Actb* mRNA level and normalized by Bio-Rad CFX Manager 3.1.

12. The authors postulates RACK1 acetylation as a key effector of NAT10 in T cell homeostasis. It would be compelling to determine if the RACK1 K185Q mutation can rectify the deficiencies in NAT10-deficient T cell functions, including metabolic activity (Figure 5E, F) and cytokine production (Figure 4C), thereby strengthening the manuscript's conclusions.

Response: Based on the insightful recommendation from the reviewer, we compared the energy metabolism and functional cytokine production levels of T cells transfected with either RACK1 WT or the acetylation-mimic mutant RACK1 K185Q. The result demonstrated that NAT10-deficient T cells overexpressing the K185Q RACK1 mutant exhibited enhanced glycolysis and FAO metabolism, and cytokine production (**Figure 7N-S**).

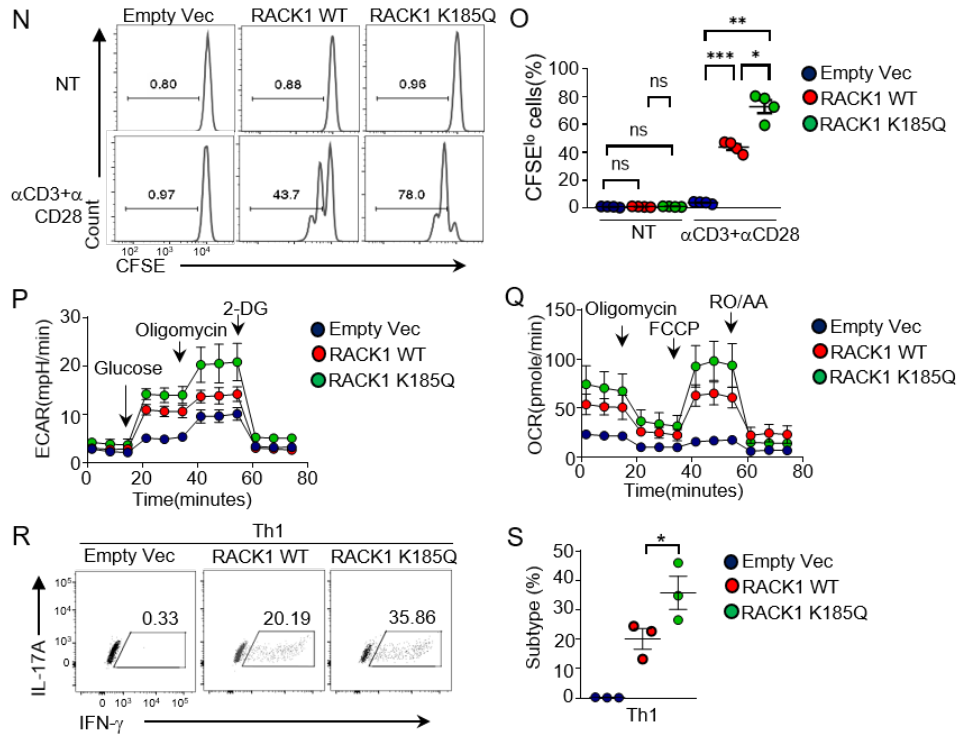


Figure 7 (N-O) mCherry⁺ WT and RACK1 K185Q mutant-transfected naïve NAT10-deficient CD4⁺ T cells were labeled with CFSE, activated with anti-CD3/anti-CD28. The proliferation of these cells was measured with CFSE dilution by FACS and after 48h and presented as bar graph. **(P-Q)** ECAR and OCR of mCherry⁺ WT and RACK1 K185Q mutant-transfected naïve NAT10-deficient CD4⁺ T cells stimulated with anti-CD3 plus anti-CD28 for 18h were measured by Agilent Seahorse XF Analyzers. **(R-S)** mCherry⁺ WT and RACK1 K185Q mutant-transfected naïve NAT10-deficient CD4⁺ T cells were polarized under Th1 differentiation condition. The expression of IFN- γ were analyzed by FACS after 96h.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors responded to each observation and suggestion and supported their conclusions.

Reviewer #2 (Remarks to the Author):

The authors have addressed my primary concerns with the experimental data. However, the authors should be careful in proof reading the manuscript as the abstract mentions "RCAK1" instead of "RACK1". I believe it is the latter that is correct.

Reviewer #3 (Remarks to the Author):

The authors have addressed most of my concerns. In particular, I do appreciate the addition of functional rescue with RACK1 mutants. The lingering issue is whether NAT10 acetylates RACK1 functionally in T cells. It is understandable this question is harder to address given the technical and biological challenges described by the authors.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors responded to each observation and suggestion and supported their conclusions.

Response: We thank the reviewer's recommendation.

Reviewer #2 (Remarks to the Author):

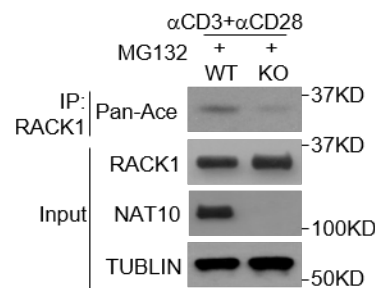
The authors have addressed my primary concerns with the experimental data. However, the authors should be careful in proof reading the manuscript as the abstract mentions "RCAK1" instead of "RACK1". I believe it is the latter that is correct.

Response: We thank the reviewer's nice remind, and corrected this typo.

Reviewer #3 (Remarks to the Author):

The authors have addressed most of my concerns. In particular, I do appreciate the addition of functional rescue with RACK1 mutants. The lingering issue is whether NAT10 acetylates RACK1 functionally in T cells. It is understandable this question is harder to address given the technical and biological challenges described by the authors.

Response: This is an excellent question. To address the reviewer's concern, we compared the acetylation level of RACK1 between WT and NAT10-deficient CD4⁺ T cells. The results indicated that NAT10 was partially required for acetylation level of RACK1 during CD4⁺ T cells activation.



Supplementary Figure 7 (c) IB analyses of α CD3 + α CD28-triggered pan-acetylation of RACK1 in WT and NAT10-deficient CD4⁺ T cells. RACK1 was isolated by IP and followed by IB detection of pan-acetylation.