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Article

N^6-methyladenosine promotes TNF mRNA degradation in $CD4^+$ T lymphocytes

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Abstract

N^6-methyladenosine (m^6A) is a RNA modification that can regulate post-transcriptional processes including RNA stability, translation, splicing, and nuclear export. In $CD4^+$ lymphocytes, m^6A modifications have been demonstrated to play a role in early differentiation processes. The role of m^6A in $CD4^+$ T cell activation and effector function remains incompletely understood. To assess the role of m^6A in $CD4^+$ T lymphocyte activation and function, we assessed the transcriptome-wide m^6A landscape of human primary $CD4^+$ T cells by methylated RNA immunoprecipitation sequencing. Stimulation of the T cells impacted the m^6A pattern of hundreds of transcripts including tumor necrosis factor (*TNF*). m^6A methylation was increased on *TNF* messenger RNA (mRNA) after activation, predominantly in the 3′ untranslated region of the transcript. Manipulation of m^6A levels in primary human T cells, the directly affected the expression of TNF. Furthermore, we identified that the m^6A reader protein YTHDF2 binds m^6A-methylated *TNF* mRNA, and promotes its degradation. Taken together, this study demonstrates that TNF expression in $CD4^+$ T lymphocytes is regulated via m^6A and YTHDF2, thereby providing novel insight into the regulation of T cell effector functions.

Lay Summary

T helper cells are immune cells of the adaptive immune system. These cells are activated by antigen presenting cells that have engulfed invading pathogens. When the T helper cell is activated, it will produce and excrete signaling molecules (cytokines) that activate other immune cells in order to eradicate these pathogens. Cytokines are formed after translation of RNA molecules that encode for these cytokines. In this study it was found that a modification (N^6-methyladenosine) on RNA molecules is involved in the regulation of the life cycle of these RNA molecules. It was found that the degradation of RNA encoding for cytokine tumor necrosis factor (TNF) was mediated through N^6-methyladenosine and its "reader" protein YTHDF2 in activated T helper cells. As TNF promotes inflammation, reduction of TNF production through this mechanism dampens the immune response and therefore prevents chronic inflammation.

Keywords: adaptive immunity, epitranscriptomics, RNA methylation, t cell activation, tumor necrosis factor

1. Introduction

Post-transcriptional regulation, e.g. by RNA-binding proteins or RNA modifications, is a precise and complex mechanism by which both RNA and ultimately protein expression can be regulated. N^6-methyladenosine (m^6A) is the most abundant messenger RNA (mRNA) modification in eukaryotes, and is highly conserved among species.[1,2] This post-transcriptional modification was first described in the 1970s, but recent technological and methodological advancements to identify and manipulate m^6A have boosted m^6A research.[3,4] m^6A is a dynamic modification added to the RNA by a "writer" complex consisting of the main components METTL3,[5,6] METTL14, and WTAP[6] during pre-mRNA processing. The methyl groups are installed on specific "DRACH'-mRNA sequences (D = A, G, or U; R = G or A; H = A, C, or U). m^6A can be removed from the RNA by "eraser" proteins (demethylases) fat mass and obesity-associated protein (FTO),[7] or ALKBH5.[8] Specific m^6A-binding proteins called "readers" determine the fate of methylated RNAs. m^6A-binding proteins from the YTH family were found to regulate RNA stability (YTHDF1, YTHDF2, and YTHDF3),[9] splicing (YTHDC1),[10] and nuclear export of mRNA transcripts (YTHDC1).[11]

m^6A methylation has been demonstrated to regulate the function of different cell types including immune cells.[12–25] This modification has been demonstrated to be involved in early $CD4^+$ T lymphocyte differentiation. *Mettl3*$^{lox/lox}$ CD4-Cre mice displayed impaired naïve T lymphocyte proliferation and differentiation as a result of increased expression of *Socs1*, *Socs3*, and *Cish*, thereby suppressing interleukin (IL)-7/STAT5 signaling and enhancing ERK and AKT signaling.[20] Yao et al.[21] showed that METTL3 stabilizes *Tcf7* mRNA transcripts in T cells via m^6A modification. *Tcf7* is a cell signature gene of T follicular helper (T_{FH}) cell function and differentiation; therefore, METTL3 can promote T_{FH} differentiation and function. m^6A demethylase ALKBH5 was found to have a checkpoint role in the early differentiation of both γδ T cells and

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αβ T cells.[22] Here, ALKBH5 was found to modulate Jagged1/Notch2 signaling. Strong signaling of this pathway drives the double-negative lymphocytes toward the αβ lineage, while reduced signaling results in γδ T cells. There is an increased understanding of the function of m^6A in $CD4^+$ T lymphocyte differentiation; however, the implications of m^6A in $CD4^+$ T cell activation and effector functions remain incompletely understood.

Here, we sought to gain insight in the role of m^6A in $CD4^+$ T lymphocyte activation. We demonstrate that the expression of tumor necrosis factor (TNF), a key cytokine expressed in activated T cells, is regulated through m^6A modification. m^6A enrichment was found on the 3′ untranslated region (UTR) of *TNF* mRNA transcripts, and the m^6A reader protein YTHDF2 was shown to associate with m^6A methylated *TNF* transcripts. Manipulation of m^6A levels resulted in altered TNF expression levels. Taken together, these data demonstrate that TNF expression in stimulated $CD4^+$ T lymphocytes is regulated via m^6A and its regulatory proteins, providing novel implications of m^6A in T cell function.

2. Methods

2.1 Cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donor blood by using Ficoll-Paque Plus (Cytiva) density gradient media. $CD4^+$ T lymphocytes were isolated by magnetic-activated cell sorting using the human $CD4^+$ T cell isolation kit (Miltenyi Biotec). Cells were cultured in RPMI 1640 medium (Fisher Scientific) containing 10% heat-inactivated human AB serum, 1% L-glutamine (Life Technologies), and 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco) supplemented with 50 U/mL IL-2. Jurkat cells (Clone E6.1; ATCC) were cultured in Dulbecco's Modified Eagle Medium with GlutaMax (Gibco), supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich) and 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). All cells were cultured at 37 °C in 5% CO_2. For transient overexpression, cells were transfected with 1.5 µg DNA using polyethylenimine MAX (Polysciences Europe GmbH). After 18 h, cells were washed and cultured for 24 h in fresh medium. For the manipulation of FTO and METTL3, 50 µM entacapone (Sigma-Aldrich; SML0654-10MG) and 30 µM STM2457 (MedChemExpress; 2499663-01-1) were used. For CRISPR/Cas9 genome engineering, single guide RNAs were introduced in the lentiviral pSicoR-CRISPR-PuroR vector (RP-557) as described previously.[26] The CRISPR RNA sequence used for METTL3 knockout was gAGCTGGACTCTCTGCGGGAG. CRISPR/Cas9 knockout of *METTL3* in Jurkat cells was performed via lentiviral transduction. Second-generation lentiviral particles were produced in HEK 293T cells using polyethylenimine MAX, and Jurkat cells were transduced using 5 µg/mL polybrene (Santa Cruz Biotechnology). Transduced cells were single cell cultured and selected with 2 µg/mL puromycin (Merck).

2.2 Methylated RNA immunoprecipitation sequencing and quantitative polymerase chain reaction

$CD4^+$ T lymphocytes were isolated from healthy donor PBMCs by using magnetic-activated cell sorting. The cells were either activated for 4 h with PMA (20 ng/mL; Sigma-Aldrich) and ionomycin (1 ug/mL; Calbiochem) or stimulated overnight with CD3/CD28 Dynabeads (Invitrogen). Cells were lysed in TRIzol LS reagent (Fisher Scientific), and RNA was isolated according to the manufacturer's protocol. For methylated RNA immunoprecipitation sequencing (meRIP-seq) only, mRNA was isolated and subsequently fragmented by using NEXTflex Rapid directional mRNA sequencing bundle (5138-10). Subsequently, Protein A/G magnetic beads (Fisher Scientific) were coated with mouse anti-m^6A (Synaptic Systems GmBH; 202-111) or mouse anti-IgG2b, kappa isotype control (BD; 556577). mRNA was added to the coated beads and immunoprecipitation was performed. mRNA was washed by using a high salt/low salt washing method. To elute the bound RNA, the beads were mixed with RLT lysis buffer from the RNeasy Mini Kit (Qiagen). This kit was subsequently used for purification of the mRNA. meRIP-seq samples were supplemented with sequences from the EpiMark N6-Methyladenosine Enrichment kit (New England Biolabs) and sequenced 50 bp single-end on Illumina NextSeq500 sequencer (Utrecht DNA Sequencing Facility). Sequencing reads were aligned using STAR (version 2.7.1)[27] to the human genome (version GRCh38) with transcriptomic information from ENSEMBL (version GRCh38.v100) supplemented with sequences from the EpiMark N6-Methyladenosine Enrichment kit. Unique reads were selected and expression was quantified at the gene level using htseq-count (version 0.11.4).[28] Per-sample expression was normalized using TMM normalization.[29] Complementary DNA synthesis was performed on the meRIP quantitative polymerase chain reaction (qPCR) samples using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed with SYBR Select Master Mix (Life Technologies) in a QuantStudio 12 K Flex (Thermo Fisher Scientific) according to the manufacturer's protocol. A list of primers used in this study can be found in Supplementary Table 1.

2.3 Flow cytometry

Cells were washed in fluorescence-activated cell sorting buffer (phosphate-buffered saline with 2% fetal bovine serum, 0.1% NaN_3), fixated and permeabilized with Fixation/Permeabilization Solution Kit (BD), and stained with antibodies (Supplementary Table 2). Measurements were performed using the BD FACSCanto II flow cytometer, and FlowJo v10 (TreeStar) was used for data analysis.

2.4 Western blot

Cells were lysed in Laemmli buffer (0.12 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 0.05 µg/µL bromophenol blue, 35 mM β-mercaptoethanol). Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel and transferred to a polyvinylidene difluoride membrane (Merck). After blocking with 5% milk powder in 1% Tris-buffered saline and Tween, the membrane was probed with antibodies indicated in Supplementary Table 3 and analyzed using enhanced chemiluminescence (Thermo Fisher Scientific).

2.5 Statistics

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software). The statistical tests used to test significance are specified in the figure legends.

3. Results

3.1 Stimulation of $CD4^+$ T lymphocytes alters transcriptome-wide m^6A methylation pattern

To assess how T cell activation affects the transcriptome-wide m^6A landscape in human $CD4^+$ lymphocytes, meRIP-seq was performed. Stimulation of $CD4^+$ T lymphocytes altered the m^6A pattern of hundreds of transcripts. Gene Ontology term analysis of the transcripts for which the m^6A signal was increased the most by activation revealed that these genes are associated with

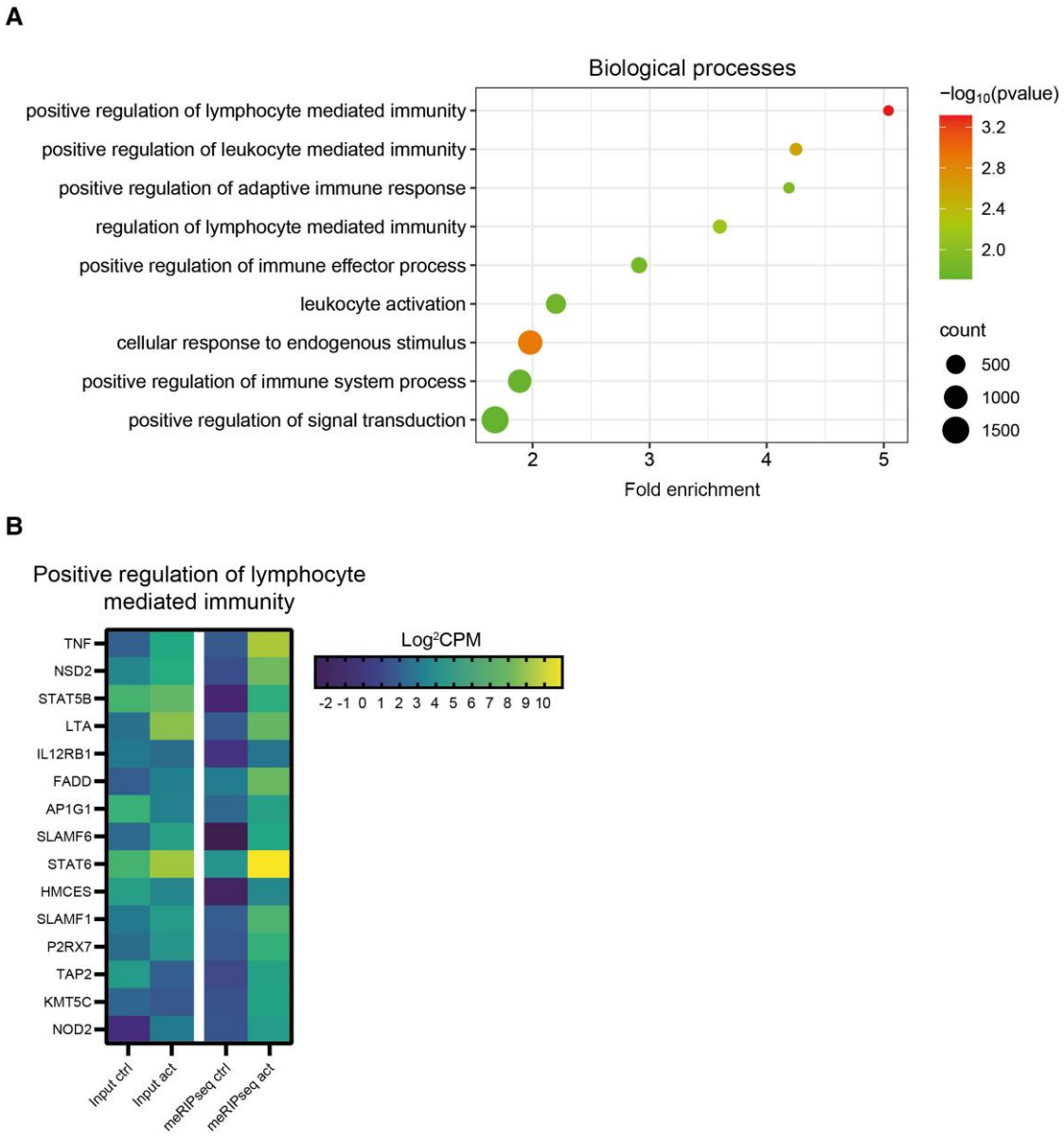

Fig. 1. Stimulation of CD4+ T lymphocytes alters the transcriptome-wide m6A enrichment pattern. (A) Gene Ontology term analysis based on log2FoldChange values following meRIP-seq in CD4+ T lymphocytes. Cells were activated overnight with CD3/CD28 beads. (B) Heat map of m6A enrichment in mRNAs involved in the positive regulation of lymphocyte mediated immunity (threshold foldchange value of 1.5). Values are expressed in log2CPM following meRIP-seq in CD4+ T lymphocytes with and without stimulation.

lymphocyte mediated immunity and adaptive immune responses (Fig. 1A). In addition, we observed that the mRNA of various transcripts was methylated; therefore, we specifically examined the m6A enrichment for mRNA transcripts encoding proteins associated with positive regulation of lymphocyte mediated immunity (Fig. 1B). m6A methylation of *TNF* mRNA was, among others, increased in activated CD4+ T cells compared with the other cytokine transcripts. TNF is an important proinflammatory cytokine expressed by activated T lymphocytes. Also, TNF is implicated in many autoimmune-related disorders. Therefore, we focused on TNF for further assessment.

3.2 m6A enrichment is increased on *TNF* mRNA in activated CD4+ T lymphocytes

Because we identified that m6A methylation is increased on *TNF* mRNA upon T cell activation, m6A enrichment patterns were studied in more detail. The m6A modifications are predominantly situated in the 3′ UTRs (Fig. 2A). To validate these results, meRIP qPCR was performed on T cells activated with either PMA/ionomycin or anti-CD3/CD28. Similar to our previous findings, m6A was significantly enriched on *TNF* mRNA of stimulated CD4+ T cells (Fig. 2B). We could furthermore confirm our observation using a publicly available m6A CLIP datasets of murine CD4+ T cells in which m6A methylation of *TNF* was enriched in the 3′ UTR (Fig. 2C).[23] The similarity in *TNF* m6A enrichment patterns of human and mouse CD4+ T lymphocytes indicate that this is a conserved mechanism to regulate TNF mRNA in CD4+ T cells.

To assess whether the increased levels of m6A methylation of TNF is the result of altered expression of m6A regulators upon CD4+ T lymphocyte activation, we examined if m6A regulators were differentially expressed in activated human CD4+ T lymphocytes by RNA-seq. No m6A writer and eraser genes were found differentially expressed in this experiment (Supplementary Fig. 1A),

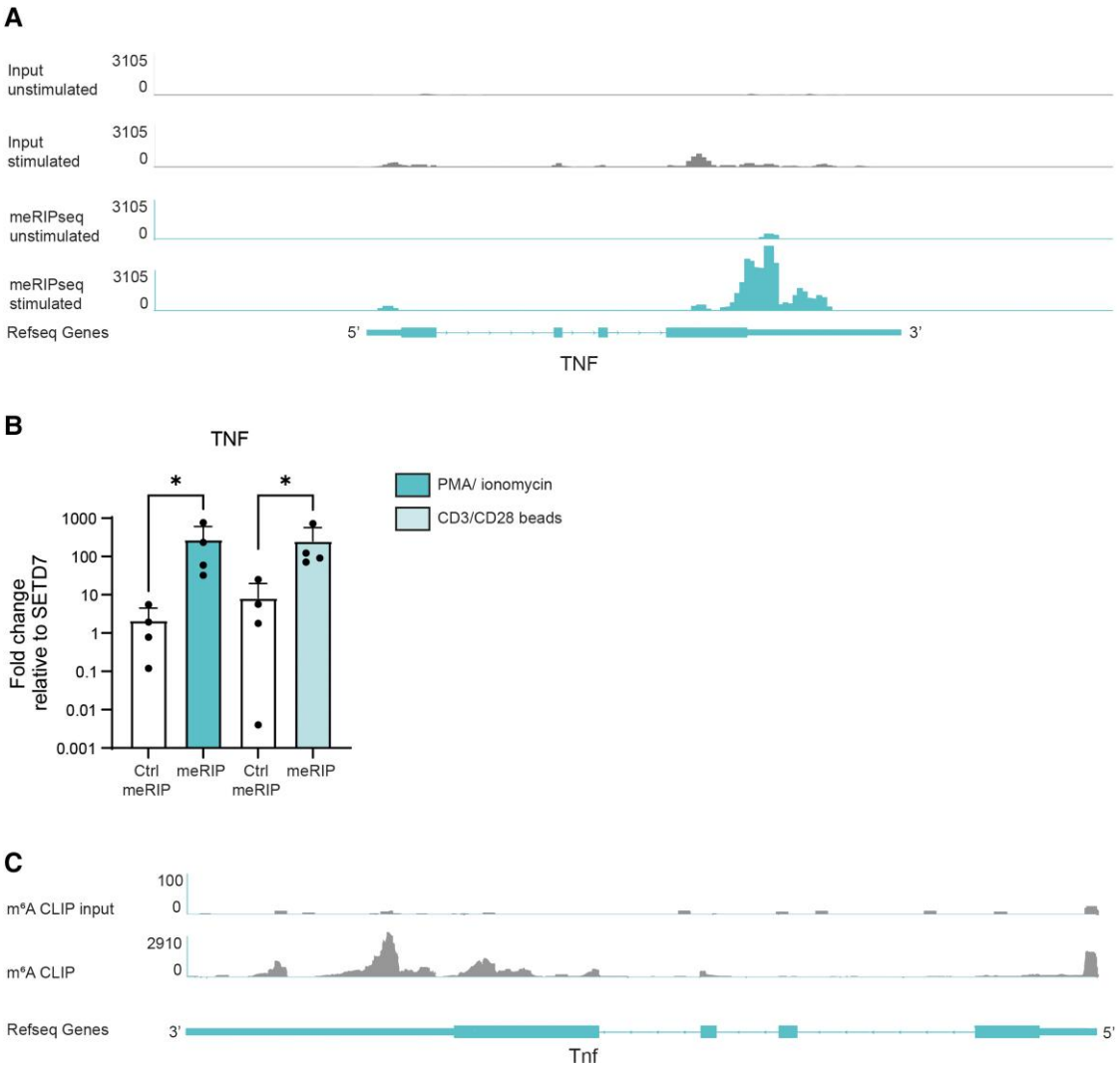

Fig. 2. m^{6}A enrichment is increased on *TNF* mRNA in activated CD4$^+$ T lymphocytes. (A) Integrative Genomics Viewer tracks of meRIP-seq in CD4$^+$ T lymphocytes. Cells were activated overnight with CD3/CD28 beads (n = 1). (B) meRIP qPCR in CD4$^+$ T lymphocytes. Cells were activated with either 4 h PMA and ionomycin or overnight with CD3/CD28 beads. *P* values were calculated using a Mann-Whitney *t* test: *$P < 0.05$. n = 4. (C) Integrative Genomics Viewer tracks of m^{6}A CLIP read distribution in naïve mouse T lymphocytes by Ito-Kureha et al.[23] Data were obtained from 3 biological replicates.

qPCR analysis confirmed these results (Supplementary Fig. 1B). Also, protein expression levels of these m^{6}A regulators were not altered by activation (Supplementary Fig. 1C) Taken together, the increased m^{6}A enrichment on *TNF* mRNA upon CD4$^+$ T cell activation is not the result of altered m^{6}A writer or eraser expression.

3.3 TNF expression in activated CD4$^+$ T lymphocytes is regulated via m^{6}A "eraser" FTO

As we demonstrated that *TNF* mRNA can be m^{6}A methylated, the next step was to determine the function of m^{6}A on these transcripts. To increase m^{6}A levels, an inhibitor of FTO was utilized. Human CD4$^+$ T lymphocytes were treated with the FTO inhibitor entacapone, activated,[30] and subsequently TNF protein expression was determined by flow cytometry. TNF expression was increased upon stimulation of the CD4$^+$ T lymphocytes, but both the amount of TNF-expressing cells and the amount of TNF expressed by each cell was decreased by FTO inhibition (Fig. 3A and Supplementary Fig. 2). *TNF* mRNA expression levels were determined in the same samples by using qPCR (Fig. 3B). Also at the mRNA level, inhibition of FTO significantly decreased the expression of *TNF*. To determine whether the decreased expression of TNF is the direct result of *TNF* m^{6}A methylation or decreased *TNF* transcription we also assessed the expression of pre-*TNF* mRNA by qPCR. The expression of *TNF* pre-mRNA was not changed upon FTO inhibition, indicating that TNF degradation is regulated by m^{6}A eraser protein FTO (Fig. 3C).

3.4 METTL3 regulates TNF expression in activated CD4$^+$ T lymphocytes

To further validate that m^{6}A is involved in the post-transcriptional regulation of TNF expression, human CD4$^+$ T lymphocytes were treated with an inhibitor of METTL3, thereby reducing m^{6}A levels.[31] The amount of TNF-expressing cells as well as the amount of TNF expressed by each cell was increased after incubation with the METTL3 inhibitor in comparison to a dimethyl sulfoxide control (Fig. 4A and Supplementary Fig. 3). To validate that the inhibitor is indeed METTL3 specific, METTL3 expression was manipulated. Here, CRISPR/Cas9 knockout of *METTL3* in Jurkat cells resulted in increased TNF expression levels (Fig. 4B). Taken

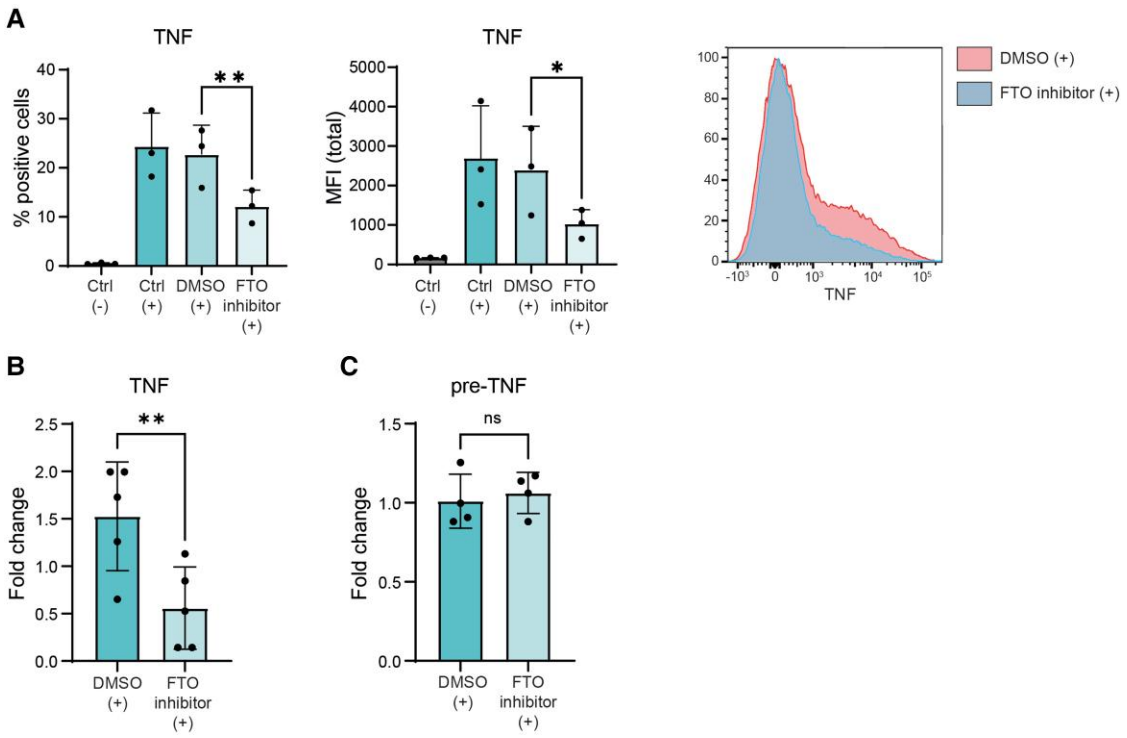

Fig. 3. TNF expression in activated CD4+ T lymphocytes is regulated via m6A "eraser" FTO. (A) Human CD4+ T lymphocytes were preincubated for 48 h with 50 μM entacapone. The cells were subsequently activated with CD3/CD28 beads overnight. Flow cytometry on TNF protein and representative histograms of TNF protein reduction after entacapone incubation. *P* values were calculated using an ordinary 1-way analysis of variance with Šídák's multiple comparisons test: $^{*}P < 0.05$, $^{**}P < 0.01$. (B) CD4+ T lymphocytes were preincubated for 48 h with 50 μM entacapone. The cells were subsequently activated with CD3/CD28 beads overnight. qPCR was performed on *TNF* mRNA. *P* values were calculated using a paired *t* test: $^{**}P < 0.001$. (C) CD4+ T lymphocytes were preincubated for 48 h with 50 μM entacapone. The cells were subsequently activated with CD3/CD28 beads overnight. qPCR was performed on pre-*TNF* mRNA. *P* values were calculated using a paired *t* test: *P* = ns. Ctrl = control; DMSO = dimethyl sulfoxide.

together, these results indicate that TNF expression in stimulated CD4+ T lymphocytes is regulated via METTL3.

3.5 TNF expression is regulated via YTHDF2-mediated destabilization

The m6A reader protein YTHDF2 has been identified to increase destabilization and degradation of target mRNA by promoting the localization of these methylated mRNA transcripts to P-bodies where mRNA is degraded.[9,32,33] To determine whether YTHDF2 can associate with methylated TNF mRNA, we analyzed a YTHDF2 iCLIP dataset previously published by Ito-Kureha et al.[23] These data show that YTHDF2 can associate with the *TNF* mRNA at locations where m6A sequences are also present, suggesting that *TNF* mRNA degradation is indeed mediated via YTHDF2 (Fig. 5A). To further test this hypothesis, we generated a HEK293T cell line stably expressing full-length *TNF* mRNA including both the 5′ and 3′ UTRs (containing the m6A-specific DRACH sequences). Overexpression of YTHDF2 in these cells resulted in significantly lower expression of TNF protein by these cells (Fig. 5B). Taken together, these results confirm that TNF expression in CD4+ T lymphocytes is regulated via YTHDF2-mediated decay of the *TNF* mRNA.

4. Discussion

Previous studies have demonstrated a role for m6A in different CD4+ T cell processes, with a focus on differentiation and T cell homeostasis.[20–22] However, implications of m6A in mature human CD4+ T lymphocyte function remains poorly understood. In this study, we demonstrate that m6A is directly involved in CD4+ T lymphocyte activation and effector functions. We show that upon CD4+ T cell activation, m6A methylation on *TNF* mRNA is increased. Manipulation of m6A either by inhibiting METTL3 or FTO significantly modulated TNF protein levels upon activation. We also demonstrate that the m6A reader YTHDF2 binds to m6A-specific sequences on the *TNF* mRNA, promoting its mRNA degradation. Taken together, this study provides novel insights regarding the mechanisms that control T cell function.

TNF is a proinflammatory cytokine rapidly produced and excreted by CD4+ T cells upon activation. Similarly, TNF expression must eventually be downregulated in order to prevent hyperactivation of the immune system.[34–37] Our data demonstrate that the degradation of *TNF* mRNA can be regulated via m6A methylation and its reader protein YTHDF2. We propose that m6A-mediated regulation of TNF expression is a negative feedback mechanism (i.e. able to rapidly decrease the amount of TNF) excreted in early stages of the immune response. This fast regulatory mechanism would prevent chronic TNF production and associated tissue damage and immunopathology. Our experiments were predominantly performed in vitro in human CD4+ T cells isolated from PBMCs of healthy donors. Additional in vivo studies could further help to understand how TNF expression is regulated by m6A during inflammation and in other cell types.

CD4+ T cell activation did not affect the expression of m6A regulatory proteins. To understand how T cell activation results in increased m6A methylation of *TNF* mRNA, more research needs to be performed. However, we speculate that either increased writer

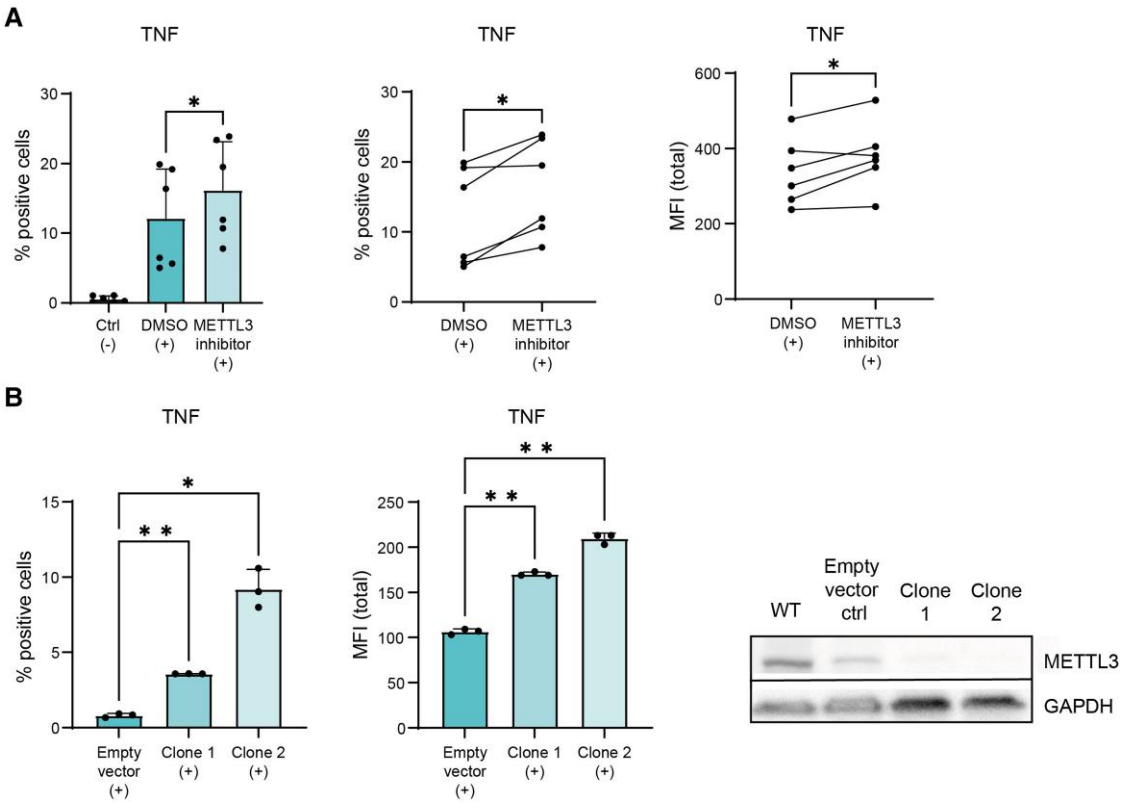

Fig. 4. METTL3 regulates TNF expression in activated CD4⁺ T lymphocytes. (A) CD4⁺ T lymphocytes were preincubated for 48 h with 30 μM STM2457 (METTL3 inhibitor). The cells were subsequently activated with CD3/CD28 beads overnight (indicated with +). Flow cytometry was performed on TNF protein (B) (Left) Flow cytometry of *METTL3* knockout Jurkat cells 4 h stimulated with PMA and ionomycin compared with empty vector control. (Right) Western blot of CRISPR/Cas9 knockout of *METTL3* in Jurkat cells compared with control (2 single-cell clones). *P* values were calculated using a paired *t* test: $^{*}P < 0.05$, $^{**}P < 0.01$. Ctrl = control; DMSO = dimethyl sulfoxide; MFI = Mean Fluorescence Intensity; WT= wild-type.

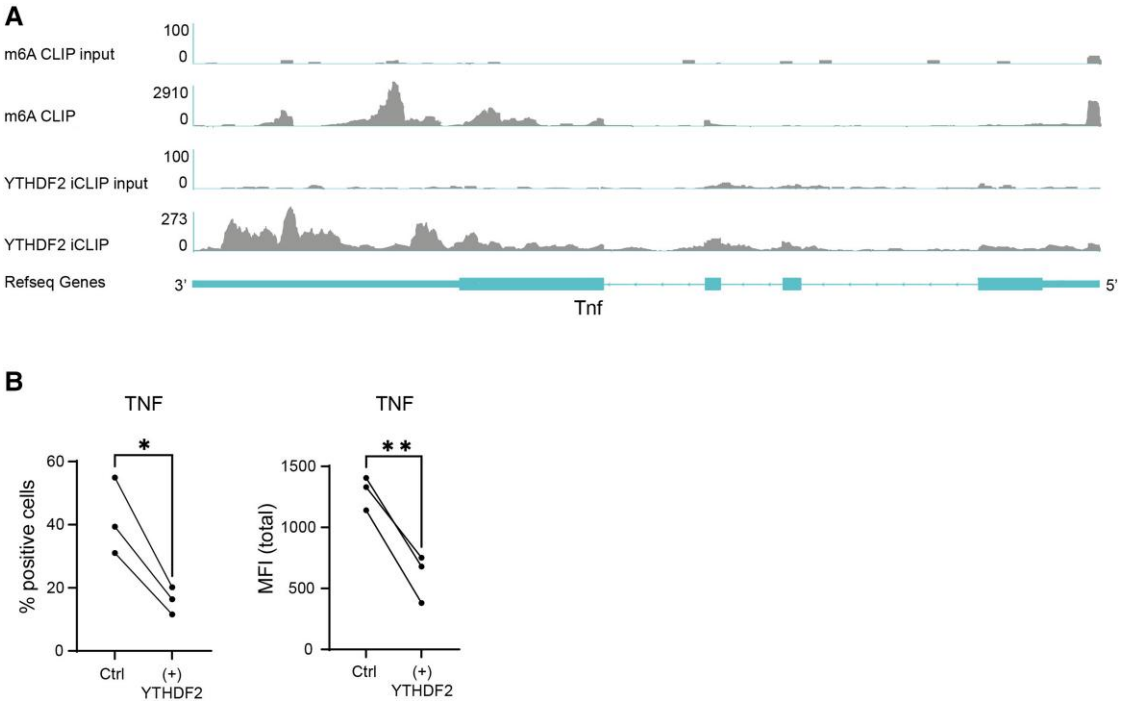

Fig. 5. TNF expression is directly regulated via YTHDF2-mediated destabilization. (A) YTHDF2 iCLIP data in naïve mouse T lymphocytes by Ito-Kureha et al.[23] (B) Overexpression of YTHDF2 in HEK293T cells with stable expression of TNF. Flow cytometry was performed on TNF protein. *P* values were calculated using a paired *t* test: $^{*}P < 0.05$, $^{**}P < 0.01$.

complex activity or increased availability of DRACH sequences on the *TNF* mRNA may cause increased m^{6}A enrichment in activated T cells.

In this study, the FTO inhibitor entacapone was used to manipulate the function of FTO and thereby inhibit the demethylation of m^{6}A-modified mRNA. In the research of Peng et al.,[30] it was demonstrated that entacapone is a specific inhibitor of FTO, blocking both cofactor and substrate binding. However, in previous research, it was demonstrated that FTO functions not only as an eraser of m^{6}A, but also of m^{6}A$_m$.[38] m^{6}A$_m$ is an m^{6}A modification on the 5′ cap of processed mRNA. The anti-m^{6}A antibody used for meRIP-seq and qPCR experiments recognizes both m^{6}A and m^{6}A$_m$. In our meRIP-seq data, it can indeed be observed that TNF mRNA in activated T cells is enriched for m^{6}A at the 5′ cap. To validate that the observed effects with FTO inhibition are m^{6}A specific, the METTL3 inhibitor STM2457 was used to decrease m^{6}A levels and validate our observations on TNF expression. METTL3 is known as a specific methyltransferase for m^{6}A.[39] Additionally, we demonstrate that YTHDF2 promotes TNF degradation. Because YTHDF2 associates with m^{6}A and not with m^{6}A$_m$, this further validates that the observed effect of the FTO inhibitor on TNF is very likely regulated via m^{6}A modifications and its regulatory proteins.

Here, we demonstrate that m^{6}A RNA methylation of TNF can promote its degradation, and we speculate that modulating TNF methylation by FTO inhibition might be leveraged for therapeutic purposes. Transcriptome-wide m^{6}A mapping studies have demonstrated that 1 out of every 3 mRNA transcripts in human cells contains m^{6}A modifications.[3,4] Therefore, manipulation of m^{6}A RNA methylation levels is expected to modulate the expression of various cytokines and inflammation-related genes, as long as the encoding mRNA is m^{6}A methylated. Potentially, the expression of all the mRNAs involved in the regulation of lymphocyte-mediated immunity, shown in the meRIP-seq data of Fig. 1, could be manipulated by inhibiting FTO or METTL3 function. Additional research is needed to assess how m^{6}A influences these other transcripts related to lymphocyte-mediated immunity and how transcript specificity for m^{6}A modulation can be achieved in the future.

The role of m^{6}A in the regulation of TNF expression in vivo and in TNF-related diseases is unfortunately incompletely understood. A limitation of the current study is the absence of in vivo experiments. However, there are studies using in vivo (disease) models that suggest a correlation between m^{6}A-methylation and TNF expression. METTL3 expression was found to be decreased in osteoarthritis.[40] In this same study, METTL3 overexpression in SW1353 (fibroblast-like) cells decreased TNF protein expression. In rheumatoid arthritis, it was demonstrated that increased expression of METTL3 can suppress lipopolysaccharide-induced expression of inflammatory cytokines such as TNF by inhibiting nuclear factor κB signaling,[41] but perhaps this effect could also be mediated via direct TNF m^{6}A methylation. Taken together, these models support a role for m^{6}A methylation in TNF expression. However, as direct degradation of TNF by YTHDF2 was not the focus of these studies, additional in vivo research is essential to fully understand how m^{6}A methylation impacts TNF expression in these models.

In conclusion, we demonstrated that TNF expression in CD4$^+$ T cells is regulated via m^{6}A modifications and its regulatory proteins. These data increase our understanding regarding the role of RNA modification m^{6}A in CD4$^+$ T lymphocyte activation and function. Because TNF is an central proinflammatory cytokine implicated in many autoimmune-related disorders, this research helps to pave the way to explore the therapeutic potential of m^{6}A manipulation in the treatment of autoimmune diseases.

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Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

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Conflict of interest statement. None declared.

Data availability

The meRIP-seq data have been deposited online. Read count data and per gene TMM normalized log2CPM expression values from meRIP-seq are accessible at 10.5281/zenodo.8016876. Not all raw RNA sequencing data used in this study are publicly available due to research participant privacy/consent. Therefore, these data are only available upon request and after signing a Data Sharing Agreement within a specially designed UMC Utrecht–provided environment.

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