The abundant expression of IFNγ in Th-inducing POK (ThPOK)-deficient CD4⁺ T cells requires the activation of Eomesodermin (Eomes); however, the underlying mechanism of this phenomenon remains unclear. Here we report that ThPOK binds directly to the promoter region of the Eomes gene to repress its expression in CD4⁺ T cells. We identified the histone acetyltransferase TIP60 as a co-repressor of ThPOK-target genes, where ectopically expressed TIP60 increased ThPOK protein stability by promoting its acetylation at its Lys³⁶⁰ residue due to then augment the transcriptional repression of Eomes. Moreover, knockdown of endogenous TIP60 abolished the stabilization of ThPOK in CD4⁺ T cells, which led to the transcriptional activation of Eomes and increased production of IFNγ. Our results reveal a novel pathway by which TIP60 and ThPOK synergistically suppress Eomes function and IFNγ production, which could contribute to the regulation of inflammation.

TIP60² (Tat-interactive protein, 60 kDa) was identified as an HIV-1 TAT-interacting protein that augments TAT-mediated transactivation of the HIV-1 promoter (1). Further studies characterized TIP60 as a histone acetyltransferase that acetylates H2A, H3, and H4, but not H2B of the core histones to regulate gene expression (2). However, TIP60 is not only a chromatin modifier that regulates transcription as it also plays a role in DNA repair, cell apoptosis, oncogenesis, and serum deprivation-induced autophagy through exerting its function as a transcriptional co-regulator or through its enzymatic activity on transcription factors such as FOXP3, STAT3, c-Myc, p53, and protein kinases including ATM and UKL1 (3–9).

TIP60 deficiency in mice is lethal, whereas heterozygous deletion of TIP60 leads to haplo-insufficient tumor suppression (10). However, the role of TIP60 in regulating immune responses remains largely uncharacterized. In CD4⁺ CD25⁺ regulatory T (Treg) cells, we have shown that TIP60 binds directly to the prolinc-rich domain of the forkhead family transcription factor FOXP3, which is essential for FOXP3-mediated repression of IL-2 expression (8). In CD4⁺ Th helper 2 (Th2) and Th17 cells, the proinflammatory cytokine IL-9 activates STAT3 to modulate its downstream gene expression (11) and is regulated by TIP60 through its interaction with both IL9Rα and STAT3 (9, 12).

The TCR signal-induced zinc finger and BR-C, Ttk, and Bab (BTB) domain-containing protein family transcription factor Th-inducing POK (ThPOK) is dominantly expressed in CD4⁺ T cells (13) and is necessary for T helper cell differentiation (14, 15). ThPOK binds to the silencers within the CD4 and ThPOK gene loci and inhibits silencer activity (13, 16). Moreover, a number of studies have shown how ThPOK regulates CD4 versus CD8 T cell lineage commitment by suppressing classical CD8 lineage genes such as CD8, Perforin, Granzyme B, and RUNX3 (14, 17–19). Others have also shown how the function of Eomesodermin (Eomes), a T-box transcriptional activator of IFN-γ, negatively correlates with ThPOK expression (17, 18, 20).

Here we report a previously uncharacterized mechanism by which the gene transcription of Eomes is directly repressed by ThPOK and how TIP60 is a cofactor for ThPOK-mediated repression of Eomes expression. This pathway in turn mitigates the activation of Eomes target genes such as IFNγ in human
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CD4+ T cells. As ThPOK contains a proline-rich domain, we hypothesized that TIP60 might also bind to its proline-rich domain to mediate T cell lineage differentiation and function and modulate inflammation through regulating the transcriptional induction of Eomes; however, we found that the C-terminal region of ThPOK interacted with TIP60 and is acetylated at the Lys360 residue. Our results thus reveal a direct molecular link between TIP60 function and the modulation of CD4+ T cell-mediated inflammation through cytokine production.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK 293T cells were cultured in DMEM containing 10% FBS and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Jurkat cells were maintained in RPMI 1640 medium containing 10% FBS. Transfection of Jurkat cells with plasmid DNA was performed by electroporation on a Gene Pulser X cell apparatus (Bio-Rad Laboratories). Jurkat cells were activated using soluble antibodies against CD3 (1 μg/ml, Hit3a; Biolegend) and CD28 (2 μg/ml, CD28.2; Biolegend).

Immunoprecipitation and Immunoblotting—Cells were washed with ice-cold PBS and lysed on ice for 30 min in 1× RIPA buffer (50 mM Tris-HCl, pH 7.5, 135 mM NaCl; 1% Non-ident P-40; 0.5% sodium DOC; 1 mM EDTA, 10% glycerol) containing protease inhibitor (1:100, P8340; Sigma-Aldrich), 1 mM NaF, and 1 mM PMSF. Cell lysates were cleared by centrifugation, and supernatants were immunoprecipitated with the indicated antibodies (Abs) using protein A/G-agarose beads at 4°C. After washing, 2× sample loading buffer was added to the immunoprecipitates. Samples were then used for immunoblot analysis with the indicated antibodies.

Antibodies and Reagents—The following antibodies were used for flow cytometry analysis: anti-CD4-FITC (RPA-T4; Biolegend), anti-CD8-APC (RPA-T8; BD Biosciences), anti-TCRβ-PE (IP26; eBioscience), and anti-IFN-γ-APC (45-83B; eBioscience). Fixable viability dye eFluor 780 was purchased from eBioscience. Fixable HA (F-7), anti-ThPOK (A-4), anti-TIP60 (N-17), and goat IgG (sc-2028) were from Santa Cruz Biotechnology. Anti-FLAG (M2), anti-β-actin, and anti-α-tubulin were from Sigma-Aldrich and Tianjin Sungen Biotech (China), respectively. Mouse IgG was from Millipore. Anti-acetylysine Ab was obtained from Immunechem Pharmaceuticals (Canada). Protein A/G-agarose beads (A10001) were purchased from Abcam (China). Cycloheximide (C7698-5G) and nicotinamide (72340-100G) were purchased from Sigma-Aldrich. EX-527 (S1541) was purchased from Selleck. Human ThPOK was cloned into the pLP-HA2 vector, and pCMV2-FLAG-TIP60 has been described previously (8). Mutagenesis was carried out according to the manufacturer’s instructions using the Toyobo mutagenesis kit. ThPOK was cloned into the FUGW plasmid (kindly provided by Lan Ke, Institut Pasteur of Shanghai, Chinese Academy of Sciences).

Luciferase Reporter Assay—The 1000-bp region upstream of the human Eomes transcriptional starting site (NCBI: human, chromosome 3 NC_000003.11; mouse, chromosome 9 NC_000075.6) was cloned into the pGL3-Basic vector to generate the pGL3-Eomes-Luc reporter construct. Jurkat cells were co-transfected with the reporter plasmid and a Renilla luciferase encoding plasmid as a control, and/or FLAG-TIP60 as indicated. 48 h later, cells were lysed, and luciferase assays were performed using the Dual-luciferase reporter kit (Promega).

Chromatin Immunoprecipitation—Primary human CD4+ T cells were stimulated using anti-CD3/CD28 dynal beads (Invitrogen) at a cell to bead ratio of 1:1 for the indicated time periods. Cells were then cross-linked with formaldehyde, and the chromatin was sonicated into ~500-bp fragments. The sheared chromatin was immunoprecipitated with anti-ThPOK antibody, and mouse IgG was used as a negative control. The pulled down DNA fragments were subjected to qPCR analysis. Primers that were used are as follows: Probe N forward, 5′-cagctcagaggggtgcctg-3′ and reverse, 5′-tgaatgggctccctcttgg-3′; Probe B forward, 5′-agtcacaggctgtcatc-3′ and reverse, 5′-agatctgttgcacccatcc-3′.

Quantitative Real Time PCR—Total RNA was extracted using TRIzol reagent (Invitrogen). cdNA was synthesized using a reverse transcriptase kit (TaKaRa, Japan), followed by qRT-PCR analysis (SYBR Green; TaKaRa). The primers that were used are as follows: CD8 forward, 5′-ctgagcaactcactagt-3′ and reverse, 5′-gatcagccaggaattgca-3′; CD4 forward, 5′-ggtaaatgacctcaag-3′ and reverse, 5′-ctgaaacgcttggagac-3′; ThPOK forward, 5′-gtctgaccaacagac-3′ and reverse, 5′-tcgagctgtcaggac-3′; TIP60 forward, 5′-agatctggctgtgacc-3′ and reverse, 5′-tctctctgagcagc-3′; Eomes forward, 5′-tgcaaagctctctagac-3′ and reverse, 5′-ctctctgactgagac-3′; Tbx21 forward, 5′-cctcgactgctcagat-3′ and reverse, 5′-attcccacagatgtac-3′; IFNγ forward, 5′-aaggagatgactcct-3′ and reverse, 5′-atttggagcagtcacctc-3′; β-actin forward, 5′-cctctctcagctctctctt-3′ and reverse, 5′-caggcagttctctctt-3′.

T Cell Isolation—Primary human CD4+ and CD8+ T cells from healthy donors were isolated by FACS on a BD FACs ARIA II sorter (BD Biosciences). Primary T cells were expanded using anti-CD3/CD28 dynal beads (Invitrogen) at a cell to bead ratio of 1:1 in X-VIVO-15 medium (Lonza, Switzerland) supplemented with 10% human AB serum, 1% GlutaMax (GIBCO), 1% sodium pyruvate (GIBCO), and 1% Pen/Strep (GIBCO).

Intracellular Staining—In brief, CD4+ T cells were restimulated with phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (1 μM), and Golgi Stop for 4 h. At the end of stimulation, cells were stained with fixable viability dye eFluor 780 and anti-CD4-FITC, then washed with PBS. Staining of IFNγ was carried out with the IC Fixation Buffer (eBioscience catalog number 00-8222) and Permeabilization Buffer (10×) (eBioscience catalog number 00-8333) according to the manufacturer’s instructions.

Lentiviral Constructs and Transduction—The shRNA lentiviral vector pLKO.1 shTIP60, pLKO.1 shThPOK, or pLKO.1 shCK was transduced into HEK 293T cells via calcium phosphate transfection with the lentivirus packing vector Delta 8.9 and VSVG envelope glycoprotein. Viral supernatants were harvested after 48 h. Primary T cells were transduced with virus along with anti-CD3/28 stimuli (1 cell to 1 bead). The following shRNA sequences were used: shCK, 5′-caacaatagaaaagagca-
RESULTS

Differential Expression of TIP60 in Human CD4+ and CD8+ T Cells—CD4+ and CD8+ T cells were isolated from human peripheral blood mononuclear cells of healthy donors (Fig. 1A) and subsequently examined for TIP60 mRNA and protein expression levels alongside the CD4 T cell lineage-specific transcription factor ThPOK as a control. We found that TIP60 mRNA was highly expressed in CD4+ T cells compared with CD8+ T cells (Fig. 1B). Also, protein expression was found to be consistent with the mRNA levels (Fig. 1C). Therefore, both TIP60 and ThPOK are higher expressed in CD4+ T cells.

Short Term TCR Stimulation Stabilizes ThPOK in CD4+ T Cells—Previous studies indicated that long term TCR stimulation triggers ThPOK transcription in CD4+CD8lo thymocytes (13). However, it remains unclear how short term TCR stimulation regulates ThPOK expression in CD4 single positive (SP) cells. To reveal the role of TCR signaling, resting CD4 SP T cells were stimulated with soluble antibodies against CD3 and CD28 for a short period (within 8 h) prior to the analysis of ThPOK protein and mRNA levels. We observed an increasing level of ThPOK protein upon TCR stimulation, as well as TIP60 (Fig. 1D). However, the transcription of both genes was slightly repressed (Fig. 1E), which indicated that ThPOK and TIP60 were stabilized posttranslationally. Consistent with the previous find-
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TIP60 is a lysine acetyltransferase, which acetylates not only histones but also nuclear transcription factors such as c-Myc, p53, and FOXP3. We were interested in investigating whether TIP60 could similarly modify ThPOK by lysine acetylation. We co-transfected the HA-ThPOK-expressing plasmid with increasing amounts of FLAG-TIP60-expressing plasmid into HEK 293T cells; cells were then treated with protein deacetylase inhibitors. We found that the overexpression of TIP60 promoted ThPOK acetylation (Fig. 2C). To elucidate the role of TIP60 in primary T cells, lentivirus-mediated shRNA knockdown of endogenous TIP60 was performed. Loss of TIP60 significantly reduced the acetylation level of ThPOK (Fig. 2D).

Previously, we found that short term TCR stimulation could stabilize ThPOK. Others have also shown that the acetylation of ThPOK increases its stability (21); therefore, we decided to check the acetylation level of ThPOK after TCR stimulation. As expected, short term TCR stimulation promoted the interaction between TIP60 and ThPOK, which most likely increases the acetylation of ThPOK (Fig. 2E).

We then further tested the relationship between acetylation and protein stability. We observed that the increase in ThPOK protein level positively correlated with TIP60 level, which indicated that the overexpression of TIP60 facilitated the stabilization of ThPOK (Fig. 3A). To fully confirm this result, we treated the cells with protein synthesis inhibitor cycloheximide and examined ThPOK protein stability. HA-ThPOK was co-transfected with or without FLAG-TIP60, and cells were then treated with cycloheximide for the indicated time periods. Here, we confirmed that ThPOK stability could be positively regulated by TIP60 (Fig. 3B). To further examine TIP60-mediated stabilization of ThPOK under more physiological conditions, knockdown of endogenous TIP60 was performed in primary human CD4+ T cells. The protein level of ThPOK decreased noticeably in TIP60-silenced CD4+ T cells. Meanwhile, CD4+ T cells transduced with shRNA constructs targeting ThPOK were tested as a control (Fig. 3C). We then mapped by co-IP for the regions of ThPOK that associates with TIP60 to the C-terminal domain (residues 310–539), but not the N-terminal subdomain, which contains the proline-rich region (Fig. 3, D and E). These results indicated that the acetylated lysines were likely at the C-terminal region. Furthermore, TIP60 specifically stabilized the C-terminal region (C1) of ThPOK but not the N-terminal region (N1) (Fig. 3F). TIP60 preferentially acetylates the lysine of G(X)KG motif of histones (22). When we screened all of the residues of ThPOK, we identified that lysine 360 (GAGK motif) was contained in the C-terminal region, and mutation of lysine 360 into arginine abrogated TIP60-mediated acetylation (Fig. 3G). We found that a point mutation of lysine 360 into arginine abrogated TIP60-mediated stabilization, whereas combinatorial mutation of Lys203, Lys206, and Lys312 (lysines located in the proline-rich domain) could not (Fig. 3F). Further experiments showed that the ThPOK-K360R mutant abolished TIP60-mediated acetylation, which indicates that lysine 360 is the target of TIP60 (Fig. 3G).

TIP60 Associates with ThPOK to Promote Its Acetylation and Stability—As ThPOK contains a proline-rich domain that could be potentially recognized by TIP60, we tested whether TIP60 and ThPOK could interact with each other by reciprocal immunoprecipitation. HA-tagged ThPOK (HA-ThPOK) and FLAG-tagged TIP60 (FLAG-TIP60) were ectopically expressed in HEK 293T cells, followed by reciprocal immunoprecipitation. We found a positive interaction between TIP60 and ThPOK (Fig. 2A). We then further showed that endogenous TIP60 could co-immunoprecipitate ThPOK in human primary CD4+ T cells (Fig. 2B).

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Previous studies have revealed how Eomes activity is up-regulated in the absence of ThPOK or in the presence of insufficient ThPOK expression (17, 18). Overexpression of ThPOK in CD8+ T cells specifically represses Eomes but not T-bet (20). Thus, we intended to clarify whether ThPOK could bind to the Eomes gene promoter to repress its transcription in CD4+ T cells.

FIGURE 3. TIP60-mediated acetylation of lysine 360 stabilizes ThPOK. A, HA-ThPOK was co-transfected with increasing amounts of FLAG-TIP60 into HEK 293T cells; cell lysates were analyzed by Western blotting (IB). B, HA-ThPOK was expressed in HEK 293T cells in the presence or absence of FLAG-TIP60. Cells were treated with 20 μg/ml cycloheximide (CHX) for the indicated periods. Levels of ThPOK were determined by Western blotting. C, CD4+ T cells were transduced with lentivirus containing shRNA sequences targeting CK (control), TIP60, or ThPOK. Cells were cultured with anti-CD3/CD28 dynal beads and selected with puromycin for 5 days. Selected cells were lysed for the detection of ThPOK and TIP60 levels by Western blotting. D, domain structure of ThPOK protein. The potential acetylated lysines by TIP60 were labeled as indicated. E, FLAG-TIP60 was expressed in HEK 293T cells with HA-tagged ThPOK or its deletion variants. Cell lysates were immunoprecipitated (IP) with anti-HA antibody and detected by WB with the indicated antibodies. F, FLAG-TIP60 was expressed in HEK 293T cells with HA-tagged ThPOK, its lysine mutants, or deletion variants. Cell lysates were analyzed by Western blotting. G, HEK 293T cells were transfected with HA-tagged ThPOK, its lysine mutants, or FLAG-TIP60 as indicated. 1 mM nicotinamide and 50 μM Ex-527 were added 6 h before cell harvesting. Cell lysates were immunoprecipitated with anti-HA antibody and detected by Western blotting with the indicated antibodies.
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the indicated time periods (Fig. 4A). We then examined the mRNA levels of Eomes and IFNγ. Both genes were down-regulated in ThPOK-Jurkat cells (Fig. 4A). We aligned the 1-kb region (−1,000 to 0) of the Eomes promoter from mouse and human. The alignment result showed that there was a consen-
sus ThPOK binding sequence CCCCTCCC (19, 23, 24), which suggested that Eomes is potentially a direct transcriptional tar-
get of ThPOK (Fig. 4B). We next subcloned the 1-kb region of
the human Eomes promoter into the pGL3-Basic Luciferase
reporter plasmid into Jurkat or ThPOK-Jurkat cells. We found
that the Eomes-Luc reporter activity was significantly repressed
(Fig. 4C). To further confirm the involvement of ThPOK in Eomes gene transcription we ana-
alyzed whether ThPOK could bind to the Eomes promoter in
primary CD4+ T cells using a ChIP assay. Human CD4+ T cells
were stimulated with anti-CD3/CD28 dynal beads for the indi-
cated periods, and the binding of ThPOK to the promoter
region (Probe B region with a consensus ThPOK binding site) was
notably higher after anti-CD3/CD28 stimulation (Fig. 4D). Fur-
thermore, mutation of the ThPOK binding site could reverse
ThPOK-mediated repression of Eomes-Luc reporter activity
(Fig. 4E), but not completely, perhaps due to other less consen-
sus binding sites that may be responsible for ThPOK binding
and activity at the Eomes promoter.

As we found that TIP60 could stabilize ThPOK in CD4+ T
cells we decided to test whether TIP60 could promote ThPOK-
mediated repression of Eomes expression. Jurkat and ThPOK-
Jurkat cells were transfected with the Eomes luciferase reporter
and FLAG-TIP60-encoding plasmids as indicated. We found
that TIP60 stabilized ThPOK and increased the repression of
the Eomes promoter as indicated by the decrease in luciferase
activity (Fig. 4F).

TIP60 Positively Regulates ThPOK-mediated Repression
of Eomes in CD4+ T Cells—ThPOK was reported to bind to the
silencers of the CD4 and ThPOK gene loci to abolish their activ-
ity (16). We hypothesized that TIP60 could positively regulate
ThPOK-mediated transcriptional regulation of CD4, ThPOK,
and Eomes. To test this, primary CD4+ T cells were transduced
with shRNA constructs targeting CK (control), TIP60, or
ThPOK and then subjected to analysis by qRT-PCR or flow
cytometry (Fig. 5A). Total RNA was then extracted, and the
mRNA levels of CD8, CD4, ThPOK, Eomes, Tbx21, and
IFNγ were determined by qRT-PCR. No CD8 transcripts were
detected, which indicated that the knockdown of either TIP60
or ThPOK did not induce CD8 expression in CD4+ CD8− T
cells (Fig. 5B). Both CD4 and ThPOK were down-regulated
upon knockdown of TIP60 or ThPOK, whereas Eomes and
IFNγ were both noticeably up-regulated (Fig. 5B). However,
the expression of Tbx21, another T-box transcriptional activator
of IFN-γ, was not dramatically affected (Fig. 5B). The protein
levels of CD4 and IFNγ were further tested by flow cytometry.
Knockdown of either TIP60 or ThPOK in CD4+ CD8− T cells
slightly decreased CD4 protein level, which was consistent with
the previous mRNA expression result (Fig. 5C). Meanwhile, we
observed a higher percentage of CD4+ IFNγ+ T cells in cells
depleted of TIP60 or ThPOK (Fig. 5D). To test whether the
up-regulation of Eomes mRNA in TIP60-depleted cells was
ThPOK-dependent, we overexpressed ThPOK in TIP60-de-
pleted cells. The overexpression of ThPOK significantly pro-
moted the repression of Eomes (Fig. 5E), which indicated that
the loss of ThPOK activity in TIP60-depleted CD4+ T cells was
responsible for the increase in transcription of Eomes. Our data
suggest that TIP60 and ThPOK could significantly repress
Eomes expression and play a potential role in modulating
inflammation (Fig. 6).

DISCUSSION

Eomes was originally characterized as an inflammation-in-
ducing gene in CD8+ T cells and Natural Killer cells, indispen-
sible for IFNγ production during host defense responses
against intracellular bacteria and virus infections (25–28).
Recently, more attention has been paid to the function of
Eomes in CD4+ T helper cells; Eomes is required for Th1 cell
differentiation and is generally repressed in other T helper cell
lineages. For example, Eomes directs naïve CD4+ T cell polar-
ization toward the Th1 lineage versus Th17, where TGF-β
(required for Th17 skewing) suppresses Eomes expression via
the c-Jun N-terminal kinase (JNK-c-Jun signaling pathway
(29–31). Eomes is also suppressed in effector Th2 cells;
GATA3-deficient naïve CD4+ T cells produce a significant
amount of IFNγ during Th2 differentiation, which is dependent
on Eomes but not T-bet (32). GATA3 was reported to induce
ThPOK expression (33) and may suppress Eomes in a ThPOK-
dependent manner. Moreover, in memory Th2 cells, Eomes is
highly expressed and interacts with GATA3 to prevent its bind-
ing to the IL-5 promoter (34). Here, we have identified another
novel molecular pathway which could explain the molecular
basis by which Eomes is repressed in CD4+ T helper cells in a
TIP60- and ThPOK-dependent manner.

As we postulated, TIP60 was found to augment ThPOK-me-
diated repression of Eomes through stabilizing ThPOK; we
speculate that TIP60 may also recruit histone deacetylases
to ThPOK transcriptional complexes to epigenetically silence
Eomes by the deacetylation of its promoter, such as HDAC7/9
and SIRT1, which have been reported to associate with TIP60
(8, 9, 35). In addition, ThPOK can recruit HDAC4/5 to the CD8
gene loci to impair CD8 transcription (19). However, it needs to
be further substantiated as to whether these histone deacetyl-
lases could be recruited to the promoter of Eomes in CD4+ T
cells.

The uncontrollable overproduction of IFNγ by CD4+ T
helper cells may coincide with the development of autoimmune
disease (36, 37). CD4+ CD25− CD127low FOXP3+ regulatory T
cells are essential for peripheral tolerance and the prevention of
autoimmune diseases (38). Strong evidence shows that autore-
active Treg cells can suppress adaptive immune responses and
dampen inflammation (39). Therefore, TIP60, through positive
regulation of its function, may be a potential anti-inflammation
target because it inhibits IFNγ production in CD4+ T helper
cells and also strengthens the suppressive function of Treg cells
by promoting FOXP3-mediated transcriptional repression as
we had previously identified (8). Although TIP60 has been
shown to be involved in DNA repair, apoptosis, and Treg func-
tion, here we give a new insight into the molecular mechanism
by which TIP60 suppresses Eomes and attenuates the produc-
FIGURE 4. Overexpression of TIP60 augments ThPOK-mediated repression of Eomes. A, a Jurkat cell line stably expressing ThPOK (ThPOK-Jurkat) was established. Jurkat and ThPOK-Jurkat cells were stimulated with α-CD3 and α-CD28 antibodies for the indicated time periods. Total RNA was extracted, and the mRNA levels of Eomes, IFNγ, and ThPOK were measured by qRT-PCR. Data show the mean of three separate experiments. Error bars indicate S.D. B, The 1-kb region (−1,000 to 0) of the Eomes promoter from mouse and human were aligned online using NCBI Blast (blast2seq). One consensus ThPOK binding site CCCTCCC was found on the Eomes promoter from both mouse and human. C, reporter transfection assay of the repressive activity of Eomes promoter by ThPOK in Jurkat and ThPOK-Jurkat cells. Cells were transfected with the pGL3-Eomes-Luc reporter which carries a 1,000-bp region of the Eomes promoter. 48 h later, luciferase activity was analyzed. D, CD4+ T cells were stimulated with anti-CD3/CD28 dynal beads for the indicated time periods. The binding of ThPOK to the Eomes promoter was determined by a ChIP assay with probe B, and probe N was used as a control. E, Jurkat or ThPOK-Jurkat cells were transfected with either pGL3-Eomes-Luc reporter or mutated reporter (CCCTCCC to CATTCCC) as indicated. The luciferase activity was analyzed 48 h later. Data show the mean of three separate experiments. Error bars indicate S.D. F, the pGL3-Eomes-Luc reporter was co-transfected with or without FLAG-TIP60 into Jurkat or ThPOK-Jurkat cells as indicated. The luciferase activity in the transfected cells was then determined. Error bars of C, D, and F show the S.D. from the mean in one experiment representative of three independent experiments.
FIGURE 5. TIP60 positively regulates ThPOK-mediated repression of Eomes in CD4⁺ T cells. A, timeline showing the knockdown of TIP60 and ThPOK in CD4⁺ T cells. On day 0, CD4⁺ T cells were transduced with lentivirus. 48 h later, cultures were treated with puromycin and replenished every other day. On day 7, selected CD4⁺ T cells were subjected to analysis by FACS or qRT-PCR. B, CD4⁺ T cells transduced with lentivirus-containing shRNA constructs targeting CK (control), TIP60, or ThPOK. Cells were cultured with anti-CD3/CD28 dynal beads and selected with puromycin for 5 days prior to RNA extraction. Total RNA was extracted, and mRNA levels of CD8, CD4, TIP60, ThPOK, Eomes, Tbx21, and IFN-γ were determined by qRT-PCR. Data show the mean of three separate experiments. Error bars indicate S.D. C, expression of CD4 in selected CD4⁺ T cells described in B. Surface CD4 level was analyzed by flow cytometry. The shaded area represents the isotype control. D, IFN-γ production by CD4⁺ T cells described in B. IFN-γ level was observed after restimulation of cells with phorbol 12-myristate 13-acetate, ionomycin, and Golgi Stop for 4 h. E, CD4⁺ T cells transduced with lentivirus-containing shRNA constructs targeting CK (shCK) or TIP60 (shTIP60) as described in A. On day 4, shTIP60-transduced CD4⁺ T cells were also transduced with lentivirus-encoding ThPOK protein and GFP under the internal ribosome entry site. These cells (shTIP60-ThPOK) were cultured with anti-CD3/CD28 dynal beads and selected with puromycin for another 3 days. Both GFP⁺ and GFP⁻ cells were selected by FACS prior to RNA extraction. Total RNA was extracted and mRNA levels of ThPOK, TIP60, Eomes, and Tbx21 were determined by qRT-PCR. Data show the mean of three separate experiments. Error bars indicate S.D.
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FIGURE 6. Working model describing the role of TIP60 for ThPOK-mediated repression of Eomes. In human CD4+ T cells, short term TCR stimulation promotes TIP60 association with and acetylation of ThPOK to prevent its degradation. TIP60-mediated stabilization of ThPOK augments the transcriptional repression of Eomes, a T-box transcriptional activator of IFNγ, which leads to the reduced production of IFNγ. Thus, TIP60 and ThPOK may contribute to the dampening of Th1-type inflammation.

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