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Long noncoding RNA derived from CD244 signaling epigenetically controls CD8$^+$ T-cell immune responses in tuberculosis infection

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Molecular mechanisms for T-cell immune responses modulated by T cell-inhibitory molecules during tuberculosis (TB) infection remain unclear. Here, we show that active human TB infection up-regulates CD244 and CD244 signaling-associated molecules in CD8$^+$ T cells and that blockade of CD244 signaling enhances production of IFN-γ and TNF-α. CD244 expression/signaling in TB correlates with high levels of a long noncoding RNA (lncRNA)-BC050410 [named as lncRNA-AS-GSTT1(1-72) or lncRNA-CD244] in the CD244$^+$CD8$^+$ T-cell subpopulation. CD244 signaling drives lncRNA-CD244 expression via sustaining a permissive chromatin state in the lncRNA-CD244 locus. By recruiting polycomb protein enhancer of zeste homolog 2 (EZH2) to *infg*/*tnfa* promoters, lncRNA-CD244 mediates H3K27 trimethylation at *infg*/*tnfa* loci toward repressive chromatin states and inhibits IFN-γ/TNF-α expression in CD8$^+$ T cells. Such inhibition can be reversed by knock down of lncRNA-CD244. Interestingly, adoptive transfer of lncRNA-CD244–depressed CD8$^+$ T cells to *Mycobacterium tuberculosis* (MTB)-infected mice reduced MTB infection and TB pathology compared with lncRNA-CD244–expressed controls. Thus, this work uncovers previously unidentified mechanisms in which T cell-inhibitory signaling and lncRNAs regulate T-cell responses and host defense against TB infection.

tuberculosis | lncRNA | CD8$^+$ T cells

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) infection remains a leading public health threat with high morbidity and mortality around the world (1, 2). CD4$^+$ T cells, CD8$^+$ T cells, and γδ T cells played critical roles in mounting adaptive immune response against MTB infection (3–8). Deciphering the molecular mechanisms for host responses linked to TB pathogenesis and prognosis is of great importance for developing new vaccines and therapeutics and for diagnosis.

Activation and effector functions of T cells are regulated by CD3/T-cell receptor (TCR) signal upon antigenic engagement and by a group of signals from costimulatory molecules, including CD28, cytotoxic T-lymphocyte–associated protein 4 (CTLA4), inducible T-cell costimulator (ICOS), programmed death-1 (PD-1), T cell immunoglobulin mucin-3 (Tim-3), and CD244 (2B4) (9–14). Accumulating evidence suggests that a variety of pathogens, including HIV, simian immunodeficiency virus, hepatitis C virus (HCV), lymphocytic choriomeningitis virus, and *Plasmodium*, induce immune evasion by up-regulating costimulatory molecules such as PD-1, CTLA4, and Tim-3 as a result of repeated antigenic stimulation of T cells. However, T-cell immune responses regulated by these "inhibitory receptors" during TB infection appear to be more complex than what have been observed in chronic viral infections. In fact, we have recently reported that up-regulation of the T cell-inhibitory molecule Tim-3 would lead to an enhanced but not inhibitory anti-TB effector function during active human TB (15), and others have also found that Tim-3 signaling may benefit innate immunity against intracellular MTB (16). Thus, it is necessary to elucidate the mechanisms by which T cell-inhibitory molecules regulate T-cell effector functions producing cytokines during active microbial infection.

Although heritable changes in gene regulation that occur via modification of the DNA without changes to the DNA sequence are often referred to as epigenetic programming, noncoding RNA (ncRNA)-mediated transcriptional or posttranscriptional regulation is one of the major regulation mechanisms for epigenetic programming (17–30). Recent studies have identified thousands of long ncRNAs (lncRNAs) (17, 23) in mammalian genomes that regulate gene expression in a variety of immunological processes (31–35), such as differentiation of T cells (33, 36) and dendritic cells (DCs) (35). Thus, diverse functions among T-cell subpopulations may manifest through highly dynamic changes in lncRNA-regulated epigenetic programming, and lncRNA-regulated epigenetic reprogramming is emerging as a novel mechanism to explain functional plasticity and diversity of T cells. Despite these advances, it remains unclear whether and how lncRNAs act as regulators of T-cell immune response during TB infection.

CD244 (2B4) is a costimulatory receptor regulating immune functions of natural killer (NK) cells (37), and it may also

Significance

Tuberculosis (TB) infection induces up-regulation of T cell-inhibitory molecules on CD8$^+$ T cells, which may induce impairment of CD8$^+$ T-cell immunity. However, how T cell-inhibitory molecules regulate CD8$^+$ T-cell immune responses during TB infection remains unclear. Here, we demonstrate that CD244, a T cell-inhibitory molecule, mediates inhibition of IFN-γ and TNF-α expression through inducing expression of a long noncoding RNA (lncRNA)-CD244. lncRNA-CD244 physically interacts with a chromatin-modification enzyme, enhancer of zeste homolog 2 (EZH2), and mediates modification of a more repressive chromatin state in *infg* and *tnfa* loci. Knock down of lncRNA-CD244 significantly enhances IFN-γ and TNF-α expression and improves protective immunity of CD8$^+$ T cells. This study therefore uncovers a previously unknown mechanism for T-cell immune responses regulated by lncRNA during TB infection.

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The authors declare no conflict of interest.

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provide a negative signal that counters the activation signal provided by TCR engagement in $CD8^+$ T cells (38). It has recently been shown that CD244 is expressed on virus-specific $CD8^+$ T cells (9, 39–41) and that CD244 signaling correlates with viral persistence of hepatitis B virus (HBV) and HCV in humans (9, 39, 40). However, little is known about the molecular mechanism and consequence for CD244 regulation of T-cell effector functions during active TB infection. In this study, we examine whether and how CD244 signaling regulates T-cell effector function and impacts homeostasis and host defense against MTB infection. We hypothesize that sustained CD244 signaling directly or indirectly induces epigenetic changes to regulate the expression of proinflammatory cytokines by TB-specific T cells. Our data provide previously unidentified insights into the mechanism by which T cell-inhibitory signaling-derived lncRNA acts as an epigenetic regulator of IFN-γ and TNF-α production in $CD8^+$ T cells and impacts $CD8^+$ T-cell immunity against active MTB infection.

Results

Active TB Infection Induces CD244 Signaling Cascades in $CD8^+$ T Cells. To determine whether CD244 signaling is involved anti-TB immune responses, we first examined CD244 expression levels in $CD4^+$ T cells, $CD8^+$ T cells, and NK cells. Flow cytometric analysis showed that, compared with healthy controls, active TB infection induced significant increases in $CD244^+CD8^+$ T cells but not $CD244^+CD3^-CD56^{Bright}$ NK cells (Fig. 1 *A* and *B*). In addition, percentages of $CD244^+CD8^+$ T cells were much higher than those of $CD244^+CD4^+$ T cells in peripheral blood mononuclear cells (PBMCs) from either healthy controls or patients with active TB, regardless of ex vivo stimulation with MTB lysates (Fig. 1 *A* and *B*). Furthermore, ex vivo stimulation with MTB lysate induced further increases in percentages of $CD244^+CD8^+$ T cells during active TB infection, suggesting that up-regulation of CD244 in $CD8^+$ T cells is at least partially TB-driven (Fig. 1 *A* and *B*). These data collectively suggested the importance of CD244 signaling in regulating $CD8^+$ T-cell immune responses during active TB infection. The role for $CD8^+$ T cells in anti-TB immunity (5) led us to examine the expression of molecules associated with the CD244 signaling pathway in $CD8^+$ T cells during active TB infection. Because signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) and EWS-Fli1-activated transcript 2 (EAT-2) are potential downstream molecules associated

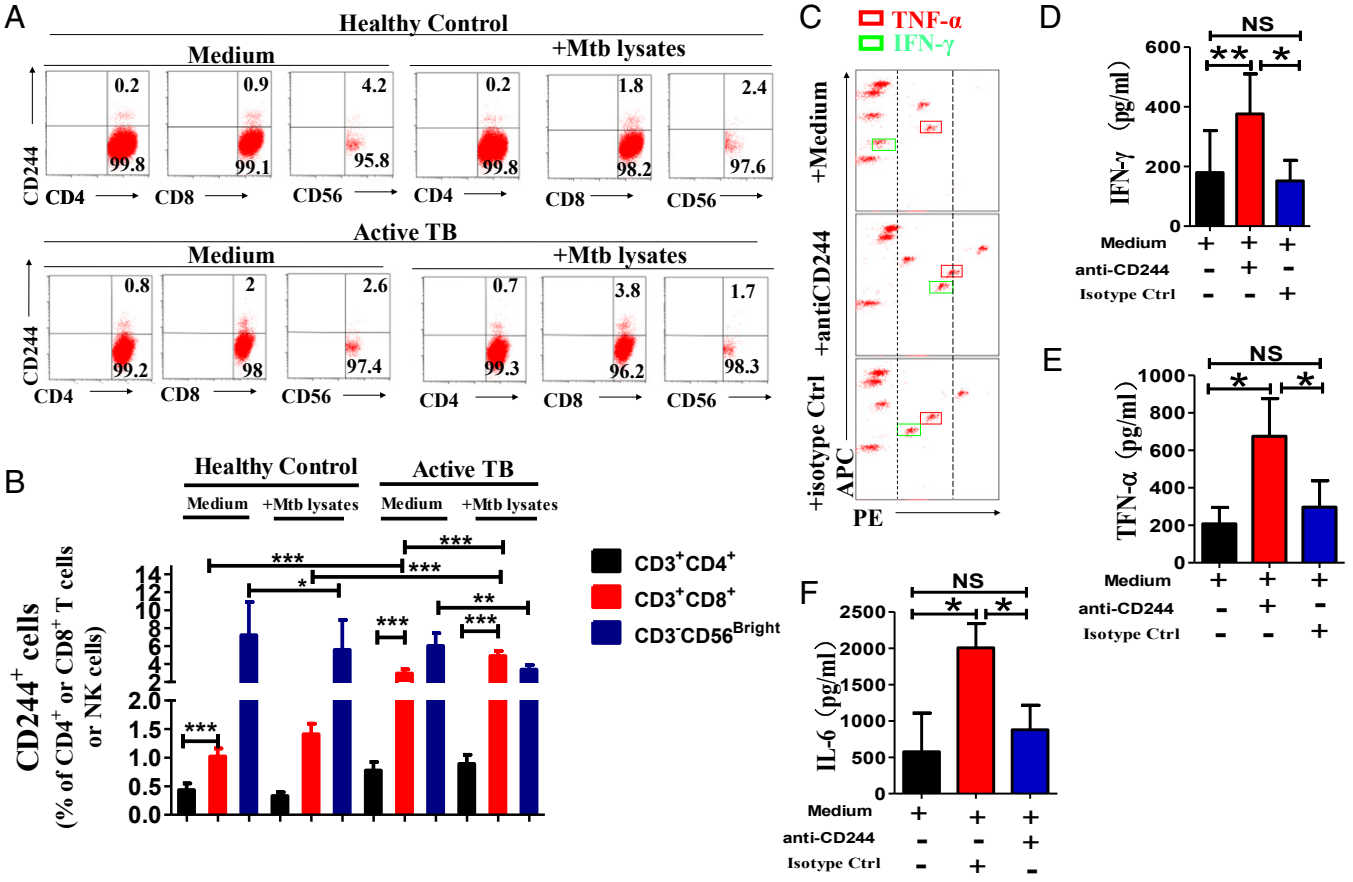


Fig. 1. CD244 is preferentially up-regulated on $CD8^+$ T cells during active MTB infection, and blockade of CD244 signaling enhances production of IFN-γ and TNF-α by $CD8^+$ T cells. (*A*) Representative flow cytometric dot plots show the ex vivo expression of CD244 on $CD4^+$ and $CD8^+$ T cells and CD3-$CD56^{Bright}$ NK cells from one healthy control and one patient with active TB. Data were gated on $CD3^+CD4^+$, $CD3^+CD8^+$, and CD3-$CD56^{Bright}$. Percentages of $CD244^+$ T (or NK) cells are shown in the upright quadruple in each dot plot. PBMCs were treated either with or without ex vivo restimulation with MTB lysates. (*B*) Pooled data show the percentages of $CD244^+CD4^+$ T cells, $CD244^+CD4^+$ T cells, or $CD244^+$NK cells among total $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and NK cells ($n = 15$). Error bars represent SEM. (*C*) Representative CBA assays of a patient with active TB showing that treatment of anti-CD244 mAb induced significant increase of concentration of IFN-γ and TNF-α in culture supernatants of $CD8^+$ T cells purified from PBMCs of patients with active TB. The red and green squares mark the TNF-α and IFN-γ, respectively. The dashed lines mark relative fluorescent intensity of TNF-α and IFN-γ. Treatment of anti-CD244 mAb increased the concentrations of TNF-α and IFN-γ (i.e., the fluorescent intensity of phycoerythrin (PE) increased, and squares shift toward right). (*D–F*) Pooled data show the concentrations of IFN-γ, TNF-α, and IL-6 in the presence of indicated antibody treatment ($n = 7$). $^*P < 0.05$; $^{**}P < 0.01$; NS, no statistical significance. Error bars represent SEM from three independent experiments.

with CD244 signaling (42), we sought to determine whether SAP and EAT-2 could be affected by CD244 signaling in $CD8^+$ T cells during active TB infection. To address this, PBMCs from patients with active TB were transfected with siRNA targeting CD244 (siRNA-CD244), siRNA control (siRNA-Ctrl), or transfection medium. Although we detected significant percentages of SAP^+CD8^+ T-cell subsets and $EAT\text{-}2^+CD8^+$ T-cell subsets in $CD8^+$ T cells from patients with active TB (Fig. S1), siRNA-CD244 transfection significantly decreased percentages of $CD244^+CD8^+$ T, $CD244^+SAP^+CD8^+$ T, and $CD244^+EAT\text{-}2^+CD8^+$ T-cell subsets in total $CD8^+$ T cells (Fig. S1). The data support the idea that SAP and EAT-2 (42–44) may be associated with CD244 signaling in $CD8^+$ T cells during active TB infection.

Anti-CD244 mAb Modulation of CD244 Signaling in $CD8^+$ T Cells from TB Patients Leads to Increased Production of IFN-γ and TNF-α. We then examined the role of CD244 signaling in mediating the effector function of $CD8^+$ T cells. We found that anti-CD244 mAb but not control IgG significantly increased concentration of IFN-γ, TNF-α, and IL-6 in supernatants of cultured $CD8^+$ T cells from patients with active TB (Fig. 1 *C*–*F*). These data suggested that antibody modulation of CD244 on $CD8^+$ T cells of patients with active TB could signal an increase in effector function for cytokine production.

CD244 Signaling Epigenetically Inhibits EZH2 Expression, but the Differential EZH2 Expression Itself Is Not Efficient Enough to Inhibit IFN-γ and TNF-α Production in $CD8^+$ T Cells. We then sought to explore whether CD244 expression or signaling in $CD8^+$ T cells correlated with altered expression of regulatory molecules in active TB infection. We purified $CD244^+CD8^+$ T cells and $CD244^-CD8^+$ T cells from PBMCs of patients with active TB for differential expression of genes, and the hierarchical clustering analysis revealed differential gene expression profiles between $CD244^+CD8^+$ T and $CD244^-CD8^+$ T cells (Fig. 2*A*). Particularly, two genes encoding histone-modification enzymes, including polycomb protein enhancer of zeste homolog 2 (EZH2) and histone deacetylases 11 (HDAC11), were differentially expressed between $CD244^+CD8^+$ T and $CD244^-CD8^+$ T cells (Fig. 2*B*). Confocal microscopic images of immunofluorescence staining of EZH2 verified that $CD8^+$ T cells expressed significant amounts of EZH2 in the nucleus (Fig. 2*C*).

It has been suggested that EZH2 forms two closely related PRC2 complexes that can trimethylate H3K27 (45, 46); this process marks a repressive chromatin state coinciding with gene silencing (47). We thus examined whether differential EZH2 expression between $CD244^+$ and $CD244^-$ subpopulations might impact effector functions of $CD8^+$ T cells during active TB. Indeed, quantitative (q)PCR validation indicated that $CD244^-CD8^+$

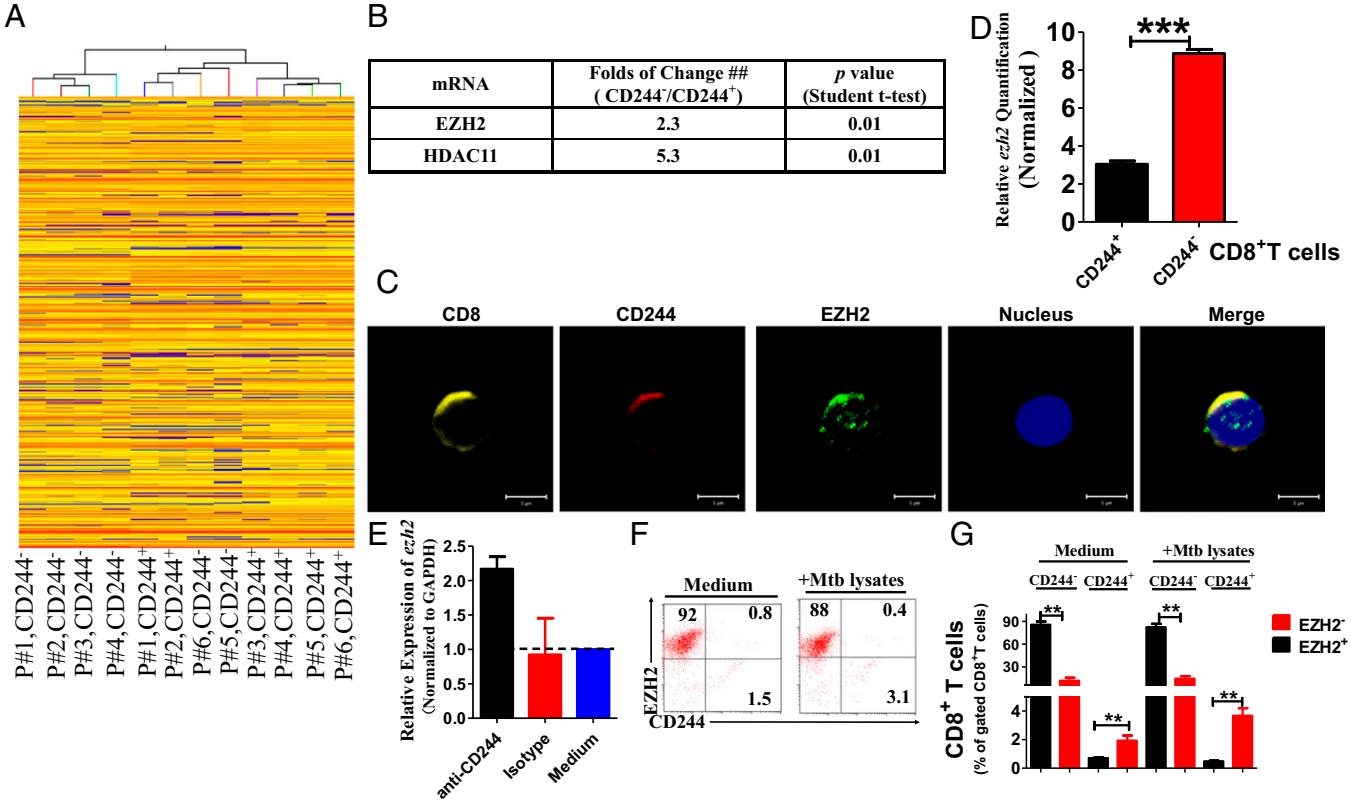
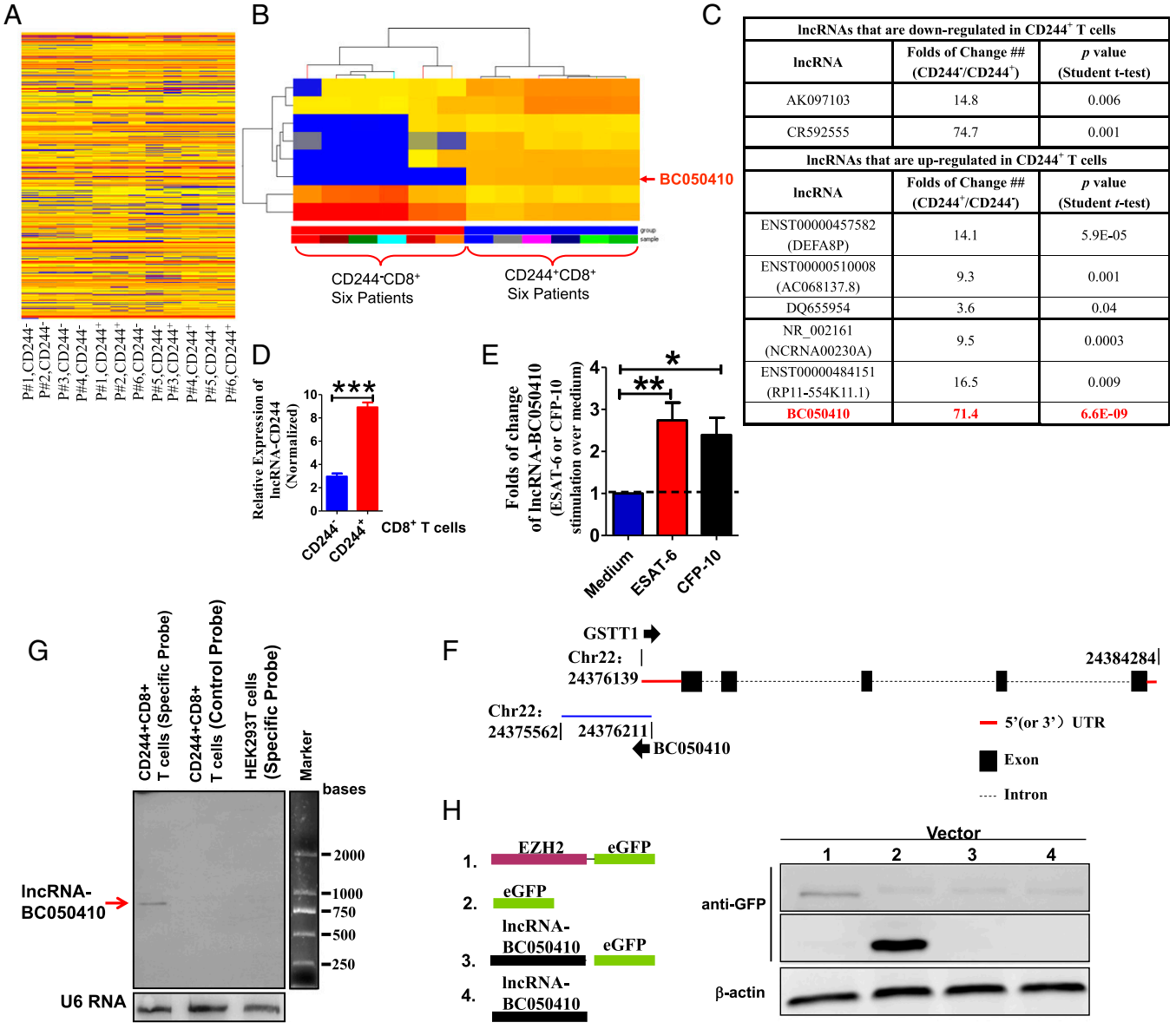


Fig. 2. EZH2 correlates negatively with CD244 signaling. (*A*) Unsupervised clustering analysis of differentially expressed genes between $CD244^+CD8^+$ T cells and $CD244^-CD8^+$ T cells that were purified from PBMCs of patients with active TB. Individual squares represent the relative gene expression intensity of the given genes (rows) in each of six patients (columns), with red indicating an increase in expression and blue a decrease. (*B*) A table shows the fold of changes of expression of EZH2 and HDAC11 in $CD244^+CD8^+$ T cells overexpression in $CD244^-CD8^+$ T cells. (*C*) Typical confocal microscopic images show the expression of EZH2 in $CD244^{high}$ $CD8^+$ T cells in PBMCs derived from patients with active TB. (Scale Bar: 5 μm.) (*D*) qPCR validation of differential expression of EZH2 gene between $CD244^+CD8^+$ T cells and $CD244^-CD8^+$ T cells. (*E*) qPCR analysis of the EZH2 gene in PBMCs from patients with active TB treated with anti-CD244 mAb or control antibody for 5 d. Data are presented as relative expression levels of *ezh2* in PBMCs treated with anti-CD244 mAb or control antibody over expression levels of *ezh2* in PBMCs treated with medium ($n = 7$). Data were normalized to GAPDH. (*F*) Representative flow cytometric dot plot data showing expression of EZH2 and CD244 in $CD8^+$ T cells of PBMCs with or without ex vivo restimulation with MTB lysates. Data were gated on $CD8^+$ T cells. (*G*) Pooled data show the frequency of $EZH2^+CD244^-$, EZH2-$CD244^+$, and $EZH2^+CD244^+$ subpopulations of $CD8^+$ T cells over total $CD8^+$ T cells ($n = 6$). $^{**}P < 0.01$; NS, no statistical significance. Except for *A*, error bars represent SEM from two independent experiments.

T cells expressed much higher levels of EZH2 than their $CD244^+$ counterparts (Fig. 2*D*). Consistently, ex vivo anti-CD244 mAb modulation of CD244 signaling in PBMCs of patients with active TB significantly enhanced *ezh2* gene expression (Fig. 2*E*). In addition, immune costaining of CD244 and EZH2 in $CD8^+$ T cells and flow cytometric analysis showed that percentages of $EZH2^+CD244^-CD8^+$ T-cell subsets or $EZH2^-CD244^+CD8^+$ T-cell subsets are much higher than those of $EZH2^+CD244^+CD8^+$ T-cell subsets regardless of MTB lysate ex vivo restimulation, suggesting that EZH2 and CD244 tend to be expressed in distinct $CD8^+$ T-cell subpopulations during active TB infection (Fig. 2 *F* and *G*). Thus, these results suggest that CD244 signaling negatively regulates EZH2 expression in $CD8^+$ T cells during active TB infection.



lncRNAs that are down-regulated in $CD244^+$ T cells		
lncRNA	Folds of Change ## ($CD244^-/CD244^+$)	*p* value (Student t-test)
AK097103	14.8	0.006
CR592555	74.7	0.001
lncRNAs that are up-regulated in $CD244^+$ T cells		
lncRNA	**Folds of Change ## ($CD244^+/CD244^-$)**	***p* value (Student *t*-test)**
ENST00000457582 (DEFA8P)	14.1	5.9E-05
ENST00000510008 (AC068137.8)	9.3	0.001
DQ655954	3.6	0.04
NR_002161 (NCRNA00230A)	9.5	0.0003
ENST00000484151 (RP11-554K11.1)	16.5	0.009
BC050410	71.4	6.6E-09

Fig. 3. lncRNA-CD244 is highly expressed in $CD244^+CD8^+$ T cells during active TB. (*A*) Unsupervised clustering analysis of differentially expressed lncRNAs between $CD244^+CD8^+$ T cells and $CD244^-CD8^+$ T cells that were purified from PBMCs of patients with active TB. Individual squares represent the relative lncRNA expression intensity of the given lncRNAs (rows) in each of the patients (columns), with red indicating an increase in expression and blue a decrease. (*B*) Supervised clustering analysis using differentially expressed lncRNAs that can distinguish $CD244^+CD8^+$ T cells from $CD244^-CD8^+$ T cells. (*C*) A table shows the folds of change and *P* values (Student *t* test) of eight lncRNAs that could distinguish $CD244^+CD8^+$ T-cell subpopulation from $CD244^-CD8^+$ T-cell subpopulation of six patients with active TB. (*D*) qPCR validation of differential expression of lncRNA-CD244 between $CD244^+CD8^+$ T cells and $CD244^-CD8^+$ T cells. (*E*) qPCR analysis of lncRNA-CD244 expression in $CD244^+CD8^+$ T cells purified from PBMCs of patients with active TB upon stimulation with MTB ESAT-6 (10 μg/mL) or CFP-10 (10 μg/mL) peptide pools for 5 d in vitro. (*F*) Schematic diagram of the lncRNA-CD244 genomic locus in human chromosome 22. The bars represent exons, dashed lines represent intron, red line represents 5′ (or 3′) UTR, and arrows indicate the direction of transcription. The length of lncRNA-CD244 is 796 bases, which overlapped 5′ UTR of GST θ1 (GSTT1) by 72 bases. No homologs of lncRNA-CD244 were found in mouse (Fig. S3 *A* and *B*). (*G*) Northern blot analysis show the expression of full-length lncRNA-CD244 in $CD244^+CD8^+$ T cells from patients with active TB using specific probe but not antisense control probe. No lncRNA-CD244 was detected in HEK293T cells. U6 RNA served as a control. (*H*) Plasmids as schematically shown at *Left* were transfected to HEK293T cells (*Right*). Immunoblotting using antibody specific to EGFP and fluorescent imaging (Fig. S5) showed that lncRNA-CD244-EGFP plasmid and lncRNA-CD244 plasmid did not express GFP. Error bars represent SEM. Data shown in *D*, *E*, *G*, and *H* are representative of at least two independent experiments.

We then determined whether differential expression of EZH2 contributes to inhibition of IFN-γ and TNF-α production by $CD8^+$ T cells during active TB infection. PBMCs from patients with active TB were transfected with siRNA targeting EZH2 (siRNA-EZH2), control siRNA (siRNA-Ctrl), or transfection medium only. Compared with PBMCs transfected with siRNA-Ctrl or medium, transfection of siRNA-EZH2 did not significantly increase production of IFN-γ and TNF-α in PBMC culture supernatants or percentages of IFN-γ^+CD8^+ T cells and TNF-α^+ $CD8^+$ T cells among total $CD8^+$ T cells (Fig. S2 *A*–*G*). Consistently, ChIP-qPCR also showed that, compared with siRNA-Ctrl, siRNA-EZH2 was not able to induce a significant decrease in the amounts of H3K27Me3 at promoter regions of *infg* and *tnfa* loci (Fig. S2*H*). Thus, the differential expression of EZH2 itself in $CD244^+$ T cells was not efficient enough to depress production of IFN-γ and TNF-α by $CD8^+$ T cells in active TB.

CD244 Signaling Positively Correlates with High-Level Expression of TB-Specific lncRNA-BC050410 [lncRNA-AS-GSTT1(1-72) or lncRNA-CD244] in $CD8^+$ T Cells. Because differential EZH2 expression was not efficient enough to inhibit production of IFN-γ and TNF-α by $CD8^+$ T cells, we then investigated the possibility that EZH2 might be recruited to the promoters of IFN-γ and TNF-α in $CD8^+$ T cells to induce repressive chromatin states at *infg* and *tnfa* loci in $CD244^+$ $CD8^+$ T cells. This consideration was supported by the finding that lncRNA might mediate targeted recruitment of repressive histone-modifying activities to epigenetically silence transcription (48–52). We used human lncRNA microarray and hierarchical clustering analyses to compare lncRNA expression in $CD244^+CD8^+$ T cells and $CD244^-CD8^+$ T cells. The comparative analysis between these two subsets allowed us to display a distinct lncRNA expression profile in $CD244^+CD8^+$ T cells (Fig. 3*A*). The supervised hierarchal clustering segregation analysis then identified dominant groups of lncRNAs selectively expressed in $CD244^+CD8^+$ T cells (Fig. 3*B*). Interestingly, lncRNA-BC050410 [named as lncRNA-AS-GSTT1(1-72) based on its genomic context (53) and termed as lncRNA-CD244 here for simplicity] with genomic overlapping to 5′ UTR of GST θ1 (GSTT1) was one of the eight lncRNAs that could distinguish the $CD244^+$ $CD8^+$ T-cell subpopulation from its $CD244^-$ counterpart, with a largest-fold difference and a most-significant *P* value (Fig. 3 *C* and *E* and Fig. S3 *A* and *B*). Note that lncRNA-CR592555, a lncRNA mostly down-regulated in $CD244^+CD8^+$ T cells, was located between 79,946,861 bp and ~79,947,776 bp in chromosome 5 (Fig. S4). Such differential expression of lncRNA-CD244 in $CD244^+CD8^+$ T cells in active TB was also validated by qPCR (Fig. 3*D*). Furthermore, stimulation with peptide pools of 6-kDa early secretory antigenic target (ESAT-6) or 10-kDa culture filtrate protein (CFP-10) of MTB induced higher expression levels of lncRNA-CD244 in purified $CD244^+CD8^+$ T cells, suggesting that expression of lncRNA-CD244 is at least partially TB-specific (Fig. 3*E*). Also, expression of full-length lncRNA-CD244 was confirmed by Northern blot analysis (Fig. 3*F*). We then assessed whether lncRNA-CD244 has protein-coding capability. As with lncRNA-DC (35), analysis based on coding potential calculator (CPC) (Fig. S3 *C*–*E*) suggests that the overall protein-coding potential of lncRNA-CD244 is weak [e.g., the hit score of lncRNA-CD244 (~23.56) is as small as that of lncRNA-DC (~24.35), which suggests that lncRNA-CD244, like lncRNA-DC (35), is unlikely protein-coding; lncRNA-CD244 has five potential ORFs (Fig. S3*F*), but ORF coverage of lncRNA-CD244 (~33.54%) is much smaller than that of lncRNA-DC (~50.72%), which suggests that the ORF quality of lncRNA-CD244 is worse than that of lncRNA-DC]. In addition, analysis of the ratio of the number of nonsynonymous substitutions per nonsynonymous site to the number of synonymous substitutions per synonymous site (Ka/Ks or dN/dS) between two different species of Hominoidea (e.g., dN/dS ratio between human and chimpanzee is 0.48; $P = 0.068 > 0.05$) (Fig. S3*G*) suggests that the ORFs of lncRNA-CD244 lack negative selection in species of Hominoidea. Thus, no bioinformatics evidence implicates

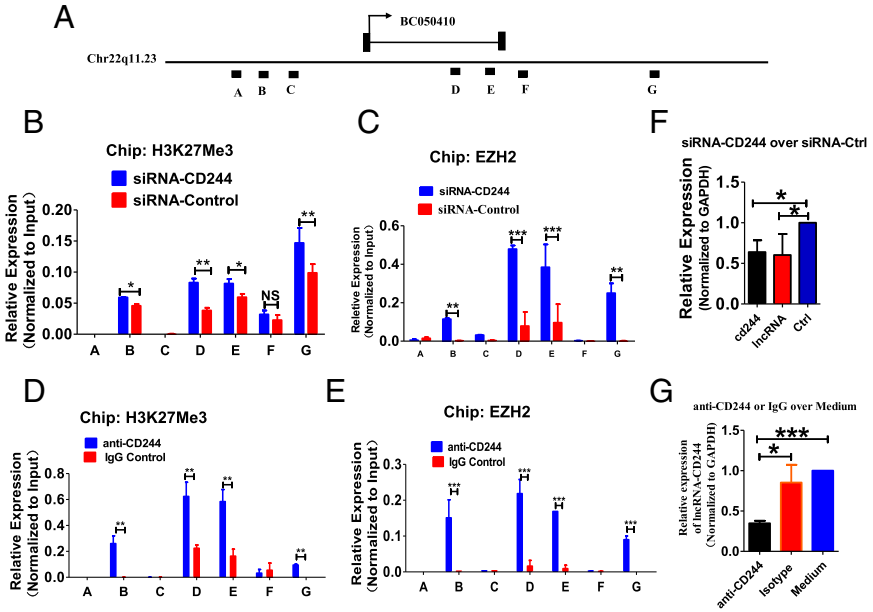


Fig. 4. Knock down of CD244 or blockade of CD244 signaling induces a more repressive chromatin state in lncRNA-CD244 locus and inhibits expression of lncRNA-CD244. Seven regions (capital letters A to G) across lncRNA-CD244 locus, as shown in *A*, were analyzed in ChIP-qPCR analyses for H3K27Me3 (*B* and *D*) histone modification and EZH2 (*C* and *E*) in PBMCs from patients with active TB. PBMCs were transfected with siRNA-CD244 or siRNA-Ctrl or treated with anti-CD244 mAb or IgG control as indicated in each of subfigure. Values derived from three independent experiments were normalized by background signals and input chromatin. (*F* and *G*) qPCR analysis of lncRNA-CD244 and/or the *cd244* gene in PBMCs from patients with active TB transfected (or treated) with indicated siRNAs (*F*) or antibodies (*G*). Data are presented as relative expression levels of lncRNA-CD244 (or *cd244*) (normalized to GAPDH) in siRNA-CD244–transfected (or anti-CD244–treated) PBMCs over expression levels of lncRNA-CD244 (or *cd244*) in siRNA-Ctrl–transfected (or IgG-treated) PBMCs ($n = 7$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, no statistical significance. Error bars represent SEM from three independent experiments.

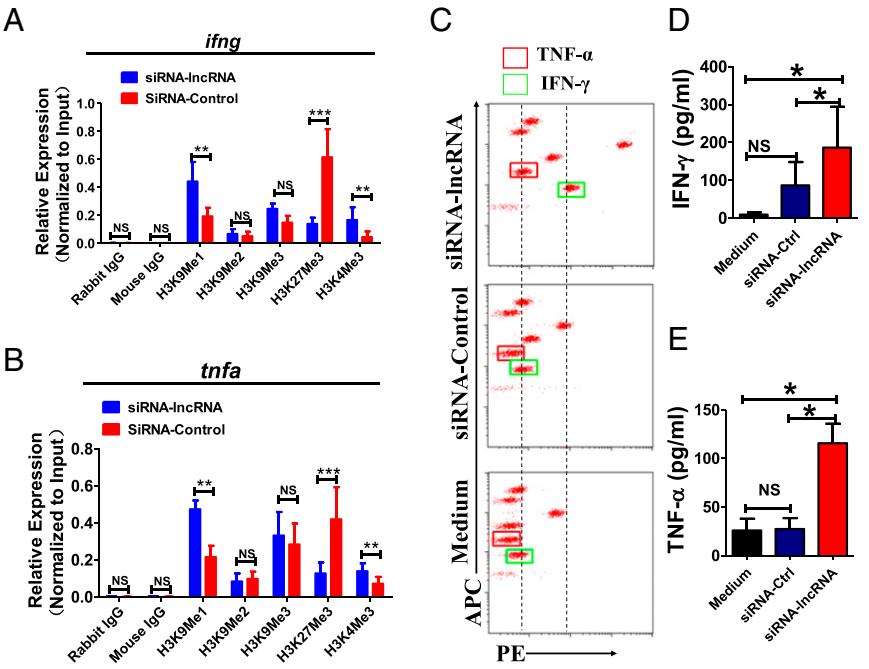


Fig. 5. lncRNA-CD244 regulates IFN-γ and TNF-α expression in active TB infection. (*A* and *B*) ChIP-qPCR analysis of H3K9Me1, H3K9Me2, H3K9Me3, H3K4me3, H3K27Me3, and control antibodies at the promoters of IFN-γ (*A*) and TNF-α (*B*) in $CD8^+$ T cells transfected with indicated siRNAs. (*C*) Representative CBA assays of PBMCs from a patient with active TB showing that, compared with siRNA-Ctrl or transfection medium, siRNA-lncRNA-CD244 induced significant increases in concentrations of IFN-γ/TNF-α. (*D* and *E*) Pooled data showing the concentrations of IFN-γ/TNF-α upon transfection of indicated siRNAs or medium ($n = 7$). *$P <$ 0.05; **$P < 0.01$; ***$P < 0.001$; NS, no statistical significance. Error bars represent SEM from three independent experiments.

lncRNA-CD244 as having protein-coding capability. Furthermore, a vector construct comprising full-length lncRNA-CD244 and EGFP tag was developed and assessed for expression using immunoblotting (IB) and fluorescent imaging. The lncRNA-CD244 did not have any detectable protein-coding ability because neither lncRNA-CD244-EGFP vector nor lncRNA-CD244 vector expressed EGFP (Fig. 3*H* and Fig. S5). Thus, lncRNA-CD244 preferentially expressed in $CD244^+CD8^+$ T cells during active human TB infection.

CD244 Signaling Drives lncRNA-CD244 Expression via Sustaining a More Permissive Chromatin State in lncRNA-CD244 Locus. To determine the mechanisms underlying the preferential expression of lncRNA-CD244 mediated by CD244 signaling, PBMCs of patients with active TB were transfected with siRNA targeting CD244 (siRNA-CD244) and control siRNA (siRNA-Ctrl) or treated with anti-CD244 and control IgG. ChIP-qPCR analysis showed that EZH2 and trimethylation at H3K27, a histone modification that negatively regulates transcription, markedly increased in lncRNA-CD244 loci after treatment with siRNA-CD244 but not siRNA-Ctrl (Fig. 4 *A*–*C*). Consistently, siRNA-CD244, not siRNA-Ctrl, decreased expression of the *cd244* gene and lncRNA-CD244 (Fig. 4*F*). Furthermore, anti-CD244 but not IgG control induced significant increases of H3K27Me3 and EZH2 in lncRNA-CD244 loci (Fig. 4 *A*, *D*, and *E*), and anti-CD244 but not IgG significantly decreased the expression of lncRNA-CD244 (Fig. 4*G*). Taken together, knock down of CD244 or blockade of CD244 signaling induces a more repressive chromatin state in lncRNA-CD244 locus and reduces expression of lncRNA-CD244. Thus, these results implicate CD244 signaling as driving expression of lncRNA-CD244 in $CD8^+$ T cells most likely through sustaining a more permissive chromatin state in the lncRNA-CD244 locus during active TB infection.

lncRNA-CD244 Epigenetically Regulates Expression of IFN-γ and TNF-α by $CD8^+$ T Cells. We then determined whether lncRNA-CD244 could mediate epigenetic regulation of IFN-γ and TNF-α expression in $CD8^+$ T cells during active human TB infection. Accumulative evidence suggests that H3 trimethylation on lysine4 (H3K4me3) and histone H3 methylation on lysine9 (H3K9Me1) are indicative of permissive chromatin states (54). In contrast, histone H3 trimethylation on lysine 27 (H3K27Me3) and histone H3 methylation and dimethylation on lysine9 (H3K9Me1, H3K9Me2) are indicative of repressive chromatin states (54). We hypothesized that lncRNA-CD244 might affect IFN-γ and TNF-α production at the transcriptional level by epigenetic regulation of histone modification states at *infg* and *tnfa* loci. To address this hypothesis, $CD8^+$ T cells isolated from PMBCs of patients with active TB were transfected with siRNA-lncRNA-CD244 to knock down the expression of lncRNA, with transfection of siRNA-Ctrl as a control. The amounts of H3K9Me1, H3K9Me2, H3K9Me3, H3K27Me3, and H3K4Me3 at the promoters of *infg* and *tnfa* were determined by ChIP-qPCR. Interestingly, transfection with siRNA-lncRNA, but not siRNA-Ctrl, led to a significant increase in H3K9Me1 and H3K4Me3 and decrease in H3K27me3 at *infg* and *tnfa* loci (Fig. 5*A* and *B*). Consistently, much greater concentrations of IFN-γ and TNF-α were detected via cytometric bead array (CBA) analysis in culture supernatants of PBMCs transfected with siRNA-lncRNA compared with siRNA-Ctrl or medium controls (Fig. 5 *C*–*E*). Also, $CD8^+$ T cells purified from PBMCs of patients with active TB were transduced with lentiviral (LV) vector encoding GFP and shRNA targeting lncRNA-CD244 (LV-lncRNA) or LV vector encoding GFP only (LV-Ctrl). We found that transduction with LV-lncRNA, but not LV-Ctrl or medium, in $CD8^+$ T cells induced significant increases in IFN-γ and TNF-α in culture supernatants (Fig. S6), suggesting that lncRNA-CD244 indeed regulated expression of IFN-γ and TNF-α by $CD8^+$ T cells. Moreover, such enhanced production of IFN-γ and TNF-α upon knock down of lncRNA-CD244 was unlikely caused by the impact of lncRNA-CD244 on GSTT1 expression, because we found no significant changes in GSTT1 expression upon knock down of lncRNA-CD244 (Fig. S7*A*) and no significant enhancement of IFN-γ

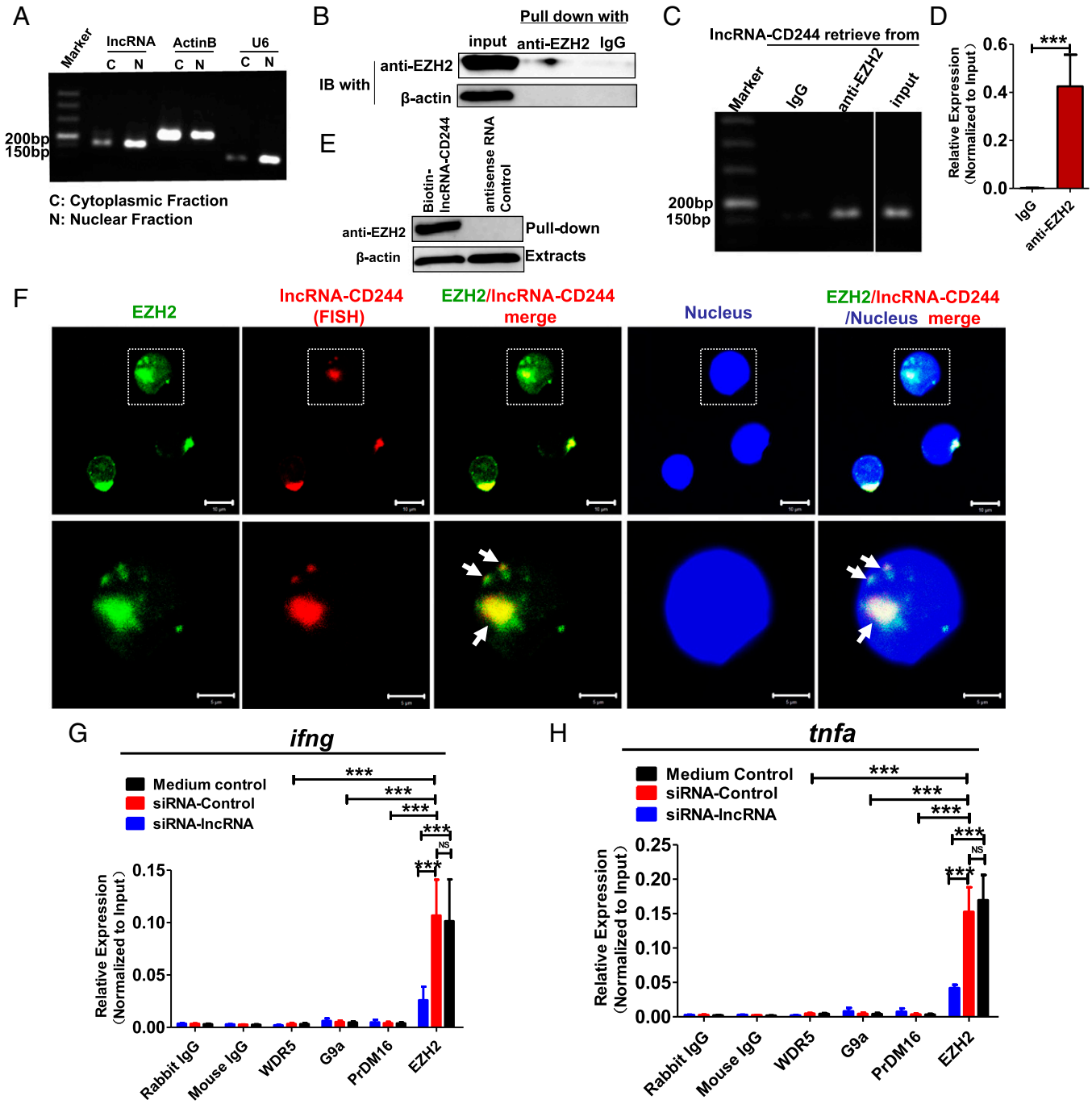


Fig. 6. lncRNA-CD244 interacts directly with EZH2 and recruits EZH2 to *ifng* and *tnfa* promoters. (*A*) Gel electrophoresis of lncRNA-CD244 extracted from nucleus and cytoplasm of $CD8^+$ T cells purified from PBMCs of patients with active TB. As controls, more actin B expressed in cytoplasm and more U6 expressed in nucleus, respectively. (*B*) IB analysis of EZH2 in the IP by IgG or anti-EZH2–specific antibody from $CD8^+$ T-cell lysates of patients with active TB. (*C* and *D*) Gel electrophoresis (*C*) and qPCR analysis (*D*) of lncRNA-CD244 retrieve in IP by IgG or anti-EZH2–specific antibody from $CD8^+$ T-cell lysates of patients with active TB. The levels of qRT-PCR products were expressed as a percentage of input RNA. (*E*) Biotinylated lncRNA-CD244 or antisense RNA control was incubated with nuclear extracts of $CD8^+$ T cells from patients with active TB and targeted with streptavidin-conjugated magnetic beads (MB), and associated proteins were assessed with Western blot using anti-EZH2–specific antibody. (*F*) Confocal microscopic images of RNA FISH assay of lncRNA-CD244 and immunofluorescence analysis of EZH2 show that EZH2 colocalizes with lncRNA-CD244 in nucleus of $CD8^+$ T cells from patients with active TB. Lower images were cropped from the squares in the upper images. (Scale bars: 10 μm in *Upper* and 5 μm in *Lower*.) More than 30 cells were examined and had similar results. White arrowheads mark the EZH2/lncRNA-CD244 colocalization. (*G* and *H*) ChIP-qPCR analysis of WDR5, G9a, Prdm16, EZH2, and control antibodies at the promoters of IFN-γ and TNF-α in $CD8^+$ T cells transfected with siRNA-lncRNA and siRNA-Ctrl ($n = 7$). ***$P < 0.001$. Error bars represent SEM from three independent experiments.

and TNF-α production upon GSTT1 knock down by shRNA (Fig. S7 *B* and *C*). Furthermore, such enhanced production of IFN-γ and TNF-α was not attributable to changes in viability of $CD8^+$ T cells because $CD8^+$ T cells did not exhibit enhanced apoptosis after lncRNA-CD244 knockdown (Fig. S7 *D* and *E*). Thus, these results collectively suggest that whereas TB-driven up-regulation

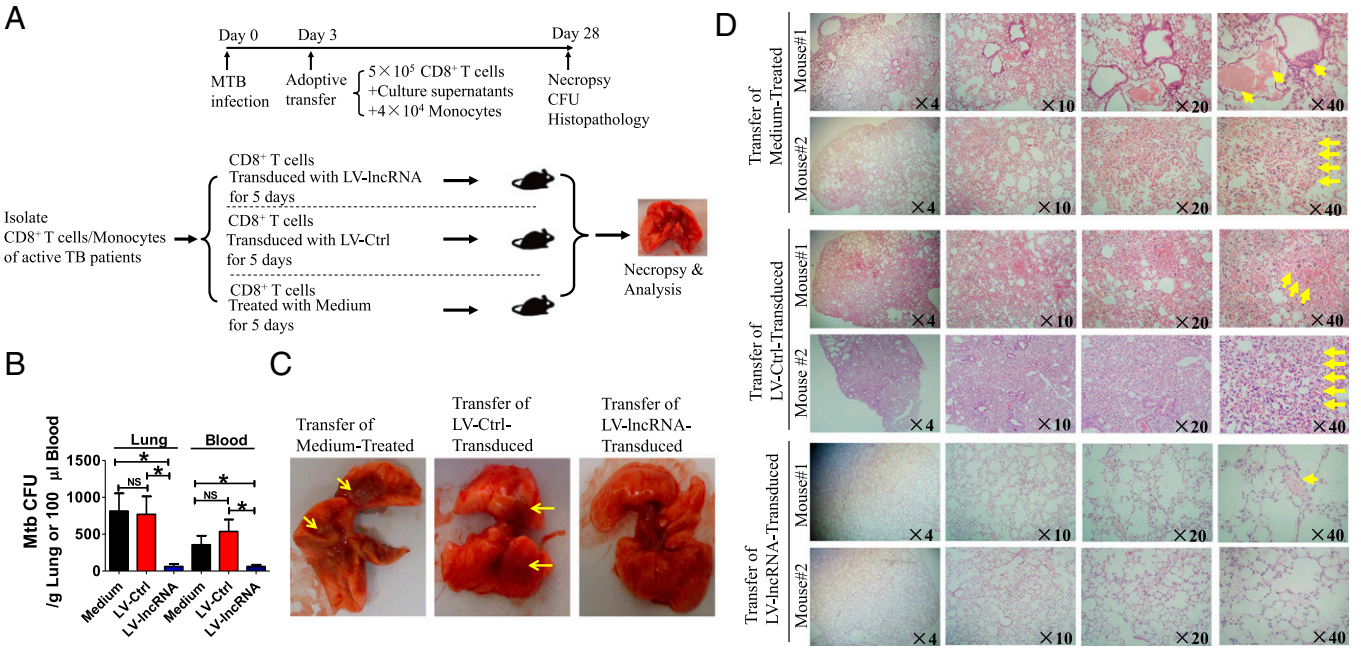


Fig. 7. Adoptive transfer of lncRNA-CD244–depressed CD8$^+$ T cells to MTB-infected SCID mice reduced MTB infection and TB pathology compared with lncRNA-CD244–expressed controls. (*A*) Schematic diagram shows the experimental strategy of adoptive transfer of CD8$^+$ T cells with inhibited lncRNA-CD244 expression, culture supernatants of indicated CD8$^+$ T cells, and monocytes in MTB-infected mice. (*B*) Bacterial load (CFU, H37Rv) in the lungs and peripheral blood of mice indicated in *A* ($n = 8$ mice per group). (*C*) Representative digital camera images of lungs collected from mice receiving transfer of indicated cells with or without knock down of lncRNA-CD244. Yellow arrows mark the necrosis. (*D*) H&E-stained lung sections derived from two representative mice in each group of mice indicated in *A*. Yellow arrows mark the infiltration of red blood cells or damage of pulmonary structure. The magnification is shown at the lower right of each image. *$P < 0.05$. Error bars represent SEM from two independent experiments.

of lncRNA-CD244 might influence repressive chromatin states at *infg* and *tnfa* loci, silence or down-regulation of lncRNA-CD244 could confer permissive chromatin states at *infg* and *tnfa* loci and enhance expression of IFN-γ and TNF-α.

lncRNA-CD244 Associates Physically with EZH2 and Mediates Recruitment of EZH2 to *ifng* and *tnfa* Loci for Repressive Chromatin States. Next, we sought to examine the interrelation of lncRNA-CD244, repressive chromatin states at *infg* and *tnfa* loci, and altered expression of EZH2 (Fig. 2). We presumed that lncRNA-CD244 could mediate the recruitment of the histone-modifying enzyme EZH2, which catalyzed the trimethylation modification of H3K27 at promoters of *infg* and *tnfa*. Of note, while performing gel electrophoresis of RNA extracts of nuclear and cytoplasmic fractions of CD8$^+$ T cells from patients with active TB, we found that most of the lncRNA-CD244 localized in the nuclear fraction of CD8$^+$ T cells (Fig. 6*A*). In addition, when we performed immunoprecipitation (IP) of EZH2 in extracts of CD8$^+$ T cells from TB patients or in HEK293T cells with exogenous expression of EZH2 and full-length lncRNA-CD244, we found that EZH2-specific mAb, but not control IgG, could actually coprecipitate lncRNA-CD244 molecules, as detected by qRT-PCR (Fig. 6 *B–D* and Fig. S8). Furthermore, biotinylated lncRNA-CD244 and an antisense control RNA were incubated with nuclear extracts of CD8$^+$ T cells from patients with active TB, and Western blotting showed that lncRNA-CD244 but not antisense control RNA specifically bound to EZH2 (Fig. 6*E*). Consistently, when we performed confocal microscope-based FISH analysis of lncRNA-CD244 and immunofluorescent analysis of EZH2, we found that significant amounts of EZH2 colocalized with lncRNA-CD244 in the nucleus of CD8$^+$ T cells from patients with active TB (Fig. 6*F*). These results suggest that lncRNA-CD244 is physically associated with EZH2 during active TB. Furthermore, transfection of CD8$^+$ T cells with siRNA-lncRNA-CD244, but not siRNA-Ctrl, led to significant decreases in EZH2, but not WDR5, Prdm16, and G9a, at either the *infg* or *tnfa* promoter (Fig. 6 *G* and *H*). ChIP-qPCR analysis also demonstrated that EZH2 accumulated at promoters of *infg* or *tnfa* at much greater levels than WDR5, PRDM16, and G9a in CD8$^+$ T cells from patients with active TB (Fig. 6 *G* and *H*). These results suggest that EZH2 and lncRNA-CD244 complex formation may lead to trimethylation of H3K27, which contributes to inducing repressive chromatin states at *infg* or *tnfa* loci in CD8$^+$ T cells during active TB infection. The data also suggest a hypothetical model in which expression of lncRNA-CD244 may physically recruit EZH2 to control H3K27Me3 at the *ifng* and *tnfa* loci and therefore allow chromatin to program repressive states and inhibit transcription of *infg* and *tnfa* genes in CD244$^+$CD8$^+$ T cells.

lncRNA-CD244 Is a Major Regulator of CD8$^+$ T-Cell Immune Response During in Vivo MTB Infection. Finally, we sought to determine whether the lncRNA-CD244 could regulate immune response of CD8$^+$ T cells in vivo. Because no mouse homolog of lncRNA-CD244 was found (Fig. 3*F* and Fig. S3 *A* and *B*), we developed an adoptive transfer strategy to determine the in vivo role of lncRNA-CD244 in regulating CD8$^+$ T-cell immune response during MTB infection (Fig. 7*A*). CD8$^+$ T cells and CD14$^+$ monocytes were first purified from PBMCs of patients with active TB. CD8$^+$ T cells were then transduced with LV-lncRNA to knock down the expression of lncRNA-CD244 and enhance production of IFN-γ and TNF-α (Fig. S6). LV-lncRNA–transduced (lncRNA-CD244–depressed) CD8$^+$ T cells were then adoptively transferred to SCID mice on the third day after MTB infection (Fig. 7*A*). LV-Ctrl–transduced (lncRNA-CD244–expressed) CD8$^+$ T cells and medium-treated (lncRNA-expressed) CD8$^+$ T cells served as controls, respectively. At the same time of CD8$^+$ T-cell transfer, each mouse also received autologous monocytes from TB patients to facilitate MTB infection (Fig. 7*A*). Interestingly, mice infused with LV-lncRNA–transduced (lncRNA-CD244–depressed) CD8$^+$ T cells showed significantly lower MTB bacterial burdens in lungs and blood compared with

control mice infused with LV-Ctrl–transduced (lncRNA-CD244–expressed) $CD8^+$ T cells or medium-treated (lncRNA-CD244–expressed) $CD8^+$ T cells (Fig. 7*B*). The control mice infused with LV-Ctrl–transduced $CD8^+$ T cells or medium-treated $CD8^+$ T cells exhibited severer lung necrosis (Fig. 7*C*) and histopathology characterized by significant hemorrhages or infiltration of RBCs in alveoli and apparent damage of pulmonary structures (Fig. 7*D*). In contrast, only mild changes in alveoli were observed in lung sections of mice infused with LV-lncRNA-CD244–transduced $CD8^+$ T cells (Fig. 7*D*). Thus, lncRNA-CD244–depressed $CD8^+$ T cells can more potently control in vivo MTB infection than lncRNA-CD244–expressed $CD8^+$ T cells in SCID mice, suggesting that lncRNA-CD244 may play a role in regulating in vivo immune response of $CD8^+$ T cells during MTB infection.

Discussion

Molecular mechanisms for CD244 modulation of T-cell responses in infections remain unknown, although CD244 appears to regulate NK cell function that correlates with HBV or HCV persistence in humans (40, 41). In this study, we find that human TB can upregulate CD244 and CD244 signaling-related molecules in $CD8^+$ T cells. Whereas the $CD244^+CD8^+$ T-cell subset from TB patients expresses much higher levels of lncRNA-CD244 than its $CD244^-$ $CD8^+$ T-cell subset counterpart, siRNA or shRNA knock down of lncRNA-CD244 leads to increased production of IFN-γ and TNF-α. The blockade of CD244 signaling by anti-CD244 mAb can similarly lead to in vitro enhancement of IFN-γ and TNF-α production. Thus, we demonstrate the interrelation between CD244 signaling, lncRNA-CD244 expression, and regulation of effector function in $CD8^+$ T cells at epigenetic levels in TB patients.

The current study provides a previously unidentified mechanism by which CD244 signaling regulates IFN-γ and TNF-α production in human $CD8^+$ T cells in MTB infection. CD244 signaling during active MTB infection can exploit lncRNA and histone-modifying enzymes to regulate the effector functions of $CD244^+CD8^+$ T cells. Often, CD244 expression/signaling in $CD244^+CD8^+$ T cells from TB patients leads to remarkable increases in expression of lncRNA-CD244. Interestingly, lncRNA-CD244 appears to physically interact with a polycomb protein, EZH2. This interaction mediates recruitment of EZH2 to *infg* and *tnfa* loci and trimethylates H3K27 at promoters of *infg* and *tnfa* toward repressive chromatin states and suppression of *infg* and *tnfa* expression. Our findings support the hypothesis that ncRNA can interact with chromatin and mediate targeted recruitment of repressive histone-modifying activities to epigenetically silence transcription (49, 51). In fact, it has been recently shown that a number of intronic RNA sequences are capable of binding to the core component EZH2 and regulating the transcriptional output of its genomic counterpart (55). In addition, the ncRNAs *HOTAIR* (56), *Xist* (57), and *RepA* (58) might recruit the polycomb complex to the *HoxD* locus or the X chromosome, respectively, where they mediate trimethylation of H3K27 and induce heterochromatin formation and repression of gene expression. Furthermore, EZH2 is also shown to bind strongly to genes encoding the transcription factors T-bet, Eomes, and Gata3; controls differentiation into Th1 and Th2 effector cells; and regulates plasticity of these subsets after differentiation (59). However, we cannot rule out the possibility that lncRNA-CD244 in our model may regulate the repression of *infg* and *tnfa* via other undefined epigenetic mechanisms (48, 50).

The data from the current study also suggest that down-regulation of lncRNA-CD244 in $CD8^+$ T cells from TB patients can yield a favorable in vivo consequence during MTB infection at SCID model. Adoptive transfer of lncRNA-CD244–depressed $CD8^+$ T cells with the enhanced IFN-γ/TNF-α production could attenuate MTB infection and TB pathology in SCID mice compared with lncRNA-CD244–expressed controls. Enhanced CTL activity and IFN-γ/TNF-α production of lncRNA-CD244–depressed $CD8^+$ T cells may contribute to inhibition of intracellular MTB replication in infused autologous monocytes/macrophages. This notion is supported by the previous studies demonstrating $CD8^+$ T-cell-mediated anti-TB immunity (5). Thus, the identification of the CD244 and lncRNA-CD244 axis in the modulation of IFN-γ and TNF-α expression provides an lncRNA-driven epigenetic program of T-cell immunity against MTB infection.

lncRNAs have recently been in the spotlight for their critical roles in human biology and diseases (21, 22, 34, 50, 60, 61). They have been explored as biomarkers for cancers and potential targets for disease or dysregulated expression of genes or phenotypes (49). Here, we demonstrate that lncRNA-CD244 serves as one of the mechanisms by which CD244 signaling regulates the ability of human $CD8^+$ T cells to produce IFN-γ and TNF-α at epigenetic levels in MTB infection. To our knowledge, this is the first evidence that lncRNA is one of the major epigenetic factors modulating Th1 immune response in human TB.

Thus, this study allows us to demonstrate that CD244 signaling in active human TB regulates repression of IFN-γ and TNF-α through a mechanism in which lncRNA-CD244 modulates recruitment of EHZ2 to promoters of IFN-γ and TNF-α for potential trimethylation of H3K27 and repression of *infg* and *tnfa* expression (Fig. S9). The CD244 signaling and lncRNA-CD244 modulation of IFN-γ and TNF-α expression presents an lncRNA-driven epigenetic program of T-cell immunity against microbial infection. Our findings also suggest that lncRNA-CD244 may be a potential target for therapeutic intervention of TB.

Materials and Methods

Study Subjects. Active TB infections were confirmed based on clinical symptoms, chest radiography, sputum staining for acid-fast bacilli (AFB), and laboratory culture and PCR for MTB that were carried out in the Institute for Chronic Diseases Prevention of Huadu District in Guangzhou, China. All patients were not receiving anti-TB therapy at the time of analysis. Patients with active TB were recruited in our study and gave written informed consent according to the protocols approved by the institutional review and the ethics boards of the Zhongshan School of Medicine of Sun Yat-sen University (SYSU).

Animal study protocols were also reviewed and approved by the SYSU Institutional Animal Care and Use Committee.

Statistics. Statistical analysis was performed using GraphPad Prism. Statistical significance was determined with Student *t* test. A value of $P < 0.05$ was considered significant. Asterisks in the figures represent the following: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. "NS" in the figures indicates no statistical significance.

Full materials and methods and any associated references for following experiments are described in detail in *SI Materials and Methods*: isolation of PBMCs; antibodies for flow cytometry; CBA analysis of the cell culture supernatants; blocking experiments using anti-CD244 mAb; intracellular cytokine staining (ICS) and flow cytometric assay; siRNA transfection; purification of monocytes, $CD8^+CD244^-$ T cells, and $CD8^+CD244^+$ T cells; mRNA and lncRNA microarray analysis, data analysis, and statistics; lentivirus-mediated knock down of lncRNA-CD244 or GSTT1; qPCR; ChIP-qPCR; RNA IP (RIP)-qPCR; nuclear and cytoplasmic extraction of lncRNA; RNA FISH and immunofluorescence microscopy; confocal microscopic analysis; bioinformatics analyses of evolutionary conservation and coding potential of lncRNA-CD244 and plasmid constructions; Northern blot assay of lncRNA; lncRNA pull-down assay; MTB infection of mice; adoptive transfer; and histopathological, bacterial, and immune analyses of MTB-infected mice.

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