Title *Mafb* Deficiency in Myeloid Cells Increases Susceptibility to *Mycobacterium*

***tuberculosis* Infection in Mice**

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Abstract

v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*)

is a candidate gene associated with early tuberculosis onset identified by a genome-wide

association study. Here, we investigated the role of *Mafb* in susceptibility to

Mycobacterium tuberculosis (*Mtb*) infection in myeloid-specific *Mafb*-knockout (*Mafb*-

cKO) mice. We infected bone marrow-derived macrophages (BMMs) from *Mafb*-cKO

mice and *Mafb*-cKO mice with *Mtb*. The absence of *Mafb* promoted *Mtb* proliferation in

BMMs. RNA sequencing (RNA-seq) revealed activation of the metabolic process and

impairment of the response to type I interferons (IFNs) in *Mtb*-infected BMMs from

Mafb-cKO mice, which conforms to our previous findings in *Mtb*-infected human

macrophages with *MAFB* knockdown. *Mafb* deficiency increased mortality and

bacterial burden in the lungs and spleens during *Mtb* infection in mice. RNA-seq

revealed weakened leukocyte or lymphocyte chemotaxis in *Mtb*-infected *Mafb*-cKO

mouse lungs. Flow cytometry demonstrated an alteration in the proportion of immune

cells in *Mtb*-infected mouse lungs due to *Mafb* deficiency. Together, *Mafb* in myeloid

cells is involved not only in the functional antibacterial process of macrophages but also

in immune cell recruitment in the lungs, thereby contributing to host defense against

Mtb infection.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) infection, has resurged as the leading infectious disease, with 8.2 million newly diagnosed cases and 1.2 million deaths in 2023 alone (1). The newly proposed spectrum of TB categorizes this disease into five stages based on the level of infectiousness, presenting symptoms, and macroscopic pathology (2). In this category, subclinical TB is accountable for half the TB burden in any community, making it potentially infectious without any symptoms (3). Therefore, early diagnosis, preventive therapy, or treatment of subclinical TB is critical to prevent further transmission and to ultimately achieve global TB elimination (4). Several studies have attempted to estimate the activation risk based on gene signatures or transcriptional biomarkers (5). Notably, it is important to investigate the factors that determine the trajectory of infection in TB patients or drive susceptibility to TB so as to understand this complex disease as well as to accelerate research in drug and vaccine development.

To date, numerous genome-wide association studies (GWASs) have been conducted to investigate the host genetic factors in TB susceptibility. However, only a few associations have proven reproducibility owing to the modest population sizes, variability in phenotyping across studies, population-specific effects, or complex population structures under certain high-burden settings (6). A meta-analysis combining two GWASs in Thai and Japanese populations did not replicate the association of 25 selected single-nucleotide polymorphisms (SNPs) (7). However, the age-stratified analysis from the same dataset revealed a significant locus on chromosome 20q12 linked to the younger onset group. This locus is located approximately 450-kb upstream of *v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B* (*MAFB*). Early-

onset of TB implies the relatively sooner development after exposure to *Mtb*. The

GWAS result suggests that *MAFB* plays a role in the host immunity toward controlling

Mtb infection. With this background, we investigated the role of *MAFB* as a promising

candidate gene involved in TB susceptibility.

MAFB belongs to the large Maf family of transcription factors characterized by

a conserved basic leucine zipper (bZip) enabling specific DNA binding to Maf-

recognition elements (MAREs) (8). *Mafb* was first identified as a causative gene for

segmentation abnormalities in the hindbrain and in defective inner ear development (9).

Lethal phenotypes observed in *Mafb*-null mice, including respiratory failure, renal

dysgenesis, and parathyroid abnormalities, indicate the critical role of *Mafb* in

organogenesis (10). *Mafb* has been demonstrated to control the expression of apoptosis

inhibitor of macrophages (AIM) and promote atherosclerosis through inhibiting foam-

cell apoptosis (11). In addition, *Mafb* plays diverse roles in various biological processes

(BPs), including the myeloid commitment of hematopoietic stem cells, self-renewal of

macrophages, generation of thymus, or the creation of hair cuticles (10).

In the context of immune regulation and infectious disease, *MAFB* has been

reported to control antiviral response and macrophage polarization (12, 13). Previously,

we investigated the function of *MAFB* in *Mtb*-infected human macrophages to explore

the biological mechanism underlying *MAFB* in macrophages (14). Our gene knockdown

(KD) experiments revealed that MAFB regulates the gene expression related to

interferon (IFN) responses in *Mtb*-infected macrophages. In the present study, we

investigated the role of *MAFB*, particularly in disease outcomes and dynamic immune

cell interactions in organisms by using myeloid-specific *Mafb*-knockout (*Mafb*-cKO)

mice (15). We monitored the survival and bacterial burden in the murine organs and

found that *Mafb*-cKO mice had higher mortality and bacterial burden during the *Mtb*

infection. RNA sequencing (RNA-seq) of *Mtb*-infected *Mafb*-cKO mouse lungs

revealed a disrupted chemotaxis. These results were consistent with altered immune cell

populations in the lungs of *Mtb*-infected *Mafb*-cKO mice. Taken together, this study

highlights *MAFB* as an important gene in macrophages that contributes to protective

immunity against *Mtb* infection.

Materials and Methods

Ethics statement

Animal experiments in this study were approved by the Animal Care and Use

Committee of the Research Institute of Tuberculosis (RIT) (permit number: No. 2021-

04). Animals were treated in accordance with the ethical guidelines of RIT. The

endpoints were set to determine whether the mice were imminently dying of *Mtb*

infection and/or required compassionate euthanasia: bodyweight loss >20% of the initial

bodyweight at the time of infection.

Mice

Macrophage-specific *Mafb* conditional-knockout (*Mafb*$^{f/f}$::LysM-Cre$^{+/+}$ or

Mafb$^{f/f}$::LysM-Cre$^{+/-}$, *Mafb*-cKO) and *Mafb*$^{f/f}$ control mice were used (15). *Mafb*-cKO

and control mice (*Mafb*$^{f/f}$) were maintained in a filtered-air laminar-flow cabinet and

provided with sterile bedding, water, and mouse chow at an RIT animal facility. Wild-

type (WT) C57BL/6J mice were obtained from The Jackson Laboratory Japan, Inc.

Specific pathogen-free status was verified by testing sentinel mice housed within the

colony.

Mtb culture

The *Mtb* strain Erdman was used and stored as previously described (16-18).

For determining the bacterial burden in macrophages, the infected cells were lysed with

PBS containing 0.1% SDS. Infected lungs or spleens were homogenized using a

ShakeMaster Neo (Bio Medical Science). The resulting cell lysates or homogenates

were serially diluted and plated in duplicate on 7H10 or 7H11 agar plates supplemented

with 10% Middlebrook OADC (BD Bioscience) and 0.5% glycerol. *Mtb* colony-

forming units (CFUs) were determined by calculating the mean CFU count from the

two plates at each dilution.

Bone marrow-derived macrophage (BMM) isolation

BMMs were differentiated as described previously (19), with some

modifications. Briefly, bone marrow was isolated from the hind legs of *Mafb*-cKO and

control mice (6 weeks), washed, and suspended into a single cell by passing through a

cell strainer. The bone marrow cells were then incubated at a concentration of 2×10^6

cells/mL in DMEM (Sigma-Aldrich) supplemented with 10% inactivated-fetal bovine

serum (FBS, JRH Biosciences Inc.) and 10% of L929-conditioned medium in a 12-well

plate for 7 days. Differentiated macrophages in DMEM containing 10% FBS were

infected with *Mtb* at a multiplicity of infection (MOI) of one. At one day postinfection

(p.i.), BMMs were collected for mRNA sequencing (mRNA-seq). At 1, 3, and 7 days

p.i., the number of the intracellular bacteria within BMMs was determined by CFU.

Mtb infection in mice

The experimental mice (age: 6–10 weeks) were transferred to a biosafety level

3 animal facility at RIT. The mice were infected with *Mtb* via the aerosol route using an

inhalation exposure system (Glas-Col). This method routinely gave *Mtb* infection at

100–200 CFU per lung one day p.i.

Survival study

WT and *Mafb*-cKO mice infected with *Mtb* were monitored for 315 days. The

mice that survived throughout the experiments or met the endpoint were euthanized by

exsanguination under anesthesia with 0.75 mg/kg of medetomidine, 4.0 mg/kg of

midazolam, and 5.0 mg/kg of butorphanol *via* the intraperitoneal route (20). Survival

probabilities between the two groups were analyzed using Kaplan–Meier analysis and

the log-rank test.

mRNA-seq

mRNA-seq was performed as described previously (18). Briefly, infected

BMMs or lungs were homogenized with TRIzol Reagent (Invitrogen), followed by

RNA purification using an RNeasy Mini kit (Qiagen). Total RNA qualified and

quantified by a 4150 TapeStation system (Agilent) and a Qubit 4 Fluorometer

(Invitrogen), respectively, was subjected to construct cDNA libraries using NEBNext®

Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and NEB Next

Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs). All the cDNA

libraries were examined for quality using a 4150 TapeStation system and quantified

with a Qubit 4 Fluorometer. The libraries were sequenced with a NextSeq1000 system

(Illumina).

Data processing

 For read-quality trimming, raw reads were processed with Trim Galore v0.6.7 (https://github.com/FelixKrueger/TrimGalore). The expressions of transcripts were estimated by Salmon v0.12.0 directly from the processed reads (21). Transcript-level expression data was then summarized into gene-level data by the R package tximport v1.30.0 (https://github.com/thelovelab/tximport) in R v4.3.3(22). The analysis for differentially expressed genes (DEGs) was performed by edgeR v4.0.16 (23) using generalized linear models and quasi-likelihood tests (24). The DEGs were further utilized to perform Gene Ontology (GO) enrichment analysis to identify enriched BPs using the R package clusterProfiler v4.10 (25). To reduce redundancy among the identified GO BP categories, a simplification method in clusterProfiler was used. The gene concept network of the top 3 upregulated and downregulated GOBP categories was visualized by cnetplot in clusterProfiler. KEGG pathway enrichment analysis was conducted using ShinyGO v0.82 (26), an online gene set enrichment tool (http://bioinformatics.sdstate.edu/go/). Adapted KEGG pathway diagrams were visualized using Pathview v1.42.0 in R software (27). Pathway source: KEGG (https://www.kegg.jp). Gene set enrichment analysis (GSEA) was performed locally using the GSEA desktop application (Broad Institute, v4.2.3) with the WikiPathway gene sets (c2.cp.wikipathways.v2024.1.Hs.symbols.gmt) obtained from the Molecular Signature Database (MSigDB). MafB ChIP-seq peaks (GEO GSM1964739/SRA SRX1465586) (28) were downloaded via ChIP-Atlas (accessed 27 June 2025) (29) and compared with the DEGs identified in the present study. The ChIP-seq Atlas is accessible at https://chip-atlas.org/.

Flow cytometry

Infected lung cells were obtained using the Lung Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, cell suspensions from the lungs were incubated with ACK buffer to lyse red blood cells. The cells were washed and diluted in MACS buffer (PBS supplemented with 2mM EDTA and 2% FBS) to achieve 1–3×10^6 cells/mL. The cells were incubated with TruStain FcX™ PLUS (anti-mouse CD16/32) (Biolegend), followed by staining with antibodies against CD4, CD8, CD45R, CD3, SiglecF, CD64, CD11b, CD45, or Ly6G (BioLegend). The stained cells were then fixed with the fixation buffer (BioLegend) to inactivate infected *Mtb* for 24 h at 4°C. The cells were analyzed on a BD FACSLyric™ using analysis software BD FACSuite™ Application V1.4.0.7047 and FlowJo™ Software v10.10 (BD Biosciences).

Results

***Mafb* deficiency on mycobacterial killing in macrophages**

In our previous study, we demonstrated impaired inflammatory responses in *MAFB* knockdown, PMA-stimulated THP-1 cells (*MAFB*-KD macrophages). However, no significant difference in bacterial burden was observed between *MAFB*-KD macrophages and control macrophages at 24 h or 48 h p.i., suggesting that the knockdown effect and/or the duration of infection was insufficient to detect intracellular bacterial proliferation (14). In this study, we investigated the effect of *Mafb* deficiency on mycobacterial proliferation in BMMs (**Figure 1**). For this purpose, we infected BMMs of *Mafb*^f/f^::LysM-Cre^+/-^ (*Mafb*-cKO) or *Mafb*^f/f^ (control) with *Mtb* at an MOI of one for 1, 3, and 7 days. At 3 and 7 days p.i., *Mafb*-cKO BMMs exhibited a higher

bacterial burden compared to control BMMs. Notably, the intracellular number of *Mtb*

in *Mafb*-cKO BMMs increased significantly more rapidly from day 3 to day 7,

suggesting that the absence of *Mafb* transforms macrophages into a more permissive

environment for *Mtb* proliferation.

***Mafb*-cKO BMMs demonstrated functional changes in metabolic process and**

immune response during *Mtb* infection

When macrophages are exposed to *Mtb*, they internalize the bacteria, and *Mtb*

begins adapting to the intracellular environment by 24 h p.i. During this period,

macrophages undergo robust transcriptional changes, indicating active host–pathogen

interactions (30). To investigate the transcriptional function of *Mafb* in *Mtb*-infected

macrophages, we infected BMMs from *Mafb*^f/f::LysM-Cre^+/- (*Mafb*-cKO) and *Mafb*^f/f

(control) with *Mtb* and conducted mRNA-seq at 24 h p.i. mRNA-seq comparing

between *Mtb*-infected BMMs from *Mafb*-cKO and control mice identified 1223 DEGs

(**Figure 2A**). GO analysis for BP (GOBP) identified 974 significantly enriched GOBP

terms in 614 upregulated DEGs in *Mtb*-infected *Mafb*-cKO BMMs, including leukocyte

cell–cell adhesion, reactive oxygen species (ROS) metabolic process, or nucleotide

metabolic process (**Figure 2B**). In 609 downregulated DEGs, 493 significantly enriched

GOBP terms were identified, including response to virus, defense response to symbiont,

or regulation of innate immune response (**Figure 2B**). To visualize the interactions of

the genes annotated to each GO term, we constructed gene networks (**Figure 2C**).

Upregulated genes annotated to leukocyte cell–cell adhesion include *Itgb2*, *Itgb7*, *Itgal*,

F11r, *Selplg*, *Cd44* (adhesion molecules or integrins), *Ccr2*, *Cx3cr1*, *Cd86*, *Cd74*,

Cd24a, *Cd177*, *Cd300a* (leukocyte surface receptors), *H2-Ab1*, *H2-Aa*, *H2-Eb1*, *H2-*

DMa, *H2-DMb1* (MHC molecules), and *Arg2*, *Thbs1*, *Alox5*, *Sirpb1b*, *Sirpb1c* (immune

modulation), suggesting that the macrophages are in the state where they are actively

participating in immune surveillance, cellular communication, and antigen presentation.

Genes annotated to ROS metabolic process include *Cybb*, *Cyba*, *Ncf2* (ROS

generation), *Prdx1*, *Prdx5*, *Prdx6*, *Txn1*, *Txnrd1*, *Sod2*, *G6pdx*, *Gpx3*, *Nnt* (ROS

detoxification, antioxidant defense), and *Thbs1*, *Rhoa*, *Atg5* (oxidative stress

modulation). The downregulated genes annotated to top significantly enriched GOBP

terms were highly overlapped: *Apobec1*, *Adar*, *Ifi204*, *Ifi203*, *Ifi205*, *Ifi206*, *Ifi207*,

Ifi208, *Ifi209*, *Ifi211* (RNA editing and modification), *Ifih1*, *Dhx58*, *Rigi*, *Ddx60*, *Stat1*,

Stat2, *Irf3*, *Irf7*, *Tbk1*, *Trim25*, *Trim5*, *Trim21*, *Trim30a*, *Trim30c*, *Trim30d*, *Trim34a*,

Trim35, *Trim12c*, *Trim8*, *Isg15*, *Isg20*, *Oas1a*, *Oas1g*, *Oas2*, *Oas3*, *Oasl2*, *Ifit1*, *Ifit2*,

Ifit3, *Ifit3b*, *Ifit1bl1*, *Mx1*, *Mx2*, *Bst2*, *Dtx3l*, *Rnasel*, *Parp9*, *Shfl*, *Slfn8*, *Slfn9*, *Gbp3*,

Gbp5, *Gbp7*, *Rnf135*, *Nt5c3* (innate immune sensors and IFN-stimulated genes (ISGs)),

Irgm1, *Irgm2*, *Igtp*, *Mov10* (autophagy and intracellular pathogen defense), *Pou2f2*,

Il10rb, *Il15*, *Tlr9*, *Tlr1*, *Cd37*, *Cd180*, *Cd36*, *Cd300ld3*, *Pik3ap1*, *Rtp4*, *Sash1*, *Trafd1*,

Appl2 (transcription factors and signal transduction), *Eif2ak2*, *Pml*, *Bcl2l1*, *Trex1*, *Xaf1*,

Htra2, *F830016B08Rik* (cell cycle, apoptosis, and DNA repair), *Lacc1*, *Apoe*, *Adam8*,

and *Hyal1* (metabolism and miscellaneous functions). These downregulated DEGs

suggested weakened pathogen sensing and reduced IFN response or inflammatory

signaling, indicating a potential shift to an anti-inflammatory phenotype in *Mtb*-infected

BMMs of *Mafb*-cKO mice. KEGG pathway enrichment analysis revealed that oxidative

phosphorylation and chemical carcinogenesis-ROS were enriched in the upregulated

DEGs (**Supplementary Figure 1A**), whereas ECM-receptor interaction, lysosome, and

endocytosis were enriched in downregulated DEGs (**Supplementary Figure 1B**). As

depicted in the lysosome pathway diagram, the proton pump ATPeV, which plays a

critical role in lysosomal acidification, is downregulated (**Supplementary Figure 2)**

(31). By GSEA using all expressed genes, a pathway of immune response to TB

(https://www.wikipathways.org/instance/WP4197) exhibited impairment in *Mtb*-

infected *Mafb*-cKO BMMs (**Supplementary Figure 3**). These enriched BPs and

pathways are consistent with the previous results obtained from mRNA-seq of *Mtb*-

infected *MAFB*-KD macrophages (14). IFN-gamma inducible chemokines (*Cxcl11*,

Ccl2, *Ccl7*, *Cxcl9*, *Cxcl10*) were downregulated in *Mtb*-infected *MAFB*-KD

macrophages, as well as in *Mtb*-infected BMMs from *Mafb*-cKO mice (**Supplementary**

Figure 4). Thus, we proposed that MAFB in human and murine macrophages has a

similar role during *Mtb* infection.

Mafb **deficiency in macrophages increased mortality during** *Mtb* **infection in mice**

To examine whether *Mafb* deficiency in macrophages influences the outcome

of *Mtb* infection in mice, we conducted aerosol infections in *Mafb*-cKO mice and WT

mice and monitored them for 315 days. We found that *Mafb*-cKO mice started

succumbing after 20 weeks, and no *Mafb*-cKO mice survived by the end of the

experiment (**Figure 3A**). The survival probability between the two groups was analyzed

by Kaplan–Meier analysis and the log-rank test. The median survival of *Mafb*-cKO

mice was 211 days, which was significantly shorter than that of WT mice.-We next

determined the bacterial burden in the murine organs after *Mtb* infection. At 10 and 20

weeks p.i., *Mafb*-cKO mice exhibited significantly higher burden in the lungs, but not at

4 weeks p.i. (**Figure 3B**). The spleens of *Mafb*-cKO mice also showed a higher burden

at 10 and 20 weeks p.i., demonstrating the involvement of *Mafb* in the control of the

bacterial burden in the lung and spleen (**Supplementary Figure 5**).

Transcriptomics of *Mtb*-infected *Mafb*-cKO mouse lungs

To investigate whether *Mafb* deficiency in macrophages alters BPs in the lungs

during *Mtb* infection, we performed mRNA-seq on the lungs of *Mtb*-infected *Mafb*-cKO

and control mice at 10 or 20 weeks p.i., respectively. At 10 weeks p.i., 89 genes were

identified as DEGs in the lungs of *Mtb*-infected *Mafb*-cKO mice (**Figure 4A**). Among

these 89 genes, 48 genes were upregulated and 41 genes were downregulated. GOBP of

DEGs demonstrated that cell–cell adhesion, leukocyte proliferation, or the regulation of

T-cell activation were activated, whereas complement activation, cellular response to

type II IFN, synapse pruning, and response to protozoan were suppressed in the lungs of

Mafb-cKO mice (**Figure 4B**). Concept gene network for GO categories visualized that

Cd1d1, *Cdkn2a*, *Tarm1*, *Havcr2*, and *Slfn1* were the key genes for T-cell regulation

(**Figure 4C**). KEGG pathway enrichment analysis demonstrated the enrichment of

osteoclast differentiation (**Supplementary Figure 6A**). Complement components such

as *C1qa*, *C1qb*, or *C1qc*, identified in suppressed GO categories, played a central role in

complement activation. The involvement of *Mafb* in regulating complement

components was consistent with the previous report (15). In addition to complement

components, downregulated DEGs included cytokine ligands such as *Ccl8*, which is

also known as *monocyte chemoattractant protein 2* (MCP2), *Ccl12*, known as *monocyte*

chemoattractant protein 5 (MCP5), or *Pf4,* known as *Cxcl4*. KEGG pathway

enrichment analysis demonstrated that complement and coagulation cascade, and

chemokine signaling pathway were enriched in downregulated DEGs (**Supplementary**

Figure 6BC). At 20 weeks p.i., 267 DEGs were identified (**Figure 5A**), of which DEGs

found at 10 weeks p.i. were included. Among the 267 genes, 110 genes were

upregulated and 157 genes were downregulated. GOBP showed that myeloid leukocyte

activation, myeloid leukocyte differentiation, the regulation of macrophage activation,

the regulation of endocytosis, and the regulation of angiogenesis were upregulated,

whereas leukocyte migration, leukocyte chemotaxis, leukocyte proliferation, and

immune response cell-surface receptor-signaling pathway were downregulated in the

lungs of *Mtb*-infected *Mafb*-cKO mice (**Figure 5B**). Concept gene network revealed

Csfs (GM-CSF), a key regulator for macrophage and dendritic cell function, *Mmp8*,

Cd177, genes associated with neutrophil activation and migration, or *Sirpb1 family* for

phagocytosis and immune modulation in upregulated DEGs, highlights strong

differentiation and the activation of myeloid-derived immune cells (**Figure 5C**). Down

regulated DEGs included *Ccl22*, *Ccl8*, *Ccl5*, *Cx3cr1*, *Pf4*, and *Ccr7*, which are involved

in chemokine signaling or leukocyte migration; *P2rx7*, *Nfatc2*, and *Ptpn22*, regulatory

genes in T-cell activation and immune tolerance, *Cd22* or *Icosl*, which are involved in B

cell-mediated immune response, suggesting reduced adaptive immune activation and

leukocyte or lymphocyte recruitment in the lungs of *Mtb*-infected *Mafb*-cKO mice

compared to those of control mice (**Figure 5C**).

Immune cell recruitment in the lungs of *Mtb*-infected *Mafb*-cKO mice

328   Transcriptomics of the lungs of *Mtb*-infected *Mafb*-cKO mice suggested altered

recruitment of immune cells during *Mtb* infection (**Figure 4 and 5**). Therefore, we

investigated the proportion of immune cells in the lungs of *Mafb*-cKO mice during *Mtb*

infection by flow cytometry (**Figure 6**). The frequencies of both CD4$^+$ and CD8$^+$ T-cells

were high at 10 weeks p.i., and then decreased at 20 weeks p.i. in control mice, whereas

they were at the same levels in *Mafb*-cKO mice during infection, suggesting blocked

early recruitment of CD4^{+} and CD8^{+} T-cells in the infected lungs of *Mafb*-cKO mice in

line with the transcriptomic feature of negative T cell regulation at 10 weeks p.i.

(**Figure 6**). The frequency of B-cells remained the same from 10 weeks to 20 weeks p.i.

in the control mice; however, it decreased in *Mafb*-cKO mice at 20 weeks p.i., which

also supports the transcriptomics data. Despite the impaired chemokine signaling, the

frequency of neutrophils was significantly higher in *Mafb*-cKO at 10 or 20 weeks p.i.

compared to that in control mice. Although the frequency of macrophages was slightly

lower in *Mafb*-cKO mice, the difference was not statistically significant due to the high

inter-sample variability. Nonetheless, these results indicate that *Mafb* deficiency in

macrophages affects the recruitment of various immune cells to the lungs of *Mtb*-

infected mice.

Discussion

We have demonstrated that *MAFB* deficiency activates oxidative

phosphorylation and impairs type I and II IFN responses in *Mtb*-infected human

macrophages (14). For a deeper understanding of *MAFB*'s role in *Mtb* infection, we

investigated its function using a murine model of myeloid-specific *Mafb* conditional-

knockout (*Mafb*-cKO) mice. Transcriptomics of BMMs from *Mafb*-cKO mice revealed

that a ROS metabolic process and oxidative phosphorylation were activated, whereas

the IFN responses were suppressed during *Mtb* infection (**Figure 2**), which is consistent

with our previous results (14). Therefore, we assume that *MAFB* in human and murine

macrophages acts similarly in response to *Mtb* infection.

We found that BMMs from *Mafb*-cKO mice failed to control intracellular *Mtb*

proliferation (**Figure 1**). In fact, *Mtb* infection typically induces the production of ROS

in infected macrophages to reduce the intracellular bacterial load (32). However, an

imbalance between ROS and antioxidants leads to oxidative stress, which contributes to

the onset and progression of pulmonary diseases, including TB (33, 34). In BMMs from

Mafb-cKO mice, the ROS metabolic process was activated, and its related genes were

identified. CYBB, CAMK2B, and ITPR1 were involved in ROS production, and SOD2,

GPX3, and CAT were involved in ROS clearance (35). CYBB is the major catalytic

subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, encoding

NOX2 that possesses antimicrobial activity against *Mtb* (36). The superoxide dismutase

expressed by *Sod2* detoxifies the major ROS to protect host cells from the damage

caused by excessive ROS. Paradoxically, the overexpression of *Sod2* promotes the

intracellular survival of *Mtb* (37). During *Mtb* infection, genes related to both ROS

production and clearance were upregulated in BMMs derived from *Mafb*-cKO mice,

implying that BMMs from *Mafb*-cKO mice generate ROS to clear pathogen while

maintaining redox balance to prevent self-damage during infection, thereby actively

attempting to eliminate the excessive ROS.

Mtb infection also activates antiviral responses, including the induction of type

I IFNs, in infected macrophages (38, 39). During infection, mycobacterial DNA is

initially released from the phagosomes into the cytosols, where it is recognized by

cyclic GMP-AMP synthase (cGAS), initiating type I IFN production. This recognition

triggers the activation of the cGAS-STING-TBK1 cascade and transcription factors

IRF3 and IRF7, followed by the production of type I IFNs and other cytokines (40).

Activated IRF3 translocates into the nucleus and binds to IFN-stimulated response

element (ISRE) in the promoters of type I IFNs and proinflammatory genes for further

transcription induction (41). It has been shown that IRF3 is essential for downstream

genes, such as *Cxcl10* and *Ifit1*, which are induced by IFN-β and IFN-γ (42). In *Mtb*-

infected BMMs from *Mafb*-cKO mice, *Tbk1*, *Irf3*, *Irf7*, *Stat1*, *Stat2,* and other genes

with ISRE (*Adar*, *Bst2*, *Cxcl10*, *Gbp2*, *Ifit1*, *Ifit2*, *Ifit3*, *Irf7*, *Irf9*, *Isg15*, *Isg20*, *Mx1*,

Mx2, *Oas2*, *Oas3*, *Rsad2*, *Sp100)* and type I IFNs (*IFNα*, *IFNβ*) were significantly

downregulated (**Figure 2**, **Supplementary Figure 7A**), suggesting that *Mafb* can be a

positive regulator in the cGAS-STING-TBK1 cascade.

In mycobacterial infection, type I IFNs can be detrimental to the host,

considering the type I IFN-driven susceptibility in the mouse model carrying the

sensitive allele of the *Sst1* locus (43). Contrary to the results of BMMs from *Mafb*-cKO

mice, type I IFNs (IFNα) or genes with ISRE were found to be upregulated in *Mtb*-

infected *Mafb*-cKO mouse lungs **(Supplementary Figure 7BC)**. This result may

explain the increased mortality and higher bacterial burden in the organs of *Mtb*-

infected *Mafb*-cKO mice (**Figure 3**). A possible explanation for this higher bacterial

burden in BMMs from *Mafb*-cKO mice is weakened lysosome biogenesis demonstrated

by a pathway analysis (**Supplementary Figure 1B**, **2**). In addition, genes related to the

immune response to the TB pathway were downregulated in *Mafb*-cKO BMMs

compared to those in control BMMs (**Supplementary Figure 3**). Together with the

imbalanced ROS metabolism, we proposed that *Mafb*-deficient BMMs lack an effective

antibacterial response that would contain intracellular *Mtb*.

As expected from the failure of *Mtb* containment at a cellular level, *Mafb*

depletion in macrophages causes increased mortality and a higher bacterial burden in

the organs of *Mtb*-infected mice (**Figure 3**). To gain an insight into the mechanism

underlying the susceptibility of *Mafb*-cKO mice, we performed RNA-seq of *Mafb*-cKO

mouse lungs. We identified 1223 DEGs by comparing between *Mtb*-infected BMMs

from *Mafb*-cKO and control mice. In the lungs of *Mtb*-infected *Mafb*-cKO mice, 89 or

267 genes were identified as DEGs at 10 weeks or 20 weeks p.i., respectively. MafB is a

transcription factor that binds to Maf recognition elements (MAREs) in gene promoters

(8). We obtained MafB ChIP-sequencing data (28) to compare with the DEGs identified

in the present study. Among the 1,223 DEGs in *Mtb*-infected *Mafb*-cKO BMMs, 413

genes (33.8%) overlapped with MafB target genes, including 175 upregulated and 238

downregulated genes (**Table 1).** In the lungs of *Mtb*-infected *Mafb*-cKO mice, 28 DEGs

(31.5%) at 10 weeks p.i. and 55 DEGs (20.6%) at 20 weeks p.i. overlapped with MafB

target genes, respectively (**Table 1**). The proportion of DEGs directly bound by MafB

was comparable between *Mafb*-cKO BMMs and lungs at 10 weeks p.i.; however, this

proportion was lower at 20 weeks p.i., despite a greater number of DEGs being

detected. This suggests that secondary effects of *Mafb* deficiency may contribute to the

increased mortality observed in *Mafb*-cKO mice during the later stage of infection.

  Transcriptomics of the lungs of *Mtb*-infected *Mafb*-cKO mice displayed

activated myeloid-derived immune cells and differentiation (**Figure 4**). This finding is

consistent with the reports that low MafB levels activate self-renewal in resident

macrophages(28, 44). Vanneste *et al.* exhibited that myeloid-specific *Mafb* deletion

increased both the proliferative ability and cell death in macrophages, decreasing

number of macrophages in the mouse lungs (45). We also demonstrated a slightly

decreased population of CD64$^+$ macrophages among immune cells at 10 weeks p.i. but

not at 20 weeks p.i. (**Figure 6**). We displayed a significantly higher frequency of

neutrophils in *Mafb*-cKO mouse lungs (**Figure 6**). The result is consistent with the

necrosis of macrophages and bacillary replication induce neutrophil recruitment (46). In

fact, neutrophil accumulation correlates with increased disease severity, suggesting that

excessive neutrophils may exacerbate TB pathology (47). *Mtb*-infected *Mafb*-cKO mice

displayed a higher bacterial burden at 10 weeks p.i. in the lungs. Neutrophil recruitment

is further enhanced by their release of mediators in response to *Mtb* (48). These findings

explain that neutrophils can drive further neutrophil recruitment to the lungs of *Mafb*-

cKO mice. Kanai *et al.* also demonstrated an increased myeloid-cell infiltration,

including neutrophils, in an ischemic acute kidney injury (AKI) model in *Mafb*-cKO

mice, suggesting that *Mafb* is involved in myeloid-cell migration both in the site of

infection and injury. (49) Considering that *Mafb* regulates thermogenesis in brown

adipose tissue in *Mafb*-cKO mice under cold conditions (50), it is suggested that *Mafb*

controls various homeostatic functions in macrophages under infections, injuries, or

cold conditions.

We demonstrated that *Mafb* deficiency in macrophages impaired the cell

signaling for leukocyte migration and the recruitment of CD4$^+$ and CD8$^+$ T-cells in the

lungs of *Mtb*-infected mice, suggesting weakened adaptive immunity at an early stage

of infection. Several studies have shown the importance of macrophage activation by

IFN-γ produced from CD4$^+$ T-cells for protective immunity against *Mtb* in mice (51-

53). The depletion of CD4$^+$ T-cells leads to increased bacterial loads and increased

severity of the infection in *Mtb*-infected C57BL/6 mice (54). In a macaque model, CD4$^+$

T-cells display an "innate-like" defense system and serve as master helper cells to

recruit other Th-like effector cells, thereby successfully preventing early

extrapulmonary *Mtb* dissemination (55).

In summary, the present study provides evidence that *Mafb* depletion in

myeloid cells not only impairs macrophage bactericidal activity but also disrupts

immune cell recruitment, leading to failed bacterial control and higher mortality in *Mtb*-

infected mice.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any

commercial or financial relationships that could be construed as a potential conflict of

interest.

Author Contributions

HH, SS, MHijikata and NK designed the project. HH, SS, HN, and SO

performed the experiments. MHamada, and ST provided the experimental materials.

HH and SS analyzed the data. HH, SS, MHijikata and NK wrote and revised the

manuscript. All authors approved the manuscript.

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Figure legends

Figure 1. Mycobacterial proliferation on *Mafb*-deficient macrophages.

Mafb^f/f^::LysM-Cre^+/-^ (*Mafb*-cKO) and *Mafb*^f/f^ (control) bone marrow-derived

macrophages (BMMs) were infected with *Mycobacterium tuberculosis* (*Mtb*). At 1, 3,

and 7 days postinfection (p.i.), the numbers of the intracellular bacteria were determined

by colony forming units (CFUs). *$P < 0.01$ using Welch's t-test, with Holm–Bonferroni

correction applied for multiple comparisons.

Figure 2. Transcriptomics of *Mtb*-infected *Mafb*-cKO bone marrow-derived

macrophages (BMMs)

mRNA sequencing (mRNA-seq) of *Mtb*-infected *Mafb*-cKO BMMs was performed.MA

plot showing 1223 differentially expressed genes (DEGs) in *Mtb*-infected *Mafb*-cKO

BMMs compared to those in *Mtb*-infected *Mafb*^f/f^ control BMMs, marked in red (FDR

< 0.01). Each dot represents expressed genes in the sample. Log FC, log fold change.

LogCPM, log count per million. (B) Gene Ontology (GO) analysis for upregulated or

downregulated DEGs. Enriched GO biological process (BP) categories in *Mtb*-infected

Mafb-cKO BMMs can be seen. (C) Gene concept networks of the top 3 upregulated

(Up) and downregulated (Down) GOBP categories in *Mtb*-infected *Mafb*-cKO BMMs.

Upregulated GOBP categories are leukocyte cell–cell adhesion, reactive oxygen species

metabolic process, and nucleotide metabolic process, colored in salmon pink.

Downregulated GOBP categories are response to virus, defense response to symbiont,

and regulation of innate immune response, as shown in blue.

Figure 3. Effect of macrophage-specific *Mafb* deficiency on TB susceptibility in

mice

(A) *Mafb*$^{f/f}$::LysM-Cre$^{+/+}$ (*Mafb*-cKO) mice and wild-type (WT) mice (*n*=9/group) were

aerosol- infected with *Mtb*, and their survival was monitored for 315 days. Survival

probability between the two groups was analyzed by Kaplan–Meier analysis and the

log-rank test. The median survival of *Mafb*-cKO mice was 211 days. Most of the WT

mice survived when the experiment was ended. *$P = 0.00011$. (B) Bacterial loads in the

lungs of *Mtb*-infected *Mafb*$^{f/f}$::LysM-Cre$^{+/-}$ (*Mafb*-cKO) and control mice were

determined by CFU at 4, 10, and 20 weeks p.i. Data from individual mice is shown. *P

< 0.01 using Tukey–Kramer test.

Figure 4. Transcriptomics of *Mtb*-infected *Mafb*-cKO mouse lung at 10 weeks p.i.

Mafb-cKO mice and control mice were aerosol-infected with *Mtb* for 10 weeks. (A) MA

plot showing 89 DEGs in *Mtb*-infected *Mafb*-cKO mouse lungs compared to those in

Mtb-infected control mouse lungs, marked in red (FDR < 0.01). Each dot represents

expressed genes in the sample. Log FC, log fold change. LogCPM, log count per

million. (B) GO analysis for DEGs. Enriched GOBP categories in *Mtb*-infected *Mafb*-

cKO lungs are shown. (C) Gene concept network of the top 3 upregulated (Up) and

downregulated (Down) GOBP categories in *Mtb*-infected *Mafb*-cKO mouse lungs.

Upregulated GOBP categories include regulation of leukocyte cell–cell adhesion,

regulation of T-cell activation, leukocyte cell–cell adhesion, and leukocyte proliferation,

colored in salmon pink. Downregulated GOBP categories are defense response to

protozoan, response to protozoan, cellular response to type II interferon, synapse

pruning, and complement activation, and classical pathway, as shown in blue.

Figure 5. Transcriptomics of *Mtb*-infected *Mafb*-cKO mouse lung at 20 weeks p.i.

Mafb-cKO mice and control mice were infected with an aerosol of *Mtb* for 20 weeks.

(A) MA plot showing 267 DEGs in *Mtb*-infected *Mafb*-cKO mouse lungs in comparison

with control mouse lungs marked in red (FDR < 0.01). Each dot represents expressed

genes in the sample. Log FC, log fold change. LogCPM, log count per million. (B) GO

analysis for DEGs. Enriched GOBP categories in *Mtb*-infected *Mafb*-cKO lungs were

shown. (C) Gene concept network of the top 3 upregulated (Up) and downregulated

(Down) GOBP categories in *Mtb*-infected *Mafb*-cKO mouse lungs. The upregulated

GOBP categories include myeloid leukocyte activation, regulation of endocytosis,

myeloid leukocyte differentiation, regulation of angiogenesis, and regulation of

macrophage activation colored in salmon pink. Downregulated GOBP categories are

leukocyte proliferation, lymphocyte proliferation, immune response cell surface

receptor signaling pathway, and leukocyte chemotaxis, shown in blue.

Figure 6. Population of immune cells in *Mtb*-infected Mafb-cKO mice

The proportion of the immune cell population in the lungs of *Mtb*-infected *Maf*b-cKO or

control mice was determined by flow cytometry at 10 or 20 weeks p.i. The proportions

of $CD45^+$, B-cells, neutrophils, macrophages, $CD4^+$ T-cells, $CD8^+$ T-cells, and alveolar

macrophages are shown. *$P < 0.05$ using the Wilcox test for each time point.

Table 1. Comparison of MafB ChIP-seq peaks and the DEGs

MafB ChIP-seq peaks are compared with the DEGs identified in *Mtb*-infected *Mafb*-

cKO mouse BMMs, *Mtb*-infected *Mafb*-cKO mouse lungs at 10 weeks p.i., or *Mtb*-

infected *Mafb*-cKO mouse lungs at 20 weeks p.i. The ChIP-seq data (GEO

GSM1964739/SRA SRX1465586) (28) was downloaded via ChIP-Atlas (accessed 27

June 2025) (29)

		Upregulated	Downregulated	Total
Mafb-cKO mouse BMMs	DEGs	614	609	1223
	MafB ChIP-seq peaks	175	238	413
		28.5%	39.1%	33.8%
Mafb-cKO mouse lungs, 10w	DEGs	48	41	89
	MafB ChIP-seq peak	14	14	28
		29.2%	34.1%	31.5%
Mafb-cKO mouse lungs, 20w	DEGs	110	157	267
	MafB ChIP-seq peak	24	31	55
		21.8%	19.7%	20.6%

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