

Lipid Peroxidation and Type I Interferon Coupling Fuels Pathogenic Macrophage Activation Causing Tuberculosis Susceptibility

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eLife Assessment

Yabaji et al. reports a **fundamental** study highlighting the mechanistic connection for susceptibility to TB infection via the *sst1* locus, this was shown to involve increased IFN and Myc production causing the down-regulation of anti-oxidant defence genes and chronic lipidation. Ultimately, lipid peroxidation may underlie infectivity and macrophage dysfunction. Overall, the data presented are **compelling**, supported by a well designed multi-omics approach and the findings will be of broad interest to researchers investigating the molecular mechanisms of TB infection.

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Abstract

A quarter of human population is infected with *Mycobacterium tuberculosis*, but less than 10% of those infected develop pulmonary TB. We developed a genetically defined *sst1*-susceptible mouse model that uniquely reproduces a defining feature of human TB: the development of necrotic lung granulomas and determined that the *sst1*-susceptible phenotype was driven by the aberrant macrophage activation. This study demonstrates that the aberrant response of the *sst1*-susceptible macrophages to prolonged stimulation with TNF is primarily driven by conflicting Myc and antioxidant response pathways leading to a coordinated failure 1) to properly sequester intracellular iron and 2) to activate ferroptosis inhibitor enzymes. Consequently, iron-mediated lipid peroxidation fueled *Ifn* β superinduction and sustained the Type I Interferon (IFN-I) pathway hyperactivity that locked the *sst1*-susceptible macrophages in a state of unresolving stress and compromised their resistance to Mtb. The accumulation of the aberrantly activated, stressed, macrophages within granuloma microenvironment led to the local failure of anti-tuberculosis immunity and tissue necrosis. The upregulation of Myc pathway in peripheral blood cells of human TB patients was significantly associated with poor outcomes of TB treatment. Thus, Myc dysregulation in activated macrophages results in an aberrant macrophage activation and represents a novel target for host-directed TB therapies.

Introduction

Thousands of years of co-evolution with modern humans has made *Mycobacterium tuberculosis* (Mtb) arguably the most successful human pathogen (Orgeur & Brosch, 2018 [DOI](#)). It currently colonizes approximately a quarter of the global population (WHO, 2022 [DOI](#)). Most Mtb-infected people develop latent TB, in which the host responses either eliminate or sequester the bacteria inside a granuloma structure (Pagan & Ramakrishnan, 2018 [DOI](#); Pai et al., 2016 [DOI](#)). However, about 5-10% of Mtb-infected individuals will eventually develop active TB either within a year after the primary infection, or later in their life after re-activation of persistent bacteria or re-infection (Behr et al., 2021 [DOI](#); Drain et al., 2018 [DOI](#); Horsburgh & Rubin, 2011 [DOI](#); Reichler et al., 2018 [DOI](#)). A plethora of genetic, developmental and environmental factors contribute to TB progression in individuals that initially resisted the pathogen (Cohen et al., 2022 [DOI](#); Simmons et al., 2018 [DOI](#)).

Infection of the lung is central to Mtb's evolutionary success because it allows the pathogen to spread among human hosts via aerosols. After systemic dissemination from primary lesions, Mtb can be found in many human organs (Bussi & Gutierrez, 2019 [DOI](#); Ulrichs et al., 2005 [DOI](#)). However, approximately 85% of the disease develops in the lungs (Pai et al., 2016 [DOI](#)). Necrotic lesions are the major pathologic manifestation of pulmonary TB ranging from central necrosis in organized granulomas during primary TB to massive necrotizing pneumonia and the formation of cavities in post-primary pulmonary TB (Hunter, 2011 [DOI](#); Pai et al., 2016 [DOI](#)). The necrotic lung lesions develop in immunocompetent hosts despite the presence of active T cell mediated immune response (Cohen et al., 2022 [DOI](#)).

Existing mechanistic concepts explaining the lesion necrosis fall into two main categories:

1. *inadequate* local immunity that allows exuberant bacterial replication and production of virulence factors that drive tissue necrosis, vs. ii) *excessive* effector immunity that results in immune-mediated tissue damage (Ernst, 2018 [\[1\]](#); O'Garra et al, 2013 [\[2\]](#); Ogongo & Ernst, 2023 [\[3\]](#)). Although both scenarios are credible, they are mechanistically distinct and would require different therapeutic strategies. Therefore, in-depth understanding of mechanisms of pulmonary TB progression in immunocompetent hosts is necessary for accurate patient stratification and for the development of personalized approaches to immune modulation (DiNardo et al, 2021 [\[4\]](#)).

Mouse models have been successfully used for mechanistic studies of Mtb infection, although classical inbred mouse strains routinely used in TB research, such as C57BL/6 (B6) and BALB/c, do not develop human-like necrotic TB lesions (Apt & Kramnik, 2009 [\[5\]](#)). Nevertheless, even in these models, Mtb predominantly replicates in the lungs irrespective of the route of infection (North & Jung, 2004 [\[6\]](#)). Mouse models that recapitulate the necrotization of pulmonary TB lesions have also been developed (reviewed in (Kramnik & Beamer, 2016 [\[7\]](#)). We have previously found that C3HeB/FeJ mice develop necrotizing granulomas after infection with virulent Mtb and mapped several genetic loci of TB susceptibility using a cross of the C3HeB/FeJ with the resistant B6 mice (Kramnik et al, 1998 [\[8\]](#); Kramnik et al, 2000 [\[9\]](#); Yan et al, 2006 [\[10\]](#)). A single locus on chromosome 1, *sst1* (super-susceptibility to tuberculosis 1), was specifically responsible for the control of the necrotization of TB lesions (Sissons et al, 2009 [\[11\]](#)). The *sst1*-susceptible mice develop necrotic lung lesions irrespective of the route of infection – aerosol, intravenous or intradermal (Kramnik et al., 1998 [\[8\]](#); Kramnik et al., 2000 [\[9\]](#); Pan et al, 2005 [\[12\]](#); Pichugin et al, 2009 [\[13\]](#); Yabaji et al, 2023 [\[14\]](#)) – thus demonstrating a common underlying mechanism.

We further found that the *sst1* locus primarily controls innate resistance to intracellular pathogens (Boyartchuk et al, 2004 [\[15\]](#); He et al, 2013 [\[16\]](#); Pan et al., 2005 [\[12\]](#); Yan et al, 2007 [\[17\]](#)), and the *sst1*-mediated susceptibility was associated with the hyperactivity of the type I interferon (IFN-I) pathway in activated macrophages in vitro and in vivo (Bhattacharya et al, 2021 [\[18\]](#); He et al., 2013 [\[16\]](#); Ji et al, 2019 [\[19\]](#)). Of note, the hyperactivity of IFN-I pathway has been associated with TB susceptibility and the disease progression both in human patients and experimental models (Donovan et al, 2017 [\[20\]](#); Moreira-Teixeira et al, 2018 [\[21\]](#); O'Garra et al., 2013 [\[2\]](#); Stanley et al, 2007 [\[22\]](#)). However, mechanisms that underlie the IFN-I hyperactivity and their roles in susceptibility to TB were insufficiently elucidated. Thus, the *sst1*-mediated susceptibility recapitulates the morphologic and mechanistic hallmarks of human TB disease and provides a genetically defined mouse model to study both the upstream mechanisms responsible for the IFN-I pathway hyperactivity (Bhattacharya et al., 2021 [\[18\]](#)) and its downstream consequences (Kotov et al, 2023 [\[23\]](#)).

The *sst1* locus encodes the Sp110 (Pan et al., 2005 [\[12\]](#)) and Sp140 (Ji et al, 2021 [\[24\]](#)) proteins – known as interferon inducible chromatin binding proteins (Fraschilla & Jeffrey, 2020a [\[25\]](#)). The expression of mRNAs encoding both proteins is greatly diminished in mice that carry the *sst1* susceptibility allele and protein expression is undetectable for both (Bhattacharya et al., 2021 [\[18\]](#); Ji et al., 2021 [\[24\]](#)). Both proteins were shown to be involved in regulation of type I interferon pathway (Ji et al., 2021 [\[24\]](#); Lee et al, 2012 [\[26\]](#)). The overexpression of Sp110b in macrophages increased their resistance to intracellular bacteria in vitro (Pan et al., 2005 [\[12\]](#)), while the *Sp140* gene knockout dramatically increased the mouse susceptibility to several intracellular bacteria including virulent Mtb (Ji et al., 2021 [\[24\]](#)). It was suggested that Sp140 plays a dominant role in TB susceptibility, via direct regulation of the interferon beta gene (*Ifnb1*) mRNA stability (Witt et al, 2024 [\[27\]](#)). In humans, the *Sp140* hypomorphic alleles were associated with susceptibility to multiple sclerosis and Crohn's disease (Matesanz et al, 2015 [\[28\]](#); Mehta et al, 2017 [\[29\]](#)). Moreover, the *Sp140* mutations predicted the responsiveness of Crohn's disease patients to anti-TNF therapy, suggesting its role in regulating TNF response. Mechanistically, Sp140 was implicated in silencing "lineage-

inappropriate” and developmental genes, maintenance of heterochromatin in activated macrophages (Mehta et al., 2017 [↗](#)) and downregulating transcriptional activity by inhibiting topoisomerases (Amatullah et al, 2022 [↗](#)).

Previously, we found that prolonged TNF stimulation of the *sst1*-susceptible B6.Sst1S macrophages in vitro uniquely induced an aberrant response that was characterized by the *Ifnb1* superinduction, and a coordinated upregulation of interferon-stimulated genes (ISGs), markers of proteotoxic stress (PS) and the integrated stress response (ISR). The upregulation of all these pathways was prevented by a reactive oxygen scavenger (BHA) (Bhattacharya et al., 2021 [↗](#)) suggesting that oxidative stress was driving the aberrant activation of the *sst1*-susceptible macrophages. Current literature provides ample evidence of the crosstalk between the above pathways: 1) stress kinase activation by oxidative stress (Blaser et al, 2016 [↗](#); Kamata et al, 2005 [↗](#)); 2) promotion of type I interferon (IFN-I) responses by stress kinases (Bocconi et al, 2022 [↗](#); Buskiewicz et al, 2016 [↗](#); Karin & Gallagher, 2005 [↗](#)); 3) suppression of AOD by IFN-I (Lei et al, 2021 [↗](#); Riedelberger et al, 2020 [↗](#)); 4) suppression of IFN responses and AOD by Myc (Levy & Forman, 2010 [↗](#); Torti & Torti, 2002 [↗](#); Zimmerli et al, 2022 [↗](#)) (Fig.1A [↗](#)). However, the dynamic interactions and regulatory dependencies of these pathways in homeostasis and disease-specific contexts remain poorly understood.

In this study, we specifically addressed connectivity of the AOD and IFN-I pathways in *sst1*-susceptible macrophages persistently activated by TNF, a cell state relevant to TB granuloma microenvironment (Flynn & Chan, 2022 [↗](#)). We determined that their aberrant response to prolonged TNF stimulation was primarily fueled by conflicting Myc and anti-oxidant responses leading to *Ifnb1* superinduction and the IFN-I pathway hyperactivity that locked the *sst1*-susceptible macrophages in a state of persistent oxidative stress. This unresolving stress compromised the macrophage resistance to virulent Mtb in vitro, and the accumulation of stressed macrophages within TB lesions was associated with the failure of Mtb control within pulmonary TB lesions and their necrotization in vivo.

Results [↗](#)

1. The *sst1* locus controls diverse trajectories of macrophage activation by TNF

To explore the spectrum of TNF responses in the B6 and B6.Sst1S BMDMs, we compared gene expression in the BMDM populations either naïve, or after 24 h of stimulation with TNF (10 ng/ml) using single-cell RNA sequencing (scRNA-seq) (Fig.1B [↗](#)). The naïve macrophage populations of both backgrounds were similar. All BMDMs responded to TNF stimulation, as evidenced by the de novo formation of clusters 1-6, where cluster 3 appeared exclusively in the resistant (B6) and clusters 4 and 5 in the susceptible (B6.Sst1S) macrophage populations (Fig.1C [↗](#) and Suppl.Fig.1A [↗](#)). After TNF stimulation, the expression of the *sst1*-encoded *Sp110* and *Sp140* genes coordinately increased only in the B6 macrophages (Fig.1D [↗](#) and Suppl.Fig.1B [↗](#)). The IFN pathway was dramatically upregulated in TNF stimulated B6.Sst1S cells that lacked the *Sp110* and *Sp140* expression, but it was upregulated to a lesser degree in cluster 3 exclusive for TNF-stimulated *Sp110/140*-positive B6 macrophages (Fig.1E [↗](#) and Suppl.Fig.1C [↗](#)).

To determine the relatedness of the diverse macrophage subpopulations, as they emerge during TNF stimulation, we performed trajectory analysis that clearly demonstrated partial overlap and the divergence between the trajectories of TNF-stimulated resistant (RT) and susceptible (ST) subpopulations (Fig.1F [↗](#)). Subpopulations sp1 and sp2 of the B6 and B6.Sst1S macrophages were closely related and were characterized by the upregulation of cell division pathways in both backgrounds (Fig.1G [↗](#)). Cell cycle analysis demonstrated that TNF stimulation increased the fraction of macrophages in S phase (Suppl. Table 1 [↗](#)).

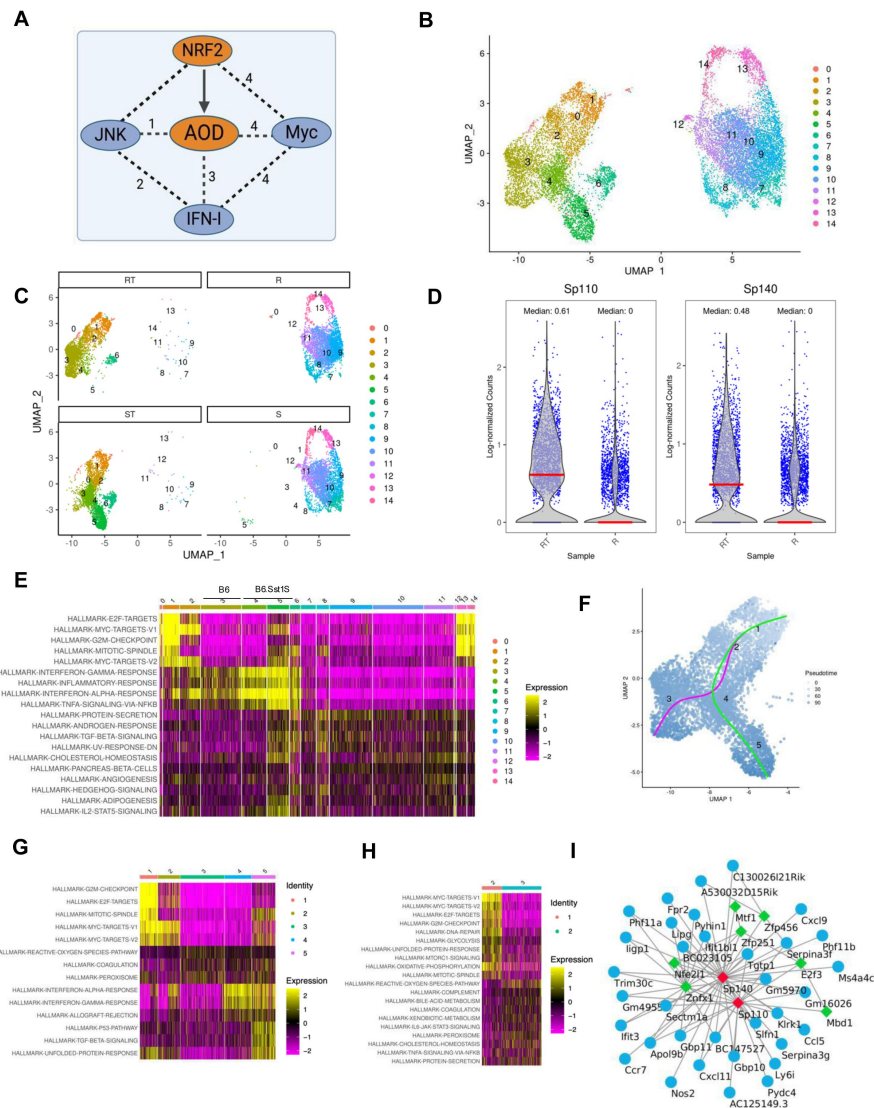


Figure 1.

Single cell RNAseq analysis of the population dynamics of B6 and B6.Sst1S macrophages after TNF stimulation.

(A). Connectivity of antioxidant defense (AOD) with the Myc-, Nrf2-, JNK-, and IFN-I-regulated pathways: 1) stress kinase activation by oxidative stress; 2) promotion of IFN-I responses by stress kinases; 3) suppression of AOD by IFN-I; 4) inhibition of Nrf2, AOD and IFN responses by Myc. (B and C). scRNA-seq analysis (UMAP and individual clusters) of B6 (R) and B6.Sst1S (S) BMDMs either naïve (R and S) or after 24 h of stimulation with TNF (RT and ST, respectively). (D). Expression of the *sst1*-encoded *Sp110* and *Sp140* genes in the population of either naïve (R) or TNF-stimulated (RT) B6 BMDMs. (E). Heatmap showing differentially expressed pathways in all cell clusters identified using scRNA-seq. Rows represent pathways and columns represent individual clusters with color intensity indicating the relative expression. (F). Reconstruction of the activation trajectories of TNF-stimulated resistant (RT) and susceptible (ST) macrophage populations using pseudotime analysis. magenta line indicates B6 and green line indicates B6.Sst1S BMDMs. (G). Heatmap showing differentially expressed pathways in subpopulations 1 to 5 identified using pseudotime analysis. Rows represent pathways and columns represent individual subpopulations with color intensity indicating the relative expression. (H). Pathway heatmap representing transition from subpopulation 2 to unique subpopulation 3 in TNF-stimulated B6 macrophages. (I). The *Sp110* and *Sp140* gene regulatory network analysis. The mouse macrophage gene regulatory network was inferred using GENIE3 algorithm from mouse macrophages gene expression data sets obtained from Gene Expression Omnibus (GEO). First neighbors of *Sp110/Sp140* genes were selected to infer a subnetwork of *Sp110/Sp140* co-regulated genes. Green nodes represent transcription factors, blue nodes denote their potential targets.

The susceptible (ST) and resistant (RT) activation trajectories diverged as the RT transitioned from sp2 to sp3, but the ST transitioned from sp2 to sp4 and 5. The transition from sp2 to sp3 in the resistant B6 BMDMs, was characterized by an increase of the G1/S ratio, the downregulation of anabolic pathways involved in cell growth and replication (E2F and Myc), and the upregulation of anti-oxidant genes (**Fig.1G** and **1H**). In contrast, in the susceptible B6.Sst1S macrophages, the sp2 transitioned to subpopulations sp4 and sp5 that were characterized by the decreased G1/S ratios as compared to sp3, signifying an increased G1 – S transition (**Suppl. Table 2**). The sp4 represented an intermediate state between sp2 and sp5 and was primarily characterized by the upregulation of the IFN pathway (**Fig.1G**), as evidenced by the upregulation of known IFN-I pathway activation markers (*Rsad2*, *IL1rn*, *Cxcl10*) (**Suppl.Fig.1C and D**).

The ST macrophages in sp5 uniquely demonstrated a coordinated upregulation of the IFN, TGF β , Myc, E2F, and stress response (p53 and UPR) pathways (**Fig.1G**). They upregulated the Integrated Stress Response (ISR) genes (*Ddit3/Chop10*, *Atf3*, *Ddit4*, *Trib1*, *Trib3*, *Chac1*) in parallel with markers of immune suppression and apoptosis (*Cd274/PD-L1*, *Fas*, *Trail/Tnfsf10*, *Id2*, and a pro-apoptotic ligand – receptor pair *Tnfsf12/Tweak* and *Tnfrsf12a/Tweak* receptor) (**Suppl.Fig.1D**). Of note, IFN-I pathway genes were upregulated in all TNF-stimulated B6.Sst1S macrophages in agreement with the paracrine effect of Ifn β , whose increased production by TNF-stimulated B6.Sst1S macrophages we have described previously (Bhattacharya et al., 2021). Interestingly, the expression of *Irf7* and several IFN-inducible genes, such as *B2m*, *Cxcl10*, and *Ube2l6* was reduced in the sp5 cells, suggesting partial dampening of their IFN-I responsiveness (**Suppl.Fig.1C**).

Taken together, the single cell trajectory analysis revealed that sp5 represented a terminal state of the aberrant B6.Sst1S macrophage activation by TNF that was characterized the coordinated upregulation of stress, pro-apoptotic and immunosuppression genes. Unexpectedly, the stress escalation coincided with paradoxical activity of Myc and E2F pathways. Transition to this state in the susceptible macrophage population was preceded by IFN-I pathway upregulation (sp2 – sp4). In contrast, the sp2 – sp3 transition in the wild type macrophages was coincident with upregulation of both the *Sp110* and *Sp140* genes and was accompanied by the termination of cell cycle and the upregulation of antioxidant defense pathways. Therefore, we concluded that in resistant TNF-stimulated macrophages the *sst1* locus-encoded genes promoted the activation of the AOD pathway either directly, or by suppressing the IFN-I pathway.

To begin exploring the hierarchy and crosstalk of these pathways, we used an unbiased computational approach to define the *Sp110* and *Sp140* regulatory networks. First, we inferred a mouse macrophage gene regulatory network using the GENIE3 algorithm (Huynh-Thu & Geurts, 2018) and external gene expression data for mouse macrophages derived from Gene Expression Omnibus (GEO) (Clough & Barrett, 2016). This network represents co-expression dependencies between transcription factors and their potential target genes, calculated based on mutual variation in expression level of gene pairs (Huynh-Thu et al, 2010; Zhernovkov et al, 2019). This analysis revealed that in mouse macrophages the *Sp110* and *Sp140* genes co-expressed with targets of Nfe2l1/2 (Nuclear Factor Erythroid 2 Like 1/2) and Mtf (metal-responsive transcription factor) TFs that are involved in regulating macrophage responses to oxidative stress and heavy metals, respectively (**Fig.1I**). Taken together, our experimental data and the unbiased network analysis suggested that in TNF-stimulated macrophages the *sst1*-encoded *Sp110* and/or *Sp140* gene(s) might be primarily involved in regulating AOD.

2. Dysregulated AOD activation in B6.Sst1S macrophages

Next, we compared the expression of upstream regulators of AOD in B6 and B6.Sst1S macrophages during TNF activation. Our previous studies demonstrated that the earliest differences between the B6 and B6.Sst1S BMDMs occurred between 8 and 12 h of TNF stimulation, concomitant with the upregulation of the *Sp110* protein in the B6 macrophages and heat shock proteins in the mutant cells (Bhattacharya et al., 2021). Comparing the time course of major transcriptional

regulators of AOD in TNF-stimulated B6 and B6.Sst1S macrophages during this critical time interval, we observed higher upregulation of Nrf2 protein in TNF stimulated B6 BMDMs (**Fig.2A**). The Nrf1 levels were not substantially upregulated after TNF stimulation and were similar in B6 and B6.Sst1S BMDMs (**Suppl.Fig.2A and B**).

The Nrf2 difference was observed both in cytoplasmic and nuclear fractions (**Fig.2B** and **C**, respectively). The levels of Nrf2 negative regulators Keap1 and β -TrCP (**Suppl. Fig.2A**), and Bach1 (**Fig.2B** and **C**) were similar in both backgrounds and did not notably change after TNF stimulation. Quantitative microscopy confirmed that at 12 h of TNF stimulation the cytoplasmic and nuclear Nrf2 levels significantly increased in B6 but not in B6.Sst1S BMDMs (**Fig.2D**, **E** and **Suppl.Fig.2C**). In contrast, the levels of *Nfe2l1/2* mRNA induced by TNF were higher in the mutant macrophages suggesting post transcriptional regulation (**Fig.2F**). Therefore, we measured the rates of Nrf2 protein degradation 6 – 8 h after TNF stimulation but found no difference (**Fig.2G** and **2H**).

Using EMSA, we demonstrated that binding activity of nuclear Nrf2 to its target DNA at 8 and 12 h after TNF stimulation was greater in the resistant B6 BMDMs (**Fig.2I**). To identify core pathways controlled by the *sst1* locus during this critical period, we compared global mRNA expression profiles of the B6 and B6.Sst1S macrophages after 12 h of TNF stimulation using bulk RNA-seq. This analysis confirmed that the *Sp110* and *Sp140* genes were strongly upregulated by TNF stimulation exclusively in the B6 macrophages (**Fig.2J**). Gene set enrichment analysis (GSEA) of genes differentially expressed between TNF-stimulated B6.Sst1S and B6 macrophages at this critical junction revealed that the IFN response, Myc, E2F target gene, Hypoxia, UV response and DNA repair pathways were upregulated in the mutant macrophages, while the detoxification of reactive oxygen species, cholesterol homeostasis, fatty acid metabolism and oxidative phosphorylation, peroxisome and lysosome pathways were downregulated (**Suppl.Table 3**). Functional pathway profiling using KEGG and Reactome databases also highlighted the upregulation of genes involved in oxidative stress and cellular senescence in mutant macrophages. In contrast, the wild type macrophages upregulated genes involved in detoxification of reactive oxygen species, inhibition of ferroptosis and peroxisome function (**Suppl.Table 4**). Supporting these findings, the total antioxidant capacity of the B6 macrophages after TNF stimulation increased to significantly higher levels, as compared to the B6.Sst1S (**Suppl.Fig.2D**).

Transcription factor binding site analysis of genes specifically upregulated by TNF in B6, but not B6.Sst1S, macrophages (B6-specific cluster) revealed an enrichment of Nfe2l1/Nfe2l2, Bach1 and Mafk sequence motifs, i.e. binding sites of transcription factors regulating AOD. In contrast, overrepresentation of E2F, Egr1 and Pbx3 transcription factor binding sites was found for genes in the Sst1S-specific cluster (**Suppl.Table 5**). A master regulator analysis using Virtual Inference of Protein Activity by Enriched Regulon Analysis (VIPER) algorithm also revealed a key role for Nfe2l1/2 (NF-E2-like) transcription factors (TFs) as regulators of genes differentially induced by TNF in B6 and B6.Sst1S BMDMs (**Suppl. Table 6**).

To further investigate this inference, we analyzed the expression of a gene ontology set “response to oxidative stress” (GO0006979, 416 genes) and observed clear separation of these genes in two clusters in a *sst1*-dependent manner (**Fig.2J** and **Suppl.Fig.2G**). This analysis demonstrated that the response to oxidative stress in the *sst1* mutant macrophages was dysregulated, but not paralyzed. For example, the upregulation of well-known Nrf2 target genes Heme oxygenase 1 (*Hmox1*) and (NAD(P)H quinone dehydrogenase 1 (*Nqo1*) were similar in B6 and B6.Sst1S BMDMs (**Suppl.Fig.2E and 2F**, respectively).

A subset of antioxidant defense genes whose expression was concordant with *Sp110* and *Sp140* in B6 macrophages represented genes that are known to be involved in iron storage (ferritin light and heavy chains, *Ftl* and *Fth*), ROS detoxification and maintenance of redox state (*Cat*, *G6pdx*, *Sod2*, *Gstm1*, *Gpx4*, *Prdx6*, *Srxn1*, *Txn2* and *Txnrd1*) (**Fig.2J**). We hypothesized that their

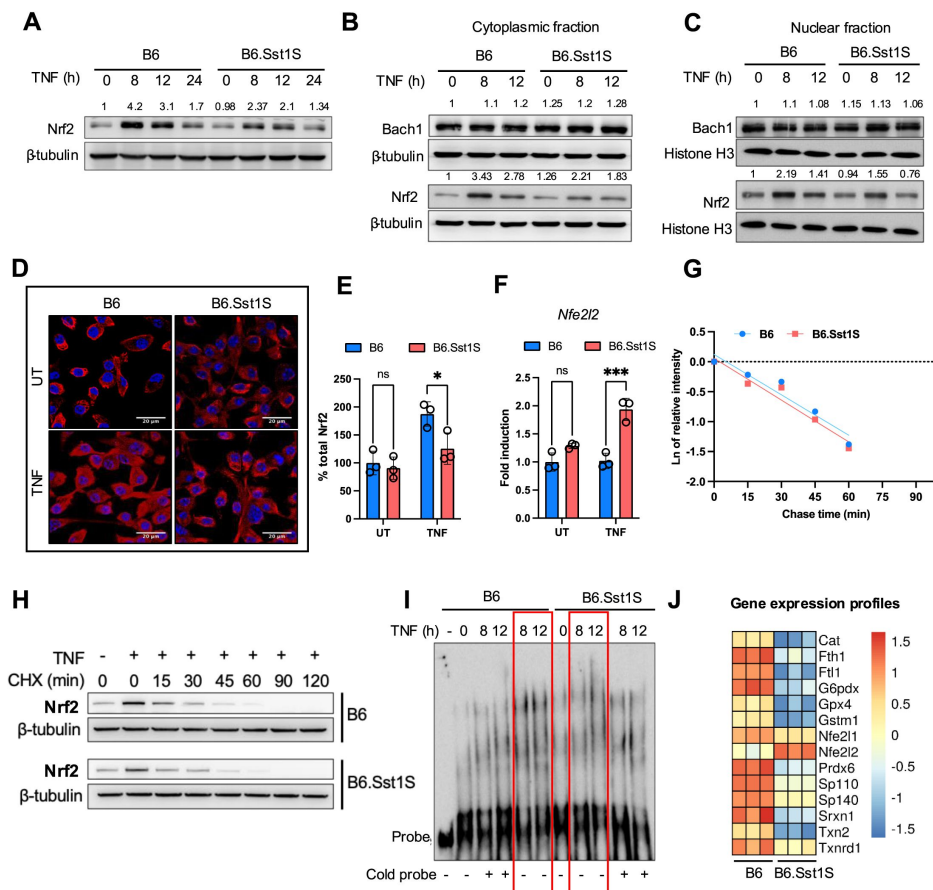


Figure 2

Gene expression profiling comparing B6 and B6.Sst1.S BMDMs stimulated with TNF and regulation of NRF2.

(A). Total level of Nrf2 protein in B6 and B6.Sst1.S BMDMs stimulated with TNF (10 ng/ml) for 8, 12 and 24 h (Western blotting). Average densitometric values from 2 independent experiment were included above the blot. (B). Cytoplasmic Nrf2 and Bach1 proteins in B6 and B6.Sst1.S BMDMs stimulated with TNF (10 ng/ml) for 8 and 12 h (Western blotting). Average densitometric values from 2 independent experiment were included above the blot. (C). Nuclear Nrf2 and Bach1 protein levels in B6 and B6.Sst1.S BMDMs treated with TNF (10 ng/ml) for 8 and 12 h (Western blotting). Average densitometric values from 2 independent experiment were included above the blot. (D and E). Confocal microscopy of Nrf2 protein in B6 and B6.Sst1.S BMDMs stimulated with TNF (10 ng/ml) for 12 h (scale bar 20 μ m). The data shows staining with Nrf2-specific antibody and performed area quantification using ImageJ to calculate the Nrf2 total signal intensity per field. Each dot in the graph represents the average intensity of 3 fields in a representative experiment. The experiment was repeated 3 times. (F). B6 and B6.Sst1.S BMDMs were stimulated with TNF (10 ng/ml) for 8 h. The *Nfe2l2* mRNA levels were quantified using quantitative RT-PCR. Fold induction was calculated by DDCT method, and b-actin was used as internal control and normalized the fold change using B6 UT. (G and H). The Nrf2 protein stability in TNF-stimulated (10 ng/ml) B6 and B6.Sst1.S BMDMs. BMDMs were stimulated with TNF. After 6 h, 25 μ g/ml of cycloheximide (CHX) was added and cells were harvested after 15, 30, 45, 60, 90, and 120 min. The Nrf2 protein levels after TNF stimulation and degradation after cycloheximide addition were determined by Western blotting. I - Linear regression curves of Nrf2 degradation after addition of CHX. Band intensities were measured by densitometry using ImageJ. No significant difference in the Nrf2 half-life was found: B6: 15.14 $\pm$ 2.5 min and B6.Sst1.S: 13.35 $\pm$ 0.6 min. (J). Nuclear Nrf2 binding to target sequence. Nuclear extracts were prepared from BMDMs treated with TNF (10 ng/ml) for 8 and 12 h. The binding activity of Nrf2 was monitored by EMSA using biotin conjugated Nrf2 specific probe (hot probe, red frames). Competition with the unconjugated NRF2 probe (cold probe) was used as specificity control. (J). Anti-oxidant genes co-regulated with *Sp110* and *Sp140* after stimulation with TNF (10 ng/ml) for 12 h. The heatmap was generated using FPKM values obtained from RNA-seq expression profiles of B6.Sst1.S and B6 BMDMs after 12 h of TNF stimulation. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. The statistical significance was performed by two-way ANOVA using Tukey's multiple comparison test (Panel E, F). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

coordinate downregulation in TNF-stimulated B6.Sst1S macrophages sensitized the mutant cells to iron-mediated oxidative damage and played a pivotal role in shaping their divergent activation trajectory.

3. Persistent TNF stimulation of B6.Sst1S macrophages leads to increased accumulation of lipid peroxidation products and IFN-I pathway hyperactivity

To test this hypothesis, first we explored the intracellular iron storage. Both the ferritin light (*Ftl*) and heavy (*Fth*) chain genes were dysregulated in TNF-stimulated B6.Sst1S BMDMs. While the *Fth* mRNA was upregulated by TNF in B6 BMDMs, it remained at a basal level in the B6.Sst1S cells (**Fig.3A** [↗](#)). The *Ftl* mRNA level was significantly reduced after TNF stimulation in B6.Sst1S macrophages but remained at the basal level in B6 (**Fig.3B** [↗](#)). Accordingly, the expression of Ftl protein was reduced in B6.Sst1S BMDMs after 12 h of TNF stimulation, and both Ftl and Fth proteins were reduced at 24 h (**Fig.3C** [↗](#)). In parallel, the levels of Gpx1 and Gpx4 proteins were also substantially reduced by 24 h in B6.Sst1S (**Fig.3D** [↗](#)). The glutathione peroxidase 4 (Gpx4) protein plays a central role in preventing ferroptosis because of its unique ability to reduce hydroperoxide in complex membrane phospholipids and, thus, limit self-catalytic lipid peroxidation (Stockwell et al, 2017 [↗](#)). Thus, TNF-stimulated B6.Sst1S BMDMs had reduced intracellular iron storage capacity accompanied by the decline of the major lipid peroxidation inhibitor Gpx4. Accordingly, we observed increases in an intracellular labile iron pool (LIP, **Fig.3E** [↗](#)), an intracellular accumulation of oxidized lipids (**Fig.3F** [↗](#)), a toxic terminal lipid peroxidation (LPO) products malondialdehyde (MDA, **Fig.3G** [↗](#)) and 4-hydroxynonenal (4-HNE, **Fig.3H** [↗](#) and **Suppl.Fig.3A** [↗](#)). Treatment with the LPO inhibitor Ferrostatin-1 (Fer-1) prevented the 4-HNE accumulation (**Fig.3H** [↗](#) and **Suppl.Fig.3F** [↗](#)). The levels of the labile iron pool (LIP) and LPO remained significantly elevated in B6.Sst1S macrophages after 48h of TNF stimulation (**Suppl.Fig.3B – F** [↗](#)). We also observed an increased ROS production in the B6.Sst1S macrophages during persistent TNF stimulation (**Fig.3I** [↗](#), **3J** [↗](#), and **Suppl. Fig.3G** [↗](#)). The ASK1 - JNK - cJun stress kinase axis was upregulated in B6.Sst1S macrophages, as compared to B6, 12 – 36 h of TNF stimulation, i.e. during the aberrant activation stage confirming persistent stress (**Fig.3K** [↗](#)). By 48h of TNF stimulation, we noted moderate cell death in B6.Sst1S macrophage cultures (**Fig.3L** [↗](#), **Suppl. Fig.3H** [↗](#)). Treatments with Fer-1, the antioxidant butylated hydroxyanisole (BHA), or IFNAR1 blockade each prevented the cell death (**Fig.3M** [↗](#)) suggesting that both persistent stress and macrophage death were mediated by ROS, lipid peroxidation and IFN-I pathway.

Next, we wanted to test whether the IFN-I pathway hyperactivity in TNF stimulated B6.Sst1S macrophages was responsible for the initial dysregulation of the *Ftl*, *Fth* and AOD gene expression, i.e. at 8 – 12 h of TNF stimulation. The IFNAR1 blockade, however, did not restore the Nrf2 and Ftl protein levels, or the *Fth*, *Ftl* and *Gpx1* mRNA levels to the wild type B6 levels (**Fig.4A – C** [↗](#)). Additionally, we observed that LPO production was increased as early as 6 h under TNF stimulated condition in both B6 and B6.Sst1S prior to *Ifnb1* superinduction (**Suppl. Fig.4A** [↗](#) and **B**). These data suggest that type I IFN signaling does not initiate LPO in our model.

Of note, the IFNAR1 blockade did not prevent the *Ifnb1* mRNA superinduction (**Fig.4D** [↗](#)), thus rejecting a hypothesis that the *Ifnb1* superinduction in B6.Sst1S macrophages was driven via an *Infβ* – IFNAR1 positive feedback (Ivashkiv, 2014 [↗](#)). The mRNA expression of the interferon-inducible gene *Rsad2*, however, was suppressed, demonstrating the efficiency of the IFNAR1 blockade (**Fig.4E** [↗](#)).

In contrast, treatment of B6.Sst1S macrophages with Fer-1 or the iron chelator DFO during initial TNF stimulation inhibited both the *Ifnb1* and *Rsad2* mRNAs upregulation (**Fig.4F** [↗](#) and **Suppl. Fig.4C** [↗](#), respectively). Importantly, Fer-1 treatment also reduced the *Ifnb1* and *Rsad2* levels in B6.Sst1S macrophages when added at 18 h after TNF stimulation, i.e. during established aberrant

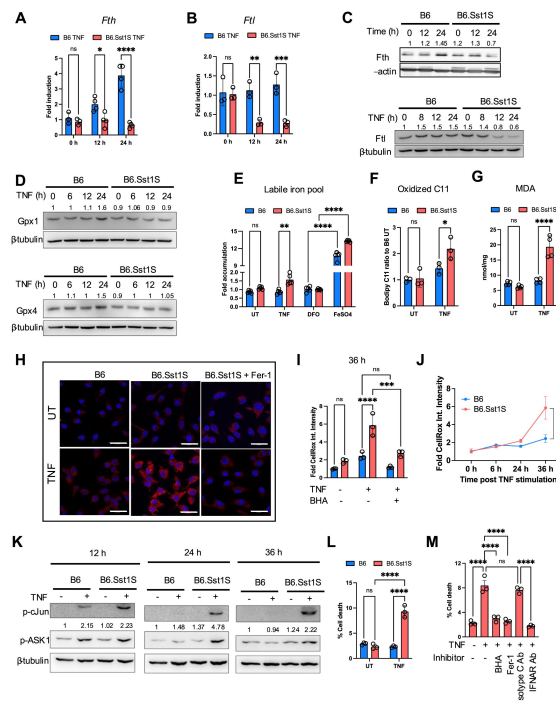


Figure 3.

Regulation of iron and lipid peroxidation in B6 and B6.Sst1.S BMDMs.

(A and B). The expression of *Fth* and *FtI* genes in B6 and B6.Sst1.S BMDMs treated with 10 ng/mL TNF for 12 and 24 h was determined using qRT-PCR. Fold induction was calculated normalizing with B6 untreated control using $\Delta\Delta Ct$ method, and 18S was used as internal control. (C). The *Fth* and *FtI* protein levels in B6 and B6.Sst1.S BMDMs treated with 10 ng/mL TNF for 0, 12 and 24 h (*Fth*) and 0, 8, 12 and 24 h (*FtI*) (Western blot). Average densitometric values from 3 independent experiment were included above the blot. (D). The *Gpx1* and *Gpx4* protein levels in B6 and B6.Sst1.S BMDMs stimulated with TNF (10 ng/mL) for 0, 6, 12, and 24 h (Western blot). Average densitometric values from 2 independent experiment were included above the blot. (E). The labile iron pool (LIP) in TNF-stimulated B6 and B6.Sst1.S BMDMs from were treated with 10 ng/mL TNF for 24 h. UT - untreated control. The LIP was determined using Calcein AM method and represented as fold change as compared to B6 untreated. DFO was used as negative control and FeSo4 was used as positive control. (F). The lipid peroxidation levels were determined by fluorometric method using C11-Bodipy 581/591. BMDMs from B6 and B6.Sst1.S were treated with 10 ng/mL TNF for 30 h. UT - untreated control. (G). Production of lipid peroxidation metabolite malondialdehyde (MDA) by B6 and B6.Sst1.S BMDMs treated with 10 ng/mL TNF for 30 h. UT - untreated control. (H). The accumulation of the intracellular lipid peroxidation product 4-HNE in B6 and B6.Sst1.S BMDMs treated with 10 ng/mL TNF for 48 h. The lipid peroxidation (ferroptosis) inhibitor, Fer-1 (10 μ M) was added 2 h post TNF stimulation in B6.Sst1.S macrophages. The 4HNE accumulation was detected using 4-HNE specific antibody and confocal microscopy. (I). Reactive oxygen species (ROS) levels were observed using the CellROX assay and quantified by automated microscopy in B6 and B6.Sst1.S BMDMs either treated with TNF (10 ng/mL) or left untreated for 36 h. BHA (100 μ M) was used as a positive control. Data are presented as fold mean fluorescence intensity (MFI) normalized by B6 UT, representing ROS levels. (J). Time course of ROS accumulation in B6 and B6.Sst1.S BMDMs during TNF stimulated condition. Reactive oxygen species (ROS) levels were observed using the CellROX assay after 0, 6, 24 and 36 h of TNF stimulation and quantified by automated microscopy. (K). Induction of c-Jun and ASK1 phosphorylation by TNF in B6 and B6.Sst1.S BMDMs. The B6 and B6.Sst1.S BMDMs were treated with TNF (10 ng/ml) or left untreated for 12, 24, and 36 h and the c-Jun and ASK1 phosphorylation was determined by Western blot. Average densitometric values from 2 independent experiment were included above the blot. (L). Cell death in B6 and B6.Sst1.S BMDMs stimulated with 50 ng/mL TNF for 48 h. Percent of dead cells was determined by automated microscopy using Live-or-DyeTM 594/614 Fixable Viability stain (Biotium) and Hoechst staining. (M). Inhibition of cell death of B6.Sst1.S BMDMs stimulated with 50 ng/mL TNF for 48 h using IFNAR1 blocking antibodies (5 μ g/mL), isotype C antibodies (5 μ g/mL), Butylated hydroxyanisole (BHA, 100 μ M) or Fer-1 (10 μ M). Percent cell death was measured as in L. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Šidák's multiple comparison test (Panels A, B, F, G and M) and Tukey's multiple comparison test (Panel E, I, J, L). Statistical significance is indicated by asterisks: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

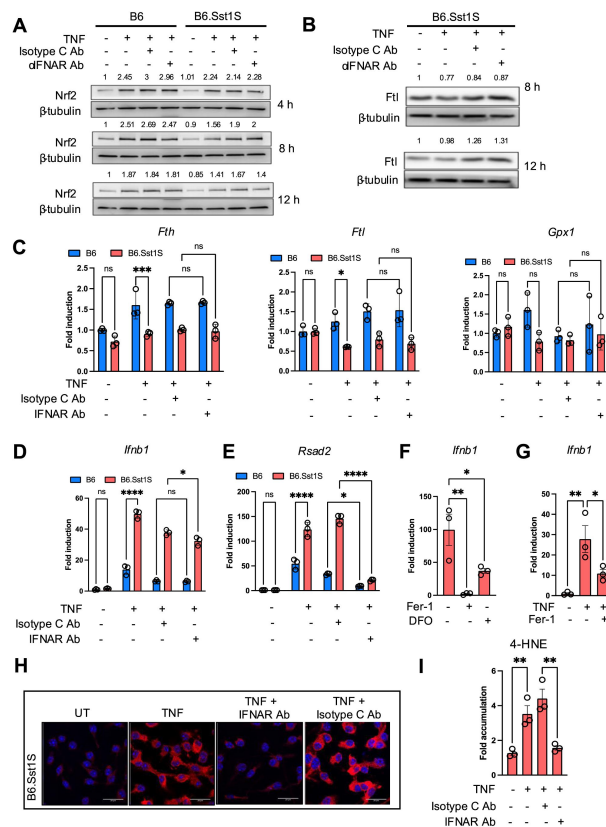


Figure 4.

Crosstalk of the IFN-I and AOD pathways.

(A) IFNAR1 blockade does not enhance Nrf2 upregulation in TNF-stimulated B6.Sst1S macrophages. B6 and B6.Sst1S BMDMs were treated with 10 ng/mL TNF, with or without IFNAR1-blocking antibodies or isotype control (Isotype C Ab) at 5 µg/mL concentration for 4, 8, and 12 hours. Nrf2 protein levels were quantified by Western blot. Average densitometric values from two separate experiments were included above the blot. (B) IFNAR1 blockade does not increase Ftl expression in TNF-stimulated B6.Sst1S macrophages. B6.Sst1S BMDMs were treated with 10 ng/mL TNF, with or without IFNAR1-blocking antibodies (5 µg/mL) or Isotype C Ab (5 µg/mL), for 8 and 12 hours. Ftl protein levels were quantified by Western blot. Average densitometric values from 2 independent experiment were included above the blot. (C) IFNAR1 blockade does not increase mRNA levels of *Fth*, *Ftl*, and *Gpx1*. B6 and B6.Sst1S BMDMs were treated with 10 ng/mL TNF, with or without IFNAR1-blocking antibodies or Isotype C Ab, for 12 hours. Blocking antibodies (5 µg/mL) or isotype C antibodies (5 µg/mL) were added 2 hours after TNF stimulation. Fold induction was calculated using B6 untreated control as average one-fold by utilizing the $\Delta\Delta C_t$ method with β -actin as the internal control. (D and E) IFNAR1 blockade reduces *Rsad2* mRNA levels (E) but does not affect *Ifnb1* mRNA levels (D). B6 and B6.Sst1S BMDMs were treated with 10 ng/mL TNF, with or without IFNAR1-blocking antibodies or Isotype C Ab for 16 hours. Blocking antibodies (5 µg/mL) or isotype C antibodies (5 µg/mL) were added 2 hours after TNF stimulation. Fold induction was calculated using B6 untreated control as average one-fold by utilizing the $\Delta\Delta C_t$ method with β -actin as the internal control. (F) Lipid peroxidation inhibition prevents the superinduction of *Ifnb1* mRNA. B6.Sst1S BMDMs were treated with 10 ng/mL TNF, and the lipid peroxidation inhibitor (Fer-1) was added 2 hours post-TNF stimulation. *Ifnb1* mRNA levels were measured using qRT-PCR after 16 hours of TNF treatment. Fold induction was calculated using untreated control as average one-fold by utilizing the $\Delta\Delta C_t$ method with 18S as the internal control. (G) Lipid peroxidation inhibition reverses the superinduction of *Ifnb1* mRNA. B6.Sst1S BMDMs were stimulated with 10 ng/mL TNF for 18 h, then the LPO inhibitor (Fer-1) was added for the remaining 12 h. *Ifnb1* mRNA levels were measured using qRT-PCR. Fold induction was calculated using untreated control as average one-fold by utilizing the $\Delta\Delta C_t$ method with 18S as the internal control. (H and I) IFNAR1 blockade reduces 4-HNE accumulation in B6.Sst1S BMDMs treated with TNF (10 ng/mL) for 48 hours. Blocking antibodies (5 µg/mL) or Isotype C Ab (5 µg/mL) were added 2 hours post-TNF stimulation. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. The statistical significance was performed by two-way ANOVA using Tukey's multiple comparison test (Panel C-E), Ordinary one-way ANOVA using Bonferroni's multiple comparison test (Panel F-G and I). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

response (**Fig. 4G** [↗](#) and **Suppl. Fig.4D** [↗](#)). Thus, lipid peroxidation was involved in both the initial *Ifnb1* superinduction and in maintenance of the and IFN-I pathway hyperactivity driven by prolonged TNF stimulation.

Next, we wanted to test whether continuous IFN-I signaling was required for the accumulation of 4-HNE. during prolonged macrophage activation. Indeed, the blockade of type I IFN receptor (IFNAR1) after 2, 12 or 24 h after TNF stimulation prevented the 4-HNE accumulation at 48 h (**Fig.4H and I** [↗](#), and **Suppl.Fig.4E - G** [↗](#)). Thus, *Ifnb1* super-induction and IFN-I pathway hyperactivity in B6.Sst1S macrophages follow the initial LPO production, and maintain and amplify it during prolonged TNF stimulation.

The *sst1*-encoded *Sp110* and *Sp140* genes were described as interferon-induced genes, and Sp140 protein was implicated in maintenance of heterochromatin silencing in activated macrophages (Fraschilla & Jeffrey, 2020a [↗](#); Mehta et al., 2017 [↗](#)). Therefore, we hypothesized that their deficiency in the TNF-stimulated B6.Sst1S mutants may lead to the upregulation of silenced transposons and, thus, trigger the *Ifnb* upregulation via intracellular RNA sensors, i.e. by an autonomous mechanism unrelated to the AOD dysregulation. We examined the transcriptomes of B6 and B6.Sst1S macrophages before and after TNF stimulation for the presence of persistent viruses or transposons using a custom bioinformatics pipeline (Ma et al, 2021 [↗](#)). No exogenous mouse viral RNAs were detected. A select set of mouse LTR-containing endogenous retroviruses (ERV's) (Jayewickreme et al, 2021 [↗](#)), and non-retroviral LINE L1 elements were expressed at a basal level before and after TNF stimulation, but their levels in the B6.Sst1S BMDMs were similar to or lower than those seen in B6 (**Suppl.Fig.5A - C** [↗](#)). We also tested the accumulation of dsRNA using deep sequencing of macrophage small RNAs and failed to detect evidence of transposon-derived dsRNAs (**Suppl.Fig.5D** [↗](#)). We concluded from these findings that the majority of the basal transposon RNAs in macrophages exist primarily as single-stranded mRNAs that evade triggering interferon pathway. The above analyses allowed us to exclude the overexpression of persistent viral or transposon RNAs as a primary mechanism of the IFN-I pathway hyperactivity.

Taken together, the above experiments allowed us to reject the hypothesis that IFN-I hyperactivity caused the *sst1*-dependent AOD dysregulation. In contrast, they established that the hyperactivity of the IFN-I pathway in TNF-stimulated B6.Sst1S macrophages was itself driven by the *initial* dysregulation of AOD and iron-mediated lipid peroxidation. During prolonged TNF stimulation, however, the IFN-I pathway was upregulated, possibly via ROS/LPO-dependent JNK activation, and acted as a potent amplifier of lipid peroxidation.

4. Hyperactivity of Myc in susceptible macrophages after TNF stimulation fuels lipid peroxidation

We wanted to determine whether the AOD genes were regulated by the *sst1*-encoded genes directly or indirectly via an intermediary regulator. Previously, we identified two transcription factors whose binding activities were exclusively upregulated in the susceptible macrophages after 12h of TNF stimulation: Myc and HSF1 (Bhattacharya et al., 2021 [↗](#)). The bulk RNA-seq analysis at this timepoint (12 h) also demonstrated the upregulation of Myc pathway along with E2F target genes and stress responses specifically in TNF-stimulated B6.Sst1S BMDMs, as compared to B6 (**Fig.2** [↗](#)). Of note, the scRNA-seq analysis above also demonstrated the association of Myc and stress response pathways in the mutant cells at 24 h (**Fig.1** [↗](#)). Therefore, we hypothesized that in susceptible macrophages Myc might be involved in the dysregulation of AOD and iron storage.

First, we observed that Myc was regulated in an *sst1*-dependent manner: in TNF-stimulated B6 wild type BMDMs, *c-Myc* mRNA was downregulated, while in the susceptible macrophages *c-Myc* mRNA was upregulated (**Fig.5A** [↗](#)). The *c-Myc* protein levels were also higher in the B6.Sst1S cells in unstimulated BMDMs and 6 – 12 h of TNF stimulation (**Fig.5B** [↗](#)). Next, we tested whether

suppression of Myc activity could “normalize” the susceptible phenotype using Myc-Max dimerization inhibitor 10058-F4 (F4). Indeed, this treatment increased the levels of Fth and Ftl proteins in TNF-stimulated susceptible macrophages (**Fig.5C**) and decreased the labile iron pool (**Fig.5D**). Accordingly, the levels of MDA, oxidized lipids and 4-HNE also significantly decreased (**Fig.5E – G**, **Suppl.Fig.6A and B**), as well as the levels of *Ifnb1*, *Rsad2* and the ISR markers *Trib3* and *Chac1* (**Fig.5H**). Possibly, the ISR activation serves as an alternative pathway of Myc inhibition, as it is known to inhibit the oncogene protein translation (Wolfe et al, 2014).

Next, we wanted to determine whether the upregulation of Myc is driven by TNF alone or in synergy with CSF1, a growth factor that also stimulates Myc. In vitro, we observed the upregulation of Myc shortly after the addition of fresh CSF1-containing media, but no difference in the Myc protein dynamics between B6 and B6.Sst1S BMDMs in the absence of TNF (**Suppl.Fig.6C**). In addition, we tested whether CSF1R inhibitors could prevent the superinduction of *Ifnb1* and *Rsad2* mRNAs in TNF-stimulated B6.Sst1S macrophages. The CSF1R inhibitors were used at concentrations that did not cause macrophage death (**Suppl. Fig.6D**). Neither of these inhibitors prevented the *Ifnb1* and *Rsad2* upregulation (**Suppl.Fig.6E** and **F**). Because Myc induction by CSF1 and TNF is conducted via distinct relays of receptor signaling and transcription factors, we concluded that the *sst1* locus specifically controls Myc expression induced by inflammatory signaling. Indeed, Myc promoter has multiple predicted NF- κ B and/or AP-1 transcription factors binding sites. To test this hypothesis, we used specific JNK inhibitor D-JNK1, but it only partially reduced Myc protein levels in TNF-stimulated B6.Sst1S macrophages (**Fig.5I and J**). Because both *Sp110* and *Sp140* mRNAs and proteins are upregulated during extended TNF stimulation, one of them may participate in feedback regulation of TNF-induced Myc, either directly or via yet unknown intermediates.

5. Myc hyperactivity and lipid peroxidation compromise the cell autonomous and T cell-mediated control of Mtb infection by B6.Sst1S macrophages

Next, we tested whether the described facets of the aberrant macrophage activation conferred by the *sst1S* allele were relevant to Mtb susceptibility. After macrophage infection with virulent Mtb in vitro, gradual accumulation of LPO product 4-HNE was observed in BMDMs of both B6 and B6Sst1S genetic backgrounds at 3 – 5 days post infection (dpi). It occurred either in the presence or absence of exogenous TNF (**Fig.6A**, and **Suppl.Fig.7A and B**). By day 5 post infection, TNF stimulation significantly increased LPO accumulation only in the B6.Sst1S macrophages (**Fig.6A**). Both Mtb-infected and bystander non-infected B6.Sst1S macrophages showed 4-HNE accumulation (**Fig. 6B**).

TNF stimulation improved the control of Mtb growth in B6 but not in B6.Sst1S macrophages, as quantified using an Mtb replication reporter strain (**Fig.6B**, **Suppl. Fig.7C and D**) and quantitative PCR of Mtb genomes (**Fig.6C**). The LPO inhibition using Fer-1 improved the survival of the Mtb-infected BMDMs (**Fig.6D – E**) and prevented the intracellular Mtb growth (**Fig.6F**) during the five-day in vitro infection. The iron chelator DFO also significantly reduced the Mtb growth, although restricting iron availability may also directly affect the bacterial replication. Importantly, the survival of Mtb-infected BMDMs was also improved, and the bacterial loads were significantly reduced by the Myc inhibitor, F4 (**Fig.6G and H**). Of note, the effects of the lipid peroxidation inhibitors became prominent between days 3 and 5 post infection (**Suppl.Fig.7E**) suggesting that these inhibitors do not directly boost the bacterial killing by activated macrophages, but rather prevent macrophage damage.

Next, we tested whether the *sst1* susceptible allele compromised responsiveness of Mtb-infected macrophages to mycobacteria-specific T cells. The immune T cells were isolated from the regional lymph nodes of the resistant B6 mice vaccinated with live attenuated BCG vaccine, and added either to the B6 or B.Sst1S BMDM monolayers infected with Mtb the day before. The BMDMs were

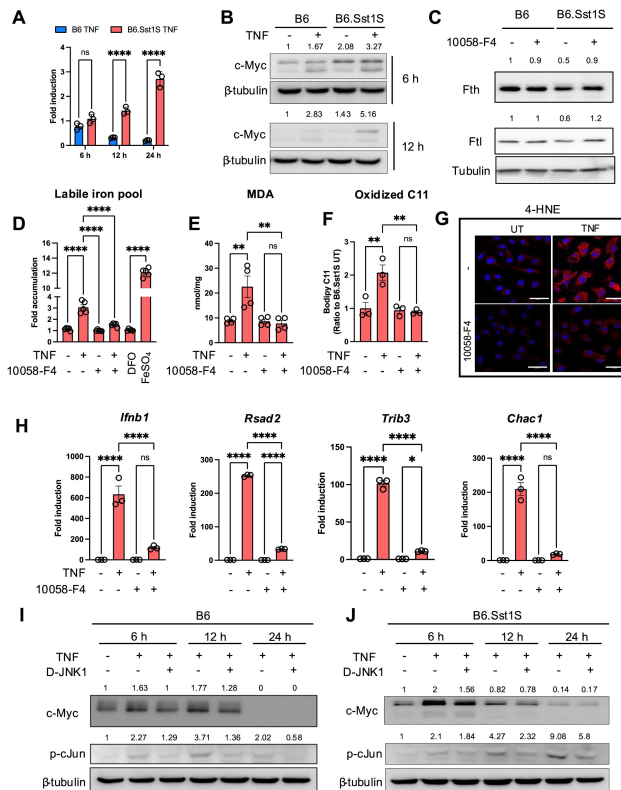


Figure 5.

Myc dysregulation drives the aberrant state of macrophage activation.

(A). The lack of *Myc* mRNA downregulation after prolonged TNF stimulation in B6.Sst1S macrophages. BMDMs from B6 and B6.Sst1S were treated with 10 ng/mL TNF for 6, 12 and 24 h. Expression of *Myc* was quantified by the $\Delta\Delta C_t$ method using qRT-PCR and expressed as a fold induction compared to the untreated B6 BMDMs. β -actin was used as the internal control. (B). *Myc* protein levels expressed by B6 and B6.Sst1S BMDMs during the course of stimulation with TNF(10 ng/mL) for 6 and 12 h. (Western blot). Average densitometric values from 2 independent experiment were included above the blot. (C). *Myc* inhibition restored the levels of Fth and Ftl proteins in TNF-stimulated B6.Sst1S macrophages to the B6 levels. B6 and B6.Sst1S BMDMs from were treated with 10 ng/mL TNF alone or in combination with *Myc* inhibitor, 10058-F4 (10 μ M) for 24 h. 10058-F4 was added 2 h post TNF stimulation. Protein levels of Fth and Ftl were observed using Western blot. Average densitometric values from 2 independent experiment were included above the blot. (D). *Myc* inhibition decreased the labile iron pool in TNF-stimulated B6.Sst1S macrophages. B6.Sst1S BMDMs were treated with 10 ng/mL TNF or left untreated for 48 h. The 10058-F4 inhibitor was added 2 h post TNF stimulation. The labile iron pool (LIP) was measured using Calcein AM method and represented as fold change as compared to untreated control. DFO was used as negative control and FeSo₄ was used as positive control. (E and F). *Myc* inhibition reduced lipid peroxidation in TNF-stimulated B6.Sst1S BMDMs. Cells were treated with 10 ng/mL TNF in presence or absence of 10058-F4 for 48 h. The inhibitor was added 2 h post TNF stimulation. The MDA production was measured using commercial MDA assay (E). The lipid peroxidation was measured by fluorometric method using C11-Bodipy 581/591 (F). (G). B6.Sst1S BMDMs were treated as above in E. The accumulation of lipid peroxidation product, 4HNE after 48h was detected by confocal microscopy using 4-HNE specific antibody. The 4HNE accumulation was quantified using ImageJ and plotted as fold accumulation compared to untreated group. (H). The BMDMs from B6.Sst1S were treated with 10 ng/mL TNF alone or in combination with *Myc* inhibitor, 10058-F4 (10 μ M) for 24 h. 10058-F4 was added 2 h post TNF stimulation. Expression of *Ifnb1*, *Rsad2*, *Trib3* and *Chac1* was quantified by the $\Delta\Delta C_t$ method using qRT-PCR and expressed as a fold induction compared to the untreated group. 18S was used as the internal control. (I and J). B6 (I) and B6.Sst1S (J) BMDMs were treated with TNF (10 ng/ml) for 6, 12, and 24 h in the presence or absence of JNK inhibitor D-JNK1 (2 μ M). The cells were harvested and the protein levels of c-Myc and p-c-Jun were determined by western blotting. JNK inhibitor D-JNK1 was added 2 h post TNF stimulation. Average densitometric values from 2 independent experiment were included above the blot. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. The statistical significance was performed by two-way ANOVA using Šídák's multiple comparison test (Panel A) and ordinary one-way ANOVA using Šídák's multiple comparison test (Panel D-F and H). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

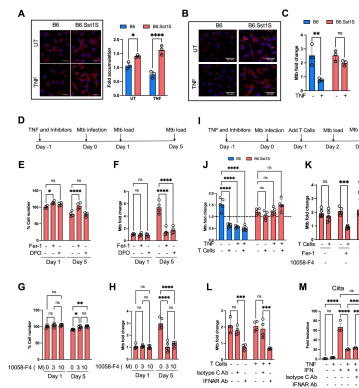


Figure 6.

Myc and lipid peroxidation compromise control of intracellular Mtb by the B6.Sst1S macrophages.

(A). Accumulation of 4-HNE in Mtb-infected B6 and B6.Sst1S macrophage monolayers infected with Mtb. BMDMs were either treated with 10 ng/mL TNF or left untreated (UT), and subsequently infected with Mtb at MOI=1. 4-HNE was detected by confocal microscopy using 4-HNE specific antibody 5 dpi. The 4HNE accumulation was quantified at 5 dpi using ImageJ and plotted as fold accumulation compared to untreated B6 (UT). (B). Naïve and TNF-stimulated B6 and B6.Sst1S BMDMs were infected with Mtb Erdman reporter strain (SSB-GFP, *smyc*::mCherry) for 5 days. The accumulation of 4HNE was detected in both Mtb-infected and non-infected B6.Sst1S cells at day 5 p.i. (C). Naïve and TNF-stimulated B6.Sst1S BMDMs were infected with Mtb at MOI=1. At days 4 post infection Mtb load was determined using a qPCR-based method. (D). Testing the effects of LPO and Myc inhibitors on the Mtb-infected B6.Sst1S BMDM survival and Mtb control: experimental design for panels E - H. (E and F). Prevention of iron-mediated lipid peroxidation improves the survival of and Mtb control by the B6.Sst1S macrophages. BMDMs were treated with 10 ng/mL TNF alone in combination with Fer-1 (3 μ M) or DFO (50 μ M) for 16 h and subsequently infected with Mtb at MOI=1. The inhibitors were added after infection for the duration of the experiment. At days 1 and 5 post infection, total cell numbers were quantified using automated microscopy (E) and Mtb loads was determined using a qPCR-based method (F). The percentage cell number were calculated based on the number of cells at Day 0 (immediately after Mtb infection and washes). The fold change of Mtb was calculated after normalization using Mtb load at Day 0 after infection. (G and H). Myc inhibition improves the survival and Mtb control by B6.Sst1S macrophages. BMDMs were treated with 10 ng/mL TNF alone or in combination with 3 μ M or 10 μ M 10058-F4 for 16 h and subsequently infected with Mtb at MOI=1. At days 1 and 5 post infection, total cell numbers were quantified using automated microscopy (G) and Mtb loads was determined using a qPCR-based method (H). The percentage cell number were calculated based on the number of cells at Day 0 (immediately after Mtb infection). The fold change of Mtb was calculated after normalization using Mtb load at Day 0 after infection and washes. (I). LPO and Myc inhibitors improve Mtb control by B6.Sst1S BMDMs co-cultured with BCG-induced T cells: experimental design for panels J - L. (J). Differential effect of BCG-induced T cells on Mtb control by B6 and B6.Sst1S macrophages. BMDMs of both backgrounds were treated with 10 ng/mL TNF or left untreated and subsequently infected with Mtb at MOI=1. T lymphocytes purified from lymph nodes of BCG vaccinated B6 mice were added to the infected macrophage monolayers 24 h post infection. The Mtb load was calculated by qPCR based method after 2 days of co-culture with T lymphocytes (3 days post infection). The dotted line indicates the Mtb load in untreated cells at day 2 post infection. (K). Inhibition of Myc and lipid peroxidation improves control of Mtb by B6.Sst1S macrophages co-cultured with immune T cells isolated from BCG-vaccinated B6 mice. BMDMs were pretreated with 10 ng/mL TNF alone or in combination with either Fer-1 (3 μ M) or 10058-F4 (10 μ M) for 16 h and subsequently infected with Mtb at MOI 1. At 24 h post infection the lymphocytes from BCG immunized B6 mice were added to the infected macrophage monolayers. The Mtb loads were determined by qPCR based method after 2 days of co-culture with T cells (3 days post infection). (L). Inhibition of type I IFN receptor improves control of Mtb by B6.Sst1S macrophages. BMDMs were pretreated with 10 ng/mL TNF alone or in combination with IFNAR1 blocking Ab or isotype C Ab for 16 h and subsequently infected with Mtb at MOI 1. At 24 h post infection the lymphocytes from BCG immunized B6 mice were added to the infected macrophage monolayers. The Mtb loads were determined by qPCR-based method after 2 days of co-culture with T cells (3 days post infection). (M). TNF stimulation inhibits and IFNAR1 blockade restores the response of B6.Sst1S macrophages to IFN γ . BMDMs were pretreated with TNF (10 ng/mL) for 18 h and the IFNAR1 blocking Abs or isotype C Ab were added two hours after TNF. Subsequently, IFN γ (10 U/mL) was added for additional 12 h. The expression of the IFN γ -specific target gene *Ciita* was assessed using qRT-PCR. 18S was used as internal control. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. The statistical significance was performed by two-way ANOVA using Bonferroni's multiple comparison test (Panel A, C, J and K) and Tukey's multiple comparison test (Panel E-H and L). One-way ANOVA using Bonferroni's multiple comparison test (Panel M). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

either treated with TNF prior to infection or not. After co-culture with the immune T cells for one or two days, Mtb loads were significantly reduced in T cell co-cultures with the resistant B6 macrophages. The susceptible B6.Sst1S BMDMs did not respond to the same T cells either in the presence or absence of exogenous TNF (**Fig.6I - J** [↗](#) and **Suppl.Fig.7F** [↗](#)). The B6.Sst1S BMDM responsiveness to T cells was improved by inhibitors of lipid peroxidation (Fer1) and Myc (F4): the bacterial loads were significantly reduced 48 h after co-culture with the immune T cells (**Fig.6K** [↗](#), **Suppl. Fig.7G** [↗](#)). This T cell-mediated Mtb control was specific for T cells isolated from BCG-immunized mice (**Suppl. Fig.7H** [↗](#)). The IFNAR1 blockade improved the ability of TNF-stimulated B6.Sst1S macrophages to control Mtb with and without T help (**Fig.6L** [↗](#), **Suppl. Fig.7I** [↗](#)).

It also restored their responsiveness to soluble Ifny that was inhibited by pre-stimulation with TNF (**Fig.6M** [↗](#)). These data demonstrate that during prolonged TNF stimulation of B6.Sst1S macrophages the Myc-driven lipid peroxidation and subsequent IFN-I hyperactivity compromise both the cell autonomous and T cell-mediated Mtb control.

6. Loss of Mtb control in pulmonary TB lesions is associated with the accumulation of lipid peroxidation products and stress escalation in intralesional macrophages

We wanted to determine whether the aberrantly activated macrophages accumulate within pulmonary TB lesions in vivo. We used a mouse model of pulmonary TB where the lung lesions develop after hematogenous spread from the primary site of infection and progress exclusively in the lungs, despite systemic immunity and control of infection in other organs. Microscopic pulmonary lesions develop in the lungs of both B6 and B6.Sst1S mice, but advanced multibacillary TB lesions develop exclusively in the B6.Sst1S ([Yabaji et al, 2025a](#) [↗](#); [Yabaji et al, 2025b](#) [↗](#); [Yabaji et al., 2023](#) [↗](#)).

Based on Mtb loads, TB lesions were classified in two categories: the Mtb-controlling paucibacillary lesions, and multibacillary lesions, in which the control of Mtb growth was compromised (**Suppl.Fig.8** [↗](#)). The 4-HNE levels dramatically increased in multibacillary lesions (**Fig.7A** [↗](#) and **Suppl. Fig.9A** [↗](#)). The majority of the 4-HNE+ cells were CD11b+ myeloid cells (**Fig.7B** [↗](#) and **Suppl. Fig.9B** [↗](#)).

To characterize macrophages in TB lesions and identify pathways associated with the loss of Mtb control, we performed spatial transcriptomics analysis of intralesional macrophages (Iba1+) using the Nanostring GeoMX Digital Spatial Profiler (DSP) system ([Merritt et al, 2020](#) [↗](#)) (**Suppl.Fig.10** [↗](#)).

Comparing the Iba1+ macrophage gene expression profiles in the multibacillary vs paucibacillary lesions, we identified 192 upregulated and 376 downregulated genes at a two and above fold change (**Suppl.Table 7** [↗](#)). Pathway analysis demonstrated a highly significant upregulation of Hypoxia, TNF and IL6/STAT3 Signaling, Glycolysis, Complement and Coagulation pathways in the multibacillary lesions consistent with the escalation of hypoxia, inflammation and macrophage activation in the advanced lesions. Mechanistically, top transcription factors associated with genes upregulated in the multibacillary lesions were NFKB1, JUN, STAT1, STAT3 and SP1 (**Suppl.Fig.11A - C** [↗](#)).

To specifically interrogate the interferon pathways in paucibacillary vs multibacillary lesions, we compiled a list of 430 interferon type I and type II inducible genes from public databases that were also included in Nanostring Whole Transcriptome Analysis (WTA) probes. Among these, 70 genes were differentially regulated between the Iba1+ macrophages in multi-vs paucibacillary lesions (**Fig.7C** [↗](#)). Among the upregulated genes were metalloprotease *Mmp12*, *IL6* and *Socs3*, chemokines *Cxcl10*, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl19*, cell stress and senescence marker p21 (*Cdkn1a*), and many known IFN-I target genes. The most upregulated pathways in Iba1+ cells within

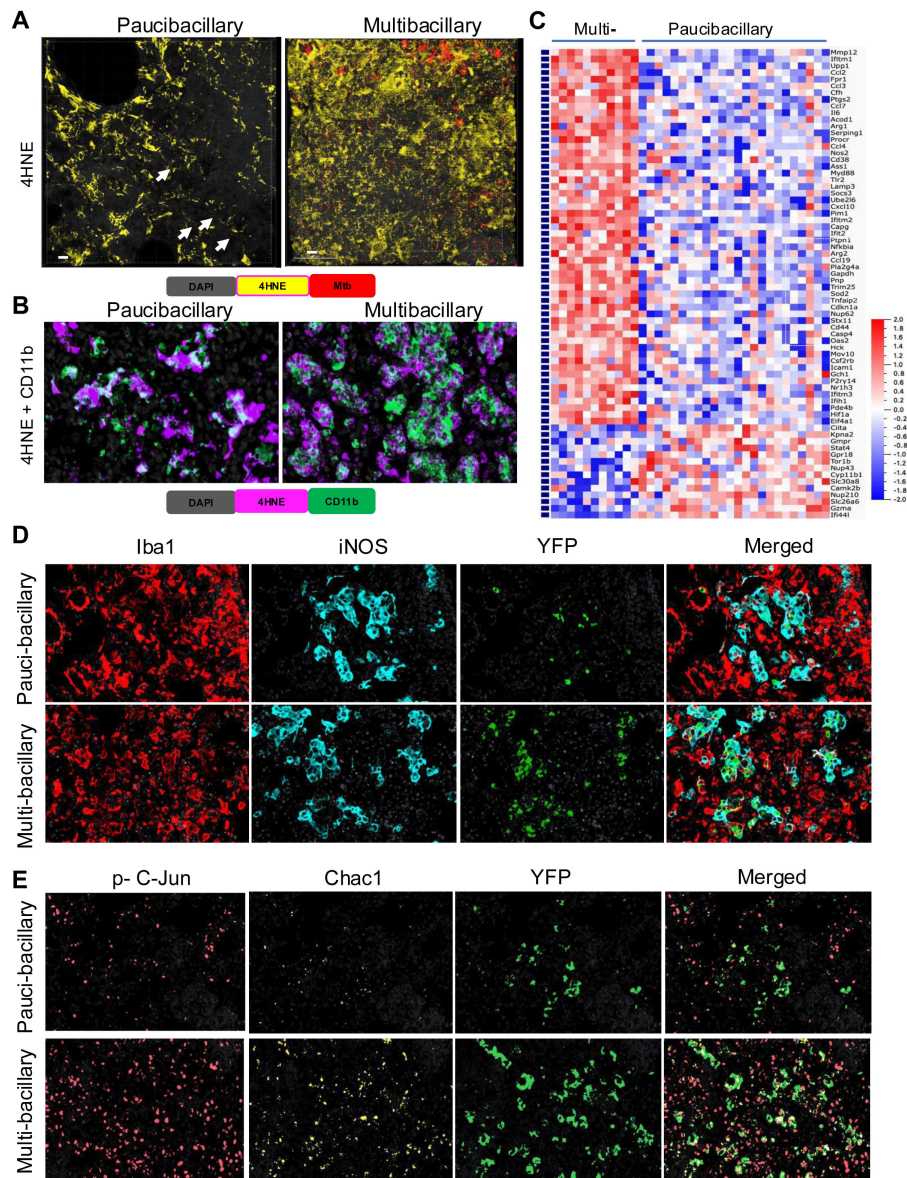


Figure 7

Accumulation of lipid peroxidation products and stress escalation in macrophages during pulmonary TB progression.

(A). Representative 3D confocal images of paucibacillary (n=16) and multibacillary (n=16) pulmonary TB lesions of B6.Sst1S, *Ifnb1*-YFP reporter mice stained with anti-4HNE antibody (yellow). Cells expressing YFP are green, Mtb reporter Mtb (*smyc'*:: mCherry) is red. Arrows indicate Mtb reporter strain expressing mCherry. The mice were infected for 20 weeks. (B). Representative Fluorescent multiplexed immunohistochemistry (fmIHC) images of pauci-bacillary and multi-bacillary PTB lesion in B6.Sst1S mice at high magnification (600X). 4-HNE (magenta), CD11b (green) and DAPI (grey). White areas showing 4-HNE and CD11b co-localization. The mice were infected for 20 weeks. (C). Heatmap of interferon inducible genes differentially expressed in Iba1+ cells within multibacillary vs paucibacillary lesions (fold change 1.5 and above). Pooled gene list of IFN type I and II regulated genes was assembled using public databases (as described in Methods). The mice were infected for 14 weeks. (D). Representative fmIHC images of IFN-I producing (YFP positive) myeloid cells in pauci-bacillary (n=8) and multi-bacillary (n=8) lesion of B6.Sst1S, *Ifnb1*-YFP reporter mice. The different markers are shown as Iba1 (red), iNOS (teal) and YFP (green) at 400X magnification. The mice were infected for 20 weeks. (E). Representative fmIHC images of IFN-I producing (YFP positive) cells accumulating stress markers in pauci-bacillary (n=6) and multi-bacillary (n=9) lesion of B6.Sst1S, *Ifnb1*-YFP reporter mice. The different markers are shown as phospho-c-Jun (peach), Chac-1 (yellow) and YFP (green) at 400X original magnification. The mice were infected for 20 weeks.

multibacillary lesions were Interferon, TNF, and IL6/STAT3 Signaling, and top transcription factors associated with upregulation of these pathways were NFKB1, STAT3, STAT1 and IRF1 (Suppl.Fig.11D – [E](#)).

Among the IFN-inducible genes upregulated in paucibacillary lesions were *Ifi44l*, a recently described negative regulator of IFN-I that enhances control of Mtb in human macrophages (DeDiego et al, 2019 ; Jiang et al, 2021) and *Ciita*, a regulator of MHC class II inducible by Ifny, but not IFN-I (Suppl.Table 8). Thus, the loss of local Mtb control in advanced pulmonary TB lesions was associated with the accumulation of aberrantly activated macrophages that contained lipid peroxidation products, with upregulation of the IFN-I pathway and downregulation of Ifny-inducible genes.

To detect the Ifn β -expressing cells within TB lesions, we introduced the *Ifnb1*-YFP reporter described previously (Scheu et al, 2008) in the B6.Sst1S background. The B6.Sst1S,*Ifnb1*-YFP reporter mice were infected with virulent Mtb (*smyc*:: mCherry) constitutively expressing the red fluorescent protein reporter (Lavin & Tan, 2022). We validated the Ifn β reporter in vitro using co-staining of TNF-stimulated B6.Sst1S,*Ifnb1*-YFP BMDMs with YFP-specific antibodies (Suppl.Fig.12A). We demonstrated the accumulation of Ifn β expressing cells in pulmonary TB lesions of B6.Sst1S, *Ifnb1*-YFP mice using both the YFP reporter and in situ hybridization with the *Ifnb1* probe (Suppl.Fig.12B). The majority of the YFP+ cells in TB lesions were Iba1+ macrophages a fraction of which were iNOS positive (Fig. 7D , Suppl. Fig.13A). The mice with paucibacillary lesions had 62% and multibacillary lesions had 80% activated macrophages expressing YFP. These findings clearly demonstrated the production of Ifn β by activated M1-like macrophages.

Next, we assessed the expression of stress markers phospho-cJun and Chac1 in total, IFN-I producing and activated macrophages in TB lesions. These markers were primarily expressed by activated macrophages (Iba1+) expressing iNOS and/or Ifn β (YFP+) (Fig.7E , Suppl. Fig.13B - F). We also documented the upregulation of PKR in the multibacillary lesions, which is consistent with the upregulation of the upstream IFN-I and downstream Integrated Stress Response (ISR) pathways, as described in our previous studies (Bhattacharya et al., 2021) (Suppl.Fig.14A).

Thus, progression from the Mtb-controlling paucibacillary to non-controlling multibacillary TB lesions in the lungs of TB susceptible mice was mechanistically linked with a pathological state of macrophage activation characterized by escalating stress (as evidenced by the upregulation phospho-cJun, PKR and Chac1), the upregulation of Ifn β and the IFN-I pathway hyperactivity, with a concurrent reduction of Ifny responses. In our in vitro experiments, these stressed macrophages were unresponsive to T cell help (Fig.6J). Consequently, the administration of BCG vaccine to Mtb-infected B6.Sst1S mice did not increase their survival (Suppl.Fig.14B).

7. Myc upregulated genes are enriched in TB patients who fail treatment

Next, we tested whether the upregulation of Myc pathway is associated with pulmonary TB progression in human TB patients. We used blood samples obtained from 41 individuals recently (<90 days) diagnosed with TB that were enrolled in the Regional Prospective Observational Research for Tuberculosis (RePORT)-India consortium. Patients were infected with drug susceptible Mtb and treated with rifampicin, isoniazid, ethambutol, and pyrazinamide, per Technical and Operational Guidelines for TB Control by the Ministry of Health and Family Welfare, Government of India, 2016. Blood samples were collected before or within a week of the antibiotic treatment commencement. Patients were monitored for two years during and post-treatment. Individuals who failed the antibiotic treatment (n=21) were identified by positive sputum culture

or clinical diagnosis of symptoms at any time after 4 full months of treatment and symptoms determined to not be from another cause. Cured TB patients (controls) remained culture-negative and symptom-negative for the two-year observation period.

Several oncogene signatures identified previously (Bild et al, 2006 [\[1\]](#)) were analyzed with the TBSignatureProfiler (Johnson et al, 2021 [\[2\]](#)) to determine their ability to differentiate between treatment failures and controls based on bootstrapped AUC scores. Myc_up ranked within the top 3 signatures with an AUC of 0.74 and p-value of 0.008 (Suppl.Fig.15A [\[3\]](#)). Boxplots of ssGSEA scores were created to determine whether the myc signatures were differentially enriched between treatment failures and controls. Myc_upregulated genes were enriched in the treatment failure group relative to the treatment control group (Suppl.Fig.15B [\[4\]](#), Suppl.Table 9 [\[5\]](#)). The ROC curve used to generate the AUC scores for myc_up is depicted in Suppl.Fig.15C [\[6\]](#), and a heatmap of the genes used to generate the ssGSEA scores is depicted in Suppl.Fig.15D [\[7\]](#), with ssGSEA scores of each patient sample. These data indicate that the upregulation of Myc pathway in peripheral blood cells of TB patients was associated with poor prognosis and TB persistence even in patients infected with antibiotic sensitive Mtb. Although pathological evaluation was not included in this study, treatment failures in immunocompetent patients are often associated with massive fibro-necrotic pulmonary TB lesions.

Discussion

This study revealed a mechanistic connection between susceptibility to TB conferred by the *sst1* locus, hyperactivity of the IFN-I pathway and unresolving stress in activated macrophages. We have shown that the aberrant activation of the *sst1*-susceptible macrophages and persistent stress in response to TNF form a vicious cycle shaped by disbalance of anabolic (Myc), homeostatic (antioxidant defense and proteostasis) and immune pathways (TNF and type I IFN).

Previous studies demonstrated that the IFN-I pathway hyperactivity underlies the Mtb susceptibility mediated by the *sst1* locus (Moreira-Teixeira et al., 2018 [\[8\]](#); O'Garra et al., 2013 [\[9\]](#)). Indeed, the IFN-I pathway inactivation in B6.Sst1S mice increased their resistance to Mtb infection, but it did not restore it to the wildtype B6 level (Ji et al., 2019 [\[10\]](#)). Thus, the IFN-I pathway hyperactivity was insufficient to explain the *sst1*-mediated susceptibility.

Exploring additional mechanisms controlled by the *sst1* locus in activated macrophages we revealed deficient activation of a fraction of the antioxidant defense genes, including genes involved in glutathione and thioredoxin antioxidant systems, NADPH regeneration and ferroptosis resistance, such as ferritin light and heavy chains *Ftl* and *Fth*, subunits of glutamate-cysteine ligase subunits *Gclm*, glutathione peroxidases *Gpx1* and *Gpx4* and stearyl-Coenzyme A desaturase 2 *Scd2*. These experimental findings are consistent with an unbiased computational analysis that considered genes co-regulated with the *sst1*-encoded *Sp110* and *Sp140* genes in mouse macrophage datasets and suggested their primary association with antioxidant response pathways. Blockade of IFNAR1 did not restore the AOD gene expression. In contrast, ROS scavengers, iron chelators and inhibitors of lipid peroxidation prevented the *Ifnb1* superinduction suggesting that the dysregulated oxidative stress response of B6.Sst1S macrophages was upstream of the IFN-I pathway hyperactivity. Boosting antioxidant defense in B6.Sst1S mice by knockout of the *Bach1* gene, a negative regulator of Nrf2, not only reduced lung inflammatory damage, but also decreased the expression of the IFN-I pathway genes (Amaral et al, 2023 [\[11\]](#)).

Taken together these data are consistent with our previous observations that the initial *Ifnb1* super-induction in the *sst1*-susceptible macrophages was driven by the synergy of TNF/NFκB and a JNK pathway activated by oxidative stress (Bhattacharya et al., 2021 [\[12\]](#)). Here we found that the prolonged TNF stimulation of the *sst1* mutant macrophages led to the IFN-I dependent accumulation of toxic low molecular weight lipid peroxidation products MDA and 4-HNE. The

Ifnb1 superinduction also resulted in the downstream activation of the integrated stress response markers *Trb3* and *Chac1*. Thus, IFN-I pathway did not initiate, but amplified the AOD dysregulation.

Among pathways upregulated in TNF-stimulated B6.Sst1S macrophages was Myc pathway. In actively growing cells, Myc promotes ribosome biogenesis, cap-dependent protein translation and also suppresses expression of ferritins, most likely to provide labile iron for anabolic metabolism (Torti & Torti, 2002 [↗](#); Wu et al, 1999 [↗](#)). Myc hyperactivity is associated with cell senescence and maladaptive activity mTOR pathway (Alic & Partridge, 2015 [↗](#); Hofmann et al, 2015 [↗](#)). In macrophages, Myc promotes the development of myeloid derived suppressor cells and alternatively activated macrophages (Kumar et al, 2016 [↗](#); Pello et al, 2012 [↗](#)). Here we have shown that Myc hyperactivity was responsible for the initiation of the aberrant macrophage activation trajectory leading to increased intracellular labile iron pool (Fe^{+2}) and lipid peroxidation. Myc inhibition also prevented the *Ifnb1* superinduction and activation of the integrated stress response markers *Trb3* and *Chac1*.

The above findings allowed us to reconstruct the regulatory cascade driving the aberrant macrophage activation (**Suppl.Fig.16** [↗](#)). During prolonged TNF stimulation of the B6.Sst1S macrophages, Myc hyperactivity and the impairment of AOD lead a coordinated downregulation of the Ferritin heavy and light chains and lipid peroxidation inhibitor genes. In the absence of terminators, the intracellular peroxidation of polyunsaturated fatty acids (PUFA) is catalyzed by ferrous iron (Fe^{+2}) via the Fenton reaction and proceeds in an autocatalytic manner damaging cell membranes containing tightly packed PUFA (Mortensen et al, 2023 [↗](#)). This autocatalytic lipid peroxidation fuels persistent oxidative stress and, likely, sustains the activity of JNK. Downstream, *Ifnb1* superinduction in B6.Sst1S macrophages leads to the upregulation of interferon-inducible genes, including PKR. The subsequent PKR-dependent ISR activation leads to the upregulation of *Chac1* (Bhattacharya et al., 2021 [↗](#)) (Bhattacharya et al., 2021 [↗](#)) - a glutathione degrading enzyme gamma-glutamylcyclotransferase 1 (Crawford et al, 2015 [↗](#)) that further compromises the AOD. This stepwise escalation eventually locks the susceptible macrophages in a state of unresolving stress which is maintained by continuous stimulation with TNF and boosted by IFN-I signaling. Thus, during prolonged stimulation of the B6.Sst1S macrophages with TNF the autocatalytic lipid peroxidation and IFN-I hyperactivity form a positive feedback loop sustaining the unresolving oxidative stress. Unlike ferroptosis, however, this did not immediately result in massive cell death but sustained a state of the aberrant macrophage activation that reduced their ability to control *Mtb* both in a cell autonomous and a T cell-dependent manner. In contrast, in the wild type B6 macrophages Myc was inhibited more readily, and ferritin proteins were upregulated to sequester free iron in parallel with the upregulation of Nrf2. Their combined effects increased stress resilience of activated macrophages. Recent in vivo studies also demonstrated that ferritins and antioxidant pathways were upregulated in inflammatory macrophages isolated from *Mtb*-infected wild type B6 mice (Pisu et al, 2020 [↗](#)).

At the molecular level, the detailed characterization of the *sst1*-mediated regulation of macrophage activation provides proximal phenotypes for deeper understanding of the functions of the *sst1*-encoded Sp110 and Sp140 proteins. The *Sp110* and *Sp140* genes and proteins are upregulated exclusively in the wild type cells 8 - 12h after TNF stimulation, i.e. the time interval during which the divergence between the wild type and mutant macrophages occurs (Bhattacharya et al., 2021 [↗](#)). Their transcription and protein stability are regulated by interferons and stress kinases (Fraschilla & Jeffrey, 2020a [↗](#)). Their activities can modulate i) responses to inflammatory stimuli via CARD domain (Fraschilla & Jeffrey, 2020b [↗](#)), ii) metabolism, via the activated nuclear receptor interaction domain LXXLL, iii) regulation of chromatin silencing (Mehta et al., 2017 [↗](#)), transcriptional elongation (Amatullah et al., 2022 [↗](#)) via chromatin interacting PHD, BRD and SAND domains and indirect regulation of the *Ifnb1* mRNA stability (Witt et al., 2024 [↗](#)). Potentially, they may interact with intracellular nucleic acids via the DNA interaction domain SAND (Bottomley et al, 2001 [↗](#); Carles & Fletcher, 2010 [↗](#)).

Our data suggest that one of them might be involved in regulation of the Nrf2 protein biosynthesis either at the levels of *Nfe2l1/2* mRNA splicing (Goldstein et al, 2016) or cap-independent translation (Li et al, 2010; Shay et al, 2012) (**Fig.2**). Recent experimental evidence suggest that a specialized translation machinery is involved in context-dependent regulation of protein biosynthesis (Shi & Barna, 2015). Ribosome modifications, such as protein composition and rRNA methylation, were shown to be involved in control of selective IRES-dependent translation in myeloid cells (Basu et al, 2011; Mazumder et al, 2003) and regulation of inflammation (Basu et al, 2014; Poddar et al, 2013; Poddar et al, 2015). Recently, enrichment of Sp100 and Sp110 proteins in ribosomes was observed during B6 macrophage activation with LPS (Susanto et al, 2023). These findings suggest that the Sp110 protein may be involved in fine tuning of protein biosynthesis in activated macrophages favoring the IRES-dependent Nrf2 translation.

More generally, the Sp110 and Sp140 proteins may serve as context-dependent regulators (possibly, both as sensors and tunable controllers) of macrophage activation and stress resilience via a coordinated upregulation of AOD and downregulating Myc and IFN-I pathways. Hypothetically, downregulating this mechanism may also play physiological roles in eliminating infected (Rothchild et al, 2019) or transformed cells (Gorbunova et al, 2012; Leonova et al, 2013). By coordinately increasing free iron pool for non-enzymatic free radical generation, and simultaneously decreasing the buffering capacity of intracellular antioxidants, this activation state promotes generation and unopposed spread of free radicals. However, the inappropriate activation of its build-in amplification mechanisms, such as IFN-I, may drive immunopathologies in the face of chronic stimulation, as occurs with *Mtb* infection.

In the mouse model of chronic TB infection, lipid peroxidation products did not appear to significantly affect the long term *Mtb* survival: the mycobacterial cell wall and multiple mycobacterial antioxidant pathways exist to allow for the survival of, at least, a fraction of mycobacterial population in highly oxidative environments (Pacl et al, 2018; Piddington et al, 2001). The accumulation of stressed macrophages and LPO products within TB lesions, however, gradually degraded local immunity that allowed for *Mtb* replication and provided a fodder for growing mycobacteria (Mahamed et al, 2017). Thus, in the mouse model that recapitulate key pathomorphological features of pulmonary TB in human patients, the aberrant macrophage activation state was responsible for both the inflammatory tissue damage and the failure of local immunity.

The above mechanistic dissection of the aberrant macrophage activation drivers provides therapeutic targets for interrupting their vicious cycle in vivo. For example, monocytes recruited to inflammatory lesions may be particularly vulnerable to the dysregulation of Myc. Prior to terminal differentiation within inflammatory milieu, these immature cells undergo several cycles of replication. Cell growth requires labile iron that is provided by Myc via downregulation of ferritins (Stockwell et al., 2017; Torti & Torti, 2002). Recently we found that lung epithelial cells embedded within TB lesions express CSF1 – a macrophage growth factor known to stimulate Myc expression (Yabaji et al., 2023). Thus, the coincidence of TNF and CSF1 – Myc stimulation may initiate and propagate the aberrant activation state in monocytes recruited to chronic pulmonary TB lesions. Similarly, growth factors produced locally within inflamed tissues may sustain Myc hyperactivity and the aberrant activation state of monocytes/macrophages in other chronic inflammatory pathologies.

Further understanding the specific roles of the SP100 family proteins, as well as other signals and checkpoints that regulate the pathological macrophage activation state transitions, will allow for their rational therapeutic modulation - to boost host defenses and mitigate the collateral tissue damage.

Materials and methods

Reagents

Recombinant mouse TNF (Cat# 315-01A) and IL-3 (Cat# 213-13) were procured from Peprotech. The mouse monoclonal antibody to mouse TNF (MAb; clone XT22), isotype control, and mouse monoclonal antibody to mouse *Ifn* β (Clone: MAR1-5A3) were obtained from Thermo Scientific. BHA (Cat# B1253) and Deferoxamine mesylate (Cat#D9533J) were sourced from Sigma Aldrich. PLX3397 (Cat# S7818), Myc inhibitor (10058-F4) (Cat# S7153), and ferrostatin-1 (Cat# S7243) were purchased from Selleckchem. D-JNK1 (Cat# HY-P0069), GW2580 (Cat# HY-10917), and BLZ945 (Cat# HY-12768) were acquired from Med Chem Express. Fetal bovine serum (FBS) for cell culture medium was sourced from HyClone. Middlebrook 7H9 and 7H10 mycobacterial growth media were purchased from BD and prepared following the manufacturer's instructions. A 50 μ g/mL hygromycin solution was utilized for reporter strains of *Mtb* Erdman (SSB-GFP, smyc':mCherry).

Animals

C57BL/6J inbred mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The B6J.C3-Sst1^{C3HeB/FeJ} Krmn congenic mice were created by transferring the *sst1* susceptible allele from C3HeB/FeJ mouse strain on the B6 (C57BL/6J) genetic background using twelve backcrosses (referred to as B6.Sst1S in the text). The B6.Sst1S.*Ifnb1*-YFP mice were produced by breeding B6.Sst1S mice with a reporter mouse containing a Yellow Fluorescent Protein (YFP) reporter gene inserted after the *Ifnb1* gene promoter (Scheu et al., 2008 [\[1\]](#)). The YFP serves as a substitute for Interferon beta (*Ifnb1*) gene expression. All animal procedures were performed with the full knowledge and approval by the Institutional Animal Care and Use Committee (IACUC) of Boston University. Mice were euthanized by CO₂ asphyxiation in accordance with IACUC-approved protocols.

BMDMs culture and Treatment

The isolation of mouse bone marrow and the cultivation of bone marrow-derived macrophages (BMDMs) were conducted following the procedures outlined in a prior study (Yabaji et al., 2022 [\[2\]](#)). BMDMs were plated in tissue culture plates, followed by treatment with TNF and incubation for 16 hours at 37°C with 5% CO₂. Various inhibitors were applied to the cells until the point of harvest.

Infection of BMDM with *M. tuberculosis*

For infection experiments, *M. tuberculosis* H37Rv (*Mtb*) was cultured in 7H9 liquid media for three days and subsequently harvested. The bacteria were then diluted in media containing 10% L929 cell culture medium (LCCM) without antibiotics to achieve the desired Multiplicity of Infection (MOI). Following this, 100 μ L of *Mtb*-containing media with the specified MOI was added to BMDMs cultivated in a 96-well plate format that had been pre-treated with TNF or inhibitors. The plates underwent centrifugation at 500xg for 5 minutes, followed by an incubation period of 1 hour at 37°C. To eliminate any extracellular bacteria, cells were treated with Amikacin at 200 μ g/ μ L for 45 minutes. Subsequently, cells were washed and maintained with inhibitors, as applicable, in DMEM/F12 containing 10% FBS medium without antibiotics at 37°C in 5% CO₂ for the duration of each experiment. Media changes and inhibitor replacements were carried out every 48 hours. The confirmation of MOIs was achieved by counting colonies on 7H10 agar plates. All procedures involving live *Mtb* were conducted within Biosafety Level 3 containment, adhering to regulations from Environmental Health and Safety at the National Emerging Infectious Disease Laboratories, the Boston Public Health Commission, and the Center for Disease Control.

Cytotoxicity and Mycobacterial growth assays

The cytotoxicity, cell loss, and Mtb growth assays were conducted in accordance with the procedures outlined by (Yabaji et al., 2022 [DOI](#)). In brief, BMDMs were subjected to inhibitor treatment or Mtb infection as previously described for specified time points. Upon harvesting, samples were treated with Live-or-Dye™ Fixable Viability Stain (Biotium) at a 1:1000 dilution in 1X PBS/1% FBS for 30 minutes. Following staining, samples were carefully washed to prevent any loss of dead cells from the plate and subsequently treated with 4% paraformaldehyde (PFA) for 30 minutes. After rinsing off the fixative, samples were replaced with 1X PBS. Following decontamination, the sample plates were safely removed from containment. Utilizing the Operetta CLS HCA System (PerkinElmer), both infected and uninfected cells were quantified. The intracellular bacterial load was assessed through quantitative PCR (qPCR) employing a specific set of Mtb and *M. bovis*-BCG primer/probes, with BCG spikes included as an internal control, as previously described (Yabaji et al., 2022 [DOI](#)). The percentage of cell numbers were calculated based on the number of cells at Day 0 (immediately after Mtb infection). The fold change of Mtb was calculated after normalization using Mtb load at Day 0 after infection and washes. We quantified the percentage of replicating Mtb using a replication reporter strain, Mtb Erdman (SSB-GFP, *smyc*::mCherry). Replicating Mtb was identified by counting the number of SSB-GFP puncta-positive Mtb, while the total number of Mtb per field was determined by counting mCherry-expressing bacteria (red).

RNA Isolation and quantitative PCR

The extraction of total RNA was carried out utilizing the RNeasy Plus mini kit (Qiagen). Subsequently, cDNA synthesis was conducted using the Invitrogen™ SuperScript™ III First-Strand Synthesis SuperMix (Cat#18080400). Real-time PCR was executed with the GoTaq qPCR Mastermix (Promega) employing the CFX-96 real-time PCR System (Bio-Rad). Oligonucleotide primers were designed using Integrated DNA Technologies. Either 18S or β -actin expression served as an internal control, and the quantification of fold induction was computed using the $\Delta\Delta C_t$ method.

Analysis of RNA sequencing data

Raw sequence reads were mapped to the reference mouse genome build 38 (GRCm38) by STAR (Dobin et al, 2013 [DOI](#)). The read count per gene for analysis was generated by featureCounts (Liao et al, 2014 [DOI](#)). Read counts were normalized to the number of fragments per kilobase of transcript per million mapped reads (FPKM) using the DESeq2 Bioconductor R package (Love et al, 2014 [DOI](#)). Pathway analysis was performed using the GSEA method implemented in the camera function from the limma R package (Wu & Smyth, 2012 [DOI](#)). Databases KEGG, MSigDB Hallmark, Reactome were used for the GSEA analysis. Transcripts expressed in either 3 conditions with FPKM > 1 were included in the pathway analyses. To infer mice macrophage gene regulatory network, we used ARCHS4 collected list of RNE-seq data for mice macrophages cells from Gene Expression Omnibus (GEO) (Lachmann et al, 2018 [DOI](#)). The total number of analyzed experimental datasets was 1960. This gene expression matrix was utilized as input for the GENIE3 algorithm, which calculated the most likely co-expression partners for transcription factors. The list of transcription factors was derived from the Animal TFDB 3.0 database (Hu et al, 2019 [DOI](#)). The Virtual Inference of Protein Activity by Enriched Regulon Analysis (VIPER) algorithm was used to estimate the prioritized list of transcription factors (Alvarez et al, 2016 [DOI](#)). The software Cytoscape (version 3.4.0) was used for network visualization (Shannon et al, 2003 [DOI](#)). The i-cisTarget web service was used to identify transcription factor binding motifs that were over-represented on a gene list (Imrichova et al, 2015 [DOI](#)). Statistical analysis and data processing were performed with R version 3.6.1 (<https://www.r-project.org/> [DOI](#)) and RStudio version 1.1.383 (<https://www.rstudio.com> [DOI](#)).

Generation and sequencing of single-cell RNA libraries

Single cell suspensions of mouse bone marrow derived macrophages were loaded on a 10X genomics chip G at concentrations varying between 1,240,000 – 1,575,000 cells/mL aiming for a targeted cell recovery of 5,000 cells per sample and processed using the 10X Genomics 3'v3.1 Dual index kit according to the manufacturer protocol (10x Genomics, CA, USA). Following the 10X Genomics Chromium controller run the Gel-beads in emulsion (GEMs) were transferred to strip tubes and subsequently put into a thermal cycler using the following parameters: 45 min at 53 °C, 5 min at 85 °C, hold at 4 °C. The samples were then stored in a –20°C freezer overnight. The next day samples were thawed at room temperature before adding the recovery agent to break the GEMs. Subsequently the cDNA was purified and amplified, and the gene expression libraries were generated and purified. The size distribution and molarity of these libraries were assessed using the Bioanalyzer High Sensitivity DNA Assay (Agilent Technologies, Lexington, MA, USA). The libraries were then pooled at 5nM and sequenced on an Illumina NextSeq 2000 instrument at a 600pM input and 2% PhiX spike in using a P3 100 cycle flow cell (Illumina, San Diego, CA, USA) resulting in 36000-58000 reads per cell.

Processing of scRNA-seq data

The 10x Genomics Cell Ranger pipeline v6.0.1 was used for demultiplexing, alignment, identification of cells, and counting of unique molecular indices (UMIs). The Cell Ranger mkfastq pipeline was used to demultiplex raw base call (BCL) files generated by Illumina sequencers into FASTQ files. The Cell Ranger count pipeline was used to perform alignment and create UMI count matrices using reference genome mm10 (Ensembl 84) and parameters `–expect-cells = 5000`. Droplets with at least 500 UMIs underwent further quality control with the SCTK-QC pipeline (Hong et al, 2022 [\[1\]](#)). The median number of UMIs was 9,927, the median number of genes detected was 2,637, the median percentage of mitochondrial reads was 5.43%, and the median contamination estimated by decontX was 0.17 across cells from all samples (Yang et al, 2020 [\[2\]](#)). Cells with less than 500 counts, less than 500 genes detected, or more than 25% mitochondrial counts were excluded leaving a total of 14,658 cells for the downstream analysis.

Clustering of single-cell data

The Seurat package was used to cluster the cells into subpopulations (Stuart et al, 2019 [\[3\]](#)). The 2,000 most variable genes were selected using the `FindVariableFeatures` function, after removing features with fewer than 3 counts in 3 cells. Cells were embedded in two dimensions with UMAP using the `RunUMAP` function. The clustering was performed at resolution 0.8 resulting in 15 clusters. Markers for each cluster were identified with the `FindAllMarkers` function using the Wilcoxon Rank Sum test and default parameters: `log fold-change threshold = 0.25` and `min.pct = 0.1`. The trajectory analysis was performed using the slingshot package; the pseudotime trajectory was computed using the `slingshot` function and selecting the PCA (considering 50 PCA) and unweighted cluster labels as inputs (Street et al, 2018 [\[4\]](#)). The pathway analysis was performed using the VAM package in combination with MSigDB's Hallmark geneset collection. The genesets were scored using the `vamForSeurat` function, and the top pathways were found by running the `FindAllMarkers` analysis on the CDF assay. The following package versions were used: Seurat v4.0.4, VAM v1.0.0, Slingshot v2.3.0, SingleCellTK v2.4.1, Celda v1.10.0.

Western Blot Analysis

Equal amounts of protein extracts were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). Following a 2-hour blocking period in 5% skim milk in TBS-T buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween20], the membranes were incubated overnight with the primary antibody at 4°C. Subsequently, bands were acquired using the chemiluminescence (ECL) kit (Thermo Scientific) and GE ImageQuant LAS-4000 Multi-Mode

imager. A loading control, either β -actin or β -tubulin, was assessed on the same membrane. Secondary antibodies used included HRP-conjugated goat anti-mouse and anti-rabbit antibodies. Densitometric analysis was performed by quantification of area using Image J.

Half-life determination of Nrf2 protein

The B6 and B6.Sst1S BMDM were stimulated with TNF (10 ng/ml) for 6 h. Cycloheximide (2 μ M) was added to the cultures and cells were harvested after 15, 30, 45, 60, 90, and 120 min. Western blotting was carried out for Nrf2 and β -tubulin as described. The densitometry analysis was performed using ImageJ software (NIH, USA). The linear regression curve was plotted using GraphPad Prism software, and the half-life of Nrf2 protein was derived.”

Electrophoretic Mobility Shift Assay

The nuclear extracts were prepared using a nuclear extraction kit (Signosis Inc.) according to the instructions provided. The EMSA was carried out using the EMSA assay kit (Signosis Inc.) following the instructions provided. Briefly, 5 μ g of nuclear extract was incubated with biotin-conjugated Nrf2 antioxidant response element (ARE) probe in 1X binding buffer for 30 min. The reaction mixture was electrophoresed in 6.5% non-denaturing polyacrylamide gel and then blotted onto a nylon membrane. The protein-bound probe and free probe were immobilized on the membrane by UV light mediated cross-linking. After blocking the membrane, the probes were detected using streptavidin-HRP mediated chemiluminescence method. The images were captured using ImageQuant LAS 4000.

Quantification of intracellular labile iron

The method used to measure Labile Intracellular Iron (LIP) involved the Calcein acetoxymethyl (AM) ester (Invitrogen) quenching technique with some modifications, as described in (Amaral et al, 2019 [\[4\]](#); Picard et al, 1998 [\[4\]](#); Thomas et al, 1999 [\[4\]](#)). BMDMs were plated in 96 well plates and washed with 1 $\times$ DPBS before being lysed with 0.05% saponin for 10 minutes. Next, the cell lysates were incubated with 125 nM calcein AM at 37°C for 30 minutes. A negative control was established by incubating the lysates with the iron chelator deferoxamine (Sigma-Aldrich) at room temperature for 30 minutes, while FeSO₄-treated cells served as the positive control. The fluorescence of Calcein AM was measured using a fluorescence microplate reader, and the differential MFI of calcein AM between untreated and deferoxamine-treated samples was calculated to determine intracellular labile iron and represented as fold change.

MDA assay

The amount of MDA in the BMDMs were measured using a Lipid Peroxidation (MDA) Assay Kit (Abcam, ab118970) according to the manufacturer’s protocols. Briefly, 2 $\times$ 10⁶ cells were lysed in Lysis Solution containing butylated hydroxytoluene (BHT). The insoluble fraction was removed by centrifugation, and the supernatant was collected, protein concentrations were estimated and used for analysis. The equal amount of supernatants (Based on protein concentration) were mixed with thiobarbituric acid (TBA) solution reconstituted in glacial acetic acid and then incubated at 95°C for 60 min. The supernatants containing MDA-TBA adduct were added into a 96-well microplate for analysis. A microplate reader was used to measure the absorbance at OD 532 nm.

Lipid peroxidation by C11-Bodipy 581/591

Briefly, the cells were washed 3 times with 1X PBS and treated with 1 μ M C11-Bodipy 581/591 dye (Invitrogen) suspended in 1X PBS for 30 min in the dark at 37°C. The LPOs were measured fluorometrically using Spectramax M5 microplate reader (Molecular Devices).

Measurement of total antioxidant capacity

The total antioxidant capacity was quantified using the Antioxidant assay kit (Cayman chemical) according to the instructions provided. A standard curve was generated by using various concentrations of Trolox (0.068-0.495 mM). 10 µg of the cell lysate was used to determine the Trolox equivalent antioxidant capacity. The percentage of induced antioxidant capacity was calculated using the formula, $(\text{Trolox equivalent}_{\text{TNF stimulated}} - \text{Trolox equivalent}_{\text{unstimulated}}) / \text{Trolox equivalent}_{\text{unstimulated}} \times 100$.

Immunofluorescence imaging

BMDMs were cultured on coverslips and subjected to treatment with or without TNF, followed by processing for inhibitor treatment or Mtb infection. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes (non-infected) or 30 minutes (Mtb infected) at room temperature and then blocked for 60 minutes with 1% BSA containing 22.52 mg/mL glycine in PBST (PBS + 0.1% Tween 20). After the blocking step, cells were incubated overnight with primary antibodies (4-HNE, Nrf2, or Nrf-1 specific), washed, and then incubated with Alexa Fluor 594-conjugated Goat anti-Rabbit IgG (H+L) secondary Antibody (Invitrogen) in 1% BSA in the dark for 1 hour. The cells were mounted using Prolong™ Gold antifade reagent (Thermo Fisher Scientific), and images were captured using an SP5 confocal microscope. All images were processed using ImageJ software.

Detection of lipid peroxidation by Click-iT linoleamide alkyne (LAA) method

To investigate the lipid peroxidation, the Click-iT™ lipid peroxidation imaging kit (C10446, ThermoFisher) was used. The BMDMs were cultured on 12 mm coverslips in 24-well plates. Following a 6 h of TNF treatment, 50 µM Click-iT linoleamide alkyne (LAA) was added to the cell culture and incubated at 37°C for 1 h under 5 % CO₂. The cells were washed, fixed, permeabilized, blocked, and stained with Alexa fluor 488 azide according to the manufacturer's instructions. Subsequently, the coverslips were mounted onto the microscope slide with ProLong™ Diamond Antifade mountant with DAPI (P36962, ThermoFisher). The images were captured using the Leica SP5 confocal microscope and further the signal quantification was carried out using ImageJ software (Version 1.53k, NIH).

Quantification of oxidative stress

B6 and B6.Sst1S cells were seeded in a 96-well plate and stimulated with TNF (10 ng/mL) or left untreated for 6, 24, and 36 hours. For ROS inhibition, cells were treated with BHA (100 µM) 2 h post TNF stimulation. At each time point, CellROX® Oxidative Stress Reagent (Cat#C10444, Invitrogen™) at 5 µM final concentration was added to the cells and incubated for 30 minutes at 37°C, protected from light. After incubation, cells were washed twice with warm PBS, and fluorescence was analyzed using a fluorescence microscope or flow cytometer. ROS levels were quantified by comparing the fluorescence intensity across treatment groups and time points.

Analysis of Small RNAs in Macrophages

Small RNA libraries were prepared from size fractionation of 10ug of total macrophage RNA on a denaturing polyacrylamide gel to purify 18-35nt small RNAs, and converted into Illumina sequencing libraries with the NEBNext Small RNA Library kit (NEB). Libraries were sequenced on the Illumina NextSeq-550 in the Boston University Microarray and Sequencing Core. We applied long RNAs and small RNAs RNAseq fastq files to a transposon and small RNA genomics analysis pipeline previously developed for mosquitoes (Dayama et al, 2022 [DOI](#); Ma et al., 2021 [DOI](#)) with the

mouse transposon consensus sequences loaded instead. Mouse transposon consensus sequences were downloaded from RepBase (Bao et al, 2015 [DOI](#)), and RNA expression levels were normalized internally to each library's depth by the Reads Per Million counting method.

Infection of mice with *Mycobacterium tuberculosis* and collection of organs

The subcutaneous infection of mice was conducted following the procedure outlined in (Yabaji et al., 2023 [DOI](#)). In brief, mice were anesthetized using a ketamine-xylazine solution administered intraperitoneally. Each mouse was subcutaneously injected in the hock, specifically in the lateral tarsal region just above the ankle (by placing the animal in the restrainer), with 50 µl of 1X PBS containing 10^6 CFU of Mtb H37Rv or Mtb Erdman (SSB-GFP, smyc::mCherry). At the designated time points, the mice were anesthetized, lung perfusion was performed using 1X PBS/heparin (10 U/ml), and organs were collected.

Female mice were used unless otherwise specified and mice were randomly assigned to experimental groups. No animals were excluded after enrollment. Investigators were blinded to group assignments during outcome assessment. Sample size was determined based on prior studies.

Confocal immunofluorescence microscopy of tissue sections

Immunofluorescence of thick lung sections (50-100 µm) was conducted following the detailed procedures outlined earlier (Yabaji et al., 2023 [DOI](#)). Briefly, lung slices were prepared by embedding formalin fixed lung lobes in 4% agarose and cutting 50 µm sections using a Leica VT1200S vibratome. Sections were permeabilized in 2% Triton X-100 for 1 day at room temperature, followed by washing and blocking with 3% BSA in PBS and 0.1% Triton X-100 for 1 hour. Primary antibodies were applied overnight at room temperature, followed by washing and incubation with secondary antibodies for 2 hours. Samples were stained with Hoechst solution for nuclei detection, cleared using RapiClear® 1.47 solution for 2 days, and mounted with ProLong Gold Antifade Mountant. Imaging was performed using a Leica SP5 spectral confocal microscope. Primary rabbit anti-4-HNE, anti-Iba1, and anti-iNOS polyclonal antibodies were detected using goat anti-rabbit antibodies labelled with Alexa Fluor 647.

Mycobacterial staining of lung sections

Paraffin-embedded 5µm sections were stained using New Fuchsin method (Poly Scientific R and D Corp., cat no. K093) and counterstained with methylene blue following the manufacturer's instructions.

Tissue inactivation, processing, and histopathologic interpretation

Tissue samples were submersion fixed for 48 h in 10% neutral buffered formalin, processed in a Tissue-Tek VIP-5 automated vacuum infiltration processor (Sakura Finetek, Torrance, CA, USA), followed by paraffin embedding with a HistoCore Arcadia paraffin embedding machine (Leica, Wetzlar, Germany) to generate formalin-fixed, paraffin-embedded (FFPE) blocks, which were sectioned to 5 µm, transferred to positively charged slides, deparaffinized in xylene, and dehydrated in graded ethanol. A subset of slides from each sample were stained with hematoxylin and eosin (H&E) and consensus qualitative histopathology analysis was conducted by a board-certified veterinary pathologist (N.A.C.) to characterize the overall heterogeneity and severity of lesions.

Chromogenic Monoplex Immunohistochemistry

A rabbit specific HRP/DAB detection kit was employed (Abcam catalog #ab64261, Cambridge, United Kingdom). In brief, slides were deparaffinized and rehydrated, endogenous peroxidases were blocked with hydrogen peroxidase, antigen retrieval was performed with a citrate buffer for 40 minutes at 90°C using a NxGen Decloaking chamber (Biocare Medical, Pacheco, California), non-specific binding was blocked using a kit protein block, the primary antibody was applied at a 1:200 dilution, which cross-reacts with mycobacterium species (Biocare Medical catalog#CP140A,C) and was incubated for 1 h at room temperature, followed by an anti-rabbit antibody, DAB chromogen, and hematoxylin counterstain. Uninfected mouse lung was examined in parallel under identical conditions with no immunolabeling observed serving as a negative control.

Multiplex fluorescent immunohistochemistry (mfIHC)

A Ventana Discovery Ultra (Roche, Basel, Switzerland) tissue autostainer was used for brightfield and multiplex fluorescent immunohistochemistry (mfIHC). In brief tyramide signaling amplification (TSA) was used in an iterative approach to covalently bind Opal fluorophores (Akoya Bioscience, Marlborough, MA) to tyrosine residues in tissue sections, with subsequent heat stripping of primary-secondary antibody complexes until all antibodies were developed. Before multiplex-IHC was performed, each antibody was individually optimized using a single-plex-IHC assay using an appropriate positive control tissue. Optimizations were performed to determine ideal primary antibody dilution, sequential order of antibody development, assignment of each primary antibody to an Opal fluorophore, and fluorophore dilution. Once an optimal protocol was established, 5µm tissue sections were cut from FFPE lung arrays. All Opal TSA-conjugated fluorophore reactions took place for 20 minutes. Fluorescent slides were counterstained with spectral DAPI (Akoya Biosciences) for 16 minutes before being mounted with ProLong gold antifade (ThermoFischer, Waltham, MA). Antibodies utilized in 4-plex 5 color (DAPI counterstained) analysis included: Iba1, iNOS, YFP, Chac1, and P-c-Jun. All rabbit antibodies were developed with a secondary goat anti-rabbit HRP-polymer antibody (Vector Laboratories, Burlingame, CA) for 20min at 37C and all mouse derived antibodies were developed with a secondary goat anti-mouse HRP-polymer antibody (Vector Laboratories).

Brightfield Immunohistochemistry

Antigen retrieval was conducted using a Tris based buffer-Cell Conditioning 1 (CC1)-Catalog # 950-124 (Roche). The MHCII primary was of mouse origin, so a goat anti-mouse HRP-polymer antibody (Vector Laboratories) was utilized. Brightfield slides utilized A ChromoMap DAB (3,3'-Diaminobenzidine) Kit-Catalog #760-159 (Roche) to form a brown precipitate at the site of primary-secondary antibody complexes containing HRP. Slides were counterstained with hematoxylin and mounted.

Multispectral whole slide microscopy

Fluorescent and chromogen labeled slides were imaged at 40X using a Vectra PolarisTM Quantitative Pathology Imaging System (Akoya Biosciences). For fluorescently labeled slides, exposures for all Opal dyes were set based upon regions of interest with strong signal intensities to minimize exposure times and maximize the specificity of signal detected. A brightfield acquisition protocol at 40X was used for chromogenically labeled slides.

Digitalization and linear unmixing of multiplex fluorescent immunohistochemistry

Whole slide fluorescent images were segmented into QPTIFFs, uploaded into Inform software version 2.4.9 (Akoya Biosciences), unmixed using spectral libraries affiliated with each respective opal fluorophore including removal of autofluorescence, then fused together as a single whole

slide image in HALO (Indica Labs, Inc., Corrales, NM).

Quantitative analysis of immunohistochemistry

View settings were adjusted to allow for optimal visibility of immunomarkers and to reduce background signal by setting threshold gates to minimum signal intensities. After optimizing view settings, annotations around the entire tissue were created to define analysis area using the flood tool and artifacts were excluded using the exclusion pen tool. To analyze lesions independently, a tissue classifier that uses a machine-learning approach was utilized to identify lesions. The outputs from this classifier were annotations for each lesion, which were then isolated into independent layers, which were analyzed separately from other layers. For quantifying myeloid markers in multiplexes, an algorithm called the HALO (v3.4.2986.151, Indica Labs, Albuquerque, NM, USA) Area Quantification (AQ) module was created and finetuned to quantify the immunoreactivity for all targets. Thresholds were set to define positive staining based on a real-time tuning feature. AQ outputted the percentage of total area displaying immunoreactivity across the annotated whole slide scan in micrometers squared (μm^2). AQ output the total percentage positive for each phenotype. AQ and HP algorithms were run across all layers for each individual lesion and exported as a .csv file.

Spatial transcriptomics

To perform spatial transcriptomics analysis, we used the Nanostring GeoMX Digital Spatial Profiler (DSP) system (Nanostring, Seattle, WA) (Danaher et al, 2022 [DOI](#); Merritt et al., 2020 [DOI](#)). To identify pathways dominating early vs. late (advanced) lesions, we selected lungs from 2 mice with paucibacillary early lesions and 2 mice with advanced multibacillary lesions with necrotic areas. Slides were stained with fluorescent CD45-, pan-keratin-specific antibodies, and Iba1-specific antibodies (sc-32725, Santa Cruz Biotechnology, CA, USA) and DAPI. Diseased regions of interest (ROI) for expression profiling of Iba1+ cells were selected to focus on myeloid-rich areas avoiding areas of micronecrosis and tertiary lymphoid tissue (**Suppl. Fig.9** [DOI](#)). Eight ROI each of normal lung, early and late lesions (respectively) were studied. The profiling used the Mouse Whole Transcriptome Atlas (WTA) panel which targets ~21,000+ transcripts for mouse protein coding genes plus ERCC negative controls to profile the whole transcriptome, excluding uninformative high expressing targets such as ribosomal subunits. A subsequent study focused on gene expression within macrophages identified by Iba1 labeling in lung sections showing uncontrolled (advanced multibacillary) lesions in comparison to controlled (paucibacillary stage). Samples from each ROI were packaged into library for sequencing (NextSeq550, Illumina) following the procedure recommended by Nanostring. After sequencing, the data analysis workflow began with QC evaluation of each ROI based on thresholds for number of raw and aligned reads, sequencing saturation, negative control probe means, and number of nuclei and surface area. Background correction is performed using subtraction of the mean of negative probe counts. Q3 normalization (recommended by Nanostring) results in scaling of all ROIs to the same value for their 3rd quartile value. The count matrix was imported into the Qlucore Genomics Explorer software package (Qlucore, Stockholm, Sweden) and log2 transformed for further analysis. Statistical analysis was then performed to identify differentially expressed genes (typically unpaired t-test with Benjamini-Hochberg control for FDR rate at $q < .05$). Lists of differentially expressed genes (DEGs) from comparisons of distinct stages were further analyzed for enrichment of pathways or predicted transcription factors using the Enrichr online tool (Chen et al, 2013 [DOI](#); Liberzon et al, 2015 [DOI](#); Xie et al, 2021 [DOI](#)). To specifically explore interferon-related gene expression in Iba1+ macrophages, we used: 1) a list of genes unique to type I genes derived by pooling four common type I and type II interferon gene list respectively and identifying the non-overlapping (unique) genes for each type (see **Suppl. Table 8** [DOI](#), **Suppl. Fig. 10** [DOI](#)); 2) using the GSEA analysis tool of the Qlucore software to evaluate enrichment of the MSigDB Hallmark collection of pathways (which include Interferon-alpha and Interferon-gamma, and other pathways of interest (Liberzon et al., 2015 [DOI](#))).

Human blood transcriptome analysis Ethics approval

Ethics approval for the study was obtained from the Institutional Ethics Committee of the participating institutions, and written informed consent was obtained prior to enrollment. This study utilized data from four longitudinal observational studies collected at five clinical sites within the Regional Prospective Observational Research for Tuberculosis (RePORT)-India consortium: Byramjee Jeejeebhoy Government Medical College (BJMC), Pune; the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER, Puducherry); National Institute for Research in Tuberculosis (NIRT), Chennai; Prof. M. Viswanathan Diabetes Research Centre (MVDRC), Chennai; and the Christian Medical College (CMC), Vellore (Ayiraveetil et al, 2020 [DOI](#); Christopher et al, 2021 [DOI](#); Gupta et al, 2016 [DOI](#); Kornfeld et al, 2016 [DOI](#)).

Blood sample collection and processing

Participant demographics including age, sex, body mass index (BMI), diabetes status, alcohol use, and tobacco use were recorded and matched for Blood collection. Blood samples were collected from 41 individuals and recently (<90 days) diagnosed with TB, within one week of treatment commencement from five clinical sites within the Regional Prospective Observational Research for Tuberculosis (RePORT)-India consortium. Individuals for this study were newly diagnosed with TB (within 90 days; sputum smear-positive or Xpert MTB/RIF assay positive (Cepheid, Sunnyvale, CA, USA)), at least 15 years of age, multi-drug-resistant negative, and had received less than one week of treatment. Sociodemographic data such as age, sex, behavioral characteristics (smoking, alcohol use (using the Alcohol Use Disorders Identification Test [AUDIT-C]), tobacco use, and body mass index (BMI) were obtained through a questionnaire and the medical history of the participants. There were no significant differences in risk factors for TB or treatment failure, including sex, risky alcohol use, tobacco use, BMI, or diabetes. Patients were monitored for two years during and post-treatment with Rifampicin, isoniazid, ethambutol, and pyrazinamide, per India national guidelines [Division CT. Technical and Operational Guidelines for TB Control in India 2016. Central TB Division, Directorate General of Health Service, Ministry of Health and Family Welfare; Government of India, New Delhi. 2016 (Bush et al, 1998 [DOI](#))]. Individuals who failed treatment (n=21) were identified by positive sputum culture or clinical diagnosis of symptoms at any time after 4 full months of treatment and symptoms determined to not be from another cause. Treatment controls were determined by those who remained culture-negative and symptom-negative for the two-year observation period.

Blood samples from each person were collected and stored in PAXgene tubes (Cat #762165, BD Biosciences, San Jose, CA, USA) at -80°C until processing using the PAXgene Blood RNA kit (Cat #762164, Qiagen, Hilden, Germany). PAXgene tubes were sent to MedGenome (Bangalore, India) for processing. Library preparation and sequencing were performed at the GenoTypic Technology Pvt. Ltd. Genomics facility in Bangalore, India, with the SureSelect Strand-Specific mRNA Library Prep kit (Cat #5190-6411, Agilent, Santa Clara, USA). One µg of RNA was used for mRNA enrichment by poly(A) selection and fragmented using RNAseq Fragmentation Mix containing divalent cations at 94 degrees C for 4 minutes. Enriched mRNA underwent fragmentation through exposure to RNAseq Fragmentation Mix with divalent cations at 94 °C for 4 min. Subsequently, single-strand cDNA was synthesized in the presence of Actinomycin D (Gibco, Life Technologies, Carlsbad, CA, USA) and purified utilizing HighPrep magnetic beads (Magbio Genomics Inc., USA). Following this step, double-stranded cDNA was synthesized, and the ends were repaired before undergoing further purification. Adenylation of the 3'-ends of cDNA preceded the ligation of Illumina Universal sequencing adaptors, which were then purified and amplified with 10 PCR cycles. The final cDNA sequencing libraries were purified and quantified using Qubit, while the fragment size distribution was assessed using Agilent TapeStation. Subsequently, the libraries were combined in equimolar proportions, and the resulting multiplexed library pools were sequenced utilizing the Illumina NextSeq 500 platform for 75 bp single-end reads.

RNA-sequencing data processing of human samples QC and alignment

Raw sequencing FASTQ files were assessed for data quality using FastQC[S. A. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute. 2010]. Trimmomatic was used to trim the reads (SLIDINGWINDOW:4:20 LEADING:3 TRAILING:3 MINLEN:36) (Bolger et al, 2014 [\[4\]](#)). Rsubread (Liao et al, 2013 [\[5\]](#)) was used to align reads to the human genome hg39 and to determine expression counts for each gene. Genes missing (or with 0 counts) in more than 20% of the samples were excluded before batch correction, as well as one outlier identified with Principal Components Analysis (PCA) before batch correction and could not be rectified using batch correction (unpublished data).

Batch correction and normalization

The RNA samples were processed sequentially in two batches. Batch effects created by combining the two batches were removed using ComBat-Seq (Zhang et al, 2020 [\[6\]](#)). The ComBat-Seq adjusted counts were normalized using a log₂-counts per million (logCPM) adjustment, and the logCPM values were used for downstream analysis.

Differential expression

Differential expression was performed on batch-corrected counts/logcpm using limma (Ritchie et al, 2015 [\[7\]](#)) and signature genes were selected from the limma results to obtain logFC, p-values, and p-adjusted values (using a Benjamini-Hochberg false-discovery rate).

Oncogene signatures

Normal Human mammary epithelial cells (HMEC) were transfected with adenoviruses containing either the gene of interest or GFP as described in (Bild et al., 2006 [\[8\]](#); McQuerry et al, 2019 [\[9\]](#)). Differentially expressed genes between transfected cells and controls with a Benjamini-Hochberg cutoff of < 0.05 were used in for the signatures of each transfected oncogene.

TBSignatureProfiler Platform

Heatmaps, boxplots, receiver-operating characteristic (ROC) curve, and area under the ROC curve (AUC) were calculated and depicted using the functions within the TBSignatureProfiler (Johnson et al., 2021 [\[10\]](#)). Bootstrapping was used to iteratively calculate AUC values using leave-one-out cross-validation to obtain mean AUCs and 95% confidence intervals (CI) for 100 repeats for each signature. We generated mean AUC values for the predictive performance of collected oncogene-specific signatures (Bild et al., 2006 [\[8\]](#); McQuerry et al., 2019 [\[9\]](#)) in terms of their ability to distinguish between treatment-failure individuals and control samples in our cohort. The single sample Gene Set Enrichment Analysis (ssGSEA) (Barbie et al, 2009 [\[11\]](#)) was used to generate enrichment scores for each signature.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 software (RRID:SCR_002798). Differences among groups involving two or more variables were assessed using two-way analysis of variance (ANOVA) with adjustments for multiple post hoc comparisons. For comparisons across multiple groups based on a single variable, one-way ANOVA with post hoc testing was applied. Two-tailed paired or unpaired t-tests were used for comparisons between two groups after verifying data normality. For non-parametric datasets, the Wilcoxon Rank Sum test (Mann-Whitney U test equivalent) was employed. Statistical significance was defined as $p < 0.05$. Significance levels are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Igor Kramnik (ikramnik@bu.edu).

Materials availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

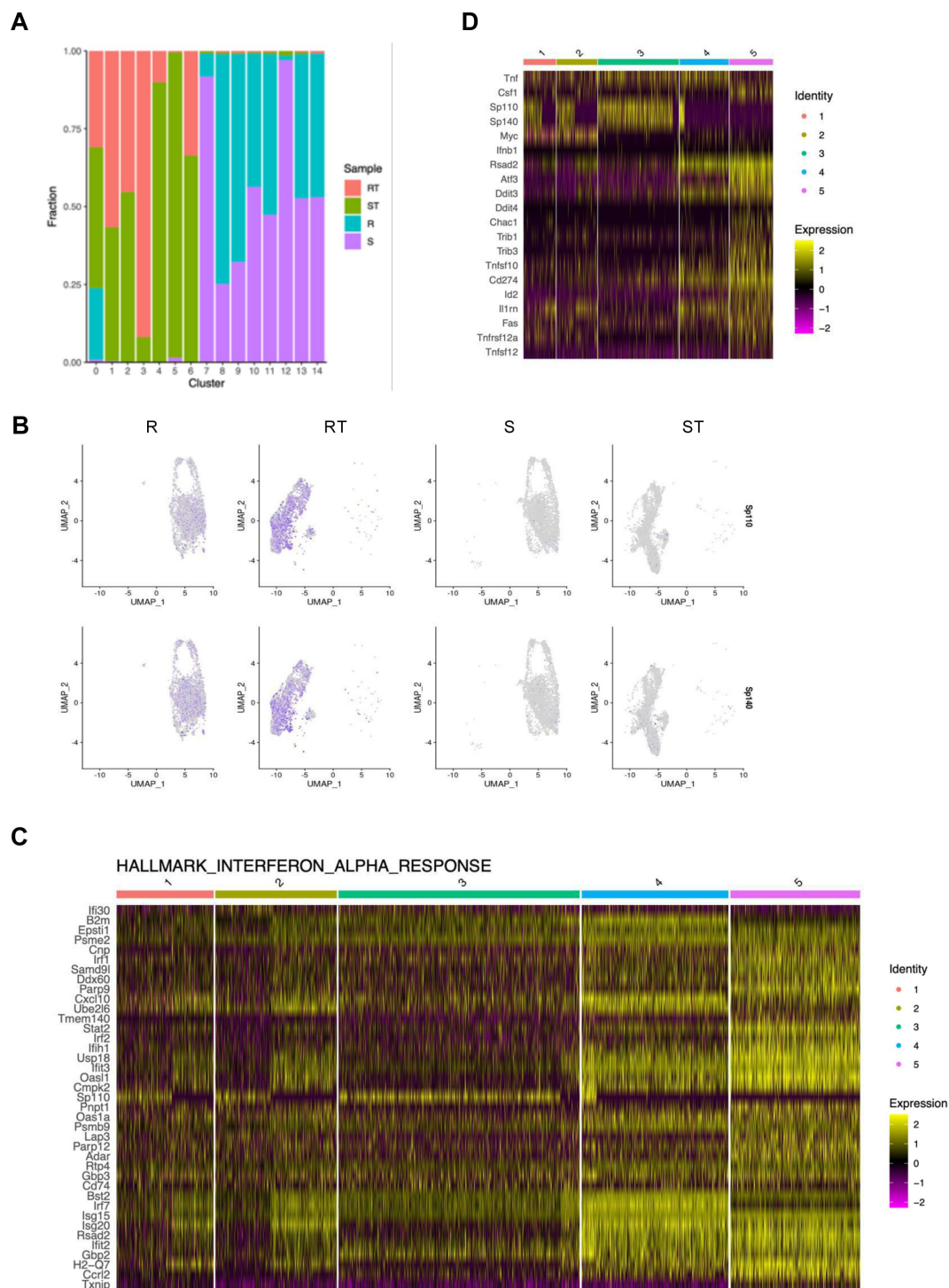
Data and code availability

This study did not generate datasets/code and any additional information will be available from the lead contact upon request.

Inclusion and Diversity

We support inclusive, diverse, and equitable conduct of research.

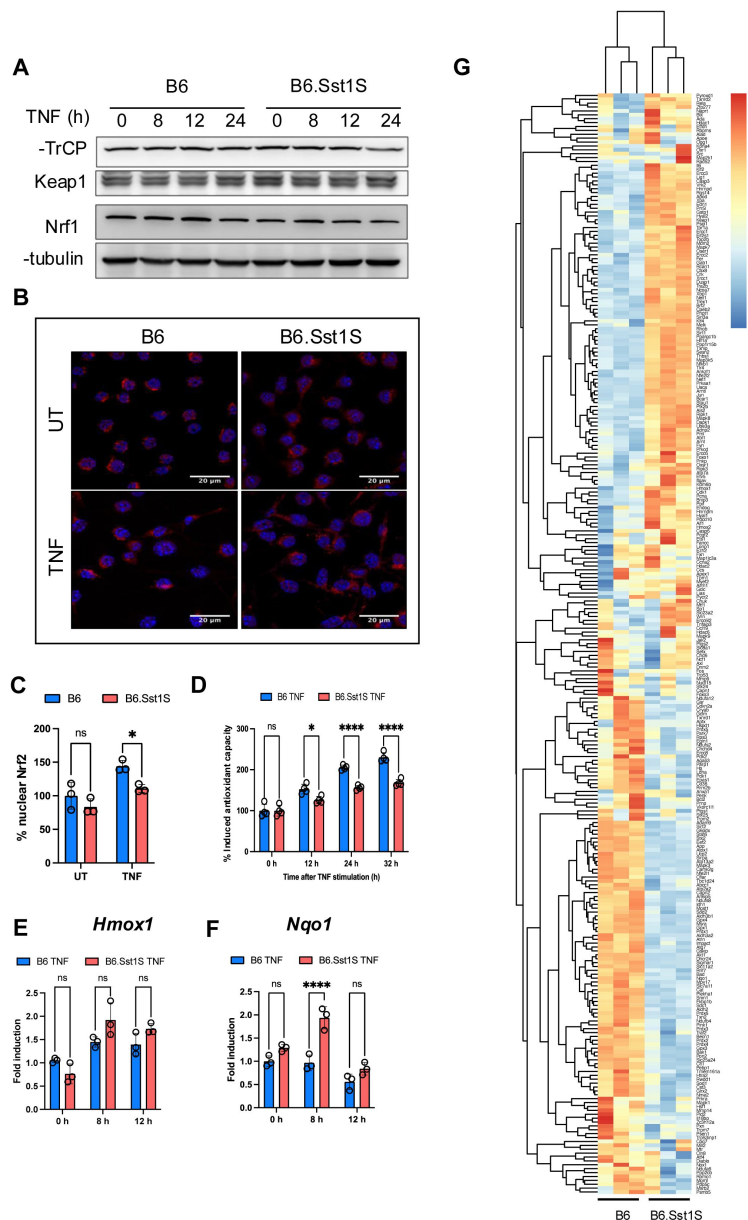
Supplemental figures and tables



Supplemental Figure 1

Single cell RNAseq analysis of the B6 and B6.Sst1S macrophages after TNF stimulation.

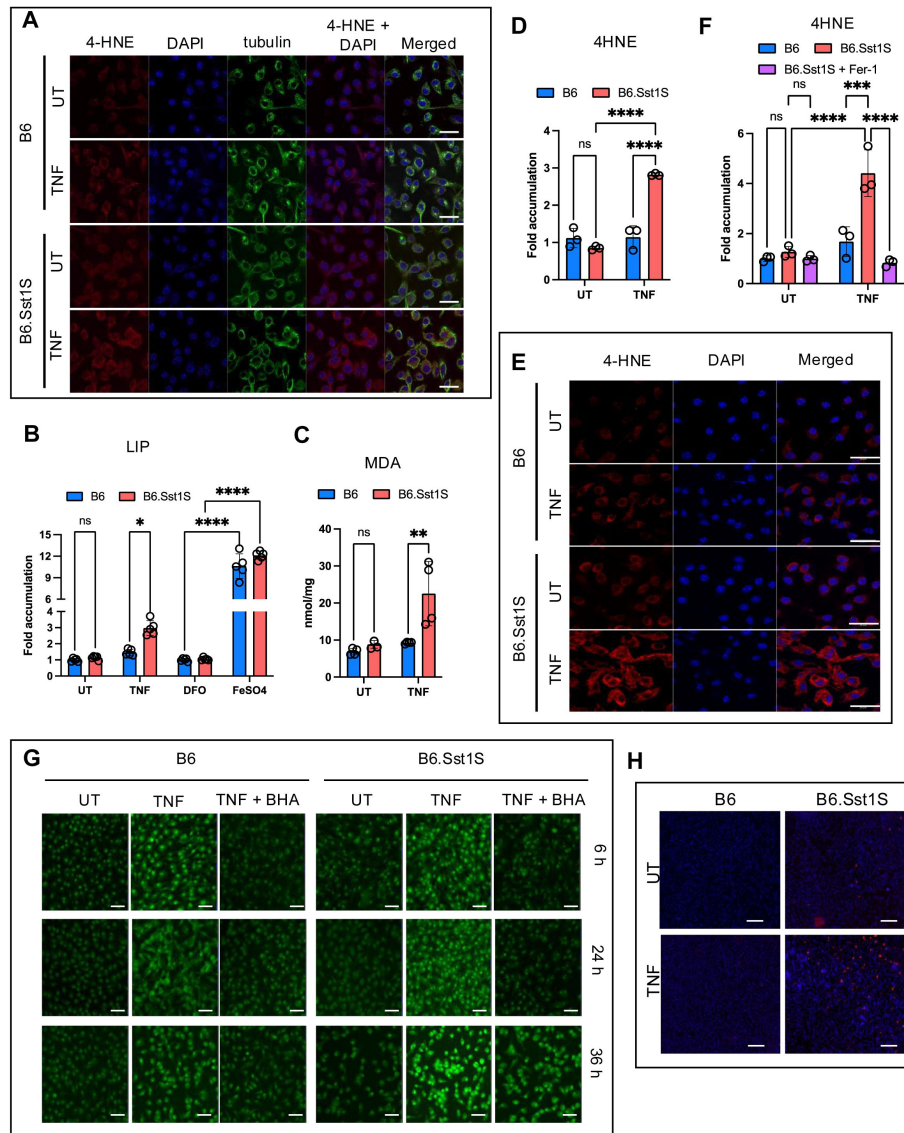
A. Relative proportions of either naive (R and S) or TNF-stimulated (RT and ST) B6 or B6.Sst1S macrophages within individual single cell clusters depicted in [Fig.1B](#). **B.** The distribution of macrophages expressing the *sst1*-encoded *Sp110* and *Sp140* genes across the populations of naive (R and S) and TNF-stimulated (RT and ST) B6 or B6.Sst1S macrophages. **C.** Heatmap of the IFN-I pathway gene expression in subpopulations 1 - 5 identified using pseudotime trajectory analysis. **D.** The expression of known *sst1*-dependent genes representing IFN-I, stress response, apoptosis and immunosuppression in subpopulations 1 - 5.



Supplemental Figure 2

Anti-oxidant response of TNF-stimulated B6 and B6.Sst1.S BMDMs.

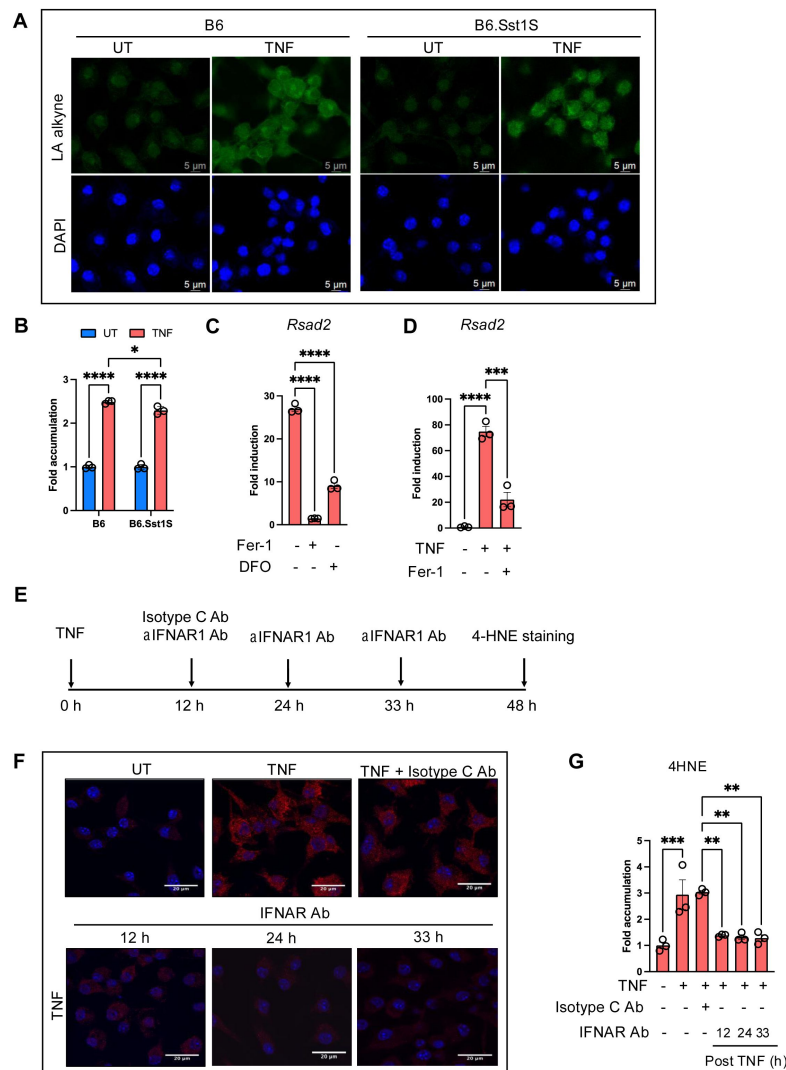
A. Total levels of Nrf1, -TrCP and Keap1 proteins in B6 and B6.Sst1S BMDMs stimulated with TNF (10 ng/ml) for 8, 12 and 24 h (Western blotting). The experiment was repeated 2 times and shown the representative blot. **B.** B6 and B6.Sst1S BMDMs stimulated with TNF (10 ng/ml) for 12 h. Total NRF1 levels were evaluated using confocal microscopy. Scale bar = 20 m. **C.** B6 and B6.Sst1S BMDMs stimulated with TNF (10 ng/ml) for 12 h. Nrf2 nuclear translocation was quantified using automated microscopy (Operetta CLS High Content Analysis System). Untreated samples considered 100%. **D.** The total antioxidant capacity of B6 and B6.Sst1S BMDMs were measured after TNF (10 ng/ml) stimulation. The percentage of induced antioxidant capacity upon TNF stimulation was plotted (Y axis). **E and F.** B6 and B6.Sst1S BMDMs stimulated with TNF (10 ng/ml) for 8 and 12 h. *Hmox1* and *Nqo1* expressions were quantified using qRT-PCR. **G.** The heatmap of all genes related to response to oxidative stress (gene ontology category GO: 0006979). The heatmap was generated using FPKM values obtained using bulk RNAseq of B6.Sst1S and B6 BMDMs after 12 h of TNF stimulation. For heatmap generation, FPKM values were scaled using Z-scores for each tested gene. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. The statistical significance was performed by two-way ANOVA using Tukey's multiple comparison test (Panels C-F). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).



Supplemental figure 3

Regulation of TNF-induced ROS, labile iron pool and lipid peroxidation in B6 and B6.Sst1.S BMDMs.

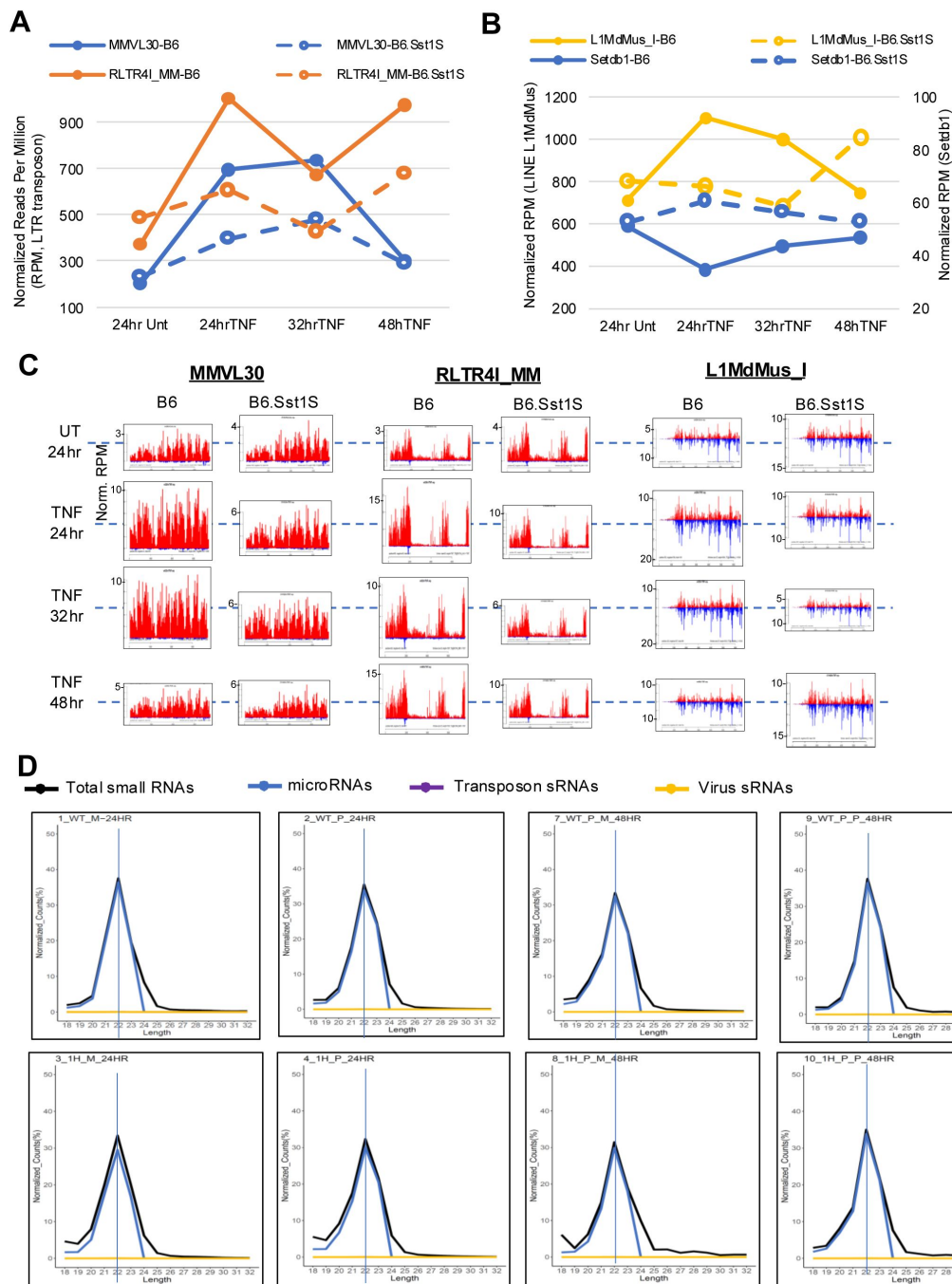
A. B6 and B6 BMDMs were treated with 10 ng/mL TNF or left untreated for 48 h. The cells were stained to observe 4-HNE accumulation and cellular structure using anti-4-HNE and anti-tubulin Ab respectively. The images were acquired using confocal microscopy. Scale bar = 20 m. **B.** The labile iron pool (LIP) in B6 and B6.Sst1.S BMDMs treated with 10 ng/mL TNF or left untreated for 48 h. LIP was calculated using Calcein AM method and represented as fold change. DFO was used as negative control and FeSO₄ was used as positive control. **C.** Production of lipid peroxidation metabolite malondialdehyde (determined by MDA assay) by B6 and B6.Sst1.S BMDMs treated with 10 ng/mL TNF for 48 h. UT - untreated control. **D - F.** The intracellular 4-HNE accumulation in B6 and B6.Sst1.S BMDMs treated with 10 ng/mL TNF for 30 h (D and E) and 48 h (F). The 4-HNE accumulation was quantified using ImageJ and plotted as a fold accumulation compared to B6 untreated group. Scale bar = 20 m. **G.** Reactive oxygen species (ROS) levels were observed using the CellROX assay and imaged by fluorescence microscopy in B6 and B6.Sst1.S BMDMs either treated with TNF (10 ng/mL) or left untreated for 6, 24, and 36 h. BHA (100 M) was used as a positive control. Scale bar = 50 m. **H.** Cell death in B6 and B6.Sst1.S BMDMs stimulated with 50 ng/mL TNF for 48 h. Imaging was performed by automated microscopy using Live-or-DyeTM 594/614 Fixable Viability stain (Biotium) and Hoechst staining. Scale bar = 100 m. The data represent the means SD of three samples per experiment, representative of three independent experiments. The statistical significance was performed by two-way ANOVA using Bonferroni's multiple comparison test (Panels B, C, D and F). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).



Supplemental figure 4

Regulation of lipid peroxide production and type I IFN expression in TNF-stimulated B6 and B6.Sst1.S BMDMs.

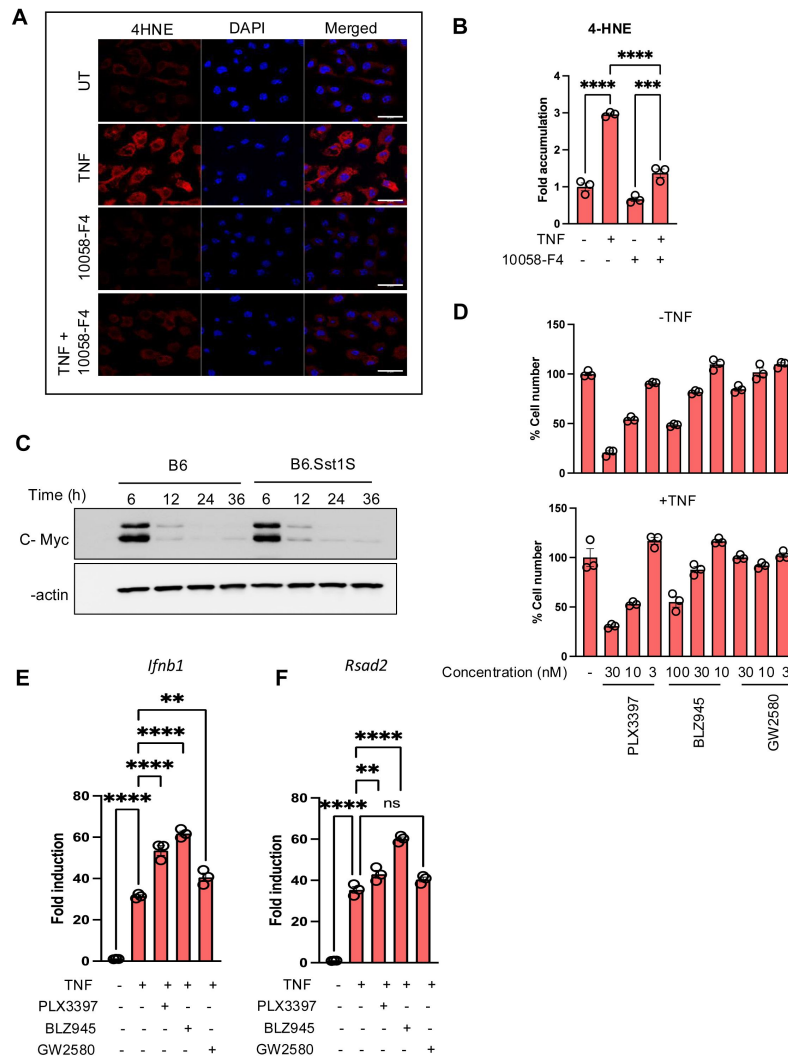
A - B. B6 and B6.Sst1.S BMDMs were either treated with 10 ng/mL TNF or left untreated for 6 h. The LPO production was observed using Click-iT linoleamide alkyne (LAA) method and imaged using confocal microscopy (A). The quantification of signal per field was performed using ImageJ (B). Scale bar = 5 μ m. **C.** Inhibition of lipid peroxidation prevented the superinduction of *Rsad2* mRNA. B6.Sst1.S BMDMs were stimulated with 10 ng/mL TNF for 2h, then the LPO inhibitor (Fer-1) or iron chelator DFO were added for the remaining 14 h. Fold induction was calculated using untreated control as average one-fold by utilizing the Ct method with 18S as the internal control. **D.** Inhibition of TNF and lipid peroxidation reversed the *Rsad2* mRNA superinduction after prolonged TNF stimulation. B6.Sst1.S BMDMs were stimulated with 10 ng/mL TNF for 18 h, then the LPO inhibitor (Fer-1) was added for the remaining 12 h. Fold induction was calculated using untreated control as average one-fold by utilizing the Ct method with 18S as the internal control. **E.** Experimental design for panels F-G. **F - G.** Inhibition of the 4-HNE accumulation in TNF-stimulated B6.Sst1.S BMDMs by IFNAR1 blocking antibodies. Cells were treated with 10 ng/mL TNF for 48 h in presence of IFNAR1 blocking antibodies. 4HNE was detected using specific antibody staining and confocal microscopy. The isotype C Ab was added 12 h post TNF and anti-IFNAR Ab was added at 12, 24 and 33 h post TNF stimulation. The 4-HNE accumulation was quantified using ImageJ and plotted as fold accumulation compared to untreated group. Isotype C Ab - isotype matched negative control antibodies. Scale bar = 20 μ m. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. The statistical significance was performed by Ordinary one-way ANOVA using Bonferroni's multiple comparison test (B-D) and two-way ANOVA using Tukey's multiple comparison test (Panel E). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).



Supplementary Figure 5.

The transient upregulation of transposon mRNAs in B6 macrophages after TNF treatment is affected in the B6.Sst1S.

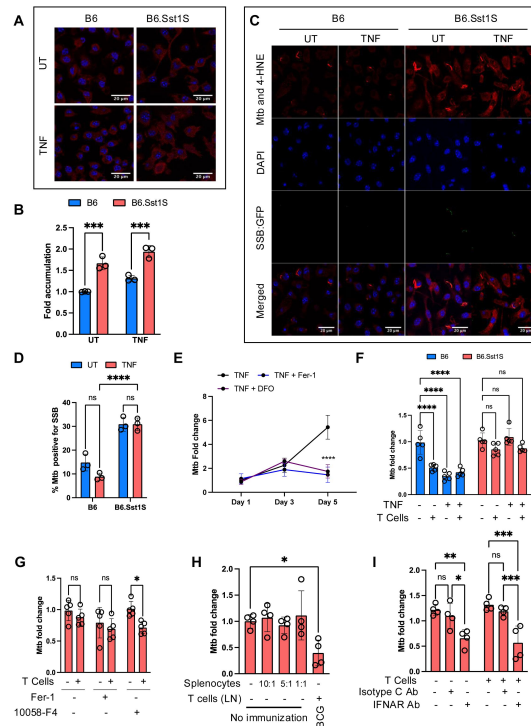
A. Line graphs displaying the expression of two mouse LTR transposons, mVL30 and RLTR4i activated in expression by TNF treatment in WT macrophages with the levels less stimulated in the B6.Sst1S. **B.** A LINE transposon L1Md expression pattern compared to the distinct expression dynamics of Setdb1 after TNF stimulation between B6 and B6.Sst1S macrophages. Setdb1 protein is implicated as the histone methyltransferase marking H3K9me3 on these two transposons. **C.** Reads coverage plots show extensive transcript representation and positive strand bias for LTR transposons, but double-stranded RNA expression from the LINE transposon. **D.** Size distribution plots with functional annotations indicate the vast majority of macrophage small RNAs are mainly microRNAs, with little detection of viral and transposon small RNAs that would be indicative of these small interfering RNAs being processed by putative longer double-stranded RNA precursors.



Supplemental Figure 6

Effects of Myc and CSF1R inhibition on B6.Sst1S macrophage activation by TNF.

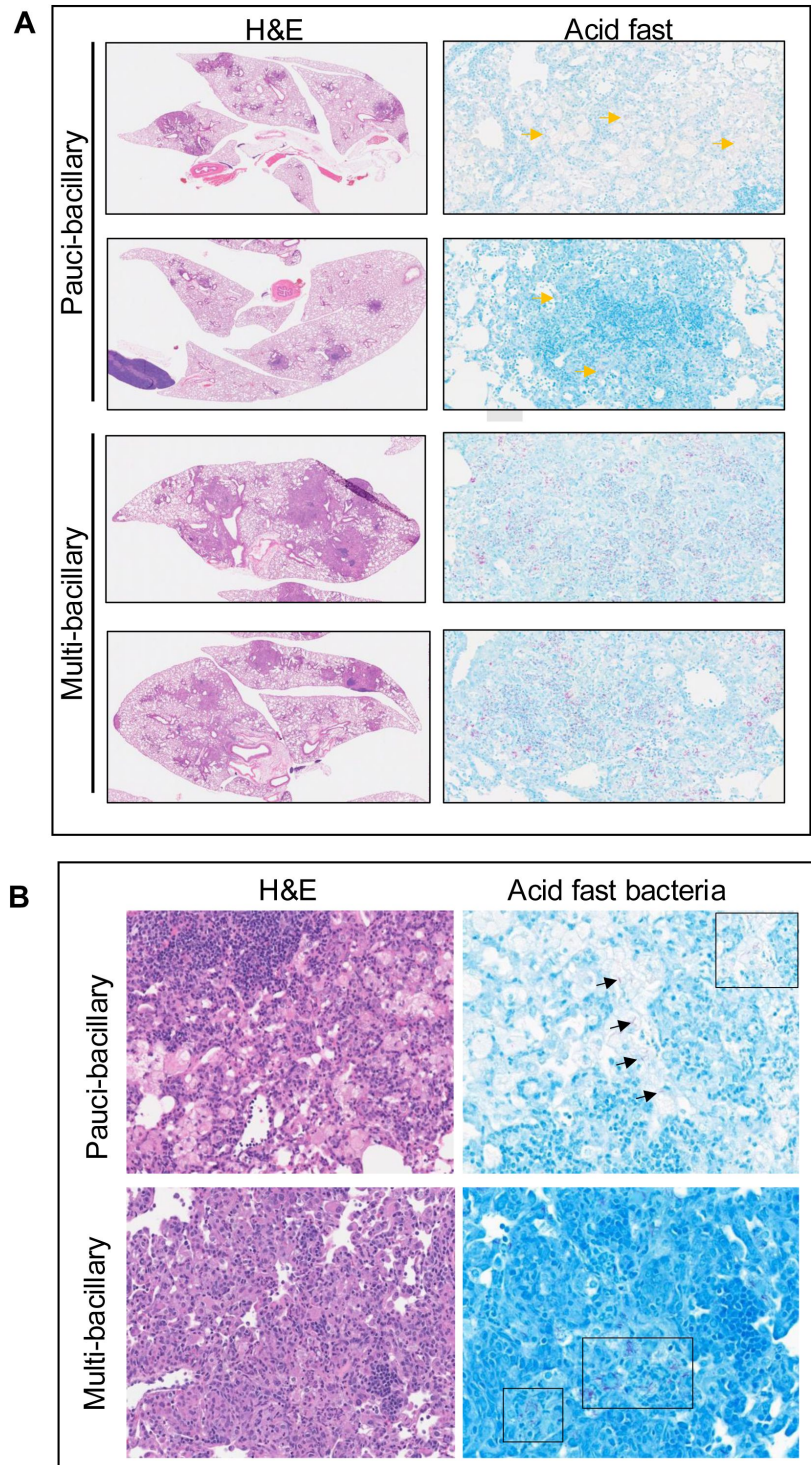
A. Myc inhibitor 10058-F4 (10) prevents the 4-HNE accumulation in TNF-stimulated B6.Sst1S BMDMs. B6.Sst1S BMDMs were treated with 10 ng/mL TNF for 48 h. The inhibitor was added 2 h post TNF stimulation. The accumulation of lipid peroxidation product, 4-HNE was detected by confocal microscopy using 4-HNE-specific antibody. Scale bar = 20 m. **B.** Myc inhibition reduced lipid peroxidation in TNF-stimulated B6.Sst1S BMDMs. Cells were treated with 10 ng/mL TNF in presence or absence of 10058-F4 for 48 h. The inhibitor was added 2 h post TNF stimulation. The accumulation of lipid peroxidation product, 4-HNE was detected by confocal microscopy using 4-HNE specific antibody. The 4-HNE accumulation was quantified using ImageJ and plotted as fold accumulation compared to untreated group. **C.** Media change induces Myc upregulation and similar downregulation in B6 and B6.Sst1S BMDMs. Myc protein levels were monitored using Western blot. **D.** Selection of non-toxic concentration of CSF1R inhibitors. BMDMs from B6.Sst1S were either left untreated or treated with 10 ng/mL TNF. Post 2 h of TNF stimulation the inhibitors of CSF1R were added at different concentrations. PLX3397 (30, 10 and 3 nM), BLZ945 (100, 30 and 10 nM) and GW2580 (30, 10 and 3 nM) for 46 h. Percent of cell number was determined by automated microscopy. **E** and **F.** CSF1R inhibitors do not prevent the IFN-I pathway hyperactivity in TNF-stimulated B6.Sst1S macrophages. BMDMs from B6.Sst1S were treated with 10 ng/mL TNF alone or in combination with CSF1R inhibitors, PLX3397 (3 nM), BLZ945 (10 nM) and GW2580 (10 nM) for 20 h. CSF1R inhibitors were added 2 h post TNF stimulation. Expression of *Ifnb1* and *Rsad2* were quantified by the Ct method using qRT-PCR and expressed as a fold induction compared to the untreated group. 18S was used as the internal control. The data are presented as means $\pm$ standard deviation (SD) from three-five samples per experiment, representative of three independent experiments. The statistical significance was performed by ordinary one-way ANOVA using multiple comparison test (Panel B, E and F). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).



Supplemental Figure 7.

Inhibition of lipid peroxidation improves Mtb control by B6.Sst1S macrophages.

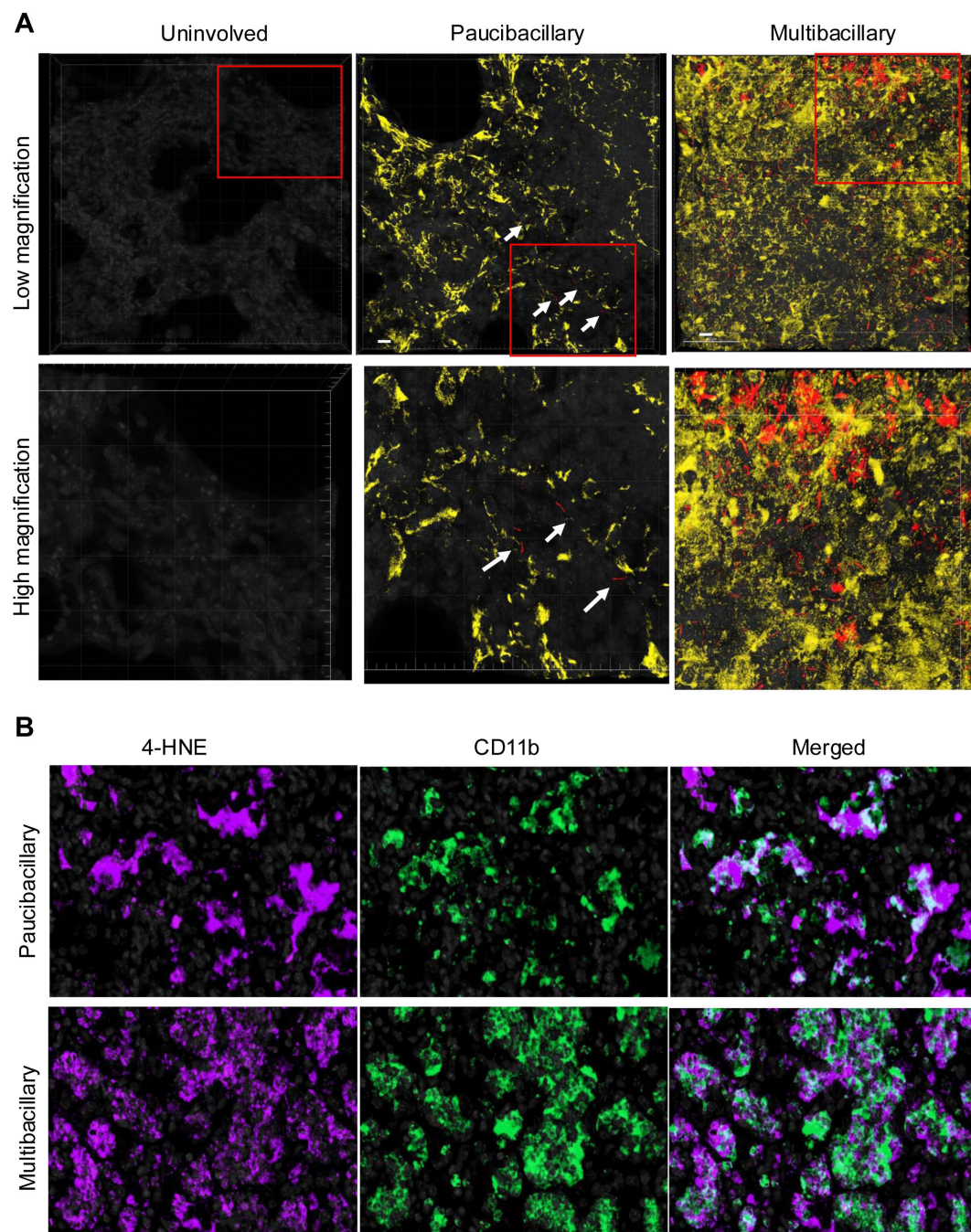
A-B. Accumulation of 4-HNE in Mtb-infected B6 and B6.Sst1S macrophage monolayers infected with Mtb. BMDMs were either treated with 10 ng/mL TNF or left untreated (UT), and subsequently infected with Mtb at MOI=1. 4-HNE was detected by confocal microscopy using 4-HNE specific antibody 3 dpi. The 4HNE accumulation was quantified at 3 dpi using ImageJ and plotted as fold accumulation compared to untreated B6 (UT). Scale bar = 20 m. **C-D.** Naïve and TNF-stimulated B6 and B6.Sst1S BMDMs were infected with Mtb Erdman reporter strain (Mtb SSB-GFP, smyc::mCherry) at MOI=1 for 5 days. The 4-HNE accumulation and Mtb SSB were observed using confocal microscopy at day 5 p.i. (C). The percent of replicating Mtb was quantified by calculating number of Mtb (red) and SSB-GFP puncta (green) (D). Scale bar = 20 m. **E.** Control of Mtb growth by B6.Sst1S BMDMs pre-treated with 10 ng/mL TNF alone or in combination with Fer-1 (3 M) or DFO (50 M). Macrophages were pretreated 16 h before Mtb infection and infected with Mtb at MOI=1. Mtb loads were determined on days 1, 3 and 5 post infection using a qPCR-based method. **F.** Differential effect of BCG-induced T cells on Mtb control by B6 and B6.Sst1S macrophages. BMDMs of both backgrounds were treated with 10 ng/mL TNF or left untreated and subsequently infected with Mtb at MOI=1. T lymphocytes purified from lymph nodes of BCG vaccinated B6 mice were added to the infected macrophage monolayers 24 h post infection. The Mtb load was calculated by qPCR-based method after 1 day of co-culture with T lymphocytes (day 2 post infection). The dotted line indicates the Mtb load in untreated cells at day 2 post infection. **G.** Inhibition of Myc and lipid peroxidation improves control of Mtb by B6.Sst1S macrophages co-cultured with immune T cells isolated from BCG-vaccinated B6 mice. BMDMs were pretreated with 10 ng/mL TNF alone or in combination with either Ferrostatin 1 (3 M) or 10058-F4 (10 M) for 16 h and subsequently infected with Mtb at MOI 1. At 24 h post infection the lymphocytes from BCG immunized B6 mice were added to the infected macrophage monolayers. The Mtb loads were determined by qPCR-based method after 1 day of co-culture with T cells (2 days post infection). **H.** BMDMs from B6 were infected with Mtb at MOI 1. At 24 h post infection the splenocytes from non-immunized mice at 10:1, 5:1 and 1:1 ratio (splenocytes:macrophages) or lymphocytes from BCG immunized B6 mice were added to the infected macrophage monolayers. The Mtb loads were determined by qPCR-based method after 1 day of co-culture with T cells (2 days post infection). **I.** Inhibition of type I IFN receptor improves control of Mtb by B6.Sst1S macrophages. BMDMs were pretreated with 10 ng/mL TNF alone or in combination with anti-IFNAR Ab or Isotype C Ab for 16 h and subsequently infected with Mtb at MOI 1. At 24 h post infection the lymphocytes from BCG immunized B6 mice were added to the infected macrophage monolayers. The Mtb loads were determined by qPCR-based method after 1 day of co-culture with T cells (2 days post infection). The data represent the means SD of three samples per experiment, representative of three independent experiments. The statistical significance was performed by two-way ANOVA using Bonferroni's multiple comparison test (Panel B, D, F, G and I), and multiple comparison test (Panel E) and One-way ANOVA using Bonferroni's multiple comparison test (Panel H). Significant differences are indicated with asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).



Supplemental Figure 8.

Pauci- and multi-bacillary pulmonary lesions of Mtb infected B6.Sst1S mice.

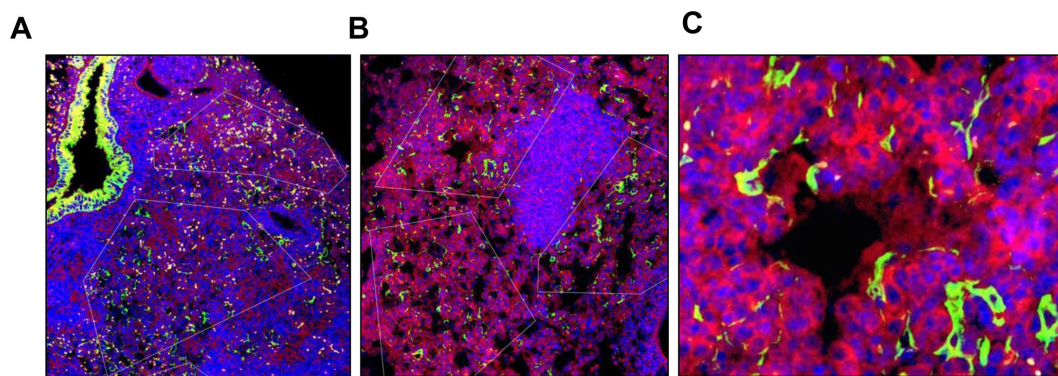
A. Representative histopathology and Acid Fast Bacteria (AFB) loads in of B6.Sst1S mouse lungs at 14 weeks after infection with 10^6 CFU of Mtb Erdman. Arrows indicate acid-fast bailli (Mtb). **B.** Left-Representative H & E staining and Right - acid fast staining of B6.Sst1S Mtb infected lungs at 200X original magnification. Arrows and boxes indicate acid-fast bailli (Mtb).



Supplemental Figure 9.

Accumulation of 4-HNE and $\text{Ifn}\beta$ producing cells in Mtb-infected B6.Sst1S mouse lung lesions.

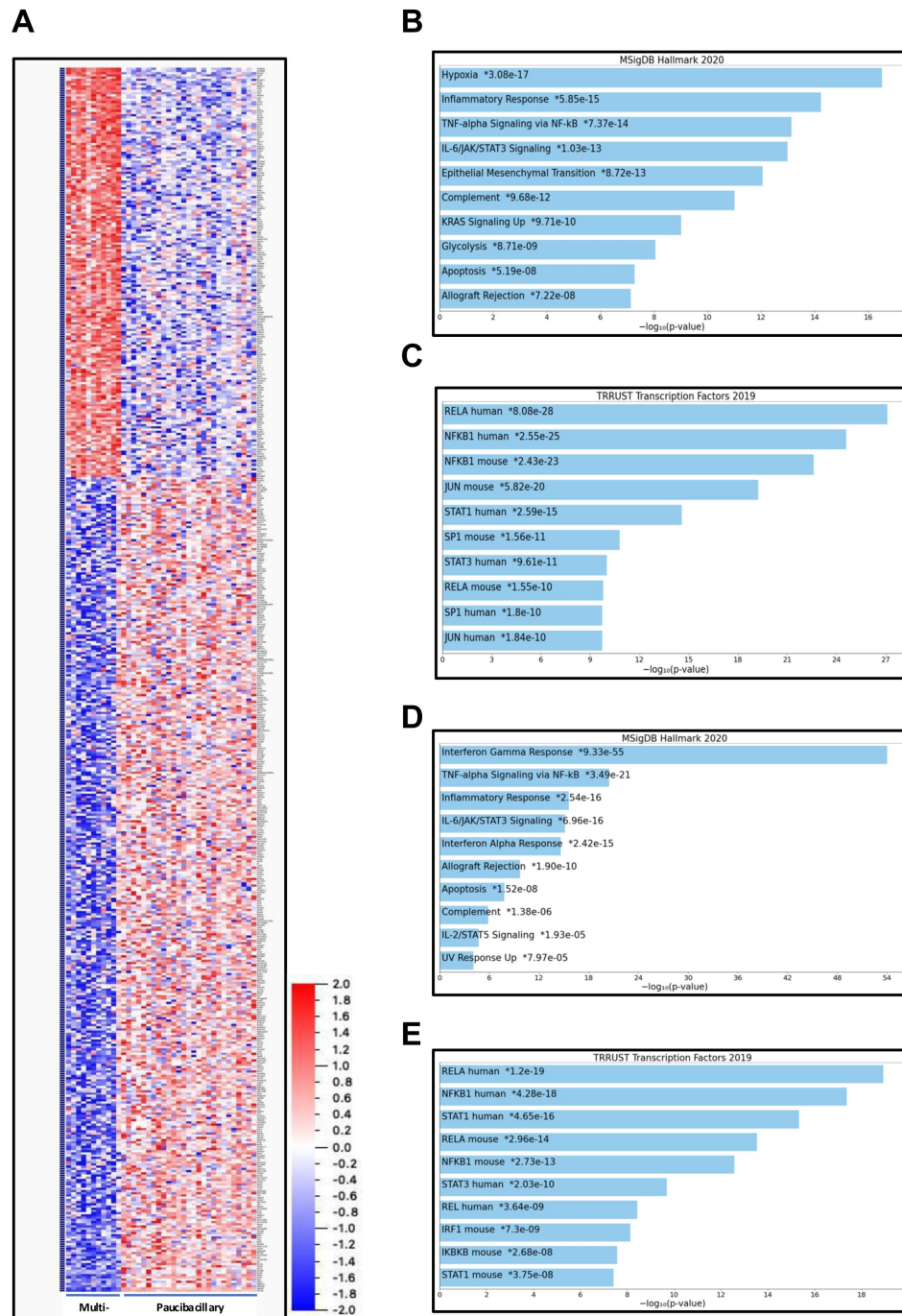
A. 3D images of uninvolved lung and pulmonary TB lesions in Mtb-infected B6.Sst1S,*Ifnb1*-YFP mice presented in [Fig.7A](#) at low and high magnification. 4HNE+ staining - is yellow, and the reporter Mtb (*smyc'*:: mCherry) is red. The mice were infected for 20 weeks. Scale bar = 10 μm . Arrows indicate Mtb reporter strain expressing mCherry. Lower panels are magnified area of insets shown in top panels. **B.** Representative fluorescent single channel images of pauci- and multibacillary PTB lesions in B6.Sst1S mice and merged images corresponding to [Fig.7B](#). 4-HNE (magenta), CD11b (green) and DAPI (grey).



Supplemental Figure 10.

Representative Images of GeoMX Region of Interests (ROIs).

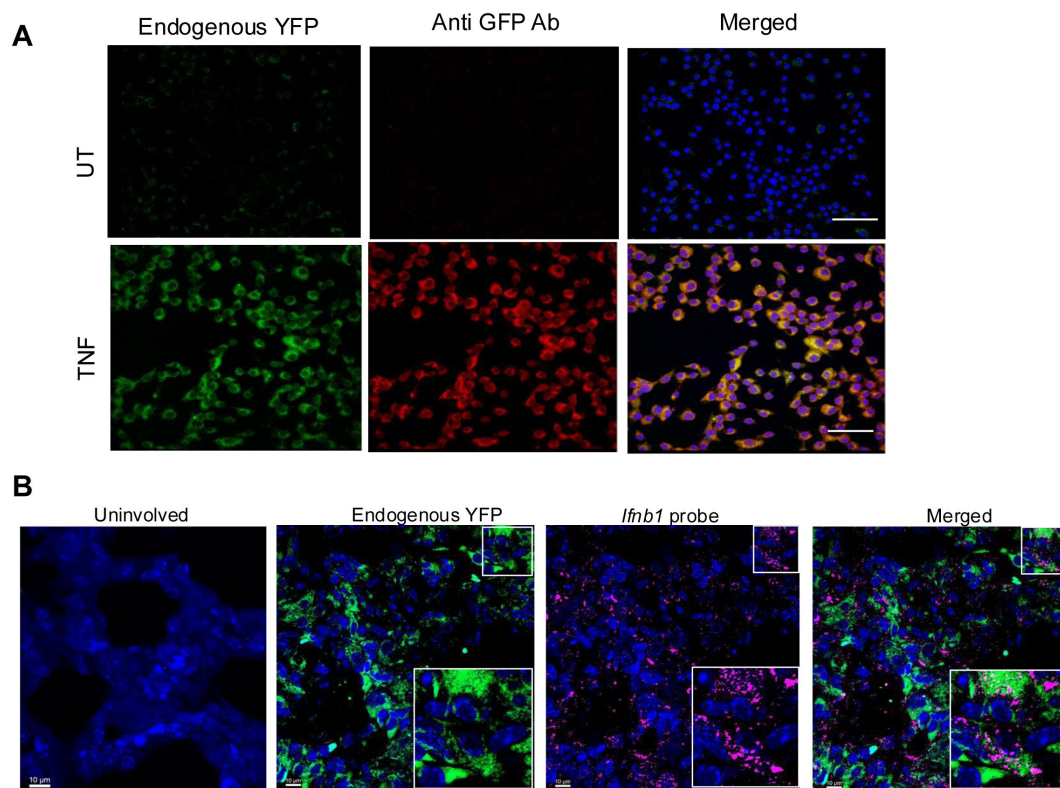
Representative Images of GeoMX ROIs (Regions of Interest) with tuberculous lesions labeled with DAPI (nuclear stain, blue), anti-pancytokeratin (epithelial cells, green) and anti-Iba1 (macrophages, red). A&B Low magnification views with two (A) or three (B) ROIs outlined (white) containing abundant macrophages (red) and scattered enlarged alveolar epithelial cells (green), as illustrated in a higher magnification view in (C). (Original magnification, A&B 40X, C 400X). The profiling used the Mouse Whole Transcriptome Atlas (WTA) panel which targets ~21,000+ transcripts for mouse protein coding genes plus ERCC negative controls, to profile the whole transcriptome, excluding uninformative high expressing targets such as ribosomal subunits. We assembled lungs from two mice with paucibacillary and two mice with multibacillary lesions to prepare a tissue array of paraffin-embedded lung tissues, and used acid fast Mtb staining on serial sections to classify individual TB lesions in the Mtb paucibacillary and multibacillary categories (**Suppl. Fig.8** [🔗](#)).



Supplemental Figure 11.

Analysis of spatial transcriptomics data from Iba1+ cells in pauci- and multi-bacillary lesions in Mtb infected B6.Sst1S mouse lungs.

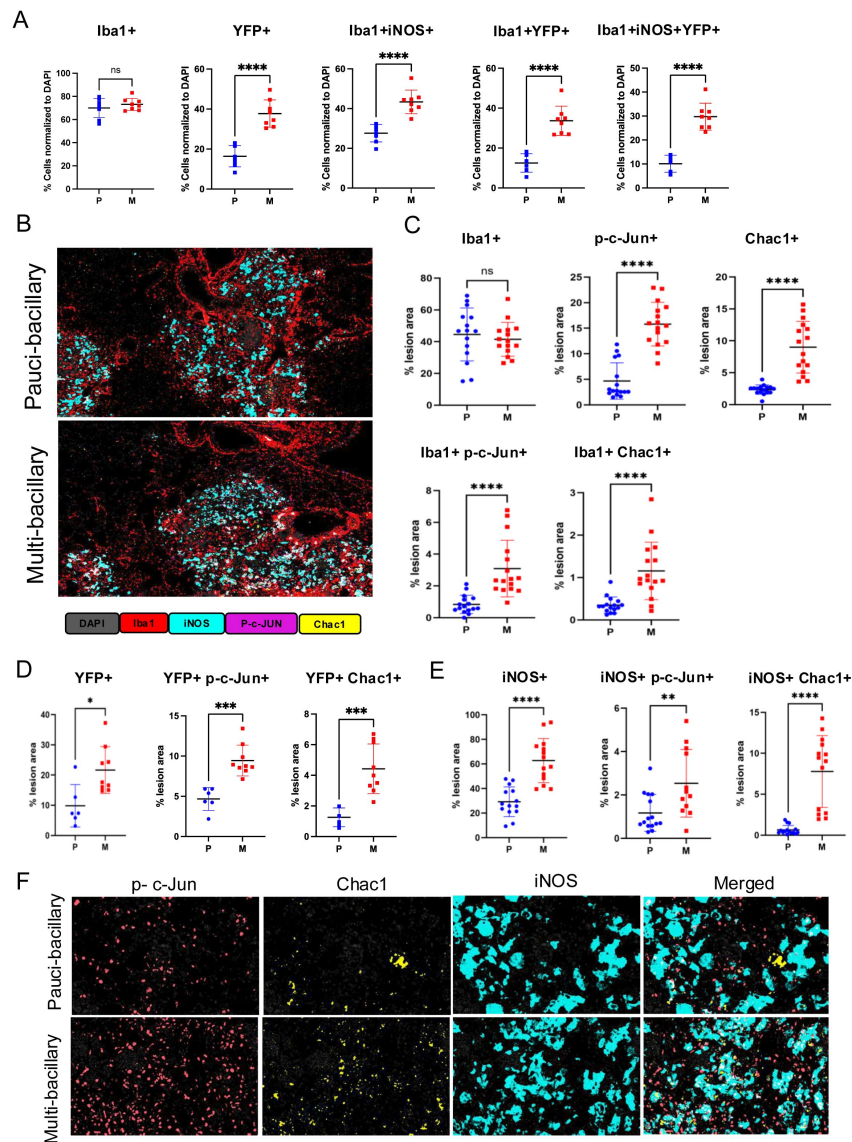
A. Heatmap of all differentially expressed genes (fold change 2 and above) by Iba1+ cells in Multibacillary vs Paucibacillary TB lesions. **B.** Top 10 Hallmark pathways upregulated in Iba1+ cells in multibacillary lesions. **C.** Top 10 transcription factors enriched in promoters of genes upregulated in Iba1+ cells in multibacillary lesions. **D.** Top 10 Hallmark pathways upregulated in the combined gene set of IFN-inducible genes expressed by Iba1+ cells in multibacillary lesions. **E.** Top 10 transcription factors enriched in promoters of genes upregulated in the list of IFN-inducible genes expressed by Iba1+ cells in multibacillary lesions.



Supplemental Figure 12.

Accumulation of 4-HNE and Ifn producing cells in Mtb-infected B6.Sst1S mouse lung lesions.

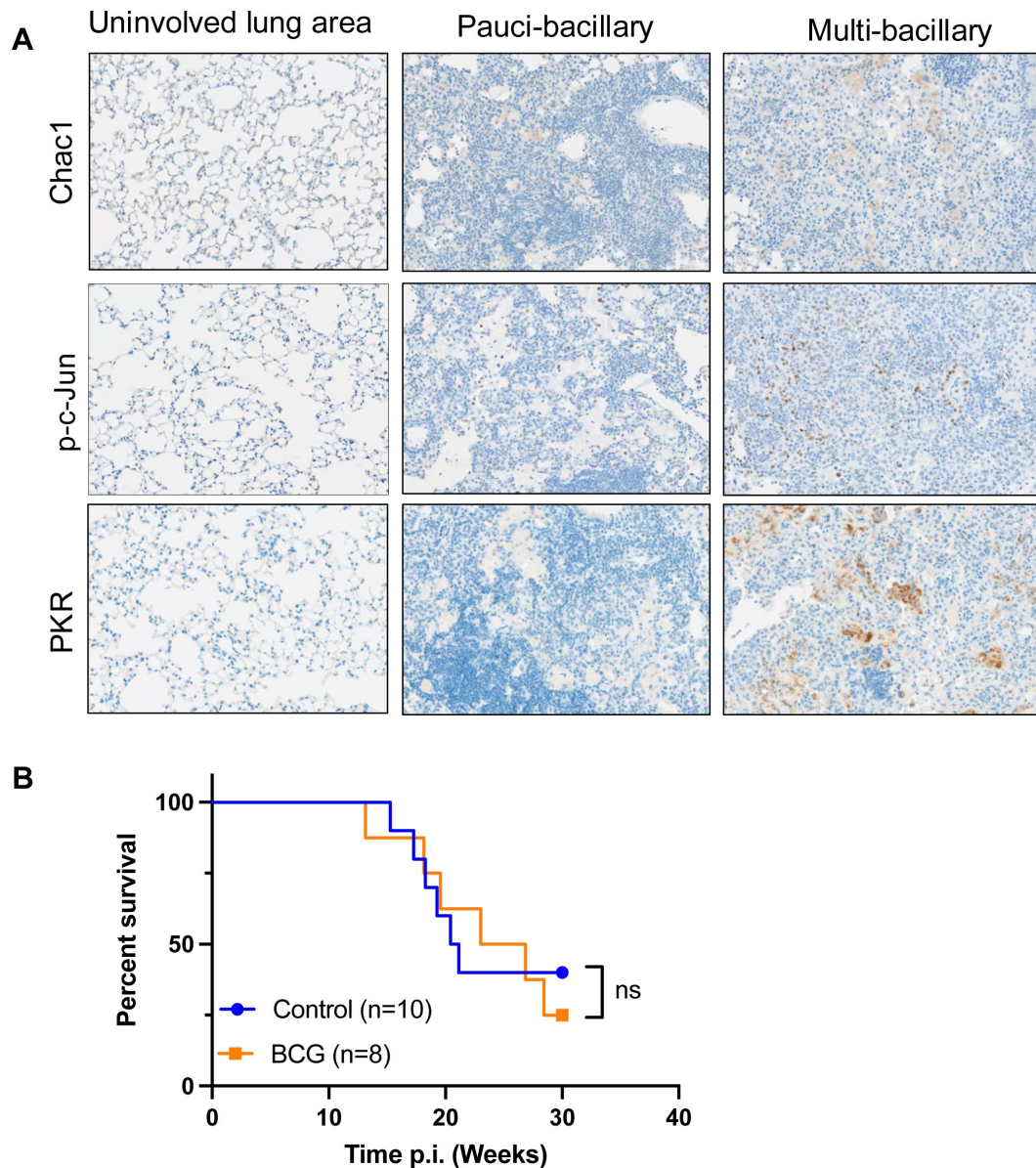
A. Confocal images of BMDMs isolated from B6.Sst1S, *Ifnb1*-YFP mice stimulated with TNF (10 ng/ml) in vitro for 24 h or left untreated. The endogenous YFP signal is shown in green. Staining with anti-YFP antibody was used to demonstrate YFP expression in activated BMDMs, as a control of specificity. Nuclei in blue (DAPI). Scale bar = 50 m. **B.** Confirmation of YFP signal from B6.Sst1S,*Ifnb1*-YFP reporter mice using *Ifnb1* mRNA in situ hybridization. 3D confocal images of florescent in situ hybridization with *Ifnb1* mRNA probes (red) showed overlap with YFP+ cells. Insets at lower right corner is an enlarged image of inset at top right corner which is showing the same cells expressing both signals. The mice were infected for 20 weeks. Scale bar = 10 m.



Supplemental Figure 13.

Fluorescent multiplexed immunohistochemistry (fmIHC) images representing increased expression of stress markers in macrophages within multi-bacillary pulmonary TB lesions of B6.Sst15 mice.

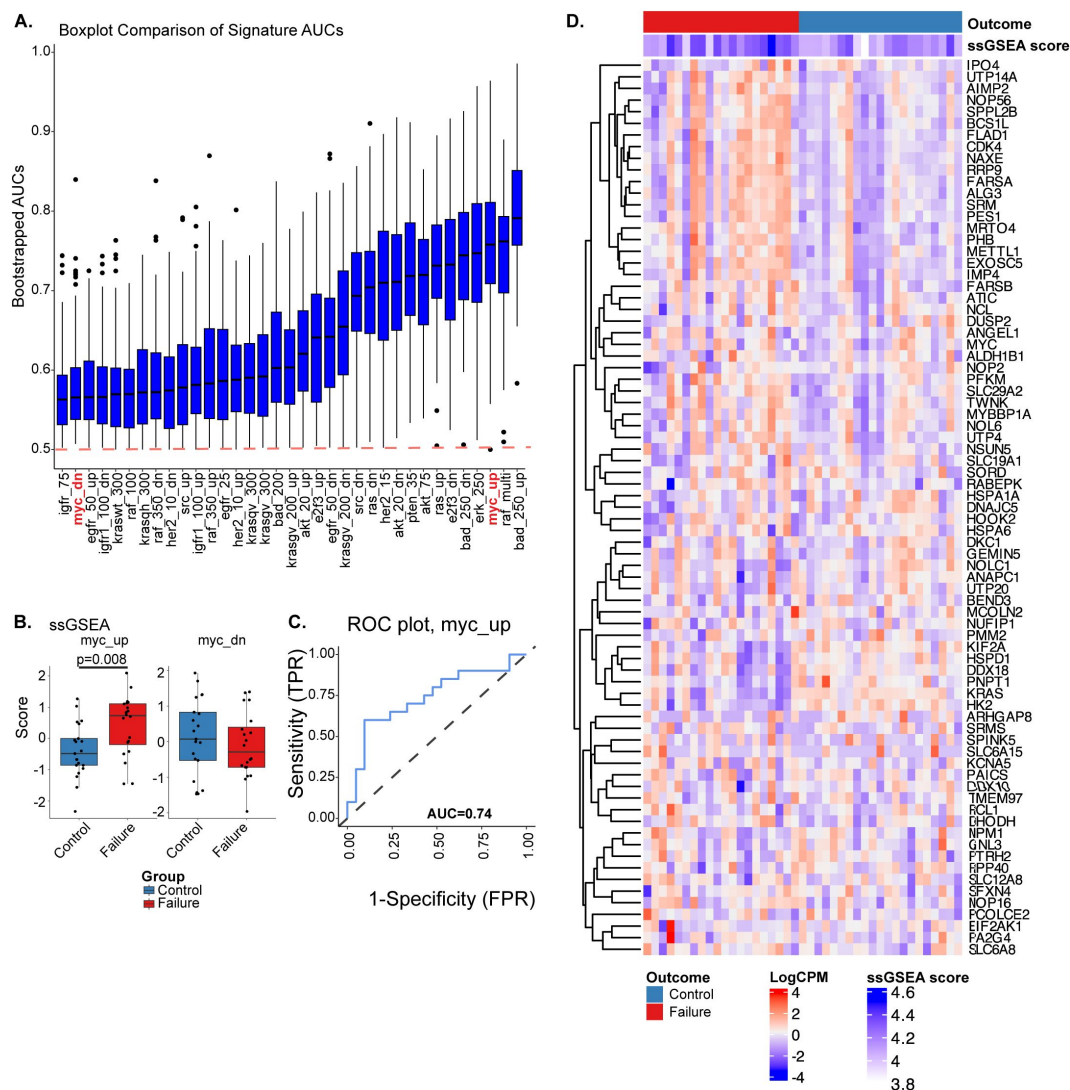
A. Quantification of IFN-I producing (YFP positive) myeloid cells in pauci-bacillary (n=8) and multi-bacillary (n=8) lesion of B6.Sst15, *Ifnb1*-YFP reporter mice. The quantification was performed by manually counting total and individual group of markers at different lesions at 400X magnification and calculated the percentage cell number as compared to total DAPI. The average total cell number (DAPI) per field was 140. **B - C.** fmIHC images and quantification of total macrophages (Iba1 positive) co-expressing stress markers in paucibacillary (n=15) and multibacillary (n=15) PTB lesions. The quantification was performed using Halo automated analysis as percent lesion area. The different markers are shown as p-c-Jun (peach), Chac-1 (yellow) an Iba1 (red) at 200X magnification. The mice were infected for 20 weeks. **D.** Quantification of IFN-I producing (YFP positive) cells accumulating stress markers in pauci-bacillary (n=6) and multi-bacillary (n=9) lesion of B6.Sst15, *Ifnb1*-YFP reporter mice using Halo automated analysis. **E F.** quantification and representative fmIHC images of activated (iNOS+) macrophages co-expressing stress markers in paucibacillary (n=14) and multibacillary (n=14) PTB lesions. The quantification was performed using Halo automated analysis and the signals are presented as percent lesion area. The different markers are shown as phospho-c-Jun (peach), Chac-1 (yellow) an iNOS (teal) at 400X magnification. The mice were infected for 20 weeks. The data represent the means $\pm$ SD and the statistical significance was performed by two-tailed unpaired t-test (Panel A, C-E). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).



Supplemental Figure 14.

Expression of stress markers in pauci- and multi-bacillary lesions of *Mtb*-infected B6.Sst1S mouse lungs.

A. Representative monochromogenic immunohistochemistry staining images of Chac1, p-c-Jun and PKR in uninvolved lung area, paucibacillary and multibacillary pulmonary lesions in *Mtb*-infected B6.Sst1S mice. Mice were infected for 14 weeks. B. No effect of post-exposure BCG vaccination on the survival of *Mtb*-infected B6.Sst1S mice. Mice were infected with *Mtb* and BCG-vaccinated two months post infection. The survival curves of vaccinated and non-vaccinated mice were compared using the Log-rank (Mantel-Cox). Numbers of mice per group indicated in parentheses. This experiment was performed once. -rank (Mantel-Cox) test was applied to determine statistical significances between the groups for panel B. Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).



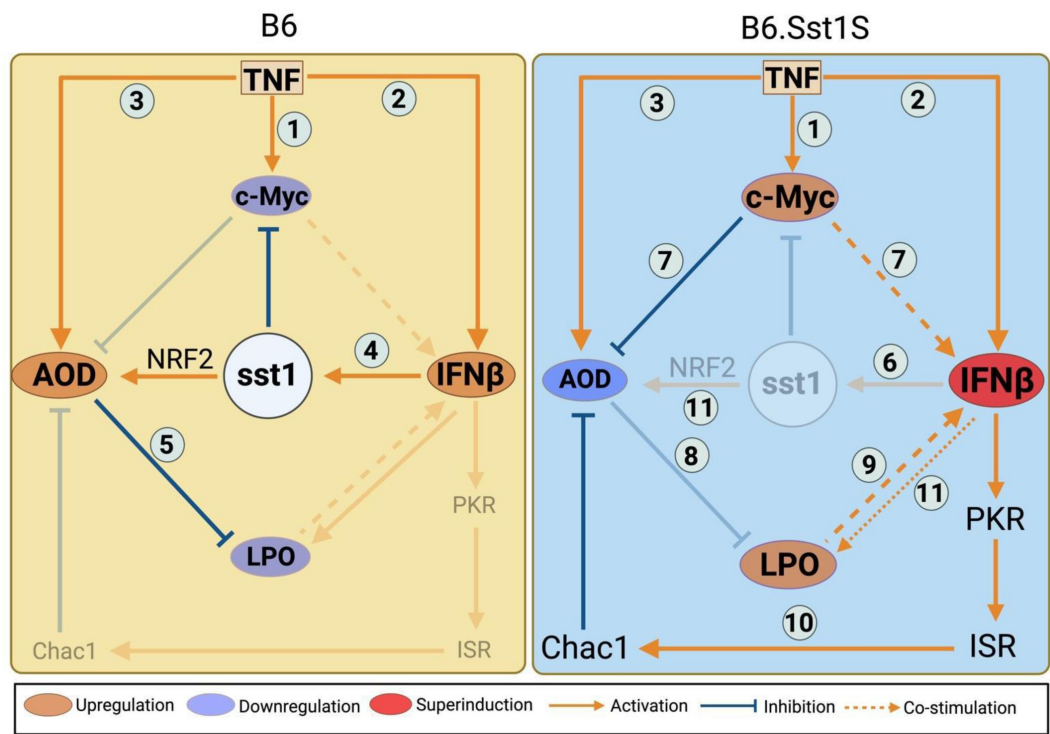
Supplemental Figure 15.

Myc upregulated gene signature in peripheral blood of TB patients is associated with treatment failures.

A. PBMC gene expression profiling of TB patients prior to TB treatment. Boxplot of bootstrapped ssGSEA enrichment AUC scores from several oncogene signatures ranked from lowest to highest area under curve (AUC) score (Y-axis), with *Myc_up* and *Myc_dn* gene sets (X-axis) are highlighted in red. **B C.** Boxplots of myc upregulated (*Myc_up*) and downregulated (*Myc_dn*) gene signatures in successful (control) or failed (failed) TB therapy groups, with the receiver-operating characteristic (ROC) curve of *Myc_up* depicted in C. **D.** Heatmap of all genes utilized in ssGSEA enrichment of the *myc_up* signature are depicted with individual ssGSEA scores for each patient sample. All plots were generated with the TBSignatureProfiler in R and p-values were determined by a t-test.

Supplemental Figure 16

Schematic representation of B6 and B6.Sst1S macrophage responses to TNF.



Supplemental Table 1.

Cell cycle analysis of B6 and B6.Sst1S BMDMS 24 h after TNF stimulation using scRNA-seq.

Sample	G1	S	G2M	G1/S
R	3257	299	507	10.9
S	3073	437	540	7
RT	1877	638	507	2.9
ST	2049	1107	367	1.9

Supplemental Table 2.

Cell cycle analysis of B6 and B6.Sst1S specific BMDM subpopulations 24 h after TNF stimulation using scRNA-seq.

Cluster	G1	S	G2M	G1/S
2	267	573	133	0.5
3	1627	269	41	6
4	943	220	17	4.3
5	718	308	29	2.3

Gene Set Enrichment Analysis

	Gene set	FDR
Overrepresented genesets for comparison B6.Sst1S.TNF vs B6.TNF	SUMOYLATION (Reactome)	0.00271
	SUMOYLATION OF DNA DAMAGE RESPONSE AND REPAIR PROTEINS (Reactome)	0.00277
	TNFA SIGNALING VIA NFKB (MSigDB Hallmark)	0.00293
	SPLICEOSOME (KEGG)	0.00816
	DNA DOUBLE-STRAND BREAK REPAIR (Reactome)	0.01653
	DNA REPAIR (Reactome)	0.03494
	HOMOLOGOUS RECOMBINATION (KEGG)	0.03746
	E2F TARGETS (MSigDB Hallmark)	0.05488
Underrepresented genesets for comparison B6.Sst1S.TNF vs B6.TNF	LYSOSOME (KEGG)	8.26E-05
	OXIDATIVE PHOSPHORYLATION (KEGG)	1.36E-05
	OXIDATIVE PHOSPHORYLATION (MSigDB Hallmark)	6.82E-05
	NONSENSE MEDIATED DECAY (NMD) INDEPENDENT OF THE EXON JUNCTION COMPLEX (EJC) (Reactome)	0.00027
	FATTY ACID METABOLISM (MSigDB Hallmark)	0.00206
	RESPONSE TO ELEVATED PLATELET CYTOSOLIC CA2+ (Reactome)	0.00212
	DETOXIFICATION OF REACTIVE OXYGEN SPECIES (Reactome)	0.00225
	FATTY ACID METABOLISM (KEGG)	0.00290
	FERROPTOSIS (KEGG)	0.00452
	MTORC1 SIGNALING (MSigDB Hallmark)	0.00642
	REACTIVE OXYGEN SPECIES PATHWAY (MSigDB Hallmark)	0.00806
	PEROXISOME (MSigDB Hallmark)	0.01475
	CHOLESTEROL_HOMEOSTASIS (MSigDB Hallmark)	0.03076

Supplemental Table 3.

Gene set enrichment analysis of differentially activated pathways in B6 and B6.Sst1S BMDMs 12 h after TNF stimulation.

Gene set enrichment analysis results for the top enriched gene sets from the KEGG, MSigDB Hallmark, Reactome databases when comparing B6.Sst1S vs B6 BMDMs stimulated with TNF for 12 h. Output includes gene set names and FDR corrected p-values. The analysis was performed using FPKM data and the camera function from the limma R package.

Supplemental Table 4.

Transcription factor binding sites analysis of differentially expressed genes in B6 and B6.Sst1S BMDMs 12 h after TNF stimulation.

The functional profiling and transcription factor binding sites analysis (TFBs) was performed for two sets of genes which represent B6.Sst1S and B6 BMDMs responses to TNF.

Functional pathway profiling

	Enriched pathways	Adjusted p.value	genes
Sst1.S response	TNF signaling pathway (KEGG)	0.0002965	Map3K5, Casp3, Jun, Nfkb1, Mapk8, Edn1, Ripk1, Il6
	Cellular Senescence (Reactome)	0.0040589	Map3K5, Jun, Cbx8, Mapk8, Mapk7, Mdm2, Ets1, Kdm6B
	MyD88-independent TLR4 cascade (Reactome)	0.0065647	Jun, Nfkb1, Mapk8, Tlr4, Ripk1, Mapk7
	Toll Like Receptor 4 (TLR4) Cascade (Reactome)	0.0129301	Jun, Nfkb1, Mapk8, Tlr4, Ripk1, Mapk7
	Oxidative Stress Induced Senescence (Reactome)	0.0247333	Map3K5, Jun, Cbx8, Mapk8, Mdm2, Kdm6B
B6 response	Detoxification of Reactive Oxygen Species (Reactome)	1.48E-15	Gpx1, Txn2, Prdx1, Gpx3, Cat, Txnrd1, Prdx2, Sod2, Sod1, Prdx3, Prdx5, Gsr
	TP53 Regulates Metabolic Genes (Reactome)	3.4733E-05	Akt1, Prdx1, G6Pdx, Txnrd1, Prdx2, Prdx5, Gsr
	Ferroptosis (KEGG)	9.9415E-05	Slc11A2, Gpx4, Slc7A11, Atg7, Prnp, Gclm
	HIF-1 signaling pathway (KEGG)	0.003628	Akt1, Mapk3, Ldha, Egln1, Pdk1, Mapk1, Camk2G
	Peroxisome (KEGG)	0.0054955	Cat, Mpv17, Idh1, Sod2, Sod1, Prdx5

Supplemental Table 5.

The list of identified transcription factors associated with differences between activated genes in response to TNF stimulation in B6 and B6.Sst1S BMDMs.

Transcription factor binding sites analysis

B6.sst1S response			B6.WT response		
Motif ID	Possible TF	NES	Motif ID	Possible TF	NES
transfac_M04823	E2f4	4.46	cisbp_M6156	Bach1	5.72
transfac_M04797	Egr1	4.33	cisbp_M1966	Nfe2l2	5.48
transfac_M04683	Nfya	4.09	factorbook_NFE2	Nfe2	5.46
taipale_TEAD4	Tead4,Cebpd	4.05	jaspar_MA0150.2	Nfe2l2	5.43
transfac_M07390	Nfe2l1	3.96	hocomoco_BACH1	Bach1	5.23
transfac_M02743	E2f3	3.94	taipale_ELK1	Elk1	4.52
cisbp_M2272	E2f4	3.91	jaspar_MA0501.1	Nfe2	4.48
factorbook_NFY	Nfyb,Nfya	3.90	transfac_M02022	Mafk	4.32
cisbp_M4473	Pbx3	3.87	cisbp_4629	Nfe2	4.28
cisbp_M4504	Egr1	3.75	transfac_M07374	Bach1	4.12

Master regulator analysis

TFs	t	p.value
Nfe2l1	12.34	0.000248
Atf5	9.49	0.000688
E2f3	4.82	0.00855
E2f6	-10.51	0.000463
Zfp523	-10.76	0.000423
Zfp623	-12.46	0.000239
Zkscan8	-13.39	0.00018
Zfp568	-16.23	8.44E-05
Zfp251	-58.59	5.08E-07

Supplemental Table 6.

Master regulator analysis of the transcription factors associated with differences between activated genes in response to TNF stimulation in B6 and B6.Sst1S BMDMs.

Master regulator analysis was performed using Virtual Inference of Protein Activity by Enriched Regulon Analysis (VIPER) algorithm, the mice macrophage gene regulatory network and log2 transformed FPKM values of genes from gene ontology category GO: 0006979. The table shows the identified list of transcription factors associated with differences between activated genes in response to TNF stimulation in B6.Sst1S vs B6 BMDMs.

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Additional information

Author contributions

Conceptualization: IK, LK, SMY, WEJ, WRB; Methodology: SMY, VZ, PBA, SL, LK, OSR, AAG, YA, JDC, NAC, NCL, SL, WEJ; Validation and formal analysis: SMY, SAA, PBA, VZ, OSR, AAG, BNK, LK, NCL, QM, GD, AV; Investigation: SMY, PBA, SL, GD, AV; Resources: IK, LK, AAG, BK, NCL, JDC, NAC, WEJ; Writing – original draft: SMY, PBA, IK, LK; Writing – review & editing: IK, LK, WRB, WEJ; Supervision: IK; Funding acquisition: IK, LK, AAG, BNK, NCL, WRB, WEJ.

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Additional files

Key Resources Table 

Supplemental Table 7. 

Supplemental Table 8. 

Supplemental Table 9. 

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Reviewer #1 (Public review):

Summary:

In this report, Yabaji et al describe studies designed to address the mechanism behind the TB susceptibility gene *sst1*. This locus is known to affect expression of IFN and synergizes with Myc to potentiate infectivity. Using a variety of molecular expression and imaging techniques, the authors demonstrate that mice harboring an *sst1* transgene (compared to B6 controls) are highly susceptible to TB infection via a mechanism involving loss of antioxidant defense systems, the down regulation of key antioxidant genes and ferritin controlling intracellular iron levels. The combination of increased iron plus decreased antioxidant defense systems in turn increases lipid peroxidation and downstream sequelae. Inhibition of peroxidation diminishes infectivity increases ferritin levels. Furthermore, the authors demonstrate that Myc activation potentiates this process and that down regulation of NRF2 antioxidant defenses accompany potentiated infectivity. Increased peroxidation products (4-HNE) may activate the ASK1/JNK system leading to IFN γ superinduction and diminished macrophage viability thereby diminishing ability to withstand TB infection. Extending these findings, additional mouse models plus some work in humans supports the peroxidation hypothesis.

Overall, the work is significant for it introduces a molecular basis for TB infectivity and presents a potential novel therapeutic opportunity.

Strengths:

- (1) Strengths of this study include a multi-omic analysis of infectivity combining gene expression analysis with biochemical and cell biological evaluation.
- (2) Novel identification of an iron-catalyzed lipid peroxidation based mechanism for why the *sst1* locus is linked to TB infection.
- (3) Parallels to human biology are included via analysis of Myc upregulation in peripheral blood from patients.
- (4) Appropriate statistical analysis

Weaknesses:

- (1) Lipid peroxidation is a broad phenotype process and the authors honed in on 4-HNE dependent processes as a likely mechanism because they can measure 4-HNE conjugated proteins. However, lipid peroxidation is a complex phenomenon and the work presented herein is largely descriptive.
- (2) The authors continually refer to increased 4HNE while they do not measure this 9 carbon lipid, they actually measure 4-HNE conjugated proteins immunochemically.
- (3) The authors do not distinguish between increased protein-HNE adducts and increased membrane peroxidation (or both) as mechanistically linked to infectivity.

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Author response:

General Statements

We are grateful for constructive reviewers' comments and criticisms and have thoroughly addressed all major and minor comments in the revised manuscript.

Summary of new data.

We have performed the following additional experiments to support our concept:

- (1) The kinetics of ROS production in B6 and B6.Sst1S macrophages after TNF stimulation (Fig. 3I and J, Suppl. Fig. 3G);
- (2) Time course of stress kinase activation (Fig. 3K) that clearly demonstrated the persistent stress kinase (phospho-ASK1 and phospho-cJUN) activation exclusively in the B6.Sst1S macrophages;
- (3) New Fig. 4 C-E panels include comparisons of the B6 and B6.Sst1S macrophage responses to TNF and effects of IFNAR1 blockade in both backgrounds.
- (4) We performed new experiments demonstrating that the synthesis of lipid peroxidation products (LPO) occurs in TNF-stimulated macrophages earlier than the IFN β super-induction (Suppl. Fig. 4A and B).
- (5) We demonstrated that the IFNAR1 blockade 12, 24 and 32 h after TNF stimulation still reduced the accumulation of LPO product (4-HNE) in TNF-stimulated B6.Sst1S BMDMs (Suppl. Fig. 4 E-G).

(6) We added comparison of cMyc expression between the wild type B6 and B6.Sst1S BMDMs during TNF stimulation for 6-24 h (Fig.5I-J).

(7) New data comparing 4-HNE levels in Mtb-infected B6 wild type and B6.Sst1S macrophages and quantification of replicating Mtb was added (Fig.6B, Suppl.Fig.7C and D).

(8) In vivo data described in Fig.7 was thoroughly revised and new data was included. We demonstrated increased 4-HNE loads in multibacillary lesions (Fig.7A, Suppl. Fig.9A) and the 4-HNE accumulation in CD11b+ myeloid cells (Fig.7B and Suppl.Fig.9B). We demonstrated that the *Ifnb* – expressing cells are activated iNOS+ macrophages (Fig.7D and Suppl.Fig.13A). Using new fluorescent multiplex IHC, we have shown that stress markers phospho-cJun and Chac1 in TB lesions are expressed by *Ifnb*- and iNOS-expressing macrophages (Fig.7E and Suppl.Fig.13D-F).

(9) We performed additional experiment to demonstrate that naïve (non-BCG vaccinated) lymphocytes did not improve Mtb control by Mtb-infected macrophages in agreement with previously published data (Suppl.Fig.7H).

Summary of updates

Following reviewers requests we updated figures to include isotype control antibodies, effects of inhibitors on non-stimulated cells, positive and negative controls for labile iron pool, additional images of 4-HNE and live/dead cell staining.

Isotype control for IFNAR1 blockade were included in Fig.3M, Fig.4C -E, Fig.6L-M Suppl.Fig.4F-G, 7I.

Positive and negative controls for labile iron pool measurements were added to Fig.3E, Fig.5D, Suppl.Fig.3B

Cell death staining images were added Suppl.Fig.3H

Co-staining of 4-HNE with tubulin was added to Suppl.Fig.3A.

High magnification images for Figure 7 were added in Suppl.Fig.8 to demonstrate paucibacillary and multibacillary image classification.

Single-channel color images for individual markers were provided in Fig.7E and Suppl.Fig.13B-F.

Inhibitor effects on non-stimulated cells were included in Fig.5 D-H, Suppl.Fig.6A and B. Titration of CSF1R inhibitors for non-toxic concentration determination are included in Suppl.Fig.6D.

In addition, we updated the figure legends in the revised manuscript to include more details about the experiments. We also clarified our conclusions in the Discussion. Responses to every major and minor comment of the reviewers are provided below.

Point-by-point description of the revisions

Reviewer #1 (Evidence, reproducibility and clarity:

Summary

The study by Yabaji et al. examines macrophage phenotypes B6.Sst1S mice, a mouse strain with increased susceptibility to M. tuberculosis infection that develops necrotic lung lesions. Extending previous work, the authors specifically focus on delineating the molecular mechanisms driving aberrant oxidative stress in TNF-activated B6.Sst1S

macrophages that has been associated with impaired control of *M. tuberculosis*. The authors use scRNAseq of bone marrow-derived macrophages to further characterize distinctions between B6.Sst1S and control macrophages and ascribe distinct trajectories upon TNF stimulation. Combined with results using inhibitory antibodies and small molecule inhibitors in *in vitro* experimentation, the authors propose that TNF-induced protracted c-Myc expression in B6.Sst1S macrophages disables the cellular defense against oxidative stress, which promotes intracellular accumulation of lipid peroxidation products, fueled at least in part by overexpression of type I IFNs by these cells. Using lung tissue sections from *M. tuberculosis*-infected B6.Sst1S mice, the authors suggest that the presence of a greater number of cells with lipid peroxidation products in lung lesions with high counts of stained *M. tuberculosis* are indicative of progressive loss of host control due to the TNF-induced dysregulation of macrophage responses to oxidative stress. In patients with active tuberculosis disease, the authors suggest that peripheral blood gene expression indicative of increased Myc activity was associated with treatment failure.

Major comments

The authors describe differences in protein expression, phosphorylation or binding when referring to Fig 2A-C, 2G, 3D, 5B, 5C. However, such differences are not easily apparent or very subtle and, in some cases, confounded by differences in resting cells (e.g. pASK1 Fig 3L; c-Myc Fig 5B) as well as analyses across separate gels/blots (e.g. Fig 3K, Fig 5B). Quantitative analyses across different independent experiments with adequate statistical analyses are required to strengthen the associated conclusions.

We updated our Western blots as follows:

(1) Densitometry of normalized bands is included above each lane (Fig.2A-C; Fig.3C-D and 3K; Fig.4A-B; Fig.5B,C,I,J). New data in Fig.3K is added to highlight differences between B6 and B6.Sst1S at individual timepoints after TNF stimulation. In Fig.5I we added new data comparing Myc levels in B6 and B6.Sst1S with and without JNK inhibitor and updated the results accordingly. New Fig.3K clearly demonstrates the persistent activation of p-cJun and pAsk1 at 24 and 36h of TNF stimulation. In Fig.5B we clearly demonstrate that Myc levels were higher in B6.Sst1S after 12 h of TNF stimulation. At 6h, however, the basal differences in Myc levels are consistently higher in B6.Sst1S and the induction by TNF is 1.6-fold similar in both backgrounds. We noted this in the text.

(2) A representative experiment is shown in individual panels and the corresponding figure legend contains information on number of biological repeats. Each Western blot was repeated 2 – 4 times.

The representative images of fluorescence microscopy in Fig 3H, 4H, 5H, S3C, S3I, S5A, S6A seem to suggest that under some conditions the fluorescence signal is located just around the nucleus rather than absent or diminished from the cytoplasm. It is unclear whether this reflects selective translocation of targets across the cell, morphological changes of macrophages in culture in response to the various treatments, or variations in focal point at which images were acquired. Control images (e.g. cellular actin, DIC) should be included for clarification. If cell morphology changes depending on treatments, how was this accounted for in the quantitative analyses? In addition, negative controls validating specificity of fluorescence signals would be warranted.

Our conclusion of higher LPO production is based on several parameters: 4-HNE staining, measurements of MDA in cell lysates and oxidized lipids using BODIPY C11. Taken together they demonstrate significant and reproducible increase in LPO accumulation in TNFstimulated B6.Sst1S macrophages. This excludes imaging artefact related to unequal 4-

HNE distribution noted by the reviewer. In fact, we also noted that the 4-HNE was spread within cell body of B6.Sst1S macrophages and confirmed it using co-staining with tubulin, as suggested by the reviewer (new Suppl.Fig.3A). Since low molecular weight LPO products, such as MDA and 4-HNE, traverse cell membranes, it is unlikely that they will be strictly localized to a specific membrane bound compartment. However, we agree that at lower concentrations, there might be some restricted localization, explaining a visible perinuclear ring of 4-HNE staining in B6 macrophages. This phenomenon may be explained just by thicker cytoplasm surrounding nucleus in activated macrophages spread on adherent plastic surface or by proximity to specific organelles involved in generation or clearance of LPO products and definitively warrants further investigation.

We also included images of non-stimulated cells in Fig.3H, Suppl.Fig.3A and 3E. We used multiple fields for imaging and quantified fluorescence signals (Suppl. Fig.3D and 3F, Suppl.Fig.4G, Suppl.Fig.6A and B).

We used negative controls without primary antibodies for the initial staining optimization, but did not include it in every experiment.

To interpret the evaluation on the hierarchy of molecular mechanisms in B6.Sst1S macrophages, comparative analyses with B6 control cells should be included (e.g. Fig 4C-I, Fig 5, Fig 6B, E-M, S6C, S6E-F). This will provide weight to the conclusions that the dysregulated processes are specifically associated with the susceptibility of B6.Sst1S macrophages.

Understanding the *sst1*-mediated effects on macrophage activation is the focus of our previously published studies (Bhattacharya et al., JCI, 2021) and this manuscript. The data comparing B6 and B6.Sst1S macrophage are presented in Fig.1, Fig.2, Fig.3, Fig.4, Fig.5A-C, I and J, Fig.6A-C, 6J and corresponding supplemental figures 1, 2, 3, 4A and B, Suppl.Fig.5, Suppl.Fig.6C, Suppl.Fig.7A-D,7F.

Once we identified the aberrantly activated pathways in the B6.Sst1S, we used specific inhibitors to correct the aberrant response in B6.Sst1S.

All experiments using inhibitory antibodies require comparison to the effect of a matched isotype control in the same experiment (e.g. Fig 3J, 4F, G, I; 6L, 6M, S3G, S6F).

Isotype control for IFNAR1 blockade were included in Fig.3M, Fig.4C-E, Fig.6L-M Suppl.Fig.4F-G, 7I.

Experiments using inhibitors require inclusion of an inhibitor-only control to assess inhibitor effects on unstimulated cells (e.g. Fig 4I, 5D-I)

Inhibitor effects on non-stimulated cells were included in Fig.5 D-H, Suppl.Fig.6A and B.

Fig 3K and Fig 5J appear to contain the same images for p-c-Jun and b-tubulin blots.

Fig.3K and 5J partially overlapped but had different focus – 3K has been updated to reflect the time course of stress kinase activation. Fig.5J is updated (currently Fig.5I and J) to display B6 and B6.Sst1S macrophage data including cMyc and p-cJun levels.

Data of TNF-treated cells in Fig 3I appear to be replotted in Fig 3J.

Currently these data is presented in Fig.3L and 3M and has been updated to include comparison of B6 and B6.Sst1S cells (Fig.3L) and effects of inhibitors in Fig.3M.

*It is stated that lungs from 2 mice with paucibacillary and 2 mice with multi-bacillary lesions were analysed. There is contradicting information on whether these tissues were collected at the same time post infection (week 14?) or whether the pauci-bacillary lesions were in lungs collected at earlier time points post infection (see Fig S8A). If the former, how do the authors conclude that multi-bacillary lesions are a progression from paucibacillary lesions and indicative of loss of *M. tuberculosis* control, especially if only one lesion type is observed in an individual host? If the latter, comparison between lesions will likely be dominated by temporal differences in the immune response to infection.*

In either case, it is relevant to consider density, location, and cellular composition of lesions (see also comments on GeoMx spatial profiling). Is the macrophage number/density per tissue area comparable between pauci-bacillary and multi-bacillary lesions?

We did not collect lungs at the same time point. As described in greater detail in our preprints (Yabaji et al., <https://doi.org/10.1101/2025.02.28.640830> and <https://doi.org/10.1101/2023.10.17.562695>) pulmonary TB lesions in our model of slow TB progression are heterogeneous between the animals at the same timepoint, as observed in human TB patients and other chronic TB animal models. Therefore, we perform analyses of individual TB lesions that are classified by a certified veterinary pathologist in a blinded manner based on their morphology (H&E) and acid fast staining of the bacteria, as depicted in Suppl.Fig.8. Currently it is impossible to monitor progression of individual lesions in mice. However, in mice TB is progressive disease and no healing and recovery from the disease have been observed in our studies or reported in literature. Therefore, we assumed that paucibacillary lesions preceded the multibacillary ones, and not vice versa, thus reflecting the disease progression. In our opinion, this conclusion most likely reflects the natural course of the disease. However, we edited the text : instead of disease progression we refer to paucibacillary and multibacillary lesions.

Does 4HNE staining align with macrophages and if so, is it elevated compared to control mice and driven by TNF in the susceptible vs more resistant mice?

We performed additional staining and analyses to demonstrate the 4-HNE accumulation in CD11b+ myeloid cells of macrophage morphology. Non-necrotic lesions contain negligible proportion of neutrophils (Fig.7B, Suppl.Fig.9B). B6 mice do not develop advanced multibacillary TB lesions containing 4-HNE+ cells. Also, 4-HNE staining was localized to TB lesions and was not found in uninvolved lung areas of the infected mice, as shown in Suppl.Fig.9A (left panel).

It is well established that TNF plays a central role in the formation and maintenance of TB granulomas in humans and in all animal models. Therefore, TNF neutralization would lead to rapid TB progression, rapid *Mtb* growth and lesions destruction in both B6 and B6.Sst1S genetic backgrounds.

Pathway analysis of spatial transcriptomic data (Suppl.Fig.11) identified TNF signaling via NFkB among dominant pathways upregulated in multibacillary lesions, suggesting that the 4-HNE accumulation paralleled increased TNF signaling. In addition, in vivo other cytokines, including IFN-I, could activate macrophages and stimulate production of reactive oxygen and nitrogen species and lead to the accumulation of LPO products as shown in this manuscript.

It would be relevant to state how many independent lesions per host were sampled in both the multiplex IHC as well as the GeoMx data. Can the authors show the selected regions of interest in the tissue overview and in the analyses to appreciate within-host

and across-host heterogeneity of lesions. The nature of the spatial transcriptomics platform used is such that the data are derived from tissue areas that contain more than just Iba1+ macrophages. At later stages of infection, the cellular composition of such macrophage-rich areas will be different when compared to lesions earlier in the infection process. Hence, gene expression profiles and differences between tissue regions cannot be attributed to macrophages in this tissue region but are more likely a reflection of a mix of cellular composition and per-cell gene expression.

We used Iba1 staining to identify macrophages in TB lesions and programmed GeoMx instrument to collect spatial transcriptomics probes from Iba1+ cells within ROIs. Also, we selected regions of interest (ROI) avoiding necrotic areas (depicted in Suppl.Fig.10). We agree that Iba1+ macrophage population is heterogeneous – some Iba1+ cells are activated iNOS+ macrophages, other are iNOS-negative (Fig.7C and D, and Suppl.Fig.13A). Multibacillary lesions contain larger areas occupied by activated (iNOS+) macrophages (Fig.7D,

Suppl.Fig.13B and 13F). Although the GeoMx spatial transcriptomic platform does not provide single cell resolution, it allowed us to compare populations of Iba1+ cells in paucibacillary and multibacillary TB lesions and to identify a shift in their overall activation pattern.

It is stated that loss of control of M. tuberculosis in multibacillary lesions was associated with "downregulation of IFNγ-inducible genes". If the authors base this on the tissue expression of individual genes, this requires further investigation to support such conclusion (also see comment on GeoMx above). Furthermore, how might this conclusion be compatible with significantly elevated iNOS+ cells (Fig 7D) in multibacillary lesions?

We demonstrated that *Ciita* gene expression is specifically induced by IFN-γ and is suppressed by IFN-I (Fig.6M). The expression of *Ciita* in paucibacillary lesions suggest the presence of the IFN-γ activated cells and its disappearance in the multibacillary lesion is consistent with massive activation of IFN-I pathway (Fig.7C).

It is appreciated that the human blood signature analyses contain Myc-signatures but the association with treatment failure is not very strong based on the data in Fig 13B and C (Suppl.Fig.15B and C now). The authors indicate that they have no information on disease severity, but it should perhaps not be assumed that treatment failure is indicative of poor host control of the infection. Perhaps independent analyses in separate cohort/data set can add strength and provide -additional insights (e.g. PMID: 35841871; PMID: 32451443, PMID: 17205474, PMID: 22872737). In addition, the human data analyses could be strengthened by extension to additional signatures such as IFN, TNF, oxidative stress. Details of the human study design are not very clear and are lacking patient demographics, site of disease, time of blood collection relative to treatment onset, approving ethics committees.

X axis of Suppl.Fig.15A represent pre-defined molecular signature gene sets (MSigDB) in Gene Set Enrichment Analysis (GSEA) database (<https://www.gseamsigdb.org/gsea/msigdb>). On Y axis is area under curve (AUC) score for each gene set. The Myc upregulated gene set *myc_up* was identified among top gene sets associated with treatment failure using unbiased ssGSEA algorithm. The upregulation of Myc pathway in the blood transcriptome associated with TB treatment failure most likely reflects greater proportion of immature cells in peripheral blood, possibly due to increased myelopoiesis.

Pathway analysis of the differentially expressed genes revealed that treatment failures were associated with the following pathways relevant to this study: NF-κB Signaling, Flt3 Signaling in Hematopoietic Progenitor Cells (indicative of common myeloid progenitor cell proliferation), SAPK/JNK Signaling and Senescence (indicative of oxidative stress). The upregulation of these pathways in human patients with poor TB treatment outcomes

correlates with our findings in TB susceptible mice. The detailed analysis of differentially regulated pathways in human TB patients is beyond the scope of this study and is presented in another manuscript entitled “Tuberculosis risk signatures and differential gene expression predict individuals who fail treatment” by Arthur VanValkenburg et al., submitted for publication.

Blood collection for PBMC gene expression profiling of TB patients was prior to TB treatment or within a first week of treatment commencement. Boxplot of bootstrapped ssGSEA enrichment AUC scores from several oncogene signatures ranked from lowest to highest AUC score, with myc_up and myc_dn genes highlighted in red.

We agree with the reviewer that not every gene in the myc_up gene set correlates with the treatment outcome. But the association of the gene set is statistically significant, as presented in Suppl.Fig.15B – C.

We updated the details of the study, including study sites and the ethics committee approval statement and references describing these cohorts.

Other comments

It is excellent that the authors provide individual data points. Choosing a colour other than black would increase clarity when black bars are used.

We followed this useful suggestion and selected consistent color codes for B6 and B6.Sst1S groups to enhance clarity throughout the revised manuscript.

Error bars are inconsistently depicted as either bi-directional or just unidirectional.

We used bi-directional error bars in the revised manuscript.

Fig 1E, G, H - please include a scale to clarify what the heat map is representing.

We have included the expression key in Fig.1E,G and H and Suppl.Fig.1C and D in the revised version.

Fig 2K, Fig S10A gene information cannot be deciphered.

We increased the font in previous Fig.2K and moved to supplement to keep larger fonts (current Suppl.Fig.2G).

Fig S4A,B please add error bars.

These data are presented as Suppl.Fig.5 in the revised version. We performed one experiment to test the hypothesis. Because the data indicated no clear increase in transposon small RNAs in the sst1S macrophages, we did not pursue this hypothesis further, and therefore, the error bars were not included. However, we decided to include these negative data because it rejects a very attractive and plausible hypothesis.

Please use gene names as per convention (e.g. Ifnb1) to distinguish gene expression from protein expression in figures and text.

We addressed the comment in the revised manuscript.

Fig S8B. Contrary to the description of results, there seems to be minimal overlap between the signal for YFP and the Ifnb1 probe. Is the Ifnb1 reporter mouse a legacy

reporter? If so, it is worth stating this and including such considerations in the data interpretation.

The YFP reporter expresses YFP protein under the control of the *Ifnb1* promoter. The YFP protein accumulates within the cells and while *Ifnb* protein is rapidly secreted and does not accumulate in the producing cells in appreciable amounts. So YFP is not a lineage tracing reporter, but its accumulation marks the *Ifnb1* promoter activity in cells, although the YFP protein half-life is longer than that of the *Ifnb1* mRNA that is rapidly degraded (Witt et al., BioRxiv, 2024; doi:10.1101/2024.08.28.61018). Therefore, there is no precise spatiotemporal coincidence of these readouts.

Please clarify what is meant by "normal interstitium" ? If the tissue is from uninfected mice, please state clearly.

In this context we refer to the uninvolved lung areas of the infected lungs. In every sample we compare uninvolved lung areas and TB lesions of the same animal. Also, we performed staining of lung of non-infected mice as additional controls.

If macrophage cultures underwent media changes every 48h, how was loss of liberated Mtb taken into account especially if differences in cell density/survival were noted? The assessment of M. tuberculosis load by qPCR is not well described. In particular, the method of normalization applied within the experiments (not within the qPCR) here remains unclear, even with reference to the authors' prior publication.

Our lab has many years of experience working with macrophage monolayers infected with virulent Mtb and uses optimized protocols to avoid cell losses and related artifacts. Recently we published a detailed protocol for this methodology in STAR Protocols (Yabaji et al., 2022; PMID 35310069). In brief, it includes preparation of single cell suspensions of Mtb by filtration to remove clumps, use of low multiplicity of infection, preparation of healthy confluent monolayers and use of nutrient rich culture medium and medium change every 2 days. We also rigorously control for cell loss using whole well imaging and quantification of cell numbers and live/dead staining.

Please add citation for the limma package.

The references has been added (Ritchie et al, NAR 2015; PMID 25605792).

The description of methodology relating to the "oncogene signatures" is unclear.

This signature was described in Bild et al, Nature, 2006 and McQuerry JA, et al, 2019 "Pathway activity profiling of growth factor receptor network and stemness pathways differentiates metaplastic breast cancer histological subtypes". BMC Cancer 19: 881 and is cited in Methods section Oncogene signatures

Please clearly state time points post infection for mouse analyses.

We collected lung samples from Mtb infected mice 12 – 20 weeks post infection. The lesions were heterogeneous and were individually classified using criteria described above.

Reference is made to "a list of genes unique to type I [interferon] genes [...]" (p29). Can the authors indicate the source of the information used for compiling this list?

The lists were compiled from Reactome, EMBL's European Bioinformatics Institute and GSEA databases. The links for all datasets are provided in Suppl.Table 8 "Expression of IFN

pathway genes in Iba1+ cells from pauci- and multi-bacillary lesions of Mtb infected B6.Sst1S mouse lungs” in the “Pool IFN I & II gene sets” worksheet.

The discussion at present is very long, contains repetition of results and meanders on occasion.

Thank you for this suggestion, We critically revised the text for brevity and clarity.

Reviewer #1 (Significance):

Strengths and limitations

Strengths: multi-pronged analysis approaches for delineating molecular mechanisms of macrophage responses that might underpin susceptibility to M. tuberculosis infection; integration of mouse tissues and human blood samples

Weaknesses: not all conclusions supported by data presented; some concerns related to experimental design and controls; links between findings in human cohort and the mechanistic insights gained in mouse macrophage model uncertain

The revised manuscript addresses every major and minor comment of the reviewers, including isotype controls and naïve T cells, to provide additional support for our conclusions. Our study revealed causal links between Myc hyperactivity with the deficiency of anti-oxidant defense and type I interferon pathway hyperactivity. We have shown that Myc hyperactivity in TNF-stimulated macrophages compromises antioxidant defense leading to autocatalytic lipid peroxidation and interferon-beta superinduction that in turn amplifies lipid peroxidation, thus, forming a vicious cycle of destructive chronic inflammation. This mechanism offers a plausible mechanistic explanation of for the association of Myc hyperactivity with poorer treatment outcomes in TB patients and provide a novel target for host-directed TB therapy.

Advance

The study has the potential to advance molecular understanding of the TNF-driven state of oxidative stress previously observed in B6.Sst1S macrophages and possible implications for host control of M. tuberculosis in vivo.

Audience

Experts seeking understanding of host factors mediating M. tuberculosis control, or failure thereof, with appreciation for the utility of the featured mouse model in assessing TB diseases progression and severe manifestation. Interest is likely extended to audience more broadly interested in TNF-driven macrophage (dys)function in infectious, inflammatory, and autoimmune pathologies.

Reviewer expertise

In preparing this review, I am drawing on my expertise in assessing macrophage responses and host defense mechanisms in bacterial infections (incl. virulent M. tuberculosis) through in vitro and in vivo studies. This includes but is not limited to macrophage infection and stimulation assays, microscopy, intra-macrophage replication of M. tuberculosis, analyses of lung tissues using multi-plex IHC and spatial transcriptomics (e.g. GeoMx). I am familiar with the interpretation of RNAseq analyses in human and mouse cells/tissues, but can provide only limited assessment of appropriateness of algorithms and analysis frameworks.

Reviewer #2 (Evidence, reproducibility and clarity):

Yabaji et al. investigated the effects of BMDMs stimulated with TNF from both WT and B6.Sst1S mice, which have previously been identified to contain the sst1 locus conferring susceptibility to Mycobacterium tuberculosis. They identified that B6.Sst1S macrophages show a superinduction of IFN β , which might be caused by increased c-Myc expression, expanding on the mechanistic insights made by the same group (Bhattacharya et al. 2021). Furthermore, prolonged TNF stimulation led to oxidative stress, which WT BMDMs could compensate for by the activation of the antioxidant defense via NRF2. On the other hand, B6.Sst1S BMDMs lack the expression of SP110 and SP140, co-activators of NRF2, and were therefore subjected to maintained oxidative stress. Yabaji et al. could link those findings to in vivo studies by correlating the presence of stressed and aberrantly activated macrophages within granulomas to the failure of Mtb control, as well as the progression towards necrosis. As the knowledge regarding Mtb progression and necrosis of granulomas is not yet well understood, findings that might help provide novel therapy options for TB are crucial. Overall, the manuscript has interesting findings with regard to macrophage responses in Mycobacteria tuberculosis infection.

However, in its current form there are several shortcomings, both with respect to the precision of the experiments and conclusions drawn.

In particular a) important controls are often missing, e.g. T-cells from non-immune mice in Fig. 6J, in F, effectivity of BCG in B6 mice in 6N; b) single experiments are shown throughout the manuscript, in particular western blots and histology without proper quantification and statistics, this is absolutely not acceptable; c) very few repetitions are shown in in vitro experiments, where there is no evidence for limitation in resources (usually not more than 3), it is not clear what "independent experiment means" - i.e. the robustness of the findings is questionable; d) data are often normalized multiple times, e.g. in the case of qPCR, and the methods of normalization are not clear (what house-keeping gene exactly?);

Moreover, experiments regarding IFN I signaling (e.g. short term TNF treatment of BMDMs to analyze LPO, making sure that the reporter mouse for IFN β works in vivo) and c-Myc (e.g. the increase after M-CSF addition might impact on other analysis as well and the experiments should be adjusted to control for this effect; MYC expression in the human samples) should be carefully repeated and evaluated to draw correct conclusions.

In addition, we would like to strongly encourage the authors to more precisely outline the experimental set-ups and figure legends, so that the reader can easily understand and follow them. In other words: The legends are - in part very - incomplete. In addition, the authors should be mindful of gene names vs. protein names and italicize where appropriate.

We appreciate a very thorough evaluation of our manuscript by this reviewer. Their insightful comments helped us improve the manuscript. As outlined below in point-by-point responses (1) we added important controls including isotype control antibodies in IFNAR blocking experiments and non-vaccinated T cells in T cell – macrophage interactions experiments; updated figure legends to indicate number of repeated experiment where a representative experiment is shown, numbers of mouse lungs and individual lesions, methods of data normalization, where it was missing. We also explained our in vitro experimental design and how we analyzed and excluded effects of media change and fresh CSF1 addition, by using a rest period before TNF stimulation and Mtb infection. The data shown in Suppl. Fig. 6C (previously Suppl. Fig. 5B) demonstrate that Myc levels induced by CSF1 return to the basal level at 12 h after media change. Our detailed in vitro protocol that

contains these details has been published (Yabaji et al., STAR Protocols, 2022). We added new data demonstrating the ROS and LPO production at 6h of TNF stimulation, while the *Ifnb1* mRNA super-induction occurred at 16 – 18 h, and edited the text to highlight these dynamics. The upregulation of Myc pathway in human samples does not necessarily mean the upregulation of Myc itself, it could be due to the dysregulation of downstream pathways. The upregulation of Myc pathway in the blood transcriptome associated with TB treatment failure most likely reflects greater proportion of immature cells in peripheral blood, possibly due to increased myelopoiesis. The detailed analysis of this cell populations in human patients is suggested by our findings but it is beyond the scope of this study.

The reviewer's comments also suggested that a summary of our findings was necessary. The main focus of our study was to untangle connections between oxidative stress and *Ifnb1* superinduction. It revealed that Myc hyperactivity caused partial deficiency of antioxidant defense leading to type I interferon pathway hyperactivity that in turn amplifies lipid peroxidation, thus establishing a vicious cycle driving inflammatory tissue damage.

Our laboratory worked on mechanisms of TB granuloma necrosis over more than two decades using genetic, molecular and immunological analyses in vitro and in vivo. It provided mechanistic basis for independent studies in other laboratories using our mouse model and further expanding our findings, thus supporting the reproducibility and robustness of our results and our lab's expertise.

Specific comments to the experiments and data:

- Fig. 1E: Evaluation of differences in up- and downregulation between B6 and B6.Sst1S cells should highlight where these cells are within the heatmap, as it is only labelled with the clusters, or it should be depicted differently (in particular for cluster 1 and 2). Furthermore, a more simple labelling of the pathways would increase the readability of the data.

For our scRNAseq data presentation, we used formats accepted by computational community. To clarify Fig.1E, we added labels above B6 and B6.Sst1S-specific clusters.

- Fig. 2D, E: The staining legend is missing. For the quantification it is not clear what % total means. Is this based on the intensity or area? What do the dots represent in the bar chart? Is one data point pooled from several pictures? If not, the experiments need to be repeated, as three pictures might not be representative for evaluation.

- Fig. 2E: Statistics comparing B6/ B6,Sst1S with TNF (different) is required: Absence of induction is not a proof for a difference!

We included staining with NRF2-specific antibodies and performed area quantification per field using ImageJ to calculate the NRF2 total signal intensity per field. Each dot in the graph represents the average intensity of 3 fields in a representative experiment. The experiment was repeated 3 times. We included pairwise comparison of TNF-stimulated B6 and B6.Sst1S macrophages and updated the figure legend.

- Fig. 3E: Positive and negative control need to be depicted in the figure (see legend).

We have added the positive and negative controls for the determination of labile iron pool to the data in Fig. 3E and related Suppl. Fig. 3B and to Fig. 5D that also demonstrates labile iron determination.

- Fig. 3I: A quantification by flow cytometry or total cell counts are important, as 6% cell death in cell culture is a very modest observation. Otherwise, confocal images of the

quantification would be a good addition to judge the specificity of the viability staining.

To validate the specificity of the viability staining method, we have provided fluorescent images as Suppl.Fig.3H. The main point of this experiment was to demonstrate a modest, but reproducible, increase in cell death in the *sst1*-mutant macrophages that suggested an IFN-dependent oxidative damage. In our study, we did not focus on mechanisms of cell death, but on a state of chronic oxidative stress in the *sst1* mutant live cells during TNF stimulation.

- Fig. 3I, J: What does one dot represent?

We performed this assay in 96 well format and each dot represent the % cell death in an individual well.

- Fig. 3K,L: For the B6 BMDMs it seems that p-cJun is highly increased at 12h in (L), while it is not in (K). On the other hand, for the B6.Sst1S BMDMs it peaks at 24h in (K), while in (L) it seems to at 12h. According to the data in (L) it seems that p-cJun is rather earlier and stronger activated in B6 BMDMs and has a weakened but prolonged activation in the B6.Sst1S BMDMs, which would not fit with your statement in the text that B6.Sst1S BMDMs show an upregulation.

These experiments need repetitions and quantification and statistics.

Fig. 3L: ASK1 seems to be higher at 12h for the B6 BMDMs and similar for both lines at 24h, which is not fitting to the statement in the text. ("Also, the ASK1 - JNK - cJun stress kinase axis was upregulated in B6.Sst1S macrophages, as compared to B6, after 12 - 36 h of TNF stimulation")

These experiments were repeated, and new data were added to highlight differences in ASK1 and c-Jun phosphorylation between B6 and B6.Sst1S at individual timepoints after TNF stimulation (presented in new Fig.3K). It demonstrated that after TNF stimulation the activation of stress kinases ASK1 and c-Jun initially increased in both genetic backgrounds. However, their upregulation was maintained exclusively in the *sst1*-susceptible macrophages from 24 to 36 h of TNF stimulation, while in the resistant macrophages their upregulation was transient. Thus, during prolonged TNF stimulation, B6.Sst1S macrophages experience stress that cannot be resolved, as evidenced by this kinetic analysis. The quantification of the band intensity was added to Western blot images above individual lanes.

Reviewer 2 pointed to missing isotype control antibodies in Fig.3 and Fig.4:

- Figure 3J: the isotype control for the IFNAR antibody is missing

- Figure 4E: It seems the isotype control itself has already an effect in the reduction of IFN γ .

- Fig. 4H: It seems that the Isotype control antibody had an effect to increase 4-HNE (compared to TNF stimulated only).

We always include isotype control antibodies in our experiments because antibodies are known to modulate macrophage activation via binding to Fc receptor. To address the reviewer's comments, we updated all panels that present the effects of IFNAR1 blockade with isotypematched non-specific control antibodies in the revised manuscript. Specifically, we included isotype control in Fig. 3M (previously Fig.3J), Fig.4I, Suppl.4E-G, Fig.6L-M), Suppl.Fig.7I (previously Suppl.Fig.6F).

- Fig.4A - C: "IFNAR1 blockade, however, did not increase either the NRF2 and FTL protein levels, or the Fth, Ftl and Gpx1 mRNA levels above those treated with isotype control

antibodies"

Maybe not above the isotype but it is higher than the TNF alone stimulation at least for NRF2 at 8h and for Ftl at both time points. Why does the isotype already cause stimulation/induction of the cells? !These experiments need repetitions and quantification and statistics!

To determine specific effects of IFNAR blockade we compared effects of non-specific isotype control and IFNAR1-specific antibodies. In our experiments, the isotype control antibody modestly increased of Nrf2 and Ftl protein levels and the *Fth* and *Ftl* mRNA levels, but their effects were similar to the effect of IFNAR-specific antibody. The non-IFN -specific effects of antibodies, although are of potential biological significance, are modest in our model and their analysis is beyond the scope of this study.

- Fig.4H Was the AB added also at 12h post stimulation? Figure legend should be adjusted.

The IFNAR1 blocking antibodies and isotype control antibodies were added at 2 h after TNF stimulation in Fig.4H and 4I, as described in the corresponding figure legend. The data demonstrating effects of IFNAR blockade after 12, 24, and 33h of TNF stimulation are presented in Suppl.Fig.4 E-G.

- Figure 4I: How was the data measured here, i.e. what is depicted? The isotype control is missing. It seems a two-way ANOVA was used, yet it is stated differently. The figure legend should be revised, as Dunnett's multiple comparison would only check for significances compared to the control.

The microscopy images and bar graphs were updated to include isotype control and presented in Suppl. Fig.4E - G of the revised version. We also revised the statistical analysis to include correction for multiple comparisons.

- Figure 4C and subsequent: How exactly was the experiment done (house-keeping gene)?

We included the details in the figure legends of revised version. We quantified the gene expression by DDCT method using b-actin (for Fig. 4C-E) and 18S (For Fig. 4F and G) as internal controls.

- Figure 4D,E: Information on cells used is missing. Why the change in stimulation time? Did it not work after 12h? Then the experiments in A-C should be repeated for 16h.

The updated Fig. 4D and E present comparison of B6 and B6.Sst1S BMDMs clearly demonstrating significant difference between these macrophages in *Ifnb1* mRNA expression 16 h after TNF stimulation, in agreement with our previous publication(Bhattacharya, et al., 2021). There we studied the time course of responses of B6 and B6.Sst1S macrophages to TNF at 2h intervals and demonstrated the divergence between their activation trajectories starting at 12 h of TNF stimulation Therefore, to reveal the underlying mechanisms we focus our analyses on this critical timepoint, i.e. as close to the divergence as possible. However, the difference between the strains in *Ifnb1* mRNA expression achieved significance only by 16h of TNF stimulation. That is why we have used this timepoint for the *Ifnb1* and *Rsad2* analyses. It clearly shows that the superinduction was not driven by the positive feedback via IFNAR, as has been shown by the Ivashkiv lab for B6 wild type macrophages previously PMID 21220349.

- Figure 4E: It would be helpful to see if these transcripts are actually translated into protein levels, e.g. perform an ELISA. Authors state that IFNAR blockages does not alter

the expression but you statistic says otherwise.

*- The data for *Ifnb* expression (or better protein level) should be provided for B6 BMDMs as well.*

We have previously reported the differences in *Ifnb* protein secretion (He et al., Plos Pathogens, 2013 and Bhattacharya et al., JCI 2021). We use mRNA quantification by qRT-PCR as a more sensitive and direct measurement of the *sst1*-mediated phenotype. The revised Fig.4D and E include responses of B6 in addition to the B6.Sst1S to demonstrate that the IFNAR blockade does not reduce the *Ifnb1* mRNA levels in TNF-stimulated B6.Sst1S mutant to the B6 wild type levels. A slight reduction can be explained by a known positive feedback loop in the IFN-I pathway (see above). In this experiment we emphasized that the effect of the *sst1* locus is substantially greater, as compared to the effect of the IFNAR blockade (Fig.4D), and updated the text accordingly.

- Fig. 4F: To what does the fold induction refer to? If it is again to unstimulated cells, then why is the induction now so much higher than in (E) where it was only 50x (now to 100x).

*- Figure 4G: Again to what is the fold induction referring to? It seems your *Fer-1* treatment only contains 2 data points. This needs to be fixed.*

Yes, the fold induction was calculated by normalizing mRNA levels to untreated control incubated for the same time. Regarding the variation in *Ifnb1* mRNA levels - a two-fold variation is not unusual in these experiments that may result in the *Ifnb1* mRNA superinduction ranging from 50 -200-fold at this timepoint (16h). The graph in Fig.4G was modified to make all datapoints more visible.

- "These data suggest that type I IFN signaling does not initiate LPO in our model but maintains and amplifies it during prolonged TNF stimulation that, eventually, may lead to cell death". Data for a short term TNF stimulation are not shown, however, so it might impact also on the initiation of LPO.

- The overall conclusion drawn from Fig. 3 and 4 is not really clear with regard that IFN does not initiate LPO. Where is that shown? Data on earlier stimulation time points should be added to make this clear.

We demonstrated ROS production (new Suppl.Fig.3G) and the rate of LPO biosynthesis (new Suppl.Fig.4E-F) at 6 h post TNF stimulation, while the *Ifnb1* superinduction occurs between 12-18 h post TNF stimulation. This temporal separation supports our conclusion that IFN- β superinduction does not initiate LPO. We clarified it in the text:

“Thus, *Ifnb1* super-induction and IFN-I pathway hyperactivity in B6.Sst1S macrophages follow the initial LPO production, and maintain and amplify it during prolonged TNF stimulation”. (Previously: These data suggest that type I IFN signaling does not initiate LPO in our model). We also edited the conclusion in this section to explain the hierarchy of the *sst1*-regulated AOD and IFN-I pathways better:

“Taken together, the above experiments allowed us to reject the hypothesis that IFN-I hyperactivity caused the *sst1*-dependent AOD dysregulation. In contrast, they established that the hyperactivity of the IFN-I pathway in TNF-stimulated B6.Sst1S macrophages was itself driven by the initial dysregulation of AOD and iron-mediated lipid peroxidation. During prolonged TNF stimulation, however, the IFN-I pathway was upregulated, possibly via ROS/LPOdependent JNK activation, and acted as a potent amplifier of lipid peroxidation”.

We believe that these additional data and explanation strengthen our conclusions drawn from Figures 3 and 4.

- "A select set of mouse LTR-containing endogenous retroviruses (ERV's) (Jayewickreme et al, 2021), and non-retroviral LINE L1 elements were expressed at a basal level before and after TNF stimulation, but their levels in the B6.Sst1S BMDMs were similar to or lower than those seen in B6". This sentence should be revised as the differences between B6 and B6.Sst1S BMDMs seem small and are not there after 48h anymore. Are these mild changes really caused by the mutation or could they result from different housing conditions and/or slowly diverging genetically lines. How many mice were used for the analysis? Is there already heterogeneity between mice from the same line?

We agree with the reviewer that the data presented in Suppl.Fig.4 (Suppl.Fig.5 in the revised version) indicated no increase in single- and double-stranded transposon RNAs in the B6.Sst1S macrophages. The purpose of these experiment was to test the hypothesis that increased transposon expression might be responsible for triggering the superinduction of type I interferon response in TNF-stimulated B6.Sst1S macrophages. In collaboration with a transposon expert Dr. Nelson Lau (co-author of this manuscript) we demonstrated that transposon expression was not increased above the B6 level and, thus, rejected this attractive hypothesis. We explained the purpose of this experiment in the text and adequately described our findings as "the levels in the B6.Sst1S BMDMs were similar to or lower than those seen in B6"...and concluded that "the above analyses allowed us to exclude the overexpression of persistent viral or transposon RNAs as a primary mechanism of the IFN-I pathway hyperactivity" in the *sst1*-mutant macrophages.

- Fig. 5A: Indeed, it even seems that Myc is upregulated for the mutant BMDMs. Yet, there are only 2 data points for B6 12h.

These experiments need repetitions and quantification and statistics.

We observed these differences in c-Myc mRNA levels by independent methods: RNAseq and qRT-PCR. The qRT-PCR experiments were repeated 3 times. A representative experiment in Fig.5A shows 3 data points for each condition. We reformatted the panel to make all data points clearly visible.

- Fig. 5B: Why would the protein level decrease in the controls over 6h of additional cultivation? Is this caused by fresh M-CSF? In this case maybe cells should be left to settle for one day before stimulating them to properly compare c-Myc induction. Comment on two c-Myc bands is needed. At 12h only the upper one seems increased for TNF stimulated mutant BMDMs compared to B6 BMDMs.

We agree with the reviewer's point that cells need to be rested after media change that contains fresh CSF-1. Indeed, in Suppl.Fig.6C, we show that after media change containing 10% L929 supernatant (a source of CSF1) there is an increase in c-Myc protein levels that takes approximately 12 hours to return to baseline.

Our protocol includes resting period of 18-24 h after medium change before TNF stimulation.

We updated Methods to highlight this detail. Thus, the increase in c-Myc levels we observe at 12 h of TNF stimulation (Fig.5B) is induced by TNF, not the addition of growth factors, as further discussed in the text.

The two c-Myc bands observed in Fig.5B,I and J, are similar to patterns reported in previous studies that used the same commercial antibodies (PMIDs: 24395249, 24137534, 25351955). Whether they correspond to different c-Myc isoforms or post-translational modifications is unknown.

- Fig. 5A,B: It seems that not all the RNA is translated into protein, as c-Myc at 12h in the mutant BMDMs seems to be lower than at 6h, while the gene expression implicates it vice versa.

In addition to Fig.5B, the time course of Myc protein expression up to 24 h is presented in new panels Fig. 5I-5J. It demonstrates the gradual decrease of Myc protein levels. The observed dissociation between the mRNA and protein levels in the *sst1*-mutant BMDMs at 12 and 24 h is most likely due to translation inhibition as a result of the development of the integrated stress response, ISR (as shown in our previous publication by Bhattacharya et al., JCI, 2021). Translation of Myc is known to be particularly sensitive to the ISR (PMID18551192, PMID25079319, PMID28490664). Perhaps, the IFN-driven ISR may serve as a backup mechanism for Myc downregulation. We are planning to investigate these regulatory mechanisms in greater detail in the future.

- Fig. 5J: Indeed, the inhibitor seems to cause the downregulation of the proteins. Explanation?

This experiment was repeated twice and the average normalized densitometry values are presented in the updated Fig.5J. The main question addressed in this experiment was whether hyperactivity of JNK in TNF-stimulated *sst1* mutant macrophages contributed to Myc upregulation, as had been previously shown in cancer. Comparing effects of JNK inhibition on phospho-cJun and c-Myc protein levels in TNF stimulated B6.Sst1S macrophages (updated Fig.5J), we rejected the hypothesis that JNK activity might have a major role in c-Myc upregulation in *sst1* mutant macrophages.

- "TNF stimulation tended to reduce the LPO accumulation in the B6 macrophages and to increase it in the B6.Sst1S ones" However, this is not apparent in Sup. Fig. 6B. Here it seems that there might be a significant increase.

Suppl.Fig.6B (currently Suppl.Fig.7B) shows the 4-HNE accumulation at day 3 post infection. The data obtained after 5 days of Mtb infection are shown in Fig.6A. We clarified this in the text: "By day 5 post infection, TNF stimulation induced significant LPO accumulation only in the B6.Sst1S macrophages (Fig.6A)".

- Fig. 6B: Mtb and 4-HNE should be shown in two different channels in order to really assign each staining correctly.

What time point is this? Are the mycobacteria cleared at MOI1, since it looks that there are fewer than that? How does this look like for the B6 BMDMs? Are there even less mycobacteria?

We included B6 infection data to the updated Fig.6B and added Suppl.Fig.7C and 7D that address this reviewer's comment. The data represent day 5 of Mtb infection as indicated in the updated Fig.6B and Suppl.Fig.7C and 7D legends. New Suppl.Fig.7D shows quantification of replicating Mtb using Mtb replication reporter stain expressing single strand DNA binding protein GFP fusion, as described in Methods. We observed fewer Mtb and a lower percentage of replicating Mtb in B6 macrophages, but we did not observe a complete Mtb elimination in either background.

We used red fluorescence for both Mtb::mCherry and 4-HNE staining to clearly visualize the SSB-GFP puncta in replicating Mtb DNA. In the revised manuscript, we have included the relevant channels in Suppl. Fig.7C and D to demonstrate clearly distinct patterns of Mtb::mCherry and 4-HNE signals. We did not aim to quantify the 4-HNE signal intensity in

this experiment. For the 4-HNE quantification we use Mtb that expressed no reporter proteins (Fig.6A-B and Suppl.Fig.7A-B).

- Fig 6E: In the context of survival a viability staining needs to be included, as well as the data from day 0. Then it needs to be analyzed whether cell numbers remain the same from D0 or if there is a change.

We updated Fig.6 legend to indicate that the cell number percentages were calculated based on the number of cells at Day 0 (immediately after Mtb infection). We routinely use fixable cell death staining to enumerate cell death to exclude artifacts due to cell loss. Brief protocol containing this information is included in Methods section. The detailed protocol including normalization using BCG spike has been published – Yabaji et al, STAR Protocols, 2022. Here we did not present dead cell percentage as it remained low and we did not observe damage to macrophage monolayers. The fold change of Mtb was calculated after normalization using Mtb load at Day 0 after infection and washes.

"The 3D imaging demonstrated that YFP-positive cells were restricted to the lesions, but did not strictly co-localize with intracellular Mtb, i.e. the Ifnb promoter activity was triggered by inflammatory stimuli, but not by the direct recognition of intracellular bacteria. We validated the IFN β reporter findings using in situ hybridization with the Ifnb probe, as well as anti-GFP antibody staining (Suppl.Fig.8B - E)." The colocalization is not present within the tissue sections. It seems that the reporter line does not show the same staining pattern in vivo as the IFN β probe or the anti GFP antibody staining. The reporter line has to be tested for the specificity of the staining. Furthermore, to state that it was restricted to the lesions, an uninvolved tissue area needs to be depicted.

The Ifnb secreting cells are notoriously difficult to detect in vivo using direct staining of the protein. Therefore, lineage tracing of reporter expression are used as surrogates. The Ifnb reporter used in our study has been developed by the Locksley laboratory (Scheu et al., PNAS, 2008, PMID: 19088190) and has been validated in many independent studies. The reporter mice express the YFP protein under the control of the *Ifnb1* promoter. The YFP protein accumulates within the cells, while Ifnb protein is rapidly secreted and does not accumulate in the producing cells in appreciable amounts. Also, the kinetics of YFP protein degradation is much slower as compared to the endogenous *Ifnb1* mRNA that was detected using in situ hybridization. Thus, there is no precise spatiotemporal coincidence of these readouts in Ifnb expressing cells in vivo. However, this methodology more closely reflect the Ifnb expressing cells in vivo, as compared to a Cre-lox mediated lineage tracing approach. In the revised manuscript we demonstrate that both YFP and mRNA signals partially overlap (Suppl.Fig.12B). In Suppl.Fig.12B. we also included a new panel showing no YFP expression in the uninvolved area of the reporter mice infected with Mtb. The YFP expression by activated macrophages is demonstrated by co-staining with Iba1- and iNOS-specific antibodies (new Fig.7D and Suppl.Fig.13A). Our specificity control also included TB lesions in mice that do not carry the YFP reporter and did not express the YFP signal, as reported elsewhere (Yabaji et al., BioRxiv, <https://doi.org/10.1101/2023.10.17.562695>).

- Are paucibacillary and multibacillary lesions different within the same animal or does one animal have one lesion phenotype? If that is the case, what is causing the differences between mice? Bacterial counts for the mice are required.

The heterogeneity of pulmonary TB lesions has been widely acknowledged in clinic and highlighted in recent experimental studies. In our model of chronic pulmonary TB (described in detail in Yabaji et al., <https://doi.org/10.1101/2025.02.28.640830> and <https://doi.org/10.1101/2023.10.17.562695>) the development of pulmonary TB lesions is not synchronized, i.e. the lesions are heterogeneous between the animals and within individual animals at the same

timepoint. Therefore, we performed a lesion stratification where individual lesions were classified by a certified veterinary pathologist in a blinded manner based on their morphology (H&E) and acid fast staining of the bacteria, as depicted in Suppl.Fig.8.

- "Among the IFN-inducible genes upregulated in paucibacillary lesions were *Ifi44l*, a recently described negative regulator of IFN-I that enhances control of *Mtb* in human macrophages (DeDiego et al, 2019; Jiang et al, 2021) and *Ciita*, a regulator of MHC class II inducible by IFN γ , but not IFN-I (Suppl.Table 8 and Suppl.Fig.10 D-E)." Why is Sup. Fig. 10 D, E referred to? The figure legend is also not clear, e.g. what means "upregulated in a subset of IFN-inducible genes"? Input for the hallmarks needs to be defined.

These data is now presented in Suppl.Fig.11 and following the reviewer's comment, we moved reference to panels 11D – E up to previous paragraph in the main text, where it naturally belongs. We also edited the figure legend to refer to the list of IFN-inducible genes compiled from the literature that is discussed in the text. We appreciate the reviewer's suggestion that helped us improve the text clarity. The inputs for the Hallmark pathway analysis are presented in Suppl.Tables 7 and 8, as described in the text.

- Fig. 7C: Single channel pictures are required as it is hard to see the differences in staining with so many markers. Why is there no iNOS expression in the bottom row? What does the rectangle indicate on the bottom right? As black is chosen for DAPI, it is not visible at all. In case the signal is needed a visible a color should be chosen.

We thoroughly revised this figure to address the reviewer's concern about the lack of clarity. We provide individual channels for each marker in Fig.7D – E and Suppl.Fig.13F. We have to use DAPI in these presentation in gray scale to better visualize other markers.

- "In the advanced lesions these markers were primarily expressed by activated macrophages (*Iba1*+) expressing iNOS and/or *Ifny* (YFP+)(Fig.7D)" *Iba1* is needed in the quantification. Based on the images, iNOS seems to be highly produced in *Iba1* negative cells. Which cells do produce it then? Flow cytometry data for this quantification are required. This would allow you to specifically check which cells express the markers and allow for a more precise analysis of double positive cells.

Currently these data demonstrating the co-localization of stress markers phospho-c-Jun and Chac1 with YFP are presented in Fig.7E (images) and Suppl.Fig.13D (quantification). The co-localization of stress markers phospho-cJun and Chac1 with iNOS is presented in Suppl.Fig.13F (images) and Suppl.Fig.13E (quantification). We agree that some iNOS+ cells are *Iba1*-negative (Fig.7D). We manually quantified percentages of *Iba1*+iNOS+ double positive cells and demonstrated that they represent the majority of the iNOS+ population(Suppl.Fig.13A). Regarding the required FACS analysis, we focus on spatial approaches because of the heterogeneity of the lesions that would be lost if lungs are dissociated for FACS. We are working on spatial transcriptomics at a single cell resolution that preserves spatial organization of TB lesions to address the reviewer's comment and will present our results in the future.

- Results part 6: In general, can you please state for each experiment at what time point mice were analyzed? You should include an additional macrophage staining (e.g. MerTK, F4/80), as alveolar macrophages are not staining well for *Iba1* and you might therefore miss them in your IF microscopy. It would be very nice if you could perform flow cytometry to really check on the macrophages during infection and distinguish subsets (e.g. alveolar macrophages, interstitial macrophages, monocytes).

We have included the details of time post infection in figure legends for Fig.7, Suppl.Figures 8, 9, 12B, 13, 14A of the revised manuscript. We have performed staining with CD11b, CD206 and CD163 to differentiate the recruited and lung resident macrophages and determined that in chronic pulmonary TB lesions in our model the vast majority of macrophages are recruited CD11b+, but not resident (CD206+ and CD163+) macrophages. These data is presented in another manuscript (Yabaji et al., BioRxiv <https://doi.org/10.1101/2023.10.17.562695>).

- Spatial sequencing: The manuscript would highly profit from more data on that. It would be very interesting to check for the DEGs and show differential spatial distribution. Expression of marker genes should be inferred to further define macrophage subsets (e.g. alveolar macrophages, interstitial macrophages, recruited macrophages) and see if these subsets behave differently within the same lesion but also between the lesions. Additional bioinformatic approaches might allow you to investigate cell-cell interactions. There is a lot of potential with such a dataset, especially from TB lesions, that would elevate your findings and prove interesting to the TB field.

- "Thus, progression from the Mtb-controlling paucibacillary to non-controlling multibacillary TB lesions in the lungs of TB susceptible mice was mechanistically linked with a pathological state of macrophage activation characterized by escalating stress (as evidenced by the upregulation phospho-cJUN, PKR and Chac1), the upregulation of IFN β and the IFN-I pathway hyperactivity, with a concurrent reduction of IFN γ responses." To really show the upregulation within macrophages and their activation, a more detailed IF microscopy with the inclusion of additional macrophage markers needs to be provided. Flow cytometry would enable analysis for the differences between alveolar and interstitial macrophages, as well as for monocytes. As however, it seems that the majority of iNOS, as well as the stress associated markers are not produced by Iba1+ cells. Analyzing granulocytes and T lymphocytes should be considered.

We appreciate the reviewer's suggestion. Indeed, our model provides an excellent opportunity to investigate macrophage heterogeneity and cell interactions within chronic TB lesions. We are working on spatial transcriptomics at a single cell resolution that would address the reviewer's comment and will present our results in the future.

In agreement with classical literature the overwhelming majority of myeloid cells in chronic pulmonary TB lesions is represented by macrophages. Neutrophils are detected at the necrotic stage, but our study is focused on pre-necrotic stages to reveal the earlier mechanisms predisposing to the necrotization. We never observed neutrophils or T cells expressing iNOS in our studies.

- It's mentioned in the method section that controls in the IF staining were only fixed for 10min, while the infected cells were fixed for 30min. Consistency is important as the PFA fixation might impact on the fluorescence signal. Therefore, controls should be repeated with the same fixation time.

We have carefully considered the impact of fixation time on fluorescence and have separately analyzed the non-infected and infected samples to address this concern. For the non-infected samples, we examined the effect of TNF in both B6 and B6.Sst1S backgrounds, ensuring that a consistent fixation protocol (10 min) was applied across all experiments without Mtb infection.

For the Mtb infection experiments, we employed an optimized fixation protocol (30 min) to ensure that Mtb was killed before handling the plates, which is critical for preserving the integrity of the samples. In this context, we compared B6 and B6.Sst1S samples to evaluate the effects of fixation and Mtb infection on lipid peroxidation (LPO) induction.

We believe this approach balances the need for experimental consistency with the specific requirements for handling infected cells, and we have revised the manuscript to reflect this clarification.

- Reactive oxygen species levels should be determined in B6 and B6.Sst1S BMDMs (stimulated and unstimulated), as they are very important for oxidative stress.

We have conducted experiments to measure ROS production in both B6 and B6.Sst1S BMDMs and demonstrated higher levels of ROS in the susceptible BMDMs after prolonged TNF stimulation (new Fig.3I-J and Suppl. Fig. 3G). Additionally, we have previously published a comparison of ROS production between B6 and B6.Sst1S by FACS (PMID: 33301427), which also supports the findings presented here.

- Sup. Fig 2C: The inclusion of an unstimulated control would be advisable in order to evaluate if there are already difference in the beginning.

We have included the untreated control to the Suppl. Fig. 2C (currently Suppl. Fig. 2D) in the revised manuscript.

- Sup. Fig. 3F: Why is the fold change now lower than in Fig. 4D (fold change of around 28 compared to 120 in 4D)?

The data in Fig.4D (Fig.4E in the revised manuscript) and Suppl.Fig.3F (currently Suppl.Fig.4C) represent separate experiments and this variation between experiments is commonly observed in qRT-PCR that is affected by slight variations in the expression in unstimulated controls used for the normalization and the kinetics of the response. This 2-4 fold difference between same treatments in separate experiments, as compared to 30 – 100 fold and higher induction by TNF does not affect the data interpretation.

- Sup. Fig. 5C, D: The data seems very interesting as you even observe an increase in gene expression. Data for the B6 mice should be evaluated for increase to a similar level as the TNF treated mutants. Data on the viability of the cells are necessary, as they no longer receive MCSF and might be dying at this point already.

To ensure that the observed effects were not confounded by cytotoxicity, we determined non-toxic concentrations of the CSF1R inhibitors during 48h of incubation and used them in our experiments that lasted for 24h. To address this valid comment, we have included cell viability data in the revised manuscript to confirm that the treatments did not result in cell death (Suppl. Fig. 6D). This experiment rejected our hypothesis that CSF1 driven Myc expression could be involved in the *Ifnb* superinduction. Other effects of CSF1R inhibitors on type I IFN pathway are intriguing but are beyond the scope of this study.

- Sup. Fig 12: the phospho-c-Jun picture for (P) is not the same as in the merged one with Iba1. Double positive cells are mentioned to be analyzed, but from the staining it appears that P-c-Jun is expressed by other cells. You do not indicate how many replicates were counted and if the P and M lesions were evaluated within the same animal. What does the error bar indicate? It seems unlikely from the plots that the double positive cells are significant. Please provide the p values and statistical analysis.

We thank the reviewer for bringing this inadvertent field replacement in the single phospho-cJun channel to our attention. However, the quantification of Iba1+phospho-cJun+ double positive cells in Suppl.Fig.12 and our conclusions were not affected. In the revised manuscript, images and quantification of phospho-cJun and Iba1 co-expression are shown in

new Suppl.Fig.13B and C, respectively. We have also updated the figure legends to denote the number of lesions analyzed and statistical tests. Specifically, lesions from 6–8 mice per group (paucibacillary and multibacillary) were evaluated. Each dot in panels Suppl.Fig.13 represent individual lesions.

- Sup. Fig. 13D (suppl.Fig.15D now): What about the expression of MYC itself? Other parts of the signaling pathway should be analyzed(e.g. IFN γ , JNK)?

The difference in *MYC* mRNA expression tended to be higher in TB patients with poor outcomes, but it was not statistically significant after correction for multiple testing. The upregulation of *Myc* pathway in the blood transcriptome associated with TB treatment failure most likely reflects greater proportion of immature cells in peripheral blood, possibly due to increased myelopoiesis. Pathway analysis of the differentially expressed genes revealed that treatment failures were associated with the following pathways relevant to this study: NF- κ B Signaling, Flt3 Signaling in Hematopoietic Progenitor Cells (indicative of common myeloid progenitor cell proliferation), SAPK/JNK Signaling and Senescence (possibly indicative of oxidative stress). The upregulation of these pathways in human patients with poor TB treatment outcomes correlates with our findings in TB susceptible mice.

- In the mfiHC you he usage of anti-mouse antibodies is mentioned. Pictures of sections incubated with the secondary antibody alone are required to exclude the possibility that the staining is not specific. Especially, as this data is essential to the manuscript and mouse-antimouse antibodies are notorious for background noise.

We are well aware of the technical difficulties associated with using mouse on mouse staining. In those cases, we use rabbit anti-mouse isotype specific antibodies specifically developed to avoid non-specific background (Abcam cat#ab133469). Each antibody panel for fluorescent multiplexed IHC is carefully optimized prior to studies. We did not use any primary mouse antibodies in the final version of the manuscript and, hence, removed this mention from the Methods.

- In order to tie the story together, it would be interesting to treat infected mice with an INFAR antibody, as well as perform this experiment with a Myc antibody. According to your data, you might expect the survival of the mice to be increased or bacterial loads to be affected.

In collaboration with the Vance laboratory, we tested effects of type I IFN pathway inhibition in B6.Sst1S mice on TB susceptibility: either type I receptor knockout or blocking antibodies increased their resistance to virulent *Mtb* (published in Ji et al., 2019; PMID 31611644). Unfortunately, blocking *Myc* using neutralizing antibodies in vivo is not currently achievable. Specifically blocking *Myc* using small molecule inhibitors in vivo is notoriously difficult, as recognized in oncology literature. We consider using small molecule inhibitors of either *Myc* translation or specific pathways downstream of *Myc* in the future.

- It is surprising that you not even once cite or mention your previous study on bioRxiv considering the similarity of the results and topic (<https://doi.org/10.1101/2020.12.14.422743>). Is not even your Figure 1I and Figure 2 J, K the same as in that study depicted in Figure 4?

The reviewer refers to the first version of this manuscript uploaded to BioRxiv, but it has never been published. We continued this work and greatly expanded our original observations, as presented in the current manuscript. Therefore, we do not consider the previous version as an independent manuscript and, therefore, do not cite it.

- Please revise spelling of the manuscript and pay attention to write gene names in *italics*

Thank you, we corrected the gene and protein names according to current nomenclature.

Minor points:

- Fig. 1: Please provide some DEGs that explain why you used this resolution for the clustering of the scRNAseq data and that these clusters are truly distinct from each other.

Differential gene expression in clusters is presented in Suppl.Fig.1C (interferon response) and Suppl.Fig.1D (stress markers and interferon response previously established in our studies).

- Fig. 1F: What do the two lines represent (magenta, green)?

The lines indicate pseudotime trajectories of B6 (magenta) and B6.Sst1S (green) BMDMs.

- Fig. 1F, G: Why was cluster 6 excluded?

This cluster was not different between B6 and B6.Sst1S, so it was not useful for drawing the strain-specific trajectories.

- Fig. 1E, G, H: The intensity scales are missing. They are vital to understand the data.

We have included the scale in revised manuscript (Fig.1E,G,H and Suppl.Fig.1C-D).

- Fig. 2G-I: please revise order, as you first refer to Fig. 2H and I

We revised the panels' order accordingly

- Fig. 5: You say the data represents three samples but at least in D and E you have more. Please revise. Why do you only include at (G) the inhibitor only control?

We added the inhibitor only controls to Fig. 5D - H. We also indicated the number of replicates in the updated Fig.5 legend.

- Figure 7A, Sup. Fig. 8: Are these maximum intensity projection? Or is one z-level from the 3D stack depicted?

The Fig. 7A shows 3D images with all the stacks combined.

- Fig. 7B: What do the white boxes indicate?

We have removed this panel in the revised version and replaced it with better images.

- Sup. Fig. 1A: The legend for the staining is missing

The Suppl. Fig.1A shows the relative proportions of either naïve (R and S) or TNFstimulated (RT and ST) B6 or B6.Sst1S macrophages within individual single cell clusters depicted in Fig.1B. The color code is shown next to the graph on the right.

- Sup. Fig. 1B: The feature plots are not clear: The legend for the expression levels is missing. What does the heading means?

We updated the headings, as in Fig.1C. The dots represent individual cells expressing *Sp110* mRNA (upper panels) and *Sp140* mRNA (lower panels).

| - *Sup. Fig. 3C: The scale bar is barely visible.*

We resized the scale bar to make it visible and presented in Suppl. Fig.3E (previously Suppl. Fig.3C).

| - *Sup. Fig. 3D: There is not figure legend or the legend to C-E is wrong.*

| - *Sup. Fig. 3F, G: You do not state to what the data is relative to.*

We identified an error in the Suppl.Fig.3 legend referring to specific panels. The Suppl.Fig.3 legend has been updated accordingly. New panels were added and Suppl.Fig.3-G panels are now Suppl.Fig.4C-D.

| - *Sup. Fig. 3H: It seems you used a two-way ANOVA, yet state it differently. Please revise the figure legend, as Dunnett's multiple comparison would only check for significances compared to the control.*

Following the reviewer's comment, we repeated statistical analysis to include correction for multiple comparisons and revised the figure and legend accordingly.

| - *Sup. Fig. 4A, B: It is not clear what the lines depict as the legend is not explained. Names that are not required should be changed to make it clear what is depicted (e.g. "TE@" what does this refer to?)*

This previous Sup. Fig 4 is now Sup. Fig. 5. The "TE@" is a leftover label from the bioinformatics pipeline, referring to "Transposable Element". We apologize for this confusion and have removed these extraneous labels. We have also added transposon names of the LTR (MMLV30 and RTL4) and L1Md to Suppl.Fig.5A and 5B legend, respectively.

| - *Sup. 4B: What does the y-scale on the right refer to?*

We apologize for the missing label for the y-scale on the right which represents the mRNA expression level for the SetDB1 gene, which has a much lower steady state level than the LINE L1Md, so we plotted two Y-scales to allow both the gene and transposon to be visualized on this graph.

| - *Sup. 4C: Interpretation of the data is highly hindered by the fact that the scales differ between the B6 and B6.Sst1. The scales are barely visible.*

We apologize for the missing labels for the y-scales of these coverage plots, which were originally meant to just show a qualitative picture of the small RNA sequencing that was already quantitated by the total amounts in Sup. 4B. We have added three auto-scaled Y-scales to Sup. 4C and improved the presentation of this figure.

| - *Sup. Fig. 5A, B: Is the legend correct? Did you add the antibody for 2 days or is the quantification from day 3?*

We recognize that the reviewer refers to Suppl.Fig.6A-B (Suppl.Fig.7A-B in the revised manuscript). We did not add antibodies to live cells. The figure legend describes staining with 4HNE-specific antibodies 3 days post Mtb infection.

- *Sup. Fig. 8A: Are the "early" and "intermediate" lesions from the same time points? What are the definitions for these stages?*

We discussed our lesion classification according to histopathology and bacterial loads above. Of note, in the revised manuscript we simplified our classification to denote paucibacillary and multibacillary lesions only. We agree with reviewers that designation lesions as early, intermediate and advanced lesions were based on our assumptions regarding the time course of their progression from low to high bacterial loads.

- *Sup. Fig. 8E: You should state that the bottom picture is an enlargement of an area in the top one. Scale bars are missing.*

We replaced this panel with clearer images in Suppl.Fig.12B.

- *Sup. Fig. 11A: The IF staining is only visible for Iba and iNOS. Please provide single channels in order to make the other staining visible.*

Suppl.Fig.11A (now Suppl.Fig.13B) shows the low-magnification images of TB lesions. In the Fig. 7 and Suppl. Fig. 13F of the revised manuscript we provided images for individual markers.

- *Sup. Fig. 13A (Suppl.Fig.15A now): Your axis label is not clear. What do the numbers behind the genes indicate? Why did you choose oncogene signatures and not inflammatory markers to check for a correlation with disease outcome?*

X axis of Suppl.Fig.15A represent pre-defined molecular signature gene sets (MSigDB) in Gene Set Enrichment Analysis (GSEA) database (<https://www.gseamsigdb.org/gsea/msigdb>). On Y axis is area under curve (AUC) score for each gene set.

- *Sup. 13D(Suppl.Fig.15D now): Maybe you could reorder the patients, so that the impression is clearer, as right now only the top genes seem to show a diverging gene signature, while the rest gives the impression of an equal distribution.*

The Myc upregulated gene set myc_up was identified among top gene sets associated with treatment failure using unbiased ssGSEA algorithm. We agree with the reviewer that not every gene in the myc_up gene set correlates with the treatment outcome. But the association of the gene set is statistically significant, as presented in Suppl.Fig.15B – C.

- *The scale bars for many microscopy pictures are missing.*

We have included clearly visible scale bars to all the microscopy images in the revised version.

- *The black bar plots should be changed (e.g. in color), since the single data points cannot be seen otherwise.*

- *It would be advisable that a consistent color scheme would be used throughout the manuscript to make it easier to identify similar conditions, as otherwise many different colours are not required and lead right now rather to confusion (e.g. sometimes a black bar refers to BMDMs with and sometimes without TNF stimulation, or B6 BMDMs). Furthermore, plot sizes and fonts should be consistent within the manuscript (including the supplemental data)*

We followed this useful suggestion and selected consistent color codes for B6 and B6.Sst1S groups to enhance clarity throughout the revised manuscript.

Within the methods section:

- At which concentration did you use the IFNAR antibody and the isotype?

We updated method section by including respective concentrations in the revised manuscript.

- Were mice maintained under SPF conditions? At what age were they used?

Yes, the mice are specific pathogen free. We used 10 - 14 week old mice for Mtb infection.

- The BMDM cultivation is not clear. According to your cited paper you use LCCM but can you provide how much M-CSF it contains? How do you make sure that amounts are the same between experiments and do not vary? You do not mention how you actually obtain this conditioned medium. Is there the possibility of contamination or transferred fibroblasts that would impact on the data analysis? Is LCCM also added during stimulation and inhibitor treatment?

We obtain LCCM by collecting the supernatant from L929 cell line that form confluent monolayer according to well-established protocols for LCCM collection. The supernatants are filtered through 0.22 micron filters to exclude contamination with L929 cells and bacteria. The medium is prepared in 500 ml batches that are sufficient for multiples experiments. Each batch of L929-conditioned medium is tested for biological activity using serial dilutions.

- How was the BCG infection performed? How much bacteria did you use? Which BCG strain was used?

We infected mice with *M. bovis* BCG Pasteur subcutaneously in the hock using 10^6 CFU per mouse.

- At what density did you seed the BMDMs for stimulation and inhibitor experiments?

In 96 well plates, we seed 12,000 cells per well and allow the cells to grow for 4 days to reach confluency (approximately 50,000 cells per well). For a 6-well plate, we seed 2.5×10^5 cells per well and culture them for 4 days to reach confluency. For a 24-well plate, we seed 50,000 cells per well and keep the cells in media for 4 days before starting any treatments. This ensures that the cells are in a proliferative or near-confluent state before beginning the stimulation or inhibitor treatments. Our detailed protocol is published in STAR Protocols (Yabaji et al., 2022; PMID 35310069).

- What machine did you use to perform the bulk RNA sequencing? How many replicates did you include for the sequencing?

For bulk sequencing we used 3 RNA samples for each condition. The samples were sequenced at Boston University Microarray & Sequencing Resource service using Illumina NextSeqTM 2000 instrument.

- How many replicates were used for the scRNA sequencing? Why is your threshold for the exclusion of mitochondrial DNA so high? A typical threshold of less than 5% has been reported to work well with mouse tissue.

We used one sample per condition. For the mitochondrial cutoff, we usually base it off of the total distribution. There is no "universal" threshold that can be applied to all datasets. Thresholds must be determined empirically.

- You do not mention how many PCAs were considered for the scRNA sequencing analysis.

We considered 50 PCAs, this information was added to Methods

- You should name all the package versions you used for the scRNA sequencing (e.g. for the slingshot, VAM package)

The following package versions were used: Seurat v4.0.4, VAM v1.0.0, Slingshot v2.3.0, SingleCellTK v2.4.1, Celda v1.10.0, we added this information to Methods.

- You mention two batches for the human samples. Can you specify what the two batches are?

Human blood samples were collected at five sites, as described in the updated Methods section and two RNAseq batches were processed separately that required batch correction.

- At which temperature was the IF staining performed?

We performed the IF at 40C. We included the details in revised version.

Reviewer #2 (Significance):

Overall, the manuscript has interesting findings with regard to macrophage responses in Mycobacteria tuberculosis infection.

However, in its current form there are several shortcomings, both with respect to the precision of the experiments and conclusions drawn.

Reviewer #3 (Evidence, reproducibility and clarity):

Summary

The authors use a mouse model designed to be more susceptible to M.tb (addition of sst1 locus) which has granulomatous lesions more similar to human granulomas, making this mouse highly relevant for M.tb pathogenesis studies. Using WT B6 macrophages or sst1B6 macrophages, the authors seek to understand the how the sst1 locus affects macrophage response to prolonged TNFa exposure, which can occur during a pro-inflammatory response in the lungs. Using single cell RNA-seq, revealed clusters of mutant macrophages with upregulated genes associated with oxidative stress responses and IFN-I signaling pathways when treated with TNF compared to WT macs. The authors go on to show that mutant macrophages have decreased NRF2, decreased antioxidant defense genes and less Sp110 and Sp140. Mutant macrophages are also more susceptible to lipid peroxidation and ironmediated oxidative stress. The IFN-I pathway hyperactivity is caused by the dysregulation of iron storage and antioxidant defense. These mutant macrophages are more susceptible to M.tb infection, showing they are less able to control bacterial growth even in the presence of T cells from BCG vaccinated mice. The transcription factor Myc is more highly expressed in mutant macs during TNF treatment and inhibition Myc led to better control of M.tb growth. Myc is also more abundant in PBMCs from M.tb infected humans with poor outcomes, suggesting that

Myc should be further investigated as a target for host-directed therapies for tuberculosis.

Major Comments

Isotypes for IF imaging and confocal IF imaging are not listed, or not performed. It is a concern that the microscopy images throughout the manuscript do not have isotype controls for the primary antibodies.

Fig 4 (and later) the anti-IFNAR Ab is used along with the Isotype antibody, Fig 4I does not show the isotype. Use of the isotype antibody is also missing in later figures as well as Fig 3J. Why was this left off as the proper control for the Ab?

We addressed the comment in revised manuscript as described above in summary and responses to reviewers 1 and 2. Isotype controls for IFNAR1 blockade were included in Fig.3M (previously 3J), Fig. 4I, Suppl.Fig.4G (previously Fig.4I), and updated Fig.4C-E, Fig.6L-M, Suppl.Fig.4F-G, 7I.

Conclusions drawn by the authors from some of the WB data are worded strongly, yet by eye the blots don't look as dramatically different as suggested. It would be very helpful to quantify the density of bands when making conclusions. (for example, Fig 4A).

We added the densitometry of Western blot values after normalization above each lane in Fig.2A-C, Fig.3C-D and 3K; Fig.4A-B, Fig.5B,C,I,J.

Fig 5A is not described clearly. If the gene expression is normalized to untreated B6 macs, then the level of untreated B6 macs should be 1. In the graph the blue bars are slightly below 1, which would not suggest that levels "initially increased and subsequently downregulated" as stated in the text. It seems like the text describes the protein expression but not the RNA expression. Please check this section and more clearly describe the results.

We appreciate the reviewer's comment and modified the text to specify the mRNA and protein expression data, as follows:

"We observed that Myc was regulated in an *sst1*-dependent manner: in TNF-stimulated B6 wild type BMDMs, *c-Myc* mRNA was downregulated, while in the susceptible macrophages *c-Myc* mRNA was upregulated (Fig.5A). The *c-Myc* protein levels were also higher in the B6.Sst1S cells in unstimulated BMDMs and 6 – 12 h of TNF stimulation (Fig.5B)".

Also, why look at RNA through 24h but protein only through 12h? If c-myc transcripts continue to increase through 24h, it would be interesting to see if protein levels also increase at this later time point.

The time-course of Myc expression up to 24 h is presented in new panels Fig. 5I-5J. It demonstrates the decrease of Myc protein levels at 24 h. In the wild type B6 BMDMs the levels of Myc protein significantly decreased in parallel with the mRNA suppression presented in Fig.5A. In contrast, we observed the dissociation of the mRNA and protein levels in the *_sst1* mutant BMDMs at 12 and 24 h, most likely, because the mutant macrophages develop integrated stress response (as shown in our previous publication by Bhattacharya et al., JCI, 2021) that is known to inhibit Myc mRNA translation.

Fig 5J the bands look smaller after D-JNK1 treatment at 6 and 12h though in the text is says no change. Quantifying the bands here would be helpful to see if there really is no difference.

This experiment was repeated twice, and the average normalized densitometry values are presented in the updated Fig.5J. The main question addressed in this experiment was whether the hyperactivity of JNK in TNF-stimulated *sst1* mutant macrophages contributed to Myc upregulation, as was previously shown in cancer. Comparing effects of JNK inhibition on phospho-cJun and c-Myc protein levels in TNF stimulated B6.Sst1S macrophages (updated Fig.5J), we concluded that JNK did not have a major role in c-Myc upregulation in this context.

Section 4, third paragraph, the conclusion that JNK activation in mutant macs drives pathways downstream of Myc are not supported here. Are there data or other literature from the lab that supports this claim?

This statement was based on evidence from available literature where JNK was shown to activate oncogens, including Myc. In addition, inhibition of Myc in our model upregulated ferritin (Fig.5C), reduced the labile iron pool, prevented the LPO accumulation (Fig.5D - G) and inhibited stress markers (Fig.5H). However, we do not have direct experimental evidence in our model that Myc inhibition reduces ASK1 and JNK activities. Hence, we removed this statement from the text and plan to investigate this in the future.

Fig 6N Please provide further rationale for the BCG in vivo experiment. It is unclear what the hypothesis was for this experiment.

In the current version BCG vaccination data is presented in Suppl.Fig.14B. We demonstrate that stressed BMDMs do not respond to activation by BCG-specific T cells (Fig.6J) and their unresponsiveness is mediated by type I interferon (Fig.6L and 6M). The observed accumulation of the stressed macrophages in pulmonary TB lesions of the *sst1*-susceptible mice (Fig.7E, Suppl.Fig.13 and 14A) and the upregulation of type I interferon pathway (Fig.1E,1G, 7C), Suppl.Fig.1C and 11) suggested that the effect of further boosting T lymphocytes using BCG in Mtb-infected mice will be neutralized due to the macrophage unresponsiveness. This experiment provides a novel insight explaining why BCG vaccine may not be efficient against pulmonary TB in susceptible hosts.

The in vitro work is all concerning treatment with TNFa and how this exposure modifies the responses in B6 vs sst1B6 macrophages; however, this is not explored in the in vivo studies. Are there differences in TNFa levels in the pauci- vs multi-bacillary lesions that lead to (or correlate with) the accumulation of peroxidation products in the intralesional macrophages. How to the experiments with TNFa in vitro relate back to how the macrophages are responding in vivo during infection?

Our investigation of mechanisms of necrosis of TB granulomas stems from and supported by in vivo studies as summarized below.

This work started with the characterization necrotic TB granulomas in C3HeB/FeJ mice in vivo followed by a classical forward genetic analysis of susceptibility to virulent Mtb in vivo.

That led to the discovery of the *sst1* locus and demonstration that it plays a dominant role in the formation of necrotic TB granulomas in mouse lungs in vivo. Using genetic and immunological approaches we demonstrated that the *sst1* susceptibility allele controls macrophage function in vivo (Yan, et al., J.Immunol. 2007) and an aberrant macrophage activation by TNF and increased production of Ifn- γ in vitro (He et al. Plos Pathogens, 2013). In collaboration with the Vance lab we demonstrated that the type I IFN receptor inactivation reduced the susceptibility to intracellular bacteria of the *sst1*-susceptible mice in vivo (Ji et al., Nature Microbiology, 2019). Next, we demonstrated that the *Ifnb1* mRNA superinduction results from combined effects of TNF and JNK leading to integrated stress response in vitro (Bhattacharya, JCI, 2021). Thus, our previous work started with extensive characterization of

the in vivo phenotype that led to the identification of the underlying macrophage deficiency that allowed for the detailed characterization of the macrophage phenotype in vitro presented in this manuscript. In a separate study, the Sher lab confirmed our conclusions and their in vivo relevance using *Bach1* knockout in the *sst1*-susceptible B6.Sst1S background, where boosting antioxidant defense by *Bach1* inactivation resulted in decreased type I interferon pathway activity and reduced granuloma necrosis. We have chosen TNF stimulation for our in vitro studies because this cytokine is most relevant for the formation and maintenance of the integrity of TB granulomas in vivo as shown in mice, non-human primates and humans. Here we demonstrate that although TNF is necessary for host resistance to virulent *Mtb*, its activity is insufficient for full protection of the susceptible hosts, because of altered macrophages responsiveness to TNF. Thus, our exploration of the necrosis of TB granulomas encompass both in vitro and extensive in vivo studies.

Minor comments

Introduction, while well written, is longer than necessary. Consider shortening this section. Throughout figures, many graphs show a fold induction/accumulation/etc, but it is rarely specified what the internal control is for each graph. This needs to be added.

Paragraph one, authors use the phrase "the entire IFN pathway was dramatically upregulated..." seems to be an exaggeration. How do you know the "entire" IFN pathway was upregulated in a dramatic fashion?

(1) We shortened the introduction and discussion; (2) verified that figure legends internal controls that were used to calculate fold induction; (3) removed the word "entire" to avoid overinterpretation.

*Figures 1E, G and H and supp fig 1C, the heat maps are missing an expression key
Section 2 second paragraph refers to figs 2D, E as cytoplasmic in the text, but figure legend and y-axis of 2E show total protein.*

The expression keys were added to Fig.1E,G,H, Fig.7C, Suppl.Fig.1C and 1D and Suppl.Fig.11A of the revised manuscript.

Section 3 end of paragraph 1 refers to Fig 3h. Does this also refer to Supp Fig 3E?

Yes, Fig.3H shows microscopy of 4-HNE and Suppl.Fig.3H shows quantification of the image analysis. In the revised manuscript these data are presented in Fig.3H and Suppl.Fig.3F. The text was modified to reflect this change.

Supplemental Fig 3 legend for C-E seems to incorrectly also reference F and G.

We corrected this error in the figure legend. New panels were added to Suppl.Fig.3 and previous Suppl.Fig.3F and G were moved to Suppl.Fig.4 panels C and D of the revised version.

Fig 3K, the p-c-Jun was inhibited with the JNK inhibitor, however it's unclear why this was done or the conclusion drawn from this experiment. Use of the JNK inhibitor is not discussed in the text.

The JNK inhibitor was used to confirm that c-Jun phosphorylation in our studies is mediated by JNK and to compare effects of JNK inhibition on phospho-c-Jun and Myc expression. This experiment demonstrated that the JNK inhibitor effectively inhibited c-Jun phosphorylation but not Myc upregulation, as shown in Fig.5I-J of the revised manuscript.

Fig 4 I and Supp Fig 3 H seem to have been swapped? The graph in Fig 4I matches the images in Supp Fig 3I. Please check.

We reorganized the panels to provide microscopy images and corresponding quantification together in the revised the panels Fig. 4H and Fig. 4I, as well as in Suppl. Fig. 4F and Suppl. Fig. 4G.

Fig 6, it is unclear what % cell number means. Also for bacterial growth, the data are fold change compared to what internal control?

We updated Fig.6 legend to indicate that the cell number percentages were calculated based on the number of cells at Day 0 (immediately after Mtb infection). We routinely use fixable cell death staining to enumerate cell death. Brief protocol containing this information is included in Methods section. The detailed protocol including normalization using BCG spike has been published – Yabaji et al, STAR Protocols, 2022. Here we did not present dead cell percentage as it remained low and we did not observe damage to macrophage monolayers. This allows us to exclude artifacts due to cell loss. The fold change of Mtb was calculated after normalization using Mtb load at Day 0 after infection and washes.

Fig 7B needs an expression key

The expression keys was added to Fig.7C (previously Fig. 7B).

Supp Fig 7 and Supp Fig 8A, what do the arrows indicate?

In Suppl.Fig.8 (previously Suppl.Fig.7) the arrows indicate acid fast bacilli (Mtb). In figures Fig.7A and Suppl.Fig.9A arrows indicate Mtb expressing fluorescent reporter mCherry. Corresponding figure legends were updated in the revised version.

*Supp Fig 9A, two ROI appear to be outlined in white, not just 1 as the legend says
Methods:*

We updated the figure legend.

Certain items are listed in the Reagents section that are not used in the manuscript, such as necrostatin-1 or Z-VAD-FMK. Please carefully check the methods to ensure extra items or missing items does not occur.

These experiments were performed, but not included in the final manuscript. Hence, we removed the “necrostatin-1 or Z-VAD-FMK” from the reagents section in methods of revised version.

Western blot, method of visualizing/imaging bands is not provided, method of quantifying density is not provided, though this was done for fig 5C and should be performed for the other WBs.

We used GE ImageQuant LAS4000 Multi-Mode Imager to acquire the Western blot images and the densitometric analyses were performed by area quantification using ImageJ. We included this information in the method section. We added the densitometry of Western blot values after normalization above each lane in Fig.2A-C, Fig.3C-D and 3K; Fig.4A-B, Fig.5B,C,I,J.

Reviewer #3 (Significance):

The work of Yabaji et al is of high significance to the field of macrophage biology and M.tb pathogenesis in macrophages. This work builds from previously published work (Bhattacharya 2021) in which the authors first identified the aberrant response induced by TNF in sst1 mutant macrophages. Better understanding how macrophages with the sst1 locus respond not only to bacterial infection but stimulation with relevant ligands such as TNF will aid the field in identifying biomarkers for TB, biomarkers that can suggest a poor outcome vs. "cure" in response to antibiotic treatment or design of host-directed therapies.

This work will be of interest to those who study macrophage biology and who study M.tb pathogenesis and tuberculosis in particular. This study expands the knowledge already gained on the sst1 locus to further determine how early macrophage responses are shaped that can ultimately determine disease progression.

Strengths of the study include the methodologies, employing both bulk and single cell-RNA seq to answer specific questions. Data are analyzed using automated methods (such as HALO) to eliminate bias. The experiments are well planned and designed to determine the mechanisms behind the increased iron-related oxidative stress found in the mutant macrophages following TNF treatment. Also, in vivo studies were performed to validate some of the in vitro work. Examining pauci-bacillary lesions vs multi-bacillary lesions and spatial transcriptomics is a significant strength of this work. The inclusion of human data is another strength of the study, showing increased Myc in humans with poor response to antibiotics for TB.

Limitations include the fact that the work is all done with BMDMs. Use of alveolar macrophages from the mice would be a more relevant cell type for M.tb studies. AMs are less inflammatory, therefore treatment with TNF of AMs could result in different results compared to BMDMs. Reviewer's field of expertise: macrophage activation, M.tb pathogenesis in human and mouse models, cell signaling.

Limitations: not qualified to evaluate single cell or bulk RNA-seq technical analysis/methodology or spatial transcriptomics analysis.

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