

1 ***Mycobacterium tuberculosis* inhibits autocrine type I interferon signaling to increase**
2 **intracellular survival.**

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4 **Running title: Mtb inhibits type I IFN receptor signaling**

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19

20 **Summary**

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22 The type I interferons (IFN- α and - β) are important for host defense against viral infections. In
23 contrast, their role in defense against non-viral pathogens is more ambiguous. Here we report
24 that IFN- β -signaling in macrophages has protective capacity against *Mycobacterium*
25 *tuberculosis* (Mtb) via the increased production of nitric oxide. Furthermore, Mtb is able to
26 inhibit IFN- α/β -receptor-mediated cell signaling and the transcription of 309 IFN- β stimulated
27 genes which includes genes associated with innate host cell defense. The molecular
28 mechanism of inhibition by Mtb involves reduced phosphorylation of the IFNAR-associated
29 protein kinases JAK1 and TYK2 leading to reduced phosphorylation of the downstream targets
30 STAT1 and STAT2. Overall, our study supports the novel concept that Mtb evolved to inhibit
31 autocrine type I IFN signaling in order to evade host defense mechanisms.

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33

34 **Introduction**

35

36 Type I interferons (IFNs) are innate cytokines that are best known for their ability to induce an
37 anti-viral state in cells (1, 2). Upon binding to their shared receptor, type I IFN receptor
38 (IFNAR), a heterodimer composed of IFNAR1 and IFNAR2 transmembrane proteins, the
39 receptor-associated tyrosine kinases JAK1 and TYK2 are activated, this leads to the
40 phosphorylation and activation of STAT1 and STAT2. Activated STAT1 can homodimerize,
41 translocated to the nucleus and bind to IFN- γ -activated sites (GAS) to promote gene
42 transcription of IFN stimulated genes (ISGs). Alternatively, STAT1 will associate with STAT2
43 and IRF-9 to form the transcription factor ISGF3 which then translocates to the nucleus to bind
44 to IFN-stimulated response elements (ISRE) of ISG and induce their expression (3, 4).

45

46 While type I IFNs clearly have a protective function during viral infection, the role of these
47 cytokines during bacterial or protozoan infections is more ambiguous (2, 4-6). IFN- β is
48 detrimental to the host during *Mycobacterium tuberculosis* (Mtb) infections. (7-16) Despite the
49 various outcomes of the type I IFN response to infection it is well documented that many
50 intracellular, non-viral pathogens elicit a host response that leads to the increase in IFN- β
51 production (2, 4, 5). Multiple cell-surface (Toll-like receptors) and intracellular (e.g., retinoic
52 acid inducible gene I) receptors recognize microbial products and initiate signaling pathways
53 that activate IRF3, IRF7 or AP1 to induce transcription of type I IFN genes (2, 4, 5). In
54 particular, Mtb gains access to the host cell cytosol via their ESX-1 type VII secretion system,
55 where secreted bacterial DNA (eDNA) binds to the cyclic GMP-AMP (cGAMP) synthase
56 (cGAS) that subsequently activates the STING/TBK1/IRF3 pathway leading to the increased

57 transcription of type I IFNs genes (17-21). The secretion of bacterial c-di-AMP can also
58 mediate the cGAS-independent activation of the STING pathway (22, 23). Finally, Mtb can
59 induce IFN- β production through mitochondrial stress and subsequent release of mitochondrial
60 DNA (mtDNA) which activates the STING pathway (24).

61

62 The potential of non-viral pathogens to inhibit cell signaling via the IFNAR has not been
63 studied in great detail. One reason for this is probably that the infected host cell detects the
64 pathogen and responds by increased synthesis of IFN- β which confounds the analysis. In
65 order to overcome this problem, we used bone marrow-derived macrophages (BMDM) from
66 *Ifn β ^{-/-}* knock-out mice and investigated the effect of IFN- β on survival of Mtb and the capacity of
67 Mtb to inhibit IFNAR-mediated cell signaling.

68

69 **Materials and Methods**

70

71 **Cell Culture and Mice**

72 *Ifn-β*^{-/-} mice were originally obtained by Dr. E. Fish (University of Toronto) and are described in
73 (25). C57BL/6J and *Nos2*^{-/-} mice were obtained from The Jackson Laboratory. All animal
74 studies were approved by the IACUC and were conducted in accordance with the National
75 Institutes of Health. Bone marrow-derived macrophages (BMDMs) were prepared from bone
76 marrow cells flushed from the femurs and tibia of mice that were cultured in DMEM
77 supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, and either 20%
78 L929 supernatant for BMDMs during a period of 6 days prior to infection. The Raw264.7-
79 derived, *Irf-3* deficient and IFNAR-signaling reporter cell line (RAW-Lucia™ ISG-KO-IRF3) is
80 commercially available, and measurement of reporter activity was performed according to
81 manufacturer's protocol (Invivogen).

82

83 **Ethics statement**

84 All animals were handled in accordance with the NIH guidelines for housing and care of
85 laboratory animals and the studies were approved by the Institutional Animal Care and Use
86 Committee at the University of Maryland (RJAN1702).

87

88 **Bacteria**

89 *M. smegmatis* (mc²155), *M. bovis* BCG-Pasteur and *M. tuberculosis* H37Rv (ATCC 25618)
90 strains were obtained from Dr. W. R. Jacobs Jr. (AECOM). *M. kansasii* strain Hauduroy
91 (ATCC 12478) was obtained from ATCC. Bacterial strains were grown in 7H9 media

92 supplemented with 10% ADC, 0.5% glycerol and 0.05% Tween 80. Hygromycin (50 µg/ml) and
93 kanamycin (40 µg/ml) were added to the mutant and complemented strain cultures,
94 respectively.

95

96 ***Mycobacterium tuberculosis* (Mtb) ex vivo Infection**

97 Bacterial infections of BMDMs were performed as previously described (26). After infection
98 cells were incubated in media containing 100 µg/mL gentamicin in the absence or presence of
99 IFN-β or IFN-γ (Peprotech). For assays using the RAW-Lucia™ ISG-KO-IRF3 reporter cell line,
100 cells were infected at MOI 10 and stimulated with the indicated amount of IFN-β. For
101 measurement of bacterial survival in macrophages, a total of 0.5 million *Ifn-β*^{-/-}, *Nos2*^{-/-},
102 C57BL/6J BMDMs were seeded in 24-well plates and infected with Mtb H37Rv at MOI of 3.
103 Selected BMDMs were then treated with 1000 pg/mL IFN-β twice a day for a total of four days.
104 Cells were lysed at indicated timepoints with 0.1% Triton X-100 in PBS, and serial dilutions
105 were plated on 7H11 agar plates (Difco). Colony forming units (CFUs) were counted after 15-
106 20 days of incubation at 37°C.

107

108 **Transwell infections**

109 6-well transwells with a 0.4µM membrane (Corning) were allowed to equilibrate in medium
110 overnight before seeding cells. 3x10⁶ *Ifnβ*^{-/-} BMDMs were seeded into the upper transwell
111 and 3x10⁶ RAW-Lucia™ ISG-KO-IRF3 cells were seeded in the lower transwell. Infections
112 were performed as described earlier in the upper transwell. Selected conditions were then
113 treated with 200pg/mL IFN-β in the upper and lower transwell for the indicated timepoints.

114

115 **Western blot analysis**

116 Whole cell lysates were obtained by lysing cells with RIPA buffer containing protease and
117 phosphatase inhibitor cocktails (Roche). Protein concentration was measured using the Pierce
118 BCA protein assay kit (Thermo Scientific) and proteins were subjected to SDS-PAGE followed
119 by immunoblotting as described (26). Antibodies were detected binding using SuperSignal
120 West Femto chemiluminescent substrate (Thermo Fisher; 34095) and images were acquired
121 using the LAS-300 imaging system (Fuji). All Western blots were performed at least 3 times
122 and the image of one representative result is shown. ImageJ software (NIH) was used for
123 densitometry quantification as described in figure legends for each Western blot.

124

125

126 **Flow Cytometry**

127 After infection, BMDMs were blocked with 5% FCS and rat anti-mouse CD16/CD32 Fc Block
128 (BD Biosciences, 553141) for 15 min followed by incubation with either PE-conjugated mouse
129 anti-IFNAR1 (Biolegend, 127311) or PE-conjugated goat anti-IFNAR2 (R&D Systems,
130 FAB1083P) for 30 min on ice. PE-conjugated mouse IgG1 (Biolegend, 400111) and goat IgG1
131 (R&D Systems, IC108P) were used as isotype controls. Protein levels were quantified by
132 acquiring 25,000 cells using the Accuri C6 flow cytometer and software (BD Biosciences).
133 Histograms were processed using FlowJo software version 10 (BD Biosciences).

134

135 **Measurement of Nitric Oxide (NO) production**

136 NO production was quantified in cell culture supernatants by the Griess reagent kit which
137 measure the NO derivate Nitrite (ThermoFisher, G7921) at the indicated timepoints.

138 Absorbance was measured at 548nm using a microplate reader (BioTek). Nitrite concentration
139 was determined using a sodium nitrite standard curve (0-100 μ M). Culture supernatants were
140 pooled from three replicate wells per experiment for all infections. All samples were assayed in
141 technical duplicates and three independent experiments were performed.

142

143 **RNAseq Library Preparation and Analysis**

144 *Ifn- β* ^{-/-} BMDMs were infected or not as indicated previously with Mtb H37Rv. At 4 hours post
145 infection (hpi) cells were lysed with 1 mL Trizol (Ambion). RNA was extracted with chloroform,
146 precipitated with 100% isopropanol, and washed with 70% ethanol. Purified RNA was treated
147 with Turbo DNase (Ambion) for 1 hour.

148 RNAseq libraries were prepared using the Illumina ScriptSeq v2 Library Preparation Kit
149 according to the manufacturer's protocol. Library quality was assayed by Bioanalyzer (Agilent)
150 and quantified by qPCR (KAPA Biosystems). Sequencing was performed on an Illumina
151 HiSeq 1500 generating 100bp paired-end reads. RNA-Seq read quality was assessed using
152 FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and low-quality base-pairs
153 were removed using Trimmomatic (27). The Ensembl *Mus musculus* GRCm38 reference
154 genome (version 76) was downloaded from the Ensembl website (28) and reads were mapped
155 to the genome using TopHat2 (29). HTSeq (30) was used to quantify expression as the gene
156 level. Count tables were loaded into R/Bioconductor (31). log₂-transformed, counts-per-million
157 (CPM) and quantile normalized (31), and variance bias was corrected for using Voom (32).
158 Next, batch adjustment was performed using ComBat (33). We performed Pearson correlation,
159 Euclidean distance and PCA analyses which revealed the presence of a single outlier Mtb
160 sample (HPGL0627), which was removed from subsequent analyses (Table S1). The

161 differential gene expression was measured for each of several contrasts: 1) uninfected (UI) vs.
162 uninfected +IFN- β (UI +IFN- β) and 2) Mtb + IFN- β vs. UI + IFN- β (Table S1). In order to
163 determine which IFN- β -stimulated genes were specifically deregulated during infection with
164 Mtb, the intersection of the set of genes found to be differentially expressed in both the UI vs.
165 UI + IFN- β and Mtb + IFN- β vs. UI + IFN- β contrasts was taken (Table S1). All raw RNAseq
166 data were submitted to SRA and can found at <https://www.ncbi.nlm.nih.gov/sra/SRP130272> .

167

168 **Cytokine Measurements**

169 *Ifn- β ^{-/-}* BMDMs were infected as described previously and treated with 50 pg/mL IFN- β . An
170 additional 50 pg/mL of IFN- β was added at 3 hpi, and cell culture supernatants were collected
171 at 6 hpi. Concentrations of selected cytokines were determined using a custom ProcartaPlex
172 magnetic bead-based multiplex assay (Thermo Fisher Scientific) on the Luminex MAGPIX[®]
173 platform according to manufacturer's instructions. CCL12 and CCL3 protein levels were
174 measured using ELISAs (R&D Systems)

175

176 **Statistical Analysis**

177 Statistical analysis was performed on at least three independent experiments using GraphPad
178 Prism 7.0 software and one-way ANOVA with Tukey's post-test or Student's t-test, and
179 representative results of triplicate values are shown with standard deviation unless otherwise
180 noted in the legends. The range of p-values is indicated as follows: * 0.01<p<0.05; **
181 0.001<p<0.01, *** 0.0001<p<0.001, and **** p<0.0001.

182

183 **Results:**

184

185 **IFN- β has anti-microbial activity via induction of *Nos2***

186 The analyses of the importance of IFN- β for host defense during infection with non-viral
187 pathogens has proven to be complex since during *in vivo* infections host genetic components,
188 tissue environment and actual doses of IFN- β all can affect outcomes (2, 4, 6). In order to
189 investigate the importance of IFNAR-signaling we used a reductionist approach by eliminating
190 bystander cell, host tissue effects and the host cell production of IFN- β in response to infection
191 by using BMDM from *Ifn- β ^{-/-}* mice (25). We infected *Ifn- β ^{-/-}* BMDMs with *Mycobacterium*
192 *tuberculosis* (Mtb), in the presence or absence of 1ng/mL IFN- β . The bacterial burden was
193 measured every 24 h for a total of 96 h by counting colony forming units (CFUs). During Mtb
194 infection, we found that reduction in bacterial burden in IFN- β -treated cells was minimal at 24
195 hpi and 48 hpi, but steadily increased between 48 hpi and 96 hpi. At 96 hpi, IFN- β treatment
196 reduced bacterial burden by ~50% (Figure 1A). We also determined that IFN- β -treated, Mtb-
197 infected cells exhibited less necrosis compared to untreated infected cells since an increased
198 release in gentamycin containing medium could have otherwise accounted for the reduction of
199 CFU (Figure 1B). These results demonstrate that IFN- β treatment promotes host defense
200 against intracellular microbes during infection of primary macrophages. The direct anti-
201 microbial activity of IFNAR-signaling on Mtb viability that we show here has not been
202 demonstrated before in a system in which the infection itself does not produce IFN- β .
203 It was previously shown that type I IFN signaling may lead to the induction of nitric oxide
204 synthase 2 (*Nos2*) gene expression and subsequent nitric oxide (NO) production infected

205 BMDMs (34). In order to investigate whether NO production plays a role in IFN- β -mediated
206 clearance *ex vivo*, we determined if NOS2 was expressed at time points correlating with the
207 reduction in bacterial burden during Mtb infection. At 72 hpi, NOS2 was upregulated in Mtb-
208 infected cells, and NOS2 levels were further increased in Mtb-infected cells stimulated with
209 IFN- β (Figure 1C). At 96 hpi the NOS2 protein levels were decreased compared to 72 hpi,
210 however there was still a significant upregulation in Mtb-infected cells stimulated with IFN- β
211 compared to uninfected cells (Figure 1C). Consequently, we measured NO levels in the
212 different experimental groups in order to assess the effect of IFN- β and infection on NO
213 production. In Mtb-infected cells treated with 1ng/ml IFN- β , we noticed a sharp increase in
214 nitrite levels which is consistent with the observed increase in NOS2 levels (Figure 1D). We
215 then investigated whether or not IFN- β could promote host resistance in *Nos2*^{-/-} BMDMs. We
216 infected wild-type (WT) or *Nos2*^{-/-} BMDMs with Mtb in the presence or absence of 1ng/ml IFN-
217 β . Infected BMDMs showed a significant decrease in CFUs at 96 hpi in IFN- β treated cells
218 (Figure 1E-F). In contrast, we measured no significant decrease in IFN- β -treated *Nos2*^{-/-}
219 BMDMs at either 72 hpi or 96 hpi (Fig 1E-F). These results demonstrate that IFN- β treatment
220 promotes host resistance to Mtb via the production of NO at later time points during *ex vivo*
221 infection.

222

223 **Mtb inhibits IFNAR-signaling**

224 Viruses are well known to evade host protective IFN- β responses by suppressing
225 signaling via IFNAR (1, 2). Our results on the host protective effect of IFN- β on Mtb infection
226 prompted us to investigate the potential of Mtb to inhibit IFNAR-signaling. To this purpose we
227 used a reporter RAW264.7 cell line (Invivogen) which is deficient in *Irf-3* and has an ISG

228 promoter in front of a reporter gene for easy quantification of IFNAR-signaling. We first
229 determined the capacity of these pathogens to induce activation of the reporter in the absence
230 of an external IFN- β stimulus. Msm, Mtb H37Rv, Mtb CDC1551 did not induce activation of the
231 ISG reporter in the absence of external stimulation. We also determined that the infection with
232 the different mycobacterial species does not induce the production of IFN- β via ELISA since
233 these cells are deficient in IRF-3 (not shown). Next, we added increasing amounts of IFN- β to
234 infected or uninfected cells and normalized the response to the response obtained in
235 uninfected cells (Figure 2A). Overall, both virulent Mtb strains consistently showed about 50-
236 60% of inhibition whereas infection with Msm had only a minor effect (10-15%) at higher doses
237 of IFN- β (Figure 2A). Notably, this effect was reversed at 1000pg/ml, suggesting there is a limit
238 to the amount of signaling Mtb can inhibit. To our best knowledge the described experiments
239 are the first to demonstrate the capacity of Mtb to inhibit IFNAR-signaling. The lack of
240 investigation into this capacity of the pathogens might be explained by the proposed overall
241 role of type I IFN in exacerbation of disease outcomes (2, 4-6).

242

243 **Mtb affects the type I IFN-stimulated gene host transcriptional profile**

244 Signaling via the IFNAR induces the formation the IFN-stimulated gene factor 3 (ISGF3)
245 complex and or STAT1:STAT1 homodimerization which both translocate into the nucleus and
246 bind to ISRE or GAS, respectively, leading to the transcription of hundreds of genes involved in
247 a variety of immunological functions (3). Consequently, we hypothesized that by inhibiting type
248 I IFN signaling, Mtb can manipulate the expression of host genes to promote its intracellular
249 survival. To investigate this, we used RNA sequencing technology (RNAseq) to identify IFN- β -
250 regulated genes that are up- or down-regulated during *Mtb* infection in *Ifn- β ^{-/-}*-derived BMDMs.

251 We analyzed the following experimental groups: 1. uninfected, untreated (UI), 2. uninfected
252 IFN- β treated (UI+) and 3. Mtb H37Rv infected and treated (Mtb+) BMDMs (Table S1). The
253 RNAseq data was reproducible as indicated by principle component analysis (PCA), hierarchal
254 clustering and Pearson correlation (Figure S1). All three analyses depicted a satisfactory
255 degree of clustering between biological replicates with the exception of one outlier
256 (HPGL0627), which was excluded from further analysis (Figure S1 and Table S1). In order to
257 identify IFN- β -stimulated genes that exhibited differential expression in Mtb-infected BMDMs,
258 we first characterized all of the IFN- β -regulated genes in our experimental system. To that
259 purpose we compared the gene expression of uninfected, untreated (UI) to uninfected, IFN- β
260 treated (UI+) conditions. We identified a total of 1144 IFN- β -stimulated genes and 956 genes
261 with reduces expression (> 2 fold change) (Figure S2A, Table S1). Next, in order to identify all
262 of the genes that are impacted by Mtb-infection we compared gene expression levels between
263 the UI +IFN β condition and the Mtb H37Rv infected and treated (Mtb+) and found 1296
264 upregulated and 1294 downregulated (> 2 fold change and an adjusted p-value <0.05) genes
265 (Figure S2B, Table S1). Finally, Mtb-infection causes the deregulation of many genes that are
266 not regulated by IFN- β but will be included in the UI+ versus Mtb+ contrast. The overlap
267 between these two gene sets was determined to be 309 genes with reduced expression and
268 170 with increased expression for a total of 479 deregulated genes (Figure S2C, Table S1).
269 To extend the results of our RNAseq analysis, we determined protein expression and secretion
270 levels of selected targets in *Ifn- β ^{-/-}* BMDMs infected with Mtb in the presence or absence of
271 IFN- β (Figure 2B). The selection of candidate gene products for follow-up studies was based
272 on magnitude of deregulation and availability of antibodies (see list of 479 genes in Table S1).
273 Rnf144A and Rnf144B proteins were not significantly upregulated upon treatment with IFN- β

274 (Figure 2C) which may reflect a low fold-increase at the RNA level as illustrated in the heatmap
275 (Figure 2B), however IFI204, IFIT1, MX1 and IIGP1 were strongly induced after treatment with
276 IFN- β (Figure 2C-D). For all targets, we observed some degree of downregulation in IFN- β -
277 treated cells infected with Mtb. In addition, we used ELISA to demonstrate the strong inhibition
278 of IFN- β -driven secretion of CCL12 by Mtb infection (Figure 2E). In conclusion, these data
279 show a strong correlation of the RNAseq analysis with protein data for the subset of 309 IFN- β -
280 regulated genes that are repressed by Mtb infection (Figure 2B and Table S1). We additionally
281 investigated some of the 170 genes that were strongly upregulated in the 479 genes set (Table
282 S1) and characterized several cytokine/chemokine targets using a combination of both
283 multiplexed and regular ELISA and determined that Mtb infection of BMDMs without addition of
284 IFN- β upregulated secretion levels of all assayed proteins (Figure S3). For IL-1 β , TNF α and
285 CXCL1 there seemed to be additive effected in Mtb +IFN- β treated cells when compared to UI
286 +IFN- β alone and Mtb -IFN- β mediated cytokine induction, whereas there was an antagonistic
287 effect of the Mtb-infection for IL-27, IL-1 α , CCL5 and CCL3 suggesting that the induction of
288 these cytokines in Mtb-infected cells is independent of IFNAR-signaling and that their
289 upregulation caused by addition of IFN- β can be inhibited by Mtb (Figure S1). Overall, this
290 analysis shows that this subset of 170 of Mtb-deregulated genes after IFN- β addition that is
291 upregulated provides less insights because many of the genes seem to be upregulated by Mtb
292 infection alone.

293

294 **Mtb inhibits type-I but not type-II IFN-mediated activation of TYK2, JAK1**

295 We discovered that Mtb inhibits signaling via IFNAR and now we investigated further at
296 what level of the signaling cascade the inhibition occurs. Several viral pathogens have evolved

297 mechanisms to evade the IFN- β response by promoting the degradation of IFNAR (35, 36).
298 We found that surface expression levels of IFNAR1 and IFNAR2 using flow cytometry
299 remained unchanged by Mtb infection (Figure 3A). After stimulation of IFNAR1/R2 by IFN- β ,
300 the cytosolic protein tyrosine kinases JAK1 and TYK2 are recruited and phosphorylated. We
301 showed that Mtb-inhibited tyrosine phosphorylation of both TYK2 and JAK1 as early as 20 min
302 post infection (Figure 3B, 3C). Since JAK1 is involved in both type I and type II interferon
303 signaling, we also analyzed phosphorylation levels of JAK1 and JAK2, another IFN- γ -activated
304 protein kinase. In IFN- γ -treated and Mtb-infected cells there was no significant change in JAK1
305 or JAK2 phosphorylation levels, showing that the inhibition of JAK1 phosphorylation is specific
306 to type I IFN signaling (Figure 3D, 3E).

307

308 **Mtb inhibits downstream phosphorylation of STAT1 and STAT2**

309 Type I and type II IFNs induce transcription of different subsets of genes but STAT1
310 phosphorylation occurs in both signaling pathways (3). The canonical signaling pathways are
311 defined as follows: type I IFN induces the heterodimerization of STAT1 and STAT2, while type
312 II IFN (IFN- γ) induces homodimerization of STAT1, although type I IFN can also induce STAT1
313 homodimerization (3). We performed an infection comparing STAT1 tyrosine phosphorylation
314 levels in IFN- β -treated *Ifn- β ^{-/-}* BMDMs infected with virulent Mtb strains H37Rv or strain
315 CDC1551 and observed that both could similarly inhibit STAT1 phosphorylation, suggesting
316 that this mechanism of host cell manipulation is shared among virulent Mtb strains (Figure 4A).
317 We also showed that Mtb did not inhibit IFN- γ -dependent phosphorylation of STAT1 (Figure
318 4B) which is consistent with previously published results (37) and suggest a separate
319 molecular pathway engaged by Mtb for the inhibition of IFNAR-signaling.

320 Besides STAT1, other STAT isoforms may be stimulated by type I IFN signaling. STAT2
321 becomes phosphorylated and heterodimerizes with pSTAT1 and IRF9 to form the ISGF3
322 complex, which translocates into the nucleus to induce transcription of genes containing
323 interferon stimulated regulatory elements (ISREs) (3). In addition, it has also been shown that
324 STAT3 can be induced by type I IFNs to regulate transcription of different gene subsets (38).
325 We discovered that Mtb also inhibited the tyrosine phosphorylation of STAT2 (Figure 4C).
326 However, Mtb actually induced phosphorylation of STAT3 (Figure 4C). STAT3 phosphorylation
327 can inhibit type I IFN-mediated signaling, although this occurs at the level of nuclear
328 translocation (39, 40). Thus, STAT3 activation is most likely not involved in our observed
329 inhibition of TYK2/JAK1 phosphorylation by Mtb.

330 The levels of IFN- β production by mycobacteria-infected cells vary depending on the
331 mycobacterial species and, in the context of Mtb, the specific strain that is used for the
332 infection (24, 41, 42). We sought to test if the difference in type I IFNs production observed for
333 the different mycobacterial species correlated with their variable capacity to inhibit IFNAR-
334 mediated cell signaling. Here we observed a more modest decrease in the relative STAT1
335 phosphorylation in cells infected with the vaccine strains *M. bovis* BCG (BCG) and *M. kansasii*
336 but only a minor reduction upon infection with *M. smegmatis* (Fig 4D). Notably, the capacity of
337 Mtb to inhibit STAT1 and STAT2 phosphorylation was also reversed upon addition of a high
338 dose of IFN- β , supporting the data using our reporter cell line (Fig 4E-F).

339 340 **Mtb infection does not inhibit IFN- β signaling in bystander cells**

341 In order to determine whether inhibition of IFN- β signaling is specific to Mtb infected
342 cells, and not due to the secretion of a soluble host factor, we sought to determine if there was

343 an effect in bystander cells. To address this, we used a transwell system in which the upper
344 transwell was seeded with *Ifn-β*^{-/-} BMDMs and the lower transwell was seeded with our ISG
345 reporter cell line (Fig 5A). The upper transwell was then infected or not with Mtb, and both
346 wells were stimulated or not with IFN-β as earlier. In this system, a reduction of secreted
347 luciferase from the cells in the lower transwell would suggest that there is indeed a bystander
348 effect. In our hands, we saw equal levels of reporter activity in bystander cells exposed to both
349 uninfected and infected *Ifn-β*^{-/-} BMDMs, suggesting that the inhibition is specific to infected
350 cells (Fig 5B). Western blots of *Ifn-β*^{-/-} BMDM lysates (Fig 5C) and the ISG reporter cell line
351 (Fig 5D) confirm that we do still see inhibition of STAT1 phosphorylation in infected *Ifn-β*^{-/-}
352 cells, however, STAT1 phosphorylation of bystander cells is not affected by the infection status
353 of the *Ifn-β*^{-/-} BMDMs.

354

355 Discussion

356 While type I IFN are largely considered to be beneficial in the context of viral infections, their
357 role during bacterial infections is not completely understood and may vary depending on the
358 bacterial pathogen and the site of infection. In the context of Mtb infections, type I IFN are
359 considered to be detrimental to the host, and numerous recent studies have worked toward
360 better understanding why. Surprisingly, we discovered an anti-microbial effect of type I IFN
361 during Mtb infection in macrophages via the production of nitric oxide (NO). The role of NO in
362 host resistance to tuberculosis has been extensively investigated and its bactericidal activity is
363 attributed to the reactive nitrogen intermediates (RNI) such as NO₂⁻, NO₃⁻ and peroxynitrite
364 (ONOO⁻) (43, 44). Activation of macrophages with IFN-γ was especially powerful in
365 augmenting RNI-mediated killing of Mtb (45-47). Two recent studies demonstrate that the

366 major host protective role of NO during an *in vivo* infection with Mtb may not be its bactericidal
367 activity but its immunosuppressive activity leading to reduced host tissue pathology (48, 49).
368 NO is generated in the cell cytosol by NOS2 and it diffuses rapidly and freely at an estimated
369 5-10 cell lengths per second (50). This means that during an *in vivo* infection dilution is an
370 important factor as within 1s the NO concentration has been diluted over 200 times in the NO-
371 generating cell (50). In addition, the proteasome of Mtb has a role in resistance of the bacteria
372 to RNI stress (51). It is thus possible that NO levels in the infected cells that actually generate
373 the NO fail to accumulate to bactericidal threshold levels *in vivo* due to diffusion and dilution as
374 compared to *ex vivo* infection experiments which are in a closed system and contain mostly
375 infected cells. In any case, the potential of Mtb to inhibit IFN- β -mediated NO production will be
376 advantageous for the pathogen. We do not believe that the recently reported direct bactericidal
377 effect of IFN- β (52) plays a role in our observed bactericidal effects since otherwise the *Nos2*^{-/-}
378 cells should not be different from wild-type BMDM (Figure 1E).

379
380 It seems confounding that Mtb would want to both induce IFN- β production and simultaneously
381 inhibit it's signaling. Considering that Mtb is a facultative intracellular pathogen, we
382 hypothesize that inhibiting type I interferon signaling allows Mtb to mitigate the anti-bacterial
383 effects of IFN- β -induced autocrine signaling (Figure 1). The transcriptome analysis of IFN- β
384 regulated genes and their deregulation by Mtb infection suggests that there are some type I
385 IFN responses that are detrimental to the bacterium during infection. CCL12/MCP-5 is one of
386 the IFN- β -regulated cytokines that is strongly inhibited by Mtb infection at the mRNA and
387 protein level (Figure 2B, 2E). It is a chemoattractant for monocytes and an agonist of CCR2
388 (53). Its expression on macrophages is induced by LPS and IFN- γ (53). Interestingly, another

389 chemoattractant for monocytes, CCL5, is also upregulated via IFN- β -signaling and its
390 expression is inhibited by Mtb (Figure S3). Consequently, the repression of these chemokines
391 could lead to a reduction of monocyte recruitment within the Mtb-infected lungs. The type I
392 IFN-driven expression of chemokines has been shown to be an important signal for
393 recruitment of bone marrow monocytes to the site of infection with *Listeria monocytogenes*
394 (54). Among other top down-regulated genes for which we also have confirmed reduced
395 protein levels are the immunity-related GTPases *Ifi204*, *Ifit1* and *Iigp1* (Figure 2) (55). *IIGP1* is
396 involved in host defense against *Chlamydia trachomatis* and *Toxoplasma gondii* (56, 57). It is
397 unlikely that this protein, at least in vivo, is involved in host defense against Mtb since *Iigp1*^{-/-}
398 deficient mice do not have a phenotype (58). *IFI204* is a cytosolic DNA sensor that binds to
399 extracellular Mtb DNA and induces the cytosolic surveillance pathway via STING/TBK1/IRF3
400 signaling (17). Consequently, reduction of *IFI204* expression by Mtb will reduce the activation
401 of IRF3 and its regulon which includes *Ifn- β* transcription. This could thus represent an
402 additional mechanism, in addition to the expression of the phosphodiesterase *CdnP* (23), by
403 which Mtb regulates IFN- β production. Interestingly, *M. bovis* does not inhibit *IFI204*
404 expression and hence this host cell protein has an important role in host IFN- β production for
405 this mycobacterial species (59). It is important to highlight, though, that the main cytosolic
406 DNA sensor involved in recognition of Mtb DNA is cGAS (19-21). *IFIT1* has well established
407 anti-viral activity and is most strongly induced by IFN- β (60). Its activity is dependent upon
408 selective binding of 5'-terminal regions of *cap0*-, *cap1*- and 5'ppp- mRNAs (61) and, hence,
409 *IFIT1* is an unlikely candidate for functioning in host cell resistance against Mtb infection. The
410 expression of the *Nos2* gene is not affected by Mtb which we think is due to the early timepoint
411 (4hpi) selected for the RNAseq analysis. *Nos2* can be induced by signaling via many TLRs and

412 cytokine receptors (43, 62). In our system, Mtb infection causes a strong induction of TNF
413 secretion which is amplified by addition of IFN- β (Figure S3). Consequently, we believe that
414 TNF is the most likely cause for the observed late induction of NOS2, especially since it has
415 been noted before that IFN- β and TNF synergistically mediate the induction of *Nos2* gene
416 transcription (43, 62-64).

417

418 The capacity of Mtb to induce activation of STAT3 was recently reported (65) and the
419 importance for STAT3 activation for virulence of Mtb was recently demonstrate by showing that
420 deletion of STAT3 in myeloid cells increase resistance of mice to Mtb infection (66). STAT3
421 phosphorylation can inhibit type I IFN-mediated signaling, although this does not seem to
422 occur at the level of STAT1/STAT2 phosphorylation but rather at the level of nuclear
423 translocation (39, 40). Thus, STAT3 activation is likely not involved in our observed inhibition
424 of TYK2/JAK1 phosphorylation by Mtb. However, we cannot exclude the possibility that Mtb
425 exerts multiple strategies that synergize to prevent transcription of IFN- β -regulated genes and
426 that STAT3-mediated inhibition may play a role during later time points to sustain the initial
427 signaling inhibition. The capacity of STAT3 to inhibit *Nos2* gene expression may explain why
428 NOS2 is only detected after 72h to 96h (Figure 1E). SOCS1, SOCS3, and USP18 are
429 negative regulators of the IFNAR signaling which are typically induced at a later time point
430 during IFN- β stimulation and serve as negative feedback regulators (3). Considering that our
431 observed inhibition occurs already as early as 5 min post-infection, albeit an infection period of
432 4h, it is highly unlikely that the molecular mechanism of at least the early inhibition is
433 dependent on these common negative regulators. It is known that Mtb possesses several
434 phosphatases that have been shown to affect the host immune response by interfering with

435 several signal transduction pathways. SapM is a phosphoinositide phosphatase that is
436 essential in arresting phagosomal maturation by inhibiting phosphatidylinositol 3-phosphate
437 phosphorylation (67). The tyrosine phosphatase PtpA is also involved inhibiting phagosomal
438 maturation through inhibition of V-ATPase, and PtpB has been shown to inhibit ERK 1/2 and
439 p38 signaling cascades (67-69). Although beyond the scope of this study, it would be
440 interesting to investigate whether these proteins also regulate IFN- β -mediated signaling by
441 directly dephosphorylating TYK2 or JAK1.

442
443 We here show that Mtb is susceptible to host cell IFNAR-signaling and has evolved to
444 suppress it. In particular, Mtb inhibits IFNAR-mediated signaling at the level of the receptor-
445 associated tyrosine kinases JAK1 and TYK2 (Figure 3). Nevertheless, this inhibition can be
446 overcome by high concentrations of extracellular IFN- β , leading to reduced viability of
447 intracellular Mtb. It is well established that Mtb induces the STING/TBK1/IRF3 signaling axis
448 via extracellular Mtb DNA (eDNA) (17, 19-21), damage to host cell mitochondria leading to
449 increase in cytosolic mitochondrial DNA (mtDNA) (24) or secretion of cyclic-di-AMP (c-di-AMP)
450 (22, 23) (Figure 6). The capacity to produce IFN- β is associated with *in vivo* virulence of Mtb
451 infections in mouse models and human studies (7, 12, 13, 15, 16). The levels of IFN- β
452 production by mycobacteria-infected cells vary depending on the mycobacterial species and, in
453 the context of Mtb, the specific strain that is used for the infection (24, 41, 42). High levels of
454 IFN- β drive production of IL-10 and IL-1Ra (Figure 6) which antagonize the host protective
455 activity of IL-1 β and ultimately lead to increased host tissue destruction which establishes a
456 replicative niche for Mtb (8). In contrast to the current dogma that Mtb induces production of
457 type I IFNs in order to support its virulence, multiple studies have provided evidence that in

458 some settings type I IFNs may have detrimental effects on Mtb. For example, *Isg15* is one of
459 the most highly upregulated genes after type I IFNs stimulation of cells and it has a host
460 protective role during Mtb infection (70). Furthermore, in the absence of IFN- γ , type I IFNs can
461 promote the activation of macrophages for improved innate host response to Mtb (34). The
462 strongest evidence for a host protective element in the IFN- β response was produced by
463 showing that *Ifngr*^{-/-} *Ifnar*^{-/-} double-knock out mice are more susceptible when compared to *Ifn- γ*
464 ^{-/-} mice (6, 34, 71). IFN- β is protective during mouse infections against two non-tuberculous
465 mycobacterial species (*M. smegmatis* and *M. avium ssp. Paratuberculosis*) (72). In this
466 context, it is less surprising that Mtb has also evolved mechanisms to limit production of type I
467 IFNs, possibly to achieve the “Goldilocks principle”. Overall, Mtb infection causes less
468 production of IFN- β in *ex vivo* infected macrophages when compared to non-tuberculous
469 mycobacteria (41). Indeed, we have shown previously that Mtb can inhibit *M. smegmatis*-
470 induced IFN- β production in an ESX-1-dependent manner in bone marrow-derived dendritic
471 cells (BMDCs) (41). There are potentially several mechanisms by which this inhibition occurs.
472 The Mtb-mediated stimulation of TLR2 inhibits the induction of type I IFNs via TLR7/9
473 activation (73). In addition, the Mtb phosphodiesterase CdnP can reduce type I IFNs
474 production by hydrolyzing bacterial-derived c-di-AMP and host-derived cGAMP, thereby
475 limiting activation of the STING pathway (23). Importantly, this inhibition is relevant for full
476 virulence of Mtb since a *CdnP* transposon mutant of Mtb is attenuated in mice (23).

477

478 We propose that Mtb has evolved to inhibit autocrine IFN- β signaling and its host protective
479 effects in order to still take advantage of the benefits of paracrine IFN- β signaling on uninfected
480 bystander cells, which is not inhibited by the Mtb-infected cells (Figure 5). This model might

481 explain why in some settings IFN- β may be beneficial for the host (6); for example, in the case
482 of nontuberculous mycobacteria which cannot inhibit IFNAR-mediated signaling (e.g. *M.*
483 *smegmatis*) and who are susceptible to a host type I IFN response (72).

484

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488

489

490 **Figure Legends**

491

492

493 **Fig 1. IFN- β treatment has a broad anti-microbial activity**

494 (A) *Ifn- β* ^{-/-} BMDMs were infected with Mtb H37Rv in the presence or absence of 1000pg/ml
495 IFN- β . The number of bacteria by CFUs in treated conditions relative to untreated cells was
496 then calculated. (B) Cell necrosis was assayed at each indicated time point using an
497 adenylate kinase release assay (Toxilight bioassay, Lonza) and is represented as fold change
498 over untreated infected cells. (C) Whole cell lysates were collected at the indicated timepoints
499 and immunoblotted for NOS2. Band densities were normalized to GAPDH. (D) Nitrite levels
500 from culture supernatants were determined using the Griess reagent. (E, F) WT or *Nos2*^{-/-}
501 BMDMs were infected with Mtb H37Rv in the presence or absence of 1000pg/ml IFN- β .
502 Bacterial burden was determined as in Fig. 1A. All data shown are presented as mean \pm
503 S.E.M. of at least three independent experiments.

504

505 **Fig 2. Mtb inhibits type I IFN signaling**

506 (A) IRF-3 deficient RAW264.7 macrophages transfected with an IFN- β responsive
507 luciferase gene (Invivogen) were infected and treated with the indicated concentrations of IFN-
508 β . After 20hpi the amount of secreted luciferase was quantified. ISG induction is represented
509 as the fold change in RLU compared to uninfected cells. (B) Heatmap of down-regulated
510 genes selected for follow-up studies. Log-transformed expression ratios for Mtb + IFN β /UI +
511 IFN- β are plotted for each gene. (C-E) *Ifn- β* ^{-/-} BMDMs were infected with *M. tuberculosis*
512 H37Rv (Mtb) and treated with 50 pg/mL IFN- β for 4 hours. (C) Whole cell lysates were
513 collected and immunoblotted for IFI204, IFIT1, Rnf144A, Rnf144B. Band density was

514 normalized to β -actin. (D) Cell lysates were collected and immunoblotted for either MX1,
515 IIGP1, and normalized to GAPDH. (E) Supernatants were analyzed for levels of CCL12 using
516 ELISA. All data shown are presented as mean \pm S.E.M. of at least three independent
517 experiments.

518

519 **Fig 3. Mtb inhibits type-I but not type-II IFN-mediated activation of TYK2 and JAK1**

520 (A) *Ifn- β* ^{-/-} BMDMs were infected with Mtb H37Rv and flow cytometry was conducted at 4hpi to
521 measure surface receptor expression levels of IFNAR1 and IFNAR2. (B and C) *Ifn- β* ^{-/-} BMDMs
522 were infected as described in the presence of 300pg/ml IFN- β . Cell lysates were collected at
523 20 min post infection and immunoblotted for pJAK1 (Y1022/1023), total JAK1, pTYK2
524 (Y1054/1055), and total TYK2. (D and E) *Ifn- β* ^{-/-} BMDMs were infected as described in the
525 presence of 300pg/ml IFN- γ . Cell lysates were collected at 20 min post infection and
526 immunoblotted for pJAK1 (Y1022/1023), total JAK1, pTYK2 (Y1054/1055), and total TYK2.
527 Densitometry was performed using ImageJ software and phosphorylated protein bands were
528 normalized to total signal for each condition. Data and densities shown represent one
529 representative experiment out of three.

530

531 **Fig 4. Mtb inhibits phosphorylation of STAT1 and STAT2**

532 (A-B) *Ifn- β* ^{-/-} BMDMs were infected with either Mtb strains H37Rv or CDC1551 and treated with
533 50pg/ml IFN- β (A) or 50pg/ml IFN- γ (B). Whole cell lysates were collected at 4 hours post
534 infection and immunoblotted for pSTAT1 (Y701) and total STAT1. (C) *Ifn- β* ^{-/-} BMDMs were
535 infected with Mtb in the presence or absence of 300pg/ml IFN- β . Whole cell lysates were
536 collected at 20 min post infection and immunoblotted for pSTAT2 (Y690), total STAT2,

537 pSTAT3 (Y705), or total STAT3 as indicated. (D) Cells were infected with the indicated
538 *Mycobacteria* strains and treated with 50pg/ml IFN- β . Whole cell lysates were collected at 4
539 hours post infection and immunoblotted for pSTAT1 and total STAT1. (E-F) *Ifn- β ^{-/-}* BMDMs
540 were infected with Mtb and treated with 1ng/ml IFN- β . Whole cell lysates were collected at 4
541 hours post infection and immunoblotted for pSTAT1, total STAT1, pSTAT2 or total STAT2 as
542 indicated.

543

544 **Fig 5. Mtb does not inhibit type I IFN signaling in bystander cells**

545 (A) Schematic of transwell experiments. (B) Upper transwells were infected or not with Mtb,
546 and then the upper and lower transwells were treated with 200pg/ml IFN- β for 4 hours. Cells in
547 the lower transwell were lysed by addition of Triton-X-100, and the amount of luciferase was
548 quantified. (C) Whole cell lysates from the upper transwell of *Ifn- β ^{-/-}* BMDMs were collected at
549 4hpi and immunoblotted for pSTAT1, total STAT1 or actin as indicated. (D) Whole cell lysates
550 from the lower transwell were collected at 4hpi and immunoblotted for pSTAT1, total STAT1, or
551 actin as indicated. UI or Mtb refers to the infection condition of the upper transwell.

552

553 **Fig 6. Overview of type I IFN signaling and production in Mtb-infected cells**

554

555 Mtb induces the production of IFN- β in infected cells via release of bacterial DNA (eDNA) (17,
556 19-22) or damage to host cell mitochondria followed by increased mitochondrial DNA (mtDNA)
557 in the cytosol (24). All these factors initiate the STING/TBK1 signaling pathway that leads to
558 activation of the transcription factor IRF-3 and increased IFN- β production. The secreted IFN- β
559 acts on bystander cells to increase secretion of IL-10 and IL-1Ra which lead to increased host

560 cell necrosis and tissue damage, thus exacerbating the disease outcome (8). Here we show
561 that Mtb inhibits autocrine IFNAR-signaling which limits not only the production of IFN- β but
562 also the expression of genes with host cell defense properties such as *Nos2*.

563

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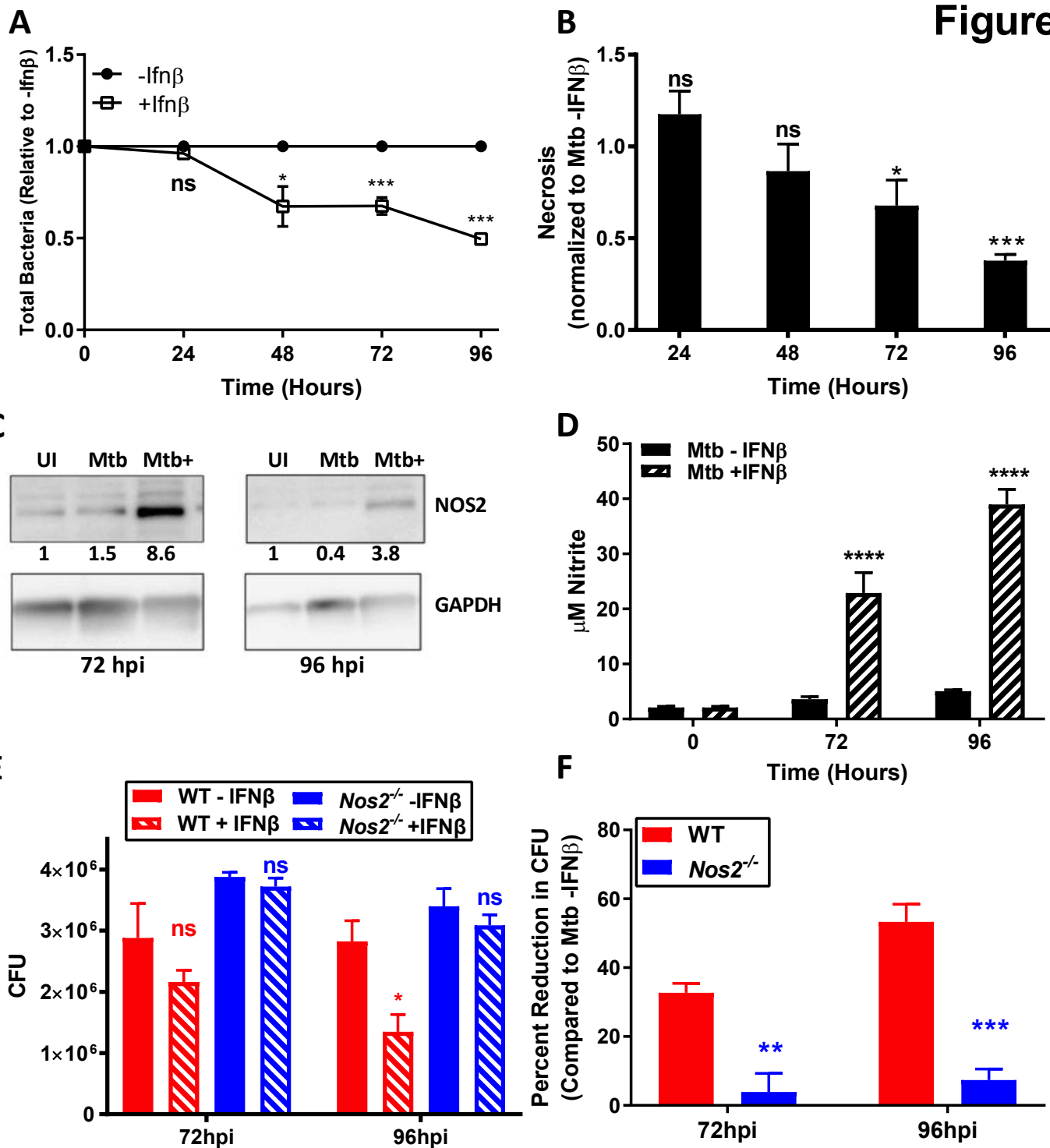
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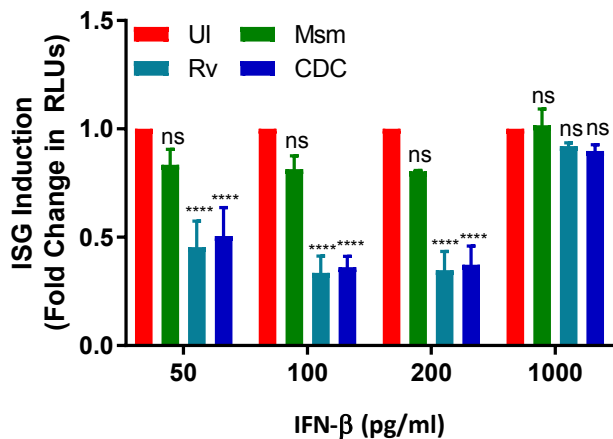
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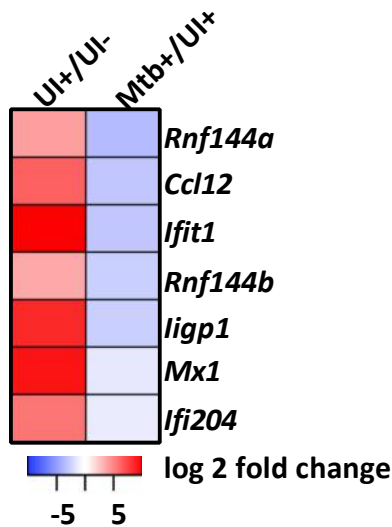
Figure 1



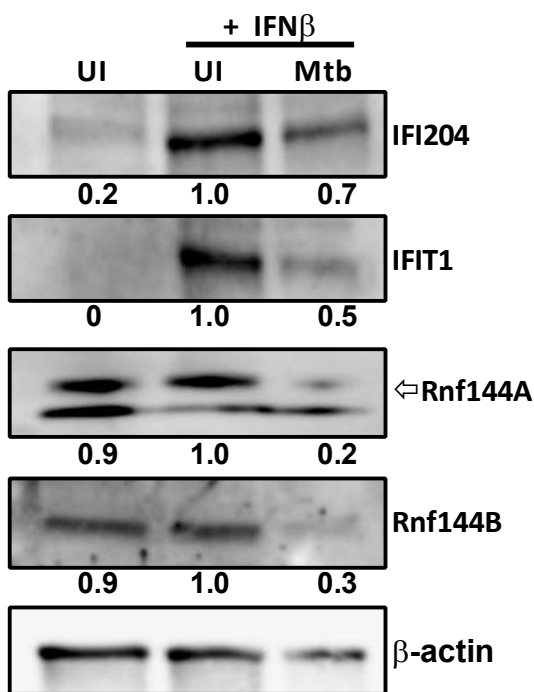
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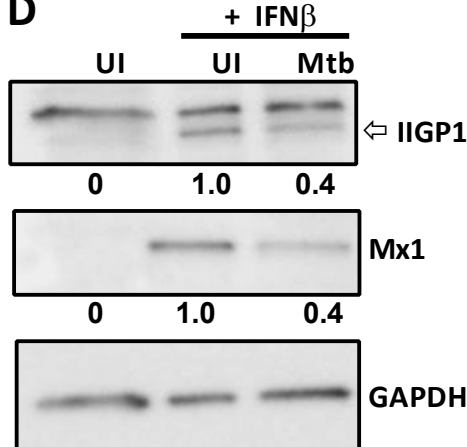
B



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D



E

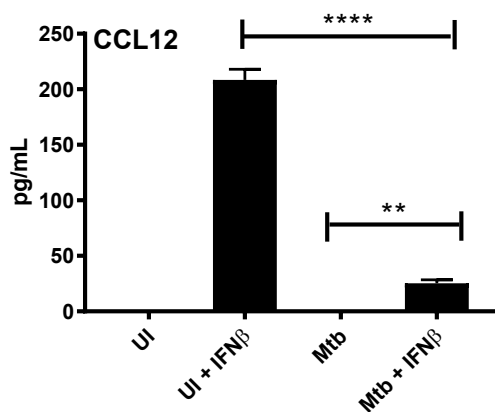


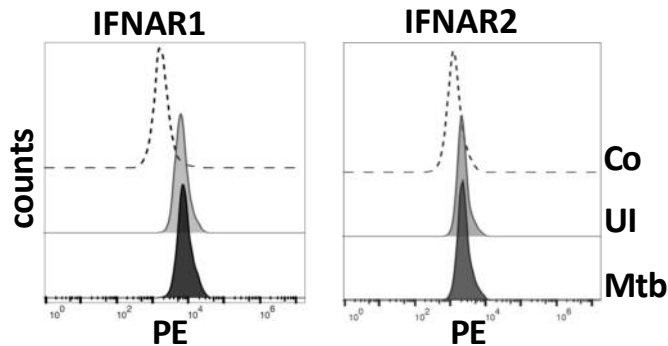
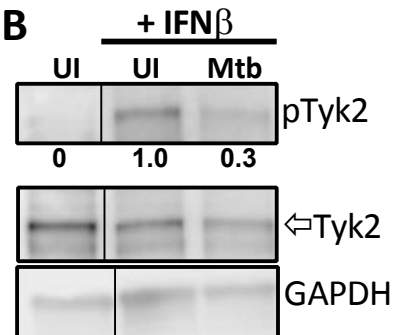
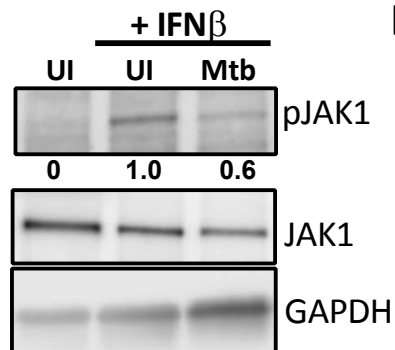
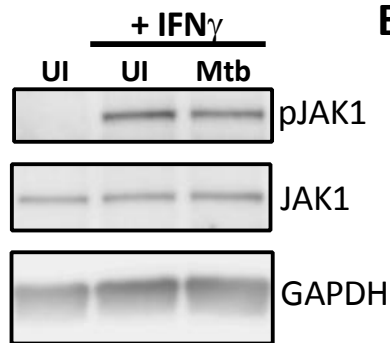
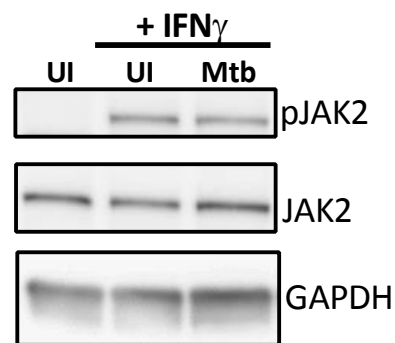
Figure 3**A****B****C****D****E**

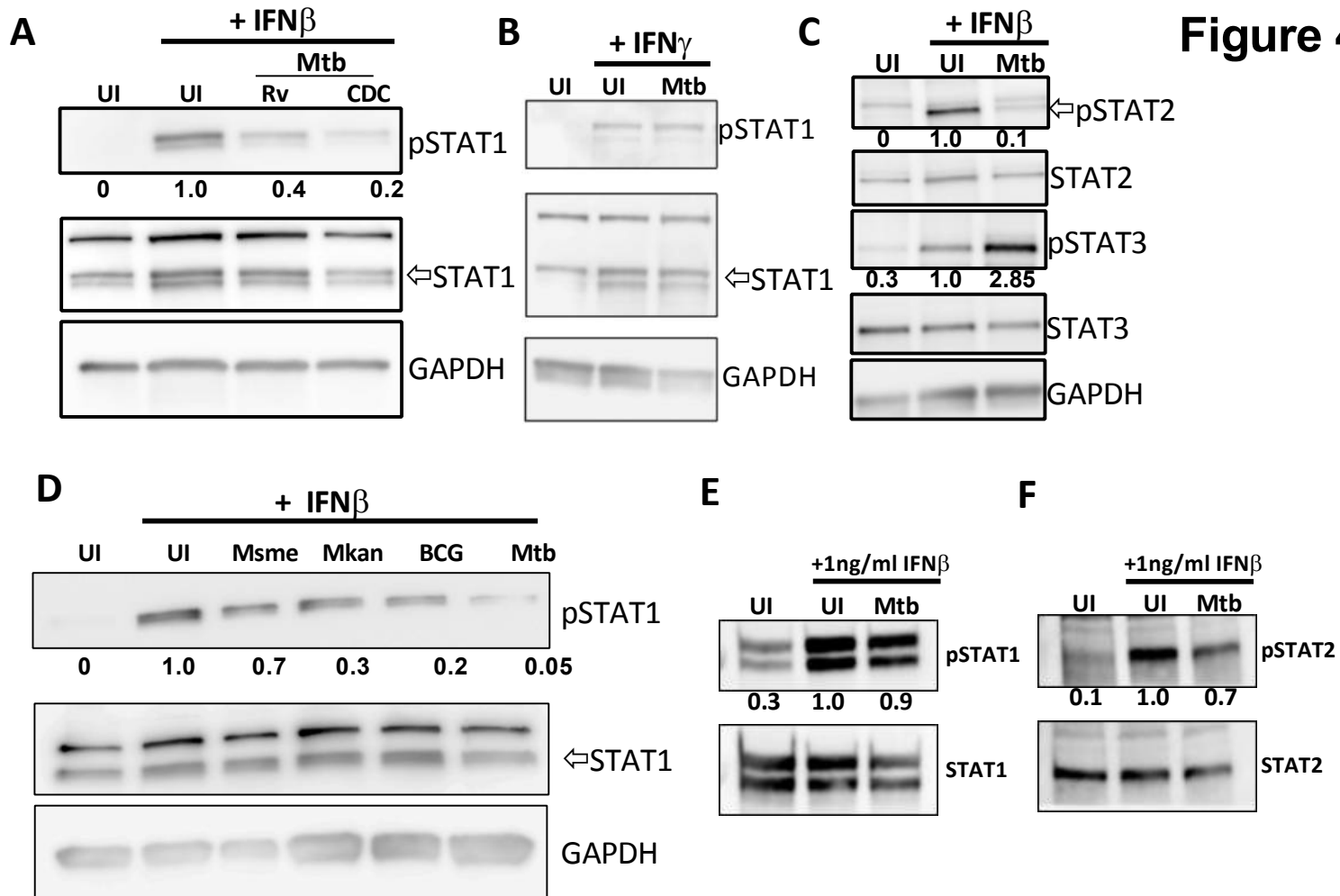
Figure 4

Figure 5

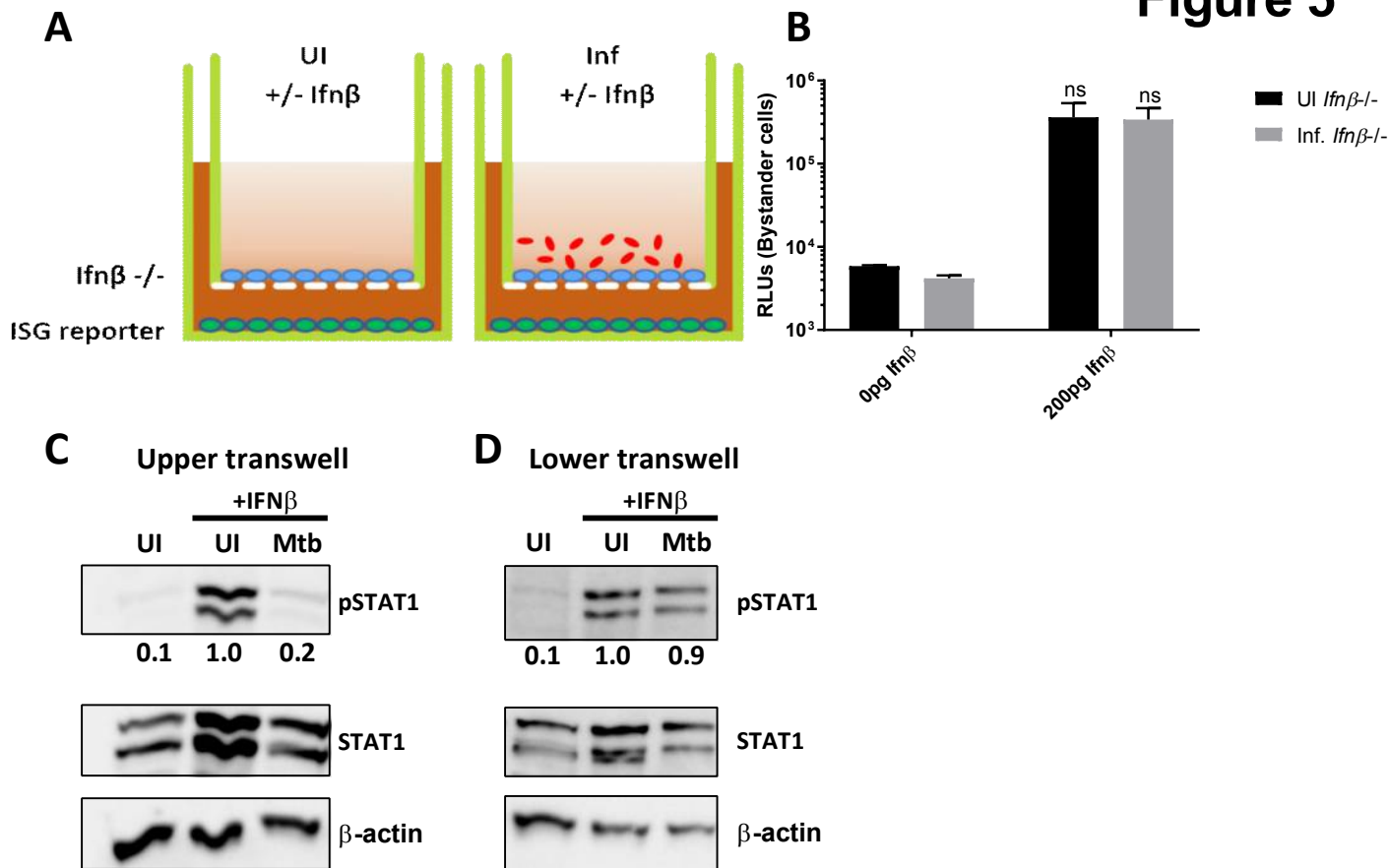


Figure 6

