

RESEARCH PAPER



cGAS-STING-TBK1-IRF3/7 induced interferon- β contributes to the clearing of non tuberculous mycobacterial infection in mice

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ABSTRACT

Type I interferons (IFN-I), such as IFN- α and IFN- β are important messengers in the host response against bacterial infections. Knowledge about the role of IFN-I in infections by nontuberculous mycobacteria (NTM) is limited. Here we show that macrophages infected with pathogens of the *Mycobacterium avium* complex produced significantly lower amounts of IFN- β than macrophages infected with the opportunistic pathogen *M. smegmatis*. To dissect the molecular mechanisms of this phenomenon, we focused on the obligate pathogen *Mycobacterium avium* ssp *paratuberculosis* (MAP) and the opportunistic *M. smegmatis*. Viability of both bacteria was required for induction of IFN- β in macrophages. Both bacteria induced IFN- β via the cGAS-STING-TBK1-IRF3/7-pathway of IFN- β activation. Stronger phosphorylation of TBK1 and higher amounts of extracellular bacterial DNA in the macrophage cytosol were found in *M. smegmatis* infected macrophages than in MAP infected macrophages. After intraperitoneal infection of mice, a strong *Ifnb* induction by *M. smegmatis* correlated with clearance of the bacteria. In contrast, MAP only induced weak *Ifnb* expression which correlated with bacterial persistence and increased number of granulomas in the liver. In mice lacking the type I interferon receptor we observed improved survival of *M. smegmatis* while survival of MAP was similar to that in wildtype mice. On the other hand, treatment of MAP infected wildtype mice with the IFN-I inducer poly(I:C) or recombinant IFN- β impaired the survival of MAP. This indicates an essential role of IFN-I in clearing infections by MAP and *M. smegmatis*. The expression level of IFN-I is decisive for transient versus persistent NTM infection.

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Introduction

Type I interferons (IFN-I) such as IFN- α and IFN- β are pleiotropic cytokines involved in various infections and inflammatory reactions as well as in cancer surveillance.¹ The protective role of IFN-I against many viral infections is well established.² IFN-I are also induced during bacterial infection. Here they have been shown to be either protective or detrimental, depending on the type of bacteria and the route of infection.³


IFN-I are produced by many cell types.² However, under conditions of bacterial infection or inflammation IFN-I are produced in high amounts by cells of the monocyte/macrophage lineage.^{4,5} A hierarchy exists for the induction of IFN-I. Usually, IFN- β is induced first which then via binding to the unique type I IFN receptor

(IFNAR) activates the IFN cascade in an autocrine and paracrine fashion.² IFN-I expression is activated by the interaction of pathogen associated molecular pattern (PAMP) with membrane-bound pattern recognition receptors (PRRs) of the Toll-like receptor (TLR) family. Alternatively, PAMPs that reach the cytosol induce IFN-I responses via cytosolic PRRs including cyclic GMP-AMP synthase (cGAS). The critical role of cGAS for IFN- β induction in bacteria infected macrophages was recently emphasized. DNA released from bacteria activates cytosolic cGAS to synthesize cyclic GMP-AMP (cGAMP). This triggers the STING-TBK-1-IRF3 signaling pathway to initiate transcription of *Ifnb*.⁶⁻⁹

Most of our knowledge of IFN-I in mycobacterial infection is derived from studies on *M. tuberculosis*. The

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bacteria have been shown to induce IFN-I response in human and murine macrophages. Even though the particular role of IFN-I during the host response against *M. tuberculosis* infection is not completely understood, most of such studies suggest that IFN-I promote rather than control *M. tuberculosis* infection.⁹⁻¹¹ At the molecular level, eDNA of *M. tuberculosis* represents the critical ligand for IFN-I induction. The ESX-1 type VII secretion system of *M. tuberculosis* appears to be essentially for higher permeability of the phagosomal membrane thus allowing eDNA to access the cytosol of host cells. By this mechanism, *M. tuberculosis* is able to induce IFN-I expression via the cGAS-STING-TBK1-IRF3 pathway.^{7,9,12,13}

Besides *M. tuberculosis*, the knowledge on IFN-I during infection with other mycobacterial species is limited. Some evidence supporting a beneficial role of IFN-I comes from experiments demonstrating that treatment of mice with recombinant murine IFN- β enhanced their resistance to systemic *M. avium* infection.¹⁴ In addition, infection of mice deficient for the IFN-I receptor (IFNAR^{-/-}) revealed that IFN-I is involved in the early control of *M. bovis* BCG infection.¹⁵ Although these experiments imply that the *M. bovis* BCG might induce host IFN-I, infection of murine macrophages and dendritic cells with BCG resulted in lower expression of IFN- β compared with *M. tuberculosis*.¹⁶ This shows that *in vitro* studies reflect only to a limited extent the sum of effects induced *in vivo*.

The group of nontuberculous mycobacteria (NTM) comprises saprophytic, non-pathogenic, opportunistic and obligate pathogenic mycobacterial species some of which cause disease in humans and animals.^{17,18} *M. smegmatis* is an environmental organism and considered as a saprophytic NTM which is not able to survive in macrophages.¹⁹ Nevertheless, *M. smegmatis* is not “non-virulent” as it was isolated from skin or soft-tissue lesions of humans.^{18,20} In addition, it was recently described that intravenous infection of mice with a high dose of *M. smegmatis* was fatal for C57BL/6 mice.²¹

In contrast, *Mycobacterium avium* ssp *paratuberculosis* (MAP) is an obligate pathogenic NTM of the *Mycobacterium avium* complex. It is the causative agent of paratuberculosis (Johne’s disease), a chronic transmural inflammation of the small intestine in ruminants. MAP has also been discussed as a possible etiological agent in the development of human diseases such as Crohn’s disease, a chronic inflammatory enteritis in humans,²² type I diabetes,^{23,24} and multiple sclerosis.²⁵ One of the key factors that enable MAP to survive inside host cells is its ability to silence the host immune response. This includes inhibition of phagosomal acidification and maturation,^{19,26} reduction of

TNF- α expression,²⁷ reduction of caspase activity,²⁸ and inhibition of CD4⁺ T cells activation.²⁹

In the present study, we aimed to study IFN- β induction upon infection with NTM by focusing on MAP or *M. smegmatis*. Using *in vitro* cell culture systems and *in vivo* mouse models, we found that both NTM induced *Ifnb* via the cGAS-STING-TBK1-IRF3/7 axis. In both cases viable bacteria were needed for *Ifnb* triggering. Higher phosphorylation of TBK1 and higher amounts of eDNA detected in macrophages infected with *M. smegmatis* indicated that this mycobacterium activated the signaling cascade more efficiently. Upon mouse infection, *M. smegmatis* but not MAP was largely cleared. However, upon infection of IFNAR^{-/-} mice *M. smegmatis* could be re-isolated in high numbers after 21 days, indicating that IFN-I contributes to clearing of mycobacterial infection. Similarly, administration of recombinant IFN- β reduced the number of MAP *in vivo*. These data suggest that the attenuation of IFN- β activation allows MAP to evade the host immune defense. Overall, our results support the hypothesis that transient vs. persistent NTM infection is determined by the extent of IFN- β induction.

Results

M. smegmatis induces higher *Ifnb* expression in macrophages than *M. avium* subspecies

To investigate the role of IFN-I in NTM infections, we infected murine RAW 264.7 macrophages (RAW) with the MAP strains MAP 6783 and MAP K10, *M. avium* ssp *hominis suis* strain 04A/1287 (MAH), *M. avium* ssp *avium* (MAA) strains 44156 and 44158, and *M. smegmatis* mc² as well as *M. bovis* BCG Pasteur for 5h. All tested mycobacterial strains induced *Ifnb* expression in RAW cells (Fig. 1A). However, compared to all other tested mycobacterial species *M. smegmatis* induced significantly higher *Ifnb* levels (Fig. 1A).

To dissect the molecular mechanism of *Ifnb* induction by the different mycobacterial strains, we focused on MAP strain MAP 6783 (MAP) and *M. smegmatis*. While abundant levels of IFN- β were secreted after *M. smegmatis* infection of RAW cells, MAP induced much lower amounts (Fig. 1B). Similar results were observed at mRNA level. *Ifnb* expression in *M. smegmatis* infected macrophages significantly increased within 5 h after infection, whereas the levels in MAP infected macrophages remained similarly low over time (Fig. 1C). Likewise, in BMDM, *Ifnb* strongly increased over time upon *M. smegmatis* and only moderately upon MAP infection (Fig. 1D). Interestingly, for both MAP and *M. smegmatis* *Ifnb* induction was dependent on the viability of the bacteria (Fig. 1C and D) and increased in a dose dependent

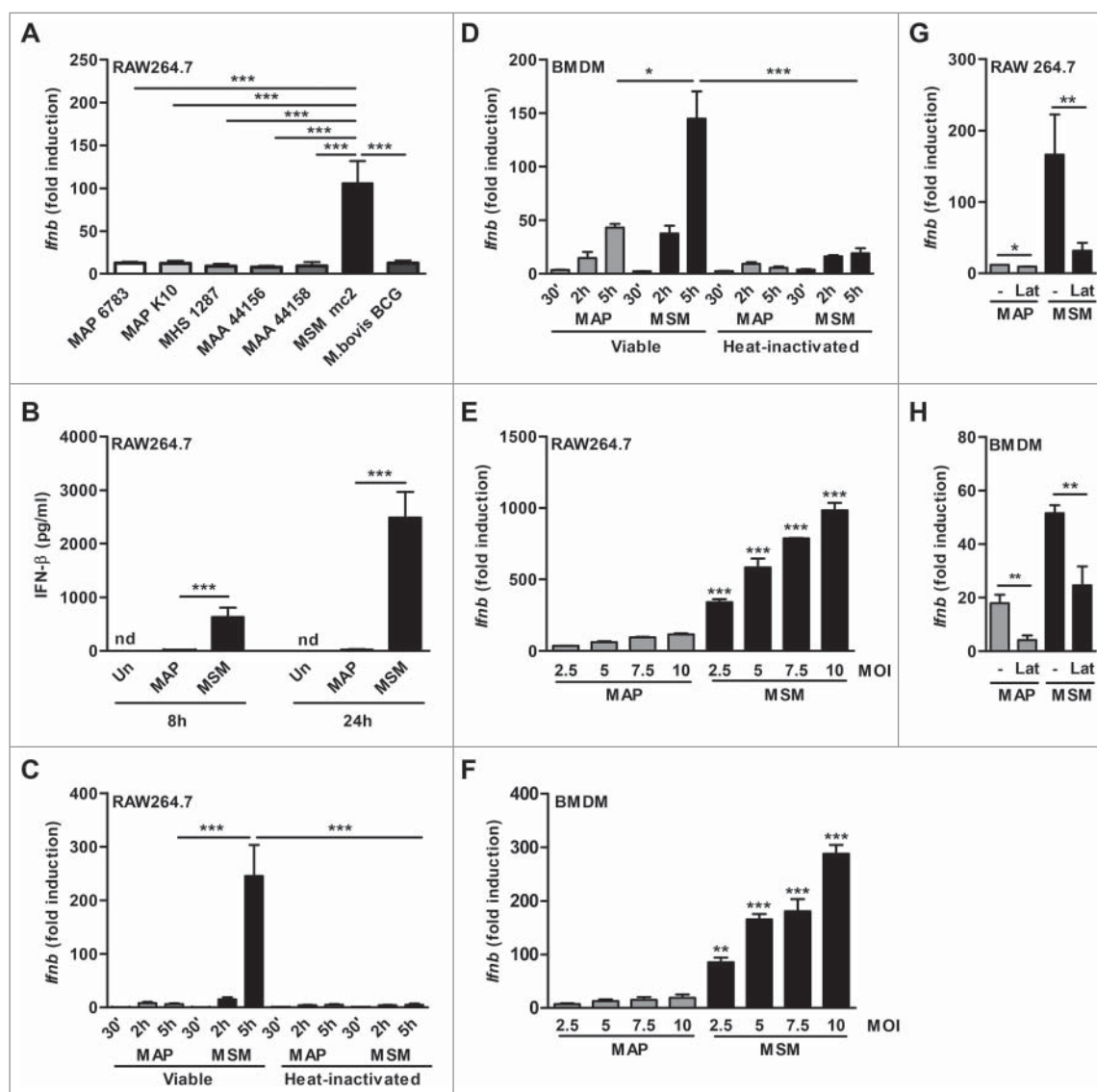


Figure 1. *Ifnb* induction by NTM mycobacteria in macrophages. (A) qPCR for *Ifnb* in RAW cells infected with viable MAP 6783, MAP K10, MAH 1287, MAA 44156, MAA 44158, *M. bovis* BCG Pasteur or *M. smegmatis* mc² (MSM) (MOI 5) for 5 h. (B) IFN-β ELISA of supernatant from RAW cells infected with MAP or MSM. (C) qPCR for *Ifnb* in RAW cells and (D) BMDM infected with viable (V) or heat inactivated (HI) MAP or MSM (MOI 5) for 30 min ('), 2 h and 5 h. (E) RAW cells and (F) BMDM were infected with MAP or MSM (MOIs 2.5, 5.0, 7.5, 10) for 5 h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Tukey post test, means \pm SEM. (G) RAW cells and (H) BMDM were pre-treated with 5 μ M latrunculin B (Lat) for 1 h and subsequently infected with MAP or MSM for 5 h. Data are representative of 3 independent experiments, * $p < 0.05$, ** $p < 0.01$ by 2 tailed student's t-test means \pm SEM.

manner (Fig. 1E and F). However, at equivalent MOIs the *Ifnb* induction by MAP was always significantly lower than by *M. smegmatis* (Fig. 1E and F). This difference in *Ifnb* triggering was not due to divergent numbers of intracellular MAP or *M. smegmatis* as observed by confocal microscopy (Fig. S1).

Endocytosis or phagocytosis was required for *Ifnb* induction. RAW cells or BMDM pre-treated with latrunculin B before both MAP and *M. smegmatis* infection exhibited significantly reduced *Ifnb* expression (Fig. 1G and H). This suggested that *Ifnb* induction by NTM is

mediated via intracellular receptors or via receptors that need to be internalized.

MAP and *M. smegmatis* induce *Ifnb* expression via the cGAS-STING-TBK1-IRF3/7 signaling pathway

Induction of *Ifnb* expression by *M. tuberculosis* has been shown to require cytosolic signaling via cGAS and the STING-TBK1-IRF3 axis.⁷ Hence, we were interested to determine whether the same pathway is involved in *Ifnb*

induction in macrophages infected with MAP or *M. smegmatis*. First, we wanted to exclude a potential contribution of TLR's. Induction of *Ifnb* was not significantly influenced in BMDM from mice lacking UNC93B, a trafficking protein required for the signaling of TLR3, 7, and 9. This indicated that intracellular TLRs did not contribute to *Ifnb* expression (Fig. 2A). Furthermore, *Ifnb* expression was independent of MyD88 and TRIF thus excluding any other TLR mediated signaling. In contrast, *Ifnb* expression was significantly reduced in IRF3^{-/-}, IFNAR^{-/-} and IRF7^{-/-} macrophages infected with both mycobacterial species (Fig. 2B), although all cells, except IRF3^{-/-}, were fully responsive to LPS stimulation (Fig. S2). IRF3 is known to be essential for induction of IFN β by LPS.³⁰ Immunoblot analysis revealed that phosphorylation of TBK1 was markedly induced in macrophages infected with *M. smegmatis*, but not with MAP (Fig. 2C).

Overall, these data suggested that cytosolic signaling might be required for *Ifnb* activation in MAP and *M. smegmatis* infected macrophages. Indeed, *Ifnb* expression was significantly reduced in STING^{-/-} (Fig. 2D) and cGAS^{-/-} macrophages (Fig. 2E) as well

as in cGAS and STING knock down RAW cells (Fig. S3). The knock out macrophages remained fully responsive to LPS stimulation (Fig. S2).

The above data showed a differential magnitude of activation of the cGAS-STING-TBK1 pathway. This might be due to distinct release of eDNA from the mycobacteria bearing phagosomes. To test this, we extracted DNA from the cytosol of infected macrophages and probed for the presence of mycobacterial eDNA by qPCR. Enhanced amounts of eDNA were detected in the cytosol of macrophages infected with viable *M. smegmatis* when compared with MAP infected macrophages. Furthermore, the amount of eDNA in the cytosol of macrophages was significantly lower when the macrophages were treated with heat-inactivated *M. smegmatis* or MAP (Fig. 2F). These data indicate that the differential release of mycobacterial eDNA into the cytosol of infected macrophages is responsible for a diverse level of activation of the cGAS-STING-TBK1 pathway by the different mycobacteria species.

It was shown before that *M. tuberculosis* activates *Ifnb* expression by releasing eDNA into the cytosol of host

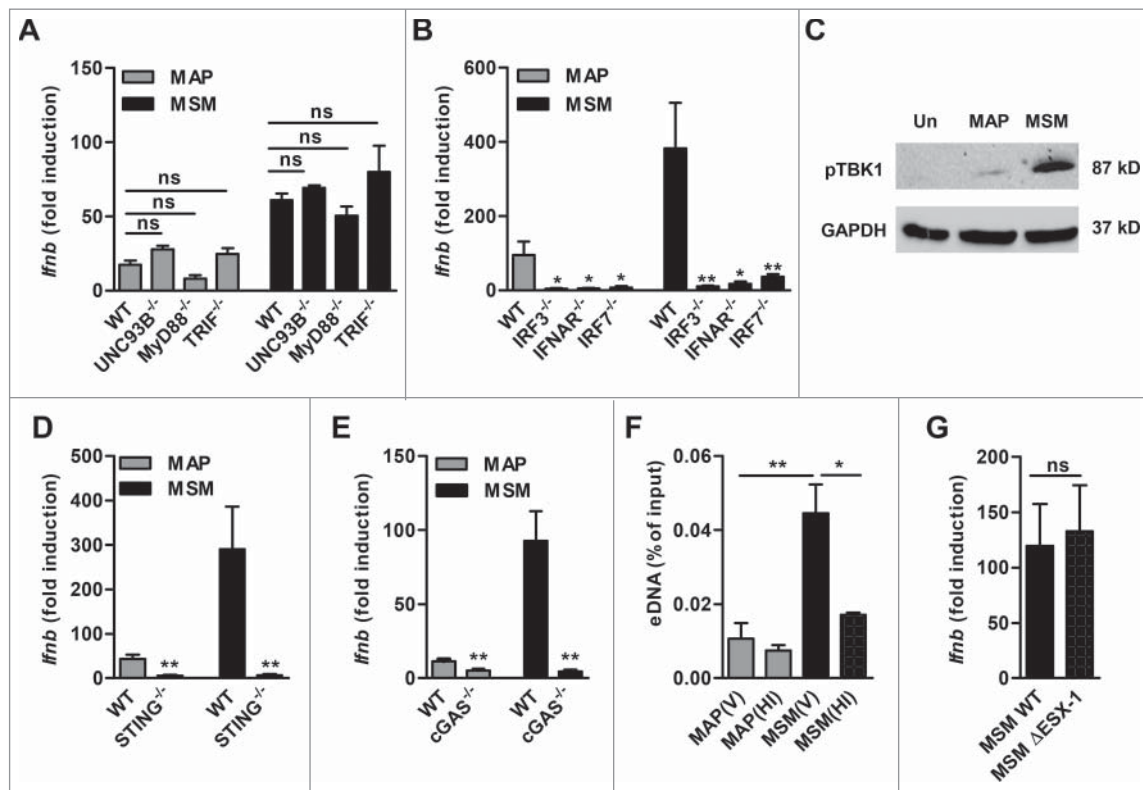


Figure 2. MAP and *M. smegmatis* (MSM) induce *Ifnb* expression via the cGAS-STING-TBK1-IRF3/7 signaling pathway. (A) qPCR for *Ifnb* in BMDM from WT, UNC93B^{-/-}, MyD88^{-/-}, TRIF^{-/-}, (B) IRF3^{-/-}, IFNAR^{-/-}, IRF7^{-/-} mice infected with MAP or MSM. * $p < 0.05$, ** $p < 0.01$, ns (non-significant) by one-way ANOVA with Tukey post test, means \pm SEM. (C) Immunoblot of phosphoTBK1 in RAW cells infected with MAP or MSM for 5 h. (D) qPCR for *Ifnb* in WT, STING^{-/-} and (E) cGAS^{-/-} mice infected with MAP or MSM for 5 h. (F) qPCR analysis of eDNA in RAW cells infected with viable (V) or heat inactivated (HI) MAP or MSM for 5 h. eDNA was extracted and shown as percentage of the input control. (G) qPCR for *Ifnb* in RAW cells infected with MSM WT or MSM Δ ESX-1 for 5 h. Data are representative of 3 independent experiments, * $p < 0.05$, ** $p < 0.01$ by 2 tailed student's t-test means \pm SEM.

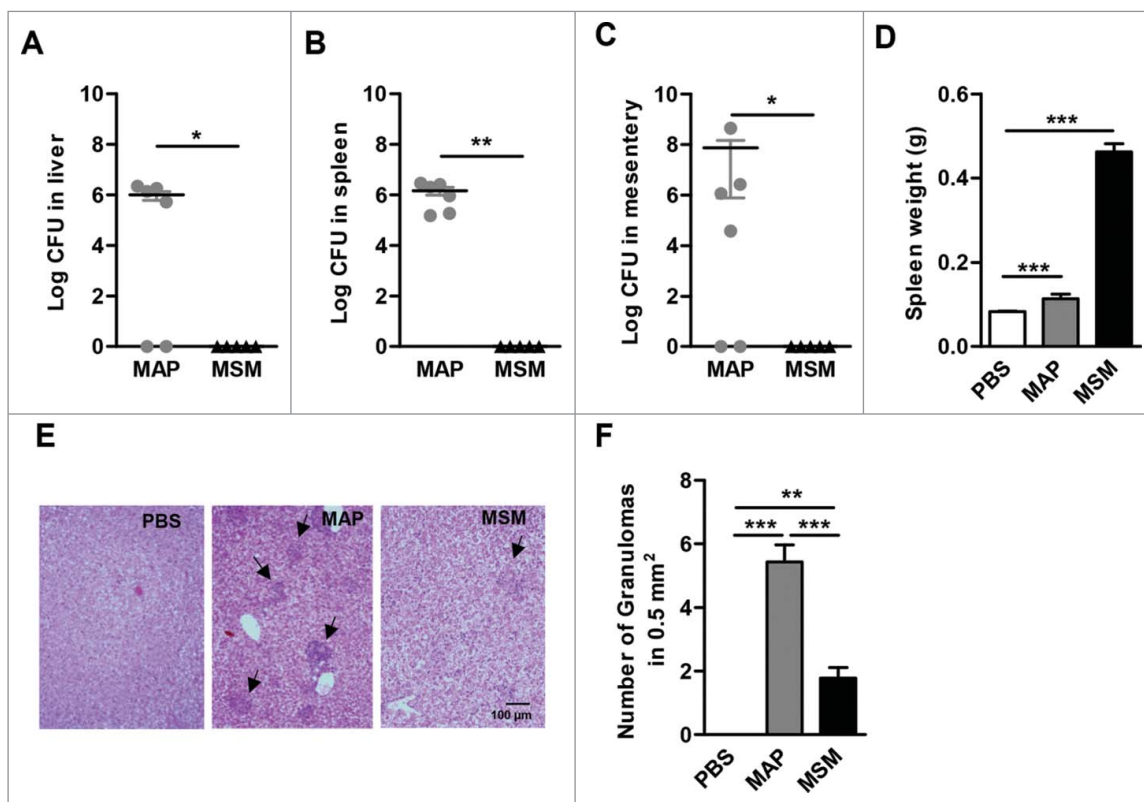


Figure 3. Pathology of MAP and *M. smegmatis* (MSM) infected mice. C57BL/6 mice ($n = 3\text{--}6/\text{group}$) were infected MAP or MSM. At day 21 post infection, (A) livers (B) spleens and (C) mesentery were plated. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney U test, means \pm SEM. (D) spleen weight was determined. (E) Representative HE stained liver sections. Arrows heads point at granulomas. (F) Numbers of granulomas in livers. Data are representative of 2 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Tukey post test, means \pm SEM.

cells in an ESX-1 dependent manner.^{7,9} *M. smegmatis* bears an ESX-1 secretion system. Therefore, by using a *M. smegmatis* ESX-1 deletion mutant, we tested whether the release of eDNA by *M. smegmatis* was dependent of ESX-1. The mutant induced *Ifnb* levels comparable to the wildtype (WT) bacteria (Fig. 2G). Moreover, no difference in bacterial burden was observed in mice infected either with *M. smegmatis* WT or the ESX-1 mutant (Fig. S4).

These results clearly demonstrate that MAP and *M. smegmatis* release DNA into the cytosol of the host cell to stimulate *Ifnb* induction via cGAS, STING, TBK1, and IRF3 which includes IFNAR and IRF7 dependent feedback signaling. Therefore, both mycobacteria appear to stimulate the cytosolic sensors cGAS and STING, but to different degrees.

***M. smegmatis* is not able to persist in infected mice**

It is well known that MAP is able to persist in intraperitoneally (i.p.) infected mice over long periods of time.³¹⁻³⁵ However, little is known for *M. smegmatis* in that respect. To better understand the fate of *M. smegmatis* after

infection of mice, C57BL/6 mice were infected i.p. with *M. smegmatis* or MAP and CFUs were determined from liver, spleen, and mesentery at 21 d post infection (dpi). MAP could be isolated from these organs at high numbers. In contrast, *M. smegmatis* was barely detectable in such tissues, indicating that the mice were able to clear *M. smegmatis*, but not MAP (Fig. 3A to C).

MAP infected mice displayed moderately enhanced spleen weight. In contrast, mice infected with *M. smegmatis* exhibited severe splenomegaly suggesting a strong inflammatory response (Fig. 3D). Granulomas were found in the liver of mice infected with both mycobacteria. However, the number and the size of granuloma in the liver of MAP infected mice were significantly larger (Fig. 3E and F). These results indicate that unlike MAP, *M. smegmatis* is efficiently cleared by the murine host.

High or low induction of *Ifnb* upon *M. smegmatis* or MAP infection, respectively, correlates with lower or higher bacterial burden in mice

We wanted to understand whether the IFN- β response in mice during MAP or *M. smegmatis*

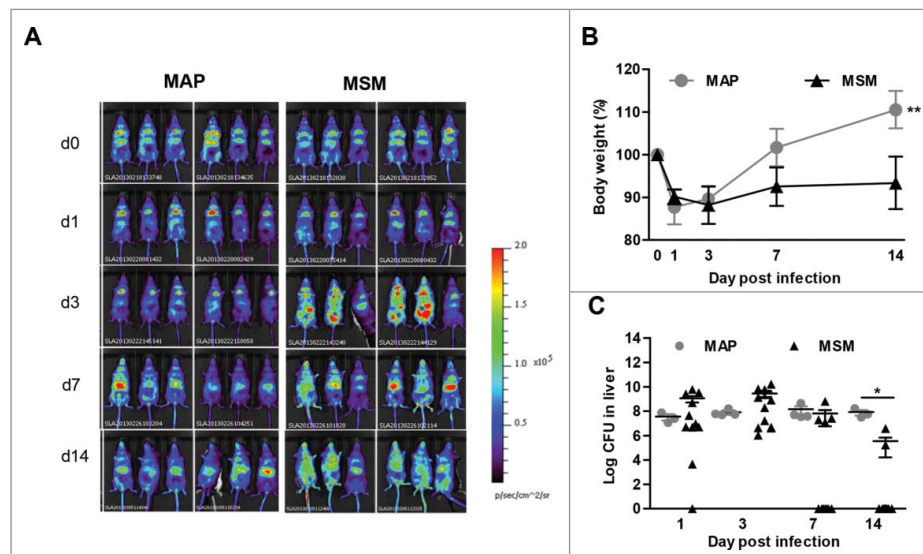


Figure 4. Induction of IFN- β after MAP or *M. smegmatis* (MSM) infection *in vivo*. (A) IFN- $\beta^{+/\Delta\beta-luc}$ ($n = 6$ /group) were infected with MAP or MSM. At day 1, 3, 7, and 14 post infection, mice were injected with D-luciferin and visualized for luciferase activity. (B) Body weights of mice infected with MAP or MSM. ** $p < 0.01$ by Two way ANOVA with Bonferroni post test, means \pm SEM. (C) C57BL/6 mice ($n = 3$ –10/group) were infected with MAP or MSM. Numbers of CFU in liver were determined. Data are representative of 2 independent experiments. * $p < 0.05$ by Mann-Whitney U test, means \pm SEM.

infection correlates with differential persistence of the bacteria. To this end, we used the firefly luciferase-based heterozygous IFN- $\beta^{+/\Delta\beta-luc}$ reporter mouse that allows *Ifnb* reporter expression concomitant with IFN- β production.³⁶ IFN- $\beta^{+/\Delta\beta-luc}$ mice were infected i.p. with MAP and *M. smegmatis* (10^8 CFUs/mouse) and luciferase expression was analyzed for up to 14 d post infection by non-invasive *in vivo* imaging. *Ifnb* promoter activity was weakly induced by MAP (Fig. 4A). In contrast, *M. smegmatis* infected mice displayed considerably higher levels of luciferase expression. In addition, in *M. smegmatis* infected mice the luciferase signal could be detected after 3 d of infection and increased activity was detectable until 14 d post infection (Fig. 4A).

To correlate the dynamics of MAP and *M. smegmatis* infections with the IFN- β response, we infected C57BL/6 mice with MAP or *M. smegmatis* and analyzed them at day 1, 3, 7, and 14 post infection. While MAP infected mice were able to recover quickly from the initial body weight loss, *M. smegmatis* infected mice did not recover during the observation period (Fig. 4B). Bacterial plating revealed that MAP was able to persist in the liver. Similar numbers of MAP could be recovered from the liver at each time point. In contrast, the bacterial load with *M. smegmatis* in the liver continuously declined (Fig. 4C). These results suggested that the increased levels of IFN- β in *M. smegmatis* infected mice might promote mycobacterial clearance.

IFNAR is necessary to clear mycobacterial infection

To demonstrate the involvement of IFN-I in the host response to mycobacterial infection, C57BL/6 WT and IFNAR^{-/-} mice which are non-responsive to IFN-I were infected with MAP or *M. smegmatis*. Mice infected with MAP initially lost weight and recovered after 2 d of infection independent of whether they were WT or IFNAR^{-/-}. In contrast, *M. smegmatis* infected mice recovered their body weights upon day 21. Remarkably, upon *M. smegmatis* infection weight loss of IFNAR^{-/-} mice was more dramatic than of WT mice and the animals did not recover during the observation period (Fig. 5A and B). At necropsy, increases in spleen weight were similar when WT to IFNAR^{-/-} mice were compared (Fig. 5C).

The analysis of tissue lysates revealed that C57BL/6 WT mice were able to clear *M. smegmatis* infection within 3 weeks. In contrast, high numbers of *M. smegmatis* could be recovered from the organs of the majority of infected IFNAR^{-/-} mice (Fig. 5D to F). These data show that functional IFN-I signaling is necessary for the host to control *M. smegmatis* infection, whereas in case of MAP infection IFN-I signaling did not play a key role.

IFN- β promotes control of MAP infections

IFN-I is obviously involved in the control of *M. smegmatis* infection. On the other hand, the low amounts of IFN- β

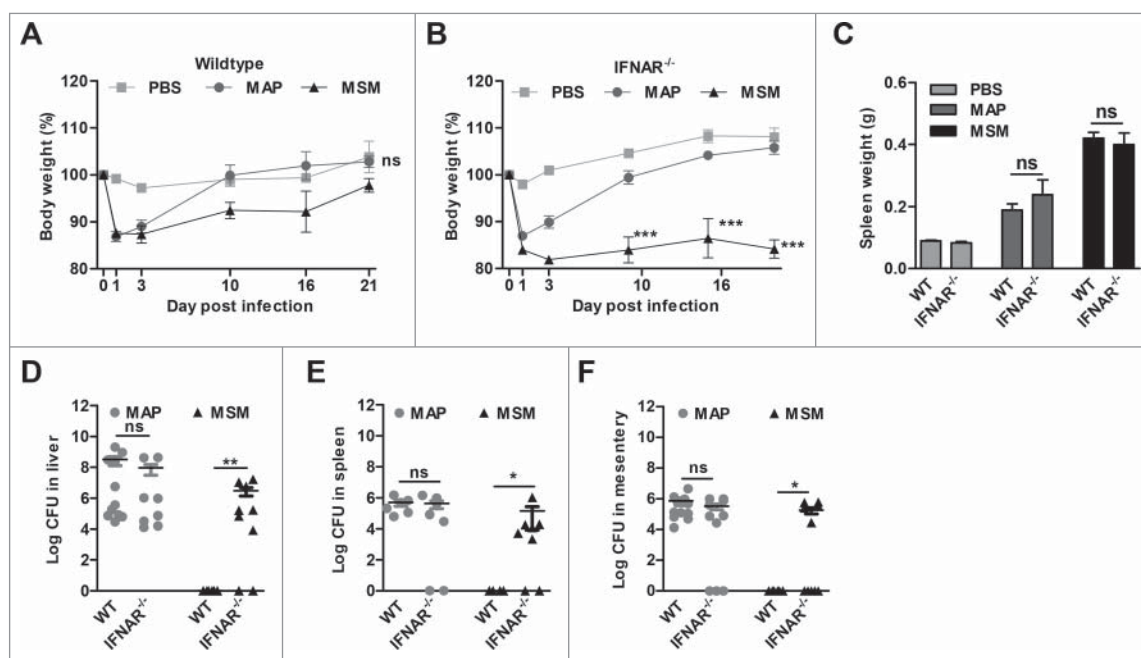


Figure 5. Clearance of *M. smegmatis* (MSM) depends on IFN-I signaling. C57BL/6 mice and IFNAR^{-/-} mice (n = 5–6/group) were injected with PBS, MAP and MSM. (A and B) body weight was monitored. ***p < 0.001 by Two way ANOVA with Bonferroni post test, means ± SEM. (C) spleen weights at 21 dpi. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA with Tukey post test, means ± SEM. (D) Numbers of CFU in liver, (E) spleen and (F) mesentery were determined by plating 21 dpi. Data are representative of 2 independent experiments, *p < 0.05, **p < 0.01, ns (non-significant), by Mann-Whitney U test, means ± SEM.

induced by MAP might not be sufficient to elicit a significant *in vivo* effect against this bacterium. Thus, we hypothesized that exogenous administration of IFN- β might help to reduce the number of bacteria. To test this, MAP infected mice were treated with either recombinant IFN- β (rIFN- β) or poly(I:C) (Fig. 6A). The spleen weights were similar in MAP infected and in MAP infected IFN- β treated mice, while the addition of poly(I:C) increased the spleen weight (Fig. 6B). Interestingly, determination of the bacterial numbers in liver, spleen and mesentery revealed that the treatment of MAP infected mice with poly(I:C) or rIFN- β led to a significant effect on bacterial persistence. In contrast to the liver, CFU in spleen and mesentery were significantly reduced compared with untreated MAP infected mice (Fig. 6C to E).

Taken together, these results clearly indicate that in case of MAP and *M. smegmatis* IFN-I contribute to mycobacterial clearance. Therefore, subversion of IFN- β induction in MAP infected macrophages and also in mice might be an immune evasion strategy of MAP and most likely of other pathogenic NTM that adds to their persistence.

Discussion

We observed that the opportunistic pathogenic *M. smegmatis* induced abundant *Ifnb* expression during

macrophage infection. This observation is in agreement with Shah et al., see ref.³⁷ who showed that IFN- β production induced by *M. smegmatis* and 2 other opportunistic pathogenic NTM, *M. fortuitum* and *M. kansasii*, was more pronounced than induction by *M. tuberculosis*.³⁷ Interestingly, *Ifnb* induction by *M. smegmatis* was significantly higher than that induced by all the slow growing pathogenic NTM or *M. bovis* BCG tested here (Fig. 1A). Remarkably, the ESX-1 secretion system is absent in the *Mycobacterium* species tested in our study besides *M. smegmatis*. The membrane-interacting activity of *M. tuberculosis* ESAT-6 secreted by ESX-1 type VII secretion system is known to be essential for *Ifnb* induction. *M. smegmatis* expresses an ESX-1 secretion system. However, it was shown recently that the membrane-interacting activity of *M. tuberculosis* ESAT-6 is not present in its ortholog in *M. smegmatis*.³⁸ Thus, it was possible that the molecular pathway of IFN- β induction and the relevance of IFN- β in the pathogenesis of infections between NTM and *M. tuberculosis* are different. This prompted us to dissect the pathways of IFN- β induction and the role of IFN- β during the host response to such infections in more detail. We focused on *M. smegmatis* and MAP.

M. smegmatis was recently found to cause fatal disease after intravenous infection of C57BL/6 mice.²¹ Similarly, we showed that even i.p. infection of mice with

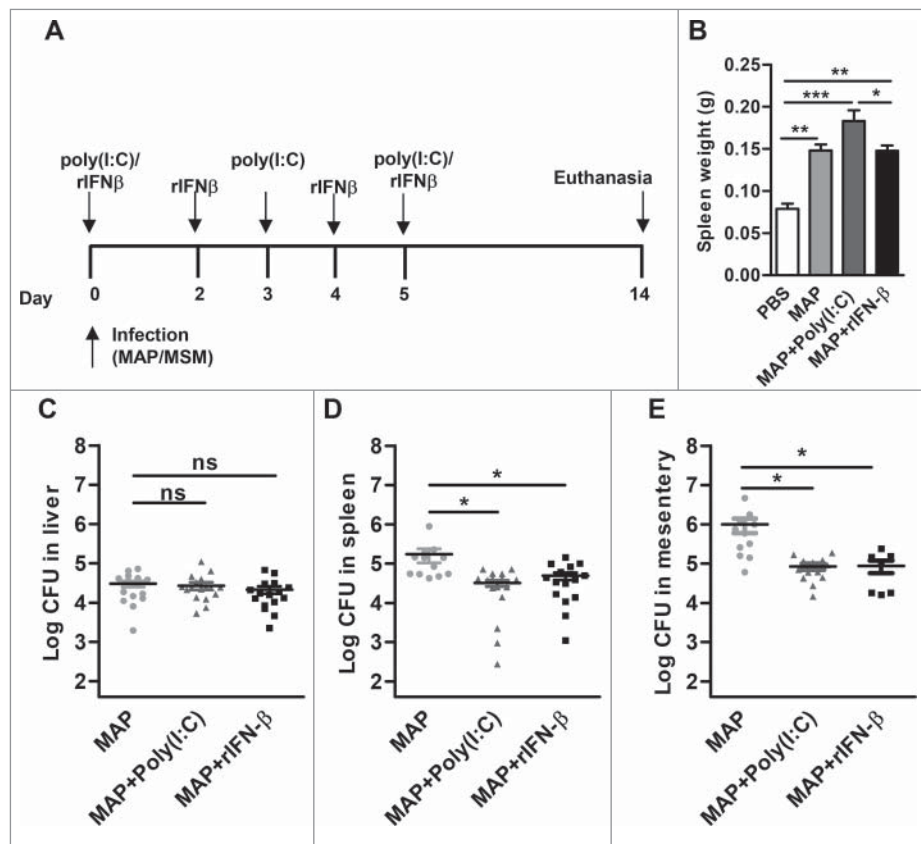


Figure 6. IFN- β supports MAP clearance. C57BL/6 ($n = 3\text{--}10/\text{group}$) were infected with PBS, MAP, MAP + 100 μg poly(I:C)/mouse, or MAP + 1000 units rIFN- β . Mice were killed at 14 dpi. (A) Schematic representation of the experiment. (B) spleen weight was determined. (C) Numbers of CFU in liver, (D) spleen and (E) mesentery were determined by plating. Data are representative of 2 independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns (non-significant), by one-way ANOVA with Tukey post test, means \pm SEM.

M. smegmatis caused severe disease, as indicated by the severe and sustained body weight loss during the first 2 weeks of infection (Fig. 4B, Fig. 5A and B). However, the animals were finally able to clear the infection by day 21 (Fig. 5D to F) and recover body weight. The clearance of the bacterial load was associated with enhanced expression of *Ifnb*. IFN- β production and signaling were essential to control the infection as IFNAR $^{-/-}$ mice lacking a functional IFN-I system were no longer able to restrict bacterial growth.

Similar to *M. tuberculosis*, the induction of *Ifnb* expression was dependent on the cGAS-STING-TBK1-IRF3 pathway of IFN- β induction (Fig. 2D and E) which appears to be activated by eDNA of *M. smegmatis*. Accordingly, high amounts of DNA of *M. smegmatis* could be detected in the cytosol of host macrophages (Fig. 2F). How this DNA is released from the phagosome into the cytosol is still unclear. We could exclude any involvement of the ESX-1 secretion system since deletion of the ESX-1 system in *M. smegmatis* neither influenced *Ifnb* expression by macrophages nor the outcome of infection *in vivo* (Fig. 2G and Fig. S4). This is in striking contrast to *M. tuberculosis* infections where the ESX-1

secretion system is required.^{9,10,12} Indeed, this situation is reminiscent of infections by extracellular pathogens such as pathogenic streptococci. In such cases, IFN- β is also produced by macrophages and is necessary for bacterial clearance.^{6,39} Streptococci induce IFN- β production in macrophages also via the cytosolic cGAS-STING-TBK1-IRF3 pathway and similar to our experiments with *M. smegmatis* this pathway requires bacterial viability, phagocytic uptake, and the release of bacterial DNA into the cytosol.^{6,39} The mechanism by which DNA breaches the vesicular membrane is also not fully resolved. It seems that streptococcal toxins partially regulate DNA access to the cytosol, although other factors are likely to play a role.⁴⁰

Pathogenicity of MAP is mostly unresolved. Similar to many other pathogenic mycobacteria, the survival and persistence of MAP is one key feature of MAP pathogenicity. In the present study, we found that similar to MAA, MAH and *M. bovis* BCG, MAP induced very weak expression of *Ifnb* in murine macrophages *in vitro* (Fig. 1A). Similarly, *in vivo* MAP induced only weak IFN- β (Fig. 4A). The weak induction correlated with MAP survival for more than 21 d and with increased

granuloma formation in the liver. MAP survival was not influenced in mice lacking IFNAR (Fig. 5D and F). This demonstrates that the low level IFN- β response induced by MAP does not substantially contribute to the infection outcome. Nevertheless, clearance of MAP can be significantly enhanced by repeated administration of rIFN- β or poly(I:C) (Fig. 6D and E). Bacterial loads of spleen and mesentery were significantly lower in this case while no difference could be found in the liver. Since the induction of IFN- β can potentially vary among different tissues,⁴¹ it is possible that the amount of *Ifnb* in the liver of mice stimulated with MAP + rIFN- β or MAP + poly(I:C) did not reach the threshold needed for effective clearance (Fig. 6C). This indicates that IFN- β , when present in sufficient amounts, contributes to clearance also of MAP. Overall these data show that IFN- β contributes to controlling NTM infection in mice. How NTM clearance is promoted by IFN- β needs further studies since IFN-I can influence a multitude of immune cells.⁴² One possible mechanism linking NTM clearance and IFN-I production might be the differential production of nitric oxide (NO) as IFNAR signaling is known to trigger NO production.⁴³ Treatment of macrophages with recombinant IFN $\alpha\beta$ results in the restriction of *M. tuberculosis* growth by NO.⁴⁴

Our *in vitro* experiments revealed that *Ifnb* induction by *M. smegmatis* and even the low response induced by MAP takes place via the cytosolic cGAS-STING-TBK1-IRF3/7 pathway. Activation required viability and cellular uptake. This indicates that mycobacterial components which are produced in the phagosome contribute to *Ifnb* induction by both mycobacterial species. The cytosolic cGAS-dependent sensing of bacterial DNA has been described for infections by *M. tuberculosis* and other intracellular pathogens, including *Listeria* and *Chlamydia*.^{7,8,45} We were able to detect mycobacterial eDNA in the cytosol of macrophages infected with MAP and *M. smegmatis* (Fig. 2F). The higher amounts in the cytosol of macrophages infected by viable *M. smegmatis* suggest that in cells infected by such bacteria the eDNA release from the phagosome is more efficient than in MAP infected cells. This might explain the stronger cGAS-STING-TBK1 activation. MAP inhibits the phagosomal maturation process and survives in macrophages. In contrast, *M. smegmatis* is found at 1 h post infection in acidified and maturing phagosomes which leads to the killing of *M. smegmatis*.¹⁹ Bacterial viability is necessary for *Ifnb* induction by *M. smegmatis*. Most probably bacterial autolysis and degradation might liberate amounts of DNA necessary to efficiently activate the signaling pathway. However, understanding the detailed molecular mechanism requires further studies.

In conclusion, our data demonstrate that IFN-I are necessary to control *M. smegmatis* and MAP infections. The persistence of each bacterium is determined by the amount of bacterial eDNA released into the cytosol of the host cell and the ability to activate IFN-I via the cGAS-STING-TBK1-IRF3/7 pathway. It seems that NTM that are unable to survive in macrophages release high amounts of eDNA by a yet unknown mechanism. This leads to the induction of high levels of IFN-I which helps to control infection. As MAP and other pathogenic NTM seem to restrict the release of eDNA, the incomplete induction of the host IFN-I response appears to be a specific immune evasion mechanism of these NTM that contributes to their persistence.

Materials and Methods

Bacterial strains and growth conditions

M. smegmatis mc²155 (ATCC 19420),¹⁹ *M. bovis* BCG Pasteur, *M. avium* ssp *avium* DSM44156, *M. avium* ssp *avium* DSM44158, *M. avium* ssp and *hominissuis* strain 04A/1287⁴⁶ were grown in Middlebrook 7H9 (MB) medium or on solid MB 7H10 agar (Beckton Dickinson) supplemented with 0.2–0.5% glycerol, 10% OADC (0.06% oleic acid/ 5% albumin/ 2% dextrose/ 0.003% catalase enrichment (Carl Roth). *M. avium* ssp *paratuberculosis* strain 6783 (DSM44135) and K10 strain (ATCC BCA-968) were grown in MB medium with mycobactin J (2 mg/l, IDVET) supplementation. The *M. smegmatis* Δ ESX-1 mutant (provided by William R Jacobs Jr, Albert Einstein College of Medicine, New York, USA) was cultured in MB medium supplemented with 50 μ g/ml hygromycin B (Roche).

Mouse strains

C57BL/6 mice were purchased from the Janvier. IRF3^{-/-},⁴⁷ IRF7^{-/-},⁴⁸ IFNAR^{-/-},⁴⁹ STING^{-/-},⁴⁵ and transgenic IFN- β -luciferase reporter mice (IFN- β ^{+/ Δ β -luc}),³⁶ cGAS^{-/-},⁵⁰ MyD88^{-/-} and TRIF^{-/-} (the Jackson Laboratory), UNC93B^{-/-}⁵¹ were bred at the Helmholtz Center for Infection Research (HZI), Braunschweig, Germany. Female mice aged between 8–12 weeks were used for all mouse infection experiments. Mouse infection experiments were approved by the Lower Saxony Federal State Office for Consumer Protection and Food Safety, Germany (LAVES: reference number 08/1504).

Macrophage infection

The mouse macrophage cell line RAW264.7 (ATCC[®] TIB-71TM) was maintained in DMEM medium (Thermo

Fisher) supplemented with 10% FCS (Biochrom), 1% glutamine (Thermo Fisher), 100 units/ml penicillin, 100 µg/ml streptomycin (Thermo Fisher). Bone marrow derived macrophages were cultured in DMEM medium in the presence of 20% L929-cell conditioned medium for 10 d. For infection experiments, macrophages were cultured in antibiotic free medium overnight and infected with a suspension containing prevalently single mycobacteria at MOI of 5 or as indicated for 5h. For some experiments, macrophages were pre-treated with 5 µM latrunculin (Calbiochem) for 1 h prior infection.

Extraction of DNA from the cytosol of macrophages and detection of eDNA

To obtain DNA from the cytosol 1×10^7 RAW cells were seeded on 10 cm cell culture dishes and infected with mycobacteria at MOIs of 5. After 5 h the cells were scraped in 1 ml sucrose containing homogenization buffer (20 mM HEPES (Thermo Fisher)/250 mM sucrose/0.5 mM EDTA (Carl Roth)).⁵² Cell membranes were disrupted with a tissue grinder and 50 µl of lysates were taken as input controls. Each remainder lysate was centrifuged for 15 min at 15,000 x g. Post-phagosomal supernatants were incubated with 5 µg/ml DNase-free RNase (Roche) for 1 h followed by incubation with 20 mg/ml proteinase K (Carl Roth) overnight at 37°C. DNA was prepared by phenol:chloroform:isoamylalcohol (25:24:1) extraction and isopropanol precipitation in the presence of 20 µg glycogen. DNA extraction of the input controls was performed by beating 4 times for 10 sec. in the presence of 0.1 mm silica beads and subjected to the extraction procedure as described above. Mycobacterial eDNA was detected by quantitative real-time PCR (qPCR) using primers to detect mycobacterial 16s rDNA (5'-for-TCCGAAGTGGAGACCGGCTTT-3', 5'-rev-TCCAGGGC TTCACACATGCT-3'). Amounts of eDNA were calculated according to the Eq. (2)^{-Ct[cytosol] - Ct[Input x DF]} x 100, where DF is dilution factor of respective input DNA.

qPCR

RNA was reverse transcribed using M-MLV reverse transcriptase (Promega) as described by the manufacturer and qPCR was performed using a Mx3005P qPCR system (Agilent Technologies). Ct values were normalized to the housekeeping gene *Rps9* (Δ ct) and expressed as fold change to the untreated control ($\Delta\Delta$ ct). Primer sequences: *Ifnb*-for_5'-ACCACAGCCCTCTCCATCAA CTA-3'; *Ifnb*-rev_5'-CTCTTCTGCATCTTCCTCCGT CAT-3'; *Rps9*-for_5'-CTGGACGAGGGCAAGATGA AG-3'; *Rps9*-rev_5'-TGACGTTGGCG GATGAGCA CA-3'.

Immunoblot analysis

Cells were lysed in cell extraction buffer (Thermo Fisher) supplemented with 1x protease inhibitor cocktail P8340 (Thermo Fisher), 0.5 mM AEBSF (CalBiochem) and 1x Halt phosphatase inhibitor cocktail (Thermo Fisher). Cells lysates were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with anti-phospho-TBK1/NAK (Ser172) (D52C2) and anti-GAPDH (Cell Signaling).

ELISA

Cell culture supernatants were collected from infected RAW cells at 8 h and 24 h. IFN- β was analyzed following the manufacturer's instruction (IFN- β ELISA Biomedical Laboratories).

In vivo infections

C57BL/6 mice were infected i.p. with 10^7 – 10^8 bacteria resuspended in 200 µl PBS (Thermo Fisher). For some experiments, mice were injected i.p. with 100 µg/ml poly (I:C) (GE Healthcare) or i.v. with 1000 units of recombinant IFN- β (Biolegend) before and after infection with MAP. Treatment with poly(I:C) and recombinant IFN- β was repeated every 3–4 and 2–3 d for 1 week, respectively.

Organ plating and histopathology

Mice were killed at 1–3 weeks. Livers, spleens and mesentery were homogenized and plated on MB agar. Number of bacteria (CFU/gram) was expressed in a logarithmic scale. Histology, hematoxylin eosin (HE), was performed in the mouse pathology platform at HZI, Braunschweig.

In vivo imaging

IFN- $\beta^{+/\Delta\beta}$ -luc mice were injected i.p. with MAP or *M. smegmatis*. Mice were anesthetized with isoflurane using the gas anesthesia system (Caliper Life Sciences). Prior to image acquisition, 3 mg of luciferin (Caliper Life Sciences) dissolved in 100 µl PBS was injected i.p. Images were obtained at the consecutive time points thereafter using the IVIS-200 system (Caliper Life Sciences). The software living image (Caliper Life Sciences) was used for image and quantification of emission intensities.

Statistical analysis

Data are expressed as means \pm SEM by using GraphPad Prism 5.03 (GraphPad, San Diego, CA, USA). Depending on the experiment, 2 tailed student's t-test, Mann-Whitney U-test, one-way ANOVA with Tukey post test and 2 way ANOVA with Bonferroni post test were used. The difference between samples and controls was considered statistically significant at a level of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

Designed experiments: NR SW RG. Performed the experiments: NR AN KA SL AS NJ KL JS. Analyzed the data: NR UK SW RG. Wrote the paper: NR SW RG.

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