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IFNγ receptor down-regulation facilitates Legionella survival in alveolar macrophages

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Interferon γ receptor downregulation facilitates *Legionella* survival in alveolar macrophages

Summary sentence: NF-κB activation following *Legionella* lung infection resulted in downregulation of IFNGR1 expression in alveolar macrophages contributing to intracellular bacterial survival.

Running Title: Interferon γ receptor downregulation during *Legionella* infection

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Abbreviations

AM Alveolar macrophage

IFNγ Interferon γ

MC Monocyte-derived cell

IFNGR1 IFNγ receptor subunit 1

LCV *Legionella* containing vacuole

PRR Pattern recognition receptor

Lp L. pneumophila

ΔflaA L. pneumophila 130b ΔflaA

ΔdotA_ΔflaA L. pneumophila 130b ΔdotA_ΔflaA

BCYE Buffered charcoal yeast extract

LB Luria-Bertani

CFU colony-forming unit

iBMDM Immortalized bone marrow derived macrophage

fGR1 FLAG-tagged IFNGR1

MOI Multiplicity of infection

PAMP Pathogen-associated molecular pattern

Abstract

Legionella pneumophila is an opportunistic human pathogen and causative agent of the acute pneumonia known as Legionnaire's Disease. Upon inhalation, the bacteria replicate in alveolar macrophages (AM), within an intracellular vacuole termed the *Legionella* containing vacuole. We recently found that, *in vivo,* interferon γ (IFNγ) was required for optimal clearance of intracellular *L. pneumophila* by monocyte-derived cells (MC), but the cytokine did not appear to influence clearance by AM. Here, we report that during *L. pneumophila* lung infection, expression of the IFNγ receptor subunit 1 (IFNGR1) is downregulated in AM and neutrophils, but not MC, offering a possible explanation for why AM are unable to effectively restrict *L. pneumophila* replication *in vivo*. To test this, we used mice that constitutively express IFNGR1 in AM and found that prevention of IFNGR1 downregulation enhanced the ability of AM to restrict *L. pneumophila* intracellular replication. IFNGR1 downregulation was independent of the type IV Dot/Icm secretion system of *L. pneumophila* indicating that bacterial effector proteins were not involved. In contrast to previous work, we found that signalling via type I interferon receptors was not required for IFNGR1 downregulation in macrophages but rather that MyD88- or Trif- mediated NF-κB activation was required. This work has uncovered an alternative signalling pathway responsible for IFNGR1 downregulation in macrophages during bacterial infection.

Introduction

Legionnaires' Disease is a life threatening infection in immunocompromised individuals and the elderly, caused predominantly by the *Legionella* species, *L. pneumophila* [1]. Acute pneumonia arises from the ability of *L. pneumophila* to infect and replicate in macrophages in the human lung [2,3]. Instead of being killed by macrophages, intracellular *L. pneumophila* secretes more than 300 effector proteins into the cytosol of the infected cells via the bacterial Dot/Icm secretion system to establish an intracellular replicative niche termed the *Legionella* containing vacuole (LCV) [4-6]. The effector proteins target numerous host cellular processes that aid LCV biogenesis, intracellular survival and bacterial replication [7-11].

In mice, innate immunity initiated by pattern recognition receptor (PRR) signalling through NF-κB mediated signalling pathways is critical for the control of *L. pneumophila* lung infection [12,13]. MyD88 signalling in particular is required for efficient production of proinflammatory cytokines and chemokines during *L. pneumophila* infection *in vitro* and *in vivo* [14,15] and these immune signalling factors drive the infiltration of neutrophils, monocytes and lymphocytes into the site of infection. Infiltrating lymphocytes in particular are responsible for the production of IFNγ in the lung, which peaks around day three after *L. pneumophila* infection [13]. IFNγ is important for the control of *L. pneumophila* infection and induces the expression of more than ~2,000 genes in cells, some of which participate in cell-autonomous killing of intracellular pathogens [16-18].

Our recent study using a mouse lung infection model, showed that viable *L. pneumophila* survived mainly in alveolar macrophages (AM) and not monocyte derived cells (MC) and neutrophils [13]. Whereas MCs clearly required IFNγ to restrict *L. pneumophila* replication, intracellular bacterial numbers were not affected in either neutrophils or AMs in IFNγ-deficient mice [16,19]. Although IFNγ can restrict *L. pneumophila* replication in macrophages *in vitro* [16], it was unclear why AM did not respond to IFNγ to restrict *L.*

pneumophila intracellular replication during mouse lung infection. Here we examined expression of the IFNγ receptor and observed downregulation of the IFNγ receptor in AM but not MC after *L. pneumophila* lung infection.

The receptor for IFNγ comprises 2 subunits, IFNGR1 and IFNFR2. Previous studies have also observed a loss of IFNGR signalling in macrophages under LPS stimulation or upon bacterial infection. This was the result of either down regulation of transcription of the IFNGR1 gene or induction of a signalling inhibitory molecule [20] and was postulated to affect the ability of macrophages to clear pathogens [21-23]. The most studied example of this phenomenon links the downregulation of IFNGR in myeloid cells to signalling mediated by type I interferons [21,24]. Type I interferon mediated downregulation of IFNGR1 during *Listeria monocytogenes* infection led to compromised clearance of the pathogen [21]. Recently, a transgenic mouse model was developed using FLAG-tagged IFNGR1 (fGR1), where IFNGR1 is constitutively expressed [21]. Here, we used fGR1 mice to demonstrate that constitutive expression of IFNGR1 improved *L. pneumophila* restriction by AM. Additionally, we found that the mechanism for IFNGR downregulation was independent of type I interferon signalling and instead dependent on NF-κB activation.

Materials and Methods

Bacterial strains and culture conditions. *L. pneumophila 130b ΔflaA* (*ΔflaA)* Str^R and *L. pneumophila 130b ΔdotA_ΔflaA* (*ΔdotA_ΔflaA*) Str^R were used in this study [25]. *L. pneumophila* strains were streaked onto buffered charcoal yeast extract (BCYE) agar supplemented with streptomycin (50 µg/mL) and cultured on BCYE agar aerobically at 37°C for at least for 3 days. Luria-Bertani (LB) broth and agar supplemented with kanamycin (100 µg/mL) were used to culture *E. coli* when required. *E. coli* strain HD5α was incubated aerobically at 37°C for 12 - 16 h, while liquid broth cultures of *E. coli* were maintained aerobically at 37°C with agitation at 180 rpm overnight.

Tissue culture. Immortalized bone marrow derived macrophages derived from wild type C57BL/6 mice (iBMDM) [26], were cultured in DMEM with GlutaMax™ culture media (Gibco, Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (HyClone Laboratories, Thermo Fisher Scientific). Cells were maintained at 37°C, 5% CO_2 and passaged 2 or 3 days to reach 90% confluency.

Mouse infection studies. Mice were infected with *ΔflaA* or *ΔdotA_ΔflaA* as described [13] and lung tissue harvested 2 days after infection. Briefly, *L. pneumophila* was cultured under optimal conditions on selective BCYE agar for 3 days. Bacterial inoculum was generated by collecting colonies from BCYE agar using PBS and adjusting via UV-spectroscopy. Mice were administered 2.5 x 10^6 CFU of *ΔflaA* or 1 x 10^8 of CFU *ΔdotA_ΔflaA* in 50 µl PBS via the intranasal route under controlled isoflurane induced anaesthesia. A higher inoculating dose of *ΔdotA_ΔflaA* was used to compensate for differences in bacterial numbers 2 days after infection between the replicating and non-replicating strains. This maintains similar CFU for *ΔflaA* and *ΔdotA_ΔflaA* at this time point. To quantitate *L. pneumophila* in lungs after infection, the right lobes of infected mice were collected and homogenised in PBS, followed by lysis with 0.1% w/v saponin for 30 minutes at 37°C and *L. pneumophila* were enumerated by serially diluting the homogenate in PBS and plating onto selective BCYE. C57BL/6 and *Ifnar1*$^{-/-}$ mice were bred and maintained at Bio21 Molecular Science and Biotechnology Institute or the Peter Doherty Institute for Infection and Immunity, The University of Melbourne under specific pathogen free conditions. FLAG-tagged IFNGR1 (fGR1) transgenic mice were bred and maintained at the Department of Immunology and Microbiology, University of Colorado School of Medicine under specific pathogen free conditions. All animal experiments were performed with the approval of the Animal Ethics Committees of both University of Melbourne and University of Colorado School of Medicine.

***L. pneumophila* infection and LPS stimulation of iBMDM.** iBMDM were seeded 12 h before infection into 24 well plates (Corning) at density 2.5 x 10^5 per well. Macrophages were infected with *ΔflaA* or *ΔdotA_ΔflaA* opsonized with anti-*L. pneumophila* antibody (Meredian life science, B65051G) at an MOI of 0.5 (*ΔflaA*) or 10 (*ΔdotA_ΔflaA*) or dosed with heat-killed *ΔflaA* (Multiplicity of infection, MOI = 0.5) or 1 μg/ml LPS from *E. coli* O111:B4 (InvivoGen, tlrl-eblps). Cells treated with bacteria or LPS were centrifuged at 1,000 rpm for 5 min at room temperature and incubated at 37°C in 5% CO_2 for various time-points.

Flow cytometry. Lung single cell suspensions for flow cytometric analysis were prepared as previously described [13]. Live cells were stained with fluorescently labelled antibodies against Siglec F, FcεRI, CD64, Ly-6G, CD11c, IFNGR1 and viability dye eFluor™ 780 (ThermoFisher Scientific, reference: 65-0865-14). Antibodies used in this study are listed in Supplementary Table 2. After washing with FACS buffer (PBS containing 2% calf serum and 1 mM EDTA), cells were stained with streptavidin conjugated with PE-CF594 (1/300) for 15 mins at 4°C in the dark. Cells were subsequently fixed and permeabilized with 200 μl 1x Fixation/Permeabilisation Buffer (eBioscience, 00-5521-00) as per the manufacturer's instructions, and then stained with a polyclonal FITC-anti-*Legionella* antibody (ViroStat, cat # 6053). Total numbers for each cell type were enumerated from the lungs by adding 2 x 10^4 APC-labelled beads (BD Calibrite, reference: 340487) into each sample prior to flow cytometry analysis. Dead cells were detected and excluded based on eFluor 780 fluorescence. Data were analysed with FlowJo software.

Cell sorting and quantification of bacterial loads in purified cells and lung tissue. Cells were prepared from whole lungs of *L. pneumophila* infected C57BL/6 or fGR1 mice as described previously [13]. For cell sorting, antibody stained cells were resuspended in 1 ml FACS buffer with 7-AAD (final 0.25 μg/ml) for flow cytometric sorting. 10^4 cells of each cell type were lysed with 200 μl of 0.05 % w/v digitonin in PBS (Sigma Aldrich) for 5 mins

at RT after which 800 µl PBS was added. 100 µl of the cell lysate was plated on selective BCYE agar. Quantification of CFU in lung tissue was performed as described previously [13].

qRT-PCR for gene expression. iBMDM or lung tissue was used for total RNA extraction via TRIsure (Bioline) according to manufacturer's instructions. 1 µg of total RNA was used for cDNA synthesis with SensiFAST™ cDNA Synthesis Kit (Bioline) according to manufacturer's recommendations. cDNA was then diluted 1:7 and 2 µl of the diluted cDNA was adopted in qRT-PCR. qRT-PCR was performed using QuantStudio 7 Flex Real-Time PCR System with 5 µl SsoAdvanced Universal SYBR Green Supermix (BioRad) and 0.2 µM of each primer in a 10 µl reaction. Primer pairs are listed in Supp Table 1. Relative transcription levels of target genes were normalised to the housekeeping gene *RNA18S5*. The equation fold change = $2^{-\Delta\Delta Ct}$ was used to calculate relative expression levels of target genes.

Immunoblot analysis. Cells were lysed in cold 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Triton X-100, 2 mM Na3VO4, 10 mM NaF, 1mM PMSF and 1 x EDTA-free Complete protease inhibitor cocktail (Roche). Cell lysate was incubated for 30 minutes on ice, then cell debris was pelleted at 13,000 rpm at 4 °C for 10 minutes. The soluble protein fraction was mixed with 4 x Bolt® LDS sample buffer (Life Technologies) and DTT (Astral Scientific) to a final concentration of 50 mM. Samples for immunoblot were subjected to electrophoresis on 4-12% Bis-Tris gels. Proteins were transferred to polyvinylidene difluoride membrane and immunodetection was performed as previously published [27]. Antibodies used were mouse monoclonal total IκBα (1:1,000) (Cell Signalling, cat# 4814), mouse monoclonal anti-β-actin (1:3,000) (Sigma Aldrich, A5441), goat anti-mouse IgG (H+L)-HRP (1:5,000) (PerkinElmer, NEF822001EA) and goat anti-rabbit IgG (H+L)-HRP (1:5,000) (Bio-Rad, 170-6515).

CRISPR/Cas9 gene mutations in iBMDM. The Optimized CRISPR Design website, crispr.mit.edu, was used for sgRNA design. Selected sgRNA sequences with a 5′ "TCCC" 4

bp overhang for the forward complementary sequence and a 5′ "AAAC" 4 bp overhang for the reverse complementary sequence were designed. The gRNAs are listed in Supp Table 3. An inducible CRISPR/Cas9 lentiviral platform was used for gene mutations [28]. Briefly, lentivirus vectors were used to express Cas9 expression and gRNAs in iBMDM. gRNA expression was induced with 1 μg/ml doxycycline treatment for 72 h. iBMDM constitutively expressing Cas9 and $Cas9^{+}gRNAs^{+}$ iBMDM were sorted based on mCherry (Cas9) and eGFP (gRNA) expression. $mCherry^{+}eGFP^{+}$ cells were sorted in to 96 well plates (one cell per well) to generate clonal cell lines. Successful gene knockout on both alleles was validated in each clone by PCR and sequencing. The PCR and sequencing primers are listed in Supp Table 4.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad In Stat Software Inc.). An unpaired, two-tailed student t-test and a two-tailed Mann-Whitney *U*-test were performed to test for differences between experimental datasets.

Results

Surface expression of IFNGR1 on AM, MC and neutrophils during *L. pneumophila* lung infection. To investigate IFNGR1 expression during *L. pneumophila* infection, C57BL/6 mice were infected via the intranasal route with *L. pneumophila ΔflaA*. Cell-surface levels of IFNGR1 on AM, neutrophils and MC were analysed by flow cytometry 2 days after infection using a gating strategy described previously [13]. Cell-surface IFNGR1 was significantly reduced in AM and neutrophils from *L. pneumophila* infected mice compared to the same cell subsets from uninfected mice (Fig 1A, B). Using an anti-*L. pneumophila* antibody, *L. pneumophila* infected cells were distinguished from uninfected cells as described previously [13]. While there was no significant reduction of cell-surface IFNGR1 in AM that had not taken up *L. pneumophila* (Lp^{-}), cell-surface levels of IFNGR1 were significantly downregulated in *L. pneumophila*-positive (Lp^{+}) AM (Fig 1A). In contrast, cell-surface expression of IFNGR1 in Lp^{+} and Lp^{-} MC were comparable (Fig 1C). Together, these data showed that IFNGR1 was

downregulated on the surface of AM and neutrophils, but not MC, upon *L. pneumophila* infection.

Contribution of the *Legionella* Dot/Icm secretion system to downregulation of surface IFNGR1. To investigate whether Dot/Icm effector proteins contributed to the downregulation of IFNGR1 in AM and neutrophils, we infected WT mice with a strain of *L. pneumophila* lacking the type IV Dot/Icm secretion system and flagellin (*ΔdotA_ΔflaA*). Cell-surface levels of IFNGR1 were measured 2 days after infection. Similar to *ΔflaA* infection, cell-surface expression of IFNGR1 in AM and neutrophils was significantly decreased in mice infected with *ΔdotA_ΔflaA* compared to uninfected mice (Fig 2A, B). Lp^+ AM, but not Lp^- AM, showed significantly lower levels of cell-surface IFNGR1 compared to AM from uninfected mice (Fig 2A). Again, MC did not show changes in IFNGR1 surface expression upon infection (Fig 2C). These results showed that downregulation of IFNGR1 in AM and neutrophils did not require the Dot/Icm secretion system of *L. pneumophila* and therefore was not effector protein-dependent. We next determined whether the downregulation of IFNGR1 in macrophages occurred *in vitro*. iBMDM were infected with *ΔflaA, ΔdotA_ΔflaA* or treated with heat-killed *ΔflaA* or LPS. We observed that cell-surface (Fig 3A, B) and mRNA expression (Fig 3C) of IFNGR1 was significantly reduced in iBMDM 1 day after *ΔdotA_ΔflaA* and *ΔflaA* infection or after treatment with heat-killed *L. pneumophila* or LPS compared to uninfected/untreated cells. However, there was no difference in IFNGR2 mRNA expression between *L. pneumophila* infected and uninfected iBMDM (Fig 3D). This suggested that the downregulation of IFNGR1 expression resulted from induction of an inflammatory response to components of *L. pneumophila*, including LPS.

Downregulation of IFNGR1 did not require type I IFN signalling. Type I IFNs have been reported to downregulate IFNGR1 expression in macrophages upon bacterial infection [22]. LPS stimulation also induces robust type I IFN expression in macrophages [29]. To investigate

whether the downregulation of IFNGR1 in macrophages was triggered by type I IFNs during *L. pneumophila* infection, we examined IFNGR1 downregulation in *Ifnar1*$^{-/-}$ mice. As shown in Fig 4A and 4B, cell-surface expression of IFNGR1 in AM and neutrophils was significantly decreased in the lungs of *Ifnar1*$^{-/-}$ mice 2 days after *ΔflaA* infection compared to AM and neutrophils from naïve *Ifnar1*$^{-/-}$ mice. *Lp*$^{+}$ AM, but not *Lp*$^{-}$ AM, also showed significant downregulation of cell-surface IFNGR1 compared to naïve AM (Fig 4A). Downregulation of IFNGR1 was not observed in MC (Fig 4C). These results indicated that downregulation of cell-surface IFNGR1 in macrophages does not require signalling through type I IFNs.

Involvement of Myd88 and Trif signalling and NF-κB activation in downregulation of IFNGR1. Following recognition of pathogen-associated molecular patterns (PAMPs), TLRs transfer signals into intracellular signalling pathways via Myd88 and/or Trif to activate NF-κB signalling [30,31,32]. To determine whether Myd88 and Trif contributed to downregulation of IFNGR1 expression in macrophages, iBMDM lacking Myd88, Trif or both proteins were treated with LPS for 24 h and cell-surface expression of IFNGR1 was examined. LPS treatment was used because we obtained stronger downregulation with this treatment. Similar to WT iBMDM, *Myd88*$^{-/-}$ iBMDM and *Trif*$^{-/-}$ iBMDM showed the loss of cell-surface IFNGR1 expression upon LPS treatment compared to untreated cells (Fig 5A-C). However, double *Myd88*$^{-/-}$*Trif*$^{-/-}$ iBMDM did not show downregulation of IFNGR1 after LPS treatment compared to untreated cells (Fig 5D). These findings suggested that signalling via Myd88 and Trif was involved in the regulation of IFNGR1 expression in macrophages.

Myd88- and Trif-mediated signalling cascades induce NF-κB activation, leading to the expression of numerous pro-inflammatory cytokines [33,34,35-38]. To investigate whether NF-κB activation contributed to downregulation of IFNGR1 in LPS treated macrophages, NF-κB activation was inhibited in iBMDM using the NF-κB inhibitor BAY 11-7085, which

irreversibly inhibits IκBα phosphorylation and prevents its degradation, thereby preventing NF-κB translocation into the nucleus [39,40]. iBMDM were treated with 10 μM of BAY 11-7085 for 1.5 h followed by 1 μg/ml LPS treatment for 20 min. Upon LPS treatment, the total amount of IκBα in BAY11-7085 treated cells was higher than in DMSO treated control cells (Supp Fig 1A), indicating that BAY 11-7805 inhibited IκBα degradation. Cell-surface expression of IFNGR1 was measured in cells pre-treated with 10 μM BAY 11-7085 for 1.5 h followed by 1 μg/ml LPS or heat-killed *L. pneumophila* stimulation. Cell-surface IFNGR1 levels were comparable between DMSO-treated and BAY 11-7085 treated macrophages without LPS or heat-killed *L. pneumophila* treatment (Fig 5E). However, downregulation of IFNGR1 was prevented in cells pre-treated with BAY 11-7085 compared to DMSO treated cells after LPS or heat-killed *L. pneumophila* treatment (Fig 5E). This suggested that NF-κB activation contributed to the downregulation of IFNGR1.

Myd88 and Trif-mediated signalling can activate receptor-interacting serine/threonine-protein kinase 3 (RipK3) and the transcription factors interferon regulatory factors 3 and 7 (Irf3 and Irf7). We examined if these molecules were required for IFNGR1 down regulation but found that iBMDM deficient in RipK3 or Irf3 and Irf7 downregulated IFNGR1 after LPS treatment in a manner similar to WT iBMDM (Supp. Fig 1B-D).

NF-κB controls expression of many genes, including transcriptional activators and repressors [35]. B-cell lymphoma 6 protein (Bcl6) is a NF-κB-inducible transcriptional repressor that has been reported to suppress IFNGR1 expression in T follicular helper cells by forming a repressive complex with metastasis-associated protein 3 (MTA3) at the promoter region of IFNGR1 [41]. We investigated whether Bcl6 contributed to the downregulation of IFNGR1 in LPS treated iBMDM. *Bcl6* was deleted in iBMDM using CRISPR/Cas9 (Supp Fig 2A-C) and *Bcl6*$^{-/-}$ iBMDM showed similar levels of cell-surface IFNGR1 compared to WT cells in steady state (Supp Fig 2D). Under LPS treatment, IFNGR1 cell-surface expression was still

downregulated in *Bcl6*$^{-/-}$ iBMDM 6 h after treatment compared to untreated cells, which was comparable to that of WT iBMDM (Supp Fig 2D). This suggested that Bcl6 does not contribute to the downregulation of IFNGR1 in macrophages during inflammatory conditions.

Constitutive expression of IFNGR1 attenuated *L. pneumophila* intracellular replication in AM *in vivo.* AM have been reported to restrict *L. pneumophila* intracellular replication in the presence of IFNγ *ex vivo* [16]. Therefore, we considered the possibility that downregulation of IFNGR1 expression in AM in the lung may result in an inability to restrict *L. pneumophila* intracellular replication. To test this hypothesis, transgenic mice that constitutively express a Flag-tagged IFNGR1 (fGR1) in myeloid cells were used[21]. WT mice and fGR1 mice were infected via the intranasal route with *ΔflaA*. The cell-surface levels of IFNGR1 were measured in Lp^+ and Lp^- AM from WT mice and fGR1 mice 2 days after infection. While Lp^+ AM from WT mice showed downregulation of IFNGR1, the expression of IFNGR1 in Lp^+ AM from fGR1 mice was not significantly changed, as expected (Fig 6A). AM were isolated from the lungs of WT mice and fGR1 mice 2 days after infection, lysed and lysates cultured to determine the number of live *L. pneumophila* in AM, as described previously [13]. We found that AM from fGR1 mice harboured significantly fewer live bacteria than AM from WT mice (Fig 6B) suggesting that constitutive IFNGR1 expression aided the ability of AM to resist *L. pneumophila* infection. However, despite a decrease in CFU recovered from AM, total CFU in lung (Fig 6C) and weight loss of the animals (Fig 6D) was not significantly different in fGR1 mice compared to WT mice.

The number of AM in WT and fGR1mice was also determined. Uninfected WT and fGR1 mice had similar numbers of AM, although 2 days after infection fGR1 mice showed significantly greater numbers of AM (Fig 6E). This increase in AM number could be due to

reduced cell death in fGR1 mice as fewer AM stained with eFluor™ 780, a viability dye used to irreversibly label dead cells (Fig 6F).

Discussion

Macrophages play a vital role in the initiation of immune responses by recognizing pathogens and other inflammatory stimuli. Due to their high phagocytic capacity, macrophages are especially vulnerable to invasion by a range of pathogenic organisms [22,23,42,43], including *L. pneumophila* [13]. IFNγ is one of the cytokines that plays an important role in the control of *L. pneumophila* intracellular replication [44,45]. Signalling via the IFNGR induces nearly 2,000 IFN-stimulated genes (ISGs) in human and mouse [18]. Clusters of ISGs, such as immunity-related GTPases (Irgs) and guanylate-binding proteins (Gbps) are of importance in cell-autonomous immunity against intracellular pathogens in mammals [17,18,46,47]. ISGs can lyse pathogen-containing vacuoles, which leads to the activation of autophagy and the inflammasome [46].

In cell culture, AM were reported to restrict *L. pneumophila* intracellular replication in the presence of IFNγ [16]. However, we found that, *in vivo*, IFNγ had no impact on the survival of intracellular *L. pneumophila* in AM and neutrophils [13]. We found that IFNGR1 was significantly downregulated in AM and neutrophils upon *L. pneumophila* lung infection and that downregulation only occurred in AM infected with *L. pneumophila* rather than uninfected AM. This has also been observed in *Mycobacterium tuberculosis* and *L. monocytogenes* infected macrophages [21,23].

IFNGR1 expression was not downregulated in MC after *L. pneumophila* infection, which could partly explain why MC restrict *L. pneumophila* in response to IFNγ while AM appear to be poorly bactericidal in the presence or absence of IFNγ [13]. IFNGR1 surface expression was also downregulated in neutrophils, yet these cells continue to clear bacteria in the absence of IFNγ [13], suggesting neutrophils kill *L. pneumophila* via IFNγ-independent

pathways. This includes the induction of NADPH oxidase 2, which generates ROS in response to the translocation of *L. pneumophila* Dot/Icm effector proteins [48].

To avoid fusion with lysosomes and bactericidal killing, *L. pneumophila* establishes the LCV, which allows the bacteria to survive and replicate intracellularly [49-51]. Effector proteins delivered by the Dot/Icm secretion system of *L. pneumophila* are critical for bacterial intracellular replication and interfere with critical host cell functions such as protein synthesis and stress responses [52-55]. We tested whether Dot/Icm secretion system deficient *L. pneumophila* mutant (*ΔdotA_ΔflaA*) also induced IFNGR1 downregulation in AM and found that IFNGR1 expression was still downregulated upon *ΔdotA_ΔflaA* infection. Furthermore, we observed that heat killed *L. pneumophila* or LPS treatment also led to downregulation of IFNGR1 in iBMDM *in vitro*. This suggested that downregulation of IFNGR1 was associated with cellular inflammatory signalling rather than *L. pneumophila* effector protein activity or live infection.

Type I IFNs have been shown to downregulate IFNGR1 expression in macrophages facilitating *L. monocytogenes* infection [22,24]. One mechanism for this may be that IFNβ treatment reduces the aggregation of activated RNA polymerase II and the accumulation of acetylated histones H3 and H4 at the *Ifngr1* promoter in macrophages, thereby affecting expression [24]. However, in our study, deficiency of IFNAR1, which ablates all signalling by type I IFNs, did not attenuate downregulation of IFNGR1 in AM after *L. pneumophila* infection. Instead we observed that LPS induced downregulation of IFNGR1 in iBMDM required both MyD88 and Trif, which connect TLR recognition of PAMPs with transcription factors, such as Irf3, Irf7 and NF-κB [33,34]. Irf3 and Irf7 are highly homologous and are the key transcription factors responsible the induction of type I IFNs [56]. NF-κB is a crucial and central regulator of gene expression for cell survival, cell death, inflammation and immune response [35-38]. Inhibition of NF-κB activation using BAY 11-7085 also significantly reduced

downregulation of IFNGR1 in macrophages upon treatment with LPS or heat-killed *L. pneumophila*, suggesting that MyD88- and/or Trif-mediated NF-κB activation was required for the reduction in IFNGR1 expression in infected or stimulated macrophages. Given the broad nature of these signaling pathways, we do not expect this finding to be specific for *Legionella* infection. Indeed *E. coli* LPS was also effective at inducing IFNGR1 downregulation, suggesting a conserved component such as Lipid A may be the stimulus. Other TLR ligands may also produce similar effects on IFNGR1 expression so further experiments are needed using diverse pathogens to determine the range of signaling events that lead to IFNGR1 downregulation.

The pathway downstream of NF-κB leading to IFNGR1 downregulation is still unknown. It has been reported that LPS stimulation induces suppressor of cytokine signalling 3 (SOCS3) expression and the overexpression of SOCS3 inhibits signal transducer and activator of transcription 1 (STAT1) and janus kinase 1 (JAK1) activation in macrophages treated with IFNγ [20]. However, whether the suppressive function of SOCS3 contributes to the downregulation of IFNGR1 by LPS in macrophages is still to be clarified. We found that none of RipK3, Irf3 or Irf7 contributed to the downregulation of IFNGR1 in LPS treated macrophages [15, 38, 39]. The lack of a role for Irf3 and Irf7 supports our finding that type I IFNs do not play a role in IFNGR1 down regulation in this model. We also examined whether the transcriptional repressor Bcl6, which can be induced by NF-κB [57], was involved but loss of *Bcl6* expression had no effect on the reduction of IFNGR1 expression in macrophages treated with LPS.

Other factors have been shown to influence the IFNGR1 promoter in cancer cell lines. In breast cancer cells, activating protein (AP)-2α binds to the IFNGR1 promoter region and overexpression of AP-2α decreased IFNGR1 expression, thereby inhibiting IFNγ signalling [58]. In the same report, specificity protein 1 (SP1) was also shown to bind the IFNGR1

promoter and overexpression of SP1 effectively antagonized the repressive effects of AP-2α in IFNGR1 expression [58]. In our system, LPS induced NF-κB activation may downregulate SP1 by inducing degradation [59]. However, whether SP1 and AP-2α contribute to NF-κB mediated downregulation of IFNGR1 in response to LPS needs further investigation. During *Legionella* infection, an active Dot/Icm secretion system has been associated with NF-κB activation [60-62], and we initially expected IFNGR1 downregulation to depend on live bacterial infection. However, this was not the case and it appears that the downregulation of IFNGR1 occurs after broader TLR stimulation and subsequent NF-κB activity.

Transgenic fGR1 mice are an ideal model to test the functional consequences of IFNGR downregulation as they express a tagged IFNGR1 under heterologous genetic regulatory sequences that are not suppressed after macrophage stimulation and hence constitutively express IFNGR1 in myeloid cells [21]. fGR1 mice have been used to explore the importance of IFNGR1 downregulation during *L. monocytogenes* infection, and these studies showed that fGR1 mice were more resistant to infection, despite normal type I and II IFN production [21]. We found that AM in fGR1 mice had a higher capacity to restrict *L. pneumophila* replication indicating that IFNGR1 downregulation and decreased IFNγ signalling compromised the bactericidal function of AM. It was somewhat surprising that despite fewer live bacteria being present in AM from fGR1 mice, there was no significant difference in *L. pneumophila* CFU in whole lung between WT and fGR1 mice. This may be because a major proportion of *L. pneumophila* immune control is performed by MC and neutrophils [13], and their bactericidal activities do not depend IFNγ. Alternatively, infected fGR1 mice have higher numbers of AM compared to WT, potentially increasing the replicative niche of *L. pneumophila* in fGR1 mice. A number of studies have suggested that the induction of cell death pathways in phagocytic cells after *Legionella* infection may be a host mechanism that aids in the clearance of the invading bacteria [25,63-65]. We also recently

observed that AM are rapidly depleted in lungs of mice infected with *L. pneumophila* [13], a result that was replicated here. We also found that in fGR1 mice, where IFNGR1 downregulation was prevented, the attrition of AM did not occur, which may be related to the reduced replication of *L. pneumophila* in these cells [13].

In summary, we found that IFNGR1 was significantly downregulated in AM in the lungs of *L. pneumophila* infected mice and this downregulation was not associated with live infection but rather MyD88- and Trif-mediated NF-κB activation. Preventing downregulation of IFNGR1 expression in AM increased their ability to restrict *L. pneumophila* replication. These findings help explain our previous observations on the insensitivity of AM to IFNγ in mice, a finding that was seemingly at odds with published *in vitro* data [13]. This work also emphasises that the study of immune responses needs to take all phagocytes into account. While macrophages are permissive for *Legionella* replication *in vitro* and *in vivo* and are therefore appropriate to use for the study of intracellular bacterial replication, neutrophils and monocyte derived cells restrict and kill the bacteria and so need to be considered as effector phagocytes when studying the pulmonary immune response.

Authorship

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Author Contributions

Conceptualisation, CY, SP, SB, IV, EH; Methodology, CY, DSM, SP, SB, LL, IV, EH; Formal analysis, CY, DSM, SP, IV, EH; Investigation, CY, DSM, SP; Writing - Original Draft, CY, IV, EH; Writing - Review and Editing, All; Supervision, SP, SB, LL, IV, EH; Project Administration, SP, LL, IV, EH; Funding Acquisition, LL, IV, EH.

Conflict of Interest Statement

The submitted work was carried out in the absence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

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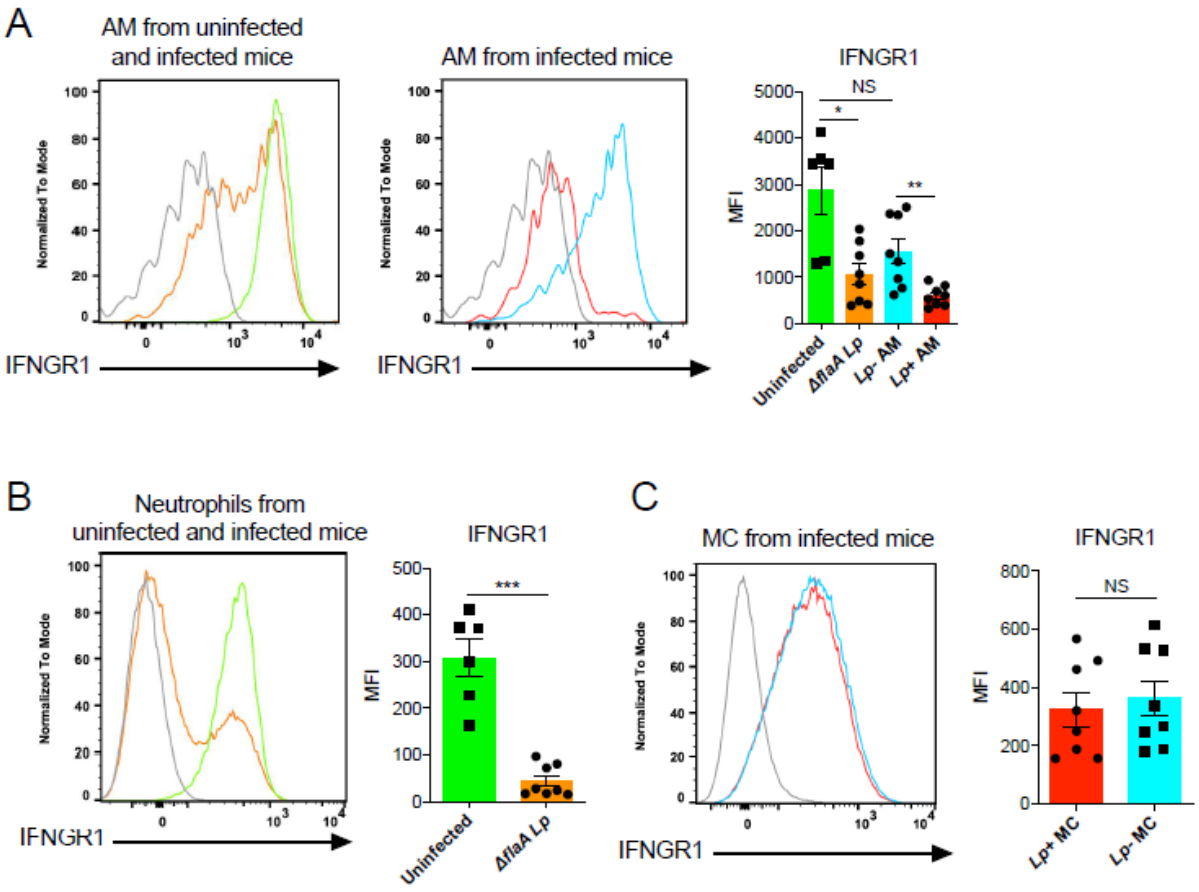

Fig 1. Cell-surface IFNGR1 is downregulated in AM and neutrophils, but not MC, after *L. pneumophila* infection. WT mice were inoculated via the intranasal route with 2.5 x 10^6 *ΔflaA* and cell-surface levels of IFNGR1 were analysed by flow cytometry in AM, neutrophils and MC, 2 days after infection. **A, B.** Representative histogram and mean fluorescence intensities (MFIs) of cell-surface IFNGR1 in total AM and neutrophils as indicated. In 'AM from uninfected and infected' and 'Neutrophils from uninfected and infected' panels, green represents cells from uninfected mice and orange cells from *L. pneumophila* infected mice. **A, C.** Cell surface IFNGR1 in Lp^+ cells (red) and Lp^- cells (blue) in AM and MC from infected mice, as indicated. Grey histograms are isotype controls. Note: In A, grey histograms in left and right panels are identical as cells were analysed in same experiment. The histograms are representative of 3 independent experiments. Each dot represents one mouse. Mean ± SEM shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS: no significance.

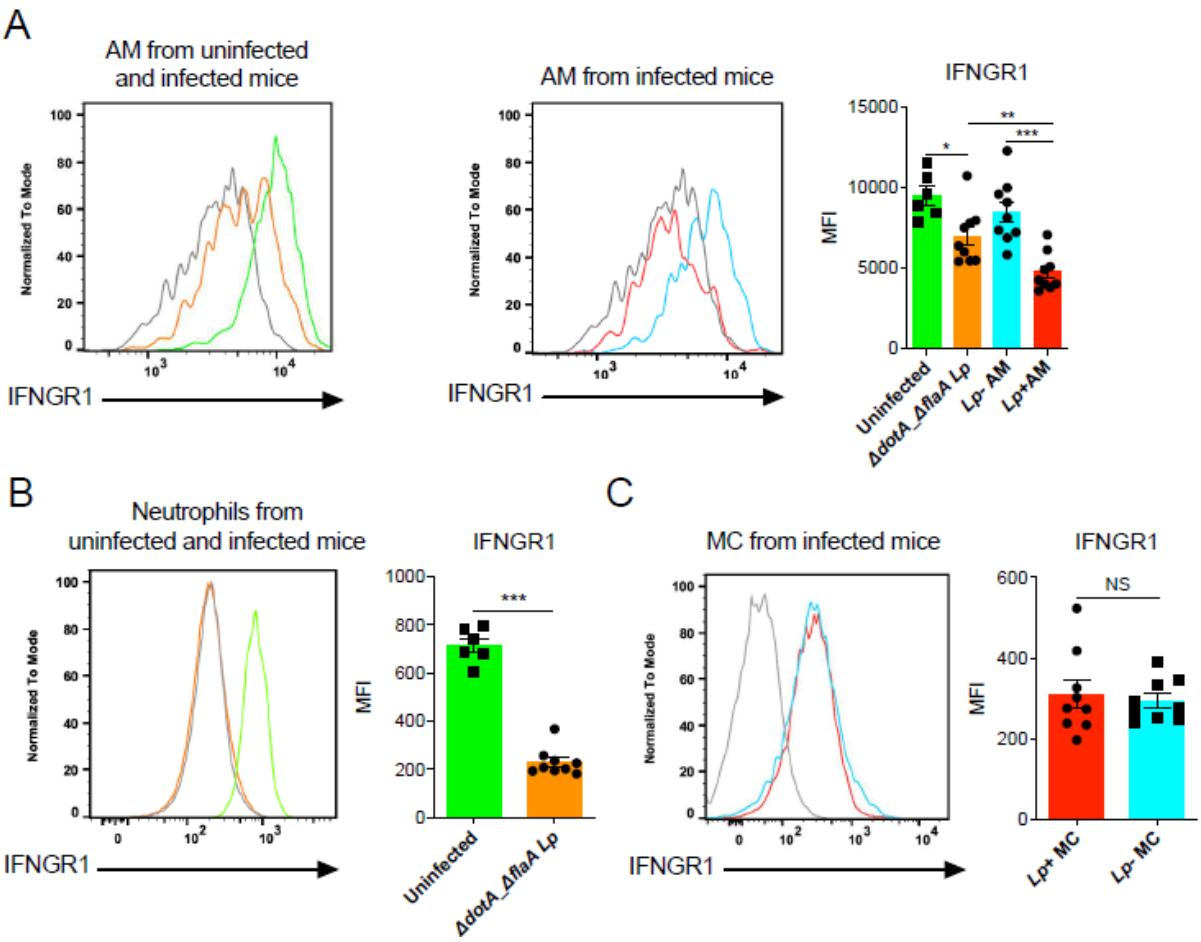

Fig 2. Downregulation of IFNGR1 was independent of the *L. pneumophila* Dot/Icm secretion system of *L. pneumophila*. WT mice were inoculated via the intranasal route with 1 x 10^8 *ΔdotA_ΔflaA* and cell-surface levels of IFNGR1 in AM, neutrophils and MC were analysed 2 days after infection by flow cytometry. **A, B.** Representative histogram and mean fluorescence intensities (MFIs) of cell-surface IFNGR1 in total AM and neutrophils as indicated. In 'AM from uninfected and infected' and 'Neutrophils from uninfected and infected' panels, green represents cells from uninfected mice and orange represents cells from *L. pneumophila* infected mice. **A, C.** Cell surface IFNGR1 in Lp^+ cells (red) and Lp^- cells (blue) in AM and MC from infected mice, as indicated. Grey histograms are isotype controls. Note: In A, grey histograms in left and right panels are identical as cells were analysed in same experiment. The histograms are representative of 3 independent experiments. Each dot

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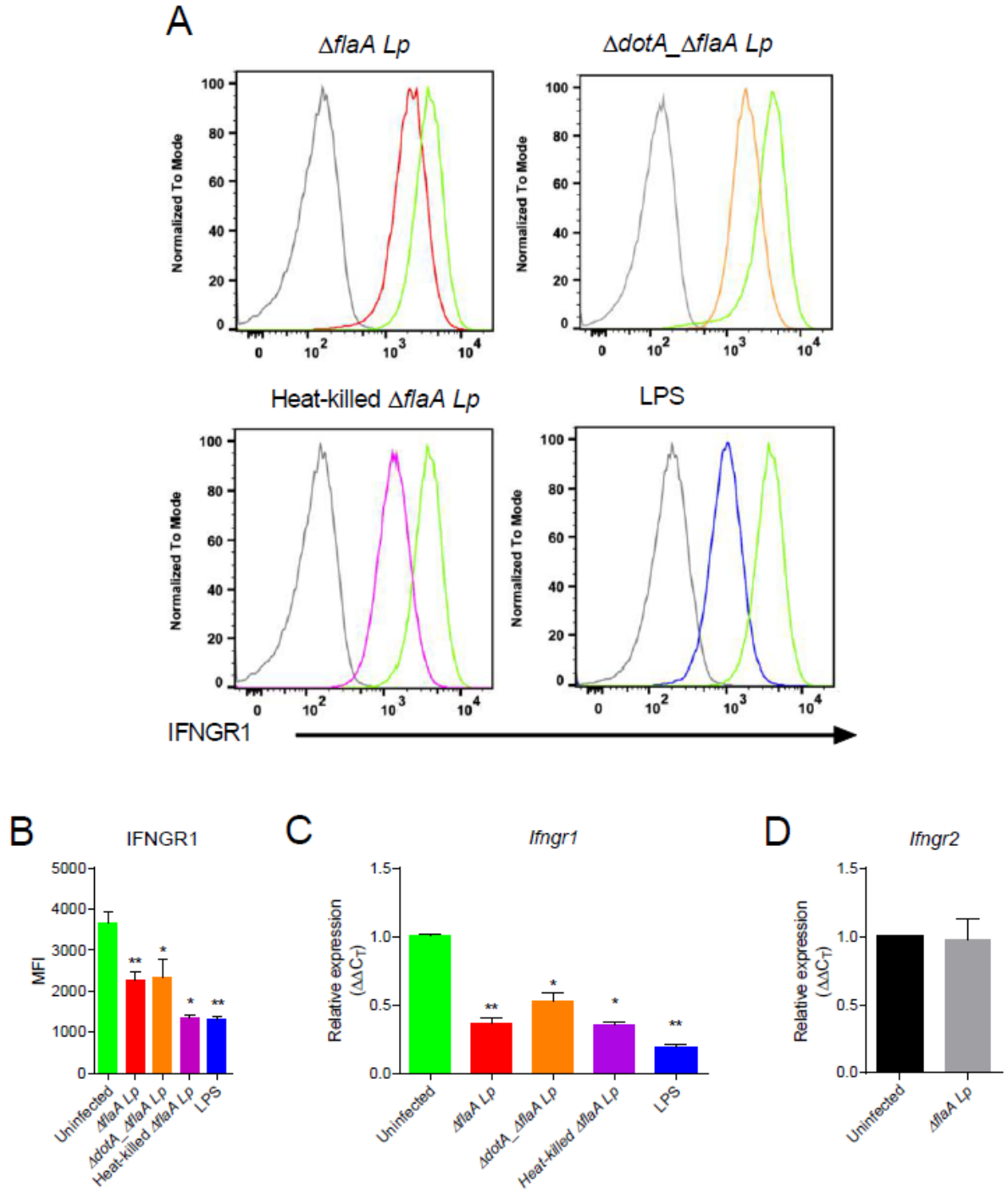

Fig 3. The downregulation of IFNGR1 in macrophages is reliant upon an inflammatory response. iBMDM were infected with *ΔdotA_ΔflaA* (MOI = 10), *ΔflaA* (MOI = 1), heat-killed *ΔflaA* (MOI = 10) or treated with 1 μg/ml LPS for 1 day. Cell-surface IFNGR1

expression was measured in iBMDM by flow cytometry (**A**, **B**) and IFNGR1 mRNA by qPCR analysis (**C**). **D**. IFNGR2 mRNA expression was measured by qPCR in iBMDM 1 day after *ΔflaA L. pneumophila* (MOI = 1) infection. Grey is isotype control (**A**). Note: In A, grey histograms in upper and lower panels are identical as cells were analysed in same experiment. The histograms are representative of at least 3 independent experiments. Mean ± SEM shown, * $p < 0.05$, ** $p < 0.01$.

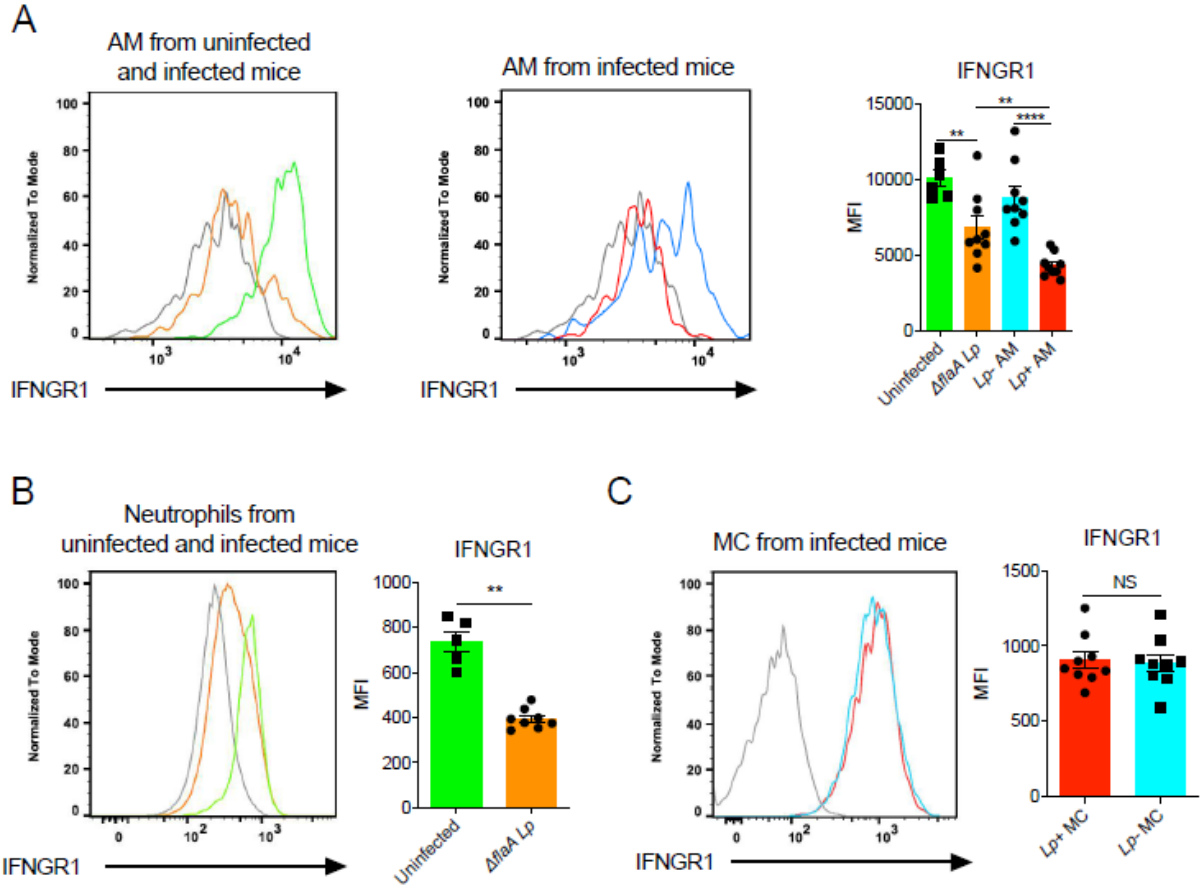

Fig 4. Downregulation of IFNGR1 in macrophages *in vivo* did not required type I IFN signalling. *Ifnar1*$^{-/-}$ mice were inoculated via the intranasal route with 2.5 x 10^6 *ΔflaA* and cell-surface levels of IFNGR1 in total AM, neutrophils populations and MC were analysed 2 days after infection by flow cytometry. **A, B.** Representative histogram and mean fluorescence intensities (MFIs) of cell-surface IFNGR1 in total AM and neutrophils as indicated. In 'AM from uninfected and infected' and 'Neutrophils from uninfected and infected' panels, green represents cells from uninfected mice and orange represents cells from

L. pneumophila infected mice. **A, C.** Cell surface IFNGR1 in Lp^+ cells (red) and Lp^- cells (blue) in AM and MC from infected mice, as indicated. Grey histograms are isotype controls. Note: In A, grey histograms in left and right panels are identical as cells were analysed in same experiment. The histograms are representative of 3 independent experiments. Each dot represents one mouse. Mean ± SEM shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS: no significance.

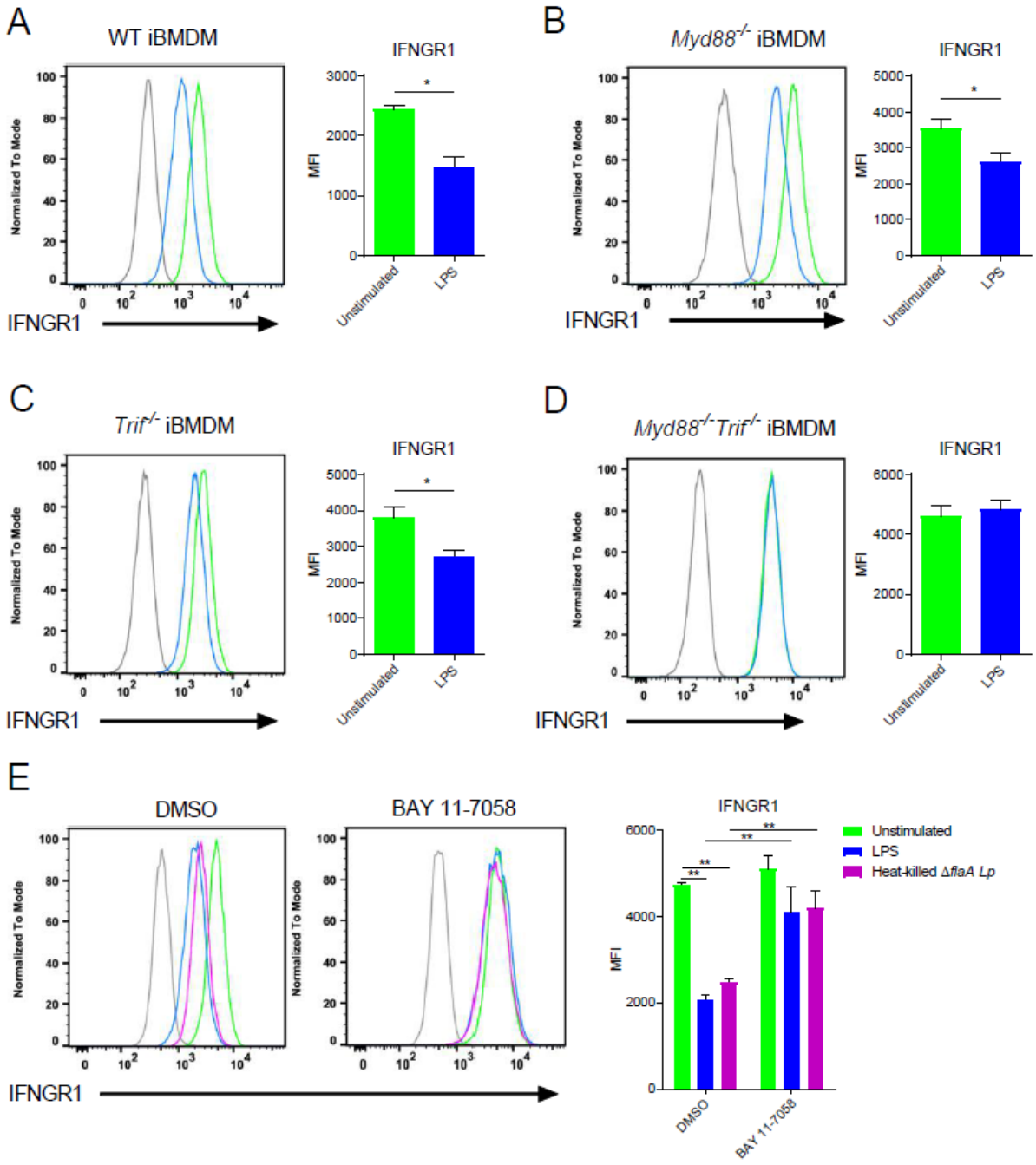

Fig 5. Both Myd88- and Trif-mediated signalling pathways and NF-κB activation were essential for the downregulation of IFNGR1 in macrophages *in vitro*. Cell-surface IFNGR1 levels were measured by flow cytometry in WT iBMDM (**A**), *Myd88*$^{-/-}$ iBMDM (**B**), *Trif1*$^{-/-}$ iBMDM (**C**) and *Myd88*$^{-/-}$*Trif1*$^{-/-}$ iBMDM (**D**) 1 day after 1 μg/ml LPS treatment. **E**. BAY 11-7085 (IC_{50} = 10 μM) or DMSO was added 1.5 h before treatment or infection and maintained in iBMDM cultures. Cell-surface IFNGR1 expression was measured by flow

cytometry 6 hours after 1 µg/ml LPS or heat-killed *ΔflaA* (MOI = 10) treatment. The histograms are representative of at least 4 independent experiments. Mean ± SEM shown. * $p < 0.05$.

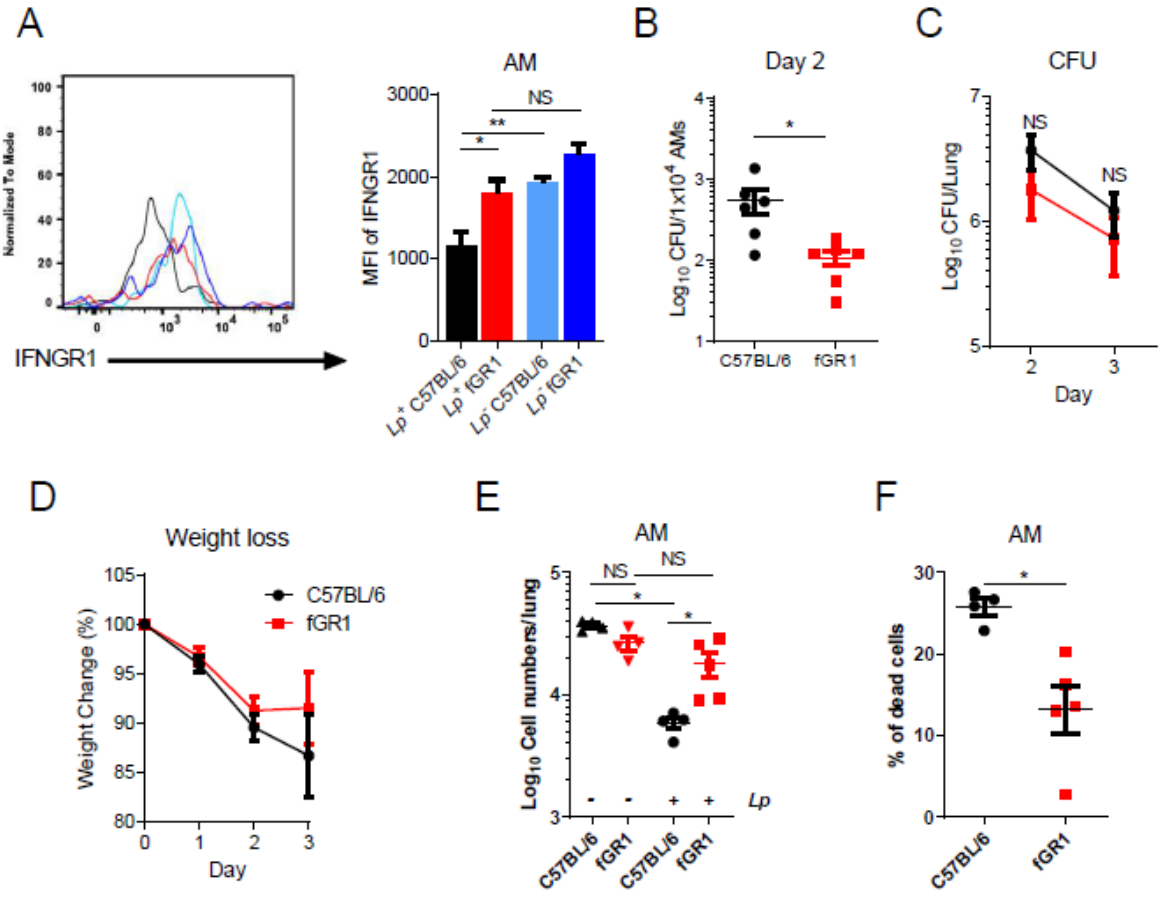

Fig 6. Constitutive expression of IFNGR1 attenuated *L. pneumophila* intracellular replication in AM *in vivo*. WT mice and fGR1 mice were inoculated via the intranasal route with 2.5 x 10^6 *ΔflaA*. **A**. Representative histogram and MFIs of cell surface levels of IFNGR1 in Lp^+ AM and Lp^- AM and analysed 2 days after infection by flow cytometry. Colour coding in histogram corresponds with that shown in the MFI graph. **B**. AM from WT mice and fGR1 mice were isolated, lysed and the lysates cultured on selective bacterial culture plates to determine the number of CFU per 10^4 cells in each cell population. The bacterial burden in the lungs (**C**) and weight loss (**D**) of WT mice and fGR1 mice in the indicated time points after infection with *ΔflaA*. The number of viable AM (**E**), and the percentage of dead AM (**F**)

in lungs of indicated mouse strains 2 days after infection. Data is pooled from 2-3 independent experiments. Each dot represents one mouse. B. Mean ± SEM is shown. * $p < 0.05$, ** $p < 0.01$, NS: no significance.

Graphical Abstract: