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MyD88 signalling in myeloid cells is sufficient to prevent chronic mycobacterial infection

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Abstract

Tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*) that is responsible for almost 1.5 million deaths per year. Sensing of mycobacteria by the host's immune system relies on different families of receptors present on innate immune cells. Amongst them, several members of the Toll-like receptor (TLR) family are involved in the activation of immune cells by mycobacteria, yet the in vivo contribution of individual TLRs to the protective immune response remains controversial. On the contrary, MyD88, the adaptor molecule for most TLRs, plays a non-redundant role in the protection against tuberculosis and mice with a complete germline deletion of MyD88 succumb very early to infection. MyD88 is expressed in both immune and non-immune cells, but it is not clear whether control of mycobacteria requires ubiquitous or cell-type specific MyD88 expression. Therefore, using novel conditional switch-on mouse models, we aimed to investigate the importance of MyD88 signalling in dendritic cells and macrophages for the induction of protective effector mechanisms against mycobacterial infection. We conclude that specific reactivation of MyD88 signalling in CD11c- or Lysozyme M-expressing myeloid cells during *Mycobacterium bovis* BCG infection is sufficient to restore systemic and local inflammatory cytokine production and to control pathogen burden.

Introduction

Since the discovery of *Mycobacterium tuberculosis* (*Mtb*) as the causal agent of tuberculosis, massive efforts have been made to understand the mechanisms underlying mycobacterial persistence. Despite current knowledge, it is still unclear why the immune response is insufficient to eradicate the bacteria and why almost 90% of *Mtb*-infected patients develop a chronic silent infection [1].

Transmission of mycobacteria occurs mostly through the respiratory tract. Here, alveolar macrophages and dendritic cells (DCs) that patrol the lungs capture mycobacteria by phagocytosis using different receptors (e.g. DC-SIGN, mannose receptor, complement receptor, scavenger receptor class A, pulmonary surfactant-associated protein A, surfactant protein A, Fc receptor, CD14, CD40 and CD44) [2-4]. Exposure of macrophages and DCs to mycobacteria or their products activates members of the TLR family, leading to a cascade of events necessary for the orchestration of an inflammatory immune response [5-7]. For example, CpG DNA sequences present in *Mtb* and *Mycobacterium bovis* BCG can be recognised by TLR9 [8-10], whereas mycobacterial lipoprotein and lipoarabinomannan can activate TLR2 [11, 12]. In addition, certain heat sensitive mycobacterial proteins can trigger the endotoxin receptor TLR4 [13]. More recently, a role for TLR8 has been also proposed [14]. In macrophages, early activation through TLRs is required for different effector functions against mycobacterial infection, including inflammatory cytokine secretion, production of ROS and reactive nitrogen intermediates (RNIs), maturation of the phagosome and autophagy, a central process for mycobacterial killing [15, 16]. In contrast, the capacity of DCs to kill BCG and *Mtb* is reduced compared to that of macrophages [17-20]. DC activation via TLRs and transport of mycobacterial products to the draining LNs is believed to be a crucial mechanism to initiate adaptive immune responses [21, 22]. Indeed, depletion of DCs using DTR-CD11c mice or altered DC migration to the LNs such as in *plt* mice, causes lower

$CD4^+$ T-cell activation and higher bacterial loads [23, 24]. In addition, DCs are an important source of IL-12, a cytokine necessary for the induction of Th1 responses [25-27]. Thus, although TLR-induced activation of macrophages and DCs is considered as one of the key effector mechanisms during the early phases of mycobacterial infection, little is known about the relevance of these cells later during infection. DCs are believed to play a role during the chronic phase of the disease by continuously sampling mycobacterial antigens to prime T cells in the LNs, thus maintaining the balance between immunity and infection [28]. But whether this mechanism requires continuous TLR signalling has not yet been investigated.

MyD88 is an adaptor protein originally described as a molecule involved in the differentiation of myeloid cells [29]. MyD88 mainly transmits signals from TLRs and most IL-1 receptor family members (e.g. IL-1, IL-18 and IL-33) [30, 31], but can also enhance IFN-γ-mediated macrophage functions [32]. MyD88 deficient mice ($MyD88^{-/-}$) have an increased susceptibility to virulent *Mtb* infection as well as to the non-virulent *M. bovis* BCG infection. Indeed, MyD88 knockout mice infected with *Mtb* succumb early to the disease [33-36], whereas *M. bovis* BCG causes a chronic persistent infection of the lungs [37]. Similarly, mice lacking components of the IL-1 axis (e.g. IL-1R, IL-1α or IL-1β) die early from *Mtb* infection [35, 38, 39], whereas TLR2/4/9 triple knockout mice have normal bacterial burden and immune responses [36]. Both MyD88 and IL-1R-deficient mycobacteria-infected mice have elevated inflammatory infiltrates composed of myeloid cells [35, 40]. However, it is not clear whether this excessive inflammatory response is an intrinsic defect of myeloid cells or whether other cells are also involved. Using novel genetic mouse models, we show here that despite a competent adaptive immune response, $MyD88^{-/-}$ mice possess an impaired capacity to clear mycobacteria that leads to chronic infection. Conversely, mice that have reactivated MyD88 signalling specifically in DCs or macrophages can control the infection similar to wild type (wt) mice. Our results suggest that during *M. bovis* BCG infection, MyD88

signalling in DCs and macrophages is essential to control bacterial burden and prevent pathogen persistence.

Results

MyD88 deficient mice fail to clear mycobacterial infection

Germline deletion of MyD88 has been associated with higher bacterial burden in the lungs of *M. bovis* BCG infected mice [37]. Similarly, here we observed that mice expressing a floxed transcriptional stop element between exons 1 and 2 of the MyD88 gene (MyD88^{OFF} mice) [41] failed to clear *M. bovis* BCG infection (Fig.1). Using the IVIS® Spectrum CT imager 5 days after infection with *M. bovis* BCG-expressing firefly luciferase (FFluc-BCG), we could observe luciferase positive regions in the lungs, liver and spleen of MyD88^{OFF}, but not in wt mice (Fig.1A). Interestingly, these differences persisted even after longer periods of time (Fig.1B). In addition, we also found higher bacterial burdens in the liver of MyD88^{OFF} mice at all time points evaluated and all infectious doses tested (Fig.1B, Supporting Information Fig.4A). Thus, similar to MyD88$^{-/-}$ mice [37], MyD88^{OFF} mice show an impaired capacity to eliminate mycobacteria.

Innate and adaptive immune responses are required for protection against mycobacterial infection

Sensing of pathogens by DCs and macrophages via TLRs and other PRRs is considered to be essential to launch immune responses against infection. However, while macrophages are primarily innate effector cells, DCs have rather an instructive function on the adaptive immune system. Therefore, we aimed to determine whether the absence of MyD88 signalling alters protective immune responses against mycobacteria by acting only via the adaptive immune system or via innate functions. For this, we crossed MyD88$^{-/-}$ to Rag1 knockout mice (Rag$^{-/-}$), lacking B and T cells, and infected them i.v. with 2x10^{6} CFUs *M. bovis* BCG.

Twenty days after infection, MyD88$^{-/-}$ mice displayed increased bacterial burden in the lungs and liver compared to wt mice (Fig.2A). However, Rag1 deficiency led to an even higher number of bacteria in the lungs and liver than MyD88 deficiency (Fig.2A). Additionally, bacterial load in the lungs and liver of MyD88$^{-/-}$ x Rag$^{-/-}$ double-deficient mice was significantly higher than in wt, Rag$^{-/-}$ or MyD88$^{-/-}$ mice (Fig.2A), suggesting that protection against *M. bovis* BCG infection depends on adaptive immune responses as well as on MyD88-dependent activation of innate immune cells.

To gain further mechanistic insights, we next evaluated the pro-inflammatory cytokine profile of wt, Rag$^{-/-}$ or MyD88$^{-/-}$ mice after *M. bovis* BCG infection. We focused on IL-12p40 and IL-1β, which are mainly produced by DCs and macrophages and serve therefore as markers of innate inflammatory responses. In addition, we evaluated IFN-γ production, which is mostly produced by IL-12-induced Th1 cells and essential for the control of mycobacterial infection [42, 43]. Consistent with the presence of APCs and the absence of T cells in Rag$^{-/-}$ mice, normal IL-12p40, but almost undetectable levels of IFN-γ were measured in the lungs of these mice (Fig.2B). Similarly, IFN-γ was only detected in the serum of wt, but not Rag$^{-/-}$ mice (Fig.2B). Yet, serum IL-12p40 levels were significantly reduced in Rag$^{-/-}$ mice compared to wt controls, indicating that local secretion is not enough to sustain systemic IL-12p40 levels. In MyD88$^{-/-}$ mice, IL-12p40 production was compromised both in the lungs and serum; however lung IFN-γ levels were significantly lower than in wt, but higher than in Rag$^{-/-}$ mice (Fig.2B). Finally, compared to wt controls, IL-1β production was significantly reduced in MyD88$^{-/-}$ and to a lesser extent in Rag$^{-/-}$ lungs, yet slightly increased in the serum of both mice, suggesting the existence of different regulatory mechanisms for the control of systemic and local IL-1β levels.

MyD88 signalling in BMDCs is required for inflammatory cytokine production and Th1 polarisation

Production of inflammatory cytokines by DCs and macrophages is a prerequisite for controlling mycobacterial infection, but its contribution to the initiation of adaptive immunity to intracellular pathogens remains controversial. To determine whether MyD88 signalling in DCs and/or macrophages is required for T helper cell differentiation, we crossed MyD88^{OFF} mice with mice expressing *cre* recombinase under the control of the CD11c (Itgax) promoter or the Lysozyme M (LysM) promoter [44-46]. Cell-type specific expression of the *cre* recombinase in the offspring of these mice, allows excision of the stop cassette and expression of MyD88 exclusively in CD11c^{+} or LysM^{+} cells (Supporting Information Fig1). Since CD11c is an alpha X integrin mostly expressed on DCs whereas Lysozyme M is an antibacterial enzyme predominantly present in macrophage, we called these mice MyD88-DC^{ON} and MyD88- macrophage^{ON} mice, respectively.

Using these novel conditional switch-on mouse models, we first tested the capacity of bone marrow-derived DCs (BMDCs) to produce inflammatory cytokines and induce Th1 and Th17 differentiation in vitro. BMDCs were infected with BCG at different MOI or treated with the TLR9 ligand CpG-B, and cytokine production was evaluated intracellularly 5h later. As expected, MyD88^{OFF} BMDCs activated with CpG-B did not produce any IL-12p40, TNFα or IL-1β (Fig.3A, Supporting Information Fig2A). Similarly, only a small percentage of the MyD88^{OFF} BMDCs produce IL-12p40, TNFα or IL-1β after infection with *M. bovis* BCG compared to wt BMDCs. In contrast, MyD88-DC^{ON} BMDCs produced IL-12p40, TNFα or IL-1β to the same levels than wt BMDCs after activation with CpG-B or infection with *M. bovis* BCG, suggesting a complete reactivation of the MyD88 pathway in these cells. Interestingly, MyD88-MACROPHAGE MACROPHAGE^{ON} BMDCs were also able to

produce comparable levels of these cytokines as wt BMDCs (Fig.3A), indicating that recombination of the MyD88 cassette also occurs in this population of myeloid cells.

Subsequently, we analysed the capacity of BMDCs to polarise $CD4^+$ Th cells in vitro. BMDCs from wt, MyD88-DC^{ON}, MyD88-$macrophage^{ON}$ or $MyD88^{OFF}$ mice were infected overnight with different MOI *M. bovis* BCG overexpressing the mycobacterial antigen Ag85B, and then co-cultured for 5 days with P25ktk $CD4^+$ T cells, carrying a specific T cell receptor that recognises the aa 240-254 of Ag85B bound to $I\text{-}A^b$ [47]. $MyD88^{OFF}$ BMDCs showed an impaired ability to induce Th1 cells as compared to wt BMDCs (Fig.3B), yet Th17 induction was only marginal in all four groups. On the contrary, the capacity of MyD88-DC^{ON} and MyD88- $macrophage^{ON}$ BMDCs to induce IFN-γ production in the proliferating $CellVio^{low}$ $CD4^+$ T cells was similar to that of wt BMDCs (Fig.3B). Altogether, our results suggest that MyD88 deficient BMDCs have a defective in vitro capacity to induce inflammatory cytokines and to polarise Th1 cells.

Reactivation of MyD88 signalling in DCs and macrophages restores IL-12p40 and IFN-γ production in vivo

To determine whether reactivation of MyD88 signalling in CD11c- or LysM-expressing cells was also sufficient to restore inflammatory cytokine production after mycobacterial infection in vivo, wt, MyD88-DC^{ON}, MyD88- $macrophage^{ON}$ or $MyD88^{OFF}$ mice were infected i.v. with $2x10^6$ CFUs *M. bovis* BCG and 20 days later, IL-12p40, IFN-γ and IL-1β analysed in sera and lung homogenates. Like $MyD88^{-/-}$, $MyD88^{OFF}$ mice showed lower levels of IL-12p40 and IFN-γ in the lungs and serum compared to wt control mice Reactivation of MyD88 signalling in $CD11c^+$ or $LysM^+$ cells was sufficient to re-establish both IL-12p40 and IFN-γ levels in the lungs, while in serum, MyD88- $macrophage^{ON}$ mice had slightly reduced IL-12p40 respect to wt mice (Fig.4A). In turn, production of IL-1β in the lungs was impaired in $MyD88^{OFF}$ mice,

but recovered to wt levels in MyD88-DC^{ON} and MyD88- macrophage ^{ON} mice, suggesting that MyD88 signalling in DCs and macrophages is sufficient to restore innate and adaptive inflammatory cytokine production.

Furthermore, we also examined the adaptive immune response after *M. bovis* BCG infection in MyD88^{OFF} mice or mice that have reactivated MyD88 signalling in CD11c^{+} or LysM^{+} cells. We did not find any differences in the percentage or total number of CD4^{+} and CD8^{+} T cells between wt, MyD88-DC^{ON}, MyD88- macrophage ^{ON} or MyD88^{OFF} infected mice (data not shown). Moreover, compared to naïve control mice, *M. bovis* BCG infection induces a strong increase in the percentage of IFN-γ-, but not IL-17A-producing CD4^{+} T cells in all four groups of mice (Fig.4B and data not shown). Thus, our results suggest that the IFN-γ deficiency observed in MyD88^{OFF} mice was not a consequence of impaired Th1 differentiation.

MyD88 signalling in DCs and MΦs is sufficient to control bacterial burden

Finally, we checked whether reactivation of MyD88 signalling in DCs and MΦs was sufficient to protect mice from mycobacterial infection. Mice were infected with 2x10^{6} CFUs *M. bovis* BCG and 20 days later, bacterial burden was determined in the lungs, liver and spleen of wt, MyD88-DC^{ON}, MyD88- macrophage ^{ON} or MyD88^{OFF} mice. As shown in Fig.5A, MyD88^{OFF} exhibited higher bacterial loads than wt mice in all organs examined, whereas mice that have reactivated MyD88 signalling in myeloid cells (MyD88-DC^{ON} or MyD88- macrophage^{ON}) have a similar capacity to eliminate bacteria than wt mice, independent of the infection doses used (Supporting Information Fig4A). Moreover, whereas MyD88^{OFF} mice still have increased bacterial burdens in the lungs and liver during the chronic phase of infection, very low CFUs were detected in MyD88-DC^{ON} and MyD88-macrophage^{ON} mice or wt mice (Fig.5B, Supporting Information Fig4B).

Discussion

MyD88 is an adaptor protein that transduces signals from TIR-containing receptors, such as IL-1R and most TLRs (except for TLR3) [48, 49]. MyD88 is expressed in a variety of cell types of hematopoietic and non-hematopoietic origin where it can modulate different inflammatory responses [41, 50-52]. Despite extensive studies on the role of TLRs and MyD88 in the immune response against mycobacteria, little is known about cell-specific functions of this key adaptor molecule. In the present study we demonstrate that while MyD88 complete deficiency leads to chronic *M. bovis* BCG infection, reconstitution of MyD88 signalling in DCs and MΦs is sufficient to control bacterial burden.

Innate immune recognition via TLRs has long been considered as a prerequisite for the initiation and maintenance of cellular adaptive immune responses during infection [53]. However, recent evidence suggests that redundancy in PRRs as well as activation of innate cells via inflammatory mediators might overcome TLR requirement [54, 55]. Host control of mycobacteria depends predominantly on Th1 cells and its master regulator, IL-12 [42, 56-58]. Surprisingly, while production of IL-12 and IL-1 by DCs and macrophages in response to mycobacterial products is MyD88-dependent [40], the relevance of these signalling molecules for the induction of Th1 and Th17 cells in vivo remains controversial [34, 36, 37, 40]. Here we show that MyD88-deficient BMDCs are impaired in their capacity to produce IL-12p40, TNFα and IL-1β in response to in vitro *M. bovis* BCG infection and to polarise naïve $CD4^+$ T cells into IFN-γ-producing cells. In vivo, although reduced levels of IL-12p40 and IFN-γ were detected in the sera and lungs of mice lacking MyD88 signalling, a high percentage of IFN-γ-producing $CD4^+$ T cells were found in both $MyD88^{OFF}$ and wt mice, compared to naïve mice. In addition, similar to the in vitro results, only marginal induction of IL-17A-producing $CD4^+$ T cells could be detected in *M. bovis* BCG infected mice compared to naïve control mice, suggesting that IL-17 does not play a significant role in *M. bovis* BCG infection. Thus, our findings reinforce the role of Th1 responses for the control of mycobacteria, but suggest that a

sustained systemic and local IL-12/IFN-γ production is required for adequate mycobacterial clearance. Our results in $Rag^{-/-}$ mice and those from others [59] demonstrate that adaptive immune responses are fundamental for protecting mice against mycobacterial infection. Yet, the higher bacterial burden observed in $MyD88^{-/-}$ x $Rag^{-/-}$ mice compared to $Rag^{-/-}$ mice, suggests that some of the protective mechanisms against mycobacteria depend exclusively on MyD88 signalling in innate immune cells. Indeed, reactivation of MyD88 signalling in DCs and macrophages restored IL-12p40 and IFN-γ levels in serum and lungs, and protected the mice from chronic mycobacterial infection. However, whether this is a direct or an indirect effect on these cells, requires further examination. IL-12p40 can form homodimers and act independently of p35 in myeloid cells [25], though it is not known if p80 homodimers can directly induce IFN-γ by APCs as it occurs in response to IL-12 and IL-18 [60]. In addition, it has recently been shown that MyD88 cooperates with IFN-γ to fully activate macrophages, a mechanism required for killing intracellular bacteria [32, 61].

Our present study suggests that despite an intact adaptive immune response in vivo, the absence of MyD88 signalling in DCs and macrophages permits mycobacterial persistence. Similar, previous studies using TLR2 or TLR4 knockout mice show that the absence of TLR signals leads to a state of chronic infection [37, 62-64]. Chronic mycobacterial infection is associated with a constant turnover of antigen-specific $CD4^+$ T cell [65], a process that requires antigen presentation as well as IL-12 production [66]. Recently, it has been shown that DCs can enter and exit chronic granulomas, suggesting that these cells might continuously sample mycobacterial antigen to prime T cells in the LNs [28]. We could not detect any differences in the IFN-γ-producing $CD4^+$ T cell responses between MyD88 deficient and wt mice, even at the chronic phase of infection. However, we cannot exclude that other effector mechanisms of the adaptive immune response are affected.

In humans, primary immunodeficiencies caused by mutations in the MyD88 pathway have been described [67]. People with defective MyD88 signalling have impaired production of

pro-inflammatory cytokines and recurrent pyogenic infections, particularly during childhood [68]. However, contrary to the mouse model, a positive correlation between MyD88 inborn mutations and susceptibility to tuberculosis could not be established [69]. It is unclear whether this is due to the low number of patients affected by MyD88- and other related deficiencies (e.g. IRAK-4 mutations), the fact that many of them die early during childhood as a consequence of their susceptibility to other infections, or the existence of compensatory mechanisms for protection against mycobacteria. In this sense, it has been proposed that the susceptibility of TLR-deficient mice to mycobacterial infection might be influenced by previous exposure to different housing conditions [36]. In contrast to MyD88 deficiency, there is consensus that certain mutations in the IL-12/IFN-γ axis cause Mendelian susceptibility to mycobacterial disease (MSMD), a rare syndrome that confers high predisposition to clinical disease caused by weakly virulent mycobacteria, such as *M. bovis* BCG, environmental mycobacteria and also *Mtb* [70-73]. Clearly, all 6 autosomal mutations found till date in humans affect IL12/IFN-γ-mediated immunity, underlining the importance of this pathway for protection against Tb.

In conclusion, using new conditional switch-on mouse models, we could demonstrate here that MyD88 signalling in DCs and macrophages is essential to control bacterial burden during *M. bovis* BCG infection. However, further studies will be necessary to dissect the mechanisms by which MyD88 affects DC *versus* MACROPHAGE MACROPHAGE function during mycobacterial disease. CD11c is predominantly expressed in DCs, but also present in alveolar macrophages and to a lower extent in activated T cells, NK cells or B cells [74, 75], whereas Lysozyme M is expressed in macrophages, but also neutrophils and a small fraction of splenic DCs [46]. Hence, more specific conditional *cre* lines will be essential to define the importance of MyD88 in DCs and macrophages for protection against mycobacteria.

Materials and methods

Mice

Mice expressing MyD88 specifically in $CD11c^+$ or $LysM^+$ cells (MyD88-DC^{ON} or MyD88-$macrophage^{ON}$) or not ($MyD88^{OFF}$) were bred and maintained under specific pathogen-free conditions at the animal facility of the Helmholtz Centre for Infection Research (HZI, Braunschweig, Germany) or the TWINCORE, Centre for Experimental and Clinical Infection Research (Hannover, Germany). Furthermore, $MyD88^{-/-}$ and $Rag1^{-/-}$ mice and P25ktk transgenic mice [47] were used and bred at the same institutions. Sex- and age-matched mice between 8- and 16-weeks of age were used in all experiments. All animal experiments were approved by the Veterinary Institute of LAVES (Lower Saxony State Office for Consumer Protection and Food Safety - Permit number: 10/0075 and 12/0732) considering the German Animal Welfare Act and all efforts were made to minimise suffering.

Mycobacteria cultures

M. bovis BCG expressing firefly luciferase was kindly provided by Dr. Brian Robertson (Imperial College London, UK) and *M. bovis* BCG overexpressing Ag85B (*M. bovis* BCG-Ag85B) by Dr. Joel Ernst, (NYU School of Medicine, USA). All strains were grown at 37°C in Middlebrook 7H9 broth (BD Biosciences) supplemented with 10% Middlebrook OADC enrichment medium (BD Biosciences), 0.002% glycerol (Roth) and 0.05% Tween-80 (Roth). Midlog phase cultures were harvested, aliquoted, and frozen at -80°C. For experimental in vitro infections, *M. bovis* BCG were prepared as previously described [76]. For in vivo infections, bacteria were prepared from frozen stocks by thawing at 37°C, resuspension in D-PBS/0.025% Tween-80 (PBS-T), and passage through a 27 gauge needle.

In vivo imaging

Mice were infected i.v. with $5x10^6$ CFUs FFluc-BCG and evaluated using an IVIS® Spectrum CT (PerkinElmer) 5 days later. Prior to assessment of bioluminescence (photons $s^{-1}cm^{-2}$ steridian $(sr)^{1}$), mice were anaesthetised using isoflurane (Abbott Laboratories). For the detection of firefly luciferase activity, 300 mg/kg of D-Luciferin (PerkinElmer) in sterile D-PBS was administered intraperitoneally (i.p.) 10 min before imaging. Mice were continuously anesthetised with isoflurane and imaged using the standard-two mice CT option of the IVIS® Spectrum CT. The CT data were combined with a bioluminescence pseudocolour image representing light intensity (blue, least intense to red, most intense) by using the Living Image software (PerkinElmer).

Colony enumeration assay

To determine bacterial burden, mice were infected intravenously (i.v) with approximately $2x10^6$ CFUs of the respective *M. bovis* BCG strain. At different time points after infection, mice were sacrificed and the lungs, liver and spleen were collected in sterile bags (Nasco) containing 1mL WTA lysis buffer (0.01% Tween-80 and 0.05% BSA (Roth) in dH_20) and mechanically disrupted. Viable cell counts were determined by plating serial dilutions of the homogenates on Middlebrook 7H11 agar plates (BD Biosciences) supplemented with 10% OADC (Difco Laboratories) and 0.5% glycerol (Roth). Plates were incubated at 37°C and CFUs were enumerated after 3 weeks. Data are presented as log10 CFUs per organ.

DC cytokine secretion assay

For generation of BMDCs, BM cells were prepared from the femurs and tibiae of wt, MyD88-DC^{ON}, MyD88- macrophage ^{ON} or $MyD88^{OFF}$ mice, and cultured for 7-8 days in complete RPMI medium (10% FCS, 10mM Hepes (GIBCO), 50µM β-mercaptoethanol (GIBCO), 100U/mL penicillin and 100µg/mL streptomycin (Biochrom)) supplemented with 5% culture

supernatant of a GM-CSF-producing cell line [77]. On day 7-8, 250.000 BMDCs/well were incubated with *M. bovis* BCG-Ag85B at different MOI for 5h (the last 3h in the presence of 5µg/mL Brefeldin A (eBioscience)), stained and analysed for intracellular cytokine production by FACS.

In vitro T-cell proliferation assay

25.000 BMDCs/well were incubated with different MOI *M. bovis* BCG-Ag85B for 18h, washed and incubated at a 1:4 ratio with $CD4^+$ T cells, obtained from the spleen and lymph nodes of P25ktk mice and enriched by negative magnetic cell sorting using the Dynal Mouse CD4 negative T cell isolation kit following the manufacturer´s protocol (Life Technologies). Co-cultures were performed for 5 days in 96-well round bottom plates (Cellstar) in 200µL complete RPMI medium. Cell proliferation was determined by staining the $CD4^+$P25ktk T cells with CellTraceViolet Cell Proliferation Kit (Life Technologies) followed by intracellular staining and FACS analysis.

Flow cytometry

The following antibodies and reagents were purchased from eBioscience: αCD11c (N418), αIL-17A (eBio17B7), αIL-12p40 (C17.8), αpro-IL-1β (NJTEN3), αCD4 (GK1.5), αIFN-γ (XMG1.2). αTNFα (MP6-XT22) was purchased from R&D Systems. To analyse intracellular cytokine production by $CD4^+$ T cells, $1x10^7$ spleen cells were stimulated with 100ng/mL PMA and 1µg/mL Ionomycin for 2h and 5µg/mL Brefeldin A was added for additional 2-3h before staining. Dead cells were excluded by Aqua fluorescent reactive dye (Life Technologies) staining. Cellular aggregates were excluded by gating singlets using SSC-A *versus* SSC-W. For intracellular cytokine staining, the cells were fixed with 2% Paraformaldehyde (PFA, Roth) for 20min and permeabilised in PBA-S buffer (0.5% Saponin and 0.25% BSA, both from Roth in PBS). Data acquisition was performed on a LSRII (BD)

or a CyAn™ ADP (Beckman Coulter) flow cytometer. Subsequent data analysis was performed with FlowJo software (Tree Star).

ELISA

Serum samples were collected and frozen at -80°C until analysis. The concentration of IL-12p40, IFN-γ and IL-1β was determined by ELISA according to the manufacturer's instructions (Duo Set, R&D Systems).

Statistical analysis

Unless otherwise stated, data analysis was performed using GraphPad Prism Software version 5.0 (GraphPad Software) and statistics were calculated by comparing groups independently using unpaired Student t-test. *P-values* were considered significant as follows: *$p<0.05$; **$p<0.01$; and ***$p<0.001$.

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Conflict of interest

The authors declare no financial/commercial conflicts of interest.

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Fig.1. MyD88 deficient mice fail to control mycobacterial infection. (A) Mice were infected i.v. with approximately $5x10^6$ CFUs FFluc-BCG and evaluated using an IVIS® Spectrum CT imager 5 days later. Left panel shows CT image with co-localisation of FFluc-BCG luminescence signal in the spleen, lungs and liver of MyD88^{OFF} (left) or wt (right) mice indicated by white arrows. Right panel depicts a ventral view of one representative mouse per group of FFluc-BCG luminescence signal. (B) Mice were infected i.v. with approx. $2x10^6$ CFUs *M. bovis* BCG and bacterial burden in the lungs (left panel) and liver (right panel) of wt (○) and MyD88^{OFF} (■) mice was determined at different days post infection (p.i.). Each symbol represents one individual mouse. Data are representative of three independent experiments. Statistic: Two-way ANOVA, Bonferroni post-test. *$p<0.05$; **$p<0.01$; and ***$p<0.001$.

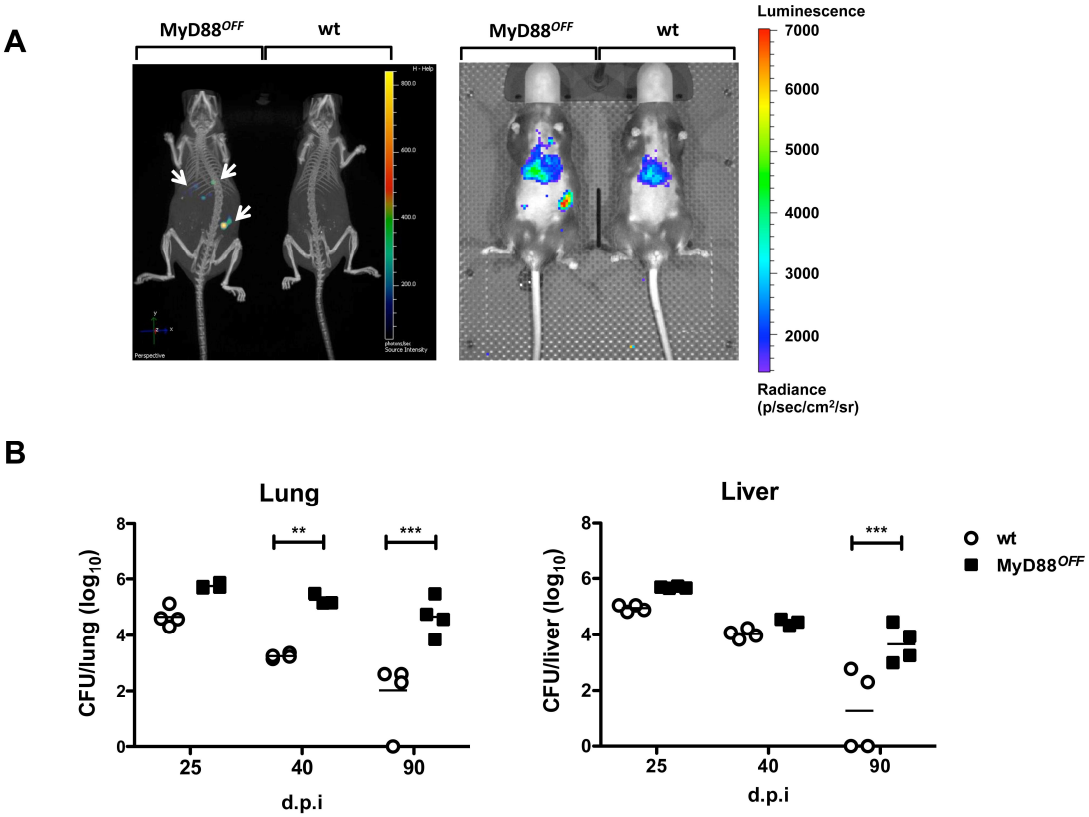

Fig.2. Mycobacterial control depends on adaptive and MyD88-dependent innate immune functions. (A) Bacterial burden in the lungs (left panel) and liver (right panel) of wt (○), $Rag^{-/-}$ (▲), $MyD88^{-/-}$ (■) or $MyD88^{-/-}$ x $Rag^{-/-}$ (▼) mice infected with $2x10^6$ CFUs *M. bovis* BCG determined at day 20p.i.. Each symbol represents an individual mouse. Data are representative of three independent experiments. To calculate statistical significance, samples from $MyD88^{-/-}$ x $Rag^{-/-}$ mice where the CFUs were too high and could not be counted, were considered as the highest countable CFUs from the same group. (B) IL-12p40 (left panel), IFN-γ (middle panel) and IL-1β (right panel) levels measured by ELISA at day 21 p.i. in the lungs and serum of wt (○), $Rag1^{-/-}$ (▲) or $MyD88^{-/-}$ (■) mice infected i.v. with $2x10^6$ CFUs *M. bovis* BCG. Each symbol represents an individual mouse. Data represent the results of one experiment. Statistics: Student *t* test. **p<0.05; **p<0.01*; and ****p<0.001*.

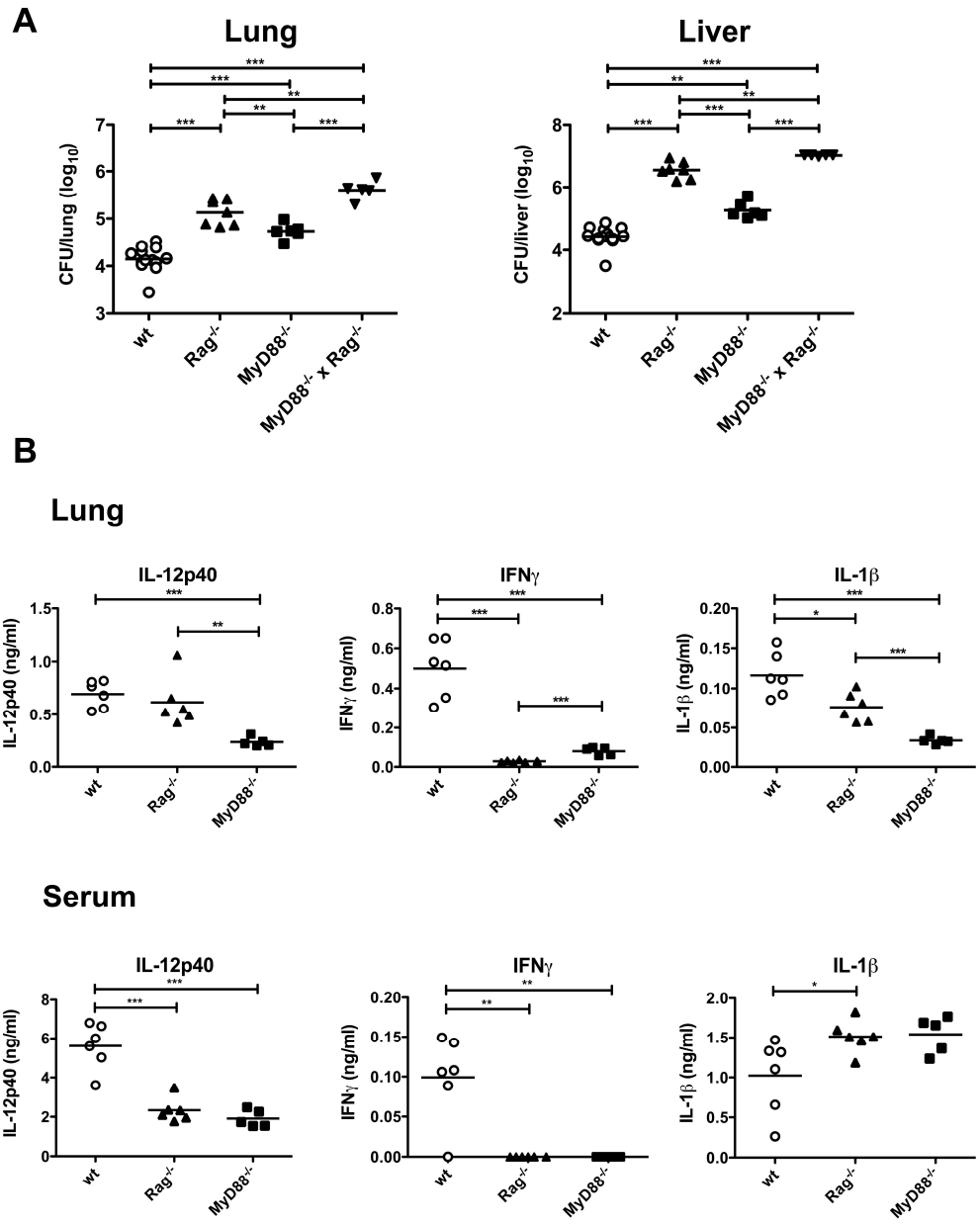

Fig.3. MyD88 controls cytokine production and Th1 cell polarisation capacity of BMDCs. (A) Intracellular IL-12p40 (left panel), TNFα (middle panel) and IL-1β (right panel) production by wt (white bars), MyD88-DC^{ON} (dark grey bars), MyD88-macrophage^{ON} (light grey bars) or MyD88^{OFF} (black bars) BMDCs infected with different MOI *M. bovis* BCG or treated with 1μM CpG-B for 5h. Bar graphs show the mean + SD of triplicate wells. Data are representative of three independent experiments. (B) Intracellular IFN-γ (right, upper panel) and IL-17A (left, lower panel) production by proliferating (CellViolet^{low}) P25ktk CD4^{+} T cells co-cultured for 5 days with wt (white bars), MyD88-DC^{ON} (dark grey bars), MyD88-macrophage ^{ON} (light grey bars) or MyD88^{OFF} (black bars) BMDCs that were previously infected with *M. bovis* BCG overexpressing the Ag85B at different MOI. FACS Plots correspond to an MOI of 5. Bar graphs represent the mean + SD of duplicate wells. Data are representative of three independent experiments.

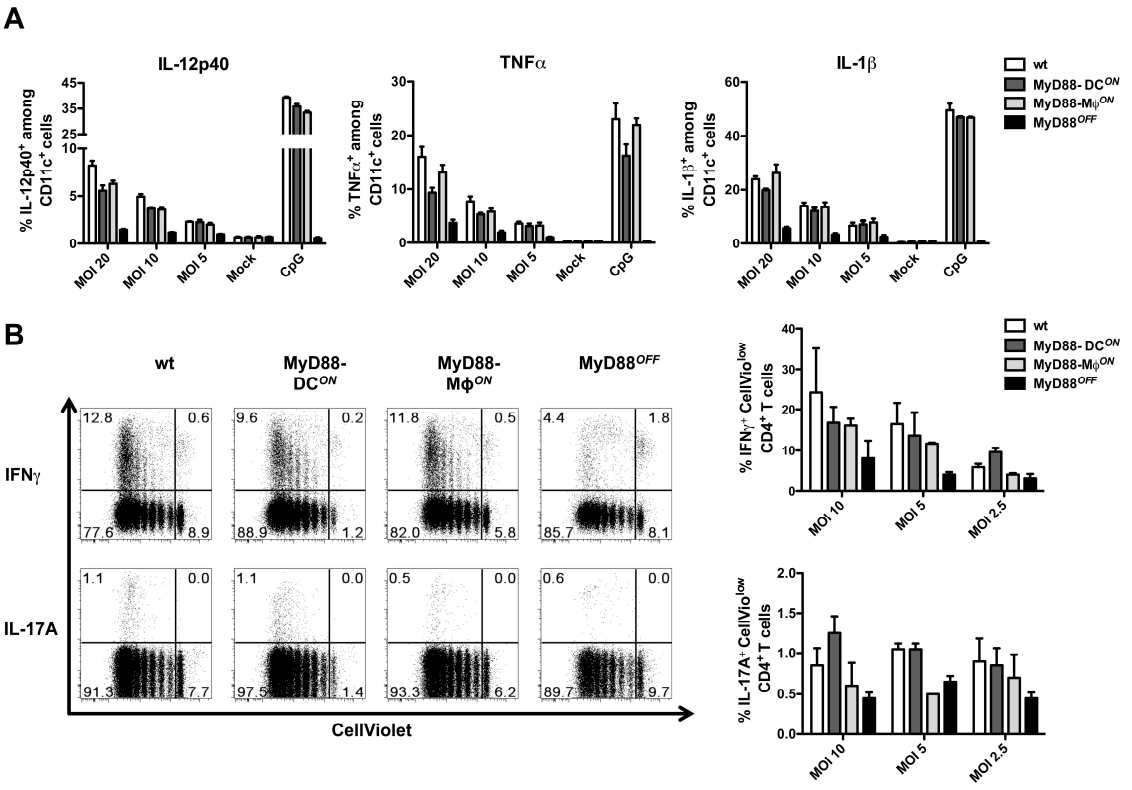

Fig.4. MyD88-signalling controls IL-12p40 and IFN-γ production in vivo, but not Th1 differentiation. (A) IL-12p40 (left panel), IFN-γ (middle panel) and IL-1β (right panel) levels measured by ELISA at day 20 p.i. in the lungs and serum of wt (○), MyD88-DC^{ON} (▼), MyD88-macrophage^{ON} (▲) or MyD88^{OFF} (■) mice infected i.v. with 2x10^6 CFUs *M. bovis* BCG. Each symbol represents an individual mouse. Data are pooled from two individual experiments and are representative of three independent experiments. Statistics: Student *t* test. **p<0.05; **p<0.01*; and ****p<0.001*. (B) Intracellular IFN-γ production by spleen CD4$^+$ T cells from wt (white bars), MyD88-DC^{ON} (dark grey bars), MyD88- macrophage ^{ON} (light grey bars) or MyD88^{OFF} (black bars) mice infected i.v. with 2x10^6 CFUs *M. bovis* BCG and analysed at day 25 p.i.. Bar graphs represent the mean + SD of 3-6 infected and 1-4 naïve mice per group and are representative of three independent experiments.

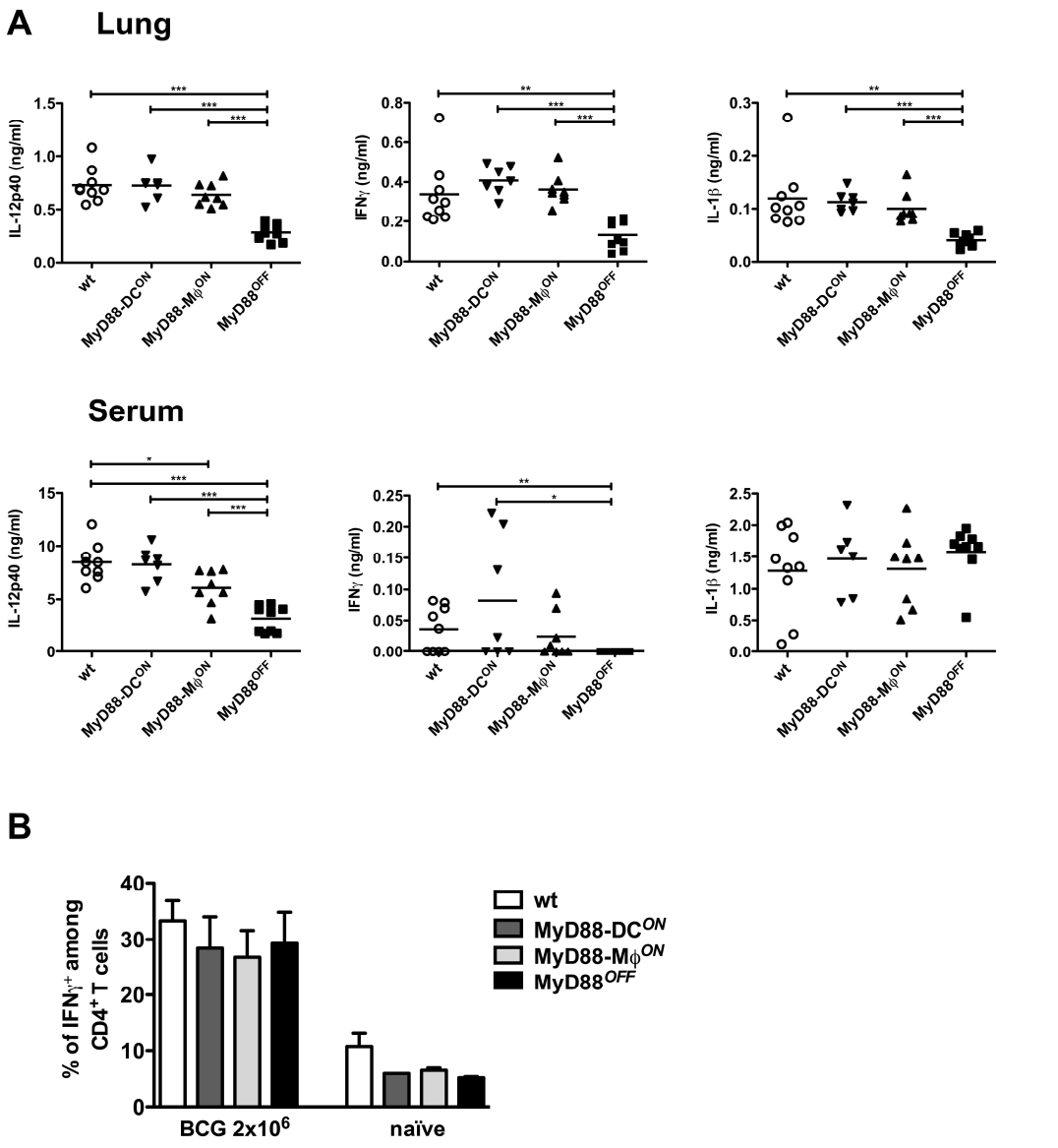

Fig.5. Reactivation of MyD88 in DCs and MΦs reduces bacterial load to wt levels. (A) Bacterial burden determined at day 20 p.i. in the lungs (left panel), liver (middle panel) and spleen (right panel) of wt (○), MyD88-DC^{ON} (▼), MyD88-macrophage^{ON} (▲) or MyD88^{OFF} (■) mice infected i.v. with 2x10^6 CFUs *M. bovis* BCG. Each symbol represents an individual mouse. Data are pooled from two individual experiments and are representative of five (lungs, liver) or two (spleen) independent experiments. (B) Mice were infected i.v. with 2x10^6 CFUs *M. bovis* BCG and bacterial burden was analysed at day 60 p.i. in the lungs (left panel) and liver (right panel) of wt (○), MyD88-DC^{ON} (▼), MyD88-macrophage^{ON} (▲) or MyD88^{OFF} (■) mice. Each symbol represents an individual mouse. Data are pooled from two (lung) or four (liver) individual experiments. Statistics: Student *t* test; **p<0.05; **p<0.01*; and ****p<0.001*.