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Cutting Edge: Mycobacterium tuberculosis but Not Nonvirulent Mycobacteria Inhibits IFN-β and AIM2 Inflammasome–Dependent IL-1β Production via Its ESX-1 Secretion System

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Mycobacterium tuberculosis extracellular DNA gains access to the host cell cytosol via the ESX-1 secretion system. It is puzzling that this extracellular DNA of M. tuberculosis does not induce activation of the AIM2 inflammasome because AIM2 recognizes cytosolic DNA. In this study, we show that nonvirulent mycobacteria such as Mycobacterium smegmatis induce AIM2 inflammasome activation, which is dependent on their strong induction of IFN-β production. In contrast, M. tuberculosis, but not an ESX-1–deficient mutant, inhibits the AIM2 inflammasome activation induced by either M. smegmatis or transfected dsDNA. The inhibition does not involve changes in host cell AIM2 mRNA or protein levels but led to decreased activation of caspase-1. We furthermore demonstrate that M. tuberculosis inhibits IFN-β production and signaling, which was partially responsible for the inhibition of AIM2 activation. In conclusion, we report a novel immune evasion mechanism of M. tuberculosis that involves the ESX-1–dependent, direct or indirect, suppression of the host cell AIM2 inflammasome activation during infection. The Journal of Immunology, 2013, 191: 3514–3518.

Interleukin-1β is important for host immune defense against Mycobacterium tuberculosis, as several studies demonstrated that IL-1β and IL-1R knockout mice are more susceptible to M. tuberculosis infections (1, 2). In macrophages and dendritic cells the production of mature IL-1β is dependent on activation of the inflammasome (3). The nucleotide-binding domain and leucine-rich-repeat–containing receptor (NLR) proteins such as NLRP3 and NLRC4 are one family of cytosolic receptors that upon ligand binding mediate inflammasome activation. In the case of M. tuberculosis the sole NLR capable of inducing inflammasome activation is NLRP3 (1, 2, 4–7).

The significance of type I IFN signaling for activation of inflammasome responses was first reported for Francisella-infected macrophages (8). Nevertheless, during the course of M. tuberculosis infections IFN-β has the opposite effect and suppresses activation of the NLRP3 inflammasome, suggesting that induction of IFN-β could correlate with increased virulence (9, 10). Mice deficient in IFN regulatory factor-3, a major signaling component of the type I IFN host cell signaling pathway, are much more resistant to M. tuberculosis infections (11). The induction of host cell type I IFN signaling after M. tuberculosis infection is dependent on the type VII secretion system (ESX-1)–mediated translocation of M. tuberculosis extracellular DNA (eDNA) into the host cell cytosol (11, 12). It is confounding that this cytosolic M. tuberculosis DNA is not recognized by the host cell inflammasome component AIM2, which should lead to subsequent inflammasome activation. AIM2 binds to dsDNA of intracellular pathogens such as Francisella and Listeria (13, 14). There is evidence that transfected M. tuberculosis dsDNA can interact with AIM2 and activate the AIM2 inflammasome and that AIM2 is important for host resistance to M. tuberculosis infection (15).

Materials and Methods

Cell culture and animals

C57BL/6 wild-type (WT) mice were obtained from The Jackson Laboratory. Nlrp3−/−, Nlrc4−/−, and Nlrp6−/− mice were provided by Dr. R. Flavell and Millennium Pharmaceuticals. Aim2−/− (16) and Aim2−/− Nlrc4−/− double knockout mice were obtained from Dr. A. I. Fitzgerald. Nlrp3−/− mice were from Dr. A. Sher (National Institutes of Health). IFN-β−/− mice were from Dr. A. Sher (National Institutes of Health).
β+/− mice were provided by Dr. S.N. Vogel. All studies were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Bacteria

Mycobacterium smegmatis (mc²155), M. tuberculosis H37Rv (ATCC 25618), and H37Ra (ATCC 25177) were obtained from Dr. W.R. Jacobs, Jr. (Albert Einstein College of Medicine), Mycobacterium fortuitum (ATCC 6841) and Mycobacterium kansasii strain Hauduroy (ATCC 12478) were obtained from the American Type Culture Collection. M. tuberculosis Δexcl and ΔexsU PAK (Pseudomonas aeruginosa) were provided by Dr. L. Gao and Dr. V. Lee, M. smegmatis Δexcl, ΔexsC mutant, and ΔexsC complemented strains were gifts of Dr. K. Derbskire (17). Francisella tularensis live vaccine strain (LVS) was obtained from Dr. Kevin McVerry.

Ex vivo infection

Bacterial infections of bone marrow–derived dendritic cells (BMDCs) and bone marrow–derived macrophages (BMDMs) were performed as described (7, 18). For induction of AIM2 inflammasome, BMDCs were pretreated with 20 ng/ml LPS (InvivoGen) for 1 h and then infected with H37Rv for 4 h. Infected BMDCs were then washed twice with PBS and transfected with 0.5 μg/ml poly(deoxyadenylic-thymidylic) acid (Sigma-Aldrich) using Lipofectamine LTX Plus reagent (Invitrogen) for 2 h. The transfection was performed according to the manufacturer’s instructions and the supernatants were harvested 2 h after transfection.

IFN-β neutralization

BMDCs from C57BL/6 mice were treated with anti–IFN-β neutralizing Ab 7F-D3 (5 μg/ml; Abcam) for 1 h and infected with M. smegmatis at a multiplicity of infection of 10:1 for 2 h as previously described. Cells were then washed with PBS and incubated for an additional 20 h in DMEM chase media. Supernatants were collected for ELISA.

Cell death assays

The adenosine kinase release assay ToxiLight bioassay (Lonza) was used to quantify necrotic cell death. The assay was performed according to the manufacturer’s instructions.

Cytokine measurement and immunoblotting

ELISA was used to measure secreted IL-1β (BD Biosciences) and IFN-β (Legend Max, BioLegend), respectively. For immunoblotting, the cell lysate preparation and Western blotting were performed as described earlier (7). The primary Abs used were anti–IL-1β (R&D Systems) at 0.15 μg/ml in 0.1% BSA, anti–caspase-1 (Santa Cruz Biotechnology) at 1:300, anti-AIM2 (Santa Cruz Biotechnology) at 1:500, and anti-tubulin (Cell Signaling Technology) at 1:1000. The above Abs were diluted in 5% milk with TBST. The secondary Abs used were donkey anti-goat (Jackson ImmunoResearch Laboratories) at 1:300, anti-rabbit (Santa Cruz Biotechnology) at 1:25,000, goat anti-mouse (Jackson ImmunoResearch Laboratories) at 1:50,000, and goat anti-mouse (Jackson ImmunoResearch Laboratories) at 1:50,000 dilutions.

Real-time PCR

BMDCs were harvested 8 h postinfection (hpi) using TRIzol (Invitrogen). Real-time PCR was done using SYBR Green PCR Master Mix (Roche) with GAPDH as the housekeeping gene. The primers used were:

- Aim2 (GenBank accession number: NM_001030320.2): 5′-GGTCCAGATGTTATGGTCCTCAGG-3′ and 5′-CACCTCAATGCGGTCGGGGC-3′.
- Gapdh (GenBank accession number: NM_017008.3): 5′-ATGGAGATTTCCCAATGTTGAACGA-3′ and 5′-CAGCACCCATGGCAAATTCC-3′.
- Δexcl (GenBank accession number: NM_017008.3): 5′-TCACCCATTGTCCCTGT-3′ and 5′-CACCCATGGCAAATTCC-3′.
- ΔexsC (GenBank accession number: NM_017008.3): 5′-GGTGCAAGCAGCCATATGAGGAAG-3′ and 5′-ACCTGTTGATCCAAATGACAG-3′.
- Mx1 (GenBank accession number: NM_017008.3): 5′-CAGGGTTTGTATCGA-3′ and 5′-GACCCATGGCAAATTCC-3′.

Statistical analysis

Statistical analysis was performed on at least three independent experiments using GraphPad Prism 5.0 software and one-way ANOVA with a Tukey posttest unless otherwise noted in the figure legends. Shown are representative results of triplicate values with SD. A p value <0.05 was considered statistically significant.

Results and Discussion

Nonviral mycobacteria induce AIM2 inflammasome activation

The nonviral mycobacterial species such as M. smegmatis induce a potent proinflammatory immune response and host cell apoptosis when compared with more virulent mycobacterial species (18), but the activation of host cell inflammasome mediated by M. smegmatis infection has not been analyzed. BMDCs of various mouse strains were infected with M. smegmatis and the amount of IL-1β in the supernatant was detected and normalized to IL-1β levels secreted by WT BMDCs (Fig. 1A). There were no differences in cell lysis as determined by adenylate kinase release assay and pro–IL-1β ELISA (not shown). Surprisingly, in NLRP3−/− cells the amount of secreted IL-1β dropped by <50%. This is unexpected because the inflammasome activation in M. tuberculosis is completely dependent on the presence of NLRP3 (1, 2, 4, 5). NLRP6, NLRP12, NLRC4, and NLRP10 did not significantly contribute to M. smegmatis–induced inflammasome activation (Fig. 1A).

Interestingly, however, the deficiency of AIM2 resulted in ~40% reduction in IL-1β response in BMDCs (Fig. 1A) and almost 75% reduction in BMDMs (Fig. 1B). The partial reductions in IL-1β secretion suggested redundancy between AIM2 and NLRP3 pathways. Consistently, the IL-1β production in Aim2/Nlrp3−/− cells was further reduced when compared with any of the single deletions; surprisingly, however, M. smegmatis–infected Aim2/Nlrp3−/− BMDCs were still able to secrete up to 40% of the IL-1β (Fig. 1A). This result suggests recognition of M. smegmatis cytosolic components by one or more unidentified NLRs. The M. tuberculosis–induced activation of NLRP3 is dependent on the M. tuberculosis ESX-1 secretion system (7, 19, 20). The core ESX-1 secretion complex is conserved in M. smegmatis, making it a compelling model to study the mechanisms of ESX-1–mediated protein secretion.
(21, 22). Similar to M. tuberculosis, a functional M. smegmatis ESX-1 secretion system is required for maximal response of AIM2/NLRP3-dependent secretion of IL-1β because two different M. smegmatis mutants with defective ESX-1 show an almost 50% reduction of IL-1β secretion when compared with WT M. smegmatis in WT BMDCs, but that difference is abolished in Aim2−/− Nlrp3−/− BMDCs (Fig. 1C).

Next, we wanted to investigate whether other mycobacterial species activate the AIM2 inflammasome. We thus infected WT and AIM2-deficient BMDCs with M. smegmatis, M. fortuitum, M. kansasii, the attenuated M. tuberculosis H37Ra, and the virulent M. tuberculosis H37Rv. We monitored cell lysis (not shown) and IL-1β secretion for all the infections (Fig. 2A). In general, there was an inverse correlation between virulence of the species and the amount of IL-1β production, with the least virulent species inducing the most IL-1β in WT BMDCs (Fig. 2A). The infection of AIM2-deficient BMDCs by these mycobacterial species allowed determining the fraction of total IL-1β secretion that was dependent on AIM2. As expected, the Francisella-induced inflammasome activation was completely dependent on AIM2, whereas ∼40–50% of the M. smegmatis, M. fortuitum, and M. kansasii induction was dependent on AIM2 (Fig. 2A). The attenuated M. tuberculosis strain H37Ra induced the lowest amount of AIM2 inflammasome activation, and the virulent M. tuberculosis strain H37Rv did not induce any AIM2 activation (Fig. 2A).

Nonvirulent mycobacteria induce IFN-β dependent AIM2 inflammasome activation

The AIM2 inflammasome activation by Francisella is stimulated by IFN-β (8). To investigate the potential importance of IFN-β in AIM2 inflammasome activation by nonvirulent mycobacteria, we analyzed the supernatants of BMDCs infected with various mycobacterial species for IFN-β production. Interestingly, the three species (M. smegmatis, M. fortuitum, and M. kansasii) that mediated the strongest AIM2 inflammasome activation also induced a very pronounced IFN-β production of ∼800 pg/ml, which was a 20-fold increase over the amount of IFN-β produced by BMDCs after infection with M. tuberculosis H37Rv (Fig. 2B). To demonstrate that this IFN-β secretion is important for AIM2 inflammasome activation, we infected BMDCs from WT and Aim2−/− mice with F. tularensis LVS and M. smegmatis in the absence or presence of neutralizing IFN-β Abs and measured the IL-1β secretion after 24 h (Fig. 2C). The addition of neutralizing IFN-β Abs significantly reduced the IL-1β secretion after infection with either F. tularensis LVS or M. smegmatis (Fig. 2C). Consistently, the production of IL-1β was reduced by similar levels when BMDCs of WT or Ifnar1−/− mice were infected (Fig. 2D). The neutralization of IFN-β had no effect on the IL-1β production after F. tularensis LVS or M. smegmatis infection of Aim2−/− BMDCs. These results suggest that IFN-β plays a similar role in the induction of AIM2 inflammasome activation by nonvirulent mycobacterial species, as it does after Francisella infections.

M. tuberculosis inhibits AIM2-dependent IL-1β production

Finally, we addressed the hypothesis that M. tuberculosis can actively inhibit AIM2 inflammasome activation by performing mixed infection experiments. BMDCs from Nlrp3−/− mice were infected with M. tuberculosis or the essA deletion M. tuberculosis mutant (M. tuberculosis ΔessA), and either 4 or 8 hpi the supernatants were harvested for analysis of IL-1β secretion (Fig. 3A, 3B). In M. smegmatis-infected BMDCs we

**FIGURE 2.** Nonvirulent mycobacteria induce IFN-β dependent AIM2 inflammasome activation. (A) WT and Aim2−/− BMDCs were infected with M. smegmatis, M. fortuitum, M. kansasii, attenuated M. tuberculosis H37Ra, and virulent M. tuberculosis H37Rv. Francisella LVS infection was used as a positive control. The secretion of IL-1β was measured at 16 hpi by ELISA. (B) IFN-β ELISA of supernatants from WT BMDCs infected with indicated mycobacterial species. (C) IL-1β ELISA of supernatants from WT and Aim2−/− M. smegmatis-infected BMDCs in the presence or absence of IFN-β neutralizing Abs. LVS was used as a positive control. (D) IL-1β ELISA of supernatants from Ifnar1−/− or WT BMDCs infected with M. smegmatis or left uninfected. Data are shown as the means ± SD of triplicate measurements of one representative experiment out of three. *0.01 < p < 0.05, **0.001 < p < 0.01, ***0.0001 < p < 0.001. Mfort, M. fortuitum; Mkan, M. kansasii; Msme, M. smegmatis; Ul, uninfected.

**FIGURE 3.** M. tuberculosis inhibits AIM2-dependent IL-1β production. Nlrp3−/− BMDCs were first infected with M. tuberculosis or the M. tuberculosis ΔessA mutant and then with M. smegmatis. Secreted IL-1β (blue, filled bars) was measured by ELISA at (A) 4 hpi or (B) 8 hpi. Necrotic cell death (red, striped bars) was assayed and is represented as fold change over uninfected. Nlrp3−/− BMDCs in the (C) absence or (D) presence of LPS pretreatment were first infected with M. tuberculosis or the M. tuberculosis ΔessA mutant and then transfected with poly(deoxyadenylc-thymidylic) acid for 2 h. Both secretion of IL-1β and necrosis were measured as before. Data are shown as the means ± SD of triplicate measurements of one representative experiment out of three. *0.01 < p < 0.05, **0.001 < p < 0.01, ***0.0001 < p < 0.001. Msme, M. smegmatis; Mtub, M. tuberculosis; Ul, uninfected.
detected ~100 pg/ml after 4 hpi and 3000 pg/ml after 8 hpi. Interestingly, when BMDCs, which had been infected with \( \text{M. tuberculosis} \), were challenged with \( \text{M. smegmatis} \) we observed a 2- to 3-fold reduction in the amount of secreted IL-1β when compared with BMDCs infected only with \( \text{M. smegmatis} \). The \( \text{M. tuberculosis} \)-mediated inhibition was dependent on a functional ESX-1 secretion system because infection with the \( \text{M. tuberculosis} \Delta \text{esxA} \) mutant did not inhibit IL-1β secretion after challenge with \( \text{M. smegmatis} \) (Fig. 3A, 3B). The rate of infection of \( \text{M. smegmatis} \) was not affected by the prior infection with \( \text{M. tuberculosis} \) as analyzed via flow cytometry using GFP-labeled \( \text{M. smegmatis} \) (Supplemental Fig. 1A). A similar inhibition by \( \text{M. tuberculosis} \) was also observed for IL-18 induced by \( \text{M. smegmatis} \) infections (Supplemental Fig. 1B). There were no differences in necrosis induction at 8 hpi (Fig. 3B). To confirm the specific inhibition of the AIM2 inflammasome, we transfected the \( \text{M. tuberculosis} \)– or \( \text{M. tuberculosis} \Delta \text{esxA} \)-infected \( \text{Nlpr3}^{−/−} \) BMDCs with 0.5 μg/ml poly(deoxyadenylic-thymidylic) acid in the absence (Fig. 3C) or presence (Fig. 3D) of LPS pretreatment. Consistent with the previous finding, analysis of IL-1β showed that \( \text{M. tuberculosis} \) does inhibit the activation of the AIM2 inflammasome when compared with \( \text{M. tuberculosis} \Delta \text{esxA} \)-infected cells. Additionally, we determined that \( \text{M. tuberculosis} \) was not able to inhibit NLRC4 inflammasome activation by \( \text{P. aeruginosa} \) (Supplemental Fig. 2).

Finally, to further support our hypothesis that \( \text{M. tuberculosis} \) mediates inhibition of AIM2 inflammasome activation, we started to investigate the mechanism of this inhibition. First, we determined that there were no significant changes of \( \text{Aim2} \) transcription mediated by \( \text{M. tuberculosis} \) infection (not shown). Consistently, immunoblots of cell lysates from infected BMDCs at 6 hpi showed that there is no difference in AIM2 protein expression (Fig. 4A). Also, the protein expression of pro–IL-1β and procaspase-1 was not affected by \( \text{M. tuberculosis} \). However, immunoblots of the corresponding superantigens showed that there is decreased secretion of p10 fragment of caspase-1 and the mature IL-1β (p17) fragment in \( \text{M. tuberculosis} \)–infected cells challenged with \( \text{M. smegmatis} \) when compared with \( \text{M. smegmatis} \) or \( \text{M. tuberculosis} \Delta \text{esxA} \) \( \text{M. smegmatis} \)-infected cells (Fig. 4A). Interestingly, \( \text{M. tuberculosis} \) infection reduced the amount of \( \text{M. smegmatis} \)-induced IFN-β secretion in an ESX-1–dependent manner (Fig. 4B). Even the addition of high amounts of exogenous IFN-β (400 ng/ml) could only partially overcome the \( \text{M. tuberculosis} \)–mediated inhibition of \( \text{M. smegmatis} \)-induced IL-1β secretion (Fig. 4C). These results demonstrate that \( \text{M. tuberculosis} \) is able to limit IFN-β production in infected host cells, which may explain some of its capacity to inhibit the IFN-β–dependent AIM2 inflammasome activation. To investigate whether \( \text{M. tuberculosis} \) may also inhibit IFN-β signaling, we used IFN-β−/− BMDCs and infected them with \( \text{M. tuberculosis} \) and \( \text{M. tuberculosis} \Delta \text{esxA} \) followed by treatment with IFN-β, and the transcription of IFN-β-inducible genes \( \text{Mx1} \) and \( \text{PKR} \) was analyzed by quantitative RT-PCR (Fig. 4D, 4E). In both cases \( \text{M. tuberculosis} \) infection reduced the IFN-β–mediated increase in transcription but this inhibition was not dependent on ESX-1, because the \( \text{M. tuberculosis} \Delta \text{esxA} \) mutant showed a similar reduction. The inhibition of IL-1β production by \( \text{M. tuberculosis} \) has been reported before (23), but the inhibition of IFN-β signaling by \( \text{M. tuberculosis} \) has not been shown previously to our knowledge.

The precise molecular mechanism of the \( \text{M. tuberculosis} \)-mediated AIM2 inflammasome inhibition remains to be elucidated. It seems unlikely that limiting IFN-β production is the only pathway for \( \text{M. tuberculosis} \) to suppress AIM2 inflammasome activation because external addition of IFN-β did not induce IL-1β secretion in \( \text{M. tuberculosis} \)–infected \( \text{Nlpr3}^{−/−} \) BMDCs (Fig. 4C) and only partially restored the \( \text{M. tuberculosis} \)–mediated inhibition of \( \text{M. smegmatis} \)-induced IL-1β. Hence, \( \text{M. tuberculosis} \) may secrete another effector that could inhibit signaling of the IFN-α/β receptor and/or directly modify AIM2 inflammasome activation. Indeed, we provide evidence that \( \text{M. tuberculosis} \) inhibits IFN-β signaling. A detailed analysis of the large number of IFN-β–regulated
genes may reveal a subset whose expression can only be inhibited by *M. tuberculosis* with a functional ESX-1 system. This subset of genes would be the most likely to contain candidates for mediating the AIM2 inflammasome inhibition. Overall, the cosecretion into the host cell cytosol of *M. tuberculosis* eDNA and a putative AIM2 inhibitor and/or IFN-β signaling inhibitor via the ESX-1 system may allow *M. tuberculosis* to take advantage of the type I IFN–mediated inhibition of the NLRP3 inflammasome without the *M. tuberculosis* eDNA inducing activation of the AIM2 inflammasome. The recent finding that *Aim2*−/− mice are very susceptible to *M. tuberculosis* infections supports the potential role of AIM2 inflammasome inhibition for optimal virulence of *M. tuberculosis* (15). Our discovery of a novel immune evasion mechanism engaged by *M. tuberculosis* opens the door for investigations into the identification of the *M. tuberculosis* genes involved in this inhibition and subsequent analysis of their importance for virulence of *M. tuberculosis*.

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**Disclosures**

The authors have no financial conflicts of interest.

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