Metallothionein 3-Zinc Axis Suppresses Caspase-11 Inflammasome Activation and Impairs Antibacterial Immunity

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Non-canonical inflammasome activation by mouse caspase-11 (or human CASPASE-4/5) is crucial for the clearance of certain gram-negative bacterial infections, but can lead to severe inflammatory damage. Factors that promote non-canonical inflammasome activation are well recognized, but less is known about the mechanisms underlying its negative regulation. Herein, we identify that the caspase-11 inflammasome in mouse and human macrophages (MØ) is negatively controlled by the zinc (Zn²⁺) regulating protein, metallothionein 3 (MT3). Upon challenge with intracellular lipopolysaccharide (iLPS), MØ increased MT3 expression that curtailed the activation of caspase-11 and its downstream targets caspase-1 and interleukin (IL)-1β. Mechanistically, MT3 increased intramacrophage Zn²⁺ to downmodulate the TRIF-IRF3-STAT1 axis that is prerequisite for caspase-11 effector function. In vivo, MT3 suppressed activation of the caspase-11 inflammasome, while caspase-11 and MT3 synergized in impairing antibacterial immunity. The present study identifies an important yin-yang relationship between the non-canonical inflammasome and MT3 in controlling inflammation and immunity to gram-negative bacteria.

Keywords: macrophage, non-canonical inflammasome, zinc, metallothionein, innate immunity, caspase-11 non-canonical inflammasome, MT3
INTRODUCTION

Gram-negative bacteria that cause more than 30% of the total healthcare-associated infections worldwide remain a global concern of morbidity and mortality (1). Assembly of inflammasome complexes during bacterial pathogenesis drives robust inflammation and shapes antibacterial immune responses. The non-canonical inflammasome is activated when innate immune cells such as Mφ sense bacterial ligands in the cytosol (2). LPS from bacterial cell walls enters the cytosol during bacterial escape from vacuoles or via rupture of outer membrane vesicles (OMV) (3). iLPS directly binds caspase-11, triggering the non-canonical inflammasome cascade, followed by activation of pro-caspase-1 to caspase-1, processing of pro-IL-1β to mature IL-1β and pyroptosis, a lytic form of programmed cell death. Activation of gasdermin D (GSDMD) by caspase-11 and caspase-1, leads to pore formation on the cell membrane facilitating the exit of IL-1β from Mφ (4). Unrestricted activation of this inflammatory cascade is a major underlying cause of tissue damage and sepsis-associated mortality (2). Thus, it is crucial to thoroughly understand the molecular cues that guard against excessive activation of the caspase-11 inflammasome. Although factors that promote non-canonical inflammasome activation have been extensively studied, less is known about the mechanisms that negatively regulate it.

MTs are Zn2+ regulating proteins induced by endogenous and exogenous stimuli including cytokines, infection, oxidative stress and heavy metals (5, 6). Intracellular availability of total Zn2+, exchangeable Zn2+ and Zn2+ redistribution among proteins is tightly regulated by MTs (7). Mice have 4 MT isoforms (MT1-4), whereas more than 16 MT isoforms are present in humans (8). Our knowledge on the role of the MT family in immune responses largely emerges from studies on MT1 and MT2. We
and others have shown that MT1 and MT2 promote antifungal and antibacterial immunity in Mφ primarily via Zn\(^{2+}\) sequestration (9, 10). MT3, on the other hand, suppresses manifestation of proinflammatory phenotypic and metabolic changes and impairs antifungal immunity in vitro in Mφ and in vivo. Mφ expression of MT3 is inducible by IL-4 stimulation. In these cells, MT3 increases intracellular free-Zn\(^{2+}\), promotes Zn\(^{2+}\) uptake by a prototypic intracellular pathogen and favors microbial survival (11). Thus, MTs and their ability to regulate Zn\(^{2+}\) homeostasis intrinsically ties Mφ inflammatory responses to antimicrobial defense.

Zn\(^{2+}\) is indispensable in many biochemical processes due to its role in structural and catalytic functions of enzymes and macromolecules. Zn\(^{2+}\) excess or deficiency compromises the development and function of immune cells including monocytes and Mφ, leading to increased risk of infection (12). Zn\(^{2+}\) also has a profound role as a signaling ion attributable to the transient changes in intracellular exchangeable Zn\(^{2+}\). LPS triggers increased Zn\(^{2+}\) import in leukocytes, monocytes and Mφ (13, 14). In peripheral blood mononuclear cells (PBMCs) and human Mφ (hMφ), LPS-induced Zn\(^{2+}\) influx promotes IL-1β production (14). In contrast, exogenous exposure to Zn\(^{2+}\) in human monocytes may reduce IL-1β production due to inhibition of nucleotide phosphodiesterases (15). Thus, the effects of Zn\(^{2+}\) on signaling and cytokine production are context and Zn\(^{2+}\) concentration-dependent. Changes in ion flux, specifically K\(^{+}\) and Cl\(^{-}\) egress and intracellular mobilization of Ca\(^{2+}\) underlie canonical nod-like receptor pyrin domain containing-3 (NLRP3) inflammasome activation (16). Intriguingly, Zn\(^{2+}\) exerts disparate effects on activation of this cascade. Long-term Zn\(^{2+}\) depletion disrupts lysosomal integrity leading to increased activation of the canonical NLRP3 inflammasome (17). On the other hand, short-term chelation of Zn\(^{2+}\) attenuates the canonical pathway due to impaired function of the pannexin-1 receptor (18).

The importance of Zn\(^{2+}\) regulation by MTs in the non-canonical inflammasome pathway remains unexplored. Given the suppressive role of MT3 in Mφ inflammatory responses (19), we hypothesized that MT3 negatively regulates the highly inflammatory caspase-11 activation cascade. Bioinformatics analysis predicted the involvement of MT3 in regulating non-canonical inflammasome-associated pathways. Using a combination of protein-protein interaction network analysis, immunological and mass-spectrometric approaches, we demonstrate that triggering caspase-11 activation results in a profound, gradual increase in the Mφ Zn\(^{2+}\) pool mediated by MT3. The increase in Zn\(^{2+}\) attenuates signaling via toll/interleukin-1 receptor (TIR) domain containing adaptor-inducing interferon (IFN)β - interferon regulatory factor 3 - signal transducer and activator of transcription factor 1 (TRIF-IRF3-STAT1), a pathway that is prerequisite for caspase-11 inflammasome activation (20). Zn\(^{2+}\) deficiency augments, whereas Zn\(^{2+}\) supplementation suppresses the non-canonical inflammasome in Mφ. Using whole-body Mt3\(^{-/-}\) and myeloid-MT3-deficient mice, we elucidate that MT3 blunts non-canonical inflammasome activation in vitro and in vivo upon challenge with iLPS or gram-negative bacteria but not gram-positive bacteria. Importantly, this function of MT3 is conserved in hMφ. Although caspase-11 and MT3 form a negative regulatory loop, we find that these two molecules synergize in compromising antibacterial immunity. Our data uncover a previously unknown yin-yang relationship whereby the MT3-Zn\(^{2+}\) axis exerts a brake on non-canonical inflammasome activation but the functions of MT3 and caspase-11 converge in crippling immunity to invading bacteria (see Graphical Abstract).

**RESULTS**

MT3 Suppresses Activation of the Non-Canonical Inflammasome In Vitro

MT3 attenuates cell death in neuronal and glial cells, but the precise underling mechanisms are not fully understood (21). As non-canonical inflammasome activation leads to pyroptotic cell death, we investigated if MT3 effector function is related to caspase-11 activation in Mφ. We explored whether MT3 is involved in inflammatory and cell-death processes using functional enrichment analysis. We assessed protein-protein interaction networks of MT3 in Mus musculus and Homo sapiens using the STRING database (22). The MT3 interaction partners significantly enriched 15 mouse and 43 human gene ontology categories for biological processes (GO BP) related to programmed cell death (PCD), LPS responses, signaling via TRIF, IL-1 and type-I IFN, cytokine responses and several immune processes. A complete network of MT3 interactions and GO BP categories is in Table 1. Supplementary Table S1, Supplementary Files S1 and S2. PCD and LPS responses are linked to inflammasome activation and more specifically, TRIF and type-I IFN signaling are tied to the non-canonical inflammasome pathway. A lack of TRIF signaling ablates non-canonical inflammasome activation in response to iLPS without impacting Mφ response to canonical NLRP3 triggers such as ATP and nigericin (20). Thus, our bioinformatics analysis together with our previously reported role for MT3 in suppressing proinflammatory responses in Mφ led us to investigate whether MT3 negatively regulates the non-canonical inflammasome pathway.

In Mφ, exposure to iLPS triggers caspase-11 activation and cell death by pyroptosis (2). We directly assessed if MT3 regulates non-canonical inflammasome activation using WT and Mt3\(^{-/-}\) mice. BMDM were exposed to iLPS or vehicle control and time-dependent changes in the gene expression of Mt1, Mt2 and Mt3 were examined. Mt3 expression increased gradually from 1 h (h) and peaked at 48h in WT BMDM challenged with iLPS (Figure 1A). The expression of Mt1 and Mt2 peaked at 6h, but receded over time in both WT and Mt3\(^{-/-}\) BMDM (Supplementary Figures S1A, B). To determine whether exogenous LPS had an effect on MT3, we stimulated WT BMDM with extracellular LPS (exLPS) for 48h. While iLPS increased Mt3 expression by 10-fold, the fold increase observed with exLPS challenge was much lower (Figure 1B).

Next, we examined if MT3 regulated non-canonical inflammasome activation by assessing pro- and active forms of caspase-11, caspase-1 and IL-1β in cell lysates and supernatants.
| SL No | Term ID   | Term Description                                | FDR     | Protein Labels                                                                 |
|------|-----------|-------------------------------------------------|---------|-------------------------------------------------------------------------------|
| 1    | GO:0060548| Negative regulation of cell death               | 2.39E-10| NGFR, MT3, RIPK2, CAT, Traf2, RIPK1, SLC40A1, SOD1, RPS27A, EGFR, APP, ALB, SNCB, FAIM2, ERBB4, GPX4, SLC30A10, TXN, MAG, UBA52, GPX1, Traf6, UBC, SOD2, AKT1, IKBKG |
| 2    | GO:0043069| Negative regulation of programmed cell death    | 6.51E-09| NGFR, MT3, RIPK2, CAT, Traf2, RIPK1, SLC40A1, SOD1, RPS27A, EGFR, ALB, SNCB, FAIM2, ERBB4, GPX4, SLC30A10, MAG, UBA52, GPX1, Traf6, UBC, SOD2, AKT1 |
| 3    | GO:0043066| Negative regulation of apoptotic process        | 2.32E-08| NGFR, MT3, RIPK2, CAT, Traf2, RIPK1, SLC40A1, SOD1, RPS27A, EGFR, ALB, SNCB, FAIM2, ERBB4, GPX4, SLC30A10, MAG, UBA52, GPX1, Traf6, UBC, SOD2, AKT1 |
| 4    | GO:0010941| Regulation of cell death                        | 2.59E-08| TNFRSF1A, NGFR, MT3, RIPK2, CAT, Traf2, RIPK1, SLC40A1, SOD1, RPS27A, EGFR, APP, ALB, SNCB, FAIM2, RTN4, ERBB4, GPX4, SLC30A10, TXN, MAG, UBA52, GPX1, CYLD, IKBKG, IKBG |
| 5    | GO:0043067| Regulation of programmed cell death             | 8.35E-08| TNFRSF1A, NGFR, MT3, RIPK2, CAT, Traf2, RIPK1, SLC40A1, SOD1, RPS27A, EGFR, APP, ALB, SNCB, FAIM2, RTN4, ERBB4, GPX4, SLC30A10, MAG, FOX30, UBA52, CYLD, GPX1, Traf6, UBC, SOD2, AKT1 |
| 6    | GO:0042981| Regulation of apoptotic process                 | 2.74E-07| TNFRSF1A, NGFR, MT3, RIPK2, CAT, Traf2, RIPK1, SLC40A1, SOD1, RPS27A, EGFR, APP, ALB, SNCB, FAIM2, RTN4, ERBB4, GPX4, SLC30A10, MAG, FOX30, UBA52, CYLD, GPX1, Traf6, UBC, SOD2, AKT1 |
| 7    | GO:0010942| Positive regulation of cell death               | 1.84E-06| TNFRSF1A, NGFR, MT3, RIPK2, CAT, Traf2, RIPK1, SLC40A1, SOD1, RPS27A, EGFR, APP, ERBB4, FOX30, UBA52, CYLD, Traf6, UBC, SOD2, AKT1 |
| 8    | GO:0055566| TRIF-dependent toll-like receptor signaling pathway | 8.19E-06| RIPK1, RPS27A, UBA52, IKBKG |
| 9    | GO:0070498| Interleukin-1-mediated signaling pathway        | 1.00E-05| RIPK2, RPS27A, IKBKG |
| 10   | GO:0045089| Regulation of innate immune response            | 2.38E-05| RIPK2, ERG, IKBKG |
| 11   | GO:0071345| Cellular response to cytokine stimulus          | 3.60E-05| RTN4, TNFRSF1A, NGFR, MT3, RIPK2, ERG, Traf2, RIPK1, SOD1, RPS27A, IKBKG |
| 12   | GO:1903209| Positive regulation of oxidative stress-induced cell death | 4.97E-05| RIPK1, SOD1, IKBKG |
| 13   | GO:0045088| Regulation of innate immune response            | 5.35E-05| RIPK2, ERG, IKBKG |
| 14   | GO:0002577| Immune response-activating signal transduction  | 0.00151 | RIPK2, RPS27A, APP, DDX58, UBA52, CYLD, Traf6, UBC, IKBKG |
| 15   | GO:0002684| Positive regulation of immune system process    | 1.02E-05| RIPK2, RPS27A, UBA52, CYLD, IKBKG |
| 16   | GO:0000695| Regulation of cytokine-mediated signaling pathway | 2.38E-05| RIPK2, ERG, IKBKG |
| 17   | GO:0000695| Apoptotic process                                | 3.60E-05| RTN4, TNFRSF1A, NGFR, MT3, RIPK2, ERG, Traf2, RIPK1, SOD1, RPS27A, MAG, UBA52, GPX1, CYLD, IKBKG |
| 18   | GO:0012350| Programmed cell death                           | 0.00031 | TNFRSF1A, NGFR, MT3, RIPK2, Traf2, RIPK1, APP, FAIM2, RTN4, ERBB4, FOX30, GPX1, SOD2, AKT1, IKBG |
| 19   | GO:1902175| Regulation of oxidative stress-induced intrinsic apoptotic signaling pathway | 0.00032 | SOD1, IKBG, IKBKG |
| 20   | GO:1902042| Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors | 0.00056 | Traf2, RIPK1, FAIM2, GPX1 |
| 21   | GO:0005778| Positive regulation of immune response           | 0.00064 | RIPK2, ERG, Traf2, RIPK1, RPS27A, RNF31, DDX58, UBA52, CYLD, Traf6, UBC, IKBKG |
| 22   | GO:1903202| Negative regulation of oxidative stress-induced cell death | 0.0011 | TXN, GPX1, SOD2, AKT1 |
| 23   | GO:2001236| Regulation of extrinsic apoptotic signaling pathway | 0.0018 | TNFRSF1A, NGFR, RIPK2, TTR, CAT, TXS, HNF1A, RIPK1, SLC40A1, PRDX1, SOD1, RPS27A, APP, RNF31, ATP5A1, DDX58, APO4, MAG, UBA52, CYLD, SERPINA1, Traf6, UBC, AKT1, IKBG |
| 24   | GO:0002376| Immune system process                           | 0.0029  | TNFRSF1A, NGFR, RIPK2, TTR, CAT, TXS, HNF1A, RIPK1, SLC40A1, PRDX1, SOD1, RPS27A, APP, RNF31, ATP5A1, DDX58, APO4, MAG, UBA52, CYLD, SERPINA1, Traf6, UBC, AKT1, IKBG |
| 25   | GO:0097300| Programmed necrotic cell death                  | 0.0034  | Traf2, RIPK1, CYLD |
| 26   | GO:0071356| Cellular response to tumor necrosis factor      | 0.0045  | TNFRSF1A, NGFR, Traf2, RIPK1, FOX30, AKT1 |
| 27   | GO:0032743| Positive regulation of interleukin-2 production | 0.0046  | RIPK2, Traf2, Traf6 |
| 28   | GO:0032755| Positive regulation of interleukin-6 production | 0.0049  | RIPK2, ERG, Traf2, Traf6 |
| 29   | GO:0010940| Positive regulation of necrotic cell death      | 0.0062  | MT3, RIPK1 |
| 30   | GO:0097190| Apoptotic signaling pathway                     | 0.0063  | TNFRSF1A, NGFR, Traf2, RIPK1, FOX30, SOD2, AKT1 |
| 31   | GO:2001233| Regulation of apoptotic signaling pathway       | 0.0066  | Traf2, RIPK1, SOD1, FAIM2, CYLD, GPX1, SOD2, AKT1 |
| 32   | GO:2001234| Negative regulation of apoptotic signaling pathway | 0.0068 | Traf2, RIPK1, FAIM2, GPX1, SOD2, AKT1 |
| 33   | GO:0050852| T cell receptor signaling pathway               | 0.0096  | RIPK2, RNF31, Traf6, IKBKG |
| 34   | GO:0097191| Extrinsic apoptotic signaling pathway           | 0.0096  | TNFRSF1A, Traf2, RIPK1, FOX30 |
| 35   | GO:2001242| Regulation of intrinsic apoptotic signaling pathway | 0.0096 | SOD1, CYLD, GPX1, SOD2, AKT1 |
| 36   | GO:0070673| Response to interleukin-18                      | 0.01    | RIPK2, AKT1 |

(Continued)
TABLE 1 | Continued

| SL No | Term ID | Term Description                                      | FDR  | Protein Labels               |
|-------|---------|-------------------------------------------------------|------|-----------------------------|
| 37    | GO:2001238 | Positive regulation of extrinsic apoptotic signaling pathway | 0.0145 | TRAF2,RIPK1,CYLD           |
| 38    | GO:0006760 | Positive regulation of response to cytokine stimulus | 0.0152 | RIPK2,TRAF2,DDX58            |
| 39    | GO:0001819 | Positive regulation of cytokine production | 0.0215 | RIPK2,EREG,TRAF2,RIPK1,SOD1,DDX58,TRAF6 |
| 40    | GO:0002824  | Positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains | 0.0476 | RIPK2,TRAF2,TRAF6        |
| 41    | GO:0045639  | Positive regulation of myeloid cell differentiation | 0.0476 | RIPK1,FOXO3,TRAF6            |
| 42    | GO:0011683  | Lipopolysaccharide-mediated signaling pathway | 0.0493 | RIPK2,AKT1                   |
| 43    | GO:2001235  | Positive regulation of apoptotic signaling pathway | 0.05  | TRAF2,RIPK1,SOD1,CYLD          |

MT3 Dampens Antibacterial Resistance and Caspase-11 Inflammasome Activation In Vitro and In Vivo

We examined whether MT3 regulated antibacterial immunity and non-canonical inflammasome activation in vitro and in vivo. WT and Mt3−/− BMDM were infected with E. coli. After 24h, bacterial survival was reduced in Mt3−/− BMDM (Figure 2E). Next, we infected WT and Mt3−/− mice in vivo intraperitoneally (i.p.) with E. coli for 6h. Compared to WT mice, MT3 deficiency bolstered bacterial elimination from the blood and moderately improved bacterial clearance in the kidney and peritoneal lavage (Figure 2F). Caspase-11, GSDMD (N-terminal) and caspase-1 activation were heightened in kidney homogenates of infected Mt3−/− mice compared to infected WT mice (Figure 2G). The decrease in pro-GSDMD of Mt3−/− mice may be explained by increased conversion of pro- to active-GSDMD form (Figure 2G). IL-1β in the peritoneal lavage was significantly elevated (p<0.01) and serum IL-1β exhibited a trend towards increase in infected Mt3−/− mice compared to WT controls (Figure 2H). To determine if this response is consistent upon LPS challenge in vivo, we primed mice i.p. with poly(I:C) for 6h and challenged them i.p. with ultrapure LPS. After 18h, IL-1β was elevated in the peritoneal lavage and serum of Mt3−/− mice compared to WT mice (Figure 2I). We further queried the impact of MT3 on LPS-induced sepsis. WT and Mt3−/− mice were challenged with ultrapure LPS (20 mg/kg) and assayed for weight loss, murine sepsis scores (MSS) as reported previously (27) and survival. MT3 deficiency resulted in greater weight loss and increased sepsis scores, but both genotypes similarly succumbed to septic shock (Supplementary Figures S2A-C).

We then investigated whether MT3 increased susceptibility to other gram-negative bacteria. WT and Mt3−/− mice were infected intranasally (i.n.) with a virulent, heavily encapsulated strain of Klebsiella pneumoniae (KP2 2-70). MT3 deficiency significantly (p<0.05) improved K. pneumoniae clearance in the spleen, but no changes were observed in the lung and kidney (Figure 2J). Gram-positive bacteria activate caspase-11 via the NLRP6 inflammasome (28). We determined whether the increased non-canonical inflammasome activation and antibacterial resistance observed in Mt3−/− mice extended to gram-positive bacterial infection. WT and Mt3−/− mice were challenged subcutaneously (s.q.) with a clinical
isolate of Group-A-Streptococcus GAS5448 (29). After 72h, Mt3-/mice manifested significantly (p<0.05) reduced GAS burden in the kidney and spleen compared to WT mice. Bacterial CFUs in the blood exhibited a similar trend (Supplementary Figure S2D).

Importantly, although Mt3-/mice exhibited higher activation of caspase-1 and IL-1β, the levels of active caspase-11 and pro-caspase-11 were diminished in GAS-infected Mt3-/mice compared to WT mice (Supplementary Figure S2E). Thus, although MT3 compromises resistance to both gram-negative and gram-positive bacteria, it specifically suppresses non-canonical inflammasome signaling in response to gram-negative microbial triggers.

Caspase-11 Synergizes With MT3 in Impairing E. coli Clearance In Vivo
Caspase-11 is crucial in antibacterial defenses particularly against gram-negative bacteria, although some studies have suggested a detrimental role for caspase-11 in bacterial elimination (30–35). MT3 suppressed antibacterial immunity as well as non-canonical inflammasome activation. Thus, we investigated whether the heightened immunity to E. coli in Mt3-/mice was due to increased non-canonical inflammasome activation. We infected WT, Casp-11−/−, Mt3−/−, and Mt3−/−Casp-11−/− mice (Supplementary Figure S3A) i.p. with E. coli and assessed bacterial burden 6h post-infection. Compared to WT mice, bacterial elimination was
enhanced in $\text{Mt3}^{-/-}$ and $\text{Casp-11}^{-/-}$ mice but this response was further exacerbated in $\text{Mt3}^{-/-}$ mice lacking caspase-11 (Figure 3A). These data indicate that the combined absence of MT3 and caspase-11 improves resistance to gram-negative bacterial infection. We then analyzed caspase-11 inflammasome mediators in kidney homogenates harvested 6h post-infection. Mice lacking MT3, or caspase-11, exhibited elevated activation of GSDMD (N-terminal), caspase-1 and IL-1β compared to WT mice (Figure 3B).
These changes were also observed in the \(Mt3^{-/-}\) \(Casp-11^{-/-}\) mice. Since GSDMD is a target of caspase-11 as well as caspase-1 (36), an elevation in active GSDMD may result from higher caspase-1 activation observed in mice lacking MT3, caspase-11 or both. Caspase-8, a pro-apoptotic caspase, collaborates with caspase-11 to mediate systemic inflammation and septic shock (37, 38). Moreover, caspase-8, in addition to caspase-1 can directly cleave IL-1β. We therefore analyzed caspase-8 in kidney homogenates from \(E. coli\) infected WT, \(Mt3^{-/-}\), \(Casp-11^{-/-}\) and \(Mt3^{-/-}Casp-11^{-/-}\) mice. Caspase-11 negatively influenced the activation of caspase-8 (Supplementary Figure S3B).

The above data demonstrate that even when caspase-11 is absent, \(Mt3^{-/-}\) mice exert heightened levels of active caspase-1, active IL-1β and antibacterial immunity. We therefore queried whether improved bacterial elimination in the absence of MT3 was facilitated by the canonical NLRP3 inflammasome. WT and

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**FIGURE 3** See also Supplementary Figure S3B

Caspase-11 synergizes with MT3 in impairing bacterial clearance. WT, Casp-11\(^{-/-}\), Mt3\(^{-/-}\) and Casp-11\(^{-/-}\)Mt3\(^{-/-}\) mice were infected i.p. with \(E. coli\) (1 X10\(^9\) CFUs/mouse) for 6h. (A) Bacterial CFUs measured in kidney, blood and peritoneal lavage, \(n = 3-6\) per group, one-way ANOVA. (B) Western blots of pro-GSDMD, active-GSDMD (p31), pro-caspase-1, active-caspase-1, pro-IL1β and active-IL-1β in kidney homogenates, \(n = 3-6\) per group, one-way ANOVA, data are mean ± SEM. (C) WT and Mt3\(^{-/-}\) mice treated i.p. with MCC950 (1 mg/mouse) or PBS and infected i.p. with \(E. coli\) (1 X10\(^9\) CFUs/mouse) for 6h. IL1β was measured by ELISA in peritoneal lavage, \(n = 6\) per group, one-way ANOVA, data are mean ± SEM. Bacterial CFUs in whole blood and kidney, \(n = 4\) per group, one-way ANOVA, data are mean ± SEM. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
Mt3<sup>-/-</sup> mice were treated i.p. with MCC950 (NLRP3 inhibitor) followed by infection i.p. with E. coli. Treatment with MCC950 reduced IL-1β levels and sharply blunted antibacterial resistance in the blood and kidney of Mt3<sup>-/-</sup> mice compared to vehicle-treated controls. Bacterial burdens were also elevated in WT mice by NLRP3 inhibition (Figure 3C). Collectively, these data demonstrate that MT3 negatively controls activation of the non-canonical inflammasome and that both MT3 and caspase-11 cripple resistance to bacterial infection.

**Myeloid MT3 Orchestrates Negative Control of the Non-Canonical Inflammasome**

To affirm that the effects on caspase-11 inflammasome activation observed in the Mt3<sup>-/-</sup> mice were dependent on myeloid-MT3, we...
generated mice specifically lacking MT3 in myeloid cells (Lys2Cre Mt3fl/fl) (Figure 4A and Supplementary Figures S4A, B). Genotyping analysis of BMDMφ and peritoneal Mφ (PMφ) from Lys2Cre, Mt3fl/fl and Lys2Cre Mt3fl/fl mice demonstrated efficient removal of the Mt3 gene from BMDMφ and PMφ only in Lys2Cre Mt3fl/fl mice (Figure 4B). To determine if myeloid MT3 deficiency augmented non-canonical inflammasome activation in vivo, we infected Lys2Cre and Lys2Cre Mt3fl/fl mice i.p. with E. coli. After 6h, caspase-11 inflammasome targets and bacterial burden were examined in kidney and blood. Lys2Cre Mt3fl/fl mice exerted increased activation of caspase-11, GSDMD (N-terminal), caspase-1, and IL-1β in kidney homogenates and improved bacterial elimination compared to Lys2Cre control mice (Figures 4C, D). Thus, myeloid MT3 facilitates subversion of non-canonical inflammasome activation and contributes to antibacterial immunity in vivo.
MT3 Exerts a Brake on the TRIF-IRF3-STAT1 Axis to Curtail Caspase-11 Signaling

Signalng via the TRIF pathway is crucial for caspase-11 activation and synergistic engagement of the NLRP3 inflammasome leading to activation of caspase-1 and IL-1β (20). Downstream of TRIF, IRF3 and IRF7 induce IFNβ production that activates STAT1 signaling and promotes transcription of inflammasome components including caspase-11 and guanylate binding proteins (GBP$s$). GBP2 and GBP5 facilitate LPS release into the cytosol from intracellular vacuoles containing bacteria (39, 40). Our functional enrichment data based on protein-protein interaction network analyses revealed a potential involvement of MT3 in LPS, TRIF, type-I IFN and IL-1 signaling (Table 1, Supplementary Table S1 and Supplementary Files S1, S2). We further examined our published RNA-seq data (NCBI SRA: PRJNA533616) to determine differentially expressed genes by comparing resting WT and Mt3$^{-/-}$ BMDM$\Phi$ (19). The derived list of differentially expressed genes significantly enriched 12 GO BP categories directly related to cytokine and chemokine signaling and regulation of inflammatory responses based on DAVID functional enrichment analysis (Figure 5A) (41). These analyses suggested that MT3 deficiency perturbed the expression of immune-related genes even at the resting state. We reported that a lack of MT3 augments IFNγ responsiveness (19). Herein, from our RNA-seq analysis, we identified 20 genes related to IFN-signaling that were upregulated in resting Mt3$^{-/-}$ BMDM$\Phi$ compared to resting WT BMDM$\Phi$ (p adj <0.05) (Figures 5B, C). Among these, Isg15, Ms1 and Iift (Iift1bl1, Iift3, Iift3h, Iift2, Iift1, Iift1bl2) family of genes are known targets of type-I IFNs (42–45). These observations led us to posit that MT3 regulated the cellular response to LPS challenge by modulating the TRIF-IRF3-STAT1 axis upstream of non-canonical inflammasome activation. LPS engages the TRIF-IRF3-STAT1 axis via toll-like receptor 4 (TLR4) signaling in Mφ. To address this hypothesis, we challenged WT and Mt3$^{-/-}$ BMDM$\Phi$ with iLPS or vehicle and examined activation of the TRIF-IRF3-STAT1 pathway. Mφ lacking MT3 exerted increased activation of phospho-IRF3 (pIRF3), pSTAT1, GBP2 and GBP5 (Figure 5D). TRIF protein levels were unaltered by MT3 deficiency (Figure 5E). Type-I IFN signaling is required for activation of the caspase-11 inflammasome cascade by gram-negative bacteria (20). As MT3 deficiency augmented the expression of genes involved in IFN signaling (Figures 5B, C), we blocked the interferon-α/β receptor (IFNAR) 1 using a monoclonal antibody prior to iLPS challenge in WT and Mt3$^{-/-}$ Mφ. IFNAR1 blockade resulted in decreased pro-caspase-11 (p43 subunit). Total STAT1 and pro-caspase-1 (p38 subunit) were not greatly affected (Supplementary Figure S5). We found robust attenuation of pSTAT1, active-caspase-11 and active-caspase-1, but secretion of active-IL-1β in both WT and Mt3$^{-/-}$ Mφ was increased upon blockade of IFNAR1 signaling. This finding corresponded with high pro-IL-1β levels in Mφ treated with the IFNAR1 antibody (Supplementary Figure S5). These data indicate that although IFNAR1 signaling is required for fueling the non-canonical inflammasome cascade and activation of caspase-1, pro-IL-1β and its activation are suppressed by IFNAR1.

We queried if MT3 exerted a brake on TRIF signaling to downmodulate non-canonical inflammasome activation. WT and Mt3$^{-/-}$ BMDM$\Phi$ were treated with scramble or Ticam1 (gene encoding TRIF) siRNA, and challenged with iLPS (Figure 5F). Ticam1 silencing reversed the effects of MT3 deficiency resulting in a sharp reduction in caspase-11 and IL-1β activation in Mφ (Figure 5F). These data reveal a central role for MT3 in attenuating the crosstalk between TRIF signaling and the caspase-11 activation cascade.

Zn$^{2+}$ Flux by MT3 Drives Suppression of the Non-Canonical Inflammasome in Mφ

MTs are master regulators of intracellular Zn$^{2+}$ availability and distribution (46, 47). We determined if negative control of the non-canonical inflammasome by MT3 was Zn$^{2+}$-dependent. First, we systematically assessed Zn$^{2+}$ changes in WT and Mt3$^{-/-}$ BMDM$\Phi$ upon challenge with iLPS over time using SEC-ICP-MS. Activation of the non-canonical inflammasome in WT Mφ was associated with profound changes in the intracellular Zn$^{2+}$ pool. iLPS exposure led to a gradual increase in total Zn$^{2+}$ largely associated with the chromatogram peak(s) between 18-21 min. that we previously identified as MTs (Figures 6A, B) (9, 11). The time-dependent elevation in Zn$^{2+}$ corresponded with kinetics of Ticam1 induction (Figures 1A and 6A, B). Mφ lacking MT3 failed to elevate total Zn$^{2+}$ and MT-associated Zn$^{2+}$ in response to iLPS (Figure 6B). In contrast to the increase in Zn$^{2+}$ pool observed in WT Mφ challenged with iLPS, resting Mt3$^{-/-}$ Mφ harbored higher Zn$^{2+}$ content that reduced over time post iLPS challenge (Figure 6B). These data indicate that MT3 drives an elevation in intracellular Zn$^{2+}$ in Mφ during non-canonical inflammasome activation.

Zn$^{2+}$ chelation in human monocytes increases IRF3 activation (48). We reasoned that if the effects of MT3 were Zn$^{2+}$ dependent, altering the intracellular Zn$^{2+}$ concentration will at least in part reverse the heightened non-canonical inflammasome signaling observed in Mt3$^{-/-}$ cells. To test this postulate, we exposed WT and Mt3$^{-/-}$ BMDM$\Phi$ to increasing amounts of ZnSO$_4$ and challenged them with iLPS in vitro. Exogenous ZnSO$_4$ supplementation remarkably reduced the ability of Mt3$^{-/-}$ Mφ to respond to iLPS. pIRF3, pSTAT1 and activation of caspase-11 were reduced in ZnSO$_4$-supplemented Mt3$^{-/-}$ Mφ (Supplementary Figure S6A). A similar effect of Zn$^{2+}$ was also observed in WT Mφ (Supplementary Figure S6A). We investigated if exposing WT Mφ to a Zn$^{2+}$-deficient environment would mimic the effects MT3 deficiency on the non-canonical inflammasome. WT BMDM$\Phi$ were cultured in Zn$^{2+}$-sufficient or Zn$^{2+}$-deficient Opti-MEM media prior to iLPS exposure. WT Mφ exposed to a Zn$^{2+}$-deficient milieu manifested higher pIRF3 and caspase-11 activation accompanied by increased activation and release of IL-1β (Figure 6C). The amount of TRIF, pro-caspase-11 and pro-IL-1β were not affected by Zn$^{2+}$ deficiency (Figures 6C and Supplementary Figure S6B).

Next, we directly addressed whether Zn$^{2+}$ is required for the suppressive function of MT3 on the caspase-11 inflammasome. We first overexpressed the MT3 gene in Mt1$^{-/-}$Mt2$^{-/-}$ Mφ and isolated the protein. Mt1$^{-/-}$Mt2$^{-/-}$ BMDM$\Phi$ were transfected with the Mt3 overexpressing vector (pCMV6-Ac-MT3-GFP) or an empty vector (pCMV6-Ac-GFP) control. Mt1$^{-/-}$Mt2$^{-/-}$ Mφ were
used so as to exclude any contribution of these MTs in the MT3 purification process. The MT-associated peak from MT3-overexpressed Mφ was identified by SEC-ICP-MS (Supplementary Figure S7) and collected. We complexed MT3 with the $^{66}\text{Zn}^{2+}$ isotope to acquire an MT3-$\text{Zn}^{2+}$ saturation of 4 $\text{Zn}^{2+}$ ions per MT3 (MT3-4$\text{Zn}^{2+}$) and 6 $\text{Zn}^{2+}$ ions per MT3 (MT3-6$\text{Zn}^{2+}$). The $^{66}\text{Zn}^{2+}$ isotope was used to monitor changes in the ratio of $^{66}\text{Zn}^{2+}$/$^{64}\text{Zn}^{2+}$ post-transfection.
of the MT3-{66}Zn2+ complexes in Mφ. We transfected apo-MT3, MT3-4Zn2+ or MT3-6Zn2+ into Mt3-/−/ BMDMφ in Zn2+-deficient media. The use of Mt3-/−/ BMDMφ and Zn2+-deficient media enabled exclusion of any possible contribution from endogenous MT and exogenous Zn2+ in our analysis. Post-transfection of apo-MT3 or MT3-Zn2+ complexes, Mφ were challenged with ILPS to activate the non-canonical inflammasome. To confirm that intracellular Zn2+ changes occurred upon transfection of the MT3-66Zn2+ complexes, we analyzed BMDMφ lysates by SEC-ICP-MS. Mt3-/−/ cells transfected with MT3-4Zn2+ and MT3-6Zn2+ but not apo-MT3 exhibited an increase in the 66Zn2+/64Zn2+ ratio in the MT-peak region at 18-21 mins. in the chromatogram indicating an elevation in the intracellular 66Zn2+ isotope (Figure 6D). These data confirm that transfection of the MT3-Zn2+ complexes resulted in an increase in intracellular 66Zn2+ in Mφ. In parallel, we isolated cell lysates and supernatants proteins from these Mφ to determine whether apo-MT3 or the MT3-Zn2+ complexes modulated the non-canonical inflammasome pathway. Transfection of MT3-4Zn2+ and MT3-6Zn2+ but not apo-MT3, dampened pIRF3, active caspase-11 and active IL-1β in response to iLPS (Figure 6E). TRIF levels were unaffected by MT3 transfection (Figure 6E). Pro-caspase-11 and pro-IL-1β were modestly diminished by MT3-4Zn2+ and MT3-6Zn2+ exposure, but these changes were not significant (Figure 6E). The effect of MT3-6Zn2+ was more profound than that of MT3-4Zn2+, indicating that a higher Zn2+ saturation on MT3 corresponded with a stronger suppressive effect on the non-canonical inflammasome (Figure 6E).

Taken together, these findings reveal a previously undescribed interplay between the non-canonical inflammasome and its negative regulator, whereby the MT3-Zn2+ axis suppresses caspase-11 inflammasome, but the two molecules concur in compromising immunological fitness of the host during bacterial pathogenesis.

DISCUSSION

Human CASPASE-4 or mouse caspase-11 are inflammatory caspsases that drive cell death via pyroptosis. These caspsases directly recognize bacterial LPS in the cytosol resulting in CASPASE-4 or caspase-11 auto-processing and synergistic activation of the NLRP3 inflammasome that culminates in caspase-1 activation, processing and release of IL-1β and IL-18 (2, 32). While the non-canonical inflammasome boosts host immunological fitness to some bacterial infections, heightened activation of this cascade poses the danger of tissue injury and organ failure. Thus far, negative regulation of IFNβ production by prostaglandin E2, immunity-related GTPases M clade, cyclic-adenosine monophosphate, and low dose oxidized phospholipid oxPAPC have been shown to thwart activation of the non-canonical inflammasome (49–52). The role of MTs in regulating inflammasome activation pathways has largely been unknown. Herein, we identify a previously undescribed function of MT3 in curtailing the highly inflammatory non-canonical inflammasome activation cascade via Zn2+ regulation. We demonstrate that while MT3 orchestrates negative regulation of the caspase-11 inflammasome, the combined presence of MT3 and caspase-11 blunts resistance to E. coli infection in vivo. These studies illuminate a central role for the MT3-Zn2+ axis in shaping the intricate balance between host antibacterial immunity and unrestrained inflammation.

MT1 and MT2 are ubiquitously expressed and can be induced by infection (53–56). Initial studies on MT3 revealed tissue-restricted expression with high levels predominantly found in the brain tissue where it inhibits neuronal cell death (21, 57). The immunological functions of MT3, particularly in the innate arm have only recently been investigated (11, 19, 21). We reported that Mt3 is inducible by the pro-resolving cytokines IL-4 and IL-13 in Mφ. One inducer of Mt3 expression is STAT6 signaling, and this MT is crucial in shaping the phenotypic and metabolic attributes of Mφ stimulated with type-2 cytokines (11, 19). Studies on MTs in response to exogenous LPS stimulation have largely focused on MT1 and MT2. Monocytes and Mφ induce MT1 and MT2 upon extracellular LPS exposure (58, 59). We found that iLPS challenge also induced Mt1 and Mt2 in Mφ, although their expression receded to baseline over time. In contrast, Mt3 expression gradually increased as non-canonical inflammasome activation progressed. Subversion of inflammatory responses in Mφ by MT3 and the delayed expression pattern in response to a non-canonical inflammasome trigger are reminiscent of waning inflammation after the initial peak of inflammasome activation has subsided (19). In line with this hypothesis, protein interaction network analysis predicted the involvement of MT3 in cellular responses related to non-canonical inflammasome activation. Mφ lacking MT3 exerted robust activation of caspase-11, caspase-1 and IL-1β. Similar to our observations with mouse MT3, a lack of human MT3 exacerbated the activation of CASPASE4 and IL-1β in hMφ. Human and mouse MT3 proteins that share 86% identity thus have consistent roles that culminate in negative regulation of the non-canonical inflammasome cascade in Mφ (60). As non-canonical inflammasome activation progressed, MT3 guarded against its unrestrained activation to avert potential inflammatory damage. Together, these observations reveal a pivotal role for MT3 in curtailing the vigor of the caspase-11 activation cascade. Although Mt3−/− mice exerted higher sepsis scores and weight loss, they succumbed to septic shock similar to WT controls, suggesting that a threshold level of caspase-11 activation may be sufficient to promote sepsis-associated mortality.

In vivo, LPS released from OMV of gram-negative bacteria triggers caspase-11 activation (2, 3). Myeloid-MT3 contributed to averting excessive activation of caspase-11 and synergistic activation of the canonical inflammasome in response to gram-negative microbial triggers. MT3 compromised antibacterial resistance to E. coli and K. pneumoniae, but this was not due to its suppressive action on the caspase-11 inflammasome in vivo. Instead, Mt3−/− and Casp-11−/− mice manifested improved antibacterial immunity, an effect that was further augmented when Mt3−/− mice lacked caspase-11. The synergism between MT3 and caspase-11 may result from
independent or combined effects of MT3 and caspase-11 in vivo. Of note, the activation of caspase-1 and caspase-8 in the absence of MT3 and caspase-11 reveal that canonical inflammasome activation was operational and both caspase-1 and caspase-8 may contribute to IL-1β activation in vivo (38).

Mφ utilize Zn²⁺ deprivation and Zn²⁺ intoxication mechanisms as strategies for antimicrobial defense (56, 61, 62). We previously showed that ablation of MT3 in Mφ augments immunity to Histoplasma capsulatum as well as E. coli. The increased antimicrobial resistance in Mt3⁻/⁻ Mφ is at least partially attributable to a decrease in the Mφ exchangeable Zn²⁺ pool and exaggerated IFNγ responsiveness (11, 19). Myeloid and non-myeloid cells may together contribute to bacterial elimination in whole-body MT3-deficient mice. Nonetheless, the augmented bacterial elimination observed in Lys2Cre Mt3⁻/⁻ mice indicates that myeloid-MT3 contributes to the suppression of antibacterial defenses in vivo. The finding that MT3 deficiency dually bolstered inflammasome activation and antibacterial immunity underpins a role for this protein in suppressing the emergence of a proinflammatory phenotype in Mφ. Our data unveil a unique crosstalk between caspase-11 and its negative regulator, whereby although MT3 keeps caspase-11 activation under control, the two synergistically compromise host immunological fitness to gram-negative bacterial infection.

Caspase-11 activation can have opposing effects on clearance of different bacteria. It improves resistance to Burkholderia thailandensis, B. pseudomallei, Brucella abortus, and Legionella pneumophila but may compromise immunity to B. cereus, Salmonella typhimurium, E. coli, Shigella flexneri, K. pneumoniae and gram-positive infections including Streptococcus pyogenes, Staphylococcus aureus and Listeria monocytogenes (28, 31–33, 35, 63–69). Lipoteichoic acid from gram-positive bacteria engages the caspase-11 inflammasome via NLRC4 (28). Likewise, GAS infection led to caspase-11 activation in vivo. Although MT3 exerted disparate effects on caspase-11 activation in gram-positive and gram-negative infections, caspase-1 and IL-1β activation was suppressed by MT3 in both infection settings. In the context of GAS infection, both the host and the pathogen contribute to canonical inflammasome activation. Surface and secreted GAS virulence factor emm, and the streptococcal pyrogenic exotoxin B (SpeB) proteins act as second signals to activate caspase-1 signaling (70–72). Our data do not exclude the role of pathogen-derived factors in contributing to the increased canonical inflammasome activation observed in Mt3⁻/⁻ mice infected with GAS. Nonetheless, our findings indicate that MT3 exerted a suppressive effect on the canonical caspase-1 pathway activated by gram-positive bacteria, while sparing negative regulation of the upstream non-canonical inflammasome activation in vivo.

The TRIF pathway is a central node in activation of the caspase-11 inflammasome in response to gram-negative infection (20). Targeting TRIF, but not IFNAR1, completely reversed the inflammatory cascade, including IL-1β activation. Blockade of IFNAR1 attenuated downstream activation of STAT1, caspase-11 and caspase-1, but both pro-IL-1β and active IL-1β levels were dramatically enhanced. This finding contrasts with the previously reported requirement of both TRIF and IFNAR1 in this pathway (20). Although that study utilized BMDM from IFNAR1⁻/⁻ mice and we used an anti-IFNAR1 monoclonal antibody, both approaches resulted in attenuation of targets downstream of IFNAR1. Emerging evidence points to an indirect inhibitory effect of type-I IFNs on inflammasome activation by decreasing pro-IL-1β transcription via IL-10 or 25-hydroxycholesterol (73, 74). The subdued activation of caspase-1 and heightened active IL-1β levels suggests that IL-1β activation occurs via a caspase-1-independent pathway when IFNAR is blocked. Interfering with IFNAR1 signaling can therefore subdue activation of critical inflammasome components including caspase-11 and caspase-1 but sustain IL-1β production and activation.

The crucial function of Zn²⁺ as a signaling molecule is well documented (13, 75–77). Changes in plasma and cellular Zn²⁺ levels regulate the production of various cytokines via NF-κB signaling (78, 79). Specifically, Zn²⁺ deficiency in humans increases the production of tumor necrosis factor (TNF)α and IL-1β by LPS-treated PBMCs ex vivo, whereas Zn²⁺ supplementation reduces it (80, 81). Therefore, a shift from physiological Zn²⁺ concentrations at the systemic or cellular level can impact proinflammatory cytokine responses and inflammation. To our knowledge, modulation of Mφ Zn²⁺ homeostasis during non-canonical inflammasome activation has not been previously demonstrated. Our data show that caspase-11 activation is accompanied by a gradual expansion of the intracellular Zn²⁺ pool driven by MT3. Zn²⁺ deficiency did not augment pro- forms of caspase-11 (p43 and p38), but specifically increased their activation. Zn²⁺ diminishes signaling via IRF3 by limiting its nuclear localization (48). Accordingly, MT3 interfered with signaling via the TRIF-IRF3-STAT1 axis by shaping the Mφ Zn²⁺ pool. The MT3-Zn²⁺ axis dampened IRF3 phosphorylation and downstream mediators without impacting TRIF levels. Although we cannot rule out the direct effect of Zn²⁺ on inflammasome components downstream of IRF3, the suppressive action of MT3 on the non-canonical inflammasome was Zn²⁺ dependent. Our data demonstrate that by manipulating the Mφ Zn²⁺ milieu, caspase-11 activation can either be triggered or averted. This finding has important implications in defining a role for Zn²⁺ in subverting caspase-11 driven hyperinflammation. Developing therapeutic strategies that temper activation of the caspase-11/4 inflammasome have garnered tremendous interest to alleviate endotoxemia. In light of this, the MT3-Zn²⁺ axis emerges as a fresh and vital candidate that guards the vigor of a caspase-11 fueled inflammatory response. In the context of gram-negative bacterial pathogenesis, our data indicate that strategies aimed at combined targeting of MT3 and the caspase-11 inflammasome may be more beneficial in infection control than targeting caspase-11 alone.

Taken together, our studies illuminate a double-edged phenomenon in inflammasome regulation whereby the MT3-Zn²⁺ axis is a sentinel of the caspase-11 inflammasome but MT3 and the non-canonical inflammasome function in concert to compromise host antibacterial resistance.
TABLE 2 | Reagents and resources.

| Name                        | Source | Identifier |
|-----------------------------|--------|------------|
| Antibodies                  |        |            |
| anti-TRIF (Ser396)          | Proteintech | Cat#2328-1-AP |
| anti-STAT1 (pY701) [M135]   | Abcam  | Cat#ab29420 |
| anti-pSTAT1 [pY701]         | Abcam  | Cat#ab29420 |
| anti-GBP5                   | Proteintech | Cat#13220-1-AP |
| anti-GBP2                   | Proteintech | Cat#11854-1-AP |
| anti-Caspase 11 (17D9)      | Abcam  | Cat#ab180673 |
| anti-CASPASE-4              | MBL    | Cat#M029-3 |
| anti-Caspase-11             | Santa Cruz | Cat#AG-20B-0042-C100 |
| anti-IL-1β/IL-1F2           | R&D Systems | Cat#AF-401-NA |
| anti-IL-1β/IL-1F2           | R&D Systems | Cat#MAB4011 |
| anti-Caspase-8 (1G12)       | Santa Cruz | Cat#sc-12742 |
| anti-β-actin                | Cell Signaling Technology | Cat#PA1183 |
| anti-β-actin                | ThermoFisher Scientific | Cat#MAB8929 |
| Mouse anti-armenian hamster IgG-HRP | Santa Cruz | Cat#sc-2789 |
| Goat anti-rabbit IgG(H+L), HRP conjugate | Proteintech | Cat#SA00001-2 |
| IRDye® 800CW goat anti-rabbit IgG | LI-COR Biosciences | Cat#926-32211 |
| IRDye® 680RD goat anti-rabbit IgG | LI-COR Biosciences | Cat#926-68071 |
| Goat anti-mouse IgG(H+L), HRP conjugate | Proteintech | Cat#SA00001-1 |
| IRDye® 680RD goat anti-mouse IgG | LI-COR Biosciences | Cat#926-68070 |
| Mouse IgG (H&L) secondary antibody | Rockland | Cat#610-1319-0500 |
| conjugated pre-adsorbed Rabbit anti-goat IgG(H+L), HRP conjugate | Proteintech | Cat#SA00001-4 |
| Goat anti-Rat IgG(H+L), HRP conjugate | Proteintech | Cat#SA00001-15 |
| IRDye® 800CW goat anti-rat IgG | LI-COR Biosciences | Cat#926-32219 |
| anti-IFNAR1 (Clone: MAR1-5A3) | BioLegend | Cat#127302 |
| anti-IFNAR1 (Clone: MOPC-21) | BioLegend | Cat#400102 |
| Bacterial Strains           |        |            |
| Escherichia coli (K12)     | Dr. Jason Gardner | N/A |
| Klebsiella pneumoniae (KP2 2-70) | Dr. Jason Gardner | N/A |
| Group A Streptococcus (Streptococcus pyogenes) | Dr. Suba Nookala | N/A |

(Continued)
### TABLE 2 | Continued

| Name                                                      | Source                           | Identifier |
|-----------------------------------------------------------|----------------------------------|------------|
| Protease & Phosphatase Inhibitor Cocktail                 | Thermo Scientific                | Cat#78442  |
| Denaturing Cell Extraction Buffer                         | Invitrogen                       | Cat#FN00091|
| XCell II® Biotin Module                                   | ThermoFisher Scientific          | Cat#E9051  |
| Glycine                                                   | Fisher Scientific                | Cat#BP581  |
| Tris-base                                                 | Fisher Scientific                | Cat#BP116  |
| Tween 20                                                  | Acros Organics                   | Cat#2336-0010|
| Triton X100                                               | Fisher Scientific                | Cat#BP151  |
| Bovine serum albumin                                      | Sigma                            | Cat#A7030  |
| Intercept® (TBS) Blocking Buffer                          | LI-COR Biosciences               | Cat#927-60001|
| Non-fat dry milk                                          | Nash Finch Co.                   | N/A        |
| Isoflurane                                                | Covetrus                         | N/A        |
| Nair                                                      | Chuich & Dwight Co Inc           | REF#PRM25444 |
| Gauze                                                     | Medine                           | SKU#836-WC |
| Puritan cotton tipped applicator                          | Puritan                          | N/A        |
| Insulin injection syringe (1mL)                           | Exelix international co.         | Cat#26029  |
| 10 ml plastic Syringe                                     | Fisherbrand                      | Cat#14965459|
| 1 ml plastic Syringe                                      | Fisherbrand                      | Cat#14965456|
| BioLite12 well multidish                                   | Thermo Scientific                | Lot# H4X4A4E106 |
| 24 well tissue culture plates                             | CellPro                          | Lot# 072219BA03 |
| 48 well cell culture plate                                | NEST                             | Lot# 121717A004 |
| Tissue culture plate 96 well flat bottom                  | Fisherbrand                      | Cat#FB012951|
| 96 well ELISA plate                                       | NEST                             | Lot#0429181A007 |
| Petri dish                                                | NEST                             | Lot#753001 |
| 15 ml Centrifuge tube                                     | Fisher Scientific                | Cat#14-965-237 |
| 50 ml Centrifuge tube                                     | Fisher Scientific                | Cat#14-965-239|
| Cell scraper                                              | SPL Life Sciences                | Lot#00030  |
| Golden Rod animal Lancet (4mm)                            | Medipoint Inc                    | N/A        |
| Omni homogenizer Th-01 and tips                           | Omni International               | Model      |
| GeneArt Platinum Cas9 Nuclease                            | ThermoFisher                     | Number#THP1115 |
| Alt-R SpCas9 Nuclease 3’LNS sgRNA                         | ThermoFisher                     | Cat#B26641  |
| TSK gel 3000SW gel filtration column                      | TOSOH BIOSCIENCE                 | Cat#05789  |
| DreamTaq DNA Polymerase Commercial Assays                 | Thermo Scientific                | REF#E07002  |
| TransIT®-TKO                                              | Mirus Bio LLC                    | Prod. No.#MIR |
| TransIT®-LT1                                               | Mirus Bio LLC                    | Prod. No.#MIR |
| Pierce Protein Transfection Reagent Kit                   | Thermo Scientific                | REF#98650  |
| Human IL-1β/IL-1F2 DuoSet ELISA                          | R&D Systems                      | Cat#DY201-05|
| Mouse IL-1β                                              | BioLegend                        | Cat# 432601 |
| IL-1α                                                    | BioLegend                        | Cat# 433401 |
| RNase Mini Kit                                            | Qiagen                           | Cat# 74104 |
| QUICK-RNA™ MINIPREP KIT                                   | Denville Scientific              | Cat# R1055 |
| Reverse Transcript System Probe Lo-Rox 2X gPCR Mix        | Promega                          | REF#FA3800 |
| CytoTox 96® NonRadioactive Cytotoxicity Assay kit Promega | RADIANT™                         | REF#G1781  |

### TABLE 2 | Continued

| Name                                                      | Source                           | Identifier |
|-----------------------------------------------------------|----------------------------------|------------|
| SuperSignal™ West Femto Maximum Sensitivity Substrate     | Thermo Scientific                | Cat#34096  |
| NEN Next Poly(A) mRNA                                    | New England BioLabs              | Cat#E7490L |
| NEN Ultra Directional RNA Library Prep Kit               | New England BioLabs              | Cat# E7420L|
| NEN Next Library Quant Kit                               | New England BioLabs              | Cat# E7630L|
| MEGAalohsontif T7 Kit                                    | Thermo Fisher                    | Cat#AM1354 |
| MEGAclear Kit                                            | Thermo Fisher                    | Cat# AM1906|
| Quick-DNA™ MiniPrep Plus Kit                             | ZYMO RESEARCH                    | Cat# D4069 |

### Experimental Models: Organisms/Strains

| Name                                                      | Source                           | Identifier |
|-----------------------------------------------------------|----------------------------------|------------|
| C57BL/6 Mice                                              | Jackson Laboratory               | Stock#00064|
| C57BL/6 Mt3-/- Mt2-/-                                      | Dr. George S. Deepe              | Stock#024698 |
| C57BL/6 Caspaas™/Casp™-/- (Casp-11/-)                     | Crossed and bred in-house        | N/A        |
| C57BL/6 Lys2Cre                                           | Dr. George S. Deepe              | N/A        |
| C57BL/6 Lys2Cre Mt3-/-                                    | Crossed and bred in-house        | N/A        |

### Instrument, Software and Algorithms

| Name                                                      | Source                           | Identifier |
|-----------------------------------------------------------|----------------------------------|------------|
| FluorChem® HD2                                            | Cell Biosciences                 | S/N#FC HD2 |
| Odyssey CLX Imaging system                                | LI-COR                           | S/N#275013253 |
| 7500 Fast Real Time PCR System                           | Applied Biosystems               | S/N#275013253 |
| RNA-Seq data analysis - DAVID Bioinformatics             | NIAID/NIH                        | (https://david.ncbi.nlm.nih.gov/summary.jsp) |
| Resources v.6.8                                           | Protein-protein interaction- STRING v.11.0 | (https://string-db.org) |
| NIH ImageJ Fiji                                           | NIH                              | (https://image.nih.gov/ij/) |

### Microbes

*E. coli* (K12) and *K. pneumoniae* were kindly provided by Dr. Jason Gardner at the University of Cincinnati. Group A Streptococcus (GAS 5448) was kindly provided by Dr. Suba Nookala at the University of North Dakota.

### Mice

All mice used in this study were on the C57BL/6 background. WT and *Casp4™/Yuan™* (*Casp-11-/-*) mice were acquired from the Jackson Laboratory. *Lys2Cre* mice were kindly provided by Dr. George S. Deepe, Jr. (University of Cincinnati). *Mt3-/-* (exon 3 deleted) and *Mt3®/®* mice were generated using clustered regularly interspaced short palindromic repeats (CRISPR) by the Transgenic Animal and Genome Editing Core facility at the Cincinnati Children’s Hospital Medical Center (CCHMC). In mice, the Mt3 gene cluster is located on chromosome 8. The Mt3

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(Continued)
gene consists of 3 exons and is preceded by Mt1 and Mt2 genes. We targeted exon 3 of Mt3 by flanking it with loxp sites (Mt3\[^{loxP}\]) using the CRISPR-Cas9 gene targeting approach. Lys2Cre Mt3\[^{loxP}\] mice that exhibit myeloid Mt3 deficiency were generated by crossing Lys2Cre mice to Mt3\[^{loxP}\] mice. Deletion of the Mt3 gene was confirmed in BMDM\[^{fl}\] and PM\[^{fl}\] of Lys2Cre Mt3\[^{loxP}\] mice by genotyping as detailed in the CRISPR/Cas9 generation of Mt3\[^{loxP}\] mice section. To confirm 3' loxp site insertion or deletion, genomic DNA amplified using forward (5' TAG GCT TCC CAC CTG TTT GG 3') and reverse (5' GCC AAG ATA AAG TCC GGG GT 3') primers. To confirm 5' loxp site insertion or deletion, genomic DNA amplified using forward (5' TCG AAC TAC TTC CAC ACA GAC AAG AAC 3') and reverse (5' TCA GTT TGG TCC AAA CGG GAT G 3') primers. To confirm Lys2Cre gene insertion, genomic DNA amplified using mutant (5' CCC AGA AGT GCC AGA TTA GG 3'), common (5' CTG GGG CTG CCA GAA TTG CTC 3') and WT (5' TTA CAG TCG GCC AGG CTG AC 3') primers. Casp-11\[^{-/-}\]Mt3\[^{-/-}\] mice were generated by crossing Casp-11\[^{-/-}\] to Mt3\[^{-/-}\] mice. Casp-11 was amplified using tail genomic DNA by mutant reverse (5' CGC TCC TTC CTG TTT AGT AGG 3') and WT reverse (5' CGG TGC CTA GTT TGT ATG G 3') primers. Mt3 was amplified using tail genomic DNA by forward (5' TGG GTC GTA GGT GAG GTT GG 3') and reverse (5' GCC AAG ATA AAG TCC GGG GT 3') primers. Mice used in this study had ad-libitum access to food and water. All mice were housed in the Department of Laboratory Animal Medicine, University of Cincinnati, accredited by American Association for Accreditation of Laboratory Animal Care (Frederick, MD) and experiments were conducted in accordance with Animal Welfare Act guidelines of the National Institutes of Health.

**CRISPR/Cas9 Generation of Mt3\[^{fl/ft}\] Mice**

The methods for the design of sgRNAs, donor oligos and the production of Mt3\[^{fl/ft}\] (loxP sites surrounding exon 3 of the murine Mt3 gene) animals were as described previously (82). The sgRNAs were selected according to the on- and off-target scores from the CRISPR design web tool (http://genome-engineering.org) as well as CRISPOR (http://crispor.tefor.net) (83). The selected sgRNA target sequences were cloned, according to the published method (84), into the pX458 vector (addgene #48138) that was modified by us to contain an optimized sgRNA scaffold (85) and a Cas9-2A-GFP. Their editing activity were validated by the T7E1 assay in mouse mK4 cells (86), compared side-by-side with Tetg sgRNA that was known to work in mouse embryos efficiently (87). Validated sgRNA was transcribed in vitro using the MEGAshterscript T7 kit (ThermoFisher) and purified by the MEGAclear Kit (ThermoFisher), and stored at -80°C. To prepare the injection mix, we incubated sgRNA and Cas9 protein (ThermoFisher) at 37°C for 5 mins. to form the ribonucleoprotein complex and then added the donor oligos to it. The initial attempt was to insert both loxp sites simultaneously via piezo-driven cytoplasmic injection (88) of 100 ng/ul Cas9 protein, 50 ng/ul 5' sgRNA, 50 ng/ul 3' sgRNA, 50 ng/ul 5' donor, and 50 ng/ul 3' donor into fertilized eggs. Injected eggs were transferred into the oviductal ampulla of pseudo-pregnant CD-1 females on the same day. Pups were born and genotyped by PCR and Sanger sequencing. However, only 3' loxp-containing mice were obtained from this attempt. After breeding them to homozygosity for the 3' loxp, a new set of 5' sgRNA and the donor oligo was designed and injected into the zygotes with the mix containing 150 ng/ul Cas9 protein, 75 ng/ul 5' sgRNA, and 100 ng/ul ssDNA donor oligo. Injected eggs were transferred into the oviductal ampulla of pseudopregnant CD-1 females on the same day. Pups were born and genotyped by PCR and Sanger sequencing. Founder mice carrying both 5' and 3' loxp sites in cis were finally obtained. Animals were housed in a controlled environment with a 12-h light/12-h dark cycle, with free access to water and a standard chow diet. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee-approved protocol of Cincinnati Children’s Hospital and Medical Center.

**Mφ Culture**

hMφ were prepared from peripheral blood mononuclear cells (PBMCs). Briefly, human blood obtained from the Hoxworth Blood Center, University of Cincinnati was diluted (1:2) with calcium- and magnesium-free 1X Dulbecco’s phosphate-buffered saline (DPBS) and inverted gently to mix. Ficoll-Paque (10 ml for the total volume of 40 ml diluted blood) was layered at the bottom of the tube. The tubes were centrifuged at 400 X g for 30 mins. without break at 20°C. The PBMC interface was transferred to sterile tubes and washed three times using 40 ml of DPBS containing 2mM EDTA and centrifuged at 120 X g for 10 mins. without break at 4°C. A final wash was performed with DPBS (without EDTA). Isolated PBMCs were resuspended in complete RPMI 1640 medium. PBMCs (5 X 10⁶) were plated in 24 well plates containing complete RPMI medium. After 24h, adherent monocytes were washed three times with DPBS and plated in complete RPMI 1640 medium (Corning) containing 10 ng/ml macrophage-colony stimulating factor (M-CSF), 10%FBS, 10 μg/ml gentamycin sulfate (Alkali Scientific Inc.) and 2-mercaptoethanol. Cells were differentiated by exposure to human recombinant M-CSF on days 0, 2 and 4. After 6 days, hMφ were washed with DBPS prior to use for the experiment. Mouse BMDMφ were prepared by differentiating bone marrow cells in complete RPMI 1640 medium containing 10 ng/ml mouse M-CSF, 10% fetal bovine serum (FBS) (HyClone Laboratories, Utah), gentamycin sulfate (10 μg/ml) and 2-mercaptoethanol. BMDMφ were fed on days 0, 2 with complete RPMI 1640 medium containing 10 ng/ml M-CSF and were supplemented on day 4 with M-CSF. After 6 days, adherent Mφ were harvested by washing with DPBS followed by trypsinization and centrifuged at 1600 rpm for 5 mins. at 4°C. Mφ were washed again with DPBS at 1600 rpm for 5 mins. at 4°C and counted under the microscope. Dead cells were excluded from enumeration using Trypan Blue stain. BMDMφ (0.5 X 10⁶ in 24 well or 1 X 10⁶ in 12 well plate) were seeded.
Bioinformatics Analysis
The STRING database was used to review the protein-protein interaction networks of MT3 in *Mus musculus* and *Homo sapiens* (22). A full network analysis was conducted based on text mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence data with a minimum required interaction score of 0.4 and a maximum of 50 interactors in the first shell and 50 interactors in the second shell. Statistical significance of the enriched biological processes (GO BP categories) in the MT3 network was set with a false discovery rate (FDR) <0.05.

Identification of differentially expressed genes in resting WT compared to resting *Mt3*−/− Mφ was based on our previously published RNA-seq data (NCBI SRA: PRJNA533616) (19). The number of biological replicates used in the analysis was 3 per group. Genes differentially expressed with a fold change FC>2 and adjusted p value q<0.05 were considered significant. The Benjamini-Hochberg correction was used to adjust p values for multiple hypothesis testing. Differentially expressed genes in *Mtg*−/− BMDMφ compared to WT BMDMφ with q<0.05 were queried using the functional annotation clustering tool DAVID to identify statistically enriched GO categories (using the GO terms, BP direct, CC direct and MF direct) in *Mt3*−/− BMDMφ compared to WT BMDMφ (41). Significance of enrichment was set to FDR <0.05.

Gene Silencing
For gene silencing, Mφ were transfected with the transfection complex (50 μl) of siRNA and TransIT-TKO™ (0.5%) transfection reagent (Mirus Bio™) in 500 μl of complete RPMI 1640 medium without antibiotics as per the manufacturer’s instructions. Concentration of siRNAs used for gene silencing were 100 nM each of the non-targeting pool (ON-TARGETplus™ scramble siRNA), human *MT3* (MT3 Silencer® Pre-designed siRNA) and mouse *Ticam1* (ON-TARGETplus SMARTpool). All siRNAs were purchased from Dharmacon (GE Healthcare). Both BMDMφ and hMφ were incubated with the siRNA containing transfection complexes for 24h in RPMI medium and washed prior to transfection with LPS in Opti-MEM medium. TRIF silencing was assessed by protein expression using Western blots. Human *MT3* silencing was assessed by gene expression using qRT-PCR.

IFNAR1 Neutralization
IFNAR1 on WT and *Mt3*−/− BMDMφ was neutralized using 10 μg/ml monoclonal anti-INFAR1 antibody (BioLegend; Clone: MARI-5A3) 1h prior and 24h after iLPS (10 μg/ml) stimulation. Negative control groups were treated with the same dose of isotype control IgG antibody (BioLegend; Clone: MOPC-21). After a total 48h, cell lysates and supernatants were harvested for the molecular analysis.

Non-Canonical Inflammasome Activation in Mφ
To activate the non-canonical inflammasome, 1 X 10⁶ Mφ were transfected with a transfection complex (50 μl) of 0.3% TransIT™-LT1 (Mirus Bio™) transfection reagent (vehicle) and 2 μg/ml or 10 μg/ml ultrapure LPS-B5 (*In vivo* Gen) prepared from *E. coli* 055:K59(B5) in 500 μl Opti-MEM medium (Thermo Fisher Scientific-US) as per manufacturer’s instructions for 24h.

Preparation of Zn²⁺-Sufficient and Zn²⁺-Deficient Opti-MEM Media
Molecular biology grade chelex-100 resin (BioRad) was washed three times with metal free ddiH₂O prior to use. To prepare Zn²⁺-deficient Opti-MEM medium, washed Chelex-100 resin (3 g per 100 ml) was added to Opti-MEM media and vigorously shaken for 1h on an orbital shaker at room temperature. After this time, media was filtered using a 0.22 μm filter and mixed with fresh washed chelex-100 resin and the same procedure was repeated for a total of 3 times to eliminate metals from Opti-MEM media. Chelex was removed from the media by a final filtration step. During each of these stages, an aliquot of the media was saved to monitor the efficiency of Ca²⁺, Mg²⁺, Mn²⁺, Co²⁺, Zn²⁺, Cu²⁺, Ni²⁺ and Fe²⁺ elimination by ICP-MS. The amount of Zn²⁺ in Opti-MEM media was decreased by 95% by the above chelation method. To prepare Zn²⁺-sufficient media, chelaxed Opti-MEM was reconstituted with Ca²⁺, Mg²⁺, Mn²⁺, Co²⁺, Cu²⁺ and Zn²⁺ at the original concentrations as measured by ICP-MS. To prepare Zn²⁺-deficient media, all measured elements except Zn²⁺ were added to the chelaxed Opti-MEM media at the original measured concentrations. Finally, the pH of Zn²⁺-sufficient and Zn²⁺-deficient Opti-MEM media was adjusted to 7.4 and filtered prior to use.

MT3 Overexpression and Purification
The pCMV6-Ac-GFP vector containing the mouse *Mt3* gene (pCMV6-Ac-MT3-GFP) and empty pCMV6-Ac-GFP vectors were acquired from Origene and dissolved in nuclease-free sterile H₂O. Plasmid DNA (5 ng) was added to 50 μl of thawed Novabluce competent *E. coli* cells (EMD Millipore) and transformation was performed as per manufacturer’s instructions. *E. coli* cells were serially diluted in S. O. C media (ThermoFisher Scientific) and plated onto Luria-Bertani (LB) plates with S. O. C media at the original measured concentrations. Finally, the pH of Zn²⁺-deficient Opti-MEM media was adjusted to 7.4 and filtered prior to use.
filter tubes and centrifuged at 13000 rpm for 5 mins. Filtered cell lysates were subjected to SEC-ICP-MS to isolate the MT3 protein as described below.

**Preparation of apo-MT3, 4Zn\textsuperscript{2+}-MT3 or 6Zn\textsuperscript{2+}-MT3**

Cell lysates from above were analyzed by SEC-ICP-MS to detect the MT3-associated peak, followed by collection of the fraction of interest (18-21 mins.). The collected fraction was concentrated by freeze drying in Millrock lyophilizer (Millrock, NY). The concentrated fraction was treated with 1 g of Chelex X-100 resin to remove the divalent metals associated to the protein. The total MT concentration was calculated by the total sulfur concentration in the sample with a 1:20 stoichiometry. The sample was divided into 3 fractions, and each fraction was incubated for 2 h in 50 mM Tris-HCl with the appropriate concentration of \(^{66}\text{Zn}\textsuperscript{2+}\) nitrate to obtain an MT3-Zn\textsuperscript{2+} saturation of 0, 4 or 6 Zn\textsuperscript{2+} ions per MT3 molecule. After incubation, samples were filtered using a 3 kDa MWCO filter to remove the unbound Zn\textsuperscript{2+} and reconstituted in 1X PBS.

**Transfection of Apo, Zn\textsuperscript{2+}-MT3 Complexes Into BMDM**

In a 24 well plate, 5 X 10\textsuperscript{5} Mt3\textsuperscript{−/−} BMDM\textsuperscript{−} were transfected with the transfection complex (10 µl) containing 500 ng of apo-MT3, 4Zn\textsuperscript{2+}-MT3 or 6Zn\textsuperscript{2+}-MT3 and Pro-Ject\textsuperscript{TM} (1.75 µl) protein transfection reagent (ThermoScientific-US) in 250 µl of 2% FBS containing antibiotic-free RPMI 1640 medium. Control M\textsuperscript{t3−} BMDM\textsuperscript{−} were treated with Pro-Ject\textsuperscript{TM} alone. Cells were incubated with the transfection complexes for 3.5h and washed two times using HBSS prior to challenge with iLPS in Zn\textsuperscript{2+} free Opti-MEM medium. At the experiment end point, cell lysates were either prepared for SEC-ICP-MS analysis or both cell lysates and supernatants were collected for the analysis of non-canonical inflammasome activation.

**SEC-ICP-MS-MS Analysis and Normalization of Data**

SEC-ICP-MS-MS analysis of WT and Mt3\textsuperscript{−/−} BMDM\textsuperscript{−} was performed as described previously (11). M\textsuperscript{ϕ} were plated in Opti-MEM media, and either left untreated or transfected with 10 µg/ml LPS for 1, 24 and 48h. After this time, M\textsuperscript{ϕ} were washed twice in HBSS and cells were lysed with 0.1% SDS on ice for 20 mins. Cell lysates were then centrifuged in 0.22 mm filter tubes at 13000 rpm for 10 mins. Filtered cell lysates were frozen at -80ºC until further analysis by SEC-ICP-MS. 50-80 µl of cell lysates were injected to the HPLC-ICP-MS system according to protein concentration. To normalize the response of ICP-MS-MS signal from SEC separations on different days, 50 µl of 0.5 mg/ml carbonic anhydrase was injected into the liquid chromatography system, and area of Zn\textsuperscript{2+} signals from samples was normalized to area of the carbonic anhydrase peak from each day. The absorbance of carbonic anhydrase at 280 nm was followed to ensure protein integrity.

The instrumentation consisted of an Agilent 1100 HPLC equipped with a degasser, a binary pump, a thermostated auto sampler, a column oven compartment and a diode array detector. For the M\textsuperscript{ϕ} lysates, a TSK gel 3000SW gel filtration column (TSK gel 3000SW) was used. The mobile phase was ammonium acetate pH 7.4, 0.05% MeOH at 0.5 ml/minute. The HPLC system was coupled to the ICP-MS-MS nebulizer via a short polyether ether ketone capillary of 0.17 mm internal diameter. An Agilent 7500ce ICP-MS system equipped with a micromist quartz nebulizer, a chilled double pass Scott spray chamber and a standard 2 mm insert quartz tor with shield torch was used for all experiments. The ICP-MS was operated by the Agilent Mass Hunter integrated chromatographic software in the helium collision mode as reported previously (11). The isotope dilution experiments were processed by exporting the chromatographic data to Origin (Origin labs, CA) and the signal, in the form of counts per second, was used to calculate the ratio of \(^{66}\text{Zn}\textsuperscript{2+}/^{64}\text{Zn}\textsuperscript{2+}\) at every point in the chromatograms. This was used to generate a new chromatogram that reflected the input of \(^{66}\text{Zn}\textsuperscript{2+}\) from every molecular mass region in the chromatogram. The total area under the chromatograms was used to calculate the concentration of total Zn\textsuperscript{2+} (\(^{66}\text{Zn}\textsuperscript{2+}\) and \(^{64}\text{Zn}\textsuperscript{2+}\) from the MT3-\(^{66}\text{Zn}\textsuperscript{2+}\) complexes (\(^{66}\text{Zn}\textsuperscript{2+}/^{64}\text{Zn}\textsuperscript{2+}\)) against a calibration curve of Zn\textsuperscript{2+} based on carbonic anhydrase.

**ICP-MS-MS and SEC-ICP-MS-MS Quality Control to Avoid External Zn\textsuperscript{2+} Contamination**

All metal analysis experiments were performed using trace metal grade reagents with acid washed plastic vials. Reagent blanks were used to correct the background signal. The analysis was performed through a metal free encased auto sampler. The concentration of Zn\textsuperscript{2+} in the blanks was always below 100 parts per trillion (ppt), the blank estimate concentration on the calibration curves was always below 50 ppt, while the detection limits were below 30 ppt.

For chromatographic analysis, the mobile phase was cleaned using a Chelex 100 resin, using the batch method. In brief, 3 g of Chelex-100 was added to a liter of mobile phase, stirred for 30 mins and passed through a 0.45 mm membrane. This decreased the Zn\textsuperscript{2+} concentration below 200 ppt (measured as total). By this method, the base line ICP-MS-MS \(^{66}\text{Zn}\textsuperscript{2+}\) signal was below 1000 counts per second, which represents sub-pb levels. The SEC column was cleaned using 10 volumes of 0.2 M NaCl and equilibrated with the mobile phase, followed by injection of 50 µl of 2% HNO\textsubscript{3} three times to remove any accumulated Zn\textsuperscript{2+} in the column. With this procedure, Zn\textsuperscript{2+} distribution in the samples never deviated more than 10% compared to the theoretical natural Zn\textsuperscript{2+} isotope distribution in the control M\textsuperscript{ϕ} samples. Four blanks and four carbonic anhydrase standards were injected after the cleaning procedure for monitoring Zn\textsuperscript{2+} signal by ICP-MS-MS to ensure optimal column performance. Typically, the column was cleaned every 30-40 samples.

**LPS Treatment In Vivo**

Thirteen-week-old WT and Mt3\textsuperscript{−/−} mice were primed with \textit{i.p.} injection of 10 mg/kg poly(I:C) for 6h followed by 2 mg/kg ultrapure LPS-B3 \textit{(In vivoGen)} prepared from \textit{E. coli} 055:K59 (B5) \textit{(i.p.} injection) for 18h. At the experiment end point, blood
was collected by cardiac puncture, allowed to clot, and centrifuged at 2000 rpm for 30 mins. at 4°C to isolate serum. Serum was used to measure cytokines by enzyme-linked immunosorbent assay (ELISA).

**In Vitro and In Vivo Infection With Gram-Negative Bacteria**

For in vitro infection, *E. coli* (K12) was grown in LB broth at 37°C overnight in an orbital shaker. The culture was pelleted, washed and resuspended with ice-cold DPBS. Optical Density (OD) of the culture was measured at 600nm using a spectrophotometer. To analyze (*) of the culture was measured at 600nm using a spectrophotometer. To analyze

In Vivo Infection With Gram-Positive Bacteria

A representative MIT1 clonal Group-A-Streptococcus GAS5448 was used for subcutaneous infections (91). GAS was grown at 37°C under static conditions in Todd-Hewitt broth (BD, MD, USA) supplemented with 1.5% yeast extract (BD Biosciences, MD, USA) and *in vivo* infections were performed as described previously (29, 92). WT and *Mt3-* mice (n=8/group) were used in this study as a model for subcutaneous GAS infections. One day prior to infection, the hair on the back of the mice was depilated (using Nair cream) and mice were injected subcutaneously with 0.1 ml of GAS suspension prepared in sterile DPBS (Ca²⁺/Mg²⁺ free, low endotoxin, Mediatech, VA, USA, DPBS) (OD₆₀₀ adjusted to yield ~1-5x10⁶ (8 CFUs). Actual inoculum was determined by plating on tryptic soy agar containing 5% sheep blood (BD Biosciences, MD, USA). Mice were monitored twice daily for body weight, lesions, and mortality. To determine GAS dissemination and load, mice were humanely euthanized 72h post-infection. Blood was drawn through cardiac puncture; necrotic skin, kidney, and spleen were recovered aseptically and weighed. One ml of DPBS was added per 100 mg tissue and homogenized (Omni International, Marietta, GA) followed by plating of ten-fold dilutions on blood agar plates. GAS burden was calculated as colony-forming units (CFUs) per ml (blood) or per mg of tissue. The remaining homogenates were centrifuged for 15 mins. at 12,000 x g at 4°C, and supernatants were stored at -80°C for western blot analysis.

**Gene Expression**

RNA was isolated from MΦ after elimination of genomic DNA using RNeasy Plus Mini kit (Qiagen) or QUICK-RNA™ MINIPREP KIT (Thomas Scientific). cDNA was prepared using Reverse Transcription Systems Kit (Promega, WI) or rAmp First Strand cDNA Synthesis Flex Kit (Thomas Scientific). Taqman primer/probe sets (Applied Biosystems, CA) were used for real-time gene expression analysis using ABI Prism 7500. For time course analysis of expression of murine *Mt* genes, MΦ were left unstimulated or stimulated with 2 µg/ml iLPS for 0h, 1h, 6h, 24h and 48h. Data are presented as fold change in gene expression normalized to unstimulated MΦ at the 0h time point. To examine the effects of extracellular ultrapure LPS (exLPS) on murine *Mt3* gene expression, BMDMΦ were left unstimulated or stimulated with 10 µg/ml exLPS. Data are presented as fold change in gene expression normalized to unstimulated MΦ at the 48h time point. For *MT2A* gene expression analysis in *Mt3* silenced hMΦ, cells were treated with either *MT3* siRNA or scramble siRNA as mentioned above. Data are presented as fold change in gene expression normalized to control siRNA treated hMΦ.
Hypoxanthine guanine phosphoribosyl transferase (Hprt) was used as an internal control to compare target gene expression.

**Western Blotting**

TRIF (Proteintech), pIRF3 (BIOSS), STAT1, pSTAT1 (Abcam), GBP2, GBP5 (Proteintech), caspase-11 (Abcam and eBioscience™), CASPASE-4 (MBL), Gasdermin D (Proteintech and Cell Signaling Technologies), caspase-1 (AdipoGen Life Sciences), IL-1β (R&D Systems) and caspase-8 (Enzo Life Sciences) were assessed in kidney homogenates of *E. coli* infected mice. Cell lysates were prepared using Denaturing Cell Extraction Buffer (Invitrogen) containing protease & phosphatase inhibitor cocktail (ThermoScientific). Culture supernatants were frozen at -80°C until use and processed using methanol-chloroform protein extraction method. Briefly, supernatants were mixed with equal volume of 100% ice-cold methanol and 0.25 times of the total volume of chloroform followed by gentle vortexing and centrifugation at 20,000 X g at 4°C for 10 min. Upper-phase was discarded without disturbing inter-phase proteins. Ice-cold methanol (500 µl) was added to the tube, gently vortexed and centrifuged at 20,000 X g at 4°C for 10 mins. Supernatants were discarded and pellet was dried at 37°C for 3-5 mins. Urea (8 M, pH-8.0) was used to dissolve the pellet and extracted proteins were stored at -80°C. Total cell lysates, supernatants proteins, cell lysates + supernatants and kidney homogenates were boiled in SDS-PAGE 1X sample buffer at 95°C for 5 mins. Kidney homogenates were centrifuged at 20,000 X g at 4°C for 15 mins. Supernatants were collected for protein analysis. Reduced proteins were run on 8%, 10% or 12% SDS-PAGE gels and transferred on to 0.22 µm nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were blocked using 5% skim milk in 1X Tris-buffered saline and 0.1% Tween 20 (1XTBST) and probed overnight with primary antibodies. Western blots were analyzed using ImageJ software and densitometry data were normalized to β-actin.

**ELISA**

Human and mouse IL-1β (BioLegend) concentration in media supernatants, serum and in peritoneal lavage and mouse IL-1α (BioLegend) in media supernatants were determined using commercial ELISA kits according to the manufacturer’s instructions.

**Quantification and Statistical Analysis**

Data were analyzed using Sigma plot or GraphPad Prism by one-way ANOVA for multiple comparisons using the indicated *ad hoc* methods with at least 3 or more independent biological replicates. Where two groups were compared, two-tailed t-test was used. For *in vivo* infection, bacterial CFUs were log-transformed for statistical analysis, p values were calculated, *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant, ND, not detected.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati and were conducted within the Department of Laboratory Animal Medicine accredited by American Association for Accreditation of Laboratory Animal Care (Frederick, MD). All animal experiments were conducted in accordance with Animal Welfare Act guidelines of the National Institutes of Health.

**AUTHOR CONTRIBUTIONS**

DC and KSV planned and conducted molecular and biochemical experiments in vitro and in vivo, generated *Casp-11<sup>–/–</sup>*Mt3<sup>–/–</sup> and *Lys2Cre Mt3<sup>–/–</sup>* mice, analyzed data, and wrote the manuscript. JG conducted *in vivo* infections with *K. pneumoniae*. AS, SN, and SM conducted *in vivo* experiments with GAS infection and analyzed data. AP assisted with bioinformatics analysis. JL conducted chromatographic and mass spectrometric analysis using ICP-MS and SEC-ICP-MS and MT3-Zn<sup>2+</sup> complex preparations, and analyzed data. KSV designed and supervised the project. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.755961/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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