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A mitochondrial micropeptide is required for activation of the Nlrp3 Inflammasome

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Abstract

Functional peptides encoded by short open reading frames (sORFs) are emerging as important mediators of fundamental biological processes. Here, we identified a micropeptide produced from a putative long-non-coding RNA that is important in controlling innate immunity. By studying lncRNAs in mice macrophages, we identified lncRNA 1810058I24Rik which was downregulated in both human and murine myeloid cells exposed to lipopolysaccharide (LPS), as well as other TLR ligands and inflammatory cytokines. Analysis of lncRNA 1810058I24Rik subcellular localization revealed this transcript was localized in the cytosol, prompting us to evaluate its coding potential. *In vitro* translation with ^{35}S-labeled methionine resulted in translation of a 47 amino acid micropeptide. Microscopy and subcellular fractionation studies in macrophages demonstrated endogenous expression of this peptide on the mitochondrion. We thus named this gene 'Mitochondrial micropeptide-47 (Mm47)'. Crispr-Cas9-mediated deletion of *Mm47,* as well as siRNA studies in mice primary macrophages showed that the transcriptional response downstream of TLR4 was intact in cells lacking Mm47. In contrast, *Mm47*-deficient or knockdown cells were compromised for Nlrp3 inflammasome responses. Activation of Nlrc4 or Aim2 inflammasomes were intact in cells lacking Mm47. This study therefore identifies a novel mitochondrial micropeptide Mm47 that is required for activation of the Nlrp3 inflammasome. This work further highlights the functional activity of sORF-encoded peptides (SEPs) and underscores their importance in innate immunity.

Introduction:

RNA molecules can be divided into two categories-messenger RNAs (mRNAs) and non-coding RNAs (ncRNAs). A large proportion of the mammalian genome is transcribed as long non-coding RNAs (lncRNAs) (1). Recent studies have demonstrated diverse physiological functions for long non-coding RNAs (lncRNAs) including a growing appreciation for the role of these molecules in the immune system, where they control the differentiation and/or activation of immune cells (2–5). In the innate immune system, our

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group and others have found that lncRNAs act as positive or negative regulators of inflammatory gene expression in a variety of immune cells including macrophages (4, 5). However, many annotated lncRNAs have short open reading frames (sORFs) that may encode for functional proteins or small peptides (6–8). These short ORF-encoded peptides (SEPs) or 'micropeptides' are now being recognized for their functions in development, differentiation, transport and other fundamental biological processes (7, 9). Indeed, a recent study has revealed that a large number of transcripts currently annotated as lncRNAs are associated with ribosomes and in some cases are translated (8). Among these, one such transcript, Aw112010 was found to be encoded from a non-canonical ORF and found to play a role in *Salmonella typhimurium* infection and intestinal inflammation (8). Similarly, humanin, an anti-apoptotic 24 aa SEP localized on the mitochondria prevents Bax translocation from the cytosol to the mitochondria (10). Other SEPs, such as Dworf and Myoregulin, have also been shown to regulate calcium transport in the endoplasmic reticulum (11). Collectively, these studies highlight important roles for SEPs in fundamental biological processes including cell death and innate immunity.

Macrophages represent the first line of defense against infection by detecting pathogens through germline-encoded Pattern Recognition Receptors (PRRs). These PRRs recognize specific pathogen associated molecular patterns (PAMPs) such as LPS and trigger the expression of pro-inflammatory cytokines and type I interferons (IFN) through the activation of the transcription factors NF-κB and IRF3 (12, 13). Macrophages also upregulate the expression of Nlrp3 and IL1β, both of which are critical for mounting rapid immune responses during infection (14). Nlrp3 forms a multiprotein complex known as the inflammasome. Inflammasomes are cytoplasmic supramolecular complexes that form in response to microbial as well as endogenous damage or danger signals (13, 15–17). Inflammasomes activate inflammatory caspases such as caspase-1, which in turn controls the protolytic maturation of the proinflammatory cytokines interleukin 1β (IL-1β) and IL-18. Caspase-1 also cleaves gasdermin D to generate an N-terminal pore forming fragment that facilitates cytokine release and pyroptotic cell death (18–20). The Nlrp3 inflammasome is activated in response to a wide variety of microbial and endogenous danger signals including nigericin, uric acid crystals, amyloid-β fibrils and extra-cellular ATP. The precise mechanisms leading to formation of the Nlrp3 inflammasome in response to these diverse ligands are still unclear although K+ efflux, and in some cases, mitochondria are important (15, 21, 22).

Here, we have identified an SEP encoded from an annotated intergenic lncRNA (lincRNA) transcript that is regulated in macrophages exposed to LPS. Loss of function studies identify a key role for this Mitochondrial micropeptide-47 (Mm47) in controlling activation of the Nlrp3 inflammasome. CRISPR-Cas9 knockout of Mm47 or siRNA mediated suppression of Mm47 expression in primary cells resulted in impaired Nlrp3 inflammasome activation, leading to reduced activation of caspase-1 and compromised IL1β secretion. The levels of Mm47 decrease following LPS stimulation suggesting that inflammasomes could be turned off by eliminating this peptide. Given its mitochondrial localization and the functional link to the Nlrp3 inflammasome, we posit that Mm47 represents a novel signaling node in the emerging crosstalk between mitochondria and the Nlrp3 inflammasome.

Materials and Methods:

Cell lines:

Primary bone marrow derived macrophages (BMDM) were generated from C57/Bl6N mice, or B6 mice expressing Cas9-GFP (Jackson labs #024858) and used, or immortalized for later. Briefly, bone marrow cells were cultured in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 20% L929 conditioned media for 7 days to differentiate into BMDM cells. Immortalized BMDM were generated using J2 transforming retroviruses expressing Raf and Myc as described (23).

Knock down and Knock out:

Knock down of Mm47 was performed using SilencerSelect small inhibitory RNAs purchased from ThermoFisher. Two non-targeting control siRNAs (ThermoFisher Cat#4390843 Cat#4390846) and three siRNAs targeting Mm47 (ThermoFisher Cat#4390771) were used. Lipofectamine and RNAiMAX (13778030) was used to transfect 30pmol siRNA in 1×10^6 BMDM cells. The cells were used for experiments at 72 hours post transfection.

CRISPR-cas9 mediated knock out of Mm47 was performed using single-guide RNAs. Non-targeting sgRNAs used were NTC Fwd GGCGAGGTATTCGGCTCCGC and NTC Rev CGCGGAGCCGAATACCTCGC. The sgRNAs targeting Mm47 genomic region were sgRNA1 Fwd CAACGTGGTTGGAATGTATC, sgRNA1 Rev GATACATTCCAACCACGTTG, sgRNA2 Fwd TGAAGAGATTAAGAAGGACC and sgRNA2 Rev GGTCCTTCTTAATCTCTTCA. Lentivirus packaging the single-guide RNAs was used to transduce immortalized BMDM expressing cas9-GFP. The cells were selected using 5ug/mL puromycin and used for respective experiments. To rescue Mm47 expression, we created a new construct in which the Pam sites were altered on the Mm47 DNA sequence to be resistant to cas9-mediated cleavage. Retrovirus was created using the empty vector or CRISPR-cas9-resistant Mm47 and used to transduce KO cells which were selected for stable lines using 2ug/mL blasticidin.

Macrophage stimulation:

The cells were treated with LPS (100ng/mL), polyinosinic-polycytidilic acid [Poly(I:C)] (25ug/mL), Pam3Csk4 (100nM), CLO97 (200ng/mL), TNFα (10ng/mL) and interferon alpha (500U/mL) purchased from Sigma-Aldrich. Cell lysate and supernatant was collected for RNA analysis and ELISA respectively. ELISA was performed for IL1b, TNFα and IL6 using kits available from R&D. RNA was extracted from cells using Biorad, Aurum kit (7326820).

RNA and Quantitative Real-Time PCR:

Total RNA was extracted from cells using Bio-rad Aurum Total RNA Mini Kit (Bio-Rad, 7326820) or TRIzol (Invitrogen, 15596026) according to the product manual. The cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad, 1708840). Quantitative PCR on the cDNA was performed in Bio-Rad CFX96 Touch Realtime PCR using gene specific primers. The genes were normalized to the housekeeping gene Gapdh.

Mouse Primers used are GAPDH Fwd TGGCAAAGTGGAGATTGTTGC, GAPDH Rev AAGATGGTGATGGGCTTCCCG, MALAT1 Fwd TTGGGACAGTGGACGTGTGG, MALAT1 Rev TCAAGTGCCAGCAGACAGCA, Mm47 Fwd GCTCAGAACTATGAAATGCCAAAC, Mm47 Rev GGTCTCAGAAGCAGGTGGAC, IL1 β Fwd GCCACCTTTTGACAGTGATGAG, IL1 β Rev GTTTGGAAGCAGCCCTTCATC, Nlrp3 Fwd CATGTTGCCTGTTCTTCCAGAC and Nlrp3 Rev CGGTTGGTGCTTAGACTTGAGA. Human primers used were GAPDH Fwd TGCAACAACCAACTGCTTA, GAPDH Rev AGAGGCAGGGATGATGTTC, Mm47 Fwd CACCGACATCATGCTCGAGT and Mm47 Rev GCCAGATACATTCCAACCACGT.

Western blots:

BMDMs stimulated as per the experiment were lysed in buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 and 5 mM EDTA with fresh 1x Halt Protease inhibitor cocktail (Promega #1861279). Homogenized lysates were resolved on 14% SDS-PAGE, and transferred to 0.2 μM PVDF membrane. Membranes were blocked with 5% non-fat dry milk (w/v) and probed with antibodies diluted in 1X PBS and 0.05% Tween-20 (v/v). The antibodies used were pro-IL1β (R&D Systems, AF-401-NA), caspase-1 (Santa Cruz Biotechnology, sc-514), gasdermin D (Abcam, AB209845), β-actin (Sigma), Nlrp3 (Enzo Life Sciences, clone cryo-2), Gapdh (Sigma, G9295), Flag (Sigma, A8592), KDEL (Enzo Life Sciences 10C3), Tom20 (Abcam, AB186734), VDAC (Cell Signaling, 4866S), HSP60 (Santa Cruz, 13115). The Mm47 antibody was custom made by Thermo Fisher against the immunogenic residue 22–47 of Mm47. Membranes were probed with horseradish peroxidase-conjugated anti-mouse (Bio-Rad, 172–1011) and anti-rabbit (Bio-Rad, 170–6515) or anti-Goat (Bio-rad, 172–1034) and developed using ECL chemiluminescent substrate (Pierce).

Immunofluorescence and Confocal Microscopy:

MitoTracker Deep Red (ThermoFisher, M22426) dye for mitochondrial staining was added to live cells as per the manual. Cells were fixed on 8-well chambered slides (Lab-Tek, 155411) using ice-cold 4% Paraformaldehyde for 15 minutes and washed with 1X PBS. The cells were permeabilized using 0.2% Triton X-100 in 1X PBS for 15 minutes and blocked using 5% Normal Goat Serum (Jackson Immunoresearch, 005–000-121) in 1X PBS and 0.2% Triton X-100. Cells were incubated with antibodies against Flag-AF488 (Cell Signaling, 5407S) or Mm47 (custom made, ThermoFisher) and anti-rabbit AF-488 (Thermo-Fisher, A11008) was used as secondary antibody. The cells were washed with PBS, and incubated with DAPI at room temperature for 10 minutes and washed again followed by imaging using Leica SP8 Lightning Confocal Microscope.

RACE:

The 5’-and 3’-ends of Mm47 transcript were determined using FirstChoice RLM-RACE kit (Ambion AM1700) according to the manufacturer’s instructions. The 1810058I24Rik gene-specific primers used were Fwd GATAGCTGCTGGGGACTCAC and Rev CGTGGTTGGAATGTATCTGGCT.

Cell fractionation:

Primary BMDM cells were suspended in resuspension buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl2). To the cell suspension, 10% Nonidet P-40 (v/v) was added, mixed and incubated on ice for 10 minutes and centrifuged at 13,000g for 30 seconds. The supernatant was used for cytosolic RNA, and the pellets were washed a few times and used to extract nuclear RNA using Qiagen RNAeasy Kit. GAPDH and MALAT1 were used as cytoplasmic and nuclear controls respectively.

Custom RT2 Profiler PCR Array:

Optimized real-time PCR primers were bound to 96-well plate by Qiagen (330171) specific for 72 annotated lincRNA genes, 8 housekeeping genes, 6 protein coding inflammatory genes, and RT-qPCR positive and negative controls. The 2^-deltaCT value for each gene was calculated against the average of β-actin and B2m for each treatment condition.

Subcellular fractionation:

Mouse embryonic fibroblast were used to perform sucrose discontinuous gradient centrifugation to separate cytosolic, ER and mitochondrial fractions as described before(24). Briefly, MEF cells were lysed in MTE buffer (270mM Mannitol + 10mM Tris + 0.1mM EDTA) and spun at 1400g for 10 minutes. The supernatant was spun at 15,000g for 10 min to separate crude ER fraction in the supernatant and mitochondrial fraction in the pellet. The crude ER fraction was further spun on a sucrose gradient of 1.3M, 1.5M and 2M at 152,000g for 74 minutes followed by collection, washing and drying of the pure ER band. Similarly, the crude mitochondrial fraction in the pellet was loaded to a sucrose gradient of 1M and 1.7M and spun at 40,000g for 30 minutes followed by collection, washing and drying of the pure mitochondrial band. All the high-speed centrifugation was performed in Beckman coulter SW40-Ti.

In vitro translation assay:

Plasmids with full wild-type sequence and frame-shifted mutant sequence of Mm47 with T7 promoter tag was prepared. The PCR primers used for cloning are Mm47 XhoI Flag Fwd ATCCTCGAG ATGGACTACAAGGACGACGATGACAAGCTCCAGTTCCTGCTTGGATTTACTT, Mm47 XhoI Fwd ATCCTCGAGATGCTCCAGTTCCTGCTTGGATTTACTT, Mm47 BglII Rev CATAGATCTTCAGGAACTAGGGGGCTTCTT, Mm47 BglII Flag Rev CATAGTCTTCACTTGTCATCGTCGTCCTTGTAGTCGGAACTAGGGGGCTTCTT, Mm47 XhoI T7 FS Fwd ATCCTCGAGTAATACGACTCACTATAGATGACTCCAGTTCCTGCTTGGATTTACTT, and Mm47 XhoI T7 promoter Fwd CTCGAGTAATACGACTCACTATAGGGACCTCTCACACCCTCCTCG. Megascript transcription kit (AM1334) was used to produce RNA. Retic lysate Kit (AM1200) and EasyTag Express^{35}S Protein Labeling Mix, 35S (Cat# NEG772002MC) were used to generated radiolabeled Mm47 peptides.

Statistical analysis:

Statistical analysis was performed with an unpaired, two-tailed Student's *t*-test in GraphPad Prism software, version 7.0.

Results:

TLR4 signaling regulates expression of long non-coding RNAs

The macrophage transcriptome is dynamically regulated in response to microbial and endogenous stimuli (12, 25). Lipopolysaccharide, which is recognized by TLR4/MD2, leads to the induction of a transcriptional program through NF-κB, IRFs and STAT proteins. Previous work from our lab and others has demonstrated that TLRs also regulate the expression of lncRNAs including both antisense lncRNAs and intergenic lncRNAs (lincRNAs). For example, lincRNAs such as lincRNA-Cox2 (Ptgs2os2) and lincRNA-EPS are regulated in response to LPS and exhibit broad regulatory effects by controlling inflammatory gene expression (26, 27). These lncRNAs were identified following transcriptional analysis of mouse bone marrow derived macrophages (BMDM) stimulated with LPS using RNA-sequencing [Fig. 1A]. To further examine the role of lncRNAs in macrophages, here we generated a custom PCR Array for an in-depth investigation of the expression of 72 lncRNAs, including genes that were TLR4-regulated (log2 fold change >2 or <−2) or abundantly expressed in BMDMs (26, 27). A heatmap showing the expression of this set of 72 lncRNAs in mouse macrophages treated with LPS (TLR4), Pam3CSK4 (TLR2), Poly I:C (TLR3), CL097 (TLR7) or the cytokines Tumor necrosis factor-α and type I IFN is shown [Fig. 1B]. Consistent with our previous studies, the expression of lincRNA-Cox2 was induced by all of these ligands, while lincRNA-EPS was downregulated in cells exposed to all of these stimuli. In addition, amongst the downregulated lncRNAs was a gene that was previously uncharacterized, called 1810058I24Rik. This transcript was downregulated in response to multiple ligands [Fig. 1B]. We confirmed the downregulation of this lncRNA in response to LPS as well as other TLR ligands as shown by quantitative RT-PCR [Fig. 1C]. The RNA virus Sendai virus (SeV) did not lead to a downregulation of this RNA.

1810058I24Rik is transcribed from mouse chromosome 6. The 1810058I24Rik transcript is spliced, and has three exons [Fig. S1A]. Using 5'-and 3'-RACE we determined the full-length sequence (580 nucleotides) of the spliced 1810058I24Rik [Fig. S1A and S1B]. qRT-PCR analysis of RNA extracted from mouse organs showed a broad expression profile of this transcript in muscle, lung, spleen, liver, kidney as well as the small and large intestine [Fig. 1D]. There was limited expression in brain and heart.

lncRNA 1810058I24Rik is down-regulated by LPS in a TRIF-dependent manner.

We next performed copy number analysis to define the abundance of this lncRNA. lncRNA 1810058I24Rik was expressed at ~1250 copies per cell in mouse macrophages and these levels were reduced to ~ 400 in response to LPS [Fig. 2A, 2B and S1C]. 1810058I24Rik is also conserved in humans where we found it was also expressed at high levels in human monocytes (~900 copies/cell) and dendritic cells, where it also exhibits a pattern of downregulation in response to LPS in both cell types [Fig. S1D (monocytes) and S1E

(monocyte derived DCs)]. We next sought to address the mechanism involved in the downregulation of 1810058I24Rik. In wild type BMDM, the expression of 1810058I24Rik was reduced in response to LPS stimulation. However, this downregulation was lost in cells lacking TLR4 or TRIF but was still observed in cells lacking MyD88, suggesting that the TLR4-TRIF pathway shuts down expression of this transcript [. 2C].

1810058I24Rik encodes a highly conserved mitochondrial micropeptide.

Understanding the subcellular localization of a lncRNA can be informative. Frequently, these RNAs are localized in the nucleus where they alter the expression of protein-coding genes by controlling gene transcription. There is also evidence that lncRNAs can be localized and function in the cytosol and act as sponges for miRNAs (4, 5). Thus, we tested the subcellular location of the 1810058I24Rik transcript by generating cytosolic and nuclear extracts, purifying RNA and performing qRT-PCR. Malat1, a well characterized lncRNA that functions in the nucleus was detected in the nuclear fraction (28). In contrast, the 1810058I24Rik transcript was predominantly cytosolic [Fig. 3A]. This was similar to the localization of the mature GAPDH mRNA, a protein coding gene that would be translated in the cytosol. Given the cytosolic location of the 1810058I24Rik transcript we next evaluated the potential protein coding capacity of this putative lncRNA, since many lncRNAs are misannotated and may encode functional proteins or short ORFs. The NCBI ORFfinder program revealed three putative ORFs in 1810058I24Rik which included a 47-aa ORF that shows high conservation across diverse species from *C. elgans* to humans [Fig. 3B]. In light of this observation, we wanted to address the possibility that 1810058I24Rik may be a protein-coding gene. To test this, we performed *in vitro* translation assays using ^{35}S labeled methionine, which confirmed that the 1810058I24Rik RNA was indeed translated into ~ 5.1 kDa peptide [Fig. 3C, S2A]. We also generated a mutant with a frameshift mutation inserted immediately after the start site, and found that this mutation ablated translation. Next, we cloned the putative ORF into a cDNA expression vector with a Flag tag at either the N-terminus, C-terminus or both. We could detect Flag-tagged translated 1810058I24Rik only with C-terminal tags in mouse embryonic fibroblast (MEF) cells and 293T human cells [Fig. 3D, S2B, S2C]. SignalP protein domain prediction suggested that the N-terminus of 1810058I24Rik contained a potential signal sequence. The addition of the N-terminal tag may not be tolerable causing degradation of 1810058I24Rik SEP. Finally, to formally define the presence of this peptide in cells, we generated an antibody to detect the endogenous 1810058I24Rik SEP. Consistent with our expression data, endogenous 1810058I24Rik SEP was expressed in BMDM. The levels of this peptide decreased in response to LPS. 1810058I24Rik SEP was abundant in macrophages and was decreased after 24 hours post LPS-treatment (Fig. 3E).

We next aimed to further understand where the 1810058I24Rik SEP was localized in cells. The SignalP program suggested that 1810058I24Rik may be localized to the mitochondria, despite the absence of a canonical mitochondrial targeting signal (29). A partial protein sequence of 1810058I24Rik had previously been reported in bovine heart and zebrafish mitochondria (30, 31). In order to determine if the 1810058I24Rik peptide localized at the mitochondria we performed immunostaining on FLAG-tagged 1810058I24Rik SEP ectopically expressed in MEFs. This analysis revealed that the 1810058I24Rik peptide co-

localized with the mitochondrial marker MitoTracker Deep Red [Fig. 4A]. Given this localization we propose to rename 1810058I24Rik-SEP as Mitochondrial Micropeptide 47 (Mm47). We next confirmed the localization of endogenous Mm47 in macrophages. Antibodies against the endogenous Mm47 again revealed mitochondrial localization in mouse BMDMs. [Fig. 4B]. The Endoplasmic Reticulum (ER) and mitochondria are known to intimately associate, which may affect the immunofluorescence imaging (32). Therefore, we employed sucrose gradient ultracentrifugation to separate pure cytosolic, ER and mitochondrial fractions (33). Using the mitochondrial membrane protein Tom20 as a positive control, western blot analysis confirmed that the Mm47 peptide is predominantly localized on the mitochondria [Fig. 4C]. Further sub-fractionation of the mitochondrial fraction revealed that Mm47 is highly enriched on the outer membrane and intermembrane fraction of the mitochondrion as indicated by the corresponding localization of VDAC, an outer membrane protein [Fig. 4D]. Overall, these studies indicate that the 1810058I24Rik transcript encodes a peptide that is localized to the mitochondrion.

Mm47 is required for Nlrp3-dependent IL1β maturation.

We next examined the possibility that Mm47 played a role in regulating the magnitude or duration of the LPS response in macrophages. Using CRISPR-Cas9 we created *Mm47* knockout immortalized mouse macrophages. Western blot analysis of BMDMs expressing a non-targeting control gRNA (NTC) and three gRNAs targeting Mm47 confirmed knockout of Mm47 in the three cell lines examined [Fig. 5A]. We then monitored LPS-dependent transcriptional responses in these cells using Nanostring technology to simultaneously detect the expression of 100 immune system-related genes. As shown in Fig. 5B, LPS treatment led to an increase in the expression of a broad panel of immune genes in the control lines after 2 and 6 hours of LPS. These responses were largely intact in macrophages lacking Mm47. These results suggest that Mm47 does not impact the transcriptional response induced in macrophages following LPS stimulation.

Given the localization of Mm47 on the mitochondria and the literature that reveals how damaged or defective mitochondria contribute to the activation of inflammation through engagement of Nlrp3, we next examined the possibility that Mm47 played a role in controlling activation of the Nlrp3 inflammasome. Immortalized BMDM were primed with LPS for 3 hours to upregulate IL1β and Nlrp3 itself, followed by stimulation with Nigericin, a potassium ionophore and bacterial pore-forming toxin that leads to formation of the Nlrp3 inflammasome complex. LPS priming and Nigericin stimulation led to robust secretion of IL1β as measured by ELISA. This response was significantly abrogated in cells lacking Mm47 [Fig. 5C]. This Mm47-dependent release of IL1β was also abrogated in cells stimulated with another Nlrp3 activator, ATP [Fig. 5D]. Indeed this effect of Mm47 was specific for the Nlrp3 inflammasome, as Mm47-deficient cells had comparable IL1β release when cells were stimulated with Poly(dA:dT), a double stranded DNA mimetic that activates the Aim2 inflammasome [Fig. 5E] or when cells were infected with *Salmonella typhimurium*, a bacterial pathogen that activates the Nlrc4 inflammasome [Fig. 5F]. We also performed rescue experiments by restoring Mm47 levels in the KO cells. This was achieved by ectopically expressing Mm47 in a form that was resistant to Cas9-directed cleavage. This approach led to restoration of Mm47 at levels observed in wild type cells [Fig. 5G]. These

restored cells were fully competent for LPS/Nigericin induced secretion of IL1β [Fig. S3A] [Fig. 5H]. Together, these observations indicate that Mm47 controls the activation of the Nlrp3 inflammasome in a highly specific manner.

Since mitochondrial ROS (mtROS) contributes to the priming of macrophages via Hif1α-mediated transcription of pro-IL1b as well as activation of the Nlrp3 inflammasome, we tested the levels of mtROS in cells (34–36). We did not observe a significant increase in mtROS in LPS treated cells and there was no difference between *Mm47*-deficient and wild type cells [Fig. S3B]. Similarly, alteration in mitochondrial electron transport chain (ETC) function affects ATP levels which in turn alter Nlrp3 activation (37). Therefore, we tested the mitochondrial oxygen consumption rate (OCR) as a proxy for mitochondrial ATP. We notice that the *Mm47*-deficient cells had reduced basal OCR. However, after the addition of chemical inhibitors of ETC we observed similar levels of OCR in both WT and Mm47 KO cells with reduced maximal OCR in LPS exposed cells [Fig. S3C]. We conclude from these studies that Mm47 does not contribute to the generation of ATP during mitochondrial ETC stress.

To further corroborate the finding that Mm47 impacts Nlrp3 inflammasome mediated IL-1β production, we employed an independent siRNA approach in primary BMDM. BMDM were transfected with two non-targeting siRNAs or with three Mm47-targeting siRNAs. Analysis of LPS and Nigericin responses in these cells showed that siRNAs targeting Mm47 led to a significant reduction in IL1β in response to Nigericin treatment [Fig. 6A]. Meanwhile, release of IL1β was comparable between non-targeting siRNA and Mm47-targeting siRNA transfected cells when cells were stimulated with double stranded DNA (poly (dA:dT)), or infected with *Salmonella typhimurium* [Fig. 6B and 6C]. Immunoblotting of cell lysates and supernatants revealed that Mm47-targeting siRNAs led to reduced processing of Caspase-1 p20 and mature IL1β (p17) [Fig. 6D]. We also measured mRNA levels of pro-IL1β or Nlrp3 itself in Mm47-siRNA targeted cells and in both cases the responses were intact [Fig. 6E and S4A]. Furthermore, the Mm47-siRNA targeted cells displayed reduced levels of processed Gasdermin D (Gsdmd) compared to controls. This effect on Gsdmd however, did not have a significant impact on cell death [Fig. S4B and S4C]. Collectively, these findings reveal that expression of Mm47 is essential to facilitate activation of the Nlrp3 inflammasome and the release of IL1β and Gsdmd processing with a modest impact on cell death.

Discussion:

An accumulating body of evidence indicates that a large number of genes are miss-annotated as long non-coding RNAs, many of which produce small proteins with important biological functions (38). Studies on the functions of these short ORF-encoded peptides are currently limited since in-depth experimental investigation of individual genes is required to uncover their functions. Recent computational and genomic advances have allowed the identification and mapping of SEPs (7). Several recent studies using Ribosome profiling and mass spectrometry-based detection of peptides have revealed that many genes currently annotated as lncRNAs produce small proteins (7, 38). These include ORFs that are translated from canonical AUG or non-canonical start sites (6, 7, 9). In macrophages, a novel gene AW112010 was recently identified as an SEP translated from a non-canonical start site of an

annotated lncRNA gene. AW112010 was shown to be regulated in innate cells such as macrophages following microbial infection, and genetic loss of function models in mice showed this SEP was important in mucosal immunity by controlling *Salmonella typhimurium* infection and altering susceptibility to colitis in mice. The same study also identified three ORFs of the *1810058I24Rik* gene in their ribosome profiling studies, two of which were predicted to be translated from non-AUG start sites (8). This study among others underscores the need for independently authenticating the coding potential of cytosolic lncRNAs and determining to what extent these RNAs produce peptides.

In a surprising twist on our initial focus on lncRNAs, here we have identified Mm47, a highly conserved 47aa long mitochondrial micropeptide, which is produced from an annotated lncRNA. Further, we provide clear genetic evidence demonstrating that Mm47 regulates Nlrp3 inflammasome function in macrophages. Mitochondria provide a signaling hub to integrate metabolic and inflammatory capability of immune cells. Such integration is provided through innate immune receptors including TLRs and the Nlrp3 inflammasome which respond to altered mitochondrial function and/or mitochondrial-derived molecules released from damaged organelles. In response to stress, mitochondria release molecules such as cardiolipin and mitochondrial DNA (mtDNA), both of which are sensed by the Nlrp3 inflammasome as well as by other innate sensors such as cyclic GMP-AMP synthase (cGAS) (22, 39, 40). *Mm47*-deficient cells exhibit impaired Nlrp3-mediated inflammasome responses. This defect was remarkably specific for Nlrp3, as neither Aim2 nor Nlrc4 inflammasomes were impacted by the loss of Mm47. Importantly, genetic-complementation of the Mm47 CDS in *Mm47* KO cells restored Nlrp3-mediated inflammasome responses. This indicates that the loss of Nlrp3 activity in the KOs is truly due to Mm47 and not an additional impairment in these cells. Further, the ability of the micropeptide ORF to rescue inflammasome defects also supports the importance of the peptide product in this biological context.

Exactly how Mm47 on the mitochondrion impacts activation of the Nlrp3 inflammasome is still an open question. Our results indicate that Mm47 does not act at the level of inflammasome priming since LPS-driven expression of pro-IL1β and of Nlrp3 were intact in *Mm47*-deficient cells as was the broader transcriptional program activated downstream of TLR4. Mm47 appears to act at the level of inflammasome licensing or activation although how this occurs is not clear from our work. Activation of Caspase-1 as measured by monitoring the formation of the p20 fragment and generation of the IL1β p17 were reduced in cell lacking Mm47. Caspase-1 cleaves pro-IL1β and Gsdmd in canonical inflammasome activation (18). The levels of IL1β p17 and Gsdmd p30 were both reduced in Mm47 targeted cells, however the kinetics of cell death was similar to non-targeted cells. Despite the reduction in Gsdmd processing, MM47 targeted cells still underwent pyroptosis. Previous studies have shown that while Gsdmd is a major executioner of cell death in the Nlrp3 pathway, Gsdmd-independent cell death pathways are also involved (18). It is possible in Mm47 targeted cells that Caspase-8 activation leads to the cell death observed (41, 42). While there has been considerable progress in understanding inflammasome biology, in particular the importance of Nlrp3 in a diverse range of cellular processes and diseases, the precise mechanisms that coordinate Nlrp3 inflammasome complex formation are very poorly understood. A unifying mechanism of how Nlrp3 is activated is not known and

therefore it is difficult to mechanistically define how Mm47 alters these processes. We also do not understand the impact of Mm47-deficiency on normal mitochondrial function. When we overexpress Mm47 in murine embryonic fibroblasts, the morphology of the mitochondria were altered. This could indicate that MM47 has a role in mitochondrial fission during stress that may directly or indirectly impact leakage of mtDNA or cardiolipins into the cytosol.

While we do not understand how Mm47 interfaces with the inflammasome, we did find that after exposure to LPS, both the 1810058I24Rik gene and the Mm47 polypeptide are reduced. At the protein level, the decrease in expression of Mm47 was observed after prolonged exposure (24–48 hours) of cells to LPS. It's possible that this temporal downregulation of Mm47 protein removes this essential factor on the mitochondrion and could represent a mechanism to curb inflammasome activation in order to resolve inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points:

a. Annotated lncRNA 1810058I24Rik encodes a conserved mitochondrial micropeptide.

b. Macrophages fail to activate Nlrp3 inflammasome in the absence of Mm47.

c. Genetic deletion of Mm47 in macrophage affects only Nlrp3 and not Aim2 or Nlrc4.

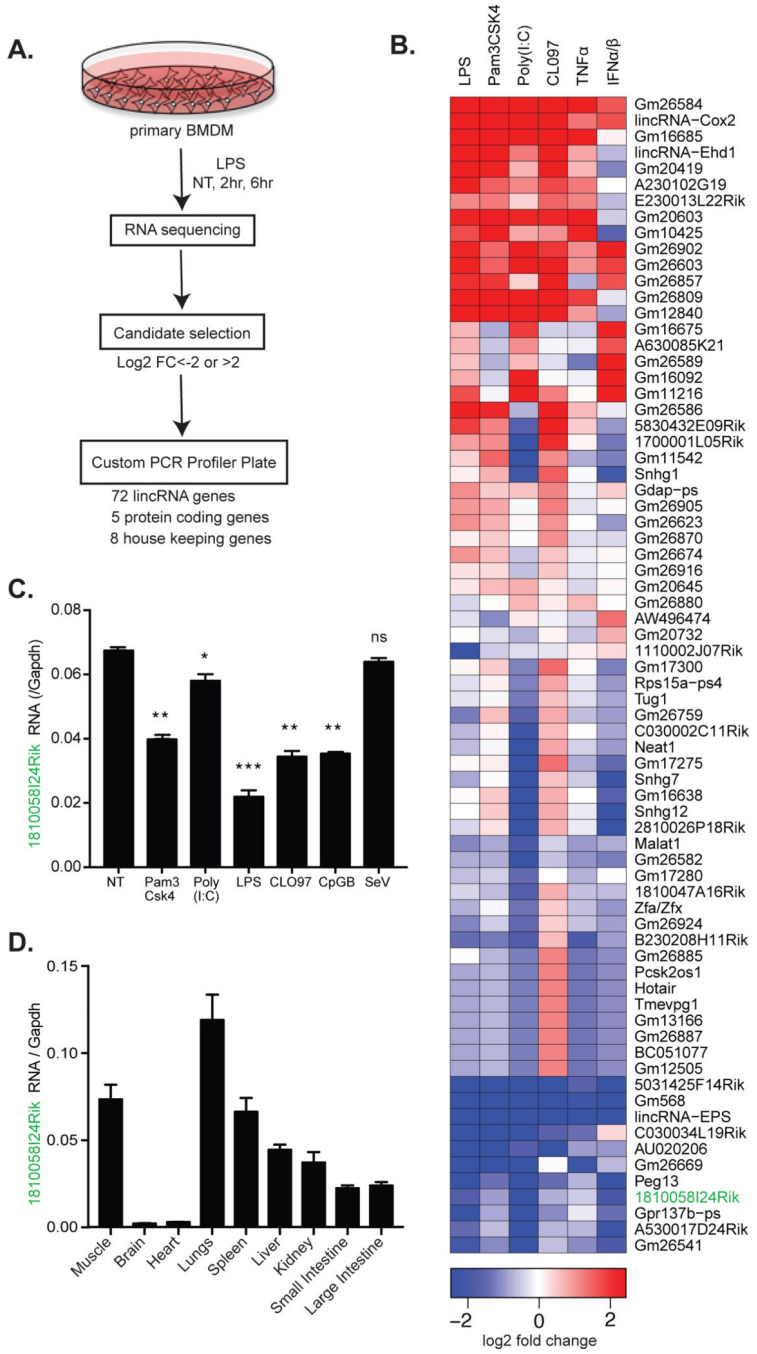

Figure 1: Identification of Mm47 as a putative lincRNA regulated in immune response

a) Schematic detailing pipeline for identification of immune regulated lincRNAs.

b) Quantitative profiling of candidate lincRNAs in response to various PAMPs and cytokines. Cells were treated with LPS (100ng/mL), Poly(I:C) (25ug/mL), Pam3Csk4 (100nM), CLO97 (200ng/mL), TNFα (10ng/mL) and interferon alpha/beta (500U/mL). Upregulated genes are in red and downregulated in blue. Mm47 is highlighted in green.

c) qRT-PCR of Mm47 relative to Gapdh in response to bacterial and viral PAMPs. Cells were treated with), Pam3Csk4 (100nM), Poly(I:C) (25ug/mL), LPS (100ng/mL), CLO97

(200ng/mL), CpGB (2ug) and Sendai virus (400HAU). Data represents mean ±SEM from three experiments.

d) qRT-PCR showing expression level of Mm47 in wild type B6 mice tissues compared to housekeeping gene Gapdh. Data represents mean ±SEM from three mice.

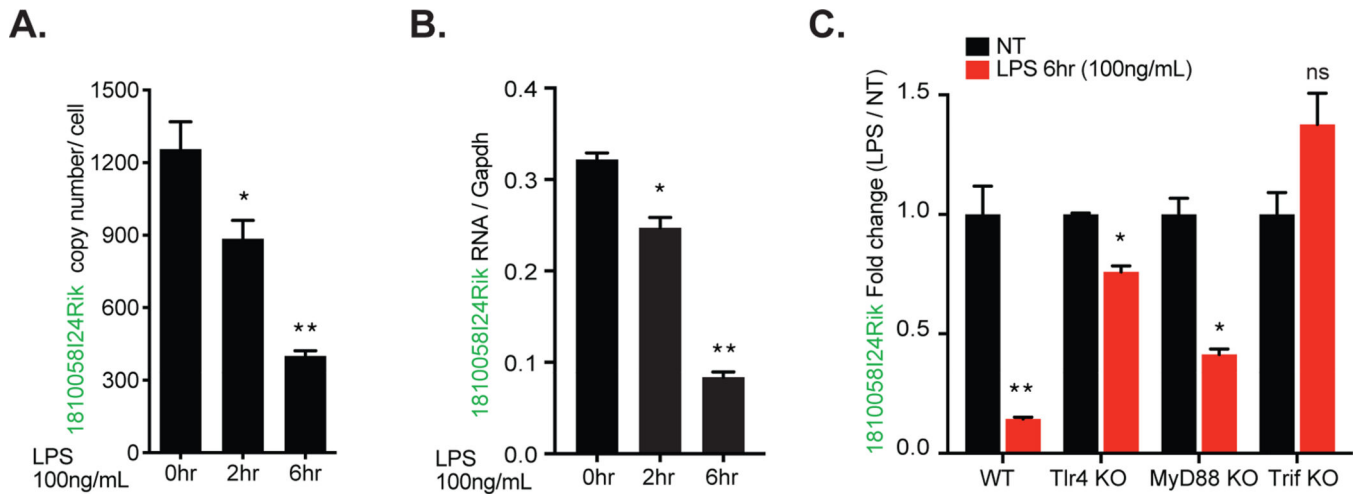

Figure 2: Mm47 transcript is downregulated in activated macrophage in Tlr4/MyD88-dependent manner

a) qRT-PCR showing copy number of mature transcripts of Mm47 in BMDM cells with and without LPS treatment.

b) qRT-PCR of Mm47 transcript with LPS treatment compared to Gapdh.

c) qRT-PCR showing Mm47 transcript downregulation in BMDM cells in response to 100 ug/mL LPS.

a-c) Data represents mean ±SEM of three experiments. *p<0.03; **p<0.002; ns, not significant.

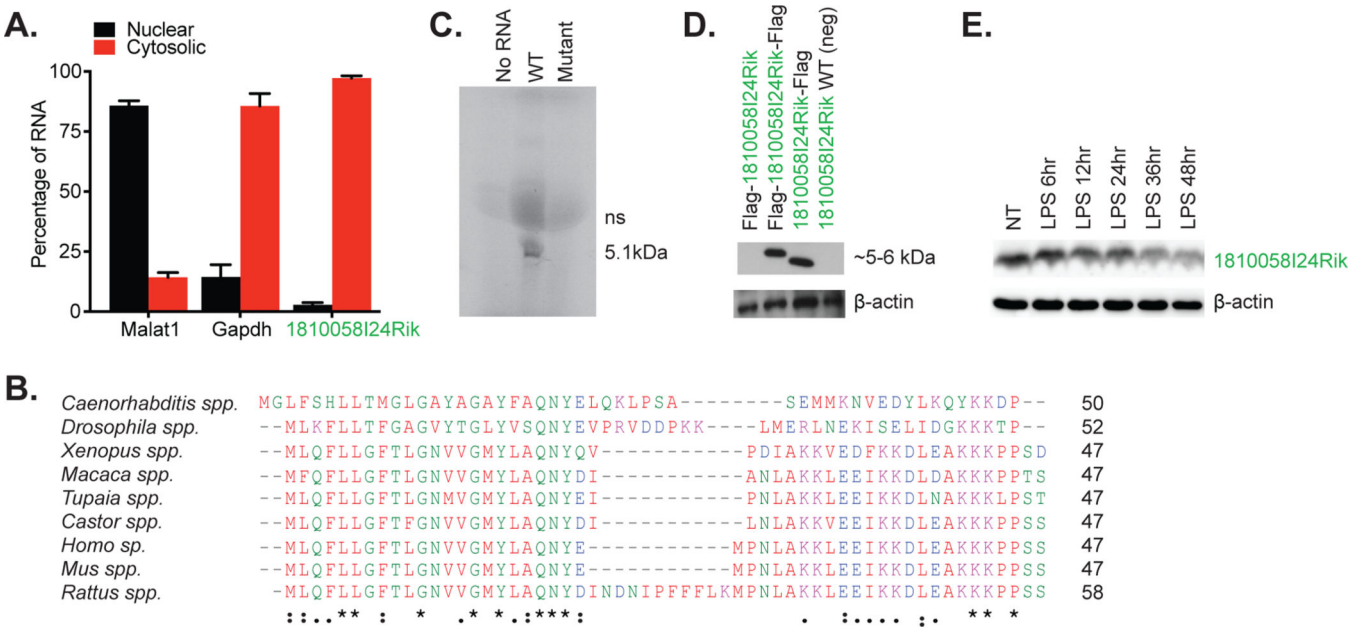

Figure 3: Mm47 encodes for a stable micropeptide

a) Subcellular fraction and qRT-PCR of Malat1, Gapdh and Mm47 to test the localization of Mm47 transcript. Data represents mean ±SEM of three independent experiments.

b) Mm47 micropeptide sequence alignment across species of different phyla.

c) *In vitro* translation assay using ^{35}S-labeled methionine and SDS-PAGE electrophoresis showing 5.1 kDa band of Mm47 micropeptide in WT sequence. Representative immunoblot of three independent experiments is shown.

d) Expression of Flag-tagged Mm47 micropeptide in MEF cells and immunoblotting for Flag-tag. Representative immunoblot of three independent experiments is shown.

e) Western blot of endogenous Mm47 micropeptide with LPS treatment. Representative immunoblot of three independent experiments is shown.

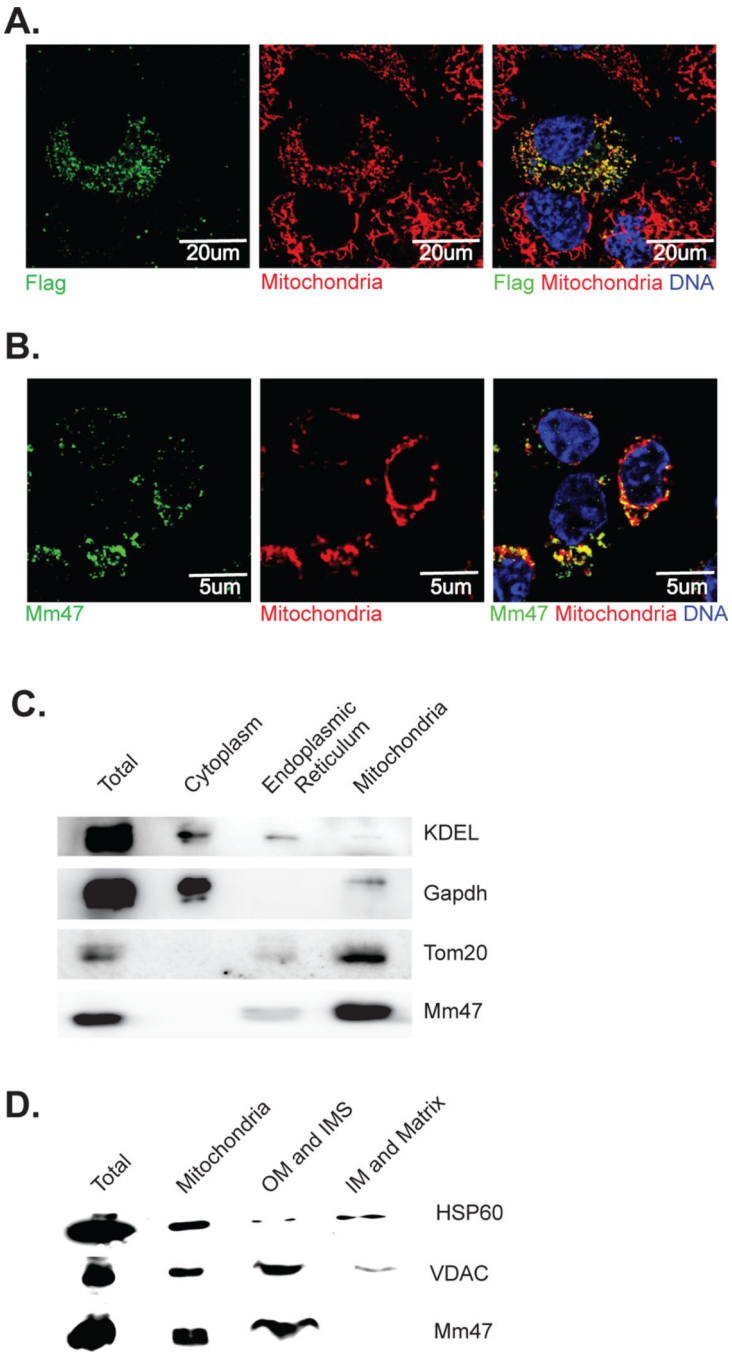

Figure 4: Mm47 micropeptide is localized on the mitochondria

a) Ectopic expression of Flag-tagged Mm47 in MEF followed by immunofluorescence imaging of Flag-Mm47 (Green), MitoTracker Deep Red (red) for mitochondria and DAPI (blue) for nucleus. Representative images from three independent experiments are shown. b) Immunofluorescence imaging of endogenous Mm47, MitoTracker Deep Red for mitochondria and DAPI for nucleus. Representative images from three independent experiments are shown.

c) Subcellular fraction of the cytosolic, ER and mitochondrial fraction followed by immunoblot for endogenous Mm47, mitochondrial outer membrane protein Tom20, ER membrane protein KDEL and cytosolic protein Gapdh. Representative immunoblot of three independent experiments is shown.

d) Sub-organelle fractionation of the outer and inner mitochondrial compartment followed by immunoblot for endogenous Mm47, outer mitochondrial membrane protein VDAC and mitochondrial matrix protein HSp60.

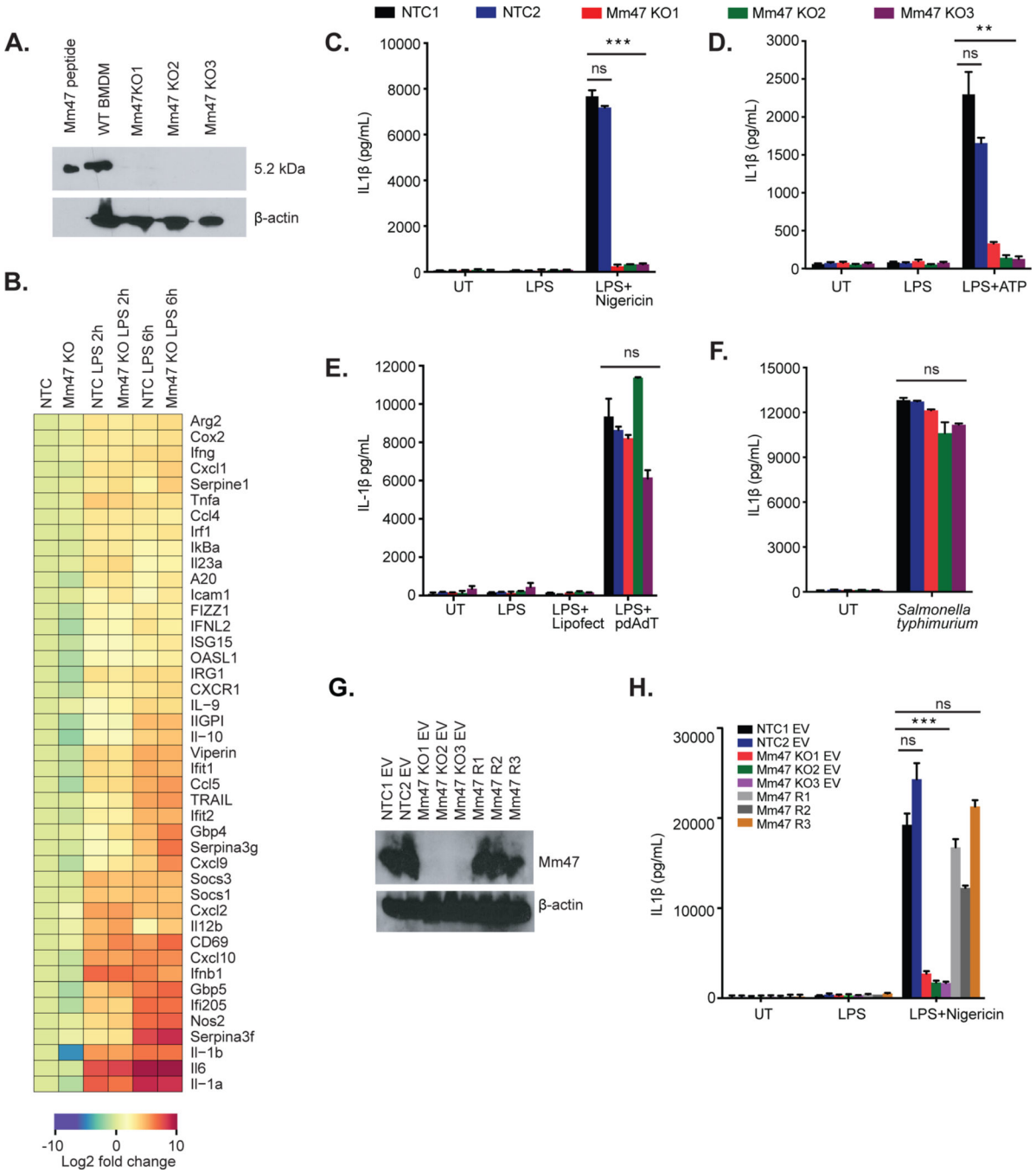

Figure 5: Mm47-deficient iBMDM have impaired Nlrp3 pathway

a) Western blot for endogenous Mm47 after CRISPR-Cas9-mediated knock out.

b) Heatmap of inflammatory genes tested using Nanostring codeset to compare response of non-targeted cells (NTC) and Mm47 KO cells to 100ng/mL LPS treatment for 2h and 6h. Transcription change shown as log2 fold change compared to NTC.

c) ELISA for secreted IL1β in non-targeted and CRISPR-cas9-mediated knockout of Mm47 in BMDM after priming with 100ng/mL LPS and stimulation with 5uM Nigericin.

d) ELISA for secreted IL1β in non-targeted and CRISPR-cas9-mediated knockout of Mm47 in BMDM after priming with 100ng/mL LPS and stimulation with 5uM ATP.
e) ELISA to test AIM2 receptor-mediated secretion of IL1β in BMDM with or without Mm47 knockout upon 100ng/mL LPS priming and 2ug Poly(dA:dT) transfection.
f) ELISA to test NLRC4 receptor-mediated IL1β secretion in primary BMDM with or without Mm47 knockout upon *Salmonella typhimurium* infection for 6 hours.
g) Western blot of Mm47 to test restoration of CRISPR-cas9-resistant Mm47 expression using lentiviral transduction. The control cells were transduced with empty vector (EV) and rescued cells (R) were transduced with CRISPR-cas9-resistant Mm47.
h) ELISA testing secreted IL1β expression in control and CRISPR-cas9-resistant Mm47 reconstituted cells upon priming with 100ng/mL LPS and stimulation with 5uM Nigericin.
a-f) Data represents mean±SEM of three experiments. *$p<0.03$; **$p<0.002$; ***$p<0.001$; ns, not significant.

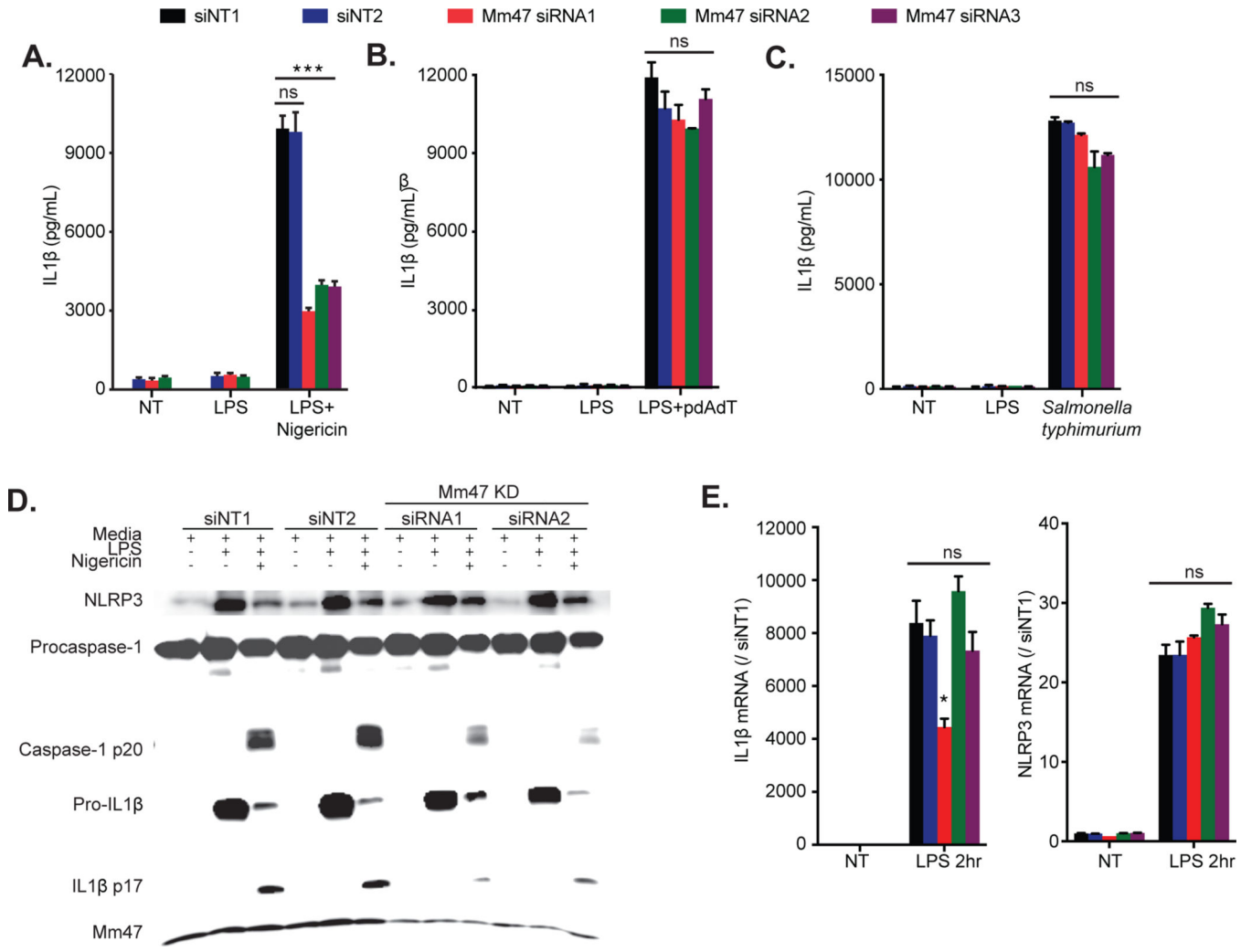

Figure 6: Mm47-deficient BMDM have impaired Nlrp3 pathway

a) ELISA testing the secreted IL1β levels upon priming with 100ng/mL LPS and treatment with 5 uM Nigericin in non-targeted or siRNA-mediated knock down of Mm47 in primary BMDM. Two nontargeting lines (NT1 and NT2) were used as control and three independent siRNA targeting Mm47 was used.

b) ELISA testing AIM2 receptor-mediated secretion of IL1β in primary BMDM with or without Mm47 knock down upon LPS priming followed by 2ug Poly(dA:dT) transfection.

c) ELISA testing NLRC4 receptor-mediated IL1β secretion in primary BMDM with or without Mm47 knock down upon *Salmonella typhimurium* infection for 6 hours.

d) Mm47 was knocked down using siRNA or non-targeting siRNA in primary BMDM cells. Cells were challenged as indicated with and Western bot was performed to pro-caspase-1, cleaved caspase-1, pro-IL1β and cleaved IL1β. Representative images from three independent experiments is shown.

e) qRT-PCR showing mRNA levels of IL1β and Nlrp3 after priming primary BMDM with 100ng/mL LPS for 2 hours with or witout Mm47 knock down.

a-d) Data represents mean ±SEM of three experiments. *p<0.03; **p<0.002; ***p<0.001; ns, not significant.