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The Role of Mitochondria in Macrophage Functionality

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Physiological Sciences

by

Matthew Quang Do

2023

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2023

ABSTRACT OF THE THESIS

The Role of Mitochondria in Macrophage Functionality

by

Matthew Quang Do

Master of Science in Physiological Science

University of California, Los Angeles, 2023

Professor Sonal Srikanth, Co-Chair

Professor Rachelle Hope Crosbie, Co-Chair

Using hybrid mouse diversity panel, our laboratory uncovered a mitochondrial membrane protein TMEM11 as a regulator of Th1 cell effector function. My research focused on examining the role of TMEM11 in macrophages. Prior research has shown that the loss of PMI in *Drosophila* results in morphological changes in mitochondria causing changes in mitochondrial inner membrane cristae architecture and cellular respiration resulting in decreased ATP production. Using mouse primary bone marrow derived macrophages (BMDMs), we were able to demonstrate that the loss of TMEM11 does not affect the ability for macrophages to polarize and does not affect the expression of key mitochondrial membrane structural proteins including MIC60 and

MIC10. Moreover, the loss of TMEM11 does not affect the expression levels of ETC complex proteins which is different from that observed in *Drosophila*. TLR stimulation-mediated cytokine response was normal in Tmem11$^{-/-}$ BMDMs. However, when infected with RNA viruses, vesicular stomatitis virus (VSV) and Influenza A virus (IAV), Tmem11$^{-/-}$ BMDMs showed reduced activation of the NLRP3 inflammasome, thereby less production of cytokines IL-1β and IL-18. Activation of the NLRP3 inflammasome ultimately leads to pyroptosis, which was reduced in Tmem11$^{-/-}$ BMDMs. Altogether, the loss of TMEM11 impairs activation of the NLRP3 inflammasome upon RNA virus infection.

The thesis of Matthew Quang Do is approved.

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2023

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Introduction/Background:

The immune system plays a vital role in the survivability of any organism; this survivability is thanks to the overwhelming complexity of the immune system and its capability to protect the body against a large number of microbes. Therefore, there is a strong motivation to not only uncover how the immune system works, in its entirety, but also to see what can interrupt it. This level of insight can help answer questions regarding 'how microbes avoid detection by our immune system?' or 'how microbes disrupt the immune system from functioning properly?' or even 'how microbes are able to turn the immune system against a host?' However, before questions like these can be answered, a more fundamental understanding of how the immune system, and the cells that play large roles within the system, is required.

There are two major branches of the immune response, innate immunity, and adaptive immunity. Although equally important, there are key differences between the two. Some major differences being the cell types involved in each type of immune response and the timing/onset of the immune response. To describe the overall immune response whenever a foreign microbe enters the body, the first defense by the immune system is the innate immune response; from here, if required, the innate immune cells will recruit and activate adaptive immune cells to initiate an adaptive immune response. Generally, the innate immune response is broad, while the adaptive immune response has more specificity. Since the innate immune cells respond to a numerous amount of varying microbes, understanding how innate immune cells function is important to understand how the immune system is able to defend against so many microbes without the host ever knowing.

Macrophages:

Macrophages are a type of innate immune cells that play a major role in the innate immune response and are the first line of defense against pathogens/microbes. When a pathogen, such as bacteria, enters the body, macrophages are among the first cells to not only recognize but also respond. Their ability to detect a wide array of pathogens is due to their high expression of pattern recognition receptors (PRRs), which identify specific and conserved molecular patterns associated with pathogens. Once macrophages detect a pathogen, they initiate a response to eliminate it. Macrophages can perform various functions, including phagocytosis, antigen presentation, and cytokine production (1): Phagocytosis, which is when macrophages engulf and digest a pathogen (2); Antigen presentation, which is a process in which macrophages present parts of the digested pathogen in order to communicate with other cells in the immune system that danger has entered; And cytokine release, which are signaling molecules that can attract other immune cells to the site of infection and help coordinate a more robust immune response, i.e inflammation.

Polarization:

Another function of macrophages is their ability to polarize, a process by which macrophages can adapt their function in response to different signals in their environment (3). Depending on the signals they receive, macrophages can polarize towards one of two different activation states, which are called M1 and M2 (3). M1 macrophages are activated in response to pro-inflammatory signals; M1 macrophages are important for the elimination of pathogens and most importantly, the initiation of the immune response. They are capable of producing pro-inflammatory cytokines and

chemokines that attract, recruit, and activate other immune cells at the site of the infection. M1 macrophages also have enhanced phagocytic and antigen-presenting abilities, which is important for recognizing and eliminating pathogens. M2 macrophages, on the other hand, are activated in response to anti-inflammatory signals. M2 macrophages are most important for repairing, remodeling, and regulating/modulating the immune response. They can produce anti-inflammatory cytokines and growth factors that contribute to their listed functions. M2 macrophages are also involved in the resolution of inflammation, as they can help to dampen the immune response and prevent tissue damage.

Inflammation:

Inflammation is a complex response of the immune system to many harmful stimuli such as infections. The purpose of inflammation is to mobilize cells in the affected area and recruit immune cells circulating through the blood to remove whatever is causing the harmful stimuli. Inflammation is characterized by the release of various proinflammatory cytokines, chemokines, and other mediators, which can recruit and activate immune cells to the site of injury or infection. Macrophages play an essential role in inflammation. Macrophages are activated by the recognition of pathogens through various PRRs such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Upon activation, macrophages will produce a range of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-18 (IL-18), and interleukin-6 (IL-6), that promote inflammation and recruit other immune cells to the site of inflammation (4).

There are multiple pathways in which macrophages can initiate inflammation, described above is what is considered the classical or canonical pathway, where pathogens are recognized by PRRs on the plasma membrane of macrophages which start a cascade of signals leading to cytokine release. Other pathways include recognition of pathogens that have been phagocytosed through expression of intracellular receptors. Although these pathways can result in inflammation, they can differ in many ways such as: the initial stimuli; the proteins/molecules involved in the cascade (i.e activation of transcription factors or inflammasomes); whether or not they result in pyroptosis.

Pyroptosis:

Pyroptosis is characterized by the formation of large pores in the cell membrane, which leads to cell swelling and lysis (5). This process is triggered by the activation of inflammasomes, such as NLRP3. The activation of inflammasomes leads to the cleavage and activation of Caspase-1, which in turn cleaves Gasdermin D, a protein that forms pores in the cell membrane. The release of intracellular contents during pyroptosis, such as IL-1β and IL-18, triggers an inflammatory response, which is important for clearing infections. Pyroptosis is a key process that macrophages undergo in order to amplify the immune response. Some are even trying to use induced pyroptosis as a form of treatment or therapy for diseases such as cancer (6).

NLRP3:

NLRP3, also known as NOD-like receptor family pyrin domain-containing protein 3, is a protein that plays a crucial role in the immune system's response to infection and inflammation. It is a member a family of intracellular PRRs, which are responsible for recognizing a variety of potential signals, i.e pathogens or damaged cells. When NLRP3

detects a signal, it triggers the assembly of a complex called the inflammasome. This inflammasome leads to the activation of Caspase-1 and the subsequent activation and release of pro-inflammatory cytokines, such as IL-1β and IL-18 (5). It has been shown that dysfunctions in the activation of NLRP3 is one of the causes for many inflammatory disorders, such as atherosclerosis, diabetes, and potentially Alzheimer's disease (7).

Caspase-1 and Gasdermin D (GSDMD):

Caspase-1 is a protease enzyme that plays a critical role in the inflammatory response and cell death processes, including pyroptosis. Caspase-1 starts as an inactive precursor, pro-caspase-1, which is activated after inflammasome formation. Specifically, the inflammasome assembly involves NLRP3, the adaptor protein ASC, and pro-caspase-1. After the inflammasome complex is formed caspase-1 is activated and cleaves pro-inflammatory cytokines precursors, i.e pro-IL-1β and pro-IL-18, into their active forms, IL-1β and IL-18 (8). As mentioned earlier, caspase-1 is also involved in the induction of pyroptosis. Activated caspase-1 is able to also cleave GSDMD, a protein that forms pores in the cell membrane, leading to the release of inflammatory mediators and ultimately causing cell death (9).

Transmembrane Protein 11:

Transmembrane Protein 11 (TMEM11) is a transmembrane mitochondrial protein that helps with the architecture of the inner membrane folds. Its potential importance and function have primarily been studied in the *Drosophila* ortholog, PMI. PMI is a gene found in *Drosophila* that encodes a protein involved in mitochondrial architecture. Specifically, PMI has been found to play a role in the assembly and stability of the mitochondrial respiratory chain, which is responsible for generating ATP, the primary

energy source of the cell (10). Mutations in the PMI gene have been shown to affect mitochondrial function in *Drosophila*, leading to defects in oxidative phosphorylation and reduced ATP production (10). In PMI deficient flies, the morphology of the mitochondria is abnormal (round and circular) and because of this change in shape, performance, especially metabolic performance of these abnormal mitochondria is significantly reduced compared to wild-type flies (11). These defects can cause a range of phenotypes, including reduced lifespan, and increased susceptibility to oxidative stress (10).

The reason why PMI mutations have these dramatic effects on mitochondrial functionality is because the protein interacts with the MICOS complex in order to help organize and structure the cristae, the inner folds, of the mitochondria (12). The MICOS complex, also known as the Mitochondrial Contact Site and Cristae Organizing System, is a large protein complex located in the inner mitochondrial membrane. Its primary function is to help organize the mitochondrial cristae, the folded structures of the inner membrane that are important for efficient energy production (13). The MICOS complex is composed of several subunits, including Mic10, Mic12, Mic19 (14), Mic25, and Mic60 (also known as Mitofilin). Mic60 is the central subunit and forms the core of the complex. Other subunits are arranged around Mic60 in a specific manner to give the complex its overall shape and function. The MICOS complex has several important roles in mitochondrial biology. It helps to stabilize the cristae and maintain their shape, which is important for the efficient function of the electron transport chain and ATP synthesis (12). It also plays a role in regulating mitochondrial dynamics, including

mitochondrial fusion and fission, and helps to mediate interactions between the mitochondria and other cellular structures, such as the endoplasmic reticulum (15,16). Since the loss of PMI has drastic effects on cellular metabolism, and since it has been shown that TMEM11, like PMI, interacts with Mic60, a key mitochondrial architecture protein (17), TMEM11 is potentially a great new tool to use as way to understand mitochondria under a different scope.

Mitochondria and Relationship with Macrophage Functionality:

Mitochondria are important for polarization of macrophages since metabolism or rather energy production is critical for polarization towards M2 (18). It has been shown that after macrophages polarize towards M1, mitochondria oxidative phosphorylation is inhibited, preventing any conversion towards M2. Furthermore, it has been shown that dysfunctions in mitochondria function push macrophage polarization towards M1 since dysfunctional mitochondria are unable to provide the energy required to maintain a M2 phenotype (19).

Through direct interaction or indirect signaling, mitochondria and mitochondrial dynamics play a role in secretion and/or activation of cytokines and inflammasomes (20). A direct interaction that involves cytokine secretion/production is the antiviral signaling protein located on the outer membrane of the mitochondria or MAVs. However, the role of MAVs will be discussed later in this paper. Indirect interactions potentially involve the role mitochondria play in ROS production, which can activate a cascade resulting in cytokine release (21, 22). Since energy production is important for any cell, mitochondria also regulate macrophage biology and any other functions that require a stable energy source, such as phagocytosis or even antigen presentation (23).

Mitochondrial Antiviral-Signaling Protein (MAVs):

MAVs stands for Mitochondrial Antiviral Signaling protein, which is a key component of the innate immune system that helps defend against viral infections. MAVS is located on the outer membrane of mitochondria and serves as a signaling hub that triggers antiviral responses upon detection of viral RNA (24). When a virus infects a cell, its RNA is detected by various sensors, which then bind to MAVs and trigger a signaling cascade that activates transcription factor or inflammasomes (e.g IRF3 and NF-κB or NLRP3) (25, 26). These transcription factors induce the expression of antiviral genes, such as type I interferons (IFNs) and other pro-inflammatory cytokines, which help to clear the virus and prevent its spread to other cells. MAVs is a critical component of the antiviral response and mutations or dysregulation of MAVs have been linked to various viral infections, including hepatitis C virus (HCV), influenza A virus (IAV), and West Nile virus (WNV) (27). Therefore, understanding the mechanisms by which MAVs functions in the antiviral response is important for developing potential new therapies for viral infections.

Results:

Loss of TMEM11 changes mitochondrial morphology in BMDMs

Using TMEM11 deficient mice **(Figure 1a-1c)** we were able to obtain and culture BMDMs. In the *Drosophila* model that lacks the TMEM11 ortholog, PMI, the most striking phenotype was the loss of mitochondrial structural integrity which resulted in mitochondria having an abnormal shape; being observed as round and circular rather than tubular and elongated (10). Therefore, to understand how the loss of TMEM11 affects mammalian cells we needed to first confirm whether or not mitochondrial morphology was affected. Using confocal imaging, we were able to show that the morphology of Tmem11$^{-/-}$ BMDMs mitochondria were abnormal, with a phenotype similar to ones described in prior research in *Drosophila* and other mammalian cells (10,28) **(Figure 1d)**. Next, we investigated whether or not this change in mitochondrial morphology has any effect on the function of macrophages.

Expression of key mitochondrial architecture proteins are unaffected by the loss of TMEM11

One of the largest factors that played a role in why the phenotype of the *Drosophila* model was so heavily affected by the loss of TMEM11 (or PMI) was that the internal cristae structure was lost or rather the MICOS complex was negatively affected (17). We performed immunoblots for two key MICOS complex proteins critical for cristae formation of the inner mitochondrial membrane, MIC60 and MIC10 **(Figure 2a)**. The loss of TMEM11 did not affect the expression levels of MIC60 or MIC10 which is different from that observed in *Drosophila*. Furthermore, the electron transport chain (ETC), sits within the cristae of the inner mitochondrial membrane and so we also

performed an immunoblot for the ETC complex **(Figure 2b)**. Similar to the expression of key MICOS complex proteins, expression levels of ETC complex proteins were unaffected by the loss of TMEM11. This is also different from observations with *Drosophila* lacking expression of *PMI*. Together these results demonstrate that although the morphology of the mitochondria in *TMEM11*$^{-/-}$ BMDMs is abnormal, similar to that observed in the *Drosophila* mutant, unlike the *Drosophila* mutant, expression of key mitochondrial proteins are unaffected by the loss of TMEM11.

Macrophage polarization is unaffected by the loss of TMEM11

To furthermore explore the role these abnormal mitochondria on the function of macrophages we sought out to check other known functions of macrophages that involve mitochondria. One function of the macrophage that has been shown to be linked to mitochondrial function is the ability for macrophages to polarize. By culturing the BMDMs and then stimulating them to stay as M0 (naive) or polarize into M1 or M2 we were able to observe whether or not the loss of TMEM11 had any effect on polarization. We performed qPCR analysis for Tnf/Il-12p40 and Arg1, which are key markers for either M1 or M2 macrophages respectively **(Figure 3a-3c)**. Relative expression of these markers were similar between control and Tmem11$^{-/-}$ BMDMs and therefore polarization does not seem to be affected by the loss of TMEM11.

Canonical NLRP3 inflammasome activation is normal in TMEM11 deficient macrophages after TLR stimulation

NLRP3 inflammasome is activated in response to various microbes, virus and particulate matters in macrophages (25, 26). A two-signal model has been proposed for canonical NLRP3 inflammasome activation. The first signal (priming) is provided by

microbial stimuli, including lipopolysaccharide (LPS), that induces NLRP3, pro-IL-1β, and pro-IL18 expression through activation of NF-κB (25, 26). The second signal (activation) is triggered by extracellular ATP, pore-forming toxins, or particulate matter. We stimulated macrophages using LPS with either ATP or Nigericin, to examine activation of the NLRP3 inflammasome, as judged by secretion of IL-1β and IL-18 **(Figure 4a-4b)**. In both methods of activation, through TLR4 stimulation, the ability for BMDMs to activate the canonical NLRP3 inflammasome is unaffected by the loss of TMEM11.

Tmem11$^{-/-}$ BMDMs cannot induce pyroptosis after RNA virus infection

Next, we checked response of *Tmem11$^{-/-}$* BMDMs to RNA virus infections. The first virus we used for infection was VSV. After stimulation with VSV for 5 hours we stained the cells with Sytox Green in order to observe pyroptotic cell death **(Figure 5a)**. What was observed was that there were significantly less Sytox Green stained cells in Tmem11$^{-/-}$ BMDMs **(Figure 5b)**. To validate these results, we infected Tmem11$^{-/-}$ BMDMs with another RNA virus, IAV, and stained once again with Sytox Green **(Figure 5c)**. After IAV infection, it was also observed that there were significantly less Sytox Green stained cells in the Tmem11$^{-/-}$ BMDMs **(Figure 5d)**. In both cases of VSV and IAV infection, *Tmem11$^{-/-}$* BMDMs were more resistant to virus-infection induced pyroptosis, which is mediated by the NLRP3 inflammasome.

NLRP3 inflammasome activation-induced cytokine secretion is significantly reduced in TMEM11 deficient macrophages after RNA virus infection.

Activation of the NLRP3 inflammasome induces secretion of IL-1β and IL18 cytokines. Since VSV infection showed a stronger effect on cellular death, we first checked

cytokine secretion levels of IL-1β and IL-18 after VSV infection at varying MOIs **(Figure 6a)**. We observed that cytokine secretion was significantly reduced in *Tmem11*$^{-/-}$BMDMs after VSV infection when compared to control. Therefore, we also checked cytokine secretion after IAV infection **(Figure 6b)**. As seen in VSV, after IAV infection, proinflammatory cytokine secretion was significantly reduced in *Tmem11*$^{-/-}$ BMDMs. This interesting observation led us to hypothesize that the form of cellular death that the *Tmem11*$^{-/-}$ BMDMs were resistant to was inflammatory based and therefore was most likely pyroptosis.

TMEM11 deficient macrophages are resistant to pyroptosis after RNA virus infections

To check whether pyroptosis is affected in *Tmem11*$^{-/-}$ BMDMs we performed immunoblots for key proteins involved in activation of pyroptosis: Gasdermin D (GSDMD) and Caspase-1, specifically their cleaved/activated forms. We checked expression levels of these two key proteins in Tmem11$^{-/-}$ BMDMs after either VSV or IAV infection **(Figure 7a-7b)**. In both situations, expression levels of Cleaved Caspase-1 and Cleaved GSDMD were reduced, confirming our assumption that after RNA virus infection *Tmem11*$^{-/-}$ BMDMs are resistant to pyroptosis. As a control we checked expression of these proteins under TLR4 stimulation, which was previously shown to be unaffected by the loss of TMEM11 **(Figure 7c)**. Expression levels of cleaved GSDMD and Cleaved Caspase-1 were relatively similar between control and Tmem11$^{-/-}$ BMDMs after LPS+ATP treatment. This data suggest that altered mitochondrial morphology due to loss of TMEM11 impairs activation of the NLRP3 inflammasome only after RNA virus infection and not after LPS+ATP treatment.

Discussion:

One of the most important factors regarding the loss of TMEM11 in murine macrophages was whether or not mitochondria morphology was altered/affected. Again, this is because previous research using *Drosophila* deficient in PMI, the TMEM11 ortholog, showed significantly altered mitochondrial function, which resulted in phenotypes observed such as a reduced lifespan, increased ROS production, and decreased ATP production (10). To validate whether TMEM11 was a good tool to be used for a mitochondrial dysfunction model we needed to confirm that the morphology was affected. We were able to show that in Tmem11$^{-/-}$ BMDMs, mitochondria were abnormal and therefore TMEM11 could be potentially used as a tool to understand the role mitochondria play in macrophage functionality. However, we were able to understand that the loss of TMEM11 in murine cells may not be as drastic as seen in *Drosophila* since we observed that the expression of key mitochondrial architecture proteins was unaffected by the loss of TMEM11, which was different from the observations in the *Drosophila* model (17). Hence we examined functions of *Tmem11*$^{-/-}$ BMDMs. We show that polarization of macrophages is unaffected by the loss of TMEM11. This was intriguing since it has been previously shown that dysfunctions in mitochondria result in the push towards proinflammatory, or M1 polarization (29). Or rather, dysfunction in mitochondria causes the inability for macrophages to polarize towards M2 since it is more reliant on mitochondrial oxidative phosphorylation as an energy source (30-32). The fact that polarization was unaffected by the loss of TMEM11 potentially demonstrates that even though the morphology of the mitochondria is significantly altered, respiratory capabilities may be unaffected.

Detecting and responding to microbes through PRRs is a key function of macrophages and part of the immune response. Specifically, cytokine secretion has been shown to be tied to proper mitochondria function (22). And so we checked response of *Tmem11*$^{-/-}$ BMDMs to virus infection. Since TMEM11 affects the morphology of mitochondria we infected macrophages with RNA viruses. RNA detection is mediated by RIG-I and MDA-5 in the cytoplasm, which then bind to the adaptor protein MAVS located on the mitochondrial membrane (33). After VSV and IAV viral infection, there was a significant reduction in the proinflammatory response of Tmem11$^{-/-}$ BMDMs. Interestingly, we did not observe any difference in canonical NLRP3 inflammasome activation using LPS+ATP and LPS+Nigercin stimulation in Tmem11$^{-/-}$ BMDMs. However, secretion of these cytokines after RNA virus infection was impaired in *Tmem11*$^{-/-}$ BMDMs, suggesting specific requirement of mitochondrial morphology/architecture in activaiton of the NLRP3 inflammasome upon RNA virus infection.

Normally, pyroptosis is induced by the cleavage of GSDMD by cleaved Caspase-1 (active Caspase-1) which results in pore formation. This GSDMD-mediated cell death is coupled with the release of proinflammatory cytokines such as IL-1beta and IL-18 resulting in pyroptosis being a proinflammatory programed-cell death (9). We showed that cleavage of Caspase-1 and GSDMD was reduced after RNA virus infection; suggesting that some part of this pathway is malfunctioning resulting in reduced immune response and a resistance to pyroptosis. Our developing hypotheses are: 1. due to the altered morphology of the mitochondria, RNA viral detection by the MAVs complex may potentially be affected or 2. due to the mitochondrial morphological

changes, the MAVs complex is unable to recruit and activate the inflammasome NLRP3, which is key to initiating an inflammatory response and initiating pyroptosis (4). At the moment the loss of TMEM11 does not seem to have as drastic effects on the phenotype of BMDMs or even the mouse itself as previously seen in *Drosophila*, however, the loss of TMEM11 in BMDMs seems to affect a very important function of these immune cells which is the response to RNA virus infections, specifically VSV and IAV.

Future Direction/Further Experiments:

There are still many experiments we want to conduct to further investigate how the loss of TMEM11 affects mitochondrial function and by extension macrophage function. Other experiments we will do is check the cellular respiratory capacity of Tmem11$^{-/-}$ BMDMs since that was a key factor in the *Drosophila* model. Similarly, we will also check ROS levels. One aspect we have started to but have not completed is how specific the resistance to pyroptosis that we observed is when infecting with other viruses. It is interesting since the two viruses used, VSV and IAV, are both negative single-stranded RNA viruses. We will be infecting TMEM11 deficient BMDMs with DNA viruses, such as HSV, and positive single-stranded viruses, such as CHIK and SINV. After these results we will be able to better understand if our observations are specific to a certain type of virus or not. We will also check if activation of type I interferons downstream of MAVs activation is influenced due to loss of TMEM11. Furthermore, other aspects we are trying to observe is what part of the pathway of the initiation of pyroptosis is being affected. We have performed immunoblots for downstream proteins such as GSDMD and Caspase-1 but since our hypotheses are that the altered morphology of the

mitochondria may be affecting either MAVs ability to detect the virus or MAVs ability to recruit and activate NLRP3 we are currently running immunoblots to see if expression of any of these upstream proteins are affected by the loss of TMEM11. Furthermore, we are checking whether, after RNA viral infection, NLRP3 and MAVs co-localize. All of these experiments will give us mechanistic insights underlying the role of mitochondrial morphology in activation of the NLRP3 inflammasome in response to RNA virus infections.

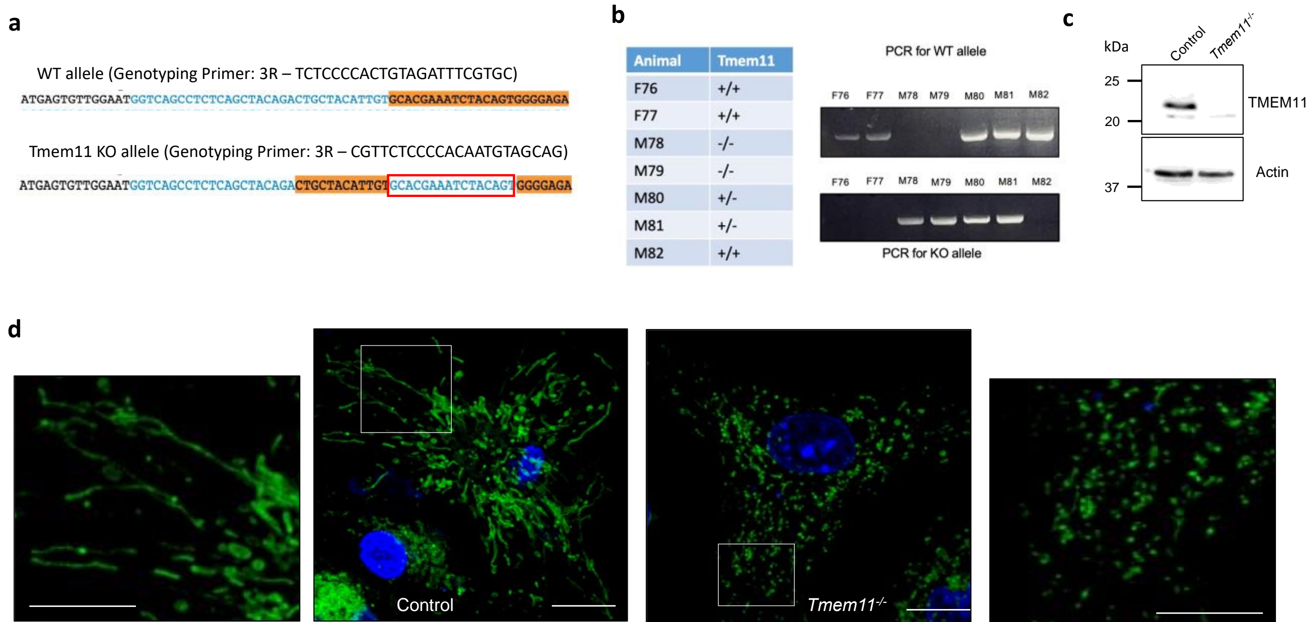
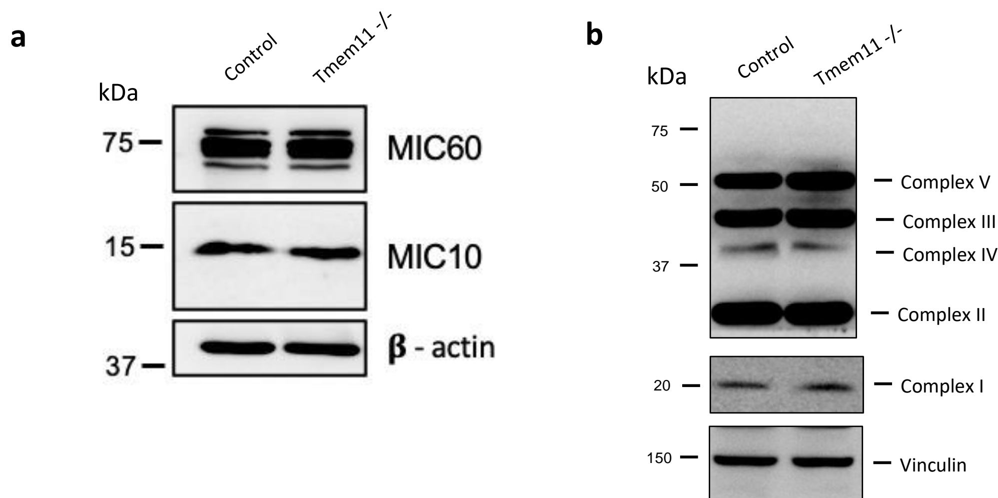
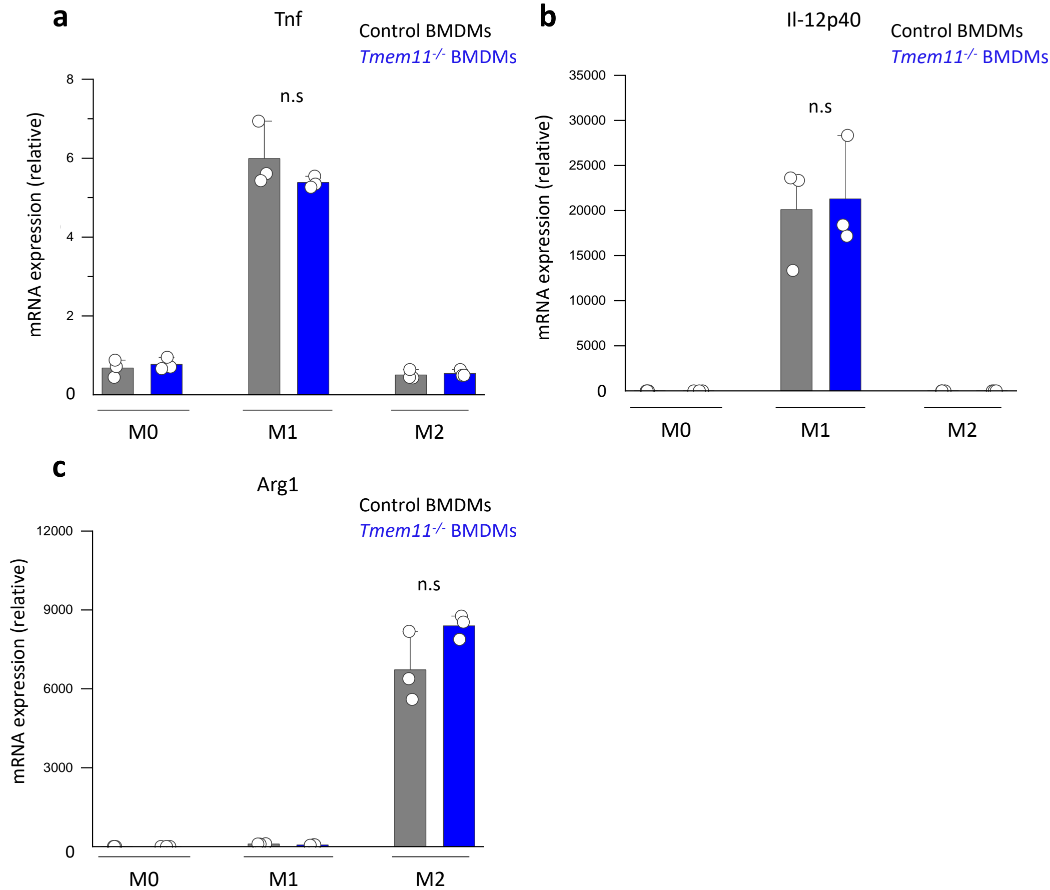


Figure 1: Loss of TMEM11 results in morphological differences in BMDMs. | **a.** Sequence of TMEM11 genomic region showing location of genotyping primers. TMEM11 knock out results in deletion of 16 nucleotides, in red box, from the coding region and a premature stop codon. The 3' reverse primer, in orange, spans the deleted region and hence the PCR would work in the presence of the knockout allele. The common 5' forward sequence used is in black. **b.** PCR and genotype of seven different mice to show deletion and efficiency of primers. **c.** Representative immunoblot showing expression of TMEM11 in WT and Tmem11$^{-/-}$ BMDMs. **d.** Representative confocal images showing mitochondrial morphology in control and Tmem11$^{-/-}$ BMDMs. Green – mitotracker green, blue- DAPI.

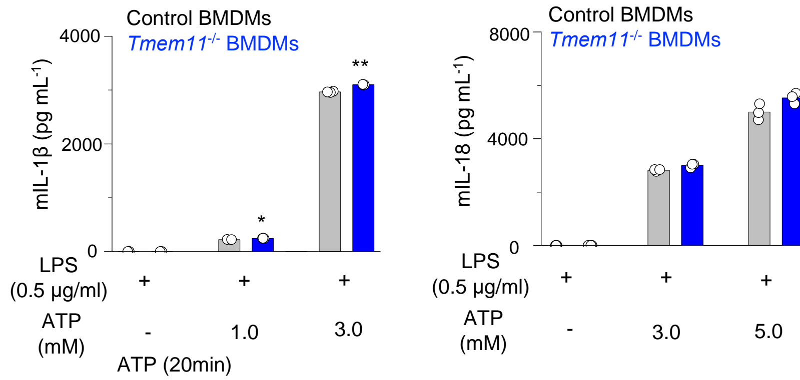


<u>Figure 2:</u> Expression of MIC60, MIC10, and Electron Transport Complex (ETC) proteins are unaffected by the loss of TMEM11. | **a.** Representative immunoblot showing expression of MIC60 and MIC10 in WT and Tmem11$^{-/-}$ BMDMs. **b.** Representative immunoblot showing expression of ETC proteins in WT and Tmem11$^{-/-}$ BMDMs.



<u>Figure 3:</u> BMDM polarization is unaffected by the loss of TMEM11. | **a-b.** qPCR analysis of TNF and IL-12p40, respectively, after WT and Tmem11$^{-/-}$ BMDMs were stimulated with 20 ng/ml IFN-γ + 1 μg/ml LPS to polarize into M1. **c.** qPCR analysis of Arg1 after WT and Tmem11$^{-/-}$ BMDMs were stimulated with 20 ng/ml IL-4 to polarize into M2. *P < 0.05, **P < 0.005, ***P < 0.0005 (unpaired/two tailed t-test for **a-c**).

a



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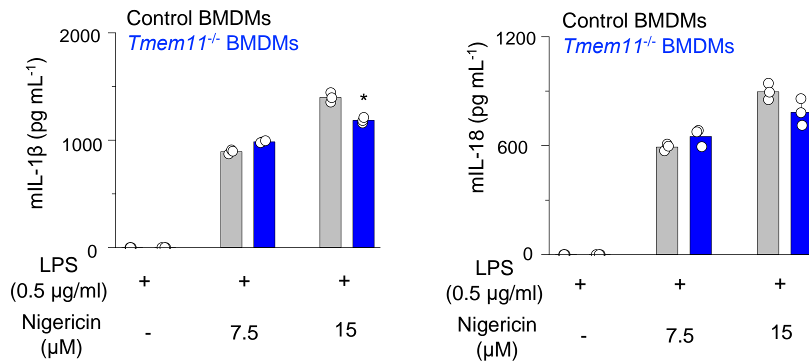
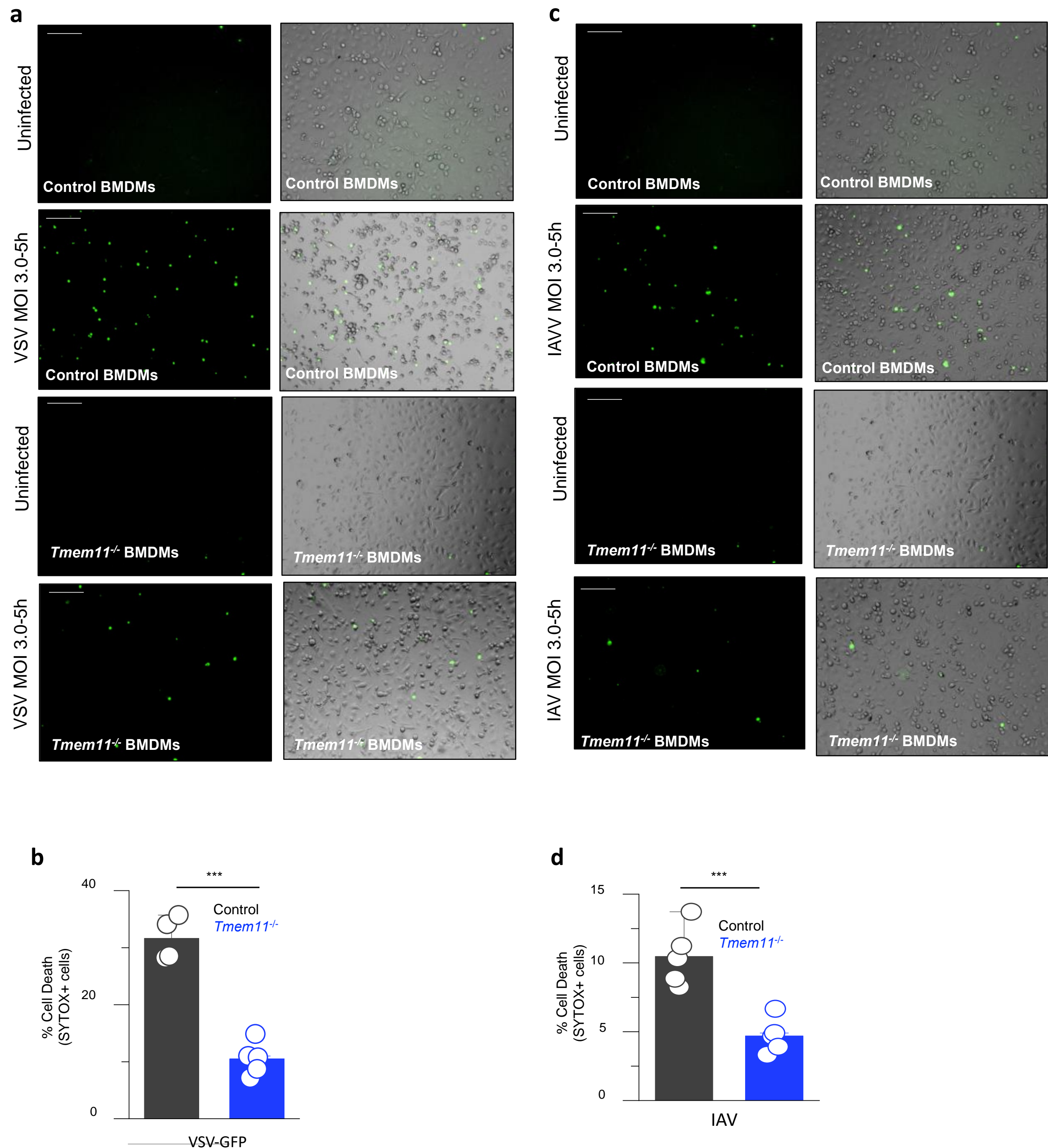


Figure 4: NLRP3 stimulation-induced cytokine production was unaffected by the loss of TMEM11 after LPS+ATP and LPS+Nigericin stimulation. | **a.** ELISA analysis for IL-1β and IL-18 of BMDMs after LPS+ATP stimulation. Cells were pre-treated with LPS (0.5μg/mL) for 3.5 h after which, 0, 1.0mM, or 3.0mM ATP was added for 30 mins **b.** ELISA analysis for IL-1β and IL-18 of BMDMs after LPS+Nigericin: LPS (0.5μg/mL) + 0, 7.5μM, 15μM Nigericin. *P < 0.05, **P < 0.005, ***P < 0.0005 (unpaired/two tailed t-test for **a** and **b**).



<u>Figure 5:</u> TMEM11$^{-/-}$ BMDMs are resistant to cellular death after VSV and IAV infection. | **a.** Representative Sytox Green and Sytox Green + bright field images of WT and Tmem11$^{-/-}$ BMDMs after 5 hours of VSV infection at MOI of 3.0. **b.** Bar graph

depicting quantification of infection in multiple fields after VSV infection. **c.** Representative Sytox Green and Sytox Green + bright field images of WT and $TMEM11^{-/-}$ BMDMs after 5 hours of IAV stimulation at a MOI of 3.0. **d.** Bar graphs depicting quantification of infection in multiple fields after IAV infection. *P < 0.05, **P < 0.005, ***P < 0.0005 (unpaired/two tailed t-test for **b** and **d**).

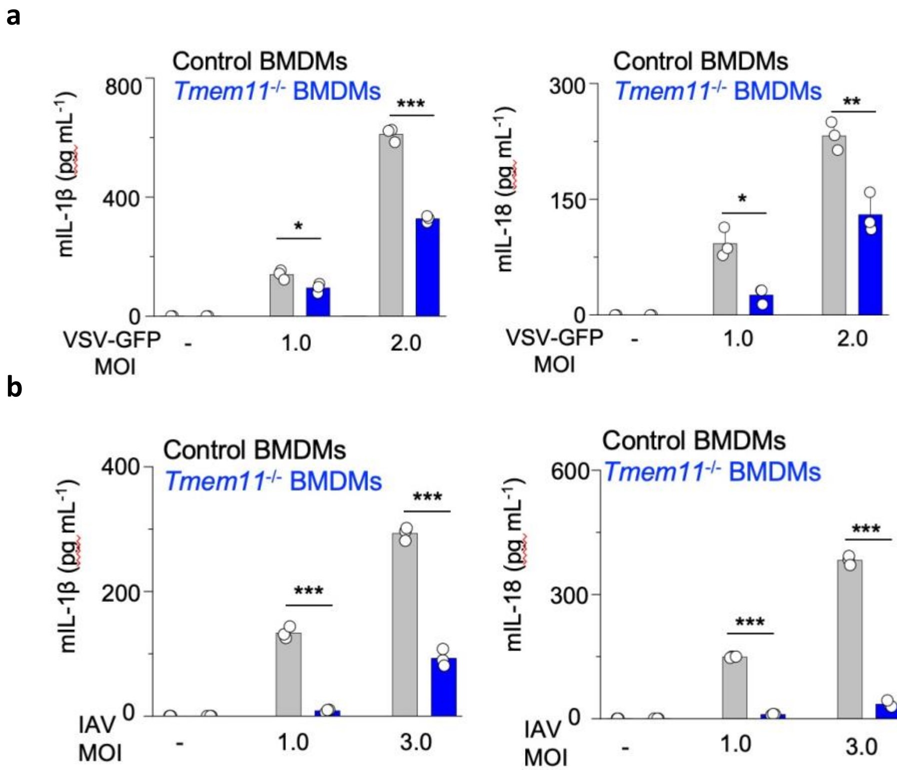
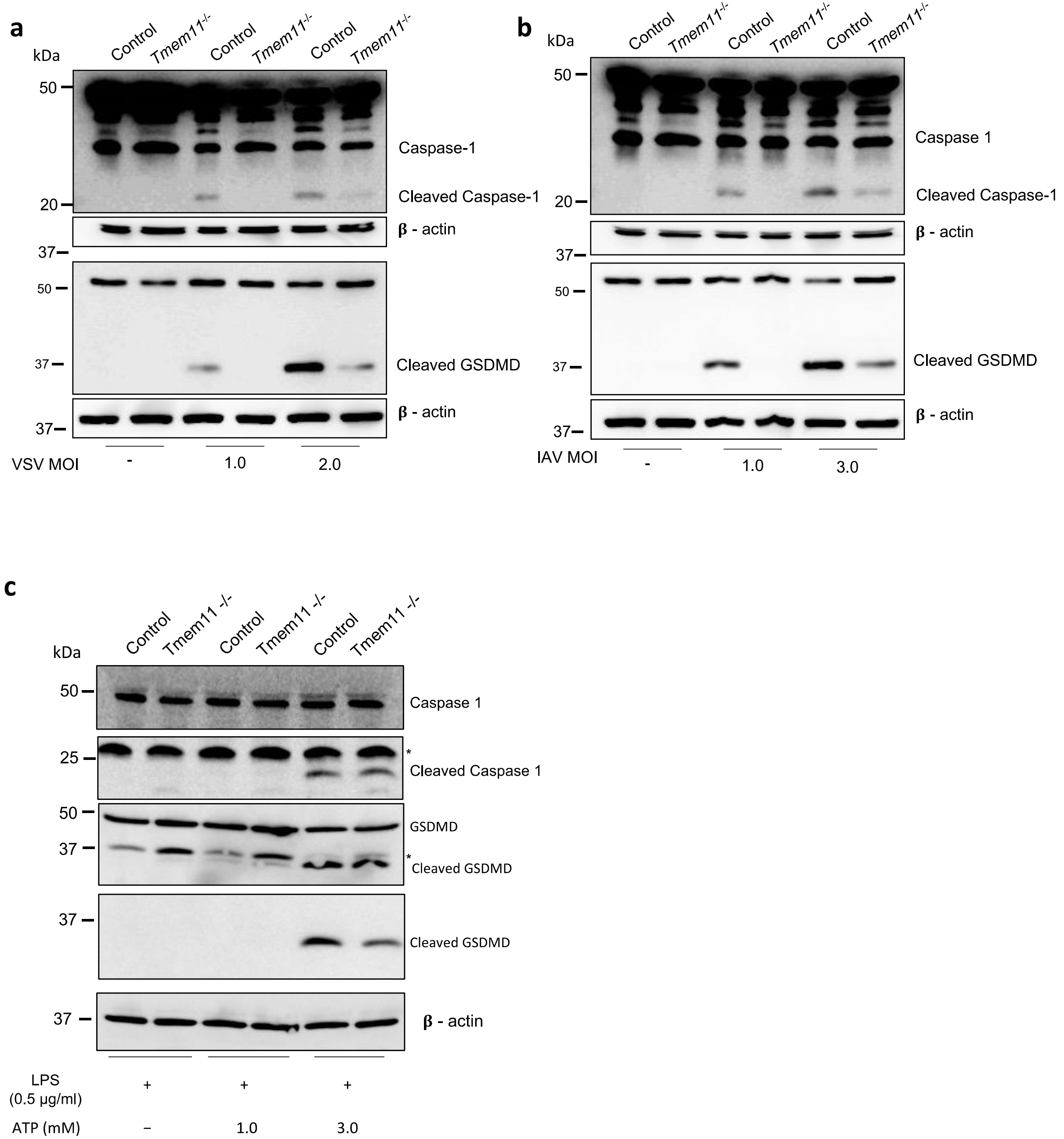


Figure 6: Cytokine production is reduced in TMEM11$^{-/-}$ BMDMs after VSV and IAV infection. | **a.** ELISA analysis for IL-1β and IL-18 of WT and Tmem11$^{-/-}$ BMDMs after 4 hours of VSV infection at MOIs of 1.0 and 3.0 **b.** ELISA analysis for IL-1β and IL-18 of WT and Tmem11$^{-/-}$ BMDMs after 8 hours IAV infection at MOIs of 1.0 and 3.0. *P < 0.05, **P < 0.005, ***P < 0.0005 (unpaired/two tailed t-test for **a** and **b**).



<u>**Figure 7:**</u> **Cleavage of Gasdermin D and Caspase-1 is reduced in TMEM11$^{-/-}$ BMDMs.** | **a.** Representative immunoblot of Caspase-1, cleaved Caspase-1, GSDMD, and cleaved GSDMD for WT and Tmem11$^{-/-}$ BMDMs after VSV infection. **b.** Representative immunoblot of Caspase-1, cleaved Caspase-1, GSDMD, and cleaved

GSDMD for WT and Tmem11$^{-/-}$ BMDMs after IAV infection. **c.** Representative immunoblot of Caspase-1, cleaved Caspase-1, GSDMD, and cleaved GSDMD for WT and Tmem11$^{-/-}$ BMDMs after LPS+ATP treatment: LPS (0.5μg/mL) + 0, 1.0 mM, 3.0 mM ATP for 20 min.

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Materials and Methods: (34, 35, 36)

Bone Marrow Isolation Protocol and Culturing of the BMDMs. For the method of dissecting out bones and flushing out bone marrow refer to the Toda et al, 2020 protocol sheet (34). After bone marrow derived stem cells are obtained, we differentiate them into macrophages in medium (RPMI) with M-CSF for 6 days before usage for experiments (at Day 3 we add 3 mL more medium into the plates).

Chemicals, antibodies, primers. LPS was purchased from InvivoGen. VSV was made in the lab. IAV was made in the lab. Antibody for detection of MitoOxphos Complex (MS604-300) was obtained from Abcam. Antibody for detection of TMEM11 (16564-I-AP) was obtained from Proteintech. Antibody for detection of Caspase-1 (AG-20B-0042-C100) from Adipogen. Antibody for Gasdermin D (EPR19828) was obtained from Abcam. Antibodies for cleaved gasdermin D (Asp276; E3E3P and cleaved caspsase-1 (Asp296; E2G2I) were obtained from Cell Signaling Technology. Antibody for the detection of MIC60 (10179-1-AP) was obtained from Proteintech. Antibody for the detection of MIC10 (C1ORF151) was obtained from Bioss Antibodies. Antibodies for the detection of Actin (C4) and Vinculin (H-10) were obtained from Santa Cruz Biotechnology. Primers for TNF, Arg1, and IL-12p40 were obtained from IDT.

Cytokine measurement by ELISA. Cytokine measurement by ELISA. ELISA was performed on cell culture supernatants from indicated cells collected from cultured BMDMs after a variety of stimulations for detection of IL-18 (ThermoFisher, no. 88-50618-22), and IL-1beta (ThermoFisher, no. 88-7013A-76).

Cell Culture. After cells were differentiated into macrophages cells were plated and stimulated under varying conditions. For treatment with LPS and ATP, BMDMs were

treated with 0.5μg/mL for 3 hours and then treated with ATP (1.0mM or 3.0mM) for 20 minutes. Supernatant was then collected for analysis by ELISA and remaining cells were collected for immunoblots. For treatment with LPS and Nigericin, BMDMs were treated with 0.5μg/mL for 3 hours and then treated with Nigericin (7.5μM and 15μM). Supernatant was then collected for analysis by ELISA and remaining cells were collected for immunoblots. For polarization of macrophages into M1, BMDMs were treated with 20 ng/ml IL-4. BMDMs were harvested in Trizol for qPCR analysis. For polarization of macrophages into M2, BMDMs were treated with 20 ng/ml IFN-g + 1 μg/ml LPS. BMDMs were harvested in Trizol for qPCR analysis.

Confocal and Fluorescence Imaging. Confocal imaging was conducted using the LSM880 confocal microscope. For staining mitochondria, BMDMs were stained with 15nm of MitoTracker Green and with 1ug/mL of Dapi as control. For Sytox Green staining, BMDMs after viral infection were stained with the recommended concentration imaged by fluorescent microscopy.

Immunoblotting. Lysate preparation for immunoblots were different for samples used for Caspase-1 and GSDMD antibody detection. For the protocols used for both samples preparations refer to Tweedell et al, 2020 protocol sheet (35).

Mice. TMEM11$^{-/-}$ animals were purchased from the International Mouse Phenotyping Consortium (colony name TCPR0855_ADGQ).

RNA isolation (kit), cDNA synthesis (script), qPCR (primers). Total RNA from cells collected in TRIzol reagent (ThermoFisher) was isolated using the Direct-zol RNA isolation kit (Zymo Research). RNA quantity and quality were confirmed with a NanoDrop ND-1000 spectrophotometer. cDNA was synthesized using 2–3 μ g of total

RNA using oligo(dT) primers and qScript cDNA SuperMix (Quantabio). Real-time qPCR was performed using PerfeCTa SYBR Green SuperMix (Quantabio)and an iCycler IQ5 system (Bio-Rad) using primers described in the Chemical, antibodies, and primers section of Materials and Methods. Threshold cycles (CT) for all of the candidate genes were normalized to those for 36B4 to obtain Δ CT and further normalized to the values obtained for WT samples to obtain Δ Δ CT (36).

VSV and IAV infection of BMDMs. After differentiation of macrophages is complete, cells are transferred and plated into a 12 well plate and left for 2-3 hours to allow for cell adhesion. The medium is then removed, and cells are washed with PBS at minimum 2 times. Add RPMI (without serum) with respective virus, VSV or IAV, at the desired MOI. Infection with VSV lasted 4 hours and infection with IAV lasted 8 hours. In both cases, after about 2 hours post infection, we add extra medium with double serum to keep the ratio of 10% FBS. After infection is completed, supernatant is collected for ELISA and remaining cells are collected for immunoblots or qPCR.

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