Myxoma virus lacking the Pyrin-like protein M013 is sensed in human myeloid cells by both NLRP3 and multiple TLRs, which independently activate the inflammasome and NF-κB innate response pathways.

Masmudur M. Rahman and Grant McFadden*

Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL 32610 USA.

*Corresponding author:
Grant McFadden
Dept of Molecular Genetics and Microbiology
College of Medicine
University of Florida
1600 SW Archer Road
PO Box 100266
Gainesville FL 32610
Phone: 352-273-6852
Fax: 352-273-6849
Email: grantmcf@ufl.edu

Running title: Sensing of vMyxM013-KO virus in human myeloid cells
Abstract

The myxoma virus (MYXV)-encoded pyrin domain containing protein M013 co-regulates inflammatory responses mediated by both inflammasomes and the NF-κB pathways. Infection of human THP-1 monocytic cells with a MYXV construct deleted for the M013 gene (vMyxM013-KO), but not the parental MYXV, activates both the inflammasome and NF-κB pathways and induces a spectrum of pro-inflammatory cytokines and chemokines, like IL-1β, TNF, IL-6 and MCP-1. Here, we report that vMyxM013-KO virus-mediated activation of inflammasomes and secretion of IL-1β was dependent on the adaptor protein ASC, caspase-1 and NLRP3 receptor. However, vMyxM013-KO virus-mediated activation of NF-κB signaling, which induces TNF secretion, was independent of ASC, caspase-1 and either the NLRP3 or AIM2 inflammasome receptors. We also report that early synthesis of pro-IL1β in response to vMyxM013-KO infection is dependent upon the components of inflammasome complex. Activation of NLRP3 inflammasome and secretion of IL-1β was also dependent on the release of cathepsin B and production of ROS. Using siRNA screening, we have further demonstrated that, among the RIG-I-like receptors and Toll-like receptors, only TLR2, TLR6, TLR7 and TLR9 contribute to the NF-κB-dependent secretion of TNF and the inflammasome-dependent secretion of IL-1β in response to vMyxM013-KO virus infection. Additionally, we demonstrate that early triggering of the MAP kinase pathway by vMyxM013-KO virus infection of THP-1 cells plays a critical common upstream role in the co-ordinate induction of both NF-κB and inflammasome pathways. We conclude that additional cellular sensor(s)/receptor(s) in addition to the known RLRs/TLRs play a role in the M013 knockout virus-induced activation of NF-κB pathway signaling, but the activation of inflammasomes entirely depends on sensing by the NLRP3 receptor in response to vMyxM013-KO infection of human myeloid cells.
**Introduction**

Invading microbial pathogens such as viruses are initially recognized by dedicated cellular receptors called pathogen recognition receptors (PRRs) that trigger the activation of the early pro-inflammatory responses responsible for the establishment of innate and adaptive immune responses. Based on recent findings, the PRRs can be grouped into four main classes: Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and the newly identified Absent In Melanoma-2 (AIM2)-like receptors (ALRs). These receptors sense pathogens or pathogen associated molecular patterns (PAMPs) such as non-self entities, mis-localized nucleic acids or over-expressed glycoproteins, either at the cell surface or within intracellular compartments. For example, exogenous cytosolic dsDNA can be sensed by endosomal TLR9 and/or intracellular ALRs (50), whereas viral envelope glycoproteins are sensed by extracellular TLR4 (36). Recent findings also suggest that the expression patterns and levels of PRRs significantly differ among various cell lineages and tissue types, and these differences govern the receptor specificity functions in a cell-, species- and pathogen-specific manner.

TLRs can sense viruses and virus-derived PAMPs at either the cell surface or within endosomal compartments. Among the TLRs, TLR3, TLR7 and TLR9, which are present in the endosomal compartment, sense both single and double stranded RNA and DNA viruses, whereas TLR2 or TLR4 recognize either DNA or RNA viruses from the cell surface. In some cases, TLRs collaborate with other receptors like the RLRs and NLRs and together they play an important co-ordinate role in triggering innate responses and facilitating adaptive immunity (23). The PAMP-activated TLRs recruit the adaptor proteins MyD88 or TRIF to activate downstream
kinases, including IκB kinase complex (IKK) and IKK-related kinases (TBK1 and IKKε), which then activate the NF-κB transcription factors and IRFs. Indeed, this NF-κB activation pathway is frequently subverted by a wide variety of pathogens (34). Members of the RLR family of RNA helicases, RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) have been shown to be critical for detecting RNA viruses by sensing ssRNAs and dsRNAs in the cytosol (52). RIG-I and MDA5 interact with the CARD domain of mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA and CARDIF) using its N-terminal CARD domain. The main function of RLR activation is the production of type I IFN by activating the IRF3, IRF7 and NF-κB pathways. In addition, RIG-I was recently described as an upstream activator of the inflammasome complex that triggers IL-1β and/or IL-18 production through caspase-1 and caspase-3 activation (33). This activation of caspase-1 initiated by the RIG-I, but not MDA5, was mediated by protein-protein interaction with the pyrin-containing cellular adaptor protein, ASC. This suggests that RIG-I uniquely mediates crosstalk between the type I IFN and inflammasome pathways.

Members of the NLR family of PAMP sensors are the major intracellular receptors involved in the activation of the inflammasome complex (21, 26, 40). To date, the NLR members NLRP1, NLRP3 and NLR family CARD-containing 4 (NLRC4) have been shown to sense viral and other pathogens infections, leading to the activation of the inflammasomes that mediate the cleavage of caspase 1 and thereby trigger release of the pro-inflammatory cytokines IL-1β and IL-18. Although both DNA and RNA viruses, such as the attenuated vaccinia virus (VACV) strain MVA and influenza A, have been shown to activate NLRP3, no direct interaction between NLRP3 and RNA or DNA has yet been established (8, 22). In addition to microbial pathogens, the NLRP3 inflammasome is also activated by cellular DAMPs, such as monosodium
urate(MSU), calcium pyrophosphate dehydrate and ATP, with the common inducer molecule being the reactive oxygen species (ROS) generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (48).

Members of AIM2-like intracellular receptors sense cytosolic DNA from either viruses or bacteria and induce the secretion of type I IFN and other pro-inflammatory mediators. AIM2 is a member of PYHIN protein family (pyrin and HIN200 domain-containing proteins; also known as p200 or HIN200 proteins) and interacts with dsDNA through its HIN domain, which then promotes the assembly of the inflammasome complex via PYD-PYD interactions with the adaptor protein ASC(6, 10, 14, 38). The AIM2 inflammasome plays a critical role in host defenses against some DNA viruses like VACV and murine CMV but not, for example, HSV-1 (11, 37). This suggests that AIM2 is either unable to recognize certain DNA viruses, or more likely, many DNA viruses have evolved mechanisms to suppress and or bypass AIM2 signaling.

Another member of PYHIN protein family, IFI16 has been recently identified as a cytosolic DNA sensor that signals via STING, TBK1 and IRF3 in human cells (49). The murine homolog of IFI16, p204, also acts as a DNA sensor in mouse cells. IFI16 and p204 each interact with STING and trigger TBK1-mediated phosphorylation of IRF3 and activation of NF-κB, which then leads to the induction of IFNα/β expression. However, IFI16 has no direct role in inflammasome activation or in IL-1β production (6, 10, 14). Apart from IFI16, cytosolic DNA is also reported to be sensed by RNA polymerase III and DAI, which then trigger the transcription of genes involved in the innate immune response (7, 46).

Myxoma virus (MYXV) is a prototype member of the leporipoxvirus genus of the Poxviridae family and causes a lethal disease called myxomatosis only in European rabbits (Oryctolagus cuniculus)(44). In most cases, myxomatosis is fatal and is accompanied by the total...
collapse of the rabbit immune system, but the virus is essentially harmless to all non-lagomorphs, such as mice and humans. The extreme virulence of MYXV in European rabbits is mediated by dozens of virus-encoded immune evasion molecules that are directed at numerous host innate and acquired immune pathways. Some of these MYXV-encoded immunomodulators are relatively rabbit-specific, depending on the degree of conservation of the host target pathways across species, but many others are capable of recognizing and inhibiting their targets in non-rabbit cells as well. To date, a wide spectrum of MYXV-encoded immunomodulators have been identified that target diverse host cytokines, host cell signaling pathways, apoptosis and other innate immune pathways (3, 24, 27).

The MYXV-encoded protein M013 is a functional ortholog of the cellular PYRIN domain (PYD) containing protein, and has been shown to be an intracellular inhibitor of both the inflammasome and the NF-κB pathway (18, 35). The cellular PYD proteins are also involved in the regulation of apoptosis, NF-κB activation and pro-inflammatory cytokine production by mediating protein-protein interactions (45). An engineered MYXV construct deleted for the M013 gene (vMyxM013-KO) was significantly attenuated in European rabbits because of decreased virus dissemination and enhanced inflammatory responses at the tissue sites of virus infection (18). The regulation of at least two distinct immune pathways by M013 is mediated by protein-protein interactions with two independent cellular regulators of the inflammasome and NF-κB signaling pathways. Firstly, M013 protein interacts with the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), a key conserved component of the cellular inflammasome complexes, and inhibits caspase-1 activation and the processing of pro-inflammatory cytokines IL-1β and IL-18 (18). Secondly, M013 protein also interacts with NF-κB1/p105, a member of the NF-κB protein family and inhibits the activation of NF-κB by
preventing the degradation of NF-κB1/p105 in response to upstream pro-inflammatory signals (35). Importantly, both of these binding/inhibitory properties of M013 are relatively species nonspecific, and thus M013 is an effective inhibitor of both pathways in human cells. Thus, infection of human myeloid cells (eg THP-1 cells) with vMyxM013-KO virus, but not wild-type MYXV, is rapidly sensed and then induces a variety of pro-inflammatory cytokine responses. However, it is not known which inflammasome sensor becomes activated by vMyxM013-KO infection, or how the NF-κB pathway is activated.

In this study, we report the mechanism(s) of inflammasome and NF-κB activation in human myeloid cells infected by vMyxM013-KO virus. We found that NLRP3, but not AIM2, together with ASC and Caspase-1, is crucial for the activation of cellular inflammasomes and the release of IL-1β by vMyxM013-KO virus. The M013 knockout virus-induced activation of the NLRP3 inflammasome is dependent upon the release of the lysosomal protease, cathepsin B, into the cytoplasm. However, unexpectedly, the activation NF-κB pathway by the M013 knockout virus infection is essentially independent of these inflammasome sensor components. Instead, the activation of NF-κB pathway is dependent on the signaling from several TLR receptors, including TLR-2, -6, -7 and -9. We also demonstrate that MEK-ERK1/2 signaling is essential for the activation of both NF-κB and inflammasomes in response to vMyxM013-KO virus infection.

Results

ASC and caspase-1 are required for vMyxM013-KO mediated release of IL-1β, but not TNF, from infected THP-1 cells

MYXV-encoded PYD containing protein M013 interacts with the adaptor protein ASC of the inflammasome complex and inhibits the activation of inflammasomes and release of pro-
inflammatory cytokines (18). In the absence of functional M013 protein, vMyxM013-KO virus infection of human macrophage-like differentiated THP-1 cells induced inflammasome activation and the cleavage of caspase-1 leading to the secretion of pro-inflammatory cytokines IL-1β and IL-18 (18). We have also demonstrated that vMyxM013-KO virus infection of THP-1 cells also rapidly activates the NF-κB signaling pathway leading to the secretion of NF-κB regulated pro-inflammatory cytokines and chemokines such as TNF, IL-6 and MCP-1 (35). Additionally, we have demonstrated that treatment of THP-1 cells with the caspase-1 inhibitor, zVAD-fmk, inhibited the vMyxM013-KO virus infection induced secretion of IL-1β (35). To investigate if these two cellular pathways (Figure 8) are co-ordinantly triggered by the same cellular sensors in response to infection with the M013-KO MYXV, we first examined the role of the common inflammasome adaptor component ASC in the vMyxM013-KO virus-mediated activation of inflammasome and NF-κB pathways. We observed that silencing ASC in THP-1 cells, using small interfering RNAs (siRNAs), completely abolished IL-1β secretion following either vMyxM013-KO virus or ATP treatment (as control), while no significant inhibition of IL-1β secretion was observed in control untreated cells or those transfected with nontargeting control siRNA (Fig 1A). In contrast, silencing of ASC expression had no significant effect on NF-κB mediated TNF secretion from the same vMyxM013-KO virus infected or LPS treated (as control) THP-1 cells (Fig 1B). To further confirm these results using transient knockdown of ASC, we generated stable knockdown clones of THP-1 cells with lentiviruses expressing shASC or shCaspase-1. The reduced expression of ASC and caspase-1 in the stable knockdown THP-1 cells, compared to wild-type (WT) or cells treated with non-target shRNA (ShControl), was confirmed by Western blot analysis (Fig 1F). When these cells were infected with vMyxM013-KO virus or treated with ATP (as control), they secreted significantly less IL-1β compared to...
WT or shControl THP-1 cells, confirming that vMyxM013-KO virus mediated IL-1β secretion relies on ASC and caspase-1 activation (Fig 1C). However, the secretion of TNF from vMyxM013-KO virus infected shASC or shCaspase-1THP-1 cells was unaffected, suggesting that ASC and caspase-1 have no direct role in the activation of NF-κB pathway (Fig 1D).

Infection of PMA-differentiated THP-1 cells with vMyxM013-KO virus upregulated the synthesis of pro-IL-1β protein and cleavage of pro-caspase-1 very rapidly which resulted in the release of mature IL-1β (Fig 1E, left 1st and 2nd panels). We examined the level of pro-IL-1β synthesis in both shASC and shCaspase-1 THP-1 cells and cleavage of caspase-1 in shASC THP-1 cells by Western blot analysis. The synthesis of pro-IL-1β was significantly delayed by the knockdown of either ASC or caspase-1, compared to the WT or shControl THP-1 cells following infection with vMyxM013-KO virus (Fig 1E). As expected, there was no cleavage of caspase-1 and formation of p20 in response to vMyxM013-KO virus infection in the ASC or caspase-1 knockdown cells compared to the shControl THP1 cells (Fig 1E). However, the level of ASC (Fig 1E) and NLRP3 (data not shown) in the Control and knock down THP-1 cells remain unchanged. These results suggest that components of inflammasome complex ASC and caspase-1 have direct and obligatory roles in the signaling that lead to the rapid induced synthesis of the pro-IL-1β protein precursor needed as substrate for caspase-1.

**NLRP3 is required for activation of inflammasomes in THP-1 cells infected with vMyxvM013-KO**

Among the intracellular PRRs identified so far, the NLRP3 and AIM2 receptors have been reported to sense infection by DNA viruses and activate the inflammasome complexes. We examined whether NLRP3 or AIM2 is involved in sensing MYXV lacking M013 and then signal the activation of the inflammasome complex in THP-1 cells. We transiently silenced the
expression of NLRP3 or AIM2 using respective siRNAs in THP-1 cells and tested the release of various indicator pro-inflammatory cytokines after virus infection. We found that silencing the expression of NLRP3 completely abolished the induced IL-1β secretion following either vMyxM013-KO virus or ATP (as control), while no significant inhibition of IL-1β secretion was observed in cells transfected with AIM2 siRNA or a nontargeting control siRNA (Fig 2A and 2C). The secretion of TNF from the NLRP3 or AIM2 knockdown THP-1 cells after vMyxM013-KO virus infection remained unaffected (Fig 2B and 2D). This observation suggests that NLRP3 receptor is involved in the M013 knockout virus-induced activation of inflammasomes and the release of IL-1β. To further study the role of NLRP3, we next generated stable knockdown of THP-1 cells using lentivirus-expressed shNLRP3 and confirmed the reduced expression of NLRP3 by Western blot analysis (Fig 2H). In these NLRP3 stable knockdown THP-1 cells, secretion of IL-1β was significantly reduced while the secretion of NF-κB-dependent TNF was unaffected (Fig 2E and 2F). We also examined the level of pro-IL-1β synthesis, the cleavage of caspase-1, and the expression of ASC in these shNLRP3 THP-1 cells at various time points after infection. The synthesis of pro-IL-1β was significantly delayed at early time points following infection by vMyxM013-KO virus infection when NLRP3 is knocked down, compared with the WT virus or shControl THP-1 cells (Fig 2G top panel). As expected, in the NLRP3 knockdown cells, there was no cleavage of caspase-1 in response to vMyxM013-KO virus infection and the level of ASC remained unchanged during the tested period of infection (Fig 2G, 2nd and 3rd panels). These results again support the conclusion that the inflammasome complex, together with the receptor NLRP3, plays a key role in the signaling events that leads to the very early synthesis of pro-IL-1β protein in response to vMyxM013-KO virus infection of human myeloid cells.
Cathepsin B and ROS activate the NLRP3 inflammasome in response to vMyxM013-KO infection

Recent studies suggest that the NLRP3 inflammasome is activated by multiple mechanisms, including lysosomal damage and release of cathepsin B from lysosomal compartments (2, 13, 15). To determine whether vMyxM013-KO virus infection mediated release of lysosomal cathepsin B into the cytoplasm might be required for NLRP3 inflammasome activation, PMA differentiated THP-1 cells were treated with increasing concentrations (25-200 µM) of the cathepsin B inhibitor Ca-074me, for 1 hour before infecting with the test virus or treatment with Alum (as control). Release of IL-1β at 6 hours post-infection was inhibited in THP-1 cells pretreated with all the different concentrations of Ca-074me, suggesting that catalytic activity of cathepsin B was required for NLRP3 inflammasome activation by vMyxM013-KO virus (Fig 3A, showing only 100µM concentration of Ca-074me). Treatment with Ca-074me also inhibited the release of TNF from vMyxM013KO -infected THP-1 cells (Fig 3B). This could be due to the role of cathepsin B in the trafficking of TNF containing vesicles to the plasma membrane, which is inhibited by the treatment of Ca-074me, as reported recently (12). This has been supported by the observation that treatment of THP-1 cells with Ca-074me inhibited the activation of NF-κB pathway, as observed by monitoring the degradation of IκBα and cleavage of caspase-1 when tested by Western blot analysis (Fig 3C). Infection of PMA activated THP-1 cells with vMyxM013-KO virus alone or treatment with LPS induced the degradation of IκBα, as reported before and included as control (Fig 3C).

The activation of the NLRP3 inflammasome by numerous stimuli depends on the production of ROS released by the NADPH oxidase. The requirement for ROS in vMyxM013-KO virus infection mediated NLRP3 activation was tested by using NADPH oxidase inhibitor
Diphenyl iodonium bromide (DPI). Release of IL-1β was inhibited in virus-infected THP-1 cells pretreated with DPI in a dose dependent manner, suggesting that ROS is required for activation of NLRP3 inflammasome activation by vMyxM013-KO virus infection (Fig 4A). Treatment with DPI also inhibited the release of TNF from the infected THP-1 cells (Fig 4B), which was reported to be an inhibitor of NF-κB pathway activation (25). The release of ROS by vMyxM013-KO infection was also confirmed by DCF fluorescence assay, and treatment with DPI significantly reduced the level of fluorescence (Fig 4C). Both Ca-074me and DPI did not significantly affect the cell viability during for the time periods tested (data not shown).

**Release of TNF and IL-1β by vMyxM013-KO virus infection of THP-1 cells is independent of RLRs but dependent on MyD88**

We have tested the potential roles of other known pathogen sensing signaling pathways to the rapid activation of NF-κB and inflammasomes in THP-1 cells by vMyxM013KO virus infection. WT MYXV is sensed by RIG-I in human primary macrophages, and this sensing leads to the co-induction of type I interferon and TNF (51), but at present it is not clear why the deletion of M013 is apparently needed in order for MYXV to induce TNF in THP-1 cells. MyD88 is an obligatory adapter molecule for the majority of TLR receptors. In order to assess which signaling pathways might contribute to the activation of NF-κB in response to vMyxM013KO virus infection of THP-1 cells, we have used siRNAs for RIG-I, MDA-5, MAVS, or MyD88 to knockdown the expression of these proteins and tested the level of TNF and IL-1β production. The knockdown of these proteins was confirmed by checking the level of protein expression by Western blotting (Fig 5A). The siRNA transfected THP-1 cells were differentiated with PMA and infected with WT MYXV or vMyxM013-KO viruses or treated with known inducers. Supernatants were collected at 6h post infection/treatment. Our results
demonstrate that knockdown of RIG-I, MDA-5 or MAVS has no effect on secretion of TNF or IL-1β (Fig 5B), suggesting that RIG-I like receptors are not required for TNF secretion from THP-1 cells in response to vMyxM013-KO virus infection. At present, the reason that RIG-I is apparently linked to MYXV-induced TNF secretion in primary human macrophages, but not THP-1 cells, is unknown. We have previously reported that in THP-1 cells, vMyxM013-KO virus infection did not induce the secretion of type I IFN (35), which has been further supported by this observation that RIG-I does not apparently trigger TNF secretion following vMyxM013-KO virus (or WT MYXV) infection of THP-1 cells. Knockdown of a key adaptor protein for TLR signaling pathways (MyD88) significantly reduced the level of TNF and IL-1β secretion from the vMyxM013-KO virus infected THP-1 cells (Fig 5C). This suggests that one or more TLRs have a role in the activation of the NF-κB signaling pathway in THP-1 cells infected with vMyxM013-KO.

Role of TLRs in activation of NF-κB and inflammasomes by vMyxM013-KO in THP-1 cells

TLRs play an important role in early detection of viruses and in the initiation of the antiviral host defense responses (5). TLR-mediated sensing of viruses is mainly linked to the rapid activation of the NF-κB signaling pathway. TLR-mediated activation of the NF-κB pathway also regulates the synthesis of pro-IL1β precursor protein, which is then utilized as substrate for cleavage into mature IL-1β by the inflammasome complex. Several TLRs have been shown to be important for sensing poxviruses, including vaccinia virus and MVA, the attenuated smallpox vaccine candidate (8, 29, 39, 54). In order to test which TLRs might be involved in sensing vMyxM013-KO virus and involved in activation of NF-κB pathway, we have used siRNAs against the all known human TLRs. Among the TLRs, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are present on the cell surface while TLR3, TLR7, TLR8 and TLR9 are present in the...
endosomes. To test which of these TLRs might contribute to the activation of NF-κB pathway, we have used siRNAs for all of them to transiently knockdown the expression of these TLRs in THP-1 cells and tested the level of TNF and IL-1β production in response to vMyxM013-KO virus infection. The siRNA transfected THP-1 cells were differentiated with PMA and infected with WT MYXV (data not shown, as there is no change compared with the mock treatment) or vMyxM013-KO viruses or treated with the known ligand inducers for TLRs. Supernatants were collected at 6h post infection/treatment for ELISA analysis of secreted cytokines. Our results demonstrate that knockdown of TLR2 and TLR6 has significant effects on secretion of both TNF and IL-1β (Fig 6B and D), suggesting that these extracellular TLRs play role in sensing vMyxM013-KO infection and activation of NF-κB signaling. Among the endosomal TLRs, TLR7 and TLR9 knockdown also had significant effects on the secretion of TNF and IL-1β by vMyxM013-KO virus infection in THP-1 cells (Fig 6E and G). The knockdown of representative TLR proteins was confirmed by checking the level of protein expression by Western blotting (Fig 6H).

The requirement of MEK-ERK1/2 for the activation of NF-κB signaling in THP-1 cells

We have previously demonstrated that vMyxM013-KO virus-mediated activation of NF-κB is dependent on the MAP kinase, but not the PI3 kinase, pathway (35). Inhibition of ERK1/2 activation by U0126 inhibited TNF secretion and NF-κB dependent synthesis of pro-IL1β protein. Inhibition of PI3 kinase using LY294002 had no effect on activation of NF-κB pathway. We have further extended this observation and studied the role of ERK1/2 in the absence of ASC, NLRP3 and caspase-1. As we have shown, constitutive knock down of the expression of ASC, NLRP3 or caspase-1 with shRNA in THP-1 cells has no effect on secretion of TNF induced by M013-KO virus infection. We then tested whether inhibition of several key kinases...
would inhibit this TNF secretion response. The cells were pretreated with U0126 or LY294002 for one hour and infected with vMyxM013-KO virus. The supernatants were collected 6 hours after infection and tested for secretion of TNF by ELISA. Treatment of shASC, shNLRP3 and shCaspase-1 THP-1 cells with U0126 totally inhibited the vMyxM013-KO virus-induced secretion of TNF (Fig 7A). The synthesis of pro-IL1β protein was also inhibited, extending out to late time points of infection in these THP-1 cells (data not shown). This is because treatment with U0126 blocked the phosphorylation of ERK1/2 and activation of NF-κB pathway, as observed by the absence of IkBα degradation in response to vMyxM013-KO virus infection (Fig 7B, lanes 1-16 in the top and bottom panels). The PI3 kinase inhibitor LY294002 had no effect on secretion of TNF from these cells as observed with THP-1 cells (Fig 7A). This was further supported by the observation that NF-κB pathway was still activated at early time points by vMyxM013-KO virus infection in the presence of a PI3 kinase inhibitor (Fig 7B, lanes 17-32 in the bottom panels). This suggests that ERK1/2 signaling plays a common central early role in sensing vMyxM013-KO virus infection via multiple TLRs and the NLRP3 inflammasome complex.

**Discussion**

Poxviruses represent a large family of DNA viruses, which replicate exclusively in the host cytoplasm. The members of poxviruses harbor unique sets of immunomodulatory proteins, which are in most cases essential for virus virulence, evasion of host immune responses, and host range functions but which are not required for virus replication in cultured cells (42). The MYXV-encoded PYD-containing protein M013 is required for viral pathogenicity in rabbits, and infection with the M013 knockout virus induces an exacerbated pro-inflammatory response that clears the virus infection (18). At the molecular level, M013 inhibits two crucial innate immune
pathways regulated by NF-κB and the inflammasome complexes in a species pan-specific fashion by virtue of binding independently to NF-κB1/p105 and the ASC adaptor protein (18, 19, 35). The present study provides insights into the mechanisms by which MYXV infection is sensed and activates these two immune pathways in human myeloid cells (Fig 8). Since MYXV replicates as well in many human cancer cells as it does in rabbit cells, but is essentially nonpathogenic to any non-lagomorph host such as mice or humans, it is being developed for cancer virotherapy and thus the specific innate interactions of MYXV with human cells of various lineages is of particular interest.

Here we show that the cellular NLRP3 receptor, in conjunction with ASC and caspase-1, plays a critical role in the sensing by activated human monocytic THP-1 cells of infection with vMyxM013-KO virus and the activation of the inflammasome-dependent release of IL-1β. In contrast, AIM2, a documented cytosolic DNA sensor that is also linked to inflammasome activation, has no apparent role in the IL-1β release in response to vMyxM013-KO virus infection in THP-1 cells (Fig 2). Furthermore, we have demonstrated that at least 4 TLR receptors, coupled with rapid triggering of the MEK-ERK1/2 pathway, are required for the NF-κB activation that is needed to induce the secretion of pro-inflammatory cytokines such as TNF (Fig 6 and 7).

Among the cytosolic inflammasome receptors identified so far, AIM2 senses certain DNA viruses, including VACV, whereas the NLRP3 inflammasome has been reported to sense both RNA and DNA viruses. The viruses sensed by NLRP3 inflammasome include Sendai virus (22), influenza A virus (1, 16, 47), adenovirus (2, 31) and the attenuated vaccinia virus strain Modified Vaccinia Ankara (MVA) (8). In vivo studies demonstrate that sensing of influenza A virus is mediated by NLRP3 (1, 47). Thus, NLRP3-deficient mice produced reduced levels of IL-
1β in response to intranasal challenge by Influenza A. In addition to NLRP3, ASC and caspase-1 deficient mice also displayed significantly reduced survival compared to wild-type mice following influenza infection (1, 47). This suggests that NLRP3, ASC and caspase-1 all play important roles in the immune responses against at least some pathogenic virus infections.

Recently, it has been reported that the influenza virus encoded M2 protein, which acts as a proton ion channel essential for viral entry and replication, was required and sufficient for NLRP3 activation by influenza A virus (17). In contrast, the available data on the innate inflammasome-linked sensors that trigger early host responses to poxvirus infections are not yet definitive. For example, MVA infection is sensed by the NLRP3 inflammasome in THP-1 cells, but the in vivo role of NLRP3 and ASC for any poxvirus infection remains to be studied. It was recently reported that AIM2 is required for caspase-1 activation and release of IL-1β in response to VACV-WR (strain Western Reserve) infection of murine bone marrow derived macrophages and DCs (37). However, in activated THP-1 cells, VACV-WR did not induce IL-1β secretion, suggesting that AIM2 sensing of poxvirus infection might only operate in a cell- or species-specific manner (unpublished observations).

Recent studies suggest that activation of the NLRP3 inflammasome and release of IL-1β requires at least two upstream signals. The NLRP3 inflammasome becomes primed when NF-κB signaling from activated TLRs upregulates the levels of the NLRP3 and pro-IL-1β proteins. An additional signal is then required to activate NLRP3 leading to the assembly of the active inflammasome complex. This can be supported by the observation that either TLR signaling or ROS generation alone is insufficient for NLRP3 inflammasome activation (28, 53). Here, we have observed that infection of activated THP-1 cells with vMyxM013-KO virus increased the level of pro-IL-1β protein very rapidly (Fig 1E). However, the protein levels of the other key

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components of the inflammasome complex, specifically NLRP3 and ASC, remained unchanged. We also observed that in the absence of NLRP3, ASC or caspase-1, the induced synthesis of pro-IL-1β in response to vMyxM013-KO virus infection is delayed, suggesting that activation of the inflammasome complex is required to initiate the rapid synthesis of pro-IL-1β protein (Fig 1E and 2G). It also suggests that NLRP3 inflammasome complex might have direct or indirect links with the NF-κB activation pathway. It has been recently demonstrated that synthesis of NLRP3 protein is dependent on NF-κB, which is induced by many other PPRs, particularly the TLRs (4).

The NLRP3 inflammasome is activated by diverse pathogens, toxins or particulates(30, 41). However, unlike the inflammasome-linked DNA sensor AIM2, direct association of NLRP3 with inflammasome activators has not been reported. Previous studies have demonstrated that release of lysosomal cathepsins into the cytoplasm can function as an additional trigger for the activation of NLRP3 inflammasome(9, 15). In addition to phagocytosed particulates such as silica particles or asbestos, intracellular bacteria and RNA or DNA viruses can also activate the NLRP3 inflammasome via the release of cathepsin B into the cytoplasm (1, 2, 9). Pharmacological inhibition or gene silencing of cathepsin B leads to the reduced NLRP3 inflammasome activation. The requirement of the NLRP3 receptor in sensing MYXV lacking M013 has been further supported by the observation that inhibition of cathepsin B results in the inhibition of release of IL-1β (Fig 3). It has been proposed that release of cathepsin B induces ROS production that might activate NLRP3 inflammasome. The release of ROS has been reported for many stimuli (including viruses) that activate NLRP3. NADPH oxidase has been shown to be a source of ROS, as inhibition of NADPH with DPI affects the release of IL-1β.

Here, we observed that DPI also inhibits vMyxM013-KO virus mediated release of both IL-1β and TNF from THP-1 cells (Fig 4).
The activation of NF-κB pathway signaling in response to virus infection, which is linked to the induction of many other pro-inflammatory cytokines such as TNF and IL-6, can also be mediated by TLRs or RLRs. TLRs in particular are capable of sensing both DNA and RNA viruses and rapidly inducing the activation of NF-κB and the IRFs needed to initiate effective antiviral immune responses. Our results suggest that at least in THP-1 cells, RLRs are not required for the release of TNF in response to vMyxM013-KO virus infection (Fig 5B).

Interestingly, primary human macrophages sense WT MYXV (ie virus that expresses M013) via RIG-I, which then induces release of both TNF and type I IFN (51), whereas WT MYXV infection of differentiated THP-1 cells does not release the secretion of TNF (35)(and this study). This suggests that MYXV is either sensed differently in primary human macrophages vs differentiated THP-1 cells, or else RIG-I integration with the TNF secretion pathway is functionally different in primary vs transformed human myeloid cells.

In this study we show that sequential transient siRNA knockdown of all known human TLRs suggest that TLR2, TLR6, TLR7 and TLR9 all have roles in sensing of vMyxM013-KO virus and inducing the release of TNF and IL-1β (Fig 6). Other members of the poxvirus family appear to be sensed by different TLRs, depending on the species or cell types. For example, MVA is a particularly immunogenic variant of VACV that is sensed by TLR2 and/or TLR6 that then drives the activation of NF-κB and synthesis of pro-IL-1β (8). VACV-WR is sensed by the TLR2-MyD88 pathway on conventional DCs (cDCs) for the production of pro-inflammatory cytokines IL-6, IL-1 and IL-12 (54); however, in murine pDCs VACV-WR is sensed by TLR8 for the activation of NF-κB and type I IFN production (29). Ectromelia virus, the causative agent of mousepox, is sensed by TLR9 in mouse DCs (39). This all suggests that the functionality of TLRs against specific poxviruses is very dependent on the host species, the cell lineage, and...
possibly even the transformed status of the cells in question. It is also possible that this variation among cell types is a result of differences in the expression levels of PRRs or adaptors molecules.

In conclusion, the present study demonstrates that MYXV is sensed by multiple pattern recognition receptors in human myeloid cells and that M013 has evolved to inhibit both the inflammasome and NF-κB signaling pathways. In human monocytic THP-1 cells, the NLRP3, ASC and caspase-1 inflammasome is required for the early synthesis and release of IL-1β precursor protein in response to vMyxM013-KO virus infection. This virus-induced activation of NLRP3 involves cathepsin B leakage and the release of ROS into the cytoplasm. We also observed that multiple TLRs, including TLR2, TLR6, TLR7 and TLR9, contribute to the activation of NF-κB, which is required for the release of other pro-inflammatory cytokines like TNF and IL-6 and for the upregulated synthesis of the pro-IL-1β precursor. MYXV is currently being developed as oncolytic virus candidate for the treatment of various human cancers and it is critical to better understand how this rabbit-specific virus is sensed by human innate sentinel cells, and how this sensing triggers the ensuing immune responses to the virus infection.

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Materials and Methods

Reagents and antibodies
Rabbit polyclonal antibodies for RIG-I, MAVS, MDA5, MyD88, IκBα, IL-1β and caspase-1 were purchased from Cell Signaling Technology. Mouse monoclonal antibody for NLRP3 from Alexis Biochemicals, rabbit polyclonal antibody for ASC from Santa cruz, rabbit polyclonal antibody for AIM2 from abcam, and mouse monoclonal antibody for β-actin was obtained from Ambion. HRP-conjugated goat anti-rabbit and anti-mouse IgG antibodies were purchased from Jackson Lab. Antibodies against human TLRs were purchased from Imgenex Corp. Adenosine triphosphate (ATP), Phorbol-12-myristate-13-acetate (PMA), and caspase-1 inhibitor zVAD-fmk were purchased from Sigma. MEK1/2 inhibitor U0126 and PI3kinase inhibitor LY294002 were purchased from Cell Signaling Technology. Cathepsin B specific inhibitor, CA074Me and NADPH oxidase inhibitor, diphenyleneiodonium (DPI) was purchased from Calbiochem. Ultrapure lipopolysaccharide from *E. coli* K12 strain (LPS-EK), poly (dA:dT), Pam3CSK4, Pam2CSK4, CL075, *E. coli* DNA, Alum Crystals, and Poly (I:C) were purchased from InvivoGen.

**Cell lines and cell culture**

The human monocytic THP-1 cell line was cultured in RPMI 1640 medium (Lonza) supplemented with 10% heat-inactivated FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin. For differentiation into macrophages, THP-1 cells were stimulated for 12-18 h with 100 ng/ml of PMA. In all experiments, THP-1 cells were activated with PMA before infection or stimulation with ligands (32). All cultures were maintained at 37°C in a humidified 5% CO₂ incubator. THP-1 cells stably expressing control shRNA, or NLRP3, caspase-1 and ASC shRNA have been described previously (8).

**Transfection of cells**
THP-1 cells were seeded at $5 \times 10^4$ cells per well in 24-well plates in growth medium without antibiotics. All the siRNAs used were ON-TARGETplus siRNA purchased from Thermo Scientific (Dharmacon). siRNA solution (50-100nM final concentration/well) was prepared in 50µl Opti-MEM I Reduced serum medium (Invitrogen). Lipofectamine RNAiMAX (Invitrogen) solution was prepared in 50µl Opti-MEM I Reduced serum medium and incubated 5 min at RT. siRNA and lipofectamine solutions were mixed and incubated for 20min at RT and added to the cells to make final volume of 500µl and incubated at 37°C in a CO2 incubator. The knock down was verified after 48-72h of transfection.

**Viral preparation**

Construction of a wild-type MYXV that express GFP under the control of a synthetic VACV early-late promoter was described previously. Construction of the vMyxM013-KO virus was described previously (18). Viruses were purified by centrifugation through a sucrose cushion and two successive sucrose gradient sedimentations as described previously (20, 43).

**Quantification of cytokine secretion by ELISA**

THP-1 cells were plated in multi-well plates in the presence of PMA (100 ng/ml) for overnight. The following day, culture media were replaced with fresh RPMI media and the cells were infected with WT-MYXV or vMyxM013-KO viruses at an MOI of 3 or treated with inducers like LPS (100ng/ml) or ATP (5mM) and the supernatants (media) were collected at different time points. Whenever mentioned, inhibitors were added one hour before infection. The level of TNF and IL-1β were determined using ELISA assay kits (eBioscience) following manufacturer protocol.

**Western blot analysis**
THP-1 cells were harvested at different time points after infection with viruses, washed with PBS and stored at -80°C or processed immediately with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 1mM PMSF, and protease inhibitor cocktail (Roche)). Amount of total proteins were estimated by Bradford assay (Bio-Rad) and equal amount of total proteins were used for Western blot analysis. Protein samples were separated on 10% SDS-PAGE gels and transferred to PVDF membrane (GE Healthcare) using a wet transfer apparatus (Invitrogen). Membranes were blocked in TBST buffer (20mM Tris, 150 mM NaCl, 0.1% Tween-20 pH 7.6) containing 5% non-fat dry milk for 1 hr at room temperature and then incubated overnight with appropriate primary antibody at 4°C. The membranes were washed three times, 15 minutes each with TBST and incubated with HRP-conjugated goat-anti-mouse (1:10,000) or goat anti-rabbit (1: 10,000) secondary antibody in TBST containing 5% non-fat dry milk for 1 hour at room temperature with gentle agitation and were then washed three times, 15 minutes each with TBST. The proteins were detected using the chemiluminescence substrate (Pierce) and exposure to X-ray film (Kodak).

Detection of ROS

The intracellular levels of ROS were measured using 2’,7’-dichlorofluorescein (DCF). Briefly, THP-1 cells were plated in multiwall plate in the presence of PMA overnight. Media was replaced with fresh complete media and infected with viruses for 6h. Cells were then loaded with 10µM of 2’,7’-dichlorofluoresceindiacetate (H$_2$DCF-DA; Molecular probes) for 30 minutes at 37°C. In some cases cells were treated with 100µM of NADPH oxidase inhibitor DPI for 1h before virus infection. H$_2$DCF-DA is a cell membrane permeable non-fluorescent compound that is hydrolyzed to DCF and becomes fluorescent when it is oxidized by ROS. Cells are then
washed and resuspended in PBS and fluorescence was measured at 520nm following excitation with 488 nm light using Appliskan plate reader (Thermo Scientific).

Statistics

Data were expressed as means ± SD and were analyzed by paired t-test. Significant difference was accepted at \( p < 0.05 \).

References

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Figure Legends

Figure 1. ASC and caspase-1 are important for activation of inflammasome and release of IL-1β by vMyxM013-KO virus infection of THP-1 cells. THP-1 cells were transfected with control siRNA or ASC siRNA, differentiated for 12-18h with PMA and then infected with MYXV or vMyxM013-KO viruses with MOI of 3 or treated with ATP (5mM) or LPS (500 ng/ml). Cell supernatants were collected after 6h and the secretion of A) IL-1β and B) TNF quantified by ELISA. THP-1 cells stably transduced with control shRNA, ASC or caspase-1 shRNAs. The cells were differentiated with PMA and were infected with WT MYXV or vMyxM013-KO viruses or treated with ATP or LPS (C-E). Cell supernatants were collected at indicated time points to quantify secretion of C) IL-1β and D) TNF by ELISA. Data are means ± SD of triplicate samples from one experiment and are representative of three independent experiments E-F) Western blots of intracellular IL-1β, caspase-1, ASC and actin (as loading control).
Figure 2. NLRP3, but not AIM2, is required for THP-1 inflammasome activation by vMyxM013-KO virus. THP-1 cells were transfected with control, NLRP3 (A-B) or AIM2 (C-D) siRNA, differentiated for 12-18h with PMA and then infected with MYXV or vMyxM013-KO viruses (MOI of 3) or treated with ATP (5mM), Poly (dA:dT) (5µg/ml) or LPS (500 ng/ml). Cell supernatants were collected after 6h to measure the secretion of IL-1β (A and C) or TNF (B and D) by ELISA. THP-1 cells stably transduced with control and NLRP3 shRNAs, differentiated with PMA and were infected with MYXV or vMyxM013-KO viruses or treated with ATP or LPS (E-G). Cell supernatants were collected at indicated time points to measure secretion of E) IL-1β and F) TNF by ELISA. Data are means ± SD of triplicate samples from one experiment and are representative of three independent experiments G-H) Western blots of intracellular IL-1β, caspase-1, ASC, NLRP3 and actin (as loading control).

Figure 3. Cathepsin B is involved in the activation of NLRP3 inflammasome activation by vMyxM013-KO virus. THP-1 cells were differentiated with PMA, treated with cathepsin B inhibitor Ca-074me (100µM) for one hour and then infected with vMyxM013-KO virus at an MOI of 3 or treated with alum (as positive control). Cell supernatants were collected after 6h to measure the secretion of IL-1β (A) and TNF (B) by ELISA. Data are means ± SD of triplicate samples from one experiment and are representative of two independent experiments C) THP-1 cells were differentiated with PMA, treated with LPS (500ng/ml) as control or infected with vMyxM013-KO virus in the presence or absence of cathepsin B inhibitor Ca-074me and Western blot analysis was done for the detection of intracellular IκBα, caspase-1 and actin (as loading control). .

Figure 4. ROS is involved in the activation of NLRP3 inflammasome activation by vMyxM013-KO virus. THP-1 cells were differentiated with PMA, treated with DPI using the indicated
concentrations for one hour and then infected with vMyxM013-KO virus at an MOI of 3. Cell supernatants were collected after 6h to measure the secretions of IL-1β (A) and TNF (B). C) PMA-differentiated THP-1 cells were mock treated or pretreated with ROS inhibitor DPI (100µM) for 1h and then infected with vMyxM013-KO virus. After 6 hrs cells were loaded with ROS sensitive fluorophore H$_2$DCF-DA (10µM) and fluorescence intensity was measured as described in the methods section. Data are means ± SD of triplicate samples from one experiment and are representative of two or three independent experiments. **P < 0.01 compared with vMyxM013-KO infection.

Figure 5. Role of RLRs and MyD88 in the activation of inflammasomes and NF-κB signaling by vMyxM013-KO. THP-1 cells were transfected with siRNAs for nontargeting control, RIG-I, MDA5, MAVS or MyD88, differentiated for 12-18h with PMA and then infected with vMyxM013-KO virus (MOI of 3), transfected with dsRNA Poly (I:C) (1µg/ml) for the indicated time periods or treated with LPS (500 ng/ml). A) Western blots showing the levels of protein knockdown using MyD88, MAVS and RIG-I siRNA. B) and C) Cell supernatants were collected after 6h or 24h of infection or treatment with the inducers to quantify the secretion of TNF and IL-1β by ELISA. Data are means ± SD of triplicate samples from one experiment and are representative of two or three independent experiments. P < 0.05 for all conditions in C.

Figure 6. Role of TLRs in the activation of inflammasomes and NF-κB signaling by vMyxM013-KO. THP-1 cells were transfected with siRNAs for nontargeting control or human TLRs (1-10), differentiated for 12-18h with PMA and then infected with vMyxM013-KO virus (MOI of 3) or treated with known ligands for TLRs; LPS (100ng/ml), Pam3CSK4 (10ng/ml), Pam2CSK4 (0.1ng/ml), CL075(5µg/ml), E.coli DNA (6µg/ml) (as control). Cell supernatants were collected after 6h of infection or treatment with LPS, Pam3CSK4, Pam2CSK4 and 24h treatment with...
CL075 and E. coli DNA to measure the secretion of TNF and IL-1β by ELISA. Results shown here are from A) TLR1, B) TLR2, C) TLR4, D) TLR6, E) TLR7, F) TLR8, G) TLR9 siRNA treated THP-1 cells. Data are means ± SD of triplicate samples from one experiment and are representative of two or three independent experiments. P < 0.05 for all conditions. H) Western blots showing the levels of protein knockdown using TLR1, TLR2, TLR4, TLR6, TLR7 and TLR9 siRNAs.

Figure 7. MEK-ERK1/2 pathway is involved in activation of both NF-κB and inflammasome by vMyxM013-KO. A) THP-1 cells stably transduced with control, ASC, caspase-1 and NLRP3 shRNAs. The cells were differentiated with PMA, treated with U0126 (20 μm) or LY294002 (20 μm) for 1 h and were infected with vMyxM013-KO virus. Cell supernatants were collected after 6 h of infection to quantify the secretion of TNF by ELISA. Data are means ± SD of triplicate samples from one experiment and are representative of three independent experiments. B) Western blots of intracellular phospho-ERK1/2 (pERK1/2), total ERK1/2 and IκBα after treatment with U0126 or LY294002 and infection with vMyxM013-KO.

Figure 8. A schematic diagram showing the mechanism(s) of activation of the NLRP3 inflammasome and NF-κB signaling pathways by vMyxM013-KO virus infection in human monocytic cell line THP-1. Infection of differentiated THP-1 cells by vMyxM013-KO virus activates the NLRP3 inflammasome by the release of ROS and cathepsin B from lysosomal destabilization. vMyxM013-KO virus activates the NF-κB pathway for the synthesis of cytokines via the activation of MEK-ERK1/2 pathway and sensed by the TLRs.
Figure 1
Figure 1 Con.
Figure 2

A) B) C) D) E) F) G) H)

mock 1h 2h 6h
vMyxM013-KO
ASC
Pro-IL-1β
actin
Caspase-1

media control siRNA NLRP3 siRNA

media control siRNA AIM2 siRNA

media control siRNA NLRP3 siRNA

media control siRNA NLRP3 siRNA

SH-1/shControl SH-1/shNLRP3

mock MYXV vMyxM013-KO ATP

mock MYXV vMyxM013-KO LPS

SH-1/shControl SH-1/shNLRP3

mock MYXV vMyxM013-KO LPS

NLRP3

actin

actin

1 2 3 4

1 2

Figure 2
Figure 3

A) B) 

C) vMyxM013-KO - + + + 
Ca-074me - + + + 
hrs 0 1 2 6 

-IkBα 
Caspase-1 
actin 

vMyxM013-KO 0 1 2 6 5 6 7 8 
LPS 0 1 2 6 9 10 11 12 

IκBα 
actin 

Figure 3
Figure 4
Figure 5

A) MyD88 
mock Control siRNA MyD88 siRNA 
Actin mock Control siRNA MAVS siRNA 
MAVS 
Actin mock Control siRNA RIG-I siRNA 
RIG-I
Actin 
1       2       3 4       5       6 7       8       9

B) 

C) 

Figure 5
Figure 6

A) B) TNF TNF TNF TNF TNF TNF IL-1β IL-1β IL-1β IL-1β IL-1β IL-1β IL-1β
C) D) E) F) G)
Figure 6 Con.
Figure 7

A) TNF (pg/ml)

B) vMyxM013-KO - + + + - + + + - + + + - + + + - + + +
U0126 - + + + - + + + - + + + - + + + - + + + - + + +
LY294002 - + + + - + + + - + + + - + + + - + + + - + + +

vMyxM013-KO - + + + - + + + - + + + - + + + - + + +
LY294002 - + + + - + + + - + + + - + + + - + + + - + + +

ERK1/2, p-ERK1/2, IκBα

Figure 7
Figure 8