



Published in final edited form as:

*Nat Immunol.* 2010 May ; 11(5): 395–402. doi:10.1038/ni.1864.

## The AIM2 inflammasome is essential for host-defense against cytosolic bacteria and DNA viruses

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### Abstract

Inflammasomes regulate the activity of caspase-1 and maturation of interleukin-1 $\beta$  and interleukin-18. Recently, AIM2 was shown to bind DNA and engage ASC to form a caspase-1 activating inflammasome. Using *Aim2*-deficient mice, we reveal a central role for AIM2 in regulating caspase-1-dependent maturation of IL-1 $\beta$  and IL-18, as well as pyroptosis in response to synthetic dsDNA. AIM2 is essential for inflammasome activation in response to *Francisella tularensis*, vaccinia virus, mouse cytomegalovirus and plays a partial role in sensing *Listeria monocytogenes*. Moreover, production of IL-18 and NK cell-dependent IFN- $\gamma$  production, events critical in early control of virus replication were dependent on AIM2 during mCMV infection *in vivo*. Collectively, these observations reveal the importance of AIM2 in sensing both bacterial and viral pathogens and triggering innate immunity.

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Optimal protection from infection requires complex immune responses involving both the innate and adaptive immune system. The innate immune system plays a key role in initiating

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**Author Contributions;** K.A.F oversaw the whole project. K.A.F, V.A.R and S.W conceived the research with assistance from S.N.V., E.L. and E.T.S. V.A.R, B.M. and V. H characterized the genetrapp integration. V.A.R and L.W conducted all genotyping, V.A.R., Z.J and S.W designed and conducted the experiments with help from S.S., L.W., S.K.V., S.G. and L.E.C who conducted the *Ft* experiments. K. A. F, V.A.R, S.S and S.W wrote the manuscript.

**Competing financial interest.** The authors declare no competing financial interest.

and orchestrating host defenses by regulating the production of pro-inflammatory cytokines, type I interferons and anti-microbial effectors<sup>1</sup>. Several distinct classes of germline-encoded pattern recognition receptors (PRRs) have been discovered and implicated in the sensing of microbial products. These include the Toll-like receptors (TLRs)<sup>2</sup>, the C-type lectin receptors (CLRs)<sup>3</sup>, the RIG-like helicases (RLRs)<sup>4</sup>, the NOD-like receptors (NLRs)<sup>5</sup> and cytosolic DNA sensors; DNA dependent activator of IRFs (DAI)<sup>6</sup>, RNA polymerase III<sup>7, 8</sup> and Absent in melanoma-2 (AIM2) (ref<sup>9-12</sup>). These individual PRRs recognize microbial products from bacteria, viruses, fungi and parasites and in most cases trigger signaling pathways which culminate in the transcription of immune response genes<sup>1</sup>.

The pro-inflammatory cytokine IL-1 $\beta$  is among the arsenal of defense measures deployed by the innate immune system<sup>13</sup>. The activity of IL-1 $\beta$  is regulated at the level of expression, processing, secretion and antagonism by a naturally occurring inhibitor, IL-1 receptor antagonist (IL1RA)<sup>13</sup>. An initial microbial stimulus acting through innate PRRs results in accumulation of intracellular stores of pro-IL-1 $\beta$ . A second stimulus then controls the activation of caspase-1, a proteolytic enzyme, which in turn leads to cleavage of pro-IL-1 $\beta$ , followed by release of the active mature 17-kDa cytokine. Interleukin-18 (IL-18) is structurally similar to IL-1 $\beta$ , and like IL-1 $\beta$  is first synthesized as a leaderless precursor requiring caspase-1 for cleavage into an active molecule<sup>14</sup>.

Multiprotein complexes containing one or more Nod-like receptors (NLRs) commonly referred to as “inflammasomes” regulate caspase-1 activity. The NLRP1 inflammasome is activated by anthrax lethal toxin<sup>15</sup>. The NLRP3 inflammasome is activated by a broad range of stimuli including bacteria, bacterial pore forming toxins, viruses, endogenous danger signals such as ATP and monosodium urate and indigestible particulates, like silica, asbestos, alum and  $\beta$ -amyloid (reviewed in<sup>16</sup>). The IPAF inflammasome is activated by flagellin or by the basal body rod component of the type 3 secretion system (T3SS) found in *Salmonella typhimurium* and other bacteria<sup>17</sup>. A fourth inflammasome, the AIM2 inflammasome was also recently identified. AIM2 is a member of the IFI202X/IFI16 (PYHIN) protein family<sup>18</sup>. AIM2 binds to DNA via a HIN200 domain, while the PYD domain associates with the adapter molecule ASC to activate caspase-1 (ref<sup>9-12</sup>). The role of AIM2 in orchestrating immune responses to DNA or in regulating immune responses to viral or bacterial pathogens *in vivo* has not been examined further.

Here we investigate the role of AIM2 *in vivo* by generating mice lacking *Aim2*. We show that *Aim2* regulates caspase-1-dependent processing of pro-IL-1 $\beta$  and pro-IL-18 in response to dsDNA in both dendritic cells and macrophages. *Aim2* does not mediate type I IFN responses to dsDNA but rather acts to negatively regulate this pathway. We also reveal a central role for *Aim2* in sensing the cytosolic bacterial pathogen, *Francisella tularensis*, live vaccine strain (LVS), as well as vaccinia virus and mouse cytomegalovirus (mCMV). Additionally, we reveal a partial role for *Aim2* in sensing *Listeria monocytogenes*. *In vivo*, *Aim2*- and *Asc*-deficient mice infected with mCMV have reduced IL-18 concentrations in the serum, and severely attenuated IFN- $\gamma$  production by NK cells, events, which are critical for the early control of viral replication. Collectively, these observations establish *Aim2* as an important anti-microbial sensor and a key determinant of protective immunity to viral pathogens.

## Results

### Generation of *Aim2*<sup>-/-</sup> mice

To study the role of the *Aim2* gene *in vivo*, we generated *Aim2*-deficient mice using a gene-trap ES cell line carrying a pGT0lxf vector insertion (CSG445;<http://www.genetrap.org>) (Fig. 1a). In this ES cell line, the pGT0lxf gene-trap vector has inserted into intron 6 of the *Aim2* gene. The fusion transcript generated by the gene-trap vector insertion directs the expression of a truncated Aim2 protein carrying only the N-terminal PYD domain fused to the β-geo cassette (Fig. 1a). The presence of the pGT0lxf vector insertion within the *Aim2* gene in the CSG445 ES cells was confirmed by RT-PCR (Supplementary Fig. 1) and the site of the pGT0lxf integration was mapped by long range PCR and sequencing and found to reside within intron 6 (Supplementary Fig. 2). Mice harboring 2 copies of the trapped *Aim2* gene were bred to homozygosity and deletion of *Aim2* confirmed by RT-PCR (Fig. 1b) and immunoblotting (Fig. 1c). *Aim2* was absent in thioglycollate-elicited macrophages (TEM), bone marrow-derived macrophages (BMDM), bone marrow-derived DCs (BMDC) and splenocytes from *Aim2*<sup>-/-</sup> mice (Fig. 1c, and data not shown). The mutant mice were born at the expected Mendelian ratios. They developed and bred normally and displayed no apparent abnormalities. *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice were compared throughout our studies and were generated from breedings of heterozygous parents.

### *Aim2* is critical for responses to cytosolic dsDNA

Consistent with previous studies, which identified human AIM2 as a cytosolic sensor for dsDNA and a key regulator of IL-1β production (ref<sup>9-12</sup>), transfection studies with the murine *Aim2* gene also led to the formation of a functional inflammasome complex. As an initial screen for the ability of *Aim2* to associate with Asc and elicit signaling, we tested the ability of transfected *Aim2* to trigger Asc-dependent NFκB signaling. Overexpression of mouse *Aim2* led to Asc-dependent NFκB reporter gene activation (Fig. 2a). We next examined whether the murine *Aim2*-Asc complex could lead to the caspase-1-dependent maturation of pro-IL-1β using a transient transfection assay with *Aim2* and Asc in the presence of caspase-1 and flag-tagged pro-IL-1β in 293T cells. *Aim2* led to maturation of pro-IL-1β in an Asc-dependent manner (Fig. 2b).

To evaluate the role of *Aim2* in the innate response to DNA, BMDM were generated from *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> littermate controls. Macrophage differentiation was examined by staining cells for CD11b and F480. Both *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> macrophages had equivalent F480/CD11b staining patterns indicating normal macrophage differentiation (Fig. 2c). Synthetic B-form dsDNA, poly(dA:dT) induced a robust IL-1β response in BMDM as measured by ELISA and this response was abolished in *Aim2*<sup>-/-</sup> macrophages (Fig. 2d). Similar results were obtained when thioglycollate-elicited macrophages and BMDCs were examined (Fig. 2e). DNA treatment also led to the proteolytic cleavage of caspase-1 and production of the mature 17kDa IL-1β cytokine and in both cases these responses were impaired in *Aim2*<sup>-/-</sup> cells (Fig. 2f). Macrophages lacking Asc or caspase-1 had a similar phenotype (data not shown). Poly(dA:dT) also induced IL-18 production and this was also *Aim2*-dependent (Fig. 2g). Finally, poly(dA:dT) induced an inflammatory form of cell death (pyroptosis) in *Aim2*<sup>+/+</sup> macrophages and this response was also attenuated in *Aim2*<sup>-/-</sup> cells

(Fig. 2h). Collectively, these data indicate that Aim2 is a non-redundant sensor regulating caspase-1 dependent maturation of IL-1 $\beta$ , IL-18 and pyroptosis in response to DNA<sup>19</sup>.

### Aim2-deficient cell responses to other inflammasome activators

To define the specificity of Aim2 for DNA, we examined inflammasome activity in response to agonists of other NLRs. *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> BMDM responded equivalently to anthrax lethal toxin which signals via Nlrp1<sup>15</sup> (Fig. 3a). IL-1 $\beta$  release in response to ATP and nigericin, activators of the Nlrp3 inflammasome was also normal (Fig 3b). Analysis of caspase-1 cleavage and IL-1 $\beta$  cleavage showed similar results (Fig 3c). Aim2-deficient cells also responded normally to *Salmonella typhimurium*, which activates the Nlr4/Ipaf inflammasome (Fig. 3d). Rig-I regulates caspase-1-dependent processing of IL-1 $\beta$  in response to some RNA viruses<sup>20</sup>. To evaluate this pathway we monitored IL-1 $\beta$  release in BMDCs from *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice that were infected with Sendai virus, a paramyxovirus which signals through RIG-I. Sendai virus induced IL-1 $\beta$  response was not impaired in *Aim2*<sup>-/-</sup> DCs (Fig. 3e). Collectively, these data indicate that Aim2 is a receptor for cytosolic dsDNA and does not contribute to inflammasome activation in response to agonists of the Nlrp1, Nlrp3, Nlr4 or Rig-I inflammasomes.

### IFN responses in Aim2-deficient macrophages

Cytosolic DNA is also a potent trigger of type I IFN production. To evaluate the role of Aim2 in this response, we measured IFN- $\beta$  production in response to poly(dA:dT) in *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice. Poly(dA:dT) induced IFN- $\beta$  in *Aim2*<sup>+/+</sup> splenocytes and thioglycollate-elicited macrophages and this response was enhanced in cells from *Aim2*<sup>-/-</sup> mice (Fig. 4a and b). These observations indicate that Aim2 is not a sensor for IFN- $\beta$  production but it may exert a negative regulatory effect on IFN responses. We also examined the responsiveness of *Aim2*<sup>-/-</sup> BMDM to type I IFNs themselves. *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> macrophages were stimulated with IFN- $\beta$  and induction of viperin, a well-characterized IFN stimulated gene was measured by immunoblotting. IFN- $\beta$  induced high levels of viperin expression in *Aim2*<sup>+/+</sup> cells and this response was still induced in *Aim2*<sup>-/-</sup> cells (Fig. 4c).

AIM2 is a type I IFN inducible gene<sup>21</sup>. While human monocytes do not express AIM2, except after IFN induction<sup>10</sup>, mouse macrophages and splenocytes express Aim2 constitutively (Fig. 4d and data not shown) and the expression of Aim2 was further increased upon treatment of BMDM with IFN- $\beta$ , poly(dA:dT) or after infection with Sendai virus (Fig. 4d). Previous work had suggested that type I IFN signaling was essential for inflammasome activation in response to *F. tularensis* and *L. monocytogenes*<sup>22, 23</sup>. To define the requirement for IFN production and/or signaling in Aim2 inflammasome activation, we monitored DNA induced IL-1 $\beta$  responses under conditions where IFN- $\beta$  was not induced. Poly(dA:dT) induced a robust IFN- $\beta$  response which was completely dependent on Irf3 and Irf7 (Fig. 4e). To determine if IFN- $\beta$  production following poly(dA:dT) treatment was required for Aim2 inflammasome activation, we monitored IL-1 $\beta$  release in these cells. In contrast to IFN- $\beta$  production, IL-1 $\beta$  production was not defective in Irf3 and Irf7-double-deficient cells in response to poly(dA:dT) (Fig. 4f). These observations indicate that

although DNA treatment leads to type I IFN production and elevated expression of Aim2, these events are dispensable for Aim2 inflammasome activity.

### Aim2-dependent IL-1 $\beta$ responses to bacteria

We next examined the role of Aim2 in regulating inflammasome activation in response to microbial pathogens. *F. tularensis* (*Ft*) is a pathogenic bacterium whose virulence is linked to its ability to replicate within the host cell cytosol<sup>24</sup>. Entry of the organism into the cytosol from the phagosome triggers the release of type I IFNs, IL-1 $\beta$  and IL-18, as well as caspase-1-dependent cell death<sup>22, 23, 25</sup>. Although production of inactive pro-IL-1 $\beta$  in response to *Ft* Live Vaccine Strain (LVS) is dependent on Tlr2<sup>22, 26</sup>, cleavage of pro-IL1 $\beta$  into its active mature form requires both entry into the cytoplasm and engagement of a previously unknown cytosolic receptor<sup>22, 23, 25</sup>. These events are dependent upon Asc<sup>25</sup> but independent of Nlrp1<sup>27, 28</sup>, Nlrc4<sup>25</sup> or Nlrp3<sup>27, 28</sup>. Additionally, an intact type I IFN response was shown to be critical for this response<sup>22, 23</sup>. To test the role of Aim2 as a candidate mediator of this response, we infected *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> macrophages with *Ft* LVS and assayed cleavage of caspase-1 and release of IL-1 $\beta$ . While *Ft* LVS strongly activated caspase-1 and IL-1 $\beta$  release in *Aim2*<sup>+/+</sup> cells, the loss of Aim2 ablated these responses (Fig. 5a and b). Priming of macrophages with LPS or other TLR ligands was not required in order to observe *Ft* LVS-induced caspase-1 activation, as *Ft* recognition by Tlr2 regulates pro-IL1 $\beta$  gene induction<sup>22, 26, 29</sup>. To confirm that the lack of secreted IL-1 $\beta$  by *Aim2*<sup>-/-</sup> cells was due to impaired inflammasome activation and not due to reduced production of pro-IL-1 $\beta$  we also evaluated the induction of IL-1 $\beta$  mRNA by qPCR. Infection with *Ft* LVS led to equivalent induction of pro-IL-1 $\beta$  mRNA in *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> cells (Fig. 5c).

*Ft* is known to induce IFN- $\beta$  and previous work has shown that IFN $\alpha$ -deficient<sup>22</sup> or IFN $\alpha$ / $\beta$ R-deficient<sup>23</sup> macrophages failed to induce inflammasome activation by *Ft*, indicating a requirement for an intact IFN response. We therefore also examined *Ft*-induced caspase-1 cleavage and IL-1 $\beta$  release in macrophages lacking Irf3 and Irf7. These cells had a blunted but not a defective response (Fig. 5a and b), in contrast to published studies with IFN- $\beta$ - or IFN $\alpha$ / $\beta$ R-deficient mice<sup>22, 23</sup>. While these combined data clearly indicate that *Ft* LVS-mediated activation of the inflammasome is dependent on type I IFN signaling and Aim2, they also suggest that in the absence of Irf3 and Irf7, *Ft* can still induce some IFN- $\beta$  production, which is sufficient to trigger Aim2. Indeed, *Ft* LVS still induced IFN- $\beta$  production albeit reduced in macrophages lacking Irf3 and Irf7 (data not shown). Precisely, how *Ft* induces type I IFNs is still unclear, although DNA sensing mechanisms have been proposed. The sensor responsible for these events has not been identified.

*L. monocytogenes* can also trigger inflammasome activity, although the mechanisms involved are not well established. Like *Ft*, *L. monocytogenes* also triggers type I IFN responses and IL-1 $\beta$  upon entry to the cytosol. The Nlrp3, Nlrc4 and another as yet-to-be-identified Asc-dependent inflammasome pathway have all been implicated<sup>30-33</sup>. To define the role of Aim2 in response to *Listeria*, macrophages from *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice were infected and caspase-1 cleavage as well as IL-1 $\beta$  maturation and release measured. In all cases, these responses were reduced but not abrogated in Aim2-deficient cells (Fig. 5d and

e). We observed similar findings in DCs (data not shown). These data indicate therefore that inflammasome activation by *L. monocytogenes* is only partially dependent on Aim2 signaling and additional pathways also contribute to this activation, consistent with published studies<sup>30,33</sup>.

### Aim2-dependent IL-1 $\beta$ responses to DNA viruses

To examine the role of Aim2 in inflammasome activation following DNA virus infection, we first examined responses in cells infected with mCMV. mCMV induced IL-1 $\beta$  release in BMDCs and this response was entirely dependent on Aim2 (Fig. 6a). Similar data were obtained when thioglycollate elicited macrophages were examined (data not shown). Activation of caspase-1 and maturation of IL-1 $\beta$  in response to mCMV were also Aim2-dependent (Fig. 6b). mCMV also induced IFN- $\beta$  and this response was not affected in *Aim2*-deficient BMDM (Fig. 6c), but was ablated in *Irf3* and *Irf7* double-deficient BMDM (Fig. 6d).

Vaccinia virus (VV) also triggered the Aim2 inflammasome. VV-induced IL-1 $\beta$  release, caspase-1 cleavage and IL-1 $\beta$  maturation were all attenuated in macrophages lacking Aim2 (Fig. 6e and f). Similar findings were made when BMDCs were tested (data not shown). Herpes Simplex Virus-1 (HSV-1) is also a strong inducer of IL-1 $\beta$  in macrophages, however, in contrast to VV and mCMV, maturation and release of IL-1 $\beta$  in response to HSV-1 was normal in cells from *Aim2*<sup>-/-</sup> mice (Fig. 6g and h). Collectively these data indicate that Aim2 is important in driving IL-1 $\beta$  maturation and release in response to DNA viruses. How Aim2 differentially senses mCMV and VV but not HSV-1 is unclear.

### Aim2-dependent responses to mCMV infection *in vivo*

IL-18 possesses biochemical similarities to IL-1 $\beta$  but functionally it is more similar to IL-12. IL-18 and IL-12 synergize to stimulate IFN- $\gamma$  production from NK cells<sup>34</sup>, events that are crucial in early defense against mCMV infection. IL-18R-deficient mice have dramatically reduced IFN- $\gamma$  amounts in both the systemic circulation and the spleen following Mcmv infection<sup>35</sup>. To define the role of Aim2 *in vivo*, we infected C57BL/6, *Asc*<sup>-/-</sup>, *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice with mCMV and first monitored IL-18 production. In agreement with published reports<sup>35</sup>, IL-18 was readily detectable in the sera of wild-type C57BL/6 mice 36 hours after infection with mCMV (Fig. 7a). IL-18 was not detected in the sera of mCMV-infected mice deficient in *Asc* (the serum concentrations in *Asc*<sup>-/-</sup> mice were below the limit of detection of this assay, 25.6 pg/ml). Serum concentrations of IL-18 during mCMV infection were reduced 6-fold ( $p = 0.026$ ,  $n = 4$ ) in *Aim2*<sup>-/-</sup> mice relative to *Aim2*<sup>+/+</sup> littermate controls (Fig. 7b), whereas systemic TNF- $\alpha$  concentrations were unaffected ( $66 \pm 30$  pg/mL (*Aim2*<sup>+/+</sup>) vs.  $56 \pm 16$  pg/mL (*Aim2*<sup>-/-</sup>),  $p = 0.56$ ,  $n = 4$ ) (data not shown). These results suggest that Aim2 and *Asc* are required for systemic induction of IL-18 but not TNF- $\alpha$  during mCMV infection *in vivo*.

Based on the important role of IL-18 in the induction of systemic and splenic IFN- $\gamma$  production during mCMV infection, we next evaluated *ex vivo* IFN- $\gamma$  production by NK cells in the spleens of mCMV-infected mice<sup>36</sup>. The percentages of NK1.1<sup>+</sup> NKp46<sup>+</sup> CD3 $\epsilon$ <sup>-</sup> NK cells in the spleens of C57BL/6, *Asc*<sup>-/-</sup>, *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice were similar following

mCMV infection (Fig. 7c and supplemental fig. 3). However, C57BL/6 and *Aim2*<sup>+/+</sup> mice had significantly higher numbers of IFN- $\gamma$  producing NK cells *ex vivo*, as determined by intracellular staining. *Asc*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> mice had substantially reduced numbers of IFN- $\gamma$  producing NK cells (29% and 52% less than controls, respectively) (Fig. 7d). Diminished numbers of IFN- $\gamma$  producing NK cells could not be attributed to reduced NK cell activation as similar proportions of splenic NK cells expressed CD69, an activation marker in response to infection in *Asc*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> relative to C57BL/6 and *Aim2*<sup>+/+</sup> controls, respectively (Fig. 7e). Of note, NK cell CD69 expression was enhanced in *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice (mixed 129 $\times$ B6 background) relative to mice on a pure C57/B16 background (supplemental fig. 3).

In C57BL/6 mice, which are resistant to mCMV, NK cell control of early virus replication is mediated in large part through recognition of mCMV m157 protein by the activating NK cell receptor, Ly49H. Although the expression (MFI) of Ly49H expression was slightly reduced in both *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> littermates on a mixed 129 $\times$ B6 background compared to C57BL/6 and *Asc*<sup>-/-</sup> mice (supplemental Fig. 3), there were no significant differences in the proportion of Ly49H<sup>+</sup> NK cells within paired groupings of 129 $\times$ B6 littermates (48  $\pm$  9 (*Aim2*<sup>+/+</sup>) vs 52  $\pm$  4 (*Aim2*<sup>-/-</sup>),  $p = 0.45$ ,  $n = 4$ ) or C57BL/6 background mice (68  $\pm$  4 (*Aim2*<sup>+/+</sup>) vs 68  $\pm$  1 (*Aim2*<sup>-/-</sup>),  $p = 0.98$ ,  $n = 4$ ). Importantly, both the Ly49H<sup>+</sup> and Ly49H<sup>-</sup> subsets of splenic NK cells displayed reduced spontaneous *ex vivo* IFN- $\gamma$  production in mCMV-infected *Asc*<sup>-/-</sup> (Fig. 7f) and *Aim2*<sup>-/-</sup> (Fig. 7g) mice relative to C57BL/6 and *Aim2*<sup>+/+</sup>, respectively. Thus, the reduced IFN- $\gamma$  response from NK cells in *Aim2* inflammasome defective mice (*Asc*<sup>-/-</sup> and *Aim2*<sup>-/-</sup>) could not be attributed to the reduction of cells that can be activated by mCMV-encoded ligands for Ly49H. Following *in vitro* PMA and ionomycin stimulation, NK cells (both Ly49H<sup>+</sup> and Ly49H<sup>-</sup>) from spleens of mCMV-infected C57BL/6 and *Asc*<sup>-/-</sup> (Fig. 7f) as well as *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> littermate mice produced similarly high amounts of IFN- $\gamma$  (Fig. 7g). These data indicate that NK cells in *Asc*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> mice are not intrinsically defective, but rather that their impaired IFN- $\gamma$  responses are likely due to the decreased systemic IL-18 production during mCMV infection.

As NK cell-derived IFN- $\gamma$  is critical for early control of mCMV replication *in vivo*, we next determined viral loads in the spleens of infected mice at 36 hours post-infection. Relative to C57BL/6 mice, *Asc*<sup>-/-</sup> mice demonstrated elevated viral titers at this early time point (Fig. 7h). Since mice of mixed background have higher viral titers relative to B6 mice, to evaluate the role of *Aim2* in viral control, we used a lower dose ( $1 \times 10^5$ , rather than  $1 \times 10^6$  PFUs of mCMV). Like the *Asc*<sup>-/-</sup> mice, *Aim2*<sup>-/-</sup> mice demonstrated dramatically elevated viral titers at this early time point (Fig. 7i). Importantly, IL-18 levels in the serum and NK cell-dependent IFN $\gamma$  production were reduced in *Aim2*<sup>-/-</sup> relative to *Aim2*<sup>+/+</sup> at this lower dose (data not shown). Collectively, these data reveal the essential role of the *Aim2*-*Asc* inflammasome in early control of mCMV infection through IL-18 and NK-cell dependent IFN- $\gamma$  production.

## Discussion

Considerable progress has been made in defining the molecular basis for the immune stimulatory activity of microbial DNA. TLR9 recognizes CpG-DNA and resides in the

endosomal compartment (reviewed in <sup>37</sup>). Additional TLR9-independent pathways have also emerged. These include an IFN inducing-pathway and a pathway leading to the conversion of the zymogens pro-IL-1 $\beta$  and IL-18 into the active mature cytokines. The IFN inducing pathway is complex and involves RNA polymerase III, and one or more redundant sensors, which collectively converge on a signaling axis involving STING<sup>38</sup>, TANK Binding Kinase-1<sup>39</sup> and the Interferon Regulatory Factors (IRF3 and IRF7) (reviewed in <sup>37</sup>). The IL-1-IL-18 pathway was recently shown to involve AIM2, which binds its ligand DNA directly and engages ASC to form a caspase-1-activating inflammasome (ref <sup>9-12</sup>). Whether AIM2 is the sole sensor for this pathway is unknown.

Herein, we have characterized the role of Aim2 in DNA responses by generating mice lacking *Aim2*. By monitoring IL-1 $\beta$  responses, our studies reveal the essential and non-redundant role for Aim2 in DNA-dependent caspase-1 activation and IL-1 $\beta$ -IL-18 maturation in both macrophages and dendritic cells. Although overproduction of IL-1 $\beta$  can have deleterious consequences for the host if not well-regulated<sup>13</sup>, the importance of IL-1 $\beta$  in anti-microbial defenses is highlighted by the enhanced susceptibility of IL-1R-deficient mice to various pathogens<sup>40, 41</sup> and by the ability of viruses to evade the production and/or action of these cytokines<sup>42-44</sup>. By studying IL-1 $\beta$  responses in *Aim2*-deficient mice, we revealed a central role for Aim2 in regulating IL-1 $\beta$  in response to *F. tularensis* LVS. Previous work had revealed the importance of type I IFNs, ASC and caspase-1 in innate responses to *Ft*<sup>25</sup>. However, the precise mechanisms by which *Ft* triggered caspase-1 activation were unclear<sup>22, 45</sup>. Our studies identify Aim2 as the sole mediator of inflammasome activation by *Ft*. *Ft* also elicits IFN- $\beta$  production, events which are not mediated by Aim2. The sensor regulating IFN- $\beta$  induction and whether DNA is also the activating ligand is unknown. Our data also reveal a contribution of the Aim2 pathway in sensing *L. monocytogenes*. In this case the role of Aim2 is partial, consistent with redundant NLR sensing mechanisms. Indeed, Aim2 and Nlrp3 appear to collaborate for sensing *L. monocytogenes*, since knockdown of Aim2 in Nlrp3-deficient macrophages abolished all inflammasome activity (S. Kim, F., Bauernfeind, A. Ablasser, G. Hartmann, K. Fitzgerald, E., Latz and V., Hornung, manuscript in press E. J. I).

The most important finding from our data is the observation that the Aim2-Asc pathway is crucial for caspase-1 dependent signaling and innate immunity to DNA viruses, particularly mCMV. Using a mouse model of mCMV, we found a critical role for Aim2 and Asc in regulating IL-18 production and NK cell-dependent IFN- $\gamma$  production. These events are critical for the early innate response to infection, as reflected by the higher viral titres in *Aim2* and *Asc*-deficient mice relative to their wild type counterparts. It is important to note that *Aim2*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> mice are on a mixed 129 $\times$ B6 background, which are more susceptible to mCMV than C57BL/6 mice, which encode *Cmv-1*, an autosomal dominant gene on chromosome 6 conferring mCMV resistance<sup>46</sup>. Defects associated with DAP12 and possibly other signaling pathways on the 129 background may also contribute to higher susceptibility of 129 $\times$ B6 mice to mCMV<sup>47</sup>. In our studies, the use of *Aim2*<sup>+/+</sup> littermate controls (derived from breedings of heterozygous parents), which showed similar expression of the NK cell receptors (NK1.1 and Ly49H; the activating NK receptor encoded by the *Cmv-1* locus on chromosome 6<sup>36, 48, 49</sup>) on both *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> littermates, permitted



us to properly control for the influence of genetic background on resistance to mCMV and assess the importance of Aim2 in early antiviral responses. Moreover, the similar reductions in systemic IL-18 and NK cell-dependent IFN- $\gamma$  production as well as the enhanced viral burden in *Asc*<sup>-/-</sup> mice fully backcrossed onto the C57BL/6 background, support our argument that the Aim2- and Asc-dependent induction of IL-18 contributes to early control of mCMV replication. Moreover, these data are consistent with published studies in IL-18R-deficient mice<sup>35</sup>. A key question rising from these studies relates to how *Aim2*, which is localized to the cytosolic compartment, gains access to mCMV DNA, since mCMV replicates in the nucleus. Whether the incoming virion or newly synthesized virions are being sensed is unclear at present. One possibility is that as virions are disassembled or assembled, DNA may gain access to the cytosol if the capsid proteins fail to perfectly protect/sequester the DNA.

While it is not surprising that organisms have evolved mechanisms to sense microbial DNA and trigger innate immunity, our understanding of how cells discriminate between self and non-self DNA is still limited. Normal defense strategies that discriminate between host and pathogenic nucleic acids are not perfect and fail under certain circumstances. This perturbation in normal control may contribute to the pathogenesis of autoinflammatory and autoimmune diseases such as Systemic Lupus Erythematosus (reviewed in<sup>37</sup>). Normally, mammalian DNA is sequestered in the nucleus or mitochondria of cells. An exception however is during replication or cell death when the integrity of the nuclear envelope is compromised thereby exposing the cytosolic compartment to free DNA. DNA found free outside cells, within endosomes or within the cytosol is degraded by DNaseI, DNaseII and DNaseIII (also called TREX1). Failure of these systems to function (due to mutation, deletion etc) can lead to aberrant accumulation of DNA and inappropriate immune activation. Whether Aim2 senses aberrant host DNA and thus contributes to the pathogenesis of SLE awaits investigation. Further characterization of the Aim2 inflammasome, will likely yield new insights into microbial pathogenesis as well as autoinflammatory and autoimmune diseases and enable the rational design of new therapies and treatments for a number of debilitating conditions.

## Materials and Methods

### Reagents

ATP, LPS, nigericin and poly(dA-dT) were from Sigma-Aldrich. Anthrax lethal toxin was from List Biologicals (5  $\mu$ g-ml<sup>-1</sup> PA and 5  $\mu$ g-ml<sup>-1</sup> LF). Vaccinia virus (Western Reserve strain) was from G. Barton (U. of Berkeley, CA). MCMV (Smith strain) was from R. Welsh (UMASS Medical School, MA). HSV-1 was from D. Knipe, (Harvard Medical School, Boston, MA). Sendai virus (Cantrell strain) was from Charles River Laboratories (Boston, MA). *Francisella tularensis* (*Ft*) Live Vaccine Strain (LVS) (ATCC 29684) was prepared as described<sup>50</sup>. *Salmonella typhimurium* (SL1344 lab strain) was from Mary O'Riordan (U. Of Michigan, Ann Arbor, MI). *L. monocytogenes* (clinical isolate 10403s) was from V. Boyartchuk (UMASS Medical School, MA). Anti-murine Aim2 polyclonal antibody was from E. Alnemri (Thomas Jefferson University, PA).

## Plasmid constructs

Full length human AIM2 was as described<sup>10</sup>. Full length murine Aim2 and murine Asc were from Invivogen (San Diego, CA). Expression plasmids (pCI) encoding human Caspase-1 and ASC-HA were from Millenium Pharmaceuticals (Cambridge, MA)<sup>10</sup>.

## Mice

ES cells harboring a genetrapp insertion in the *Aim2* gene were from the international genetrapp consortium and were microinjected into C57BL/6 blastocysts. Chimeric offspring were backcrossed to C57BL/6 mice and germline transmission was confirmed by PCR with genetrapp specific primers. Long PCRs were performed with a 5' exon5 primer combined and a 3' genetrapp vector primer to amplify the gene trap integration site in the *Aim2* gene and the amplified bands were examined by sequencing. This revealed that the gene-trap vector inserted into intron 6 of the *Aim2* gene. Due to duplication of part of *Aim2* gene at the 3' end of the integration site, we were unable to distinguish heterozygous from homozygous KO mice by PCR on genomic DNA. To definitively determine homozygous KOs, we performed reverse transcription PCR on RNA extracted from blood with following primers: F- CACACTCGACGTGGCAGATAGGAC; R1- CAGCACCGTGACAACAAGTGG; R2- TCGATGCGATCTGCGTTCTTC. Primers F and R1 amplified wild type *Aim2* transcripts and F and R2 amplifies Genetrapp-inserted *Aim2* transcripts. *Pycard* (*Asc*)<sup>-/-</sup> mice were from Millennium Pharmaceuticals and were backcrossed into C57BL/6 background. *Irf3*<sup>-/-</sup> *Irf7*<sup>-/-</sup> DKO mice were generated at UMASS Medical School from *Irf3*<sup>-/-</sup> and *Irf7*<sup>-/-</sup> mice obtained from T. Taniguchi (Tokyo, Japan). C57BL/6 mice were from Jackson Laboratories (Bar Harbor, ME) and were bred at UMASS. All mouse strains were bred and maintained under specific pathogen-free conditions in the animal facilities at the UMASS Medical School and were carried out in accordance with the guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institutional Animal Care and Use Committee.

## Cell culture and stimulation

Thioglycollate elicited peritoneal macrophages (TEM) were isolated from mice 4 days after i.p. injection of sterile 3% thioglycollate (Remel, Lenexa, KS). BMDM and BMDCs were generated as described<sup>10, 51</sup>. Cells were first primed with 200 ng/ml LPS for 3-4 h prior to treatment with different agents. ATP (5 mM) or Nigericin (10 μM) was added one h prior to harvesting supernatants. Poly(dA-dT) DNA was transfected using Lipofectamine 2000 at a concentration of 1.5 μg/ml. The doses of viruses used were as follows unless otherwise stated; vaccinia virus, MOI of 0.5 – 5; CMV, MOI of 5 for splenocytes and 10 for macrophages and DCs; HSV, MOI of 10 or 40. For experiments involving *F. tularensis* (*Ft*) LVS, *S. typhimurium* or *L. monocytogenes*, cells were cultured in antibiotic-free medium unless otherwise indicated. Macrophages were infected with *Ft* LVS (MOI = 50) for 4 h. After washing twice with PBS, infected cells were incubated for 45 min in medium containing 50 μg/ml gentamicin. Cells were washed twice with PBS and then incubated with serum free medium. For *Listeria*, cells were stimulated with MOI of 1 and 5 for 1 h and then medium was replaced with 100 μg/ml gentamicin containing medium. For *Salmonella*, 2 h post-infection, supernatants were collected and 50 μg/ml gentamicin containing medium was

added. After 1h, the medium was again replaced with 10µg/ml gentamicin containing medium and supernatants harvested after 3h.

## ELISA

Cell culture supernatants were assayed for IL-1β (BD Biosciences, Franklin Lakes, NJ), IL-18 (R & D Systems, Piscataway, NJ) by ELISA. Murine IFNβ sandwich ELISA was used as previously described<sup>52</sup>.

## Immunoblotting

Supernatants were harvested and precipitated by methanol chloroform extraction, and cell lysate was collected. Immunoblotting was conducted as previously described<sup>2,3</sup>, using anti-murine caspase-1 p10 (sc-514, Santa Cruz Biotechnology) or anti-murine IL-1β (R&D systems, Minneapolis, MN), rabbit polyclonal anti-murine Aim2 (E. Alnemri), anti-Flag (M2, Sigma), anti-Viperin (P. Cresswell, Yale, CT), anti-human β-actin (Sigma).

Quantitative real-time PCR for pro-IL1b as described previously<sup>22</sup>.

## Reporter assays

All reporter gene assays were conducted as described<sup>10</sup>.

## Cell Viability Assay

*Aim2<sup>+/+</sup>* and *Aim2<sup>-/-</sup>* cells were treated with poly(dA-dT) as described above and the cell viability was assessed at 24 h by calcein staining as described<sup>10</sup>.

## In vivo mCMV infection

The Smith strain of mCMV was propagated in vivo in salivary glands of Balb/c mice<sup>53</sup>. Infected salivary gland homogenates were diluted in HBSS and mice were inoculated intraperitoneally with  $1 \times 10^6$  plaque-forming units (PFUs) or  $1 \times 10^5$  PFUs (Fig 7i), as titrated on mouse alpha-1,3-galactosyltransferase knockout fibroblast monolayers.

## Immunofluorescence Staining and Intracellular IFN-γ Assay

Leukocytes were isolated and stained for the intracellular IFN-γ using reagents and the protocol of BD PharMingen. Two million cells were cultured in medium containing GolgiPlug at 0.2 µl per sample in the presence or absence of 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h at 37°C. Cells were washed using FACS<sup>®</sup> buffer and incubated for 5 min at 4°C in 100 µl of FACS<sup>®</sup> buffer plus 0.5 µl of anti-FcγII receptor Ab. Cells were then washed and incubated at 4°C for 30 min with combinations of anti-NK1.1-PerCPCy5.5 (BD PharMingen), anti-CD3-FITC (BD PharMingen), anti-NKp46-PE (eBioscience), anti-CD8a-Alexafluor700 (eBioscience), anti-CD69-PE-Cy7 (BD PharMingen), and anti-Ly49H-Alexafluor647 (eBioscience) mAb. Samples were washed twice, fixed and permeabilized using 100 µl of cytofix/cytoperm (BD PharMingen) solution for 20 min at 4°C. After fixation the samples were washed twice using perm/wash solution and stained with rat IgG1-Pacific Blue mAb to mouse IFN-γ (BD PharMingen) for 30 min at 4°C. Samples were then washed twice using perm/wash and once using FACS<sup>®</sup> buffer for

analysis on a LSRII. Data analysis was done using FlowJo (Treestar Inc.) software. For these analyses, 100–200,000 events were calculated, ensuring a sizable NK cell population for a valid analysis of NK subsets.

### Plaque assay

Viral loads in the spleen were titrated by plaque assay on mouse alpha-1,3-galactosyltransferase knockout fibroblast monolayers.

### Statistical analysis

Two-way analysis of variance followed by the Bonferroni post-test was performed unless otherwise stated using Prism Software. *P* values of < 0.05 were considered significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

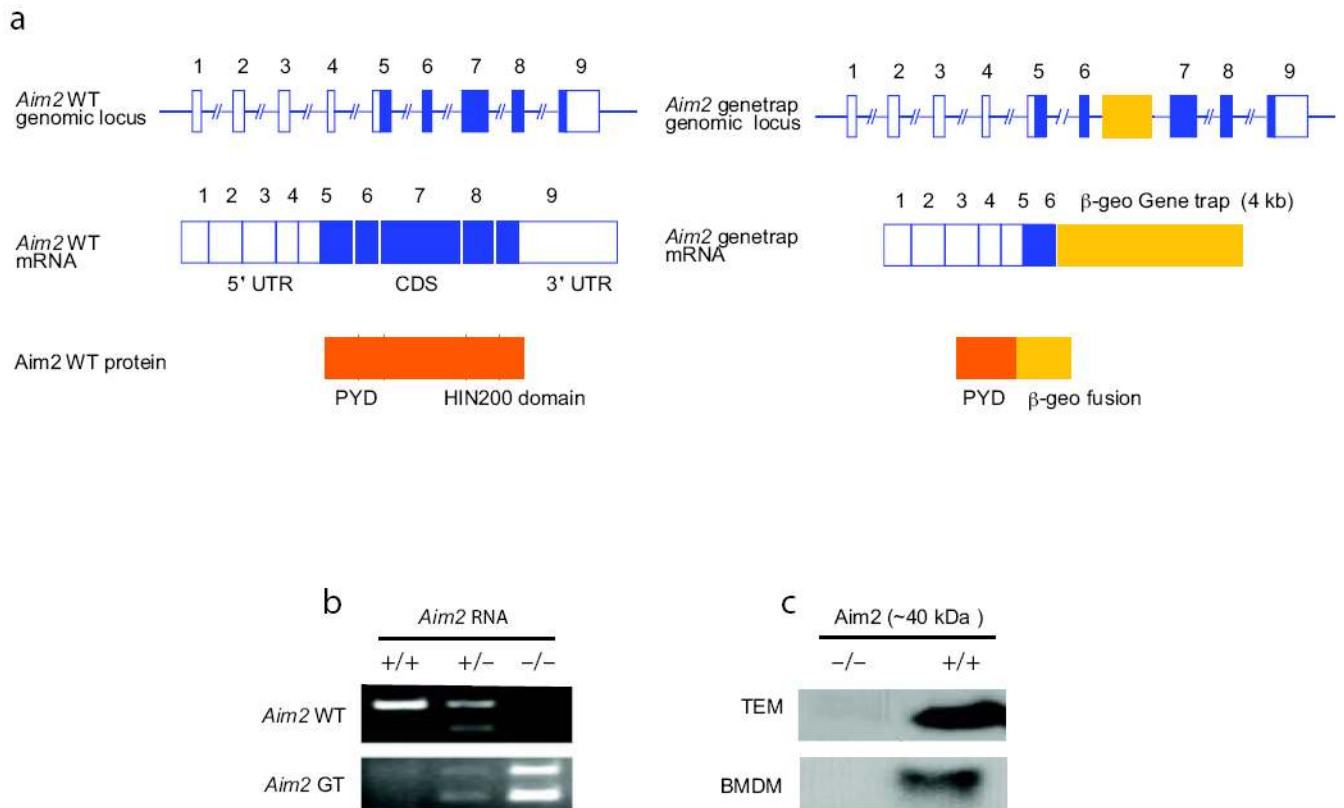
The authors would like to thank A. Cerny for animal husbandry and genotyping, A. Poltorak for guidance and help with genotyping mice, R. Barbalat and G. Barton for VV, S. Schattgen for assistance with cell viability assays, M. Pickering and T. Kowolik for helpful discussions, E. Alnemri and T. Fernandez-Alnemri (Thomas Jefferson University, Philadelphia) for helpful discussions and the anti-Aim2 antibody and E. Lien for critical reading of the manuscript. This work is supported by NIH grants AI083713 (to K.A.F and E.L), CA66644 (to E. T. S) AI07349 (to S. W), CA034461 (to R. W), U54 AI-157168 (to S.N. V) and a NERCE Fellowship to V.A.R.

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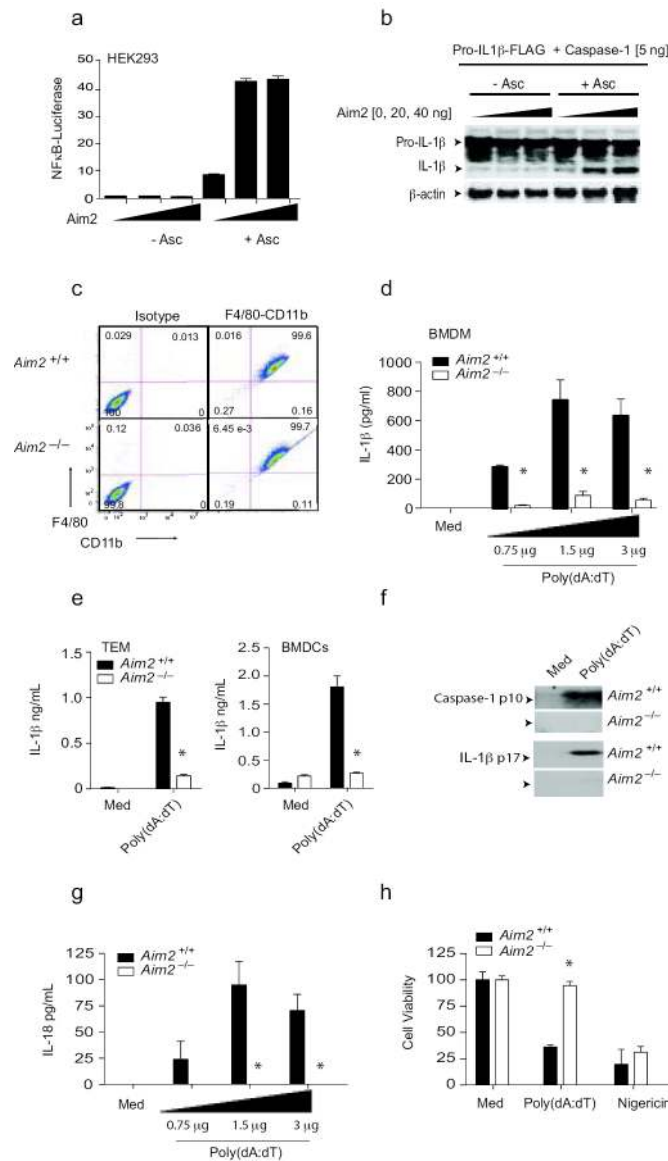
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**Figure 1.** Characterization of *Aim2*-genetrapped mice. **a** Schematic of wild-type and gene-trapped *Aim2* allele. **b**. RT-PCR analysis shown the absence of wild-type (WT) *Aim2* transcripts in the *Aim2*-genetrapped mice. WT *Aim2* RNA was detected by primers F and R1 and gene-trapped *Aim2* RNA was detected by primers F and R2. **c**. Immunoblotting of *Aim2* protein in peritoneal and bone marrow derived macrophages from *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice. Equal amounts of protein were loaded for all the samples and *Aim2* was detected using a rabbit polyclonal antibody.

**Figure 2.**

*Aim2* is essential for inflammasome activation by DNA. **a.** 293T cells were transfected with indicated plasmids together with a NF- $\kappa$ B reporter and TK Renilla-luciferase reporter gene. After 48 h, luciferase activity was measured. **b.** 293T cells were transfected with the indicated plasmids together with murine pro-IL-1 $\beta$ -Gluc/Flag. After 24 h, cell lysates were immunoblotted for pro-IL1 $\beta$ -Flag, IL-1 $\beta$ -Flag and  $\beta$ -actin. **c.** F4/80 and CD11b expression by BMDM from *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice. **d-f.** LPS (200 ng/ml) primed *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> BMDM, thioglycollate-elicited macrophages (TEM), and BMDCs were transfected with poly(dA-dT) (0.75 – 3  $\mu$ g/10<sup>6</sup> cells) for 6 h. The secreted IL-1 $\beta$  (**d-e**) and the cleaved forms of IL-1 $\beta$  and caspase 1 (**f**) in the culture supernatants were analyzed. **g.** *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> BMDM were treated as above and the IL-18 levels in the supernatants at 6 h were measured by ELISA. (**h.**) *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> TEM were treated as indicated for 24 h and cell viability was reported as % of viability of medium treated cells. Asterisks indicate *P*



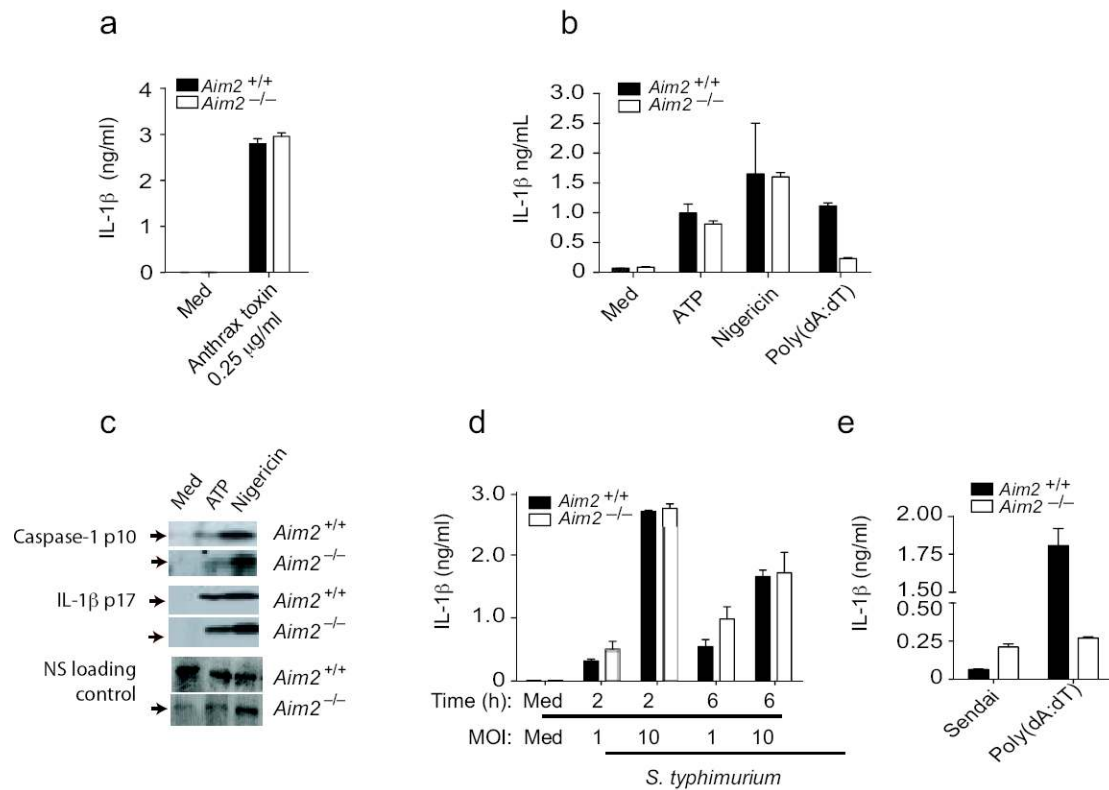
values of  $< 0.05$  for *Aim2*<sup>+/+</sup> versus *Aim2*<sup>-/-</sup>. Data are presented as mean  $\pm$  SD from one experiment representative of 3 experiments.

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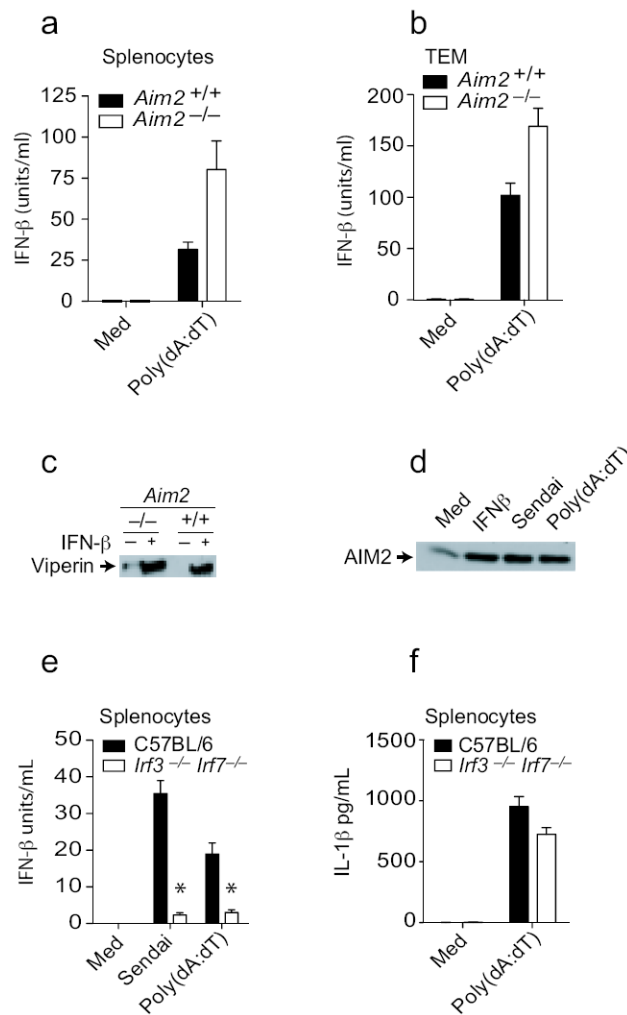
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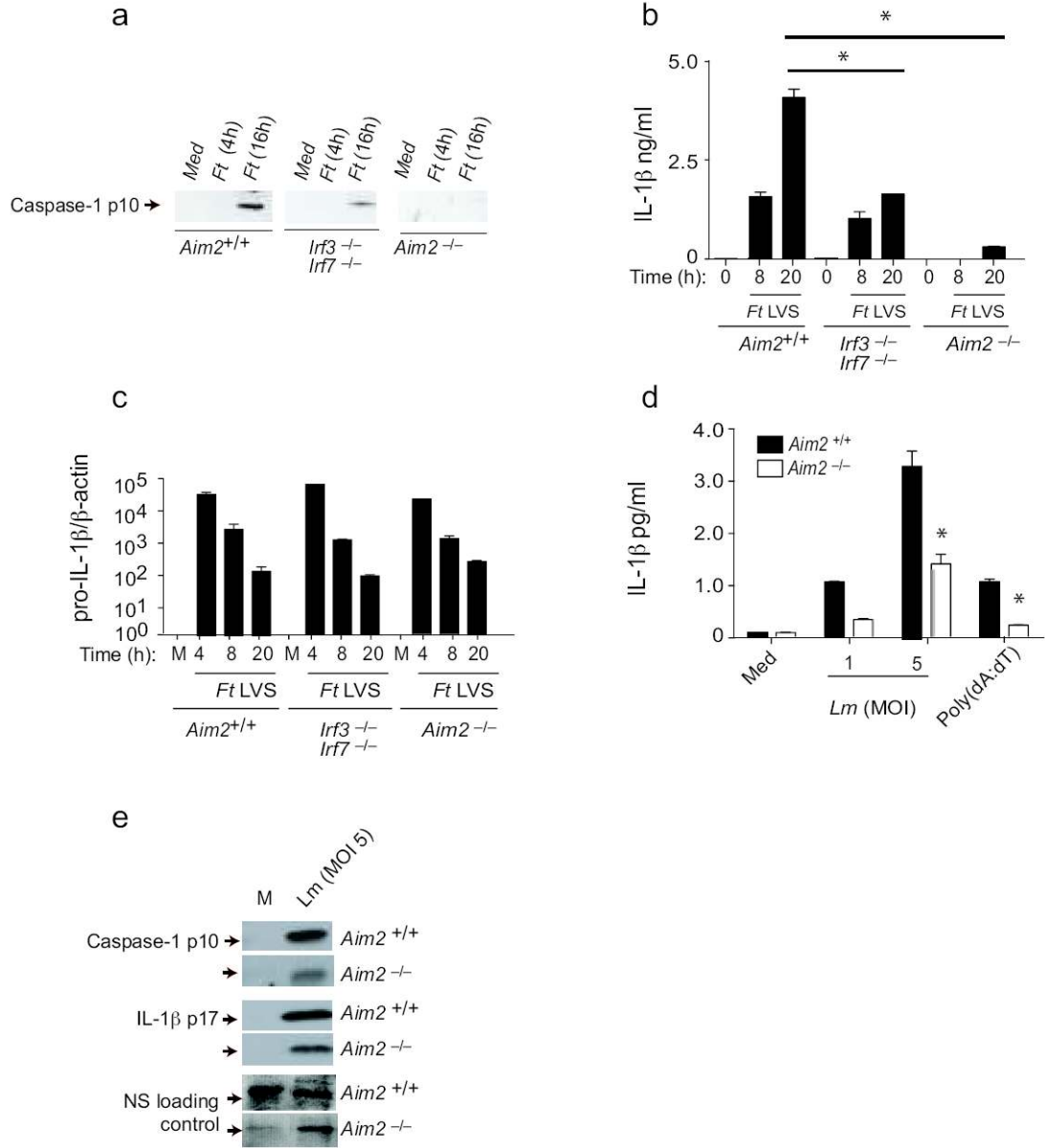
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**Figure 3.**

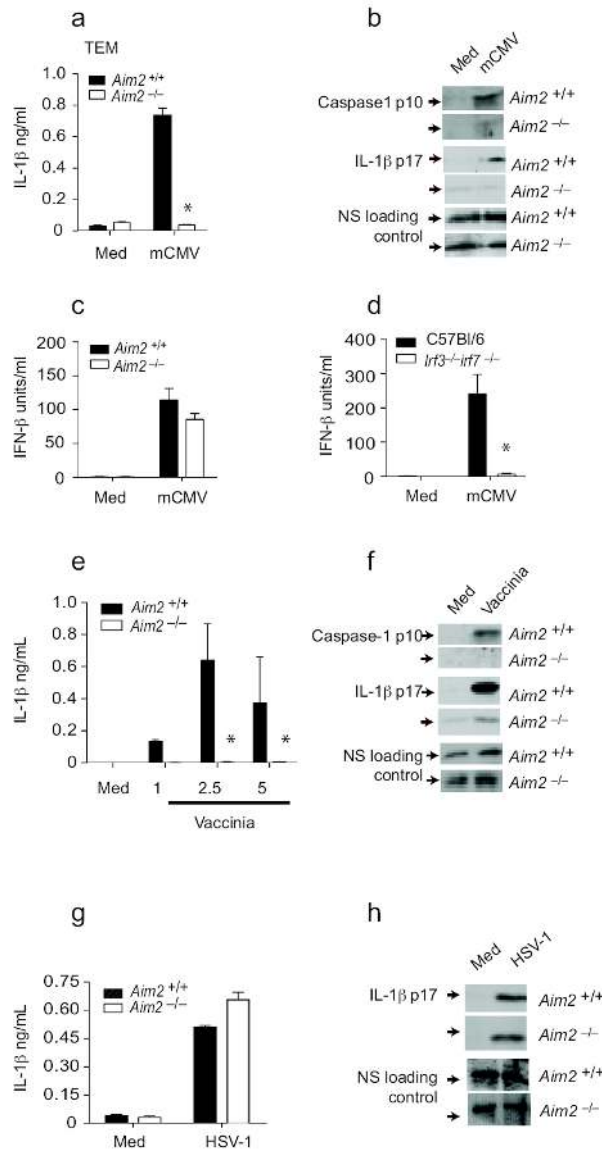
*Aim2*-deficient cells respond normally to other inflammasome activating stimuli. a. BMDM from *Aim2*<sup>+/+</sup>, *Aim2*<sup>-/-</sup>, and C57Bl/6 mice were primed with LPS (200 ng/ml) for 3 h and stimulated with anthrax toxin units PA and LF. Supernatants were harvested after 6 h and analyzed by ELISA for IL-1 $\beta$  production. b-c. LPS primed *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> TEM were treated with ATP (5 mM) and Nigericin (5  $\mu$ M) for 1 h and secretion of IL-1 $\beta$  and cleavage of IL-1 $\beta$  and caspase 1 were analyzed. d. *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> BMDM were primed with LPS (200 ng/ml) for 3 h and infected with *S. typhimurium* at MOI 1 and MOI 10. Supernatants were harvested at 2 and 6 h post infection and IL-1 $\beta$  levels were analyzed by ELISA. e. BMDCs were treated with Sendai virus (200 HA units/ml) for 18 h and IL-1 $\beta$  concentrations in the supernatant were measured by ELISA. NS; nonspecific band. Asterisks indicate *P* values of < 0.05 for *Aim2*<sup>+/+</sup> versus *Aim2*<sup>-/-</sup>. Data are presented as mean  $\pm$  SD from one experiment representative of 3 experiments.

**Figure 4.**

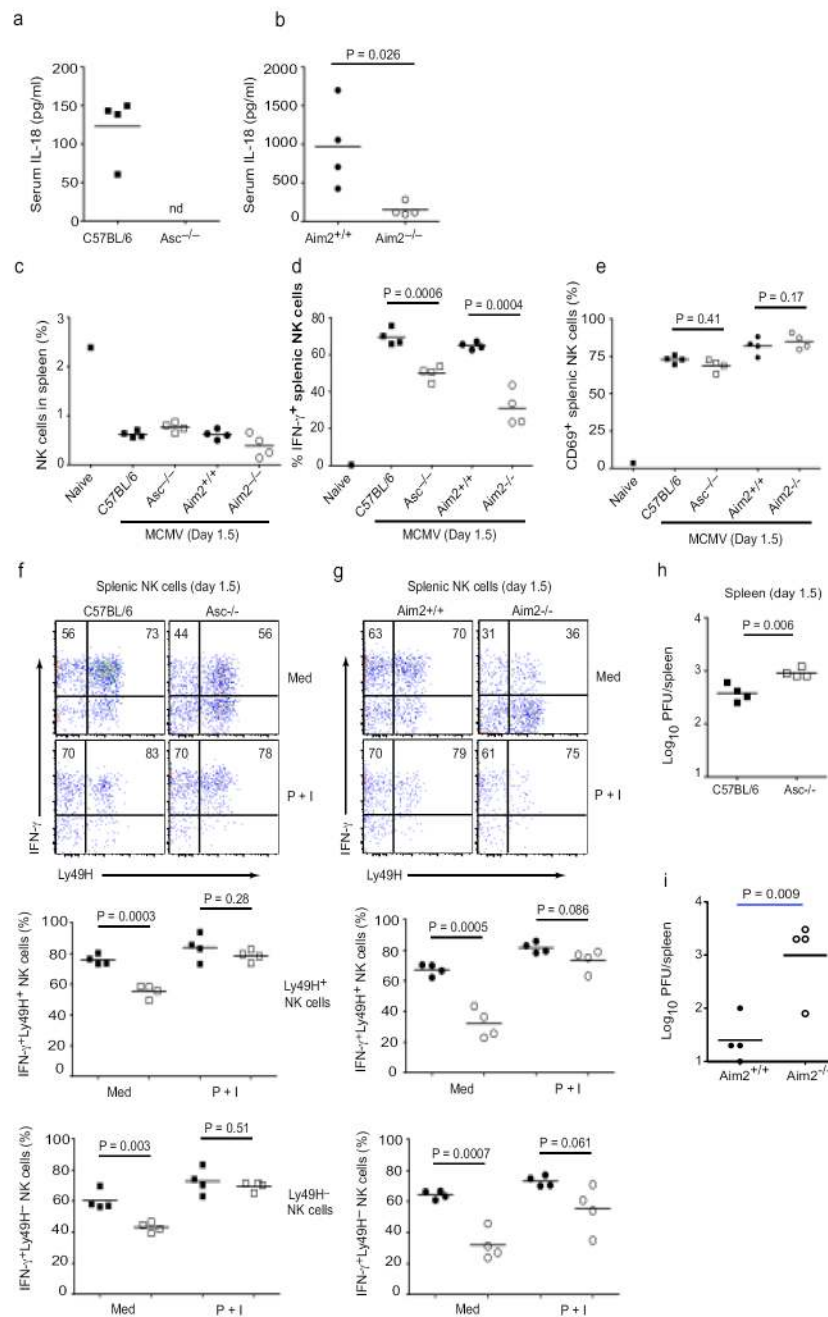
Type I interferon production and signaling remain intact in *Aim2*-deficient cells. A and e-f. Splenocytes from *Aim2*<sup>+/+</sup>, *Aim2*<sup>-/-</sup>, *Irf3*<sup>-/-</sup> *Irf7*<sup>-/-</sup> and C57BL/6 mice were plated at  $2 \times 10^5$  cells/well in 96-well plates in antibiotic free DMEM and were treated as indicated above. After 24 h, supernatants were collected and analyzed for IFN- $\beta$  concentrations. b. TEM from *Aim2*<sup>+/+</sup>, *Aim2*<sup>-/-</sup>, *Irf3*<sup>-/-</sup> *Irf7*<sup>-/-</sup>, and C57BL/6 mice were treated as indicated above and supernatants were analyzed for IL-1 $\beta$  and IFN- $\beta$  concentrations 6 h later. c. BMDM from *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice were treated with 1000 units/ml IFN $\beta$  for 18 h and the viperin protein level in the lysates was analyzed by immunoblotting. d. TEM from C57BL/6 mice were treated with 1000 units/ml IFN $\beta$ , 200 HA units/ml Sendai virus, and poly(dA-dT)1.5  $\mu$ g/ $10^6$  cells for 18 h and the Aim2 protein amounts in lysates was analyzed. Asterisks indicate *P* values of < 0.05 for *Aim2*<sup>+/+</sup> versus *Aim2*<sup>-/-</sup>. Data are presented as mean  $\pm$  SD from one experiment representative of 3 experiments.



**Figure 5.** Aim2 mediates inflammasome activation in response to bacterial pathogens. TEM from *Aim2*<sup>+/+</sup>, *Aim2*<sup>-/-</sup>, *Irf3*<sup>-/-</sup> *Irf7*<sup>-/-</sup> and C57Bl/6 mice were infected with *F. tularensis* LVS (MOI = 50) as indicated in the materials and methods. At indicated time points after infection, cleaved caspase 1 (a) and IL-1β (b) in the supernatants and the mRNA levels of pro-IL-1β (c) were measured. d-e. LPS primed *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> TEM were infected with *L. monocytogenes* at MOI of 1 and 5 and supernatants were analyzed for secreted IL-1β by ELISA (d) and cleaved caspase 1 and IL-1β by immunoblotting (e). NS; nonspecific band. Asterisks indicate *P* values of < 0.05 for *Aim2*<sup>+/+</sup> versus *Aim2*<sup>-/-</sup>. Data are presented as mean ± SD from one experiment representative of 2 or 3 experiments.

**Figure 6.**

*Aim2* is essential for inflammasome activation by DNA viruses. a-b. *Aim2*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> TEM were primed with LPS for 3 – 4 h and stimulated with mCMV at an MOI of 10 for 20 h. IL-1β concentrations were measured by ELISA and cleaved caspase 1 p10 and IL-1β p17 in the supernatants by immunoblotting. c-d. TEM from *Aim2*<sup>+/+</sup>, *Aim2*<sup>-/-</sup>, *Irf3*<sup>-/-</sup> *Irf7*<sup>-/-</sup> and C57Bl/6 mice were stimulated with CMV as indicated above and IFN-β concentrations were measured by ELISA. e-f. *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> BMDM and BMDCs were infected with vaccinia virus as described for mCMV and the supernatants were analyzed for caspase 1 and IL-1β. g-h. *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> TEM were LPS primed and treated with HSV-1 at an MOI of 40 and at 18 h post infection, IL-1β concentrations in the supernatants were determined by ELISA and immunoblotting. NS; nonspecific band. Asterisks indicate *P* values of < 0.05 for *Aim2*<sup>+/+</sup> versus *Aim2*<sup>-/-</sup>. Data are presented as mean ± SD from one experiment representative of 3 experiments.

**Figure 7.**

*Aim2*- and *Asc*-dependent IL-18 production and NK cell IFN- $\gamma$  production. **a-b.** Serum IL-18 concentrations in C57BL/6, *Asc*<sup>-/-</sup>, *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice infected with MCMV. **c** Proportions of NK1.1<sup>+</sup> NKp46<sup>+</sup> CD3<sup>-</sup> NK cells in the spleens of infected mice as well as an uninfected C57BL/6 control. **d** and **e** At 36 h p.i., splenocytes were cultured *ex vivo* in medium containing brefeldin A for 4 h with no additional stimuli. Intracellular IFN- $\gamma$  (**d**) and surface CD69 (**e**) expressions were determined on total splenic NK cells. **f-g.** Splenocytes cultured in either medium without additional stimuli or medium containing PMA (50 ng/mL) and ionomycin (500 ng/mL) (denoted P + I) were stained for surface

Ly49H and intracellular IFN- $\gamma$ . Representative staining of gated NK cells is shown for **(f)** C57BL/6 and *Asc*<sup>-/-</sup> as well as **(g)** *Aim2*<sup>+/+</sup> and *Aim2*<sup>-/-</sup> mice. Numbers in upper quadrants are proportion of Ly49H<sup>+</sup> or Ly49H<sup>-</sup> NK cells that are IFN- $\gamma$ <sup>+</sup>. Proportions of Ly49H<sup>+</sup> and Ly49H<sup>-</sup> NK cells expressing IFN- $\gamma$  are shown for individual mice in lower plots. In lower panels, black squares are C57BL/6 mice and white squares are *Asc*<sup>-/-</sup>, while black circles are *Aim2*<sup>+/+</sup> and white circles are *Aim2*<sup>-/-</sup> **(h-i)** Viral titers in the spleen at 36 h p.i. nd; not detectable. *P* values were determined by unpaired two-tailed Student's T test. Data are from one experiment representative of 2 experiments.