

# Absent in melanoma 2 inflammasome is required for host defence against *Streptococcus pneumoniae* infection

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## Abstract

*Streptococcus pneumoniae*, a leading cause of invasive pneumococcal disease, is responsible for high mortality and morbidity worldwide. A previous study showed that the NLR family pyrin domain containing 3 (NLRP3) and absent in melanoma 2 (AIM2) inflammasomes are essential for caspase-1 activation and IL-1 $\beta$  production in the host response to *S. pneumoniae* infection. The function of NLRP3 in host innate immunity to *S. pneumoniae* was studied *in vivo* and *in vitro*. However, the role of AIM2 in host defence against *S. pneumoniae* remains unclear. Here, we show that AIM2-deficient (AIM2<sup>-/-</sup>) mice display increased susceptibility to intra-nasal infection with *S. pneumoniae* in comparison to wild type mice and that this susceptibility was associated with defective IL-1 $\beta$  production. Macrophages from AIM2<sup>-/-</sup> mice infected with *S. pneumoniae* showed impaired secretion of IL-1 $\beta$  as well as activation of the inflammasome, as determined by the oligomerisation of apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 activation. Taken together, these results indicate that the AIM2 inflammasome is essential for caspase-1-dependent cytokine IL-1 $\beta$  production and eventual protection from pneumococcal infection in mice.

## Keywords

*Streptococcus pneumoniae*, AIM2, inflammasome, IL-1 $\beta$

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## Introduction

*Streptococcus pneumoniae* causes high morbidity and mortality worldwide, especially in children, so it is a pathogen of important clinical significance. *S. pneumoniae* is an opportunistic bacterial pathogen that colonises the mucosal surfaces of the human upper respiratory tract.<sup>1</sup> Local spread, aspiration or seeding to the bloodstream, the bacteria will become pathogenic, causing invasive pneumococcal disease (IPD), such as pneumonia, bacteremia, meningitis or acute otitis media.<sup>2–4</sup> The pathogenesis and clearance of bacteria during *S. pneumoniae* infection are governed by complex host inflammatory responses.<sup>5</sup> Understanding the host response to *S. pneumoniae* is critical for the prevention and treatment of IPD.

The immune system is important in host defence against pathogens, especially innate immunity, which employs PRRs to recognise invading pathogens and

leads to a subsequent series of immunity responses. Growing evidence indicates that the inflammasome, an intracellular multimeric protein complex, plays an important role in host defence against *S. pneumoniae*.<sup>6–8</sup> *In vitro* experiments showed that the NLR family pyrin domain containing 3 (NLRP3) and absent in

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melanoma 2 (AIM2) inflammasomes are activated in *S. pneumoniae*-infected macrophages, and mediate IL-1 $\beta$  maturation and secretion.<sup>9,10</sup> Studies have been carried out to address the role of NLRP3 in pneumococcal infection using NLRP3<sup>-/-</sup> mice and indicated that NLRP3 contributes to bacterial defence and lung integrity during *S. pneumoniae* infection.<sup>11,12</sup> Our previous study also found that NLRP3 deficiency significantly impaired host defence against *S. pneumoniae*, with higher mortality and bacterial colonisation in the lungs.<sup>10</sup> However, the role of AIM2 in host defence against *S. pneumoniae* *in vivo* remains to be elucidated.

In this study, we employed an intra-nasal infection model of *S. pneumoniae* in wild type (WT) and AIM2<sup>-/-</sup> mice to examine the role of AIM2 in pneumococcal infection. We found that AIM2<sup>-/-</sup> mice showed higher mortality, higher bacterial colonisation and aggravated inflammation in the lungs, which suggests that AIM2 plays a protective role in host defence against *S. pneumoniae*. Meanwhile, using primary mouse macrophages as a cell model, we showed that AIM2 inflammasome activity is required for innate immunity to *S. pneumoniae* in macrophages, which may contribute to IL-1 $\beta$  secretion in broncho-alveolar lavage fluid (BALF).

## Materials and methods

### Mice

WT C57BL/6 mice were purchased from the Chongqing Academy of Chinese Materia Medica (Chongqing, PR China). AIM2<sup>-/-</sup> mice on the C57BL/6 background were kindly provided by Feng Shao from the National Institute of Biological Sciences (Beijing, PR China). All mice were maintained in specific pathogen-free conditions and used at 8–10 wk of age. All of the animal experiments were approved by the Ethics Committee of Southwest University and following laboratory animal care principles of the National Institutes of Health, PR China.

### Bacteria

The *S. pneumoniae* D39 (serotype 2) used in this study was kindly provided by Kohsuke Tsuchiya (Kanazawa University, Kanazawa, Japan). D39 was grown and prepared as previously described.<sup>10</sup>

### Intra-nasal infection of mice

WT and AIM2<sup>-/-</sup> mice were anaesthetised with pentobarbitone (Solarbio, Beijing, PR China), and then intra-nasally infected with  $5 \times 10^7$  bacteria in 20  $\mu$ l PBS. BALF was collected 12 h post infection according to the method of our previous studies.<sup>10</sup> BALF from non-infected mice was collected as a control.

Lung tissues were obtained 48 h post infection for bacterial CFU counting and histology. Lungs were homogenised in 1 ml PBS, serially diluted in PBS and plated onto blood agar to count CFU after 12 h culture. For histology, the lungs were fixed with 10% formaldehyde, embedded in paraffin and stained with hematoxylin and eosin (H&E). Histological changes were observed by microscopy. Survival of infected mice was monitored every day until 14 d post infection.

### Macrophages

The mice were injected intraperitoneally with 2 ml 4% thioglycollate medium (Eiken Chemical, Tokyo, Japan), and 3–4 d later, peritoneal exudate cells (PECs) were collected by peritoneal lavage, as reported previously.<sup>10</sup> After being washed with RPMI 1640 medium (Gibco, Gaithersburg, MD) and suspended in RPMI 1640 with 10% FCS, the cells were incubated in 48-well tissue culture microplates ( $2.5 \times 10^5$  cells/well) at 37°C plus 5% CO<sub>2</sub> for at least 2 h. Then, the cells were washed twice with RPMI 1640 plus 10% FCS, and adherent cells were used for *ex vivo* studies.

### Abs

Biotinylated anti-mouse IL-1 $\beta$  Ab was purchased from R&D Systems. Anti-caspase-1 (p20) mAb was purchased from AdipoGen (San Diego, CA). Anti-ASC-Ab was purchased from Cell Signaling Technology (Danvers, MA). Anti-actin Ab and streptavidin-HRP-conjugated mouse anti-goat IgG were purchased from Bioss (Beijing, PR China). HRP-conjugated goat anti-mouse IgG was purchased from ComWin Biotech Co. Ltd (Beijing, PR China).

### ELISA

Cells were infected with *S. pneumoniae* D39 at a MOI of 1 bacteria per cell for 6 h, and 100  $\mu$ g/ml gentamicin (Beyotime, Beijing, PR China) was added to the cultures. The cultures were then incubated for an additional 18 h. After incubation, cell-free culture supernatants were collected for measuring cytokines (IL-1 $\beta$ , IL-12, IL-6 and TNF- $\alpha$ ) by ELISA. ELISA kits were purchased from eBioscience, Inc. (San Diego, CA) and used according to the manufacturer's instructions. Absorbance was measured at 450 nm using a 680 ELISA reader (Bio-Rad Laboratories, Hercules, CA) and the software MicroPlate Manager v5.2.

### Western blot analysis

Cells were cultured on 12-well plates ( $1 \times 10^6$  cells/well) in RPMI 1640 plus FCS for 2 h, and then the medium was replaced with Opti-MEM (Invitrogen). Cells were

infected with D39 at a MOI of 1 for 6 h, and then 100 µg/ml gentamicin was added. Culture supernatants were collected 24 h after infection. To collect cell lysate, radio-immunoprecipitation assay (RIPA) buffer (Beyotime) was added to 12-well plates (200 µl/well) to lyse cells. After the RIPA buffer was added and the cells were scraped with a pipette, adherent macrophages were almost lysed and detached from the bottom. Protein concentrations were measured using a BCA Protein Assay Kit (Beyotime). The supernatants and the cell lysates were subjected to 12–15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After the membranes were blocked with 5% nonfat milk and immunoblotted with the appropriate Ab, distinct protein bands were detected using the ECL detection reagent (Beyotime). Images were obtained using the Tanon 4600 chemiluminescence detection device (Shanghai, PR China). Actin was employed as a loading control for the cell lysates.

### ASC oligomerisation

Cells were cultured on 12-well plates ( $1 \times 10^6$  cells/well). After different treatments, culture supernatants were collected, and cells were lysed with cold PBS containing 0.5% Triton X-100. The lysates were centrifuged at 6000 g for 15 min to harvest the cell pellet. The pellet was washed twice with cold PBS and suspended in 200 µl PBS and then cross-linked using 2 mM freshly prepared disuccinimidyl suberate for 30 min at 37°C. After pelleting by centrifugation at 6000 g for 15 min at 4°C, the cross-linked pellets were redissolved in 30 µl of 1×SDS-PAGE sample loading buffer. Samples were boiled for 5 min for Western blot analysis.

### Quantitation of macrophage-associated and phagocytosed *S. pneumoniae*

Adherent macrophages were infected with *S. pneumoniae* at a MOI of 1 for 2, 4, 6 or 8 h. To enumerate *S. pneumoniae* associating with macrophages, cells were washed with chilled PBS three times to remove non-associated bacteria and lysed in PBS containing 0.1% Triton X-100. The lysates were serially diluted in PBS and plated on blood agar plates to count CFU after overnight incubation. To enumerate the phagocytosed bacteria, the cell lysates were collected after macrophages were infected with *S. pneumoniae* as above and additionally cultured for 30 min in the presence of 100 µg/ml gentamicin, and the bacterial numbers were counted. To monitor the survival of *S. pneumoniae* inside macrophages, the numbers of intracellular bacteria were determined every 30 min until 3 h after gentamicin addition (6 h post infection).

### Statistical analysis

Statistical analyses were performed using GraphPad Prism software v6 (San Diego, CA), and the data are represented as the mean with standard deviation. All of the results are representative of three independent experiments. Student's *t*-test was used to analyse the significant differences between the two groups. Multi-group comparisons of mean values were made using ANOVA and the Bonferroni post hoc test. Statistical analysis for survival curves was performed using the log-rank test. Statistical significance was defined as  $P < 0.05$  (\* $P < 0.05$  and \*\* $P < 0.01$ ).

## Results

### AIM2 protects the host from *S. pneumoniae* infection *in vivo*

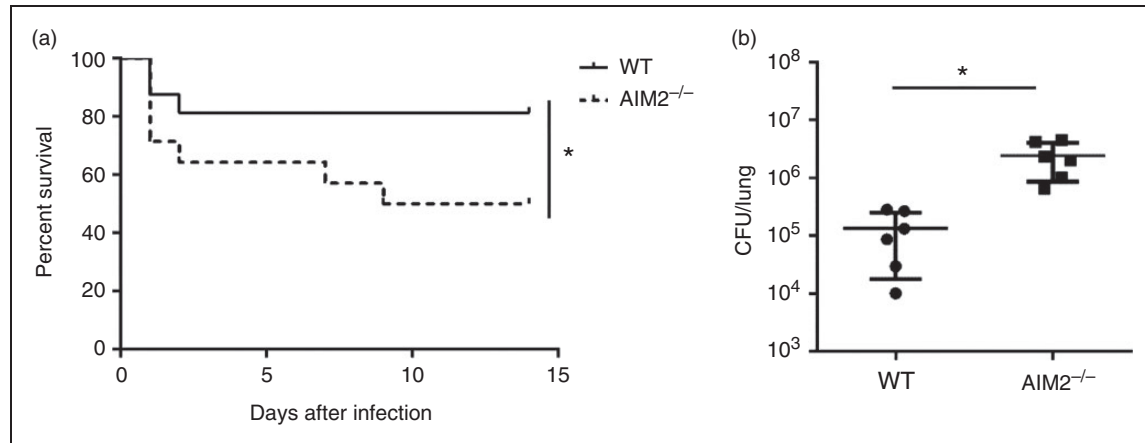
To define the role of AIM2 in host defence against *S. pneumoniae* infection, we employed an intra-nasal infection of *S. pneumoniae* in WT and AIM2<sup>-/-</sup> mice. The protective roles of AIM2 in *S. pneumoniae* infection were determined by survival curve and lung bacterial burdens. The results showed that 18.75% (3/16) of WT mice died within 2 wk, while AIM2<sup>-/-</sup> mice showed a higher mortality rate at 50% (7/14; Figure 1a). The bacterial burdens in the lungs of AIM2<sup>-/-</sup> mice were much higher than those in WT mice at 48 h after infection (Figure 1b). These results showed that AIM2 deficiency affects mouse survival and bacterial clearance upon *S. pneumoniae* infection.

Moreover, H&E staining showed that there was a more severe pulmonary inflammatory response in the AIM2<sup>-/-</sup> group (Figure 2). *S. pneumoniae* infection could cause inflammation in the lungs. In addition to oedema and haemorrhage, lungs from AIM2<sup>-/-</sup> mice displayed obvious histopathologic inflammatory reactions, such as infiltration of inflammatory cells, which suggests that AIM2 deficiency aggravates inflammatory reactions in *S. pneumoniae*-infected mouse lungs. Taken together, these results suggest that AIM2 plays an important role in protecting the host from *S. pneumoniae* infection *in vivo*.

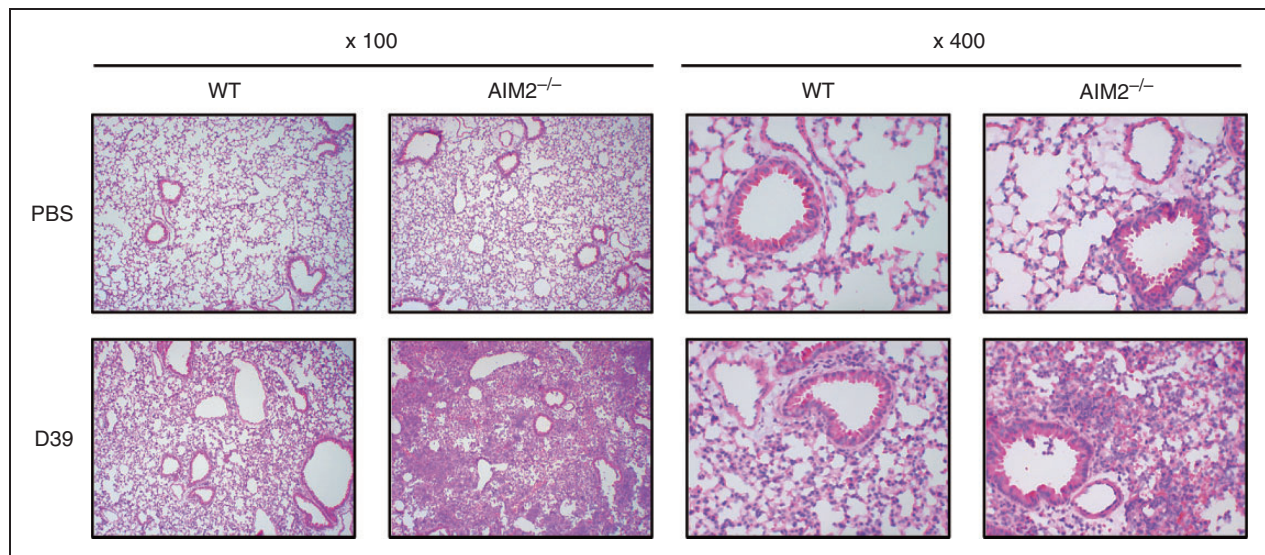
### IL-1β secretion requires AIM2 upon *S. pneumoniae* infection *in vivo*

To assess whether AIM2 has an impact on cytokine production upon *S. pneumoniae* D39 infection *in vivo*, the levels of cytokines in BALF at 12 h after infection were compared between WT and AIM2<sup>-/-</sup> mice. The concentrations of IL-1β in BALF were significantly lower in AIM2<sup>-/-</sup> mice than in WT mice after *S. pneumoniae* infection (Figure 3a), while IL-6 (Figure 3b),





**Figure 1.** Absent in melanoma 2 (AIM2) deficiency affects mouse survival and bacterial clearance upon *S. pneumoniae* infection (a and b). WT ( $n = 16$ ) AIM2<sup>-/-</sup> ( $n = 14$ ) mice were intra-nasally infected with *S. pneumoniae* D39 ( $5 \times 10^7$  CFU), and were monitored for 14 d (a). WT ( $n = 6$ ) or AIM2<sup>-/-</sup> ( $n = 6$ ) mice were intra-nasally infected with *S. pneumoniae* D39, lungs were homogenized and dilutions were plated on blood agar to count CFU 48 h post infection (b). Tests for statistical significance were performed using one-way ANOVA followed by the Bonferroni test (\* $P < 0.05$ ).



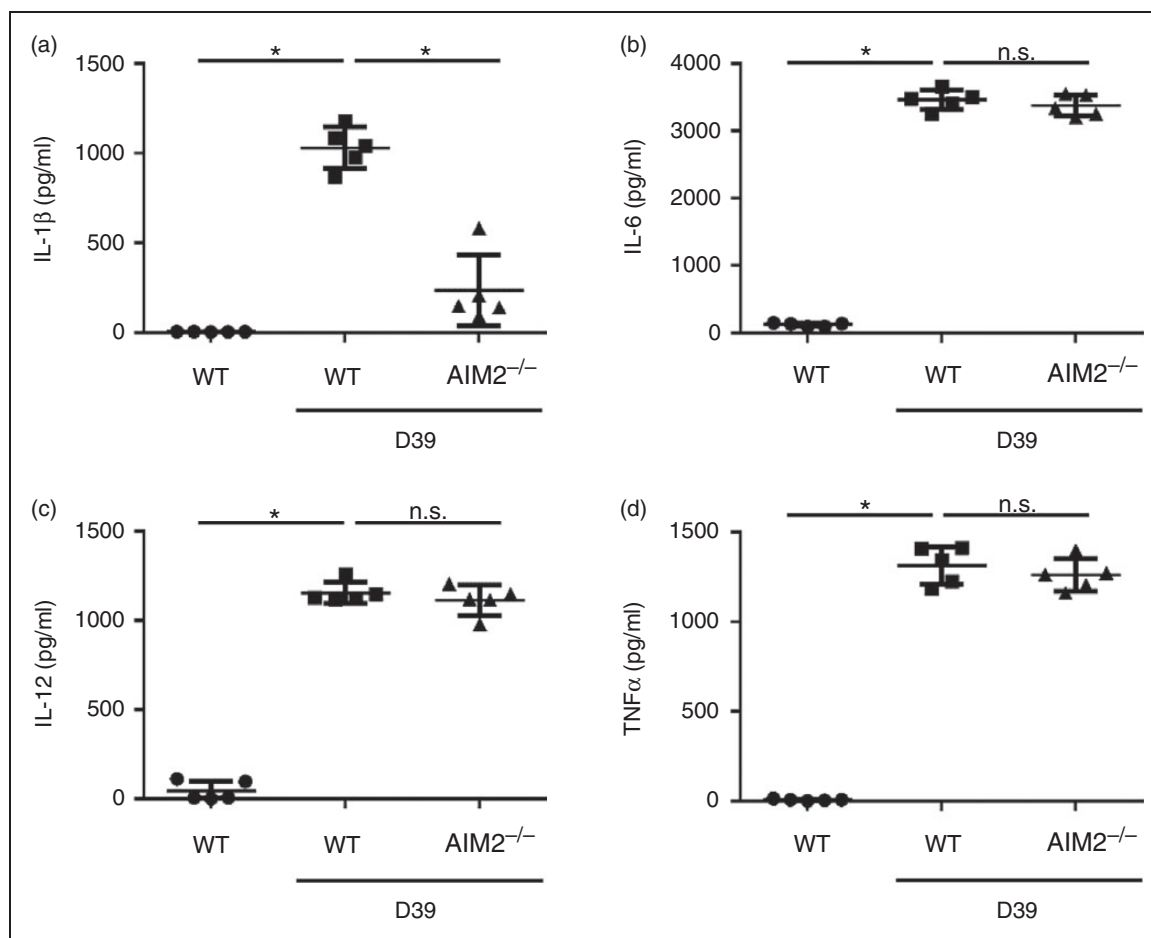
**Figure 2.** AIM2 deficiency aggravates the inflammatory reaction in *S. pneumoniae*-infected mouse lungs. WT and AIM2<sup>-/-</sup> mice were left uninfected or intra-nasally challenged with *S. pneumoniae* D39 ( $5 \times 10^7$  CFU), and the lungs were collected 48 h post infection for hematoxylin and eosin staining (original magnification 100 $\times$  and 400 $\times$ ). Pictures are representative of three mice from each group of mice.

IL-12 (Figure 3c) and TNF- $\alpha$  (Figure 3d) levels showed no difference. These results suggested that AIM2 is required for IL-1 $\beta$  production in the host response against *S. pneumoniae* infection *in vivo*.

#### AIM2 inflammasome is involved in IL-1 $\beta$ maturation and secretion in macrophages infected with *S. pneumoniae*

We next evaluated *ex vivo* IL-1 $\beta$  production by *S. pneumoniae*-infected macrophages. IL-1 $\beta$  maturation and

secretion are inflammasome dependent. So, we used primary mouse macrophages as a cell model and conducted experiments to assess whether AIM2 inflammasome is required for the secretion of IL-1 $\beta$  upon infection with *S. pneumoniae* D39. The secretion of IL-1 $\beta$  in AIM2<sup>-/-</sup> macrophages was decreased compared to that of WT macrophages, whereas TNF- $\alpha$  production was not affected (Figure 4a and b). Caspase-1 is essential for IL-1 $\beta$  maturation and secretion. As shown, a significant reduction in *S. pneumoniae*-induced ASC oligomerisation and caspase-1



**Figure 3.** AIM2 is involved in IL-1 $\beta$  secretion in the host response against *S. pneumoniae* infection *in vivo* (a–d). WT and AIM2<sup>−/−</sup> mice were left uninfected or intra-nasally challenged with *S. pneumoniae* D39 ( $5 \times 10^7$  CFU), and broncho-alveolar lavage fluid was collected 12 h post infection. The levels of IL-1 $\beta$  (a), IL-6 (b), IL-12 (c) and TNF- $\alpha$  (d) were measured by ELISA ( $n = 5$  independent experiments). Tests for statistical significance were performed using one-way ANOVA followed by the Bonferroni test (\* $P < 0.05$ ).

activation was observed in macrophages from AIM2<sup>−/−</sup> mice (Figure 4c), indicating that the AIM2 inflammasome is involved in IL-1 $\beta$  secretion in *S. pneumoniae*-infected macrophages.

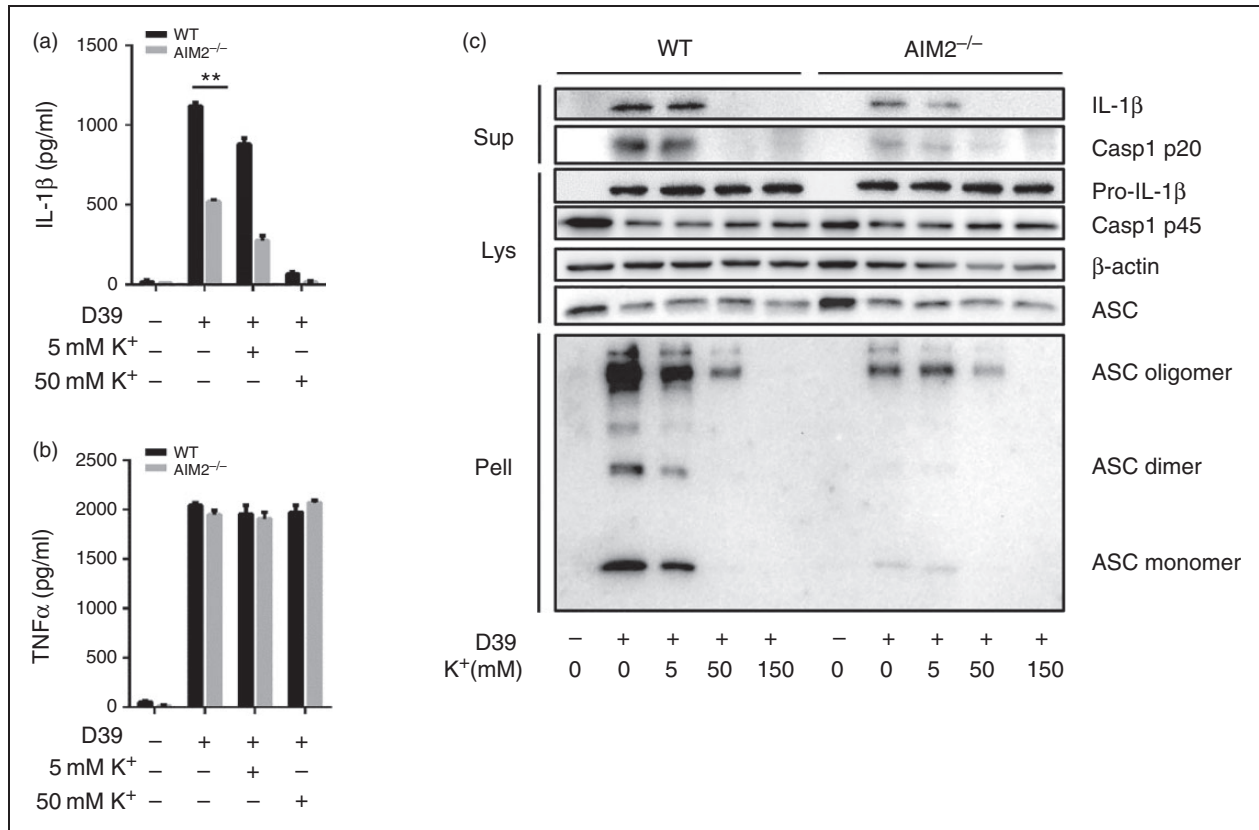
Cytoplasmic potassium efflux is critical for ASC speck formation and recruitment of caspase-1 to ASC oligomers.<sup>13</sup> Therefore, we investigated the role of cytoplasmic potassium efflux in inflammasome activation upon *S. pneumoniae* infection. Increasing the potassium concentration in the culture medium (from 5 to 150 mM) almost blocked ASC oligomerisation, caspase-1 activation and subsequent secretion of IL-1 $\beta$ , with no difference in the level of TNF- $\alpha$  (Figure 4a–c). Thus, it appears that the AIM2 inflammasome is involved in IL-1 $\beta$  maturation and secretion in macrophages infected with *S. pneumoniae*.

We have previously reported that phagocytosis is important for the caspase-1 activation and IL-1 $\beta$  secretion in *S. pneumoniae*-infected macrophages.<sup>10</sup> In order to explore whether AIM2 deficiency affects the

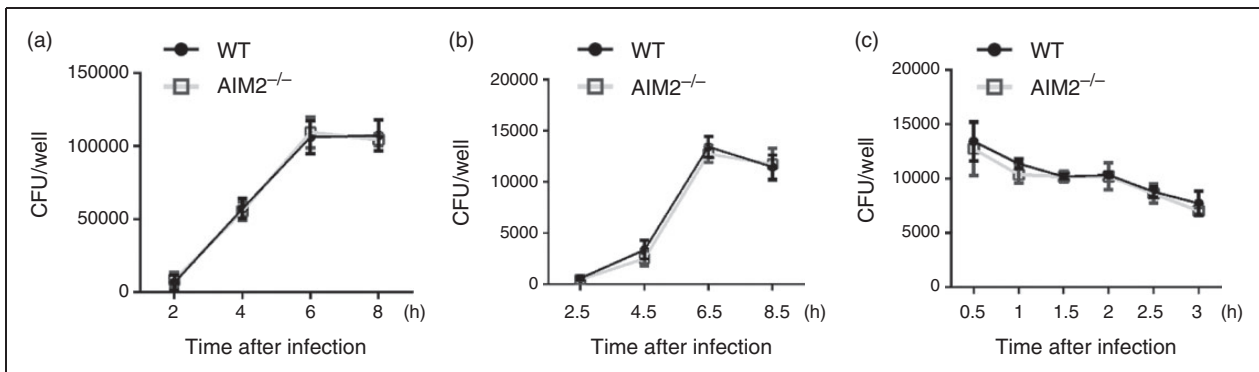
phagocytosis ability of macrophages, we challenged macrophages with D39 and enumerated bacteria associating with macrophages, phagocytosed bacteria and the bacteria viability after phagocytosis. The results showed that the fate of *S. pneumoniae* D39 post infection of AIM2<sup>−/−</sup> macrophages was almost the same as D39 post infection of WT macrophages (Figure 5a–c), which suggests AIM2<sup>−/−</sup> macrophages phagocytose and kill D39 as well as WT macrophages. These results indicate that although phagocytosis is required for caspase-1 activation and IL-1 $\beta$  secretion, AIM2 deficiency does not affect the phagocytosis ability of macrophages.

## Discussion

AIM2 is a cytoplasmic sensor that can recognise dsDNA and trigger a series of innate immune responses. In this study, we determined the important role of AIM2 in defending the host from pneumococcal infection,



**Figure 4.** The AIM2 inflammasome is involved in IL-1 $\beta$  maturation and secretion in macrophages infected with *S. pneumoniae* (a–c). Macrophages from WT or AIM2<sup>-/-</sup> mice were left uninfected or infected with *S. pneumoniae* D39 at a MOI of 1 in 5, 50 or 150 mM KCl. After 6 h, gentamicin was added to the cultures (final concentration 100  $\mu$ g/mL), and the supernatants were collected 24 h after infection. The levels of IL-1 $\beta$  and TNF- $\alpha$  in the culture supernatants were determined by ELISA (a and b). For Western blotting, the supernatants and cell lysates were collected.  $\beta$ -Actin was detected as a control. The macrophages were lysed with cold PBS containing 0.5% Triton X-100, and the pellets were harvested after centrifugation. The pellets were cross-linked with 2 mM disuccinimidyl suberate before immunoblotting (c). Casp1: caspase-1 (p20: subunit; p45: precursor); Lys: cell lysate; Sup: supernatant; Pell: pellet. All of the experiments were repeated three times. Statistical significance was determined by Student's *t*-test (\*\**P* < 0.01).



**Figure 5.** AIM2 deficiency does not affect the phagocytosis ability of macrophage (a–c). WT and AIM2<sup>-/-</sup> macrophages were infected with D39. Bacteria associating with macrophages were counted. Infected cells were washed with chilled PBS three times and lysed at 2, 4, 6, and 8 h post infection. The lysates were plated on blood agar plates to count CFU after overnight incubation (a). Phagocytosed bacteria were counted. For an additional 30 min in the presence of 100  $\mu$ g/mL gentamicin, non-associated bacteria were removed at the indicated time points, and the numbers of bacteria in cell lysates were then counted (b). The viability of phagocytosed bacteria was determined. Gentamicin was added to the cultures 6 h post infection, and the cells were additionally incubated for the indicated times. The numbers of bacteria in cell lysates were then counted (c).

since we observed that AIM2<sup>-/-</sup> mice are more susceptible to *S. pneumoniae* infection than WT mice, with higher mortality, higher bacterial colonisation and aggravated inflammation in the lungs. Collectively, we demonstrated that the AIM2 inflammasome is required for caspase-1-mediated cytokine IL-1 $\beta$  processing in *S. pneumoniae*-infected macrophages, which may participate in the control of *S. pneumoniae* infection *in vivo*.

By using *in vivo* infection model, several groups reported that ASC<sup>-/-</sup> mice were highly susceptible to pneumococcal pneumonia, while NLRP3<sup>-/-</sup> mice demonstrated only a slightly decreased resistance compared to WT mice.<sup>10,11,14</sup> These results suggest that other ASC-dependent inflammasomes or other inflammasome-independent ASC functions may contribute to host defence against *S. pneumoniae*. During the infection of a host cell, microbial DNA released into the cytoplasm can be recognised by AIM2. The assembly of the inflammasome, initiated by AIM2 activation, triggers the maturation and secretion of the cytokines IL-1 $\beta$ , which play an important role in cellular immune defence.<sup>15</sup> Several studies have demonstrated that the AIM2 inflammasome is responsible for host defence against intracellular bacteria such as *Listeria monocytogenes*, *Francisella tularensis*, *Brucella abortus* and *Mycobacterium tuberculosis*.<sup>16–19</sup> Although *S. pneumoniae* is an extracellular bacterium, once internalised by macrophages, it undergoes rapid death, and bacterial DNA is released into the cytoplasm by the pore-forming effect of pneumolysin (PLY) to activate AIM2.<sup>8,10</sup> In this study, we confirmed that the ASC-dependent AIM2 inflammasome is required for host defence against an extracellular bacterium, *S. pneumoniae*.

We previously found that *S. pneumoniae* DNA could activate the AIM2 inflammasome and that caspase-1 cleavage/IL-1 $\beta$  release was reduced in *Aim2*-silenced macrophages from mice.<sup>10</sup> In agreement with published reports, in this study, we also found that IL-1 $\beta$  secretion decreased in *S. pneumoniae*-infected AIM2<sup>-/-</sup> macrophages with reduced levels of ASC oligomerisation and caspase-1 activation, which indicates that the AIM2 inflammasome is required for cytokine production. AIM2 can bind to DNA, and the AIM2–DNA complex will serve as a platform for the recruitment of ASC and its subsequent oligomerisation.<sup>13,16</sup> Consistent with this, the results of this study also showed that depletion of intracellular potassium was required for AIM2 inflammasome assembly and ASC oligomerisation, indicating that ASC specks activate caspase-1, as described previously,<sup>13</sup> leading to IL-1 $\beta$  maturation and secretion.

Macrophages can engulf and directly kill *S. pneumoniae* by phagocytosis and secreting cytokines.<sup>20,21</sup> Moreover, IL-1 $\beta$  secretion from macrophages initiating acute inflammatory responses also plays a crucial role

in protecting the host against pneumococcal pneumonia. IL-1 $\beta$  can induce the synthesis of fibrinogen and the expression of chemokines and adhesion molecules to limit bacterial transmission and reduce the bacterial burden.<sup>22–24</sup> *S. pneumoniae* infection is associated with more death due to a greater bacterial burden and systemic infection. Given the critical role of IL-1 $\beta$  in bacterial infection, the impaired control of *S. pneumoniae* in mice lacking inflammasome components may be due to a reduction in the secretion of IL-1 $\beta$ . In line with this hypothesis, the secretion of IL-1 $\beta$  in BALF was much higher in WT mice than it was in AIM2<sup>-/-</sup> mice at 12 h after infection, which is consistent with the role of the AIM2 inflammasome in IL-1 $\beta$  production in *S. pneumoniae*-infected macrophages. Less IL-1 $\beta$  secretion in turn leads to a greater bacterial burden, which may result in a higher rate of mortality and increased inflammation in AIM2<sup>-/-</sup> mice than in WT mice during *S. pneumoniae* infection. The role of the AIM2 inflammasome in host resistance to bacterial infection in mice has also been shown with other bacterial pathogens, such as *F. tularensis*,<sup>17</sup> *B. abortus*<sup>25,26</sup> and *M. tuberculosis*.<sup>27</sup> The phenotype of *S. pneumoniae*-challenged AIM2<sup>-/-</sup> mice provides further support for the idea that the AIM2 inflammasome is critical for host defence against *S. pneumoniae* infection not only in isolated macrophages but also in a whole-animal model.

In summary, our data provide evidences that the AIM2 inflammasome is involved in host defence against *S. pneumoniae* infection, most likely by mediating IL-1 $\beta$  production to decrease the bacterial burden in tissues, thereby preventing systemic infection. Therefore, AIM2 deficiency impairs the host response to control *S. pneumoniae* infection. However, the *in vivo* environment is much more complicated than that in cell lines, and whether AIM2 has other functions contributing to the control of pneumococcal pneumonia remains to be fully elucidated. Recently, the inflammasome-independent function of AIM2 in suppressing colon tumorigenesis has been described.<sup>16,28</sup> Future studies should also be carried out to define whether there is an inflammasome-independent role of AIM2 or other PRRs in pneumococcal infection to provide prevention and treatment of *S. pneumoniae* infection.

### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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