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# Compromised NLRP3 and AIM2 inflammasome function in autoimmune NZB/W F1 mouse macrophages

Sara J. Thygesen, Karli E. Takizawa, Avril A.B. Robertson, David P. Sester, Katryn J. Stacey*

School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Qld 4072, Australia

* Corresponding author:

Katryn Stacey

School of Chemistry and Molecular Biosciences

The University of Queensland

St Lucia, Brisbane, Qld 4072

Australia

Email: katryn.stacey@uq.edu.au

Phone: +61 7 33654640

Fax: +61 7 33654273

**Running Title**

NZB/W F1 autoimmune mouse inflammasomes



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**Keywords**

Inflammasome, animal models, innate immunity, macrophages, autoimmunity, NLRP3, AIM2, lupus

**Conflict of interest**

The authors declare no commercial or financial conflict of interest.

**Abstract**

Inflammasomes are protein complexes activated by infection and cellular stress that promote caspase-1 activation and subsequent inflammatory cytokine processing and cell death. It has been anticipated that inflammasome activity contributes to autoimmunity. However, we previously showed that macrophages from autoimmune New Zealand Black (NZB) mice lack NLRP3 inflammasome function, and their AIM2 inflammasome responses are compromised by high expression of the AIM2 antagonist protein p202. Here we found that the point mutation leading to lack of NLRP3 expression occurred early in the NZB strain establishment, as it is shared with the related obese strain NZO, but not with the unrelated New Zealand White (NZW) strain. The first cross progeny of NZB and NZW mice develop more severe lupus nephritis than the NZB strain. We have compared AIM2 and NLRP3 inflammasome function in macrophages from NZB, NZW and NZB/W F1 mice. The NZW parental strain showed strong inflammasome function, whilst the NZB/W F1 have haploinsufficient expression

of NLRP3 and show reduced NLRP3 and AIM2 inflammasome responses, particularly at low stimulus strength. It remains to be established whether the low inflammasome function could contribute to loss of tolerance and the onset of autoimmunity in NZB and NZB/W F1. However, with amplifying inflammatory stimuli through the course of disease, the NLRP3 response in the NZB/W F1 may be sufficient to contribute to kidney damage at later stages of disease.

**Introduction**

Inflammasomes are multiprotein signalling complexes that form in cells in response to microbial and endogenous danger signals. Inflammasome formation leads to the activation of caspase-1 and caspase-8.[1,2] Caspase-1 cleaves pro-interleukin (IL)-1β and pro-IL-18 allowing active, pro-inflammatory IL-1β and IL-18 to be released from the cell.[1] Inflammasome activation can also lead to inflammatory lytic cell death termed pyroptosis via caspase-1, and in the absence of caspase-1, caspase-8 initiates apoptotic cell death.[1,2] Inflammasome activation requires the oligomerisation of an initiator protein. Well-characterised initiator proteins include Nod-like receptor (NLR) family member NLRP3, as well as the PYHIN/HIN-200 family member absent in melanoma 2 (AIM2).[1] The induced clustering of AIM2 or NLRP3 recruits the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) via homotypic pyrin-pyrin domain interactions and ASC subsequently recruits pro-caspases, facilitating their activation via dimerisation and proteolytic processing.[1]

A wide range of external and host-derived danger signals have been identified as stimuli for NLRP3. This includes pathogens such as *Candida albicans*, influenza A virus and *Staphylococcus aureus.*[3] NLRP3 is also activated by the bacterial ionophore nigericin and host-derived molecules that indicate cellular damage including ATP and gout-associated uric acid crystals as well as environmental irritants such as silica and asbestos.[3] These diverse stimuli generally lead to $K^+$ efflux and this is believed to induce NLRP3 clustering.[4] In contrast, AIM2 binds directly to double stranded DNA in the cytosol to initiate inflammasome formation.[5,6] AIM2 activates inflammasome responses to viruses including mouse cytomegalovirus (MCMV) and vaccinia, as well as the cytosolic bacterium *Francisella tularensis.*[7]

The NLRP3 inflammasome has been implicated in a number of diseases including hereditary periodic fevers, type II diabetes, atherosclerosis, Alzheimer's disease and gout.[8] There is also evidence to suggest a role for NLRP3 in the regulation of intestinal homeostasis.[9,10] A role for inflammasomes in autoimmunity has been proposed and has gained interest in recent years.[11] Various levels of IL-1β in systemic lupus erythematosus (SLE) patients have been reported, from undetectable to increased in a subset of patients.[12,13] Elevation of IL-18 in SLE is more clearly established[14] but can be produced by inflammasome-independent means.[15] Analysis of inflammasome function in cells from human patients is limited. Kahlenberg et al. have reported that SLE patients had higher caspase-1 activation in response to neutrophil DNA NETs and antimicrobial peptide LL37 than controls, as well as elevated IL-1β release from patient monocytes in response to ATP treatment.[16] However, other studies showed a subset of patients with a decreased NLRP3 inflammasome response.[17] A recent study in a mouse model of mild lupus-like autoimmunity suggests that NLRP3 and ASC expression actually protects against kidney disease.[18] Thus there is not a clear consensus on the role of inflammasomes in SLE.

A number of mouse models of human SLE are available, both genetic and induced. The first cross progeny of New Zealand Black (NZB) and New Zealand White (NZW) mice, termed here NZB/W F1, are considered one of the best models.[19] Disease in the NZB/W F1 mice shows a strong female bias and they develop anti-nuclear antibodies and immune complex-mediated glomerulonephritis at around 5-6 months of age, dying at around 10-12 months from kidney failure.[19] Neither of the parental strains have severe kidney disease. NZB mice develop anti-erythrocyte antibodies and are a model of autoimmune haemolytic anaemia.[20] They also have antinuclear antibodies typical of SLE but develop only mild, late-onset glomerulonephritis.[21,22] NZW mice have only a low incidence of autoimmune disease.[19] As such, both parents must pass on genetic loci that contribute to disease in the offspring.

We have recently published that NZB mice are deficient in AIM2 and NLRP3 inflammasome function.[23,24] NZB *Nlrp3* has a point mutation that leads to lack of expression and a complete loss of NLRP3 inflammasome function. In addition, reduced AIM2 inflammasome activity exists due to high

expression of p202, a PYHIN family member that binds and antagonises AIM2.[5,23,25] This demonstrates that normal NLRP3 and AIM2 inflammasome function is not required for loss of tolerance and autoantibody production in these mice. Whether the low inflammasome function actually contributes to loss of tolerance remains to be established. With respect to the tissue damage stage of disease, NLRP3 is thought to play a role in kidney damage in many but not all models of SLE.[18,26-28] It is possible that the lack of NLRP3 in NZB mice may protect them from severe glomerulonephritis. Given the profound inflammasome deficiency in NZB and the interest in the role of inflammasomes in autoimmunity, here we have investigated AIM2 and NLRP3 function in cells from NZB, NZW and NZB/W F1 mice in order to ascertain whether cells from the F1 mice that develop severe kidney damage have intact inflammasome function.

## Results

### *Intermediate expression of p202 in NZB/W F1 macrophages.*

We have previously published that NZB macrophages are deficient in AIM2-mediated responses to cytosolic DNA due to high expression of the AIM2 antagonist p202.[23,24] The level of AIM2 activity in NZW and NZB/W F1 macrophages is unknown. We first investigated expression of all the protein components of the AIM2 inflammasome. AIM2 expression was similar in C57BL/6, NZB, NZB/W F1 and NZW bone marrow derived macrophages (BMMs) and was not substantially influenced by 3 hour lipopolysaccharide (LPS) priming (Fig. 1A). p202 expression was readily detected in NZB macrophages but was undetectable in C57BL/6 and NZW macrophages. The expression of p202 in NZB/W F1 macrophages was less than NZB but readily observable. Cells from the four genotypes displayed similar expression levels of the inflammasome adaptor molecule ASC as well as pro-caspase-1 and pro-IL-1β (Fig. 1B, C). From this data, NZW cells appear competent for AIM2-mediated inflammasome responses, whilst the response of NZB/W F1 cells may be somewhat compromised by p202 expression.

***Reduced AIM2 response to cytosolic DNA and MCMV in NZB/W F1 macrophages***

Rapid loss of membrane integrity measured by propidium iodide uptake is a convenient measure of AIM2 inflammasome-mediated pyroptosis.[2] As predicted from expression analysis, NZW BMMs showed a robust pyroptotic cell death response to electroporated CT DNA, similar to that seen in C57BL/6 cells (Fig. 2A,B). NZB BMMs had lower DNA-dependent cell death, attributed to AIM2 antagonism by high p202 expression.[23,24] The cell death observed in NZB/W F1 macrophages was significantly lower than that of NZW and C57BL/6 cells. The death of NZB/W F1 and NZW cells, but not NZB cells, in response to electroporated CT DNA was blocked by pre-treatment with the caspase-1 inhibitor VX-765, indicating that it is inflammasome dependent (Fig. 2C).

Further evidence for suboptimal AIM2 function in NZB/W F1 macrophages was provided by analysis of IL-1β release in response to MCMV infection (Fig. 2D). The NZB/W F1 macrophages released an amount of IL-1β intermediate between NZW and NZB, which had a low inflammasome response to MCMV, as previously published.[24]

Together, these results suggest that the expression of p202 within the NZB/W F1 BMM is sufficient to handicap but not eliminate AIM2 inflammasome responses. BALB/c macrophages similarly express a low but functional level of p202 that limits AIM2 responses.[5]

***NZB/W F1 cells have reduced NLRP3 protein expression.***

In addition to being deficient in AIM2-mediated inflammasome responses, our previous work has shown that NZB BMMs lack NLRP3 inflammasome responses due to a point mutation causing aberrant splicing of NLRP3 transcripts.[24,29] The NZB mice were obtained from the Kew animal house (WEHI, Melbourne, Australia) where they had been breeding in isolation from stocks held at The Jackson Laboratory (ME, USA), since 1976. To ensure the mutation was not newly acquired, this region was sequenced in genomic DNA from NZB, NZW and New Zealand Obese (NZO) mice from WEHI and Jackson Laboratories. The G to A mutation previously defined[24] was present in NZB mice

from both WEHI and Jackson Laboratories (Table 1 and Supp. Fig. 1). The mutation was also present in the genome of NZO mice. NZB and NZO are related strains that diverged early in the inbreeding process.[30] This indicates that the mutation has been present in NZB since the strain was founded. The NZW genome contains the wild type G in this location like C57BL/6. The NZB/W F1 mice would thus have one wild type and one mutated copy of the *Nlrp3* gene, and are anticipated to be haploinsufficient for this gene.

NLRP3 responses in BMM *in vitro* require two signals; an initial priming signal is provided by Toll-like receptor stimuli such as LPS, followed by a triggering stimulus such as nigericin or ATP. The priming signal increases NLRP3 expression,[31] and licences the inflammasome to respond to the trigger by deubiquitination of NLRP3.[32] NLRP3 levels, under a range of LPS priming conditions, were compared in BMMs from the various mouse strains by quantitative western blot (Fig. 3). Little or no protein was detected in the NZB samples, as expected.[24] Expression and induction of NLRP3 was similar in NZW and C57BL/6 cells. The NZB/W F1 cells showed an intermediate amount of NLRP3 at baseline and with the higher dose of LPS only reached the level of NLRP3 seen in NZW and C57BL/6 cells with low priming. The level of NLRP3 is important, since induction of NLRP3 to a critical threshold by a TLR stimulus is necessary for NLRP3 function.[33]

***NZB/W F1 cells have a deficient NLRP3 inflammasome response to nigericin and ATP treatment, particularly under low priming conditions.***

In vitro experiments for NLRP3 inflammasome responses generally use priming with high doses of LPS to provide maximal sensitivity to NLRP3 triggers. However, the degree of priming relevant to conditions *in vivo* is difficult to predict. Consequently, we investigated NLRP3 function using two doses of LPS priming signal. Cells were primed with 1 or 10 ng/ml LPS and treated with nigericin or ATP for 1 hour prior to PI staining and assessment of cell viability by flow cytometry. It was found that NZB/W F1 cells had a deficient cell death response compared to C57BL/6 and NZW cells when a low priming concentration of 1 ng/ml LPS was used, but this was less clear with 10 ng/ml LPS priming (Supp. Fig. 2).

To further compare the cell death response between the strains, a plate-based assay was used to follow a time course of PI fluorescence after LPS priming and NLRP3 inflammasome stimulation. Preliminary work confirmed that cell death in C57BL/6 macrophages, primed with 10 ng/ml LPS and treated with nigericin, could be measured as an increase in fluorescence over time (Fig. 4A). This increase in fluorescence was not seen in LPS alone controls of each strain or primed and treated NZB cells that do not undergo pyroptosis. This technique was used to measure cell death in C57BL/6, NZW and NZB/W F1 macrophages that were primed with a range of LPS concentrations prior to ATP (Fig. 4B) or nigericin treatment (Fig. 4C). Cell death of all strains was reduced at lower concentrations of LPS. The NZB/W F1 cells had a deficient response compared to NZW and C57BL/6 under all conditions, but the deficiency was stronger at low LPS concentrations.

We also compared ASC speck formation in BMMs from the four mouse strains in response to both nigericin and ATP treatments. The percentage of NZB/W F1 cells with ASC specks after priming with 1 ng/ml LPS and a 30 minute nigericin treatment was much lower than that of C57BL/6 and NZW (Fig. 5A,B). This difference was reduced but still present with 10 ng/ml LPS priming. Initial experiments failed to detect ASC specks in ATP-treated NZW cells although they were readily detectable in C57BL/6 in these experiments (data not shown) and previous work.[34] The P2X7 receptor that mediates responses to ATP has several characterised alleles, and NZW, but not C57BL/6 and NZB, carry P451 conferring high sensitivity to ATP stimulation.[35,36] We reasoned that the failure to observe ASC specks in NZW could relate to a strong P2X7 response and increased cell fragility. Pre-treatment with caspase-1 inhibitor VX-765 to reduce pyroptotic lysis enabled detection of some specks within the NZW strain, although numbers were still lower than C57BL/6. Under these conditions fewer ATP-dependent ASC specks were observed in NZB/W F1 cells than NZW cells when they were primed with 1 ng/ml LPS, but no difference was observed with 10 ng/ml LPS (Fig. 5C).

IL-1β release from cells primed with 1 ng/ml LPS was not detectable by ELISA, presumably due to insufficient induction of pro-IL-1β. With 10 ng/ml LPS priming, where released IL-1β could be detected, NZB/W F1 cells had an intermediate response compared to the parental strains after

triggering with either nigericin or ATP (Fig. 5D). Unexpectedly, the levels of IL-1β released by C57BL/6 cells in response to nigericin treatment were reproducibly lower than that of the NZW and NZB/W F1 cells. The reason for this is not apparent, given that the NLRP3 response of C57BL/6 BMM is similar to NZW by other measures, and there is no difference between the cell lines in their induction of pro-IL-1β (Fig. 1C). The higher level of IL-1β release from NZW cells was still inflammasome dependent and IL-1β release from all strains in response to nigericin treatment was completely inhibited by pre-treatment with MCC950, a small molecule inhibitor of the NLRP3 inflammasome (Supp. Fig. 3)[37].

***Deficient IL-1β release in response to alum and Candida albicans in NZB/W F1 cells.***

The inflammasome response in NZB/W F1 mouse cells was checked with two further NLRP3 stimuli. Alum (aluminium hydroxide) is a commonly used vaccine adjuvant that activates the NLRP3 inflammasome.[38,39] Similar to the pattern seen with nigericin treatment, NZW macrophages produced much higher levels of IL-1β in response to stimulation with alum than C57BL/6 cells (Fig. 6A). No IL-1β was released from the NZB cells and the response from NZB/W F1 cells was much lower than NZW but higher than C57BL/6. In response to *Candida albicans* infection, NZB/W F1 macrophages were deficient in IL-1β release when compared to NZW as well as C57BL/6 (Fig. 6B). NZB BMMs released little or no IL-1β, consistent with the *in vitro* response to *C. albicans* being NLRP3-dependent.[40] At low MOI, cell death in response to *C. albicans* infection was substantially lower in NZB cells than C57BL/6 and NZW BMMs, most likely due to the lack of NLRP3-dependent pyroptosis in this line (Fig 4A). However, at the higher MOI, NZB cells died efficiently (Fig. 6C), indicating the stimulation of other death pathways. Consistent with this, *C. albicans* is reported to activate both pyroptotic and non-pyroptotic death of macrophages.[41] NZB/W F1 cells showed a trend for reduced death at the lower MOI.

**Discussion**

The development of SLE can be broadly considered as the result of two processes; (i) loss of B cell tolerance leading to autoantibody production, and (ii) tissue damage following autoantibody deposition. NZB mice clearly have a strong genetic contribution to loss of B cell tolerance, but have limited tissue damage. Our previous work demonstrated that the NZB strain has a complete lack of NLRP3 inflammasome function and low AIM2 inflammasome function due to a point mutation in the *Nlrp3* gene and high p202 expression respectively.[23,24,29] AIM2 and NLRP3 inflammasome activities are therefore clearly not necessary for the development of anti-nuclear and anti-erythrocyte antibodies observed in the NZB mice. This result may seem counterintuitive, given the publications suggesting or anticipating a role for inflammasomes in autoimmunity.[11,42] However, inflammasomes may play a role in the later stage of immune-complex-dependent tissue damage, that remains mild in the NZB mice. For this reason, we have investigated the inflammasome competence of macrophages from NZB/W F1 mice that develop profound kidney damage. The NZW parental strain showed strong AIM2 and NLRP3 inflammasome function. However, the NZB/W F1 mice still expressed enough p202 to have a reduced AIM2 inflammasome response to electroporated CT DNA and MCMV infection. In parallel with AIM2 function, the inheritance of a mutated *Nlrp3* allele from NZB mice led to haploinsufficient expression of NLRP3 in cells from the NZB/W F1 mice, and consequently function that was intermediate between NZB and NZW.

Despite deficiency in the NZB/W F1 mice, the NLRP3 inflammasome was capable of giving substantial responses under conditions of high stimulus. It is possible that in the NZB/W F1 mice, amplifying loops of tissue damage and inflammation during disease progression provide enough stimulus such that even suboptimal inflammasome systems become part of the pathological process. The NLRP3 inflammasome has been shown to be active in the kidneys of NZB/W F1 mice during disease but not at 8 weeks old, prior to disease symptoms.[26] In a number of studies treatments have been administered to NZB/W F1 mice and improvement of kidney disease symptoms has been observed alongside decreased expression and activity of NLRP3 in the kidney.[26-28] Evidence for the ability of NLRP3 to contribute to kidney damage is provided by the pristane-induced experimental

model of SLE, where mice carrying the gain of function mutation, *Nlrp3*$^{-R258W}$ developed more severe renal damage upon pristane challenge than wild type mice.[43]

Normal inflammasome function in the NZB strain is evidently not necessary for autoantibody production, but establishing whether or not the inflammasome deficiencies contribute to the loss of tolerance will require genetic manipulation of the mouse strains. AIM2 and p202 both fall within the *Nba2* lupus-susceptibility locus of NZB mice on chromosome 1.[44] This locus also contains FcγRIIb and genes for signaling lymphocytic activation molecule (SLAM) factors, which have been strongly implicated in autoantibody production, and a role for p202 has not been confirmed.[44] The NZB null allele of *Nlrp3* falls within the *lbw8* lupus susceptibility locus on chromosome 11 thought to contribute to autoantibody production[45] and is therefore a candidate gene for this effect. We have shown that prevention of aberrant *Nlrp3* mRNA splicing using targeted oligonucleotides restores NLRP3 function in NZB cells,[29] but attempts to restore *Nlrp3* expression in NZB mice by CRISPR modification have been hampered by the poor breeding performance of these mice.

Inflammasome deficiency could alter responses to both pathogens and commensal organisms, particularly given the potential role of NLRP3 in intestinal barrier function.[9,10] Loss of inflammasome-dependent cell death could lead to increased viability of cells harbouring intracellular infections, and consequently prolonged production of cytokines predisposing to loss of tolerance. Type I interferon (IFN) plays a central role in SLE, and elevated levels of IFN-responsive genes have been documented in pre-autoimmune NZB/W F1 mice.[46] Our previous work has shown that NZB macrophages make significantly more IFN-β in response to transfected DNA than C57BL/6 macrophages, and this is likely due to their lower AIM2-mediated cell death permitting ongoing cytokine release.[23] Similarly when AIM2, ASC or caspase-1 is knocked out of dendritic cells and macrophages, they have a markedly increased IFN-β response to DNA, largely due to decreased cell death.[47] Recent work has also shown that the cytosolic DNA receptor leading to IFN-β production, cGMP-AMP synthase (cGAS), is a direct cleavage target of caspase-1[48] and furthermore IL-1β has

been reported to attenuate type I IFN production and responses.[49-51] Overall it seems that type I IFN and inflammasome responses are mutually antagonistic, and so IL-1β may not be a major player in the etiology of SLE that is IFN-driven.

Unconventional roles for inflammasome components may also be relevant to autoimmunity. Recent work has shown a requirement for NLRP3 in optimal activation of the T-cell receptor in Th1, Th2 and Th17 cells[52-54] and its deficiency may have unexpected effects on T cell differentiation in NZB mice. Interestingly, against the perception that inflammasomes promote autoimmunity, Lech et al. found that knocking out NLRP3 in C57BL/6-*lpr/lpr* mice exacerbated disease phenotype.[18] Female C57BL/6-*lpr/lpr* mice develop mild, spontaneous lupus-like autoimmunity and when NLRP3 or ASC were knocked out they developed lung disease and severe lupus nephritis. Thus, in the C57BL/6-*lpr/lpr* model NLRP3 was protective against lupus nephritis, although the authors suggest this was related to an uncharacterised role in TGF-β signalling, rather than canonical inflammasome function.[18]

In summary, this study has found that the profound deficiency of AIM2 and NLRP3 inflammasomes in the parental NZB mouse strain[23,24,29] does affect inflammasome responses in macrophages of the NZB/W F1 lupus model mouse. It is possible that the moderate NLRP3 deficiency in NZB/W F1 mice contributes to the loss of tolerance while the presence of even a limited amount of NLRP3 may be acting at later stages of disease to enhance kidney damage. Being entirely deficient in NLRP3 inflammasome function may protect the NZB mice from kidney damage. This work highlights the complex role that inflammasomes could play in autoimmune disease.

## Methods

### Materials

Nigericin (N7143, Sigma-Aldrich, St Louis, USA) was dissolved in ethanol at 5 mM. ATP (adenosine 5'-triphosphate disodium salt hydrate, A2383-1G, Sigma-Aldrich) was dissolved in sterile water at 150 mM as required. CT DNA (Sigma-Aldrich) was further purified by

phenol-chloroform and Triton-X114 extraction.[55] Unless otherwise noted, LPS used was ultra-pure LPS from *E. coli* 0111:B4 (InvivoGen, San Diego, USA). *Salmonella minnesota* Re595 LPS (Sigma-Aldrich) was used only in Figure 2D. Alum was Imject Alum adjuvant (cat# 77161, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Recombinant human colony stimulating factor (CSF-1) was a gift from Chiron, Emeryville, CA. Complete RPMI 1640 is RPMI 1640 with 10% heat inactivated foetal calf serum (FCS), 1x GlutaMAX, 50 U/ml penicillin, 50 μg/ml streptomycin and 25 mM HEPES (all Life Technologies, Grand Island, USA). Propidium iodide (PI) (Life Technologies) was diluted in PBS to a stock concentration of 10 μg/ml. VX-765 (MedChemExpress, Monmouth Junction, New Jersey, USA) and digitonin (Merck, Kenilworth, New Jersey, USA) were each dissolved in DMSO to make 100 mM stocks. MCC950 sodium salt (synthesised in house using previously published procedures[56]) was prepared as a 10 mM stock in PBS.

**Mice and cell culture**

C57BL/6, NZB, NZW and NZB/W F1 mice were housed under specific pathogen-free conditions at the University of Queensland and were used under approval 353/14 from the University of Queensland Animal Ethics Committee. Female mouse bone marrow derived macrophages (BMMs) were differentiated in CSF-1 for 7-10 days as previously described.[24]

**Sequencing of Nlrp3 point mutation**

NZB, NZO and NZW genomic DNAs were kindly supplied by Tom Brodnicki (St Vincent's Institute of Medical Research, Melbourne, Australia). The region of interest was amplified by PCR using forward primer Nlrp3Intron For1 (5'-TCTTTCGTCTCCCTTCCT-3') and reverse primer mNlrp3SpliceSite R (5'-TGAATAAATGCTGGTGGTG-3'). The following cycling conditions were used: initial denaturation of 95°C for 3 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. Products were run on a 2% agarose gel and

DNA was extracted using the UltraClean 15 DNA purification kit (MoBio Laboratories, Carlsbad, USA). Products were sequenced by the Australian Genome Research Facility (North Melbourne, Australia) using mNLRP3SliceSite R primer.

**Immunoblot analysis**

Quantitative western blotting was performed as previously described.[29] Primary antibodies used were anti-NLRP3/NALP3 mAb Cryo-2 (#AG-20B-0014; Adipogen, San Diego, USA), anti-α-Tubulin (B-5-12; Sigma Aldrich), anti-S6 Ribosomal Protein rabbit mAb 5G10 (#2217; Cell Signaling Technology, Danvers, USA) and anti-mouse IL-1β (AF-401-NA; R&D Systems Inc., Minneapolis, USA). Secondary antibodies used were anti-rabbit (Dylight) 800 conjugate, anti-rabbit (Dylight) 680 conjugate, anti-Mouse (Dylight) 800 conjugate, anti-mouse (Dylight) 680 conjugate (#5151P, #5366P, #5257 and #5470P respectively; Cell Signaling Technology), and IRDye® 800CW Donkey anti-Goat IgG (H + L) (926-32214; LI-COR Biosciences, Lincoln, USA).

Non-quantitative western blotting was conducted as previously described.[24] The primary antibodies used were rabbit polyclonal anti-AIM2 (made to full-length recombinant mouse AIM2 and tested on *Aim2*$^{-/-}$ BMM extracts), goat polyclonal anti-p202 S-19 (sc-6054; Santa Cruz Biotechnology, Dallas, Texas, USA), mouse monoclonal anti-caspase-1 p20 (Casper-1; Adipogen), rabbit polyclonal anti-ASC N15-R (Sc-22514-R; Santa Cruz Biotechnology) and anti-GAPDH (2275-PC-100; R&D Systems). The HRP-linked secondary antibodies used were anti-mouse IgG, anti-rabbit IgG (#7076S and #7074S; Cell Signalling) and anti-goat IgG (HAF019; R&D systems). Blots were analysed using an Amersham Imager 600RGB (GE Healthcare Life Sciences, Chicago, Illinois, USA).

**AIM2 and NLRP3 inflammasome activation**

BMMs were electroporated with CT DNA, or infected with MCMV as previously described to activate the AIM2 inflammasome.[24] For NLRP3 inflammasome activation, BMMs were primed for 3 hours with ultra-pure LPS. Unless otherwise indicated, cells were treated with 10 μM nigericin or 5 mM ATP for 1 hour or 200 ng/ml alum for 3.5 hours at 37 °C. For protein and ELISA samples cells

were primed and treated on 24 (350,000 cells per well) or 96 (70,000 cells per well) well tissue culture plates respectively. For the ASC speck assay cells were primed on 25 well Sterilin plates (VWR International, Radnor, Pennsylvania, USA) and transferred to 1.5 ml tubes before nigericin or ATP treatment. BMM were infected with *Candida albicans* as previously described[24] but with cells seeded at 300,000 cells per well and primed for 4 hr with 1 ng/ml ultra-pure LPS.

**Measurement of cell viability**

***Propidium iodide – flow cytometry***

PI was added to samples at a final concentration of 1 μg/ml. Cells were analysed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, USA) by first gating on a side scatter vs forward scatter plot to remove debris but include the dead cells with lowered forward scatter (Fig. 2A).

***Propidium iodide- plate assay***

Cells were plated at 2 x $10^5$ per well on a black 96-well plate with clear bottom (Corning Inc, New York, USA) in complete RPMI. BMMs were primed with ultrapure LPS (10 ng/ml, 2.5 hrs). After priming the medium was replaced with 100 μl of phenol red-free, serum-free RPMI 1640 with 3 μg/ml propidium iodide (2 μg/ml final concentration following addition of stimuli). Positive controls were prepared by lysing cells from each strain with 50 μl of either 150 or 300 μM digitonin in phenol red-free, serum-free RPMI 1640. The plate was incubated for 20 min during which time the CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) was set to 37°C, 5% $CO_2$. Gain and focal height were set prior to each experiment using a well with digitonin-lysed cells. Nigericin or ATP (pre-heated to 37°C) was added to the appropriate wells immediately prior to analysis in 50 μl to give final concentrations of 10 μM or 5 mM respectively. Fluorescence readings were taken every three min for 60 min using the following settings: excitation = 535/10, emission = 617/10, bottom reading, 10 flashes per well, orbital averaging (3 mm diameter). Background fluorescence of

untreated cells with PI was subtracted from all other measurements and results are presented as a percentage of the maximum fluorescence from cells of the same strain lysed with digitonin.

***LDH assay***

Release of lactate dehydrogenase (LDH) into the cell culture medium was assessed as a measure of cell viability using the TOX7 *In Vitro* Toxicology Assay Kit (Sigma-Aldrich) according to the manufacturer's protocol. Percentage cell death is calculated relative to the total LDH released from untreated cells by detergent lysis.

**ELISA for secreted IL-1β**

Cell culture supernatants were collected and stored at -80°C. IL-1β levels in the supernatants were measured using the Mouse IL-1β/IL1F2 DuoSet ELISA kit (R&D Systems) or BD OptEIA Mouse IL-1β ELISA Set (BD Biosciences).

**ASC speck assay**

BMMs were primed with LPS and treated with ATP or nigericin as described above. Before ATP treatment VX-765 was added to the cells at a final concentration of 50 μM. After 30 min treatment cells were fixed with ethanol, immunostained for ASC and analysed for speck formation as previously described using a BD Accuri flow cytometer.[34,57] This assay involves flow cytometric assessment of the redistribution ASC based on the change in fluorescence peak height:area ratio (Fig.5A).

**Statistical Analysis**

Statistical tests described in the figure legends were conducted using Prism 7.0.

Supplementary information is available at *Immunology and Cell Biology's* website.

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**Table 1.** Genomic DNA sequencing revealed that the G to A point mutation[29] in the 7th intron of *Nlrp3* is present in NZB and NZO, but not NZW, mice from Jackson Laboratories (JAX) and The Walter and Eliza Hall Institute (WEHI).

| Strain | Location | Sequence |
|---|---|---|
| NZB | WEHI | TTTATCT**A**GATACATTT |
| NZB | JAX | TTTATCT**A**GATACATTT |
| NZO | WEHI | TTTATCT**A**GATACATTT |
| NZO | JAX | TTTATCT**A**GATACATTT |
| NZW | WEHI | TTTATCT**G**GATACATTT |
| NZW | JAX | TTTATCT**G**GATACATTT |

**Figure Legends**

**Figure 1.**

Intermediate expression of AIM2 antagonist p202 in NZB/W F1 macrophages. (**A)** Protein expression levels of p202 and AIM2 in unprimed and LPS-primed (10 ng/ml, 3h) C57BL/6, NZB, NZB/W F1 and NZW BMMs as assessed by western blot. Tubulin expression was assessed as a loading control. Results are representative of three independent protein preparations. **(B)** Protein expression levels of pro-caspase-1 and ASC in unprimed and LPS-primed (1 or 10 ng/ml, 3h) C57BL/6, NZB, NZB/W F1 and NZW BMMs as assessed by western blot. GAPDH levels were assessed as a loading control. Results are representative of three independent protein preparations. (**C**) Representative immunoblot and quantification of pro-IL-1β protein expression normalised to tubulin in unprimed and LPS-primed (10 ng/ml, 3 h) C57BL/6, NZB, NZB/W F1 and NZW BMMs. Data are from three independent protein preparations and blots. Bars are drawn to the mean of the three experiments and symbols show results of each experiment.

**Figure 2.**

Deficient AIM2 inflammasome responses in NZB/W F1 macrophages. (**A)** Representative flow cytometric plots of PI staining of C57BL/6 BMMs electroporated with and without 4 μg CT DNA. Samples are first gated to exclude debris on a SSC-area versus FSC-area plot (scatter gate). Events within the scatter gate are then viewed on a FSC-width vs FSC-area plot and gated to exclude doublets (singlet gate). Events within the singlet gate are then analysed for PI staining. (**B**) NZB/W F1 BMMs have a deficient cell death response to electroporated CT DNA. BMMs from C57BL/6, NZB, NZB/W F1 and NZW mice were untreated or electroporated with or without 20 μg of CT DNA and incubated for 30 min prior to PI staining and flow cytometric analysis. Bars are drawn to the mean of three individual experiments. Each symbol represents the mean of duplicate treatments within a single experiment. *$p < 0.05$, one-sided, paired *t* test. (**C**) Death of NZB/W F1 and NZW macrophages in response to electroporated CT DNA is largely caspase-1 dependent. BMMs from NZB, NZB/W F1 and NZW mice were treated with or without 50 μM VX-765 for 30 min prior to being electroporated with or without 10 μg of CT DNA. Cells were then incubated for 30 min, with or without VX-765, prior to PI staining and flow cytometric analysis. Bars are drawn to the mean of two independent experiments shown by different symbols, with duplicate individual samples. (**D**) Deficient IL-1β release by NZB/W F1 BMMs in response to MCMV infection. Quantification of IL-1β release by ELISA from LPS-primed C57BL/6, NZB, NZB/W F1 and NZW BMMs either mock treated or infected with MCMV (MOI 8, 6 h). LPS was from *S. Minnesota* Re595. Bars are drawn to the mean of five (C57BL/6) or six (NZB, NZB/W F1, NZW) independent experiments. Each symbol represents the result of a single experiment. **$p < 0.01$, one-tailed, paired *t* test.

**Figure 3.**

NZB/W F1 cells have intermediate NLRP3 protein expression. Quantitative western blot analysis of NLRP3 expression in C57BL/6, NZB, NZB/W F1 and NZW BMMs after 3 hrs with 0, 1 or 10 ng/ml ultra-pure LPS. NLRP3 expression was normalized to ribosomal protein S6. Data are from three

independent protein preparations and blots (except n=2 for C57BL/6 with 0 and 1 ng/ml LPS). Bars are drawn to the mean of three experiments and symbols show results of each experiment. *$p < 0.05$, one-tailed, paired *t* test.

**Figure 4.**

Reduced cell death in NZB/W F1 macrophages in response to NLRP3 stimuli, assessed by propidium iodide fluorescence using a microplate reader. **(A)** C57BL/6, NZB, NZB/W F1 and NZW BMMs were primed with LPS (10 ng/ml, 2.5 hrs). Medium alone (LPS control), nigericin or ATP was added immediately prior to commencing measurements. Data represent the mean and SD of triplicate wells. **(B)** C57BL/6, NZB/W F1 and NZW BMMs were primed with 10 ng/ml, 1 ng/ml or 0.33 ng/ml LPS and stimulated with 5 mM ATP. Data represent the mean +/- SEM of fluorescence from three (NZW) or four (C57BL/6 and NZB/W F1) independent experiments. **(C)** C57BL/6, NZB/W F1 and NZW BMMs were primed with 10 ng/ml, 1 ng/ml, 0.33 ng/ml or 0.11 ng/ml LPS and stimulated with 10 μM nigericin. Data represent the mean +/- range of fluorescence from two independent experiments. Some error bars are smaller than the symbol and cannot be seen.

**Figure 5.**

NZB/W F1 cells have reduced ASC speck formation and IL-1β release in response to nigericin and ATP. **(A)** Representative flow cytometric plots of ASC speck staining of LPS-primed C57BL/6 BMMs with and without nigericin treatment. Ethanol fixed samples are first gated to exclude debris on a SSC-area versus FSC-area plot (scatter gate). Events within the scatter gate are then viewed on a FSC-width vs FSC-area plot and gated to exclude doublets (singlet gate). These single cell events are then plotted on an ASC-height versus ASC-area plot and the population of cells with a high ASC-height:ASC-area profile, representing ASC speck positive cells, are gated (speck +ve cells). (**B**) Flow cytometric analysis of the percentage of BMMs with an ASC speck following 3 h of priming with 1 or

10 ng/ml LPS and then 30 min with or without 10 μM nigericin. Bars show the mean of three independent experiments and different symbols represent each experiment with duplicate samples. **p < 0.01, one-tailed, paired *t* test (n=3). (**C**) Flow cytometric analysis of the percentage of BMMs with an ASC speck following LPS and ATP treatment. BMMs were primed with 1 or 10 ng/ml LPS for 3 h, with 50 μM VX-765 added for the final 30 min and then treated for 30 min with or without 5 mM ATP. Bars show the mean of two independent experiments and different symbols represent each experiment with duplicate samples. (**D**) Quantification of IL-1β levels by ELISA from BMMs primed for 3 h with 10 ng/ml LPS and then either no further treatment, 5 μM nigericin or 2.5 mM ATP for 1 h. Bars show the mean of four (nigericin) or three (ATP) independent experiments and each symbol represents the mean of two treatments within a single experiment. *p < 0.05, **p < 0.01, one-tailed, paired *t* test.

**Figure 6.**

Deficient IL-1β production by NZB/W F1 cells in response to particulate and infectious stimuli. (**A**) ELISA quantification of IL-1β released by LPS-primed (10 ng/ml, 3 h) C57BL/6, NZB, NZB/W F1 and NZW BMMs treated with or without 200 μg/ml alum for a further 3.5 h. Bars are drawn to the mean of two independent experiments and each symbol represents the mean of duplicate treatments from an individual experiment. (**B**) Quantification of IL-1β release by ELISA and (**C**) cell death by LDH assay of LPS-primed (1 ng/ml, 4 h) BMMs that were uninfected or infected with *C. albicans* at the indicated MOI for 6 h. Percentage cell death is calculated relative to the total LDH released from untreated cells by detergent lysis. Bars are drawn to the mean of three (B) or two (C) independent experiments and each symbol represents the mean of duplicate treatments within a single experiment. *p < 0.05, one-tailed, paired *t* test.