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Correcting the NLRP3 inflammasome deficiency in macrophages from autoimmune NZB mice with exon skipping antisense oligonucleotides.

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Running Title: Correction of *Nlrp3* splicing in NZB macrophages

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Abstract

Inflammasomes are molecular complexes activated by infection and cellular stress, leading to caspase-1 activation and subsequent IL-1β processing and cell death. The autoimmune NZB mouse strain does not express NLRP3, a key inflammasome initiator mediating responses to a wide variety of stimuli including endogenous danger signals, environmental irritants and a range of bacterial, fungal and viral pathogens. We have previously identified an intronic point mutation in the *Nlrp3* gene from NZB mice that generates a splice acceptor site. This leads to inclusion of a pseudoexon that introduces an early termination codon and is proposed to be the cause of NLRP3 inflammasome deficiency in NZB cells. Here we have used exon skipping antisense oligonucleotides (AON) to prevent aberrant splicing of *Nlrp3* in NZB macrophages and shown that this restores both NLRP3 protein expression and NLRP3 inflammasome activity. These results indicate that the single point mutation leading to aberrant splicing is the sole cause of NLRP3 inflammasome deficiency in NZB macrophages. The NZB mouse provides a novel model for addressing a splicing defect in macrophages and could be used to further investigate AON design and delivery of AONs to macrophages *in vivo*.

Introduction

Inflammasomes are multiprotein complexes that are assembled in response to microbial and endogenous danger signals and are responsible for activating caspase-1, leading to both prointerleukin (IL)-1β and IL-18 processing and pyroptotic cell death.[1,2,3] Inflammasomes can also activate apoptotic cell death through caspase-8 activation.[4] The best-studied inflammasome is initiated by oligomerisation of the NLRP3 protein. Many external and host-derived danger signals activate the NLRP3 inflammasome, including a range of pathogens, the bacterial ionophore nigericin, host-derived molecules such as extracellular ATP and environmental irritants including silica and asbestos.[5,6,7]

Recently we demonstrated that bone marrow derived macrophages (BMMs) from NZB mice are deficient in both NLRP3 and AIM2 inflammasome responses.[8] The NZB strain is a model of autoimmune haemolytic anaemia and systemic lupus erythematosus and develops both anti-erythrocyte and anti-nuclear antibodies.[9,10] Inflammasome deficiencies could alter the interaction of the host with both microflora and pathogens, promoting cytokine release favouring the development of autoimmunity.

We proposed that the NLRP3 inflammasome defect in NZB cells is due to a point mutation found in an intron that creates a splice acceptor site.[8] The resulting pseudoexon introduces a premature stop codon producing a less stable truncated NLRP3 protein, and an almost complete lack of NLRP3 protein expression. Here exon skipping antisense oligonucleotides (AON)[11] targeting the pseudoexon restored both NLRP3 protein expression and NLRP3 inflammasome activity, demonstrating that this is the sole defect preventing NLRP3 inflammasome function in the NZB strain.

Results

To test whether the incorporation of the pseudoexon in NZB *Nlrp3* mRNA is the sole reason for the profound NLRP3 deficiency, AONs were designed to target the pseudoexon (7b) and prevent its inclusion during pre-mRNA splicing (Figure 1A). The AONs were targeted to the splice acceptor site (AON38), splice donor site (AON40) and an intra-exonic region predicted to contain several exonic splice enhancer motifs (AON39).[12,13]

Preliminary experiments demonstrated that AON39, but not AON38 or AON40, was able to substantially restore NLRP3 protein expression when electroporated into NZB BMMs (data not shown). Subsequent experiments were conducted introducing AON39 or AON40 into both NZB and C57BL/6 BMMs. After 4 h, cells were primed with LPS to up-regulate NLRP3 expression. PCR of the region encompassing the pseudoexon from cDNA showed that in the absence of AON treatment the NZB *Nlrp3* mRNA was predominantly of the longer form that includes the pseudoexon *(Nlrp3')*[8] while C57BL/6 samples had the correctly spliced form (Figure 1B). NZB cells treated with AON40 showed a small amount of correctly spliced mRNA, whilst AON39 restored correct splicing to the majority of the *Nlrp3* mRNA. Quantitative western blotting showed restoration of LPS-induced NLRP3 protein levels in NZB cells treated with AON39 to approximately 65% of the level in C57BL/6, while AON40 was largely ineffective (Figure 1C). Without LPS priming, a small amount of NLRP3 protein was detected in NZB BMMs due to the action of AON39 on the smaller amount of nascent *Nlrp3* mRNA produced constitutively during this time.

A panel of AONs were designed to microwalk around the annealing site of AON39 in an attempt to further enhance exon skipping efficiency, with four overlapping AONs (AON80-

83) designed with slight target sequence and/or length variations (Figure 2A). These were introduced into NZB BMMs and changes in *Nlrp3* expression were assessed using real time PCR primers designed for either C57BL/6 *Nlrp3* mRNA, or the NZB defective *Nlrp3'* mRNA, previously validated on cloned cDNA templates. All four new AONs promoted correct splicing, and AON83 was most efficient in suppressing the aberrant *Nlrp3'* transcript and restoring the normal *Nlrp3* transcript (Figure 2B). Concomitantly, AON83 restored NLRP3 protein levels in the NZB cells to almost 80% of that in C57BL/6 cells (Figure 2C).

The longevity of the exon skipping effect of the AONs following electroporation was investigated. AON83 and AON38 (effective and ineffective treatments respectively) were electroporated into NZB and C57BL/6 BMMs. At 0, 4, 24 or 48 h post electroporation, cells were primed for 4 h with LPS and then harvested for analysis by quantitative western blot (Figure 3). Almost complete restoration of NLRP3 expression to C57BL/6 levels was observed in NZB BMMs treated with AON83 and immediately primed with LPS, but overall levels of induced NLRP3 expression in both strains were lower than later time points, probably due to electroporation-associated stress. Effective NLRP3 restoration was observed in cells left for 4 h before priming. Thereafter the effect of the AON declined with time but was still observed 48 h post treatment.

To test the effect of AON-mediated NLRP3 protein restoration on inflammasome-induced pyroptotic cell death, cell viability was measured by MTT cleavage in response to the NLRP3 activator nigericin. A nigericin dose-dependent reduction in viability was seen in C57BL/6 cells (Figure 4A). Untreated and AON40-treated NZB cells showed a complete lack of response but AON39 treatment restored the cell death response almost to the level of C57BL/6. Subsequently, the effect of all AONs on the response to a single, high dose of

nigericin was tested. The resulting inflammasome function reflected the degree of NLRP3 protein restoration previously observed in cells, with AON83-treated cells the most sensitive to nigericin, showing that AON treatment can restore NLRP3 inflammasome function in NZB BMMs.

Discussion

Here we have used splice switching AONs to show that aberrant splicing of the *Nlrp3* gene leading to inclusion of a pseudoexon is solely responsible for the lack of NLRP3 inflammasome response observed in macrophages from NZB mice. This provides proof-of-concept for restoring NLRP3 inflammasome function in NZB mice and analysing the effect on progression and severity of autoimmune disease. In addition, NZB mice could be used as a model to investigate *in vivo* delivery of AONs to macrophages.

The AONs that targeted within the pseudoexon were effective while those targeting splice acceptor and donor sites were not. Although targeting acceptor or donor sites has worked for some genes,[14,15] our results fit with a retrospective analysis of over 400 AONs designed for the treatment of Duchenne muscular dystrophy, that showed exon-internal AONs to be the most effective for some exons.[16] Other work has suggested that the proximity of the AON target site to the 5' end of the exon and the binding energetics of the oligonucleotide to the RNA are correlated with effectiveness.[17] Exonic splice enhancer sites promote splice site recognition and are frequently targeted by effective AONs.[18] Fine-tuning of the optimal AON target sequences requires experimental validation; here this was achieved by microwalking with overlapping AONs. The five overlapping exon-internal AONs had reproducible differences in their effectiveness (Figure 2). Such differences could be due to thermodynamic properties, secondary structure of the target sequence or the masking of additional exonic

splice enhancers. We had previously shown that longer AONs (25mers) could confer substantial improvement in exon skipping efficiency, although this requires confirmation on a case-by-case basis.[19]

Apart from correction of a splicing defect, exon skipping AONs can be used experimentally as an alternative to siRNA to knock down gene expression, or to induce specific splice isoforms.[20] A consideration for experimental use is the time course of effectiveness. Here we showed that the AONs can work very quickly and must get to the nucleus, where they hybridize with pre-mRNA,[21] almost immediately.

Of all disease-causing point mutations, 15% have been predicted to fall within splice sites and it has been estimated that a further 25% of confirmed pathogenic nonsense or missense mutations alter exonic splice enhancers and silencers and hence lead to abnormal splicing.[22,23] Targeted exon skipping has potential therapeutic uses in knocking down the expression of disease causing genes or splice variants and in the restoration of normal splicing if mutations introduce deleterious pseudoexons.[24] Exon skipping is currently being tested in clinical trials for the treatment of Duchenne muscular dystrophy, where particular mutated exons of the dystrophin gene can be targeted to restore a correct open reading frame and protein expression.[25,26] Challenges that remain for exon skipping AON therapy are to define the optimal nucleic acid modifications required for stability, affinity and safety and also to optimise delivery of AONs *in vivo*. The NZB mouse provides a model for a splicing defect in macrophages, and could be used to investigate *in vivo* delivery of AONs to this compartment.

2. Materials and methods

2.1 Materials

Lipopolysaccharide (LPS) from *Salmonella minnesota* Re595 (Sigma Aldrich, St Louis, USA) was dissolved in Dulbecco′s phosphate-buffered saline (PBS (Life Technologies, Grand Island, USA))/0.1% triethylamine at 10 mg/ml. Nigericin (N7143 Sigma-Aldrich) was dissolved in ethanol at 5 mM. Recombinant human CSF1 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). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Life Technologies) was prepared as a 5 mg/ml stock in PBS.

2.2 Mice and Cell Culture

C57BL/6 and NZB mice were housed under specific pathogen-free conditions at the University of Queensland and were used under approval from the University of Queensland Animal Ethics Committee. Female mouse BMMs were differentiated as previously described[27] and used between day 7 and 10 of culture.

2.8 Treatment of BMMs with exon skipping oligonucleotides

2'-O-methyl modified AONs with a full phosphorothioate backbone were synthesized on an Expedite 8909 synthesizer using the 1micromole Thiol protocol, cleaved from the support, desalted on NAP-10 columns and stored at -20˚C. AONs had the following sequences: AON34- 5'AAUAGUUUUGGCAUCAAAAUUCU3'; AON38- 5'AUAUGGAAAUGUAUCUAGAUAAAUG3'; AON39- 5'CUAUGGGUUUUUGUGCUCCAAACUA3'; AON40- 5'CUUACCCAAAAAGUUCUUGACUUAA3', AON80- 5'GGUUUUUGUGCUCCAAACUA3', AON81-5'CUAUGGGUUUUUGUGGUCCA3',

198 AON82-5'GGUUUUUGUGCUCCAAACUAUAAGU3', AON83-
199 5'GGGUACUAUGGGUUUUUGUGCUCCA3'.
200
201 BMMs were electroporated in 400 μl of complete RPMI-1640, with or without 3.5 μM AON
202 at 260 V, 1000 μF using a Bio-Rad Gene Pulser. Cells were immediately washed with 10 ml
203 of complete RPMI-1640 without FCS, pelleted (350 x *g*, 5 min), resuspended in complete
204 RPMI-1640 and plated out for analysis. Cells were plated for: RNA extraction at 4 million
205 cells/well in 6-well plates, protein at 250,000-500,000 cells/well in 24-well plates and
206 nigericin treatment/MTT assay at 70,000 cells/well in 96 well plates. Plates were incubated
207 for 4 h and then primed with 100 ng/ml LPS for 4 h.
208
209 2.9 Qualitative and Real Time-quantitative PCR of *Nlrp3* variants.
210 RNA was extracted using the RNeasy mini-prep kit (Qiagen, Hilden, Germany). cDNA was
211 generated from each sample of RNA using oligo dT priming[28] and amplified by conventional
212 PCR using the forward primer 2706 (5'AGAAACTGTGGTTGGTGAG3') and reverse primer
213 3125 (5'TGTGGTTGTGGGTCAGAA3'). The products were visualised after electrophoresis
214 on a 2% agarose gel. Quantitative real time PCR was analysed by the ΔCt method relative to
215 *Hprt*, as described previously.[28] Primers used were NZB*Nlrp3*-For
216 5'ATGCCTTGGGAGACTCAGGA3', NZB*Nlrp3*-Rev 5'GCTGGTGGTGGGTACTATGG3',
217 C57*Nlrp3*-For 5'CAGAAGCTGGGGTTGGTGAAT3', C57*Nlrp3*-Rev
218 5'CTGAGTCCTGTGTCTCCAAGG3', *Hprt*-For 5'CAGTCCCAGCGTCGTGATTAG3' and
219 *Hprt*-Rev 5'AAACACTTTTTCCAAATCCTCGG3'.
220
221 2.3 Nigericin Treatment and MTT assays

Cells were treated with nigericin for 1 h. MTT assays of reductase activity indicating cell viability[29] were performed as previously described except that 5x MTT was added directly to 50 µL of medium that was left in the well and was not removed prior to addition of MTT solubilisation solution (isopropanol/10% Triton X-100/0.1N HCl). Plates were left overnight for maximum solubilisation of MTT formazan product prior to measurement of absorbance at 570 nm.

2.6 Quantitative Western Blotting

Cell monolayers were lysed in 100 µl 66 mM Tris pH7.4, 2% SDS. Samples were run on 15-well mini-PROTEAN TGX gels (Bio-Rad, Hercules, USA) and transferred to immobilon-FL membrane (Millipore, Billerica, USA) using a mini-trans blot system (Bio-Rad) with Tris-Glycine transfer buffer containing 10% methanol. The membrane was washed for 15 min in Tris-buffered saline (TBS) and then blocked for 1 h with Odyssey® Blocking Buffer (LI-COR, Lincoln, USA). Primary antibodies were diluted in the Odyssey® Blocking Buffer and incubated overnight at 4°C. 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) and anti-GAPDH (#2275-PC-020, Trevigen, Gaithersburg, USA). Membranes were washed with TBS containing 0.05% Tween-20 (TBS-T) and then incubated for 2 h, protected from light, at room temperature in secondary antibody diluted in the LI-COR buffer/0.1% Tween-20/ 0.01% SDS. Secondary antibodies used were anti-rabbit (Dylight) 800 conjugate, anti-rabbit (Dylight) 680 conjugate, anti-Mouse (Dylight) 800 conjugate and anti-mouse (Dylight) 680 conjugate (#5151P, #5366P, #5257 and #5470P respectively, Cell Signaling Technology, Danvers, USA). Membranes were washed with TBS-T then TBS and scanned on the Odyssey (LI-COR) and analysed with Image Studio Lite software (LI-COR).

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Antisense oligonucleotide treatment can restore normal splicing and NLRP3 expression in NZB cells. (A) Diagram showing the *Nlrp3* pseudoexon 7b, the location of the point mutation (triangle) and the position of the AONs. AON38 anneals to the last 11bp upstream of the pseudoexon and the first 14 bases of the pseudoexon. AON39 anneals entirely within the pseudoexon targeting nucleotides 21 to 46 from the 5' end. AON40 anneals to the last 20 nucleotides of the pseudoexon and the first 5 nucleotides of the downstream intron. **(B)** AON39 restores the normal *Nlrp3* mRNA splicing within NZB BMMs. BMMs from NZB and C57BL/6 mice were electroporated with AON39 or AON40 or with no addition, incubated for 4 h, then primed with 100 ng/ml LPS for 4 h. Conventional PCR using primers in exons 6/7 and 9, flanking the pseudoexon 7b, shows the mRNA variants with the pseudoexon (*Nlrp3')* or without the pseudoexon (*Nlrp3*) in NZB and C57BL/6 BMMs after AON treatment. **(C)** AON39 restores NLRP3 protein levels in NZB BMMs. Quantitative western blot of NLRP3 protein levels normalised to tubulin expression, from cells treated as in panel B, with and without LPS priming.

Figure 2. Refinement of optimal AON targeting sequence. (A) Diagram showing the position of four additional AON relative to AON39. AON80, 81, 82 and 83 all anneal within the pseudoexon, targeting nucleotides 21-40, 26-45, 16-40 and 26-50 from the 5' end respectively. A predicted exonic splice enhancer motif is shown in white text. **(B)** Effects of minor sequence changes to AON on *Nlrp3* splicing, assessed by quantitative PCR. BMMs from NZB or C57BL/6 mice were electroporated with either no addition (-), a control oligonucleotide (cont), or an AON (38-40, 80-83), incubated for 4 h, then primed with 100 ng/ml LPS for 4 h. Quantitative real time PCR analysis was performed with primers specific for *Nlrp3′* (with pseudoexon) and *Nlrp3* (correct splicing). Data were normalised to NZB without oligonucleotide for *Nlrp3′* results and C57BL/6 without oligonucleotide for *Nlrp3*

results and show the mean and range from two experiments. **(C)** Effects of minor sequence changes to AON on NLRP3 protein expression, assessed by quantitative western blotting. NLRP3 protein levels from cells treated as in panel B were assessed relative to GAPDH expression. Data shown is the mean and range of two experiments, normalised to the C57BL/6 without AON sample.

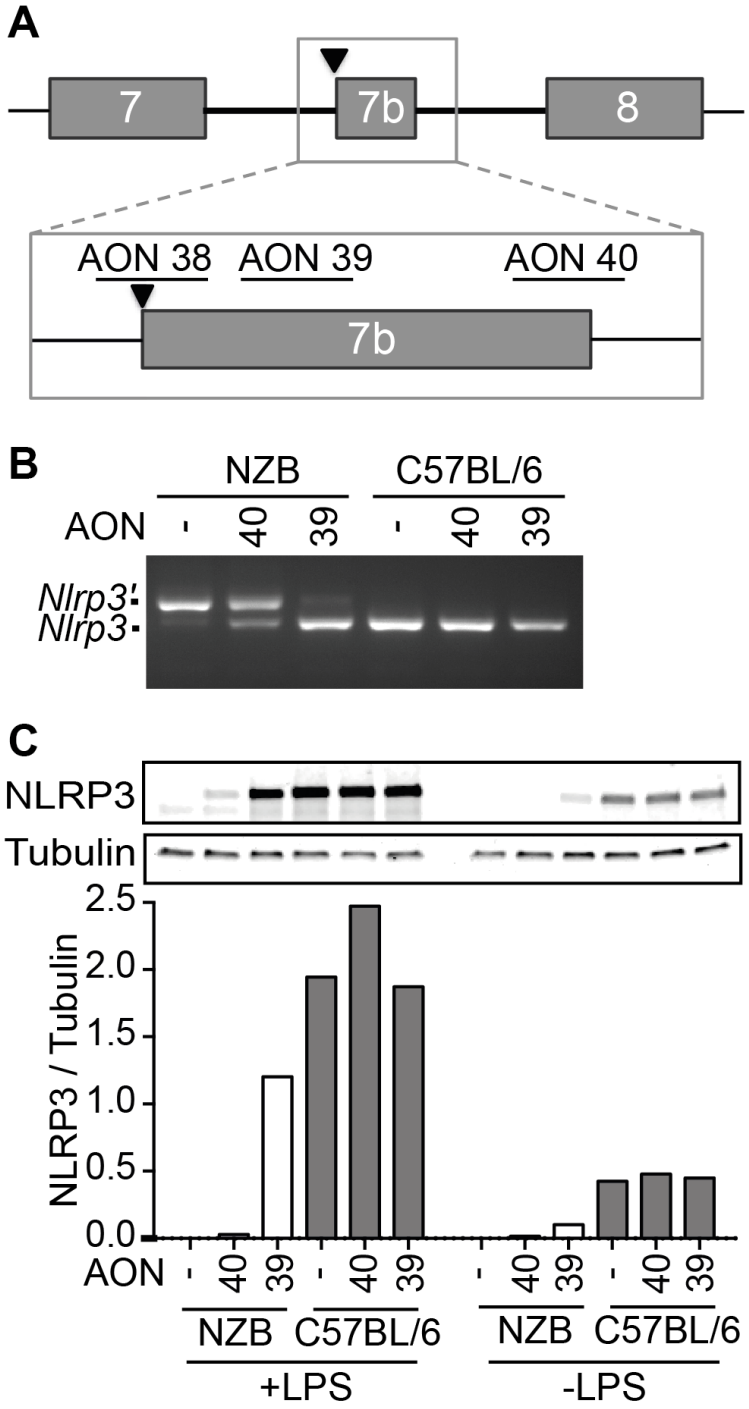
Figure 3. Exon skipping AON have diminishing potency during the 48 h post introduction into cells. BMMs from NZB and C57BL/6 mice were electroporated with no addition (-), AON38 or AON83, plated and primed with 100 ng/ml LPS for 4 h, immediately (0h) or after 4, 24 or 48 h of incubation. A quantitative western blot is shown for NLRP3 protein levels normalised to GAPDH. As samples within each experiment were analysed on two separate blots, 0h and 4h sample data is shown relative to 4h C57BL/6 without oligonucleotide and 24h and 48h sample data is shown relative to 48h C57BL/6 without oligonucleotide. Quantitative data shown is the mean and range of two experiments.

Figure 4. Inhibition of aberrant splicing restores NLRP3 inflammasome function in NZB BMMs. Relative cell viability was measured by MTT cleavage. **(A)** NZB and C57BL/6 BMMs were electroporated with nothing (-), AON40 or AON39, incubated for 4 h and then primed with 100 ng/ml LPS for 4 h. NLRP3 inflammasome was triggered by treatment with 0, 1.25, 2.5, 5 or 10 μM nigericin for 1 h. Data represent the mean and standard deviation of triplicate nigericin treatments of each AON-electroporated sample. Results are relative to the mean of the 0 μM nigericin wells for each sample, and are representative of three experiments performed. **(B)** NZB and C57BL/6 BMMs were electroporated with nothing (-), control oligonucleotide (cont), or the indicated AON, incubated for 4 h and then primed with 100 ng/ml LPS for 4 h. Cells were then treated with or without 10 μM nigericin for 1 h. Cell viability is shown relative to the unstimulated cells for each oligonucleotide treatment. Data

421 is representative of two experiments and shows the mean and standard deviation of triplicate
422 treatments from one experiment.

423

Figure 1


A
7
7b
8
AON 38
AON 39
AON 40
7b
B
NZB
C57BL/6
AON
-
40
39
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40
39
Nlrp3'
Nlrp3
C
NLRP3
Tubulin
NLRP3 / Tubulin
2.5
2.0
1.5
1.0
0.5
0.0
AON
-
40
39
-
40
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-
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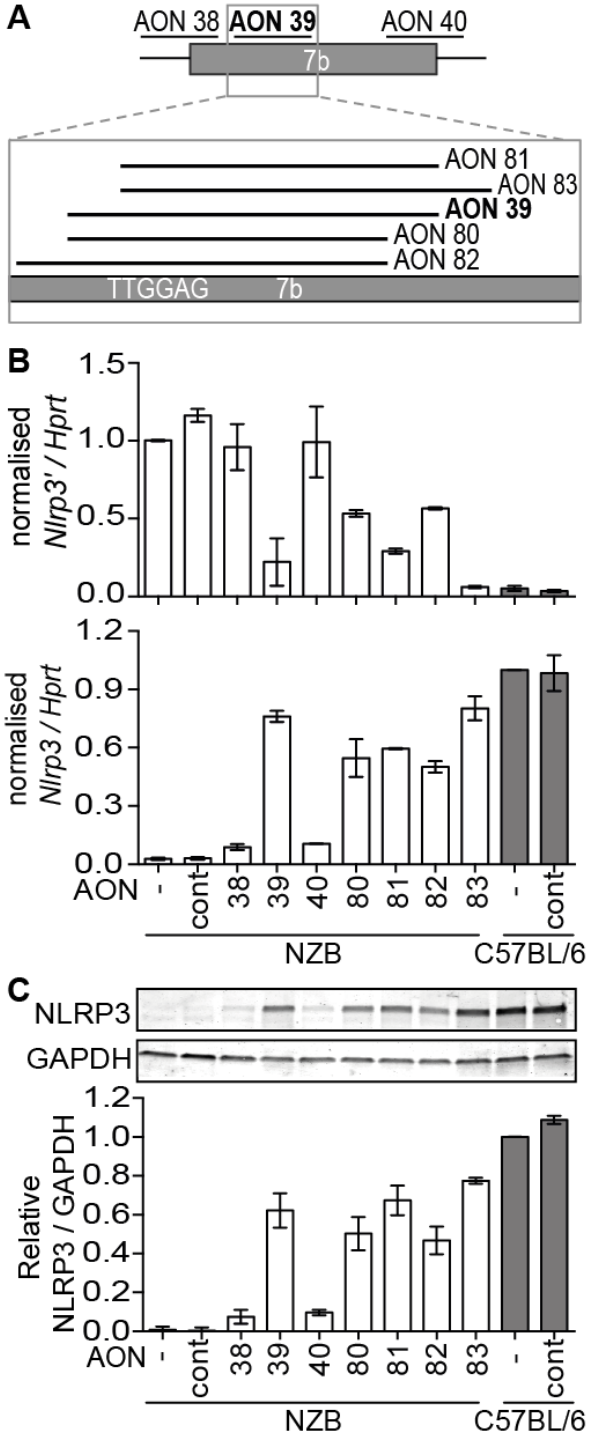
-
40
39
NZB
C57BL/6
NZB
C57BL/6
+LPS
-LPS

Figure 2


A
AON 38
AON 39
AON 40
7b
AON 81
AON 83
AON 39
AON 80
AON 82
TTGGAG
7b
B
normalised Nlrp3' / Hprt
1.5
1.0
0.5
0.0
normalised Nlrp3 / Hprt
1.2
0.9
0.6
0.3
0.0
AON
-
cont
38
39
40
80
81
82
83
-
cont
NZB
C57BL/6
C
NLRP3
GAPDH
Relative NLRP3 / GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
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AON
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cont
38
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83
-
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NZB
C57BL/6

Figure 3

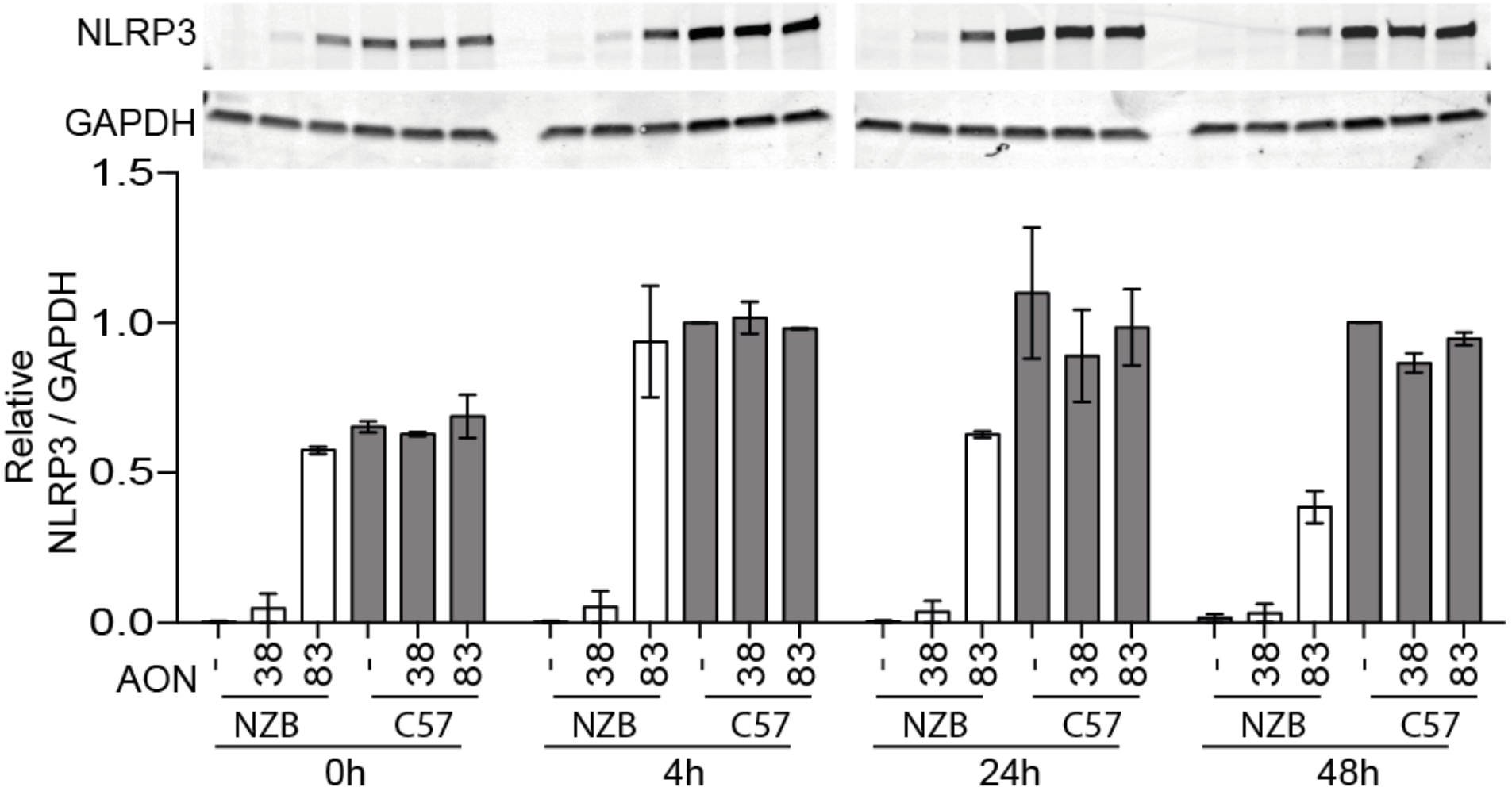
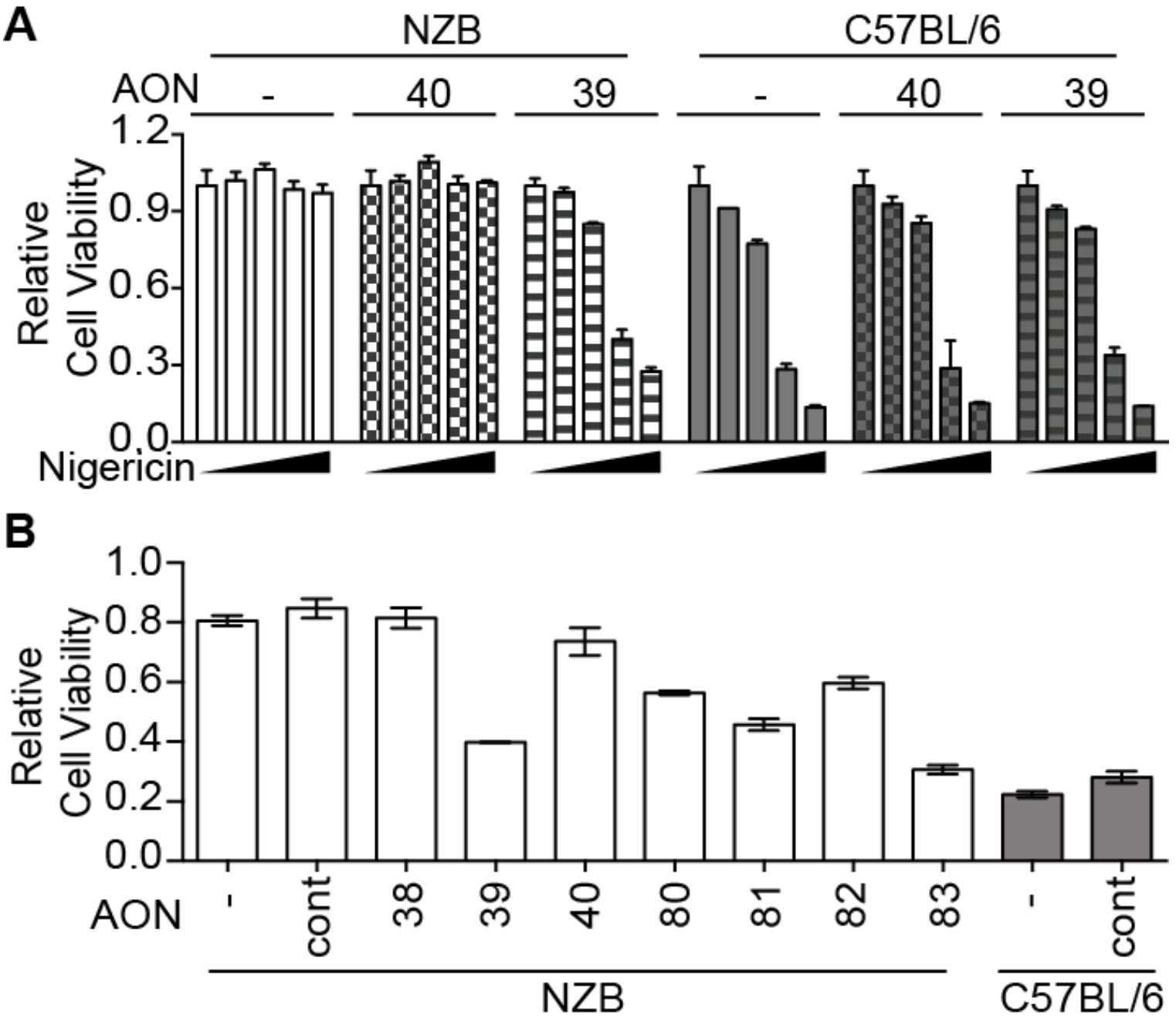

NLRP3
GAPDH
Relative NLRP3 / GAPDH
1.5
1.0
0.5
0.0
AON
-
38
83
NZB
C57
0h
4h
24h
48h

Figure 4


A
NZB
C57BL/6
AON
-
40
39
-
40
39
Relative Cell Viability
1.2
0.9
0.6
0.3
0.0
Nigericin
B
Relative Cell Viability
1.0
0.8
0.6
0.4
0.2
0.0
AON
-
cont
38
39
40
80
81
82
83
-
cont
NZB
C57BL/6