Biochimica et Biophysica Acta 1863 (2016) 244–253

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

SARM modulates MyD88-mediated TLR activation through BB-loop dependent TIR-TIR interactions

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ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form 16 November 2015
Accepted 20 November 2015
Available online 22 November 2015

Keywords:
Innate immunity
Immunomodulation
Sterile α- and armadillo-motif-containing protein (SARM)
TLR signalling
TIR domain

ABSTRACT

Toll-like receptors (TLRs) recognise invading pathogens and initiate an innate immune response by recruiting intracellular adaptor proteins via heterotypic Toll/interleukin-1 receptor (TIR) domain interactions. Of the five TIR domain-containing adaptor proteins identified, Sterile α- and armadillo-motif-containing protein (SARM) is functionally unique; suppressing immune signalling instead of promoting it. Here we demonstrate that the recombinantly expressed and purified SARM TIR domain interacts with both the major human TLR adaptors, MyD88 and TRIF. A single glycine residue located in the BB-loop of the SARM TIR domain, G601, was identified as essential for interaction. A short peptide derived from this motif was also found to interact with MyD88 in vitro. SARM expression in HEK293 cells was found to significantly suppress lipopolysaccharide (LPS)-mediated upregulation of inflammatory cytokines, IL-8 and TNF-α, an effect lost in the G601A mutant. The same result was observed with cytokine activation initiated by MyD88 expression and stimulation of TLR2 with lipoteichoic acid (LTA), suggesting that SARM is capable of suppressing both TRIF- and MyD88- dependent TLR signalling. Our findings indicate that SARM acts on a broader set of target proteins than previously thought, and that the BB-loop motif is functionally important, giving further insight into the endogenous mechanisms used to suppress inflammation in immune cells.

1. Introduction

Toll-like receptors (TLRs) are a family of type I transmembrane receptors vital for initiating the innate immune response upon pathogen recognition [1–3]. So far, a total of 13 different TLRs have been identified in mammals [4]. The TLRs recognise pathogen associated molecular patterns (PAMPs) via large leucine rich repeat (LRR) domains, and also contain a conserved C-terminal region known as the Toll/interleukin-1 receptor (TIR) domain of around 100 to 200 amino acids, located in the cytoplasmic region of the receptor [5]. Following PAMP recognition, the receptor undergoes a conformational change that allows the TIR-domain to recruit downstream TLR adaptor proteins via heterotypic protein-protein interactions, which ultimately results in the activation of immune related genes and the release of inflammatory cytokines, chemokines and interferons [6].

To date, five TLR adaptor proteins have been identified: Myeloid differentiation factor 88 (MyD88) [7], MyD88-adaptor-like protein (MAL) [8], TIR-domain-containing adaptor protein inducing IFNβ (TRIF) [9], TRIF-related adaptor molecule (TRAM) [10,11] and Sterile α- and armadillo-motif-containing protein (SARM) [12]. While MyD88, MAL, TRIF and TRAM are all known to form complexes with TLRs via TIR–TIR interactions in order to propagate downstream TLR signalling, leading to the activation of NF-κB transcription factor and secretion of multiple pro-inflammatory cytokines, SARM appears to have a different function.

SARM has been described as a negative regulator of the immune system. It inhibits NF-κB and IRF3-mediated TLR3 and TLR4 signalling [13]. It has also been shown to inhibit both lipopolysaccharide (LPS) induced TRIF- and MyD88-mediated activator protein 1 (AP-1) activation [14].

Abbreviations: ANTP, *Antennapedia* homeodomain; AP-1, activator protein 1; CV, column volume; DAPI, 4′-6-diamidino-2-phenylindole; DIC, differential interference contrast; DMEM, Dulbecco's Modified Eagle Medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione S-transferase; IEC, ion exchange chromatography; IMAC, immobilised metal-ion affinity chromatography; LRR, leucine rich repeat; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAL, myD88-adaptor-like protein; MTT, (4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MyD88, myeloid differentiation factor 88; NAD^+, nicotinamide adenine dinucleotide; PAMP, pathogen associated molecular pattern; PBS, phosphate-buffered saline; PBST, PBS supplemented with 0.05% (v/v) Tween 20; SAM, sterile α-motif; SARM, sterile α- and armadillo-motif-containing protein; TB, terrific broth; Tdp, TIR-domain protein; TIR, toll/interleukin-1 receptor; TLR, toll-like receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein inducing IFNβ.

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http://dx.doi.org/10.1016/j.bbamcr.2015.11.021
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Although the exact mechanism of inhibition is not known, a pairwise yeast two-hybrid assay demonstrated that SARM is capable of binding directly to TRIF [13]. Intriguingly, SARM appears to harbour multiple functions beyond TLR signalling [15]. For example, recent studies have revealed that the N-terminus of SARM is involved in mediating apoptosis [16], with the first 27 amino acids acting as a mitochondria-targeting signal sequence [17]. Osterloh and colleagues [18] have also demonstrated that both *Drosophilia* and mouse SARM homologues are required for activation of an injury-induced axon death signalling pathway, a function that was recently shown to involve the breakdown of nicotinamide adenine dinucleotide (NAD^+) [19,20].

SARM is the most recent of the five mammalian TLR adaptor proteins to be identified and is a highly conserved protein found amongst multiple species, including horseshoe crab [21], *Caenorhabditis elegans* [22], amphioxus [23], zebrafish [24,25] and whiteleg shrimp [26], suggesting an ancient origin for this protein. Phylogenetic studies have also revealed that SARM is closely related to bacterial TIR-proteins, suggesting a different evolutionary history compared to other members of the TLR adaptor family [27]. Over the past decade, several pathogenic bacteria have been revealed to express homologues of human TIR-domain proteins, including *Salmonella enterica* [28], *Yersinia pestis* [29,30], *Escherichia coli* CFT073 and *Brucella melitensis* [31]. Many of these bacterial TIR-domain proteins are known to suppress TLR-signalling when secreted in mammalian hosts, often by forming heterotypic TIR–TIR interactions with host TLRs or TLR adaptor proteins [32]. The apparent evolutionary relationship between SARM and bacterial TIR-domain proteins prompted us to query whether SARM suppresses innate TLR signalling in a manner similar to some of the bacterial proteins, specifically, if SARM exclusively targets the TRIF- and/or the MyD88- dependent pathway(s), and if SARM utilises TIR–TIR interactions as a mechanism of action.

In this study, we have expressed and purified the TIR-domains of all five human TLR adaptor proteins and used pulldown studies to probe the interaction between SARM–TIR and other adaptor TIR domains. The results suggest that protein-protein interactions occur between the TIR domains of SARM and TRIF, as well as SARM and MyD88. Although no SARM crystal structure has been solved to date, structures of several other mammalian TIR-domains are available, and they typically show a conserved overall core fold comprised of a central five-stranded parallel β-sheet surrounded by five α-helices, but with significant diversity in the exposed loop-regions [33]. As multiple studies have highlighted the importance of the conserved box2-region of both human and bacterial TIR-domains [29,34] that corresponds to the exposed BB-loop in the TIR structure, we performed site-directed mutagenesis across this region and identified an amino acid Gly601 critical for binding. Alanine substitution of this residue had no effect on SARM's pro-apoptotic potential, but was associated with the loss of immune suppressive function during PAMP stimulation of HEK-TLR4 and HEK-TLR2 cells. A peptide based on the SARM BB-loop region targets the MyD88TIR- domain and mildly suppressed TLR4-signalling, further underlining the importance of this region. Taken together, our findings offer insights into the mechanism used by SARM to regulate innate immune signalling.

2. Material and methods

2.1. Cell lines and general reagents

HEK293T and HEK293 cell lines stably expressing TLR2 or MD2, CD14 and TLR4 were purchased from Invivogen. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (*v*/*v*) foetal bovine serum (FBS) at 37 °C, 5% CO_2, under a humidified environment. *E. coli* 055:B5 LPS and lipoteichoic acid (LTA) were purchased from Sigma. Bacterial protein expression was performed in BL21 (DE3) cells purchased from Invitrogen. Rabbit anti-green fluorescent protein (GFP) antibody was purchased from Abcam, mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody from Santa Cruz, mouse anti-cytochrome C antibody from BD Biosciences, mouse anti-V5 and donkey anti-mouse IgG Alexa Fluor 546 conjugate antibodies were from Invitrogen, and mouse anti-polyhistidine antibody was from Sigma.

2.2. Expression vectors

All cDNA clones encoding the proteins of interest were amplified by PCR using the primers detailed in Table S1. Generation of pCDNA- and pEGFP-SARM vectors was as described previously [35]. The MyD88 clone was provided by Prof. Andrew Bowie, Trinity College Dublin. TRAM, MAL and TRIF clones were from Dr. Tom Monie, University of Cambridge.

2.3. SARM mutagenesis

Site-directed mutagenesis of SARM was performed using QuikChange® Lightning Site-directed Mutagenesis Kit (Stratagene), following the manufacturer's instructions. Oligonucleotide primers (Eurofins MWG Operon) 5′-GAGAAGCTGGAAGCAGCCAAGTTCGAGGACAAA-3′ and 5′-TTTGTCCTCGAACTTGGCTGCTTCCAGCTTCTC-3′ were used to insert a Gly601Ala mutation in the pET28a-SARM-TIR and pCDNA-SARM-V5/His expression vectors. The underlined bases indicate the mutations. Mutagenesis was confirmed by DNA sequencing (Eurofins MWG Operon).

2.4. SARM-TIR expression and purification

The pET28a-SARM-TIR construct (encoding SARM residues G559-Q700) was expressed in BL21 (DE3) *E. coli* cells, grown in terrific broth (TB). Cells were harvested by centrifugation and suspended in 5 volumes of ice-cold lysis buffer [40 mM Tris–HCl, 200 mM NaCl, 10 mM imidazole, pH 7.5] supplemented with one complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail tablet (Roche) and lysed by sonication. Following centrifugation, the supernatant was applied to Talon® metal affinity resin (Clontech) (5 mL resin per L culture) equilibrated with lysis buffer and the protein allowed to batch-bind for 1 h at 4 °C. Resin was then washed with lysis buffer, followed by protein elution with elution buffer [40 mM Tris–HCl, 200 mM NaCl, 300 mM imidazole, pH 7.5]. Purified protein was buffer-exchanged into buffer A [20 mM Tris–HCl, 5 mM dithiothreitol (DTT), 1 mM EDTA, pH 7.0] using a HiPrep™ 26/10 desalting column (GE Healthcare). The sample was further purified by anion-exchange chromatography with MonoQ resin (GE Healthcare) using a linear gradient of sodium chloride (0–1 M) in buffer A for elution. The eluted protein was finally concentrated to a volume of approximately 500 μL and purified by gel filtration with a Superdex™ 200 10/300 GL column (GE Healthcare) equilibrated with buffer B [20 mM Tris–HCl, 300 mM NaCl, 5 mM DTT, 1 mM EDTA, pH 7.0], followed by concentration of the protein to approximately 1 mg/mL. Purified protein was flash frozen in liquid nitrogen and stored at −80 °C until use.

2.5. Expression and purification of GST-tagged MyD88-TIR, TRIF-TIR, MAL and TRAM

Plasmids encoding glutathione S-transferase-MyD88-TIR (residues T148-P296), GST-TRAM, GST-MAL and GST-TRIF-TIR (residues S368-K537) were transformed into BL21 (DE3) *E. coli* cells which were then grown in LB cultures in order to express the individual proteins. Harvested cells were lysed by sonication in ice-cold lysis buffer [40 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 5 mM DTT]. Following centrifugation, the supernatants were applied to Glutathione Sepharose High Performance resin (GE Healthcare), equilibrated with lysis buffer and the proteins were allowed to batch-bind for 1 h at 4 °C. The resin was then washed with lysis buffer, followed by protein elution with

elution buffer [50 mM Tris–HCl, pH 8.0, 10 mM reduced glutathione]. Protein purity was assessed by SDS-PAGE and samples were buffer-exchanged into storage buffer [20 mM Tris–HCl, pH 7.5] and concentrated to approximately 1 mg/mL using Amicon molecular weight cut-off filters (10 kDa MWCO) (Millipore). Purified proteins were flash frozen in liquid nitrogen and stored at −80 °C until use.

2.6. Generation of SARM BB-loop peptide

Overlapping oligonucleotide primers (Eurofins MWG Operon) 5′-GATCCGTCTTCATTGATGTGGAGAAGCTGGAAGCAGGCAAGTTCGAGGACAAATAG-3′ and 5′-AATTCTATTTGTCCTCGAACTTGCCTGCTTCCAGCTTCTCCACATCAATGAAGACG-3′, coding for SARM residues S590-K606, were melted at 95 °C, followed by annealing at room temperature. The resulting DNA sequence was subcloned into a pGEX-6p-1 vector (GE Healthcare) between the BamHI and EcoRI restriction sites. The peptide was expressed as a GST fusion protein in BL21 (DE3) *E. coli* cells and purified on Glutathione Sepharose High Performance resin (GE Healthcare). For cellular experiments, a SARM BB-loop peptide tagged N-terminally with the *Antennapedia* homeodomain sequence (RQIKIWFQNRRMKWKK) for cell penetration and C-terminally with K-rhodamine for detection, was purchased from (Genemed Synthesis Inc., USA).

2.7. Determination of protein concentrations

The concentrations of all proteins were determined with a UV-spectrophotometer DU® 730 (Beckman) using extinction coefficients estimated by amino acid compositions.

2.8. GST pull down interaction assay

100 μg of purified TLR adaptor proteins: GST-MyD88-TIR, GST-TRIF-TIR, GST-MAL and GST-TRAM was selected as "baits" and individually incubated with 50 μL Glutathione Sepharose High Performance resin (GE Healthcare), equilibrated with binding buffer [20 mM Tris–HCl, 150 mM NaCl, 5 mM DTT, 1 mM EDTA, 0.1% Tergitol® Type NP-40, pH 7.5]. The total volume was 300 μL for all reactions. After being batch-bound at 4 °C for 1 h, the resins were washed three times with 20 column volumes (CVs) binding buffer. Given the fact that we get identical purification yields, and presumably binding efficiencies, for the different proteins we are confident that the amount of each GST-adaptor protein bound to the column and used as bait is very similar if not identical. Following washing 200 μg purified His-tagged SARM-TIR, the "prey", was added to the reaction mixtures. After a 2-hour incubation at 4 °C, unbound proteins were removed by washing the resin four times in 20 CVs binding buffer, after which the beads were mixed with an equal volume of SDS-PAGE sample buffer, electrophoresed and subsequently Western blotted.

2.9. Subcellular fractionation

To localise the cellular compartment where SARM-TIR might interact with other TLR–TIRs, mitochondria were extracted from homogenised cells by differential centrifugation using a mitochondria isolation kit (Pierce) following the manufacturer's instructions. Proteins in the cytosolic and mitochondrial fractions were resolved on 12% SDS-PAGE and analysed by western blot using the indicated primary antibodies.

2.10. ELISA

HEK293 cells grown in 24-well plates in 1 mL growth medium per well were transfected with increasing amounts of the indicated plasmids using Turbofect transfection reagent (Fermentas). The total plasmid concentration was normalised to 1 μg/mL growth medium using empty pCDNA plasmid. At 24 h post-transfection, growth media was exchanged for fresh media containing 0.1 μg/mL LPS or 1 μg/mL LTA, as indicated. After an additional 24 h, the absolute levels of TNF-α and IL-8 secreted into the growth medium were quantified with OptEIA human cytokine ELISA kit (BD BioSciences) following the manufacturer's instructions.

2.11. Confocal microscopy

HEK293T cells grown on coverslips were incubated with 30 nM deep red or orange mitotracker (Invitrogen) at 37 °C for 30 min, washed twice in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. After three washes with PBS supplemented with 0.05% (*v*/*v*) Tween 20 (PBST), cells were permeabilised with 0.1% Triton X-100 and immunostained with the indicated antibodies, mounted on glass slides with mounting medium containing 4′-6-diamidino-2-phenylindole (DAPI) and cured overnight at room temperature in the dark. Cells were finally viewed under LSM 510 META confocal microscopy using an EC Plan-Neofluar 100×/1.3 oil immersion objective (Carl Zeiss).

2.12. Cell viability assay

Cell viability was estimated by the (4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. HEK293T cells were grown in 96-well plates in 100 μL growth medium per well at 37 °C, 5% CO_2, in a humidified environment and transfected with the indicated amounts of plasmid. Total plasmid concentration was normalised to 100 ng per well using empty pCDNA vector. MTT tetrazolium salt (Sigma) was solubilised in PBS at 5 mg/mL. At specified timepoints, 20 μL was added to each well, followed by incubation for 4 h. The formazan crystals which formed were solubilised overnight by adding 100 μL 10% SDS in 10 mM HCl. Finally, the metabolic activity of viable cells was measured at 590 nm using a Synergy Mx microplate reader (BioTek).

2.13. Cellular apoptosis assay

Early stage apoptosis was quantified by staining harvested HEK293T cells with Annexin V (BD BioSciences) for 10 min, followed by 7-AAD (BD BioSciences) staining for 10 min to estimate overall cell death. Cells were analysed by FACS using a LSR Fortessa flow cytometry analyser (BD Biosciences) at low flow rate (12 μL/min) for 15,000 events.

2.14. Statistical analysis

Data are displayed as means ± SD of at least three independent experiments, unless otherwise stated. Differences were analysed by two-tailed Student's t-test and results with p values of <0.05 were considered significant.

3. Results

3.1. SARM and MyD88 both localise to the mitochondria

Fluorescence imaging of GFP-tagged full length SARM overexpressed in HEK293T cells showed a clear localisation to the mitochondria (Fig. 1A, upper panels), which was also confirmed by subcellular fractionation and subsequent Western blot analysis (Fig. 1B). Consistent with a previous report [17], the truncated GFP-SARM-TIR protein lacking the N-terminal region, which comprises a mitochondrial targeting sequence, was ubiquitously expressed throughout the cell without any specific pattern (Fig. 1A, lower panels). Interestingly, MyD88 was also observed to co-localise with mitochondria when overexpressed in HEK293T cells. The protein was intensely visible as condensed spots located on the mitochondria (Fig. 2A), an unusual pattern that was also

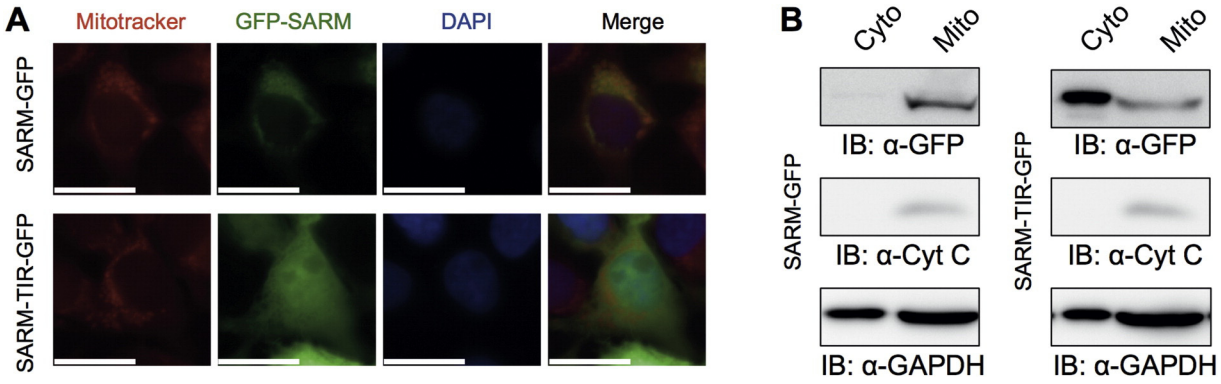

Fig. 1. SARM localises to mitochondria. (A) HEK293T cells were transfected with plasmids coding for GFP-tagged full length SARM or SARM-TIR. After 24 h incubation, cells were stained with Mitotracker, fixed and viewed under a fluorescence microscope. All scale bars, 10 μm. Original magnification X100. (B) Mitochondria from cells were isolated by differential centrifugation and solubilised in SDS-PAGE sample buffer. Mitochondrial and cytosolic fractions were subjected to Western blot analysis and probed with an anti-GFP antibody. GAPDH and Cytochrome C were used as cytosolic and mitochondrial markers, respectively.

described earlier [36]. Western blot analysis of the cytosolic and mitochondrial fractions confirmed that neither SARM nor MyD88 was freely available in the cytosol (Fig. 2B). The expression profile of the two proteins prompted us to speculate whether MyD88 and SARM might interact with each other, as heterotypic TIR–TIR interactions between TLR adaptor proteins is a requirement for downstream TLR signalling.

3.2. Glycine residue located in the TIR BB-loop is highly conserved

Multiple sequence alignment of the BB-loop region of a selection of human and bacterial TIR domains (Fig. 3A) reveals that two amino acid residues, the Pro7 and Gly8 are highly conserved. The proline has been shown to be critical for TLR4 functionality [34]. However, the SARM TIR-domain, like many of the bacterial TIR-domain proteins (Tdps), does not contain Pro7 but harbours the conserved Gly8 (residue 601 of the full length protein). A G158A mutation of the equivalent residue in the TIR-domain protein TcpB from *B. melitensis* results in a protein less effective at inhibiting upregulation of NF-kB in a cell-based assay [37] and with abolished or reduced ability to interact with TLR4 and MAL [38], suggesting that this residue may be of functional relevance. Thus we aimed to explore the role of this residue in SARM function.

3.3. Recombinant protein expression and purification

Although an intact N-terminus is required for human SARM mitochondrial localisation, the isolated TIR domain located at the C-terminus was used for biochemical characterisation as this domain is believed to mediate protein-protein interactions with other TIR-proteins [39]. Attempts to overexpress full length SARM in bacterial systems were performed, but no soluble protein was expressed. The SARM-TIR construct was designed based on the structure prediction software Phyre2 [40]. The protein was expressed in BL21(DE3) *E. coli* cells and purification by immobilised metal-ion affinity chromatography (IMAC) followed by ion exchange chromatography (IEC) resulted in highly pure His-tagged protein with a yield of around 0.3 mg/L TB culture, as shown in Fig. 3B.

GST-tagged TLR adaptor proteins MyD88-TIR, MAL, TRAM and TRIF-TIR were all successfully expressed in BL21 (DE3) *E. coli* cells and purified using a single GST affinity chromatography step, followed by buffer exchange to remove reduced glutathione from the sample buffer. Yields were approximately 1 mg purified protein/L cell culture for all 4 proteins, sufficient for conducting interaction assays with SARM.

3.4. The BB-loop of SARM-TIR mediates interaction with MyD88-TIR

Potential interactions between SARM–TIR and MyD88–TIR, TRIF–TIR, MAL, and TRAM were assessed by GST pull down interaction assay. As shown in Fig. 3C, specific interactions were observed between SARM–TIR and MyD88–TIR, with faint bands also indicating possible weak interactions with MAL and TRAM. Interaction between SARM–TIR and TRIF–TIR is consistent with previous report [13]. As isolated TIR domains were used here instead of full length proteins, we are able to delineate that SARM binds to TRIF via TIR–TIR interaction. Free GST was used as bait in a control reaction to rule out the possibility that SARM–TIR was binding to the GST-tag (of MyD88–TIR, TRIF–TIR, MAL or TRAM) or non-specifically to the resin. Interestingly, the mutation G601A resulted in complete loss of binding of SARM–TIR to all of

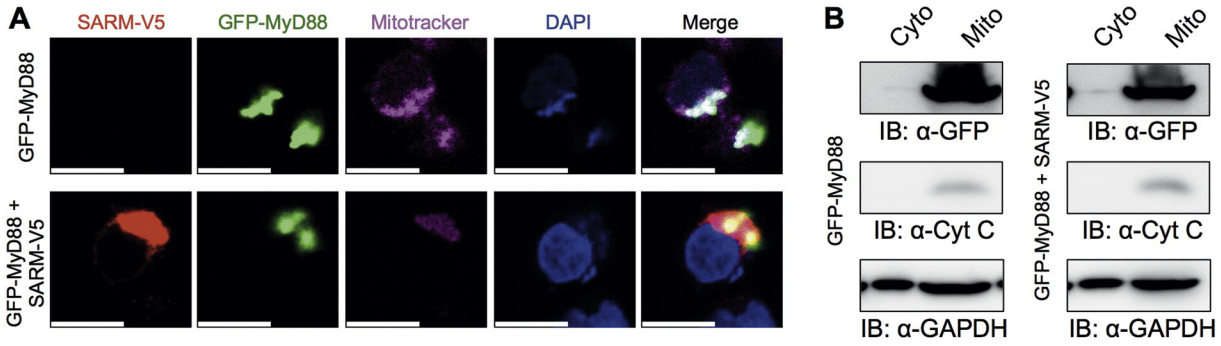

Fig. 2. MyD88 localises to mitochondria. (A) HEK293T cells were transfected with plasmids coding for V5-tagged SARM and/or GFP-tagged MyD88. At 24 h post-transfection, cells were stained with Mitotracker, fixed and viewed under a fluorescence microscope. All scale bars, 10 μm. Original magnification X100. (B) Mitochondria from cells were isolated by differential centrifugation and solubilised in SDS-PAGE sample buffer. Mitochondrial and cytosolic fractions were subjected to Western blot analysis and probed with an anti-GFP antibody. GAPDH and Cytochrome C were used as cytosolic and mitochondrial markers, respectively.

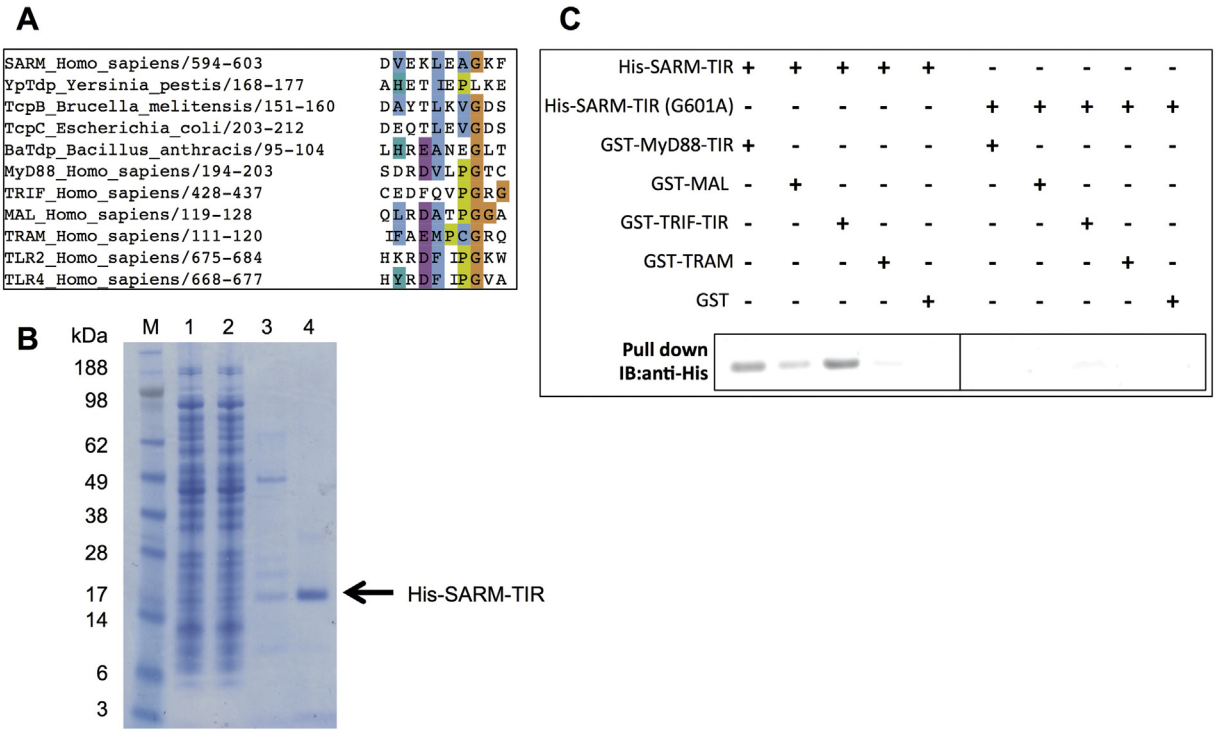

Fig. 3. The BB-loop is critical for SARM-TIR interaction with TIR domains of TRIF and MyD88. (A) Multiple sequence alignment of the amino acids in the predicted BB-loops from a selection of bacterial and human TIR domains shows that a glycine (G) residue is highly conserved in the amino acid sequence. Alignment was generated with ClustalX and visualised using Jalview. (B) SDS-PAGE of samples taken from various stages of the purification process of His-SARM-TIR. M, molecular weight marker; lane 1, soluble fraction of cell lysate; lane 2, flow through during IMAC; lane 3, eluted fraction during IMAC; lane 4, His-SARM-TIR fraction eluted from IEC. (C) Western blot analysis of samples obtained from GST pull down interaction assays between WT His-tagged SARM-TIR or the equivalent G601A mutant, and GST-tagged MyD88-TIR, MAL, TRIF-TIR and TRAM. Immunoblots were probed with anti-polyhistidine antibody. Pull down results shown are from non-adjacent lanes of the same immunoblot.

the adaptor proteins (Fig. 3C), suggesting that this residue is functionally critical for SARM's ability to form TIR-TIR complexes.

3.5. Gly601 in the BB-loop is critical for SARM-mediated immune suppression

We also assessed the effects of both full length SARM and the G601A mutant on TLR signalling. We used a HEK293 cell line stably transfected with TLR4, MD2 and CD14 (HEK-TLR4) and transiently expressing either WT or mutant SARM. Following stimulation with 0.1 μg/mL LPS for 24 h, the amounts of inflammatory cytokines, TNF-α and IL-8, in the cell supernatants were quantified by ELISA. As expected, WT SARM effectively suppressed cytokine secretion, showing significant dose-dependent reduction of IL-8 and TNF-α (Fig. 4A). Expression of SARM G601A mutant protein, on the other hand, showed a loss of anti-inflammatory activity as reflected by the lack of effect on the levels of IL-8 or TNF-α following LPS stimulation, suggesting that this specific residue is critical for SARM-mediated immune suppressive function.

As TLR4 is known to utilise both the MyD88- and TRIF-dependent pathways for immune activation, the above results do not discriminate between which pathways is affected by SARM expression. Since SARM has been reported to suppress TRIF-dependent signalling [13], it was pertinent for us to clarify whether the protein also acts on the MyD88-pathway. As a direct approach to test this, we provoked an immune response in HEK cells by overexpressing MyD88 in the cell and probed for potential co-expression of SARM-mediated reduction of cytokine activation. 100 ng of MyD88 plasmid-transfection mixture was found to be sufficient to induce cytokine activation. Interestingly, WT SARM expression was associated with reduced levels of MyD88-induced secretion of IL-8 and TNF-α suggesting that SARM dose-dependently inhibits MyD88-induced TLR signalling (Fig. 4B). In line with previous results, the G601A mutant SARM had no significant effect on immune activation, underscoring the predominant functional role of this single residue. To conclusively show that SARM acts on MyD88-mediated TLR signalling, HEK293 cells expressing TLR2 were transfected with plasmids encoding WT or G601A mutant SARM and incubated for 24 h, followed by stimulation with 1 μg/mL LTA for an additional 24 h. Cellular supernatants were analysed for IL-8 content by ELISA (Fig. 4C), and WT SARM expression was again associated with a reduction of IL-8 secretion, while expression of SARM (G601A) had no noticeable effect. As TLR2 exclusively utilises MyD88 for downstream signalling, this result further supports that SARM acts on this pathway [14].

Since SARM is pro-apoptotic in mammalian cells, one possible explanation for the apparent lack of immune suppressive effect associated with expression of the G601A mutant protein might be that the expression of WT SARM simply resulted in higher cell death than expression of the mutant protein. To investigate this possibility, we assessed the overall cell viability of HEK cells expressing either WT or mutant SARM by conducting an MTT-assay with these cells (Fig. 5A-B). While expression of both proteins was associated with lower overall cell viability compared to non-treated control cells, there was no significant difference in cell death between cells expressing SARM or SARM (G601A), suggesting that the difference in the observed cytokine secretion after immune activation is not an effect of a different rate of cell death. Annexin V and 7-AAD cellular staining of HEK293T cells expressing either WT or G601A mutant SARM confirmed that the mutation had no noticeable effect on the protein's pro-apoptotic function (Fig. 6). Although further studies are needed to describe the precise mechanism by which SARM induces apoptosis, the results suggest that the immunosuppressive and pro-apoptotic functions of SARM are likely mediated by separate regions of the protein.

3.6. SARM BB-loop peptide targets MyD88

As a mutation in the BB-loop region disrupted SARM binding to other TIR-domains, we speculated that this region may mediate

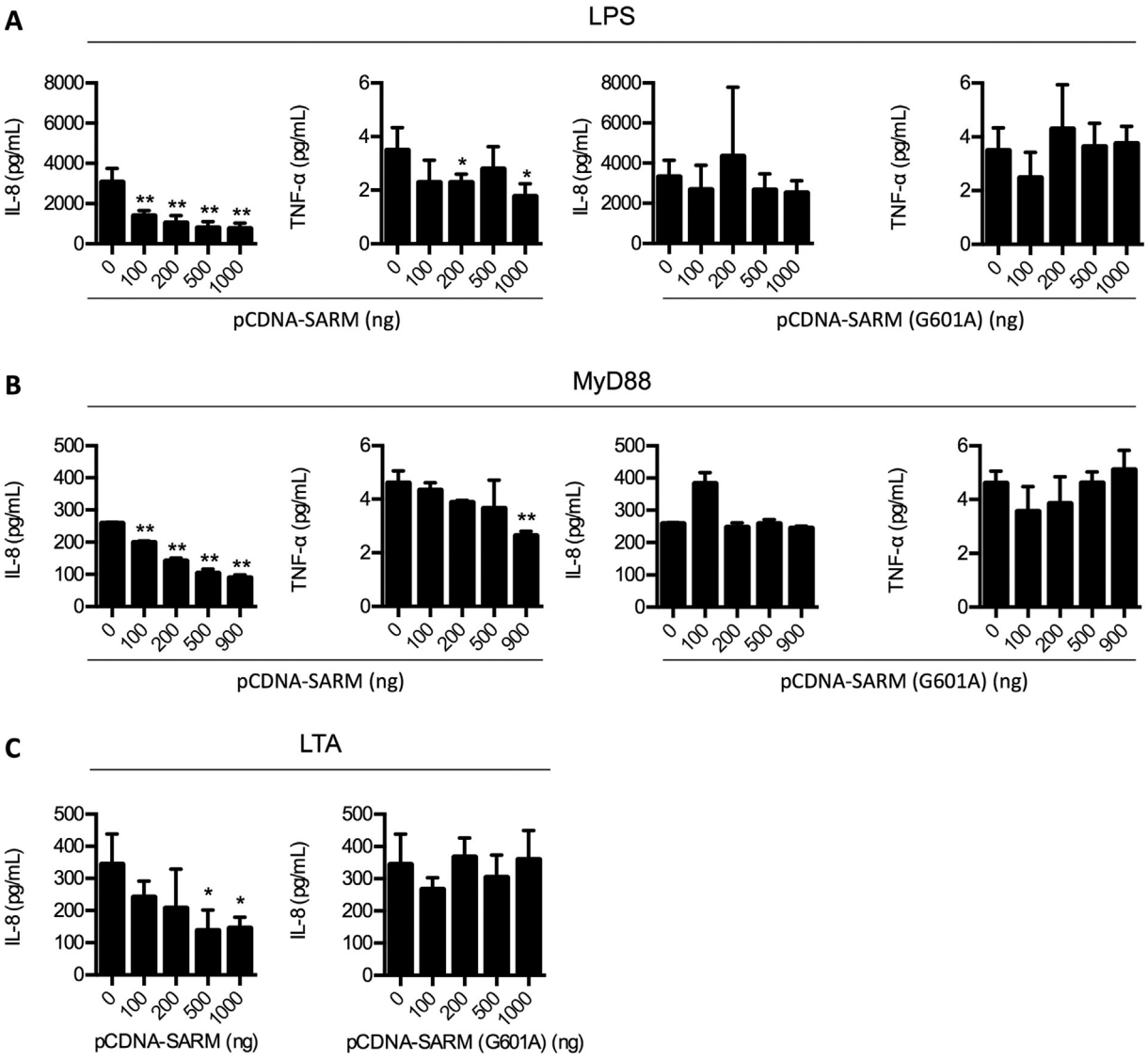

Fig. 4. SARM expression inhibits TLR4- and MyD88-induced IL-8 and TNF-α activation in HEK cells. ELISA was used to quantify secreted levels of inflammatory cytokines IL-8 and TNF-α in HEK-TLR4 cells expressing WT SARM or SARM (G601A) following stimulation with 0.1 μg/mL LPS (A) or co-transfection with 100 ng MyD88-encoding plasmid (B). (C) ELISA was also used to quantify levels of inflammatory cytokine IL-8 in HEK-TLR2 cells expressing WT SARM or SARM (G601A) following stimulation with 1 μg/mL LTA. *Error bars*, SD of triplicates; *, $p < 0.05$; **, $p < 0.01$.

interactions with other TIR domains, and decided to test this by first expressing and purifying a peptide based on this sequence, both as a GST-tagged fusion protein and as a free peptide. As shown in Fig. 7A, GST pull down confirmed that this short peptide was able to bind specifically to MyD88 (here expressed and purified as a GB1 fusion protein with a C-terminal His-tag), while the G601A mutation in the SARM BB-loop again resulted in no visible interaction between the two proteins. To ensure the BB-loop peptide crosses the cell membrane of mammalian cells and evaluate the effect it has in a cellular environment, we designed a novel peptide by fusing the SARM BB-loop region with the cell penetrating sequence of the *Antennapedia* homeodomain (ANTP-SARM-BB). The fusion peptide was also labelled with rhodamine for simple detection by fluorescence microscopy. The peptide was seen in the cytosol of HEK293T cells after just 30-min exposure, and an apparent co-localisation with GFP-tagged MyD88 could be seen (Fig. 7B). MTT assay was used to evaluate any potential cytotoxicity after overnight treatment of HEK293T cells with ANTP-SARM-BB, and a slight effect could be seen with concentrations above 10 μM (Fig. 7C). This is consistent with experiments conducted with isolated ANTP-peptide [41], suggesting that the SARM-BB region does not markedly change the cytotoxic profile of the peptide. To finally evaluate if the peptide had any immunosuppressive function, HEK-TLR4 cells were treated with up to 20 μM ANTP-SARM-BB before being stimulated with LPS overnight, and a small but significant decrease in IL-8 secretion could indeed be detected (Fig. 7D).

4. Discussion

In this study, we have characterised the interaction of human SARM TIR domain with other TLR adaptors, and delineated the critical amino acid residue in the BB-loop of SARM responsible for interaction with the MyD88 TIR domain. Furthermore, we elucidated the functional role of SARM in negatively regulating TLR signalling.

Carty and colleagues [13] first characterised SARM as a negative regulator of TLR3 and TLR4 signalling in 2006, in contrast to the other four characterised human TLR adaptor proteins, which have all been described as positive regulators. Further studies showed that SARM is more closely related to bacterial TIR-proteins than to the other mammalian TIR adaptor proteins [27], supporting the idea that it has a distinct functional role as several bacterial TIR domain proteins have been demonstrated to

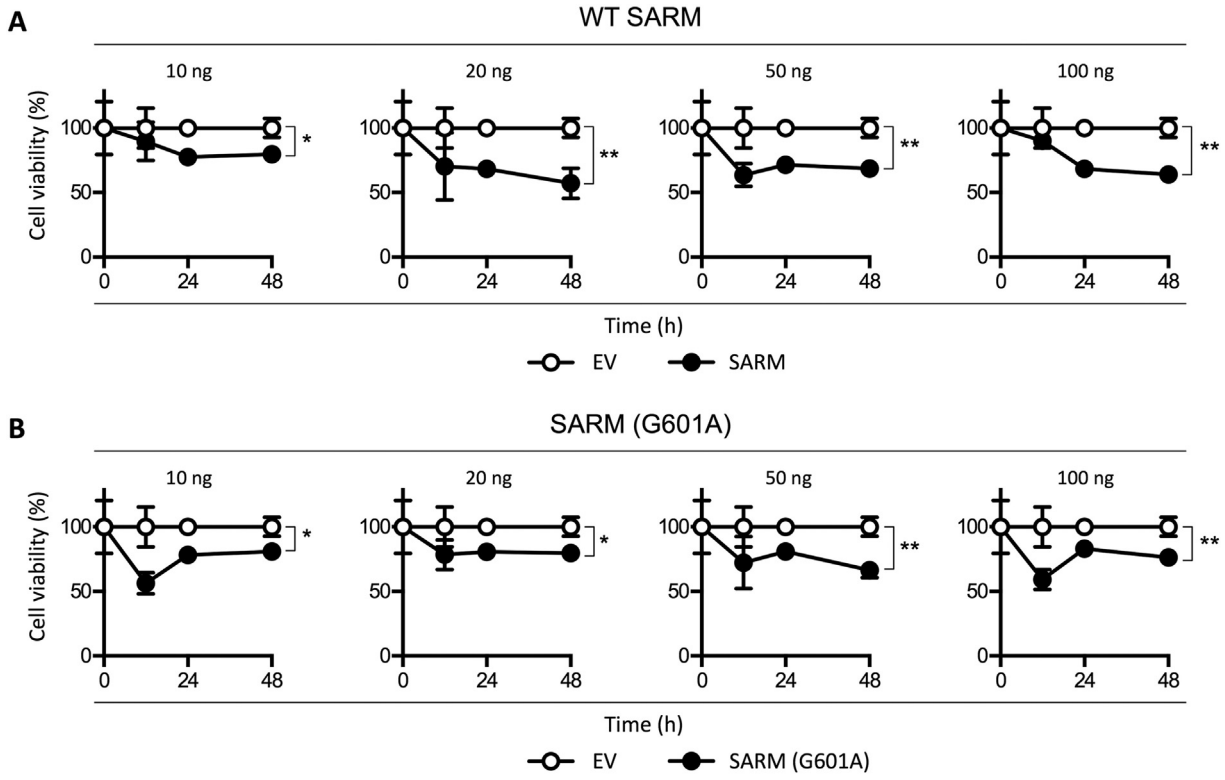

Fig. 5. Expression of WT or G601A mutant SARM resulted in reduced cell viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to quantify the overall cell viability of HEK293T cells expressing WT SARM (A) or SARM (G601A) (B), up to 48 h post-transfection. Amounts of plasmid used for transfection are indicated above each graph. Plasmid amount was normalised in each well to 100 ng. The significant differences in cell viability indicated are based on cell viabilities 48 h post-transfection. *Error bars*, SD of triplicates; *, $p < 0.05$; **, $p < 0.01$. EV, empty vector.

suppress TLR signalling [32]. In view of the evolutionary proximity of SARM to bacterial Tdps, and its presence and conservation in eukaryotes, we hypothesise that SARM could have been transferred from a microbial invader into the eukaryotic host, and through its TIR domain, SARM may be exerting a dual regulatory role: to crosstalk and regulate TLR mediated immune responses during an infection. Crosstalk within TLR signalling pathways is complex, but it can lead to an increase in specificity through collaborative engagement of multiple TLRs [42], and SARM may play an antagonistic role in this perspective.

As TLR signalling is highly dependent on the formation of heterotypic TIR–TIR interactions between the receptor and one or more cytosolic adaptor(s), any interference with the TIR–TIR interaction has the potential to inhibit or attenuate TLR signalling and influence downstream immune activation. This motivated us to investigate whether SARM, as a negative TLR adaptor, interacts with any of the other four known positively regulating TLR adaptors. An immediate problem we faced when assessing this was that TIR domain proteins are inherently difficult to express in bacterial systems, and display a low overall stability in vitro. Full length SARM did not express at all in BL21 (DE3) cells, with TRIF and MyD88 also showing very poor expression profiles, prompting us to generate a large set of constructs for expression trials. Minimal constructs comprising only the isolated TIR domains displayed the highest expression yield as well as stability, especially when fused to a solubility-tag such as GST or GB1. These results were not unanticipated, as constructs featuring isolated TIR domains have consistently been shown easier to express and purify [29,30]. For example, Ullah and colleagues recently described the expression and purification of both full length TRAM and truncated constructs comprising mainly the TIR, with the shorter constructs yielding up to three times more protein [43].

GST pulldown assays demonstrated that the SARM TIR domain interacts with both TRIF and MyD88, the two main adaptors that together transduce signalling from all human TLRs [44]. A single glycine to alanine amino acid substitution in the SARM BB-loop region was enough to fully disrupt these interactions, underlining the functional importance of an intact and flexible SARM BB-loop. As these experiments were performed with isolated TIR domains of adaptors SARM, MyD88 and TRIF, it is not certain that the full length proteins would behave similarly. It is possible that the interactions seen here may be blocked by regions of the full length proteins that were excluded in these constructs. However the results from the cell-based assays, as discussed below, supported these findings.

We found that SARM expression significantly reduced inflammatory cytokine activation by LPS-induced TLR4- or LTA-induced TLR2 stimulation in HEK cells, and this effect was completely abolished when the G601A mutation in the SARM BB-loop was introduced. In line with our binding assay, SARM expression also reduced cytokine activation caused by overexpression of MyD88, showing that the protein likely acts on both the MyD88- and TRIF-dependent pathways and not just the TRIF-dependent pathway as has been previously demonstrated. While the experiments performed in this study were performed in a model cell line, they provide important mechanistic information about SARM function. Taken together the pulldown and cell-based assay results strongly indicate that SARM can act on multiple targets to modulate TLR-signalling.

While the G601A mutation had a clear effect on the immunosuppressive function of SARM, we did not observe any notable difference in apoptotic activity between cells expressing WT or mutant SARM. This supports the findings from previous studies that have indicated that the N-terminal part of SARM is critical for mediating apoptosis [16,17], while only sterile α-motif (SAM)- and TIR-domains located near the C-terminus appear to be required for suppressing TLR-signalling [13,21]. It seems that apoptosis and inhibition of TLR-activation are mediated by discrete parts of the protein, and future studies further characterising the precise mechanisms involved would help to gain more insights into this mechanism.

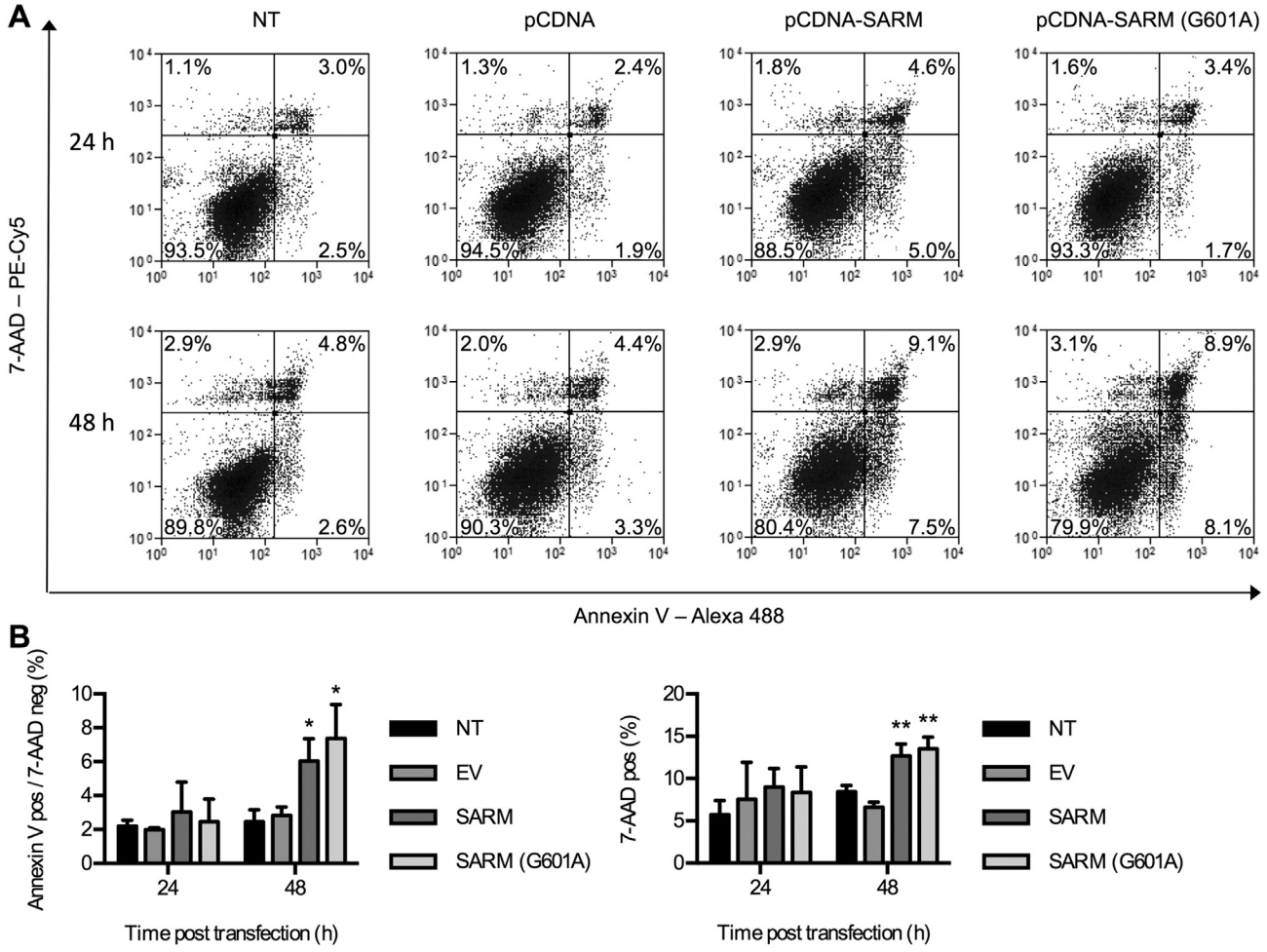

Fig. 6. The SARM G601A BB-loop mutation does not affect pro-apoptotic function. HEK293T cells transfected with 1 μg/mL pCDNA-SARM or pCDNA-SARM (G601A) were stained with Annexin V and 7-AAD 24 or 48 h post-transfection, followed by analysis by FACS. Dot plots shown (A) are representative of three independent experiments. (B) Statistical analysis of three independent experiments. *Error bars*, SD of triplicates; EV, empty vector; *, $p < 0.05$; **, $p < 0.01$.

Peptides derived from BB-loops of MyD88 [45,46], TRAM [47], TRIF [48], MAL [49], TLR4 [50], as well as *E. coli* protein TcpC [51], have all been shown to inhibit TLR-mediated immune activation in mammalian cells. Mechanisms in all cases appear to involve the peptide occupying the native binding sites on either TLRs or adaptor proteins used for homo- or hetero-dimerisation. A low molecular weight molecule modelled on the (F/Y)-(V/L/I)-(P/G) consensus sequence in the BB loops of multiple TIR domain proteins has also been demonstrated to regulate the interaction between MyD88 and IL1-R, but not the MyD88/TLR4 interaction, and was able to significantly reduce the IL-1β-induced fever response in vivo [52]. It is possible that the SARM BB-loop, both examined here as part of the full length protein or as an isolated peptide, may utilise a similar mechanism, blocking both MyD88- and TRIF-mediated signalling. A peptide derived from this motif specifically interacted with the MyD88 TIR domain during pull down studies, and a cell penetrating peptide comprising the SARM BB-loop fused to the ANTP sequence easily permeated the HEK293T cells, and co-localised with overexpressed MyD88 in the cell. Cells treated with SARM BB-loop peptide also displayed a slightly reduced ability to respond to TLR4-activation, suggesting that this region may be developed into a therapeutic immunomodulator. As the rhodamine-tagged SARM BB-peptide started to display a noticeable cytotoxic effect at concentrations around 10–20 μM, we were unable to probe if the anti-inflammatory effect would be stronger with a higher dose. As TIR–TIR interactions may be mediated by multiple parts of the protein, it is possible that other motifs could contribute further anti-inflammatory effect. Studies of fragmented TIR domains have previously indicated immunosuppressive effects from diverse sets of peptides. For example, Piao and colleagues have shown that a peptide derived from the TRAM C helix was able to potently inhibit both MyD88- and TRIF-dependent cytokine activation [47], while another peptide derived from the TRIF B helix was able to suppress TLR4 signalling in vivo and protect mice from lethal endotoxemia [48]. The availability of a SARM-TIR domain crystal structure would be pertinent to accurately predict exactly which regions of the protein are exposed. Nevertheless, the SARM-BB peptide designed in this study provided insight into its potential application in immunomodulation of MyD88- and TRIF-pathways.

As dysregulation of TLRs has been linked to several inflammatory diseases [53], the receptors have been the targets of several drugs in development over the past decade [54], and recently the intracellular TLR adaptors have also been suggested as potential therapeutic targets [55]. The native ability of SARM to interact with both MyD88 and TRIF, positions SARM strategically, as a broad target for drug development towards immune-modulation. The fact that SARM is an endogenous human protein might also indicate that drugs based on its sequence would have a lower immunogenic potential compared with artificial peptides.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.11.021.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

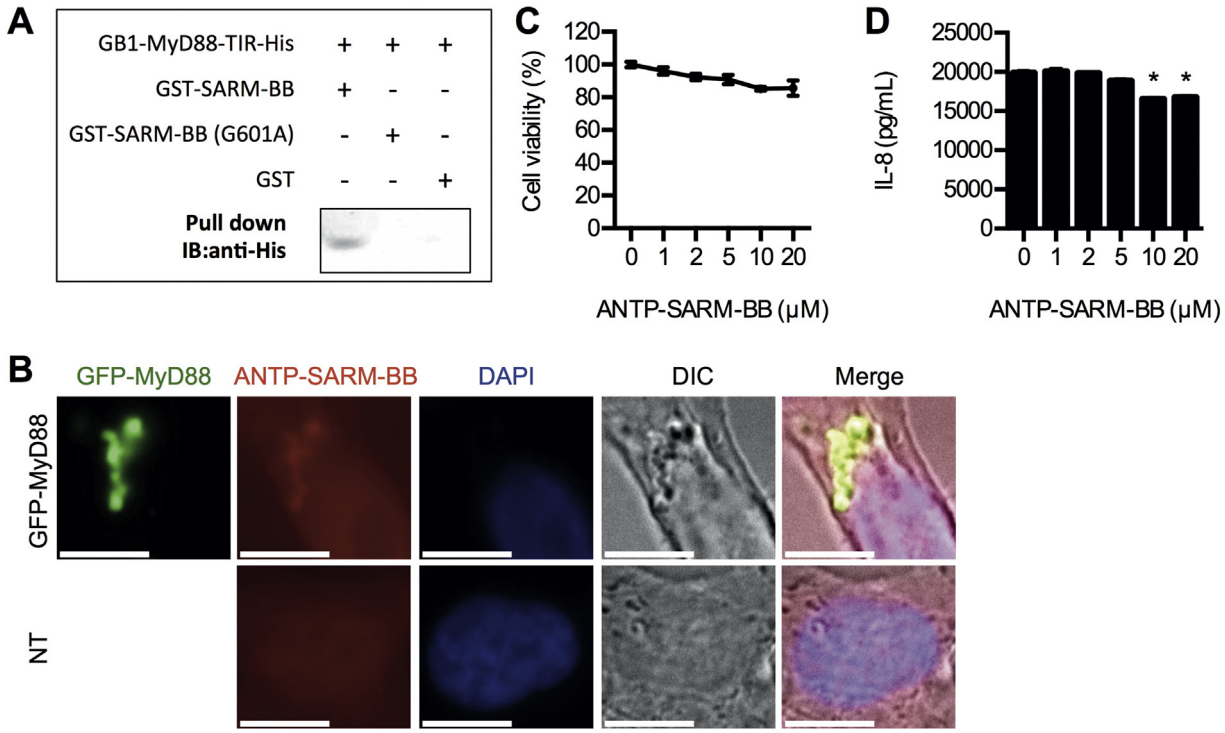

Fig. 7. SARM BB-loop peptide targets MyD88. (A) Interaction between GST-tagged BB-loop peptide and GB1-MyD88-TIR-His was probed by pull down assay. Samples were probed with anti-polyhistidine antibody. (B) HEK293T cells expressing GFP-tagged MyD88 (upper panels), or non-transfected (lower panels), were treated with 10 μM rhodamine labelled ANTP-SARM-BB peptide for 30 min, after which cells were fixed and viewed under a fluorescence microscope. All scale bars, 10 μm. Original magnification X100. NT, nontransfected cells; DIC, differential interference contrast. (C) MTT assay of HEK293T cells treated with 0–20 μM ANTP-SARM-BB peptide for 24 h. *Error bars*, SD of triplicates. (D) ELISA was used to quantify IL-8 content in HEK-TLR4 cell media following treatment with 0–20 μM ANTP-SARM-BB peptide for 30 min and stimulation with 0.1 μg/mL LPS for 24 h. *Error bars*, SD of triplicates; *, $p < 0.05$.

Acknowledgements

This work was funded by grants from the Imperial College International Joint PhD scholarship programme and the National Medical Research Council, Singapore (NMRC/CBRG/0055/2014). We thank Dr. Susan Chang for advice on MTT and cytokine secretion assays.

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