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The human adaptor SARM negatively regulates adaptor protein TRIF–dependent Toll-like receptor signaling

Michael Carty[1], Rory Goodbody[1], Martina Schröder[1], Julianne Stack[1], Paul N Moynagh[2] & Andrew G Bowie[1]

Toll-like receptors discriminate between different pathogen-associated molecules and activate signaling cascades that lead to immune responses. The specificity of Toll-like receptor signaling occurs by means of adaptor proteins containing Toll–interleukin 1 receptor (TIR) domains. Activating functions have been assigned to four TIR adaptors: MyD88, Mal, TRIF and TRAM. Here we characterize a fifth TIR adaptor, SARM, as a negative regulator of TRIF-dependent Toll-like receptor signaling. Expression of SARM blocked gene induction 'downstream' of TRIF but not of MyD88. SARM associated with TRIF, and 'knockdown' of endogenous SARM expression by interfering RNA led to enhanced TRIF-dependent cytokine and chemokine induction. Thus, the fifth mammalian TIR adaptor SARM is a negative regulator of Toll-like receptor signaling.

It is now well established that Toll-like receptors (TLRs) are pivotal in initiating the early innate immune response and in directing the later adaptive immune response[1]. Activation of TLRs by pathogen-associated molecular patterns such as lipopolysaccharide (LPS; a ligand for TLR4) or viral double-stranded RNA (a ligand for TLR3) triggers signaling cascades that alter gene expression, which is required for effective pathogen-specific immune responses. TLR engagement activates several transcription factors, including NF-κB, interferon-regulatory factor 3 (IRF3), IRF5 and IRF7, which in turn activate many genes encoding immunoregulatory molecules, including type I interferons, chemokines and inflammatory cytokines[2].

A key feature of the TLR family, which now has 13 members identified in mammals, is the presence of the conserved cytosolic protein motif TIR (Toll–interleukin 1 receptor (IL-1R)) domain through which TLRs mediate 'downstream' signaling. Extensive research over the past 5 years has demonstrated much about the molecules involved in intracellular TLR signaling. TIR domain–containing adaptor proteins are initially recruited to receptors. The specificity of signaling by distinct TLRs is achieved in part through alternate and combinatorial adaptor usage. Five such adaptor proteins have been identified, and so far four of those, MyD88, Mal, TRIF and TRAM[3], have been assigned distinct functions.

MyD88, the first adaptor to be discovered, is required for signaling by all TLRs except TLR3 (ref. 4). IL-1R also uses MyD88 and its downstream signaling components. IL-1R-associated kinases are serine-threonine kinases recruited to TLR-MyD88 complexes and are required for subsequent activation of the adaptor molecule TRAF6, which results in activation of downstream NF-κB and mitogen-activated protein kinases[3]. For TLR7 and TLR9, which respond to viral single-stranded RNA and CpG DNA, respectively, activation of MyD88 can also lead to IRF activation[2]. Although the responses to IL-1 and many TLR agonists are completely impaired in MyD88-deficient mice, for LPS-TLR4 some signals are intact, such as late-phase NF-κB activation and stimulation of the interferon pathway through IRF3 activation[5]. The discovery of the second TIR adaptor, Mal (also called TIRAP), suggested a candidate TLR4 adaptor for mediation of MyD88-independent signaling[6,7]. However, the phenotype of Mal–deficient mice is similar to that of MyD88-deficient mice for both TLR2 and TLR4 agonists, leading to the placement of Mal on the MyD88-specific pathway for those two TLRs[5]. As with the other adaptors, TRIF expression activates NF-κB, but unlike Mal and MyD88, TRIF also induces the promoter of the gene encoding interferon-β (*Ifnb*)[8,9]. That result raised the possibility that TRIF is the adaptor in the TLR4-MyD88–independent pathway as well as the 'missing' adaptor for TLR3 and would thus mediate activation of both NF-κB and IRF3. Subsequently, the generation of TRIF-deficient and TRIF-mutant mice has conclusively linked TRIF to TLR3 and also to the MyD88-independent component of the TLR4 pathway[4,10]. The fourth TIR adaptor, TRAM, is specific for TLR4 and functions 'upstream' of TRIF in the induction of MyD88-independent signaling[11].

The fifth member of this adaptor family, SARM, is a 690–amino acid protein that is highly conserved in *Caenorhabditis elegans*, drosophila and mammals[12], yet it has no known function in mammalian systems[3]. The *C. elegans* ortholog of SARM, TIR-1, has been assigned a function in worm immunity and in development. RNA-mediated interference of TIR-1 mRNA in *C. elegans* leads to diminished worm survival in response to fungal infection, partly due to reduced expression of the antimicrobial peptides NLP-29 and NLP-31 (ref. 13). Similar findings[14] have shown that the *C. elegans* p38 mitogen–activated protein kinase PMK-1, which is important in worm immunity, requires TIR-1 for activation. TIR-1 is also involved in *C. elegans* development, specifically in the asymmetric expression of

[1]School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland. [2]School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland. Correspondence should be addressed to A.B. (agbowie@tcd.ie).

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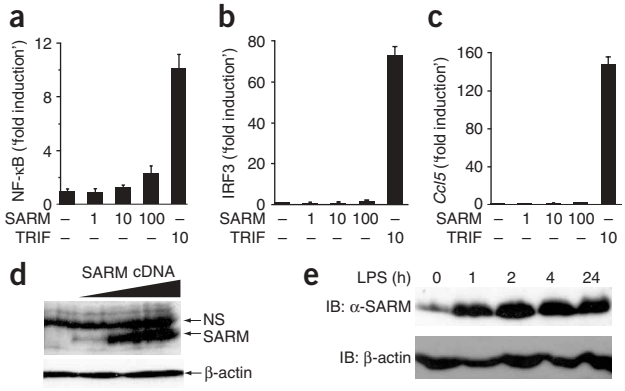

Figure 1 Expression of SARM fails to activate NF-κB or IRF3. (**a**–**c**) Luciferase assay of HEK293 cells transfected for 24 h with 1–100 ng Flag-tagged SARM or 10 ng TRIF along with reporter constructs for NF-κB (**a**), IRF3 (**b**) or the *Ccl5* promoter (**c**). (**d**) Immunoblot for expression of Flag-tagged SARM in HEK293 cells (top). NS, nonspecific. Wedge indicates increasing concentration of cDNA transfected. Bottom, immunoblot reprobed with anti-β actin (loading control). (**e**) Immunoblot (IB) for SARM in lysates of PBMCs treated with LPS (time, above lanes). Experiments are one of two repeats (error bars, s.d.).

odorant receptors of olfactory neurons[15]. Those results suggest a positive function for TIR-1 in *C. elegans* immunity. In contrast, however, a comparative study of the five mammalian adaptors has shown that mammalian SARM fails to activate NF-κB, unlike the other four adaptors[14].

Here we report that human SARM functions as a specific inhibitor of TRIF-dependent signaling, transcription factor activation and gene induction. We also identify the sequence motifs in SARM required for the inhibitory function and demonstrate that SARM and TRIF interact. Notably, small interfering RNA (siRNA) targeting of SARM mRNA led to enhanced TLR3- and TLR4-dependent gene induction in both transformed and primary human cells. Thus, we demonstrate an important function for SARM as a TRIF-specific inhibitory protein.

RESULTS

SARM expression downregulates TRIF-dependent NF-κB

Given that SARM is a cytosolic TIR domain–containing protein, we sought to determine whether it functions like other TIR domain–containing proteins. We first compared the ability of SARM and TRIF to drive transcription factor activation and gene induction. Consistent with published reports[14,16], we found that although overexpression of TRIF led to activation of NF-κB and IRF3, expression of SARM had little or no effect (**Fig. 1a**,**b**). In agreement with those results, TRIF expression led to induction of the *Ccl5* (RANTES) promoter, which requires NF-κB and IRF3 activation (**Fig. 1c**), whereas SARM expression, which we confirmed by immunoblot (**Fig. 1d**), did not activate the *Ccl5* promoter (**Fig. 1c**). Although expression of SARM did not lead to NF-κB activation, stimulation of TLR4 signaling by means of LPS treatment strongly enhanced endogenous SARM expression in primary human peripheral blood mononuclear cells (PBMCs; **Fig. 1e**). That observation led us to explore alternative functions for SARM in TLR signaling pathways.

In particular, we investigated whether SARM has a negative regulatory function, similar to that of the membrane-bound TIR domain–containing proteins SIGIRR and ST2, both of which are involved in the downregulation of IL-1R and TLR4 signaling[17,18]. All IL-1R–TLR family members except TLR4 signal via MyD88 or TRIF; TLR4 signals via both. We therefore examined the effect of SARM expression on signaling pathways emanating from MyD88 and TRIF. MyD88-induced NF-κB activation was unaffected by SARM expression (**Fig. 2a**), whereas TRIF-induced NF-κB was inhibited in a dose-dependent way by SARM expression (**Fig. 2b**). Consistent with the lack of effect on the MyD88 pathway, there was also a lack of effect on Mal, which functions upstream of MyD88 in TLR4 signaling (**Fig. 2c**). We also noted apparently selective inhibition of the TRIF but not the MyD88 pathway for ligand-induced NF-κB activation: IL-1-induced NF-κB activation, which is mediated exclusively by MyD88, was unaltered by SARM expression (**Fig. 2d**), whereas TLR3-dependent polyinosinic-polycytidylic acid (poly(I:C))–induced NF-κB, which is entirely TRIF dependent, was potently blocked by SARM expression (**Fig. 2e**). In addition to a critical function in TLR signaling, NF-κB activation is also a hallmark of tumor necrosis factor (TNF) and RNA helicase RIG-I signaling[19]; neither of those pathways was affected by SARM (**Fig. 2f**,**g**). Thus, inhibition of NF-κB by SARM expression is restricted to the TRIF pathway.

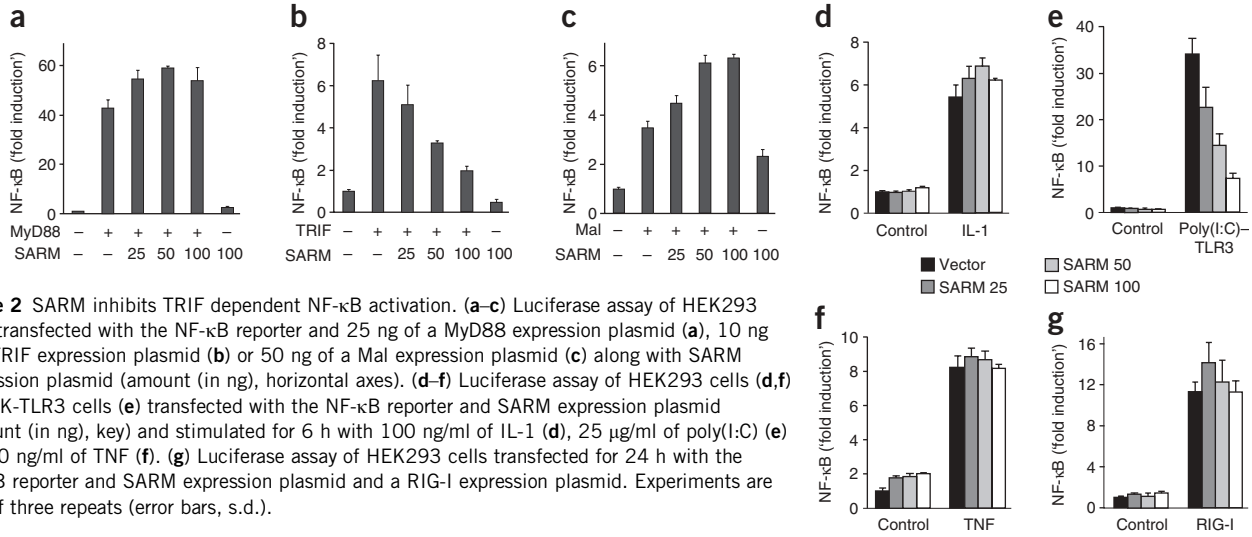

Figure 2 SARM inhibits TRIF dependent NF-κB activation. (**a**–**c**) Luciferase assay of HEK293 cells transfected with the NF-κB reporter and 25 ng of a MyD88 expression plasmid (**a**), 10 ng of a TRIF expression plasmid (**b**) or 50 ng of a Mal expression plasmid (**c**) along with SARM expression plasmid (amount (in ng), horizontal axes). (**d**–**f**) Luciferase assay of HEK293 cells (**d**,**f**) or HEK-TLR3 cells (**e**) transfected with the NF-κB reporter and SARM expression plasmid (amount (in ng), key) and stimulated for 6 h with 100 ng/ml of IL-1 (**d**), 25 μg/ml of poly(I:C) (**e**) or 100 ng/ml of TNF (**f**). (**g**) Luciferase assay of HEK293 cells transfected for 24 h with the NF-κB reporter and SARM expression plasmid and a RIG-I expression plasmid. Experiments are one of three repeats (error bars, s.d.).

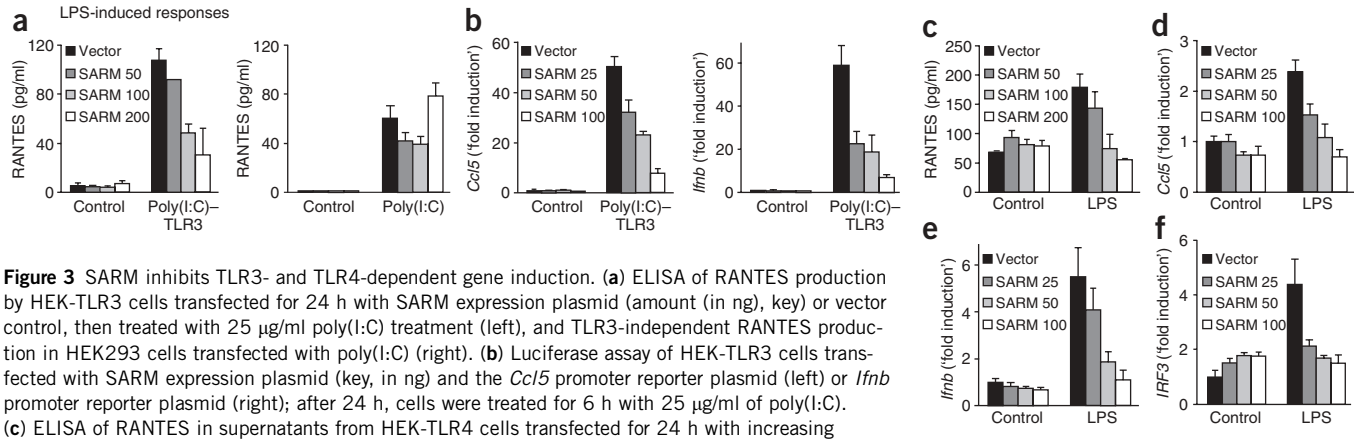

Figure 3 SARM inhibits TLR3- and TLR4-dependent gene induction. (**a**) ELISA of RANTES production by HEK-TLR3 cells transfected for 24 h with SARM expression plasmid (amount (in ng), key) or vector control, then treated with 25 μg/ml poly(I:C) treatment (left), and TLR3-independent RANTES production in HEK293 cells transfected with poly(I:C) (right). (**b**) Luciferase assay of HEK-TLR3 cells transfected with SARM expression plasmid (key, in ng) and the *Ccl5* promoter reporter plasmid (left) or *Ifnb* promoter reporter plasmid (right); after 24 h, cells were treated for 6 h with 25 μg/ml of poly(I:C). (**c**) ELISA of RANTES in supernatants from HEK-TLR4 cells transfected for 24 h with increasing amounts of SARM (key, in ng) or vector and then stimulated for 24 h with 1μg/ml of LPS. (**d**,**e**) Luciferase assay of HEK-TLR4 cells transfected for 24 h with increasing amounts of SARM (key, in ng) or vector along with the *Ccl5* promoter reporter (**d**) or *Ifnb* promoter reporter (**e**). (**f**) Luciferase assay of HEK-TLR4 cells transfected for 24 h with Gal4-IRF3 and the pFR-luciferase reporter in conditions similar to those in **d**,**e**. Experiments are one of three repeats (error bars, s.d.).

SARM inhibits induction of TLR4- and TLR3-dependent genes

We next examined the effect of SARM on TRIF-dependent signaling and chemokine induction in more detail. TLR3 induces genes exclusively via TRIF. Treatment of TLR3-expressing HEK293 cells (called 'HEK-TLR3' cells here) with poly(I:C) led to RANTES production that was inhibited in a dose-dependent way by SARM expression, whereas SARM alone had no effect on basal RANTES expression (**Fig. 3a**, left). Poly(I:C) can also signal through engagement of the double-stranded RNA–dependent protein kinase PKR and RIG-I, both of which are located in the cytoplasm[20,21]. To determine the effect of SARM on those pathways, we used HEK293 cells lacking TLR3 expression; we transfected the cells with poly(I:C) and then assessed RANTES production. In contrast to the effect of SARM on TLR3-dependent gene induction, TLR3-independent RANTES production was mostly unaltered by transient SARM expression (**Fig. 3a**, right).

We next demonstrated that the effect of SARM on TLR3-dependent gene induction was at or upstream of the level of promoter activation. SARM expression potently inhibited TLR3-induced activation of both the *Ccl5* and *Ifnb* promoters (**Fig. 3b**). Both promoters are dependent on activity of the transcription factors NF-κB and IRF3. As well as inhibiting TLR3-dependent NF-κB activation (**Fig. 2e**), SARM expression also inhibited poly(I:C)-induced activation of the interferon-stimulated responsive element but not RIG-1-stimulated activation of IRF3 (data not shown), which indicated the specificity of the SARM inhibitory effect on the TLR3 pathway for poly(I:C).

Because TLR4 also uses TRIF for signaling to NF-κB and IRF3, we next tested whether TLR4 signaling was also suppressed by SARM. Similar to the results obtained for TLR3, LPS-TLR4–induced RANTES production was also potently inhibited by SARM expression (**Fig. 3c**). RANTES production by TLR4 is known to be entirely TRIF dependent[11]. We obtained further evidence that SARM suppresses TRIF-specific signaling by examining induction of the TLR4-TRIF-IRF3–dependent promoters *Ccl5* (**Fig. 3d**) and *Ifnb* (**Fig. 3e**) and by assessing LPS-induced IRF3 activation (**Fig. 3f**); all of those were potently blocked by SARM expression.

SARM blocks TRIF-IRF7 but not MyD88-IRF7

IRF7 has been shown to be the 'master regulator' of interferon induction in response to diverse viruses[22]. IRF7 functions to upregulate interferon-α/β production, thus limiting the spread of viral infection. IRF7 can be activated by TLR3 and TLR4 via TRIF[11] or by TLR9 via MyD88 (refs. 23,24). Because of the functional importance of IRF7 and the fact that IRF7 can be activated by means of two distinct adaptors, we tested whether SARM could modulate the two TLR-IRF7 axes. Similar to the other TRIF-dependent signals, SARM inhibited TRIF-induced IRF7 activation in a dose-dependent way (**Fig. 4a**). However, SARM exerted no inhibition on MyD88-induced IRF7 activation (**Fig. 4b**). The strength of those observations was further emphasized by experiments with RAW264.7 cells in which IRF7 activation by treatment with poly(I:C) or LPS was reduced substantially by SARM expression (**Fig. 4c**). The last observation eliminated the possibility that the inhibitory effect exerted by SARM was solely HEK293 dependent.

SARM targets TRIF

The observations reported above raised the possibility that the adaptor TRIF was being directly targeted by SARM. Similar to the situation with TRIF-induced NF-κB activation (**Fig. 2b**), RANTES release, *Ccl5* (RANTES) promoter induction and IRF3 activation were all inhibited by SARM in a dose-dependent way (**Fig. 5a–c**). To confirm that the observed inhibition was specific to TRIF-related functions, we examined the sensitivity to SARM of upstream regulators and downstream effectors of TRIF. The TIR domain–containing adaptor TRAM acts as

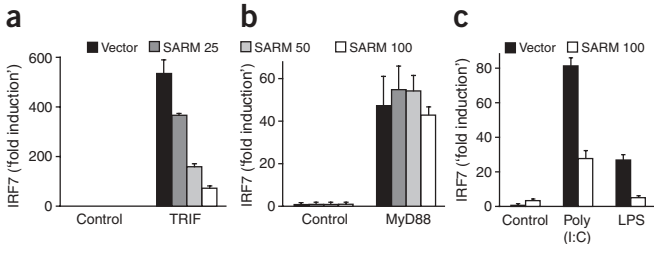

Figure 4 SARM inhibits IRF7 activation by the TRIF but not by the MyD88 pathway. (**a**,**b**) Luciferase reporter assay of IRF7 activation in HEK293 cells transfected for 24 h with 10 ng TRIF expression plasmid (**a**) or 25 ng MyD88 expression plasmid (**b**) and increasing amounts of SARM expression plasmid (key, in ng). (**c**) Luciferase reporter assay of IRF7 activation in RAW264.7 cells transfected for 24 h with 100 ng SARM expression plasmid or vector and then stimulated for 6 h with either 25 μg/ml of poly(I:C) or 1 μg/ml of LPS. Experiments are one of three repeats (**a**,**b**) or one of two repeats (**c**); error bars, s.d.

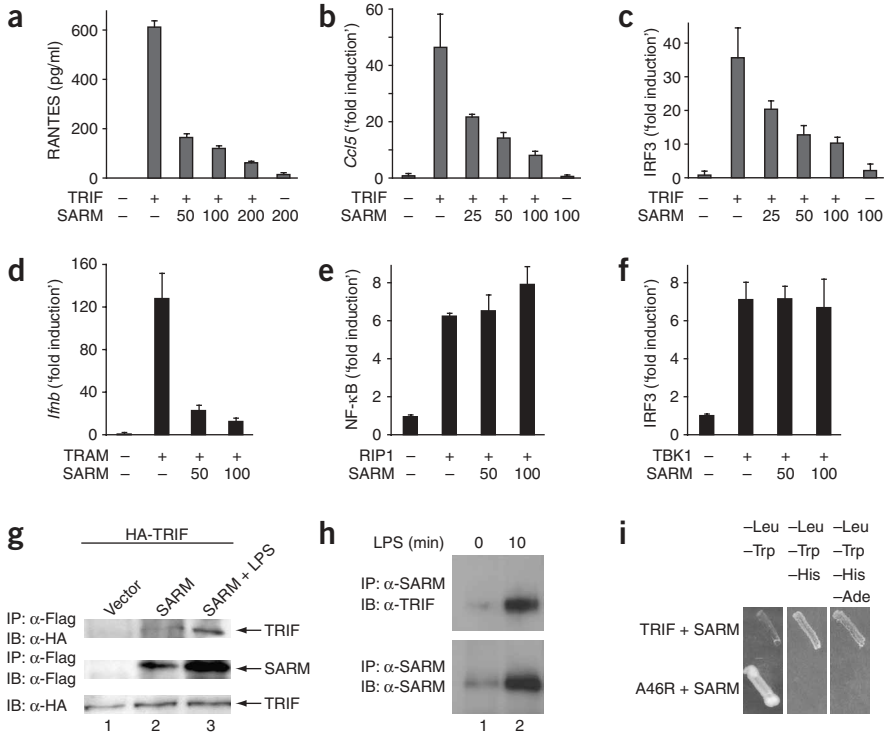

Figure 5 SARM associates with and inhibits TRIF signaling. (**a**) ELISA of RANTES production by HEK293 cells transfected for 24 h with SARM and TRIF expression plasmids. (**b**,**c**) Luciferase assay for *Ccl5* promoter induction (**b**) or IRF3 activation (**c**) in HEK293 cells transfected for 24 h with SARM expression plasmid (amount (in ng), horizontal axes) and TRIF expression plasmid. (**d**) Luciferase assay for induction of the *Ifnb* promoter in HEK293 cells transfected as in **b**,**c**, but with 50 ng TRAM expression plasmid. (**e**) Luciferase assay for NF-κB activation in HEK293 cells transfected as in **b**,**c**, but with 50 ng RIP1 expression plasmid. (**f**) Luciferase assay for IRF3 activation in HEK293 cells transfected as in **b**,**c**, but with 50 ng TBK1. (**g**) Immunoprecipitation (IP) and immunoblot (IB) analysis of lysates of HEK-TLR4 cells transfected for 36 h with hemagglutinin-tagged TRIF (HA-TRIF) and Flag-tagged SARM. +LPS, cells treated for 15 min with 10 ng/ml of LPS; α-, antibody to. (**h**) Immunoprecipitation and immunoblot analysis of lysates of human PBMCs left unstimulated (0) or treated for 10 min with 100 ng/ml of LPS (10) and then lysed in extraction buffer. (**i**) Pairwise interactions assessed in yeast cells transformed with BD-SARM and AD-TRIF or AD-A46R and grown on 'dropout' agar plates (above lanes) lacking leucine (–Leu), tryptophan (–Trp), histidine (–His) and/or adenine (–Ade). Growth on middle and right plates indicates a direct interaction. Experiments are one of three repeats (error bars, s.d.).

a bridge connecting TLR4 to TRIF and facilitates TRIF dependent signaling[4,11]. TRAM is thought to mediate signaling exclusively through TRIF. We found that activation of the *Ifnb* promoter by expression of TRAM was potently blocked by SARM expression (**Fig. 5d**). Two critical downstream mediators of TRIF signaling are RIP1 and TBK1, which are involved in activation of NF-κB and IRF3, respectively[25,26]. However, 'forced' expression of RIP1 (**Fig. 5e**) or TBK1 (**Fig. 5f**) leading to stimulation of NF-κB or IRF3 activation, respectively, was not affected by SARM expression (**Fig. 5e**,**f**). Furthermore, SARM had no effect on TRAF6-induced signaling (data not shown); TRAF6 is an important mediator of NF-κB activation downstream of both TRIF and MyD88.

The data we have reported thus far have provided compelling evidence that SARM acts at the level of TRIF. Given the presence of the TIR homotypic interaction domain in both TRIF and SARM, we speculated that SARM inhibition of TRIF might be achieved by direct interaction. To test that possibility, we did coimmunoprecipitation experiments in HEK-TLR3 and HEK-TLR4 cells. Overexpressed TRIF and SARM were immunoprecipitated together in both cell lines (**Fig. 5g** and data not shown). In HEK-TLR4 cells, we detected hemagglutinin-tagged TRIF in FLAG-tagged SARM immunoprecipitates (**Fig. 5g**, top, lane 2). The interaction between TRIF and SARM was enhanced, furthermore, after 15 min of LPS stimulation (**Fig. 5g**, top, lane 3), most likely because of higher SARM expression in the presence of LPS (**Fig. 5g**, middle). Given that human primary PBMCs expressed endogenous SARM (**Fig. 1e**), we next attempted to demonstrate an interaction between endogenous SARM and TRIF in those cells. We detected a small amount of TRIF in endogenous SARM immunoprecipitates in the absence of LPS (**Fig. 5h**, top, lane 1), and the amount of TRIF detected increased after a 10 min LPS treatment (**Fig. 5h**, top, lane 2), which again was consistent with an increase in SARM expression in the presence of LPS (**Fig. 5h**, bottom).

To obtain evidence of interaction by an independent approach and to confirm that the observed interaction between TRIF and SARM was direct, we used a pairwise yeast two-hybrid assay. Yeast transformed with both the vector bearing the SARM binding domain and the vector bearing the TRIF activation domain thrived on agar lacking histidine and on agar lacking both histidine and adenine. The ability of auxotrophic yeast strain to grow on the deficient medium was made possible by the interaction of TRIF and SARM, thus reconstituting the yeast transcription factor Gal4, leading to expression of the Gal4-responsive genes *His3* and *Ade* (**Fig. 5i**, middle and right). As a negative control, we transformed yeast with the SARM binding domain and the vaccinia TIR domain–containing protein A46R; these failed to interact (**Fig. 5i**). That result was consistent with published data demonstrating a lack of interaction between SARM and A46R in coimmunoprecipitation experiments[27].

Inhibition requires the sterile α-motif and TIR domains of SARM

SARM contains two well defined protein motifs: the TIR domain, spanning amino acids 512–690, and two tandem sterile α-motif (SAM) domains, within amino acids 371–511. It has been predicted[12] that SARM contains HEAT-armadillo motifs as well; however the existence those last motifs is more speculative. To identify the region of SARM required for the inhibition of TRIF, we generated several SARM truncations with different combinations of the TIR and SAM domains (**Fig. 6a**). We evaluated the effects of the SARM truncations on TRIF-dependent signaling using poly(I:C)-induced NF-κB activation in HEK-TLR3 cells as a 'readout'. Full length SARM (as shown in **Fig. 2e**) inhibited NF-κB activation in a dose-dependent way (**Fig. 6b**). In contrast, a SARM construct lacking the N terminus and SAM domains and a SARM construct lacking lacked the TIR domain failed to cause any inhibition. SARMΔN, which contained only the TIR domain and SAM domains and lacked the N-terminal

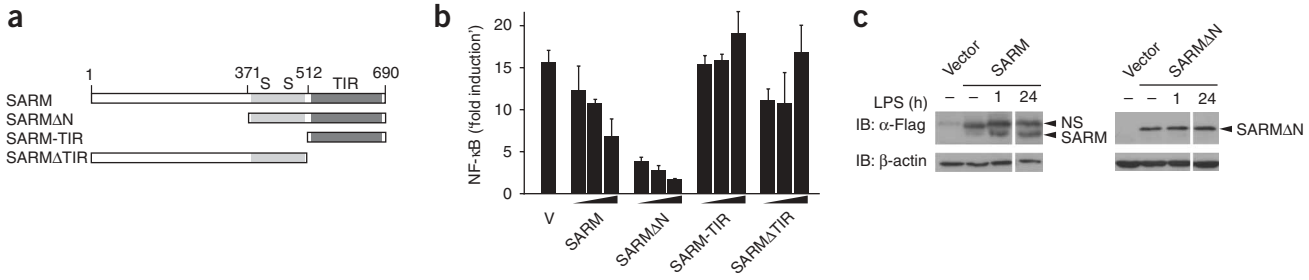

Figure 6 The TIR and SAM domains of SARM are critical for TRIF inhibition. (**a**) Protein motifs of Flag-tagged SARM and truncated SARM constructs. S, SAM; amino acid positions, above diagrams. (**b**) Luciferase assay of HEK-TLR3 cells transfected for 24 h with the NF-κB reporter plus vector (V) or increasing amounts (wedges) of SARM expression plasmid or constructs (from **a**) expressing truncation proteins; cells were collected 6 h after stimulation with 25 μg/ml of poly(I:C). (**c**) Immunoblot of lysates of HEK-TLR4 cells transfected for 24 h with SARM or SARMΔN expression plasmid and then treated with 100 ng/ml of LPS (time, above lanes). Experiments are one of three repeats (error bars, s.d.).

region, inhibited signaling in a dose-dependent way and was even more potent than full-length SARM. The strength of SARMΔN as an inhibitor was further emphasized by the finding that it completely abolished TRIF-induced NF-κB and IRF3 activation (data not shown). Examination of the expression of full-length SARM and SARMΔN provided a rationale for the more potent inhibition obtained with the latter: cells had lower expression of full-length protein than SARMΔN (**Fig. 6c**, lane 2 versus 6), whereas LPS treatment of cells for 1 h led to an increase in the abundance of full-length SARM but not SARMΔN (**Fig. 6c**, lane 3 versus lane 7), an effect that was apparent even after 24 h of LPS stimulation. These data demonstrated that the TIR domain and the two SAM domains are necessary and sufficient for inhibition of TRIF signaling and that the N terminus, although dispensable for TRIF inhibition, is required for LPS-induced enhancement of SARM expression.

SARM suppression enhances TRIF-dependent gene induction

To determine the function of endogenous human SARM in TRIF signaling, we 'knocked down' *SARM1* expression using RNA-mediated interference. In addition to the 690–amino acid form of human SARM, a second, 724–amino acid form with an extended N-terminal region has been reported (GenBank accession number AY444166). We confirmed expression of endogenous *SARM1* in HEK293 cells using RT-PCR (**Fig. 7a**) and used two siRNA oligonucleotides, S1 and S2, targeting both forms of human *SARM1* mRNA. S1 substantially reduced, whereas S2 completely abolished, *SARM1* mRNA, whereas a nontargeting siRNA had little effect on *SARM1* expression (**Fig. 7a**). Therefore, to confirm that endogenous SARM inhibited TRIF function, we examined TLR3-TRIF–dependent signaling and gene induction in the presence of S1 and S2.

Forced expression of TRIF induced the *Ifnb* promoter, which was increased approximately by a factor of three in cells treated with either S1 or S2 compared with those treated with the nontargeting control siRNA (**Fig. 7b**). There was similar enhancement of *Ifnb* promoter induction in the presence of S1 or S2 when we treated HEK-TLR3 cells with poly(I:C) to trigger TLR3 signaling (**Fig. 7c**). It has been reported that certain siRNA oligonucleotides can trigger TLR3 activation[28]. However, in our experiments here, neither S1 nor S2 alone activated the *Ifnb* promoter (**Fig. 7b,c**) or NF-κB (data not shown). We next examined the effect of 'knocking down' *SARM1* expression on

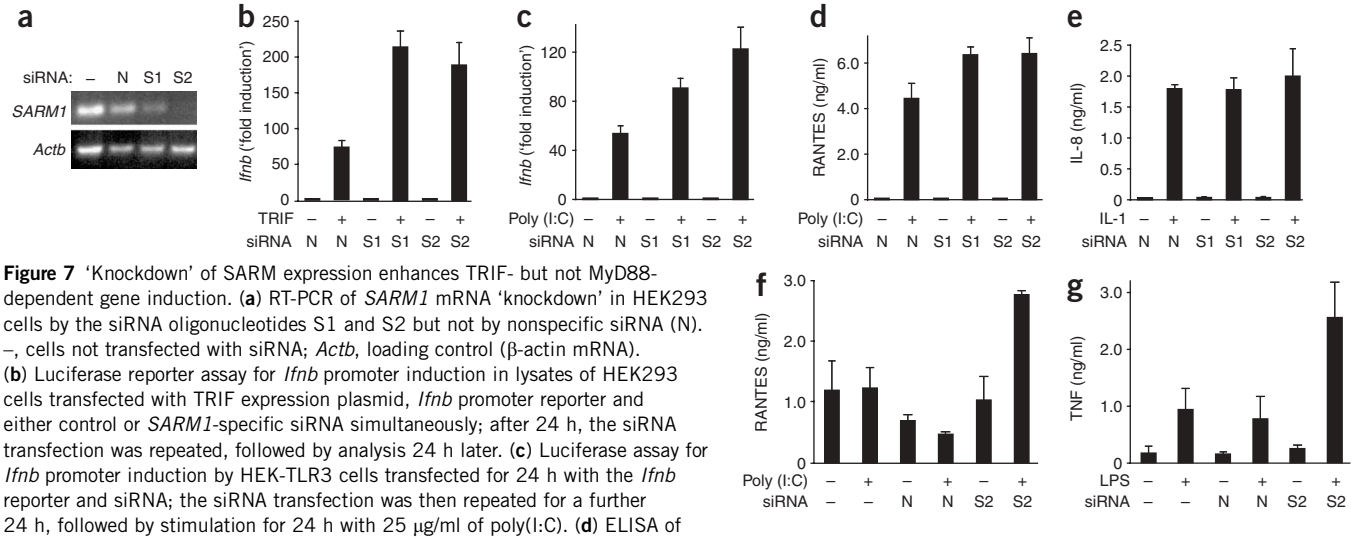

Figure 7 'Knockdown' of SARM expression enhances TRIF- but not MyD88-dependent gene induction. (**a**) RT-PCR of *SARM1* mRNA 'knockdown' in HEK293 cells by the siRNA oligonucleotides S1 and S2 but not by nonspecific siRNA (N). –, cells not transfected with siRNA; *Actb*, loading control (β-actin mRNA). (**b**) Luciferase reporter assay for *Ifnb* promoter induction in lysates of HEK293 cells transfected with TRIF expression plasmid, *Ifnb* promoter reporter and either control or *SARM1*-specific siRNA simultaneously; after 24 h, the siRNA transfection was repeated, followed by analysis 24 h later. (**c**) Luciferase assay for *Ifnb* promoter induction by HEK-TLR3 cells transfected for 24 h with the *Ifnb* reporter and siRNA; the siRNA transfection was then repeated for a further 24 h, followed by stimulation for 24 h with 25 μg/ml of poly(I:C). (**d**) ELISA of RANTES (**d**) or IL-8 (**e**) in supernatants of HEK-TLR3 (**d**) or HEK293 (**e**) cells treated with siRNA 24 and 48 h after seeding, then stimulated for 24 h with either 25 μg/ml of poly(I:C) (**d**) or 100 ng/ml of IL-1α (**e**). (**f**) ELISA of RANTES (**f**) or TNF (**g**) in supernatants of PBMCs left untreated (–) or treated with siRNA 24 and 48 h after seeding, then stimulated for 24 h with either 25 μg/ml of poly(I:C) (**f**) or 100 ng/ml of LPS (**g**). Experiments are one of three repeats (error bars, s.d.).

chemokine release in response to poly(I:C). RANTES production after poly(I:C) stimulation of HEK-TLR3 cells was enhanced by 40% by *SARM1* siRNA treatment (**Fig. 7d**). As a control, we tested MyD88-dependent release of the chemokine IL-8 in response to IL-1 stimulation; this was unaffected by 'knockdown' of *SARM1* expression (**Fig. 7e**).

Finally, we tested the effect of the *SARM1* siRNA oligonucleotides in primary human PBMCs. In the conditions of poly(I:C) stimulation used here (25 μg/ml for 24 h), we detected no RANTES release (**Fig. 7f**). Treatment of the cells with S2 facilitated RANTES release to three times above the control release (**Fig. 7f**). Again, S2 alone had no stimulatory effect on the cells. Given that TRIF is essential for LPS-induced TNF release from mouse macrophages[10], we also tested the effect of *SARM1* siRNA on this response in human PBMCs. Treatment of these cells with S2 enhanced TNF production in response to LPS by 250% (**Fig. 7g**). Thus, suppression of endogenous *SARM1* expression in primary human blood cells affected TLR3- and TLR4-dependent gene induction. These results provided conclusive evidence that SARM functions as a specific inhibitor of TRIF-dependent signaling.

DISCUSSION

Human SARM was originally identified as an ortholog of the drosophila gene CG7915, which is highly conserved in mouse and *C. elegans*[12]. High expression in the kidney and liver was demonstrated and an antisense transcript was reported[12]. As mentioned above, in *C. elegans* the SARM ortholog TIR-1 has a positive function both in development and in immunity[13–15]. In contrast, we have shown here that human SARM is a specific negative regulator of TRIF signaling and thus of certain innate immune responses. We have also demonstrated that SARM and TRIF interact directly. We have confirmed the negative function of SARM using siRNA to suppress endogenous *SARM1* expression, which led to enhanced TRIF-dependent gene induction in both transformed and primary human cells but did not affect IL-1–MyD88–induced IL-8 production. The demonstration of enhanced TNF production after treatment with SARM siRNA in PBMCs has also provided evidence of the involvement of TRIF in LPS-induced TNF production in human blood.

TRIF is critical in mediating both TLR3 and TLR4 signaling. For example, TRIF is important in resistance to cytomegalovirus infection[10], in the LPS-induced upregulation of costimulatory molecules on antigen-presenting cells[29] and in mediating the TLR4-induced cell activation program in dendritic cells[30]. The importance of TRIF in the antiviral response is emphasized by the fact that it is targeted for immune evasion by the vaccinia virus protein A46R[27] and also by the hepatitis C virus protease NS3-4A[31]. Although many diverse host mechanisms for inhibiting and regulating TLR function have been identified, most of those (such as SIGIRR, ST2, IL-1R-associated kinase-M, Tollip and MyD88s) seem to target the MyD88-dependent pathway, although at least one (A20) can inhibit both MyD88 and TRIF signaling[32]. Less is known about cellular inhibitors that are specific for the TRIF pathway, yet given the central importance of TRIF to many aspects of innate immunity, specific host regulation of TRIF function to prevent excessive activation of its downstream pathways might be expected. SARM thus provides an important regulating function for the diverse activities of TRIF. The ability of LPS to enhance SARM protein expression after just 1 h of treatment provides a mechanism whereby TLR4 signaling could lead to a rapid downregulation of the TRIF pathway. The SARM and TRIF association, relatively weak in unstimulated cells, was strongly enhanced after short LPS treatment. Poly(I:C) and LPS did not have a substantial regulatory effect on *SARM1* mRNA expression (data not shown), yet LPS stimulation led to a rapid increase in SARM protein. The SARM inhibitory activity, therefore, is not regulated by a change in the amount of mRNA but instead by ligand-induced enhancement of SARM protein expression, which in turn leads to increased interaction of SARM and TRIF. The post-transcriptional regulation of SARM might involve signal-dependent stabilization of the protein itself. The finding that deletion of the N terminus of SARM enhanced its inhibitory function and expression and reduced its sensitivity to LPS-induced expression may suggest that SARM is inherently unstable because of motifs in its N terminus and that LPS treatment somehow makes the protein more stable.

Related to the issue of SARM protein stability is the case of the *C. elegans* SARM homolog TIR-1, which when truncated to consist of only the two SAM domains and the TIR domain shows a stronger gain-of function developmental phenotype than the full-length protein[15]. Of further note, six forms of TIR-1 exist[15]. However, in all of the splice variants, the SAM and TIR domains remain intact and vary at the N terminus only. Those data are consistent with the ideas that the N terminus of SARM may be regulatory in both humans and *C. elegans* and that important functions are mediated by the SAM and TIR domains.

Although we have demonstrated that 'knockdown' of SARM enhanced TLR3- and TLR4-dependent responses and that TRIF and SARM interacted, the exact mechanism whereby SARM inhibits TRIF function is as yet unclear. One possibility is that binding of SARM to TRIF may simply physically prevent engagement of TRIF with either its upstream activators or its downstream effectors. Thus, SARM may shield TRIF from contact with TRAM, in the case of TLR4, and also prevent binding to TLR3. However, that scenario is unlikely given that SARM can inhibit signals elicited directly from TRIF. Alternatively, SARM may prevent TRIF from accessing its downstream signaling molecules, TBK1, TRAF6 and RIP1, all of which interact with specific sites on TRIF[25,33].

Another possible inhibitory mechanism is that SARM recruits an inhibitory protein (or protein complex) to TRIF. The two protein domains identified in SARM as being necessary and sufficient for TRIF inhibition were the TIR domain and the SAM domains. Thus, the TIR domain of SARM may provide the specificity required to target TRIF, whereas the SAM domains may recruit an as-yet-unidentified TLR inhibitor. SAM domains mediate the formation of homo- and heterodimers between different SAM domain–containing proteins; they can also bind to various other protein interaction motifs[34]. Unlike other protein domains, such as the TIR domain, no common function is known for SAM domains. In fact, SAM domain–containing proteins exist in all subcellular locations, are involved in many different biological processes, bind to a wide variety of proteins and have also been shown to bind to RNA[34]. Hence, it is difficult to predict what the SAM domains of SARM may interact with. Further studies, such as yeast two-hybrid screening, will be needed to identify a putative inhibitor.

METHODS

Expression vectors. Plasmids used expressed the following proteins: AU1-MyD88 (obtained from M. Muzio, Mario Negri Institute, Milan, Italy)[35]; Flag-tagged SARM, TRAM, Mal, RIP1, TBK1, RIG-I, IRF3-Gal4 and IRF7-Gal4 (obtained from K. Fitzgerald, University of Massachusetts, Worcester, Massachusetts)[14,11,26]; Flag-tagged TRIF (obtained from S. Akira, Osaka University, Osaka, Japan); hemagglutinin-tagged TRIF (obtained from C. Basler, Mount Sinai School of Medicine, New York, New York); and RIP1 (obtained from J. Tschopp, University of Lausanne, Lausanne, Switzerland)[25]. Vectors expressing various SARM truncations were made using standard PCR and cloning

techniques; the full-length construct was used as a template. Primers used are listed in **Supplementary Table 1** online.

Antibodies and reagents. Antibody to SARM (anti-SARM) was from ProSci, monoclonal anti–FLAG M2 was from Sigma, and anti-hemagglutinin was from Covance. Human recombinant IL-1α was provided by the National Cancer Institute. Human recombinant TNF was a gift from S. Foster (Zeneca Pharmaceuticals, Macclesfield, UK). TLR agonists used were poly(I:C) (Amersham Biosciences) and LPS (Alexis). Anti-TRIF was from Alexis.

Reporter gene assays. HEK293, HEK-TLR3 (ref. 11) and HEK-TLR4 cells (2×10^4 cells per well) or RAW264.7 cells (4×10^4 cells per well) were seeded into 96-well plates and were transfected 24 h later with expression vectors and luciferase reporter genes using GeneJuice (Novagen). In all cases, 20 ng/well of phRL-TK reporter plasmid (Promega) was cotransfected to allow normalization of data for transfection efficiency. The total amount of DNA per transfection was kept constant at 230 ng (HEK293) or 200 ng (RAW264.7) by the addition of pcDNA3.1 (Stratagene). After 24 h, reporter gene activity was measured[27]. Reporter genes used were for NF-κB[27] and the *Ccl5* promoter and *Ifnb* promoter. For the IRF3 and IRF7 assays, 3 ng of vector expressing IRF3-Gal4 or IRF7-Gal4 was transfected along with 60 ng of the pFR luciferase reporter plasmid[27]. All reporter assays were done in triplicate and data are expressed as 'fold induction' (mean ± s.d.) relative to control induction for a representative experiment.

Measurement of cytokine concentrations. HEK293, HEK-TLR3 or HEK-TLR4 cells were used for measurement of cytokine production. Cells (2×10^4 cells per well) transfected for 24 h with the *SARM1* expression plasmid were stimulated for 24 h with 1 μg/ml of LPS or 25 μg/ml of poly(I:C). For poly(I:C) transfection (where indicated), poly(I:C) was added to a mixture of serum-free medium and GeneJuice and then was incubated for 15 min at 25 °C before being transfected into cells. Supernatants were collected 24 h later and IL-8 or RANTES concentrations were measured by enzyme-linked immunosorbent assay (ELISA; R&D Biosystems). Experiments were done in triplicate and data are expressed as the mean ± s.d. from one representative experiment.

'Knockdown' with siRNA. The siRNA was designed to target human *SARM1* in two different regions (see **Supplementary Table 1**). For reporter gene assays, HEK293 or HEK-TLR3 cells were seeded at a density of 3×10^4 cells per well in 96-well plates and were transfected 24 h later with expression vectors and luciferase reporter genes together with siRNA, using lipofectamine 2000 (Invitrogen). The siRNA transfection was repeated 24 h later and reporter gene activity was measured 24 h thereafter. For ELISA, HEK-TLR3 or HEK293 cells were seeded at a density of 3×10^4 cells per well and siRNA was transfected twice, at 24 and 48 h after cell seeding. PBMCs from healthy donors were isolated with Lymphoprep reagent (Axis-Shield) according to the manufacturer's instruction. Then, 2×10^5 cells per well were plated in 96-well plates and cells were allowed to equilibrate for 1 h. Cells were treated with siRNA 24 and 48 h after seeding, then were treated with 25 μg/ml of poly(I:C) or 100 ng/ml of LPS; supernatants were isolated 24 h thereafter, and RANTES or TNF release was measured by ELISA.

RT-PCR. Total RNA from cells seeded in six-well plates was isolated with TRI Reagent (Sigma) according to the manufacturer's instructions. Random-primed RNA (1 μg) was reverse-transcribed with ImPROM-II reverse transcriptase (Promega) according to the manufacturer's instructions. The cDNA obtained was used in PCR with Taq DNA polymerase (Invitrogen) to determine the relative amount of SARM mRNA (see **Supplementary Table 1**).

Coimmunoprecipitation and immunoblot analysis. HEK-TLR4 cells were seeded into 175-cm^2 flasks and were grown to 60% confluence before being transfected with 8 μg of Flag-tagged SARM and hemagglutinin-tagged TRIF, using GeneJuice. The total amount of DNA was kept constant between transfections by the addition of pcDNA3.1. At 36 h after transfection, cells were lysed with 1,700 μl of Nonidet-P40 extraction buffer, consisting of 50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol and 0.5% (vol/vol) Nonidet-P40 and containing protease inhibitors. Cells were lysed on ice for 1 h, insoluble material was removed by centrifugation and the resultant lysates were immunoprecipitated for 2 h at 4 °C with anti–Flag M2 agarose (Sigma). Immune complexes were washed and eluted, were separated by SDS-PAGE and were analyzed by immunoblot with anti-hemagglutinin.

For immunoblot of PBMCs, 1.5×10^7 cells were seeded in 100-mm dishes and were treated for 1–24 h with 100 ng/ml of LPS. Cells were lysed in Lamelli sample buffer, were sonicated and boiled and were separated by SDS-PAGE. For detection of endogenous TRIF in endogenous SARM immunoprecipitates, 3×10^7 human PBMCs from buffy coats were seeded in 100-mm dishes. Cells were left to equilibrate for 1 h at 37 °C and were then treated with 100 ng/ml of LPS. Cells were scraped into medium and centrifuged. The resulting cell pellet was washed twice in ice cold PBS and was lysed on ice for 1 h with 850 μl Nonidet-P40 extraction buffer. For efficient lysis, cells were 'syringed' in extraction buffer six times with a 27-gauge needle. Insoluble material was removed by centrifugation and lysates were immunoprecipitated overnight at 4 °C with anti-SARM. Immune complexes were washed and eluted, were separated by SDS-PAGE and were analyzed by immunoblot with human anti-TRIF.

Yeast two-hybrid analysis. The MATCHMAKER GAL4 Two Hybrid System 3 (Clontech) was used to assess interaction between TRIF and SARM and between SARM and A46R. The activation-domain vector pGADT7 encoding TRIF was a gift from L. O'Neill (Trinity College, Dublin, Ireland). A46R was cloned into pGADT7, and SARM was cloned into the bait vector pGBKT7. Transformed AH109 yeast cells were plated onto agar lacking leucine and tryptophan and the resultant colonies were streaked onto plates lacking leucine, tryptophan and histidine or plates lacking leucine, tryptophan, histidine and adenine. Plates were then incubated at 30 °C for 3–4 d and the growth of yeast was recorded.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

M.C. designed and performed most of the experiments and co-wrote the paper; R.G. performed some experiments; M.S. advised on the study; J.S. performed some experiments; P.N.M co-designed and advised on the study and experiments; A.G.B. conceived and co-designed the study, advised on experiments and co-wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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