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Claudia Gürtler, Michael Carty, Jay Kearney, Stefan A. Schattgen, Aihao Ding, Katherine A. Fitzgerald and Andrew G. Bowie

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SARM Regulates CCL5 Production in Macrophages by Promoting the Recruitment of Transcription Factors and RNA Polymerase II to the *Ccl5* Promoter

Claudia Gürtler,* Michael Carty,* Jay Kearney,* Stefan A. Schattgen,[†] Aihao Ding,[‡] Katherine A. Fitzgerald,[†] and Andrew G. Bowie*

The four Toll/IL-1R domain-containing adaptor proteins MyD88, MAL, TRIF, and TRAM are well established as essential mediators of TLR signaling and gene induction following microbial detection. In contrast, the function of the fifth, most evolutionarily conserved Toll/IL-1R adaptor, sterile α and HEAT/Armadillo motif-containing protein (SARM), has remained more elusive. Recent studies of *Sarm*^{-/-} mice have highlighted a role for SARM in stress-induced neuronal cell death and immune responses in the CNS. However, whether SARM has a role in immune responses in peripheral myeloid immune cells is less clear. Thus, we characterized TLR-induced cytokine responses in SARM-deficient murine macrophages and discovered a requirement for SARM in CCL5 production, whereas gene induction of TNF, IL-1 β , CCL2, and CXCL10 were SARM-independent. SARM was not required for TLR-induced activation of MAPKs or of transcription factors implicated in CCL5 induction, namely NF- κ B and IFN regulatory factors, nor for *Ccl5* mRNA stability or splicing. However, SARM was critical for the recruitment of transcription factors and of RNA polymerase II to the *Ccl5* promoter. Strikingly, the requirement of SARM for CCL5 induction was not restricted to TLR pathways, as it was also apparent in cytosolic RNA and DNA responses. Thus, this study identifies a new role for SARM in CCL5 expression in macrophages. *The Journal of Immunology*, 2014, 192: 4821–4832.

Innate immune cells such as macrophages are key players in initiating an immune response following the detection of invading pathogens via pattern recognition receptors (PRRs). Engagement of a PRR with its microbial ligand initiates signaling cascades leading to activation of MAPKs and transcription factors, such as NF- κ B and IFN regulatory factors (IRFs) (1). These transcription factors subsequently translocate to the nucleus and bind to specific promoter elements of proinflammatory cytokines (e.g., TNF, IL-1 β), chemokines (e.g., CCL5, CCL2, CXCL10), and type I IFNs (IFN- α and IFN- β) to upregulate gene expression.

One major family of PRRs are the membrane-bound TLRs, which signal through homotypic interactions via distinct Toll/IL-1R (TIR) domain-containing adaptor proteins, namely MyD88, MyD88-adaptor-like protein (MAL), TIR domain-containing

adaptor inducing IFN- β (TRIF), and TRIF-related adaptor molecule (TRAM) (2). TLR4 is located on the plasma membrane, where it detects LPS. TLR4 utilizes all four adaptor proteins for signaling, activating a MAL/MyD88-dependent pathway from the plasma membrane, followed by TLR4 internalization and TRAM/TRIF signaling from endolysosomes. In contrast, the microbial nucleic acid-sensing TLRs, namely TLR3, TLR7, and TLR9, are expressed on endosomes, where they sense viral dsRNA, ssRNA, or CpG DNA, respectively (1). A single TIR adaptor is then recruited to mediate downstream signaling, which is TRIF for TLR3 and MyD88 for TLR7 and TLR9. Apart from the TLRs, ubiquitously expressed RNA and DNA sensors are found in the cytosol of many different cell types to detect intracellular viruses (3). Such cytosolic PRRs include the retinoic acid-inducible gene I-like receptors retinoic acid-inducible gene I and melanoma differentiation-associated gene 5, which sense viral RNA and signal via the adaptor mitochondrial antiviral signaling, and cytosolic DNA sensors such as IFN-inducible protein 16 and cyclic GMP-AMP synthase, which signal via the endoplasmic reticulum-resident protein stimulator of IFN genes (STING) (3).

Sterile α and HEAT/Armadillo motif-containing protein (SARM) has a C-terminal TIR domain and was therefore expected to function in the TLR pathway (4). Notably, SARM is highly conserved between mammals, *Drosophila*, and worms and is in fact the most evolutionary conserved member of the cytosolic TIR-containing proteins (5). Unlike the other four TIR adaptor proteins, early studies showed that overexpressed SARM did not lead to NF- κ B and IRF3 activation (6, 7). Instead, SARM was found to negatively regulate TRIF-dependent TLR3 and TLR4 signaling in human cells through direct interaction with TRIF (7). However, studies of *Sarm*^{-/-} mice have mainly revealed neuronal functions for SARM. In an initial study by Kim et al. (8) SARM was discovered to trigger stress-induced neuronal cell death in the CNS after oxygen and glucose deprivation, whereas SARM was recently also implicated in mediating neuronal cell death during

*School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland; [†]Division of Infectious Disease and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605; and [‡]Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021

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Address correspondence and reprint requests to Prof. Andrew G. Bowie, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland. E-mail address: agbowie@tcd.ie

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; MAL, MyD88-adaptor-like protein; Pol II, RNA polymerase II; poly(I:C), polyinosinic-polycytidylic acid; pre-mRNA, precursor mRNA; PRG, primary response gene; PRR, pattern recognition receptor; SARM, sterile α and HEAT/Armadillo motif-containing protein; SRG, secondary response gene; STING, stimulator of IFN genes; TIR, Toll/IL-1R; TRAM, TIR domain-containing adaptor inducing IFN- β -related adaptor molecule; TRIF, TIR domain-containing adaptor inducing IFN- β ; VACV, vaccinia virus; WNV, West Nile virus; WT, wild-type.

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infection with neurotropic La Crosse virus (9). In both cases, SARM was found to trigger neuronal cell death via apoptosis, whereas SARM was also discovered to be required for Wallerian degeneration, whereby injured neurons die via a nonapoptotic cell death pathway (10, 11). Furthermore, SARM was found to regulate neuronal morphology through controlling dendritic arborization and outgrowth (12). From an evolutionary perspective, these newly discovered functions of mammalian SARM are consistent with the role of the SARM *Caenorhabditis elegans* ortholog TIR-1 in neuronal development and anoxic death (13, 14). TIR-1 functions upstream of an MAPK cascade in the worm, and it has also been shown to have a role in worm immunity by controlling the induction of antimicrobial peptides (6, 15). SARM was also demonstrated to have a role in mammalian immunity in the CNS, for example Szretter et al. (16) reported that *Sarm*^{-/-} mice infected with neurotropic West Nile virus (WNV) displayed a defect in viral clearance and reduced TNF production in the brainstem. However, whether SARM has any role in peripheral innate immunity in the mouse has remained unclear.

Given the importance of macrophages in peripheral innate immune defense, we investigated a potential role for mouse SARM in cytokine responses in bone marrow-derived macrophages (BMDMs). We found that SARM was required for optimal CCL5 production in response to TLR4 and TLR7 stimulation, whereas the induction of TNF and of some other proinflammatory cytokines and chemokines was SARM-independent. Surprisingly therefore, the key transcription factors implicated in CCL5 induction, namely NF- κ B, IRF3, and IRF1, were activated normally in the absence of SARM, as were the MAPKs, p38, JNK, and ERK. Rather, chromatin immunoprecipitation (ChIP) analysis of *Sarm*^{-/-} BMDMs revealed a requirement of SARM for the recruitment of transcription factors and RNA polymerase II (Pol II) to the *Ccl5* promoter, a crucial step in the transcriptional induction of *Ccl5* (17). Accordingly, non-TLR pathways to CCL5 induction were also dependent on SARM. Thus, SARM has an essential role in CCL5 chemokine induction in mouse BMDMs.

Materials and Methods

Mice and cell culture

Generation of the *Sarm*^{-/-} mice on the C57BL/6 background has been previously described (8). Femurs and tibias of wild-type (WT) and *Sarm*^{-/-} mice were obtained from mice bred and maintained at the University of Massachusetts Medical School in accordance with the guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institutional Animal Care and Use Committee. Primary BMDMs were differentiated from bone marrow cells for 7 d in complete DMEM (DMEM plus GlutaMAX, 10% (v/v) FCS, 10 μ g/ml ciprofloxacin) supplemented with 15–20% (v/v) L929 supernatant as a source of M-CSF. On day 7, cells were trypsinized and seeded for experiments in complete DMEM. Immortalized WT and *Sarm*^{-/-} BMDMs were generated with J2 recombinant retrovirus carrying *v-myc* and *v-raf/mil* oncogenes as previously described (18, 19). Thioglycollate-elicited peritoneal macrophages were isolated from mice 4 d after i.p. injection of sterile 3% thioglycollate (Remel, Lenexa, KS). NIH3T3 cells and HEK293T cells were purchased from Sigma-Aldrich and the European Cell Culture Collection, respectively, and maintained in complete DMEM.

Receptor agonists and cell transfection

Ultrapure LPS from *Escherichia coli*, serotype EH100 (>99.9% pure in respect to contaminating protein and DNA with agonistic TLR activity) was from Alexis Biochemicals, CLO75 was from InvivoGen, mouse rIFN- α was from Miltenyi Biotec, polyinosinic-polycytidylic acid (poly(I:C)) was from Sigma-Aldrich. The vaccinia virus (VACV) 70-bp dsDNA oligonucleotide (dsVACV 70mer) was synthesized by MWG Biotech as previously described (20), and complementary strands were annealed by heating the mixture to 99°C and slow cooling to room temperature. Cells were transfected with poly(I:C) or dsVACV 70mer using Lipofectamine 2000 (Invitrogen).

Antibodies

Primary Abs used for immunoblotting were anti- β -actin (AC-74) and anti-Flag (M2) from Sigma-Aldrich, anti-I κ B α (a gift from Prof. R. Hay, University of Dundee, Dundee, U.K.), anti-phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵, 44-682G) from BioSource International, and anti-p38 (no. 9212), anti-phospho-p38 (Thr¹⁸⁰/Tyr¹⁸², no. 9211), anti-JNK (no. 9252), anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴, no. 4377), and anti-phospho-STAT1 (Tyr⁷⁰¹, no. 9171) from Cell Signaling Technology. The secondary Abs for immunoblotting were IRDye 680LT anti-mouse, IRDye 800CW anti-rabbit, and IRDye 680LT anti-goat (LI-COR Biosciences). Primary Abs for confocal microscopy were anti-p65 (F-6, sc-8008) from Santa Cruz Biotechnology and anti-IRF3 (no. 51-3200) from Invitrogen, whereas secondary Abs were Alexa Fluor 647 anti-mouse or Alexa Fluor 488 anti-rabbit (Invitrogen). Abs used for ChIP were anti-Pol II (N-20, sc-899X), anti-p65 (C-20, sc-372X), anti-IRF1 (M-20, sc-640X), and anti-IRF3 (C-19, sc-6058X) from Santa Cruz Biotechnology, anti-IRF3 (no. 51-3200) from Invitrogen, and isotype control rabbit IgG (Sigma-Aldrich). The neutralizing IFNAR1 Ab (MAR1-5A3, no. 16-5945) was purchased from eBioscience.

Quantitative RT-PCR

For mRNA expression analysis, cells were seeded at 4×10^5 cells/ml in 24-well plates and stimulated as indicated the next day. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche) and reverse transcribed with random hexamers (MWG Biotech) using Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. cDNA was analyzed by quantitative RT-PCR using the SYBR Green quantitative PCR Master Mix GoTaq (Promega) or KAPA SYBR FAST Universal (Kapa Biosystems) and gene-specific primer pairs (Table I). Relative mRNA expression was calculated using the comparative C_T method, normalizing the gene of interest to the housekeeping gene *\beta*-actin, and comparing it to an untreated sample as calibrator.

ELISA

Cell culture supernatants were assayed for CCL5 or TNF protein using ELISA kits (R&D Systems) according to the manufacturer's instructions.

Immunoblotting

Cells were seeded at 4×10^5 cells/ml in six-well plates and stimulated as indicated the next day. Cells were washed with ice-cold PBS, freeze-thawed once at -80°C , and then scraped into 80 μ l lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 10% [v/v] glycerol, 40 mM β -glycerophosphate, 1% [v/v] Triton X-100, containing the inhibitors 1 mM Na₂VO₄, 1 mM PMSF, and 1% [v/v] aprotinin) and left on ice for 45 min. Cleared lysates were mixed with 3 \times sample buffer (187.5 mM Tris [pH 6.8], 6% [w/v] SDS, 30% [v/v] glycerol, 0.3% [w/v] bromophenol blue, 150 mM DTT) and boiled for 5 min at 99°C . Twenty-microliter lysates were resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore), blocked for 1 h in 3% (w/v) BSA in PBS, and probed overnight with primary Ab (1:1000 dilution in blocking solution). The next day, membranes were incubated with secondary Abs (1:8000 dilution in blocking solution) and blots were visualized using the Odyssey imaging system (LI-COR Biosciences).

Confocal microscopy

Cells were seeded at 3×10^5 cells/ml on glass coverslips in 24-well plates and stimulated the next day as indicated. Cells were fixed for 12 min in 4% (w/v) paraformaldehyde and permeabilized for 15 min with 0.5% (v/v) Triton X-100 in PBS. Coverslips were blocked for 1 h in 5% (w/v) BSA/0.05% (v/v) Tween 20 in PBS and stained overnight with primary Abs (1:200 dilution in blocking solution). The following day, coverslips were incubated for 3 h with secondary Abs (1:500 dilution in blocking solution) and mounted in Mowiol 4-88 (Calbiochem) containing 1 μ g/ml DAPI. Images were obtained with an Olympus FV1000 confocal microscope using a $\times 60$ oil-immersion objective.

Reporter gene assay

The 724-aa murine SARM (SARM₇₂₄) was subcloned from pGW1-SARM1 (a gift from Y.P. Hsueh, Academia Sinica, Taipei, Taiwan), and the 764-aa murine SARM (SARM₇₆₄) was subcloned from pUNO-mSARM1B (InvivoGen). Both isoforms were cloned with a C-terminal Flag-tag attached into the mammalian expression vector pEF-BOS. The -190 to $+57$ *Ccl5* gene promoter region (transcriptional start site designated +1) was cloned from genomic DNA of primary WT BMDMs into the pGL3-control vector (Promega), replacing the SV40 promoter, and generating a pGL3-*Ccl5* promoter firefly luciferase reporter gene construct.

Table I. PCR primer sequences

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>β-actin</i> mRNA	TCCAGCCTTCCTTCTTGGGT	GCACTGTGTTGGCATAGAGGTC
<i>Ccl5</i> mRNA	CTCACCATATGGCTCGGACA	ACAAACACGACTGCAAGATTGG
<i>Tnf</i> mRNA	TCCCCAAAGGGATGAGAAGTT	GTTTGTCTACGACGTGGGCTAC
<i>Il1b</i> mRNA	GTGAAATGCCACCTTTTGACAGTGATGAG	CTGCTGCGAGATTTGAAGCTGGATG
<i>Ccl2</i> mRNA	AACTGCATCTGCCCT	ACGGGTCAACTTCAC
<i>Cxcl10</i> mRNA	TCTGAGTGGGACTCAAGGGAT	TCGTGGCAATGATCTCAACACG
<i>Ift2</i> mRNA	GACTTAGAGGTGCTGCACAG	CTCGTTGTACTCATGACTGCTG
<i>Irf7</i> mRNA	TTGGATCTACTGTGGGCCCA	CTTGCCAGAAATGATCTGGG
<i>Ccl5</i> pre-mRNA ^a	GTGTTGACCTTCCTCTCTCC	CCTCTATCCTAGCTCATCTCC
<i>Ccl5</i> mRNA ^a	CTTGCACTCGTGTGTGCTACTC	CCTCTATCCTAGCTCATCTCC
<i>Ccl5</i> promoter (ChIP)	GCAGTTAGAGGCAGAGTCATAC	CCAGGGTAGCAGAGGAAGTG
<i>Tnf</i> promoter (ChIP)	GATTCCTTGATGCCTGGGTG	GCTCTCATTAACCTCGGA
Total <i>Sarm</i> mRNA	CGCTGCCCTGTACTGGAGG	CTTCAGGAGCTGGCCAGCT
<i>Sarm</i> _724/_764 mRNA	CCTTCGCCAGCTACGCTACTTG	CTTCAGGAGCTGGCCAGCT
WT <i>Sarm</i> locus (genotyping)	ACGCTGGTTCTTACTCTACGA	GCTGGGGCCTCCTTACCTCTT
Mutated <i>Sarm</i> locus (genotyping)	CAGGTAGCCGGATCAAGCGTATGC	CCTGTCCGGTGCCCTGAATGAAC

^aTo distinguish unspliced transcripts (pre-mRNA) and spliced transcripts (mRNA), the forward primer was either designed to bind within an intron (unspliced) or intron spanning (spliced), while the reverse primer targeted an exon.

The murine (−1260) pGL3-*Tnf* promoter luciferase reporter gene construct was a gift from D.V. Kuprash (Russian Academy of Sciences, Moscow, Russia). For reporter gene assays, NIH3T3 cells were seeded at 0.8×10^5 cells/ml in 96-well plates and transfected 16 h later with 150 ng SARM_724, SARM_764, or pEF-BOS empty vector control, 60 ng pGL3-*Ccl5* or pGL3-*Tnf* promoter reporter, and 20 ng pGL3-*Renilla* luciferase using GeneJuice transfection reagent (Novagen). Forty-eight hours after transfection, cells were lysed in passive lysis buffer (Promega) and analyzed for luciferase activity. Firefly luciferase activity was normalized to *Renilla* luciferase activity to control for transfection efficiency.

Retroviral transduction

SARM_724 was subcloned from pGW1-SARM1, and SARM_764 was subcloned from pUNO-mSARM1B, with a C-terminal Flag-tag attached into the retroviral expression vector pMSCV-neo. Retroviral particles were produced in HEK293T cells: cells were seeded at 2×10^5 cells/ml in 10-cm dishes and transfected the next day with 3 μg pMSCV-SARM_724, pMSCV-SARM_764, or pMSCV empty vector control, together with 1 μg VSV-G and 1 μg Gag-Pol plasmids using 15 μl GeneJuice according to the manufacturer's instructions. Medium was then replaced 8 h later. Retroviral supernatants were harvested 48 and 72 h after transfection, centrifuged, and filtered through a 0.45-μm filter. NIH3T3 cells were seeded at 1×10^5 cells/ml in 10-cm dishes and transduced the next day with viral supernatant mixed 1:1 with fresh medium and 6 μg/ml polybrene (Sigma-Aldrich). The transduction was repeated 24 h later, and after a further 48 h cells were selected for 10 d in medium containing 1 mg/ml G418 (BD Biosciences). For transduction of immortalized BMDMs, cells were seeded at 3×10^5 cells/ml in six-well plates and spinoculated the next day with viral supernatant containing 8 μg/ml polybrene at $2000 \times g$ for 1 h at 20°C. Spinoculation was repeated 24 h later, and cells were cultured as described for NIH3T3 cells. Expression of exogenous protein was confirmed by immunoblotting.

ChIP

The protocol was adapted from Nelson et al. (21). Cells (1.5×10^7) were seeded into 15-cm dishes and stimulated as indicated the next day prior to harvesting. Formaldehyde was added to the medium to a final concentration of 1% (v/v) to fix the cells for 10 min at room temperature, and then the reaction was quenched for 5 min with 125 mM glycine (from 1 M stock solution). Cell monolayers were washed with ice-cold PBS and scraped into 10 ml PBS. Cell pellets were washed again with 1 ml PBS before lysis in 1 ml ChIP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% [v/v] Nonidet P-40, and 1% [v/v] Triton X-100, containing the inhibitors 1 mM Na₃VO₄, 1 mM PMSF, and 1% [v/v] aprotinin). Lysates were immediately centrifuged, and nuclear pellets were washed with 1 ml ChIP buffer. The pellet was resuspended in 1 ml ChIP buffer and sonicated using a Branson Sonifier 250 with a microtip attached. For BMDMs, eight cycles of 18×1 -s pulses (power output 3, 90% duty cycle), with 1 min rest on ice between each cycle, was found to generate optimal DNA fragment sizes ranging from 200 to 1000 bp. Sheared chromatin was cleared by centrifugation. For each immunoprecipitate, sheared chromatin equivalent to 2×10^6 cells was made up to 300 μl with ChIP buffer and

incubated overnight at 4°C with 2 μg specific Ab or an isotype control (IgG) while rotating. The following day, protein A- or protein A/G-Sepharose beads (Sigma-Aldrich) were blocked for 45 min with 100 μg salmon sperm DNA (Invitrogen) and 0.5 mg BSA per 1 ml beads (50% slurry in ChIP buffer), then washed once in ChIP buffer. Blocked beads (40 μl; 50% slurry) were incubated with cleared chromatin immunocomplexes for 1 h at 4°C while rotating. Then beads were washed five times with ChIP buffer (without inhibitors) before 100 μl Chelex (10% [w/v] slurry in H₂O; Bio-Rad) was added to the beads. Samples were vortexed and boiled for 10 min at 99°C. After cooling, 1 μl proteinase K (20 μg/ml; Qiagen) was added and incubated for 40 min at 55°C while shaking. Samples were boiled again, centrifuged, and 70 μl supernatant (containing DNA) was collected. In a different protocol, 250 μl 1% (w/v) SDS/0.1 M NaHCO₃ was added to the washed bead pellets instead of Chelex, and complexes were eluted for 2 h at 65°C while shaking. Eluates were collected and incubated overnight at 65°C to reverse cross-links. The next day, an equal volume of TE buffer and 1 μl RNase (100 mg/ml; Qiagen) were added and incubated for 1 h at 37°C. Then, samples were incubated with 3 μl proteinase K for 2 h at 55°C, followed by boiling. DNA fragments were purified with Wizard SV Gel and PCR Clean-Up System (Promega) and eluted in 40 μl H₂O. DNA (2 μl) was applied to quantitative RT-PCR using SYBR Green KAPA Universal and primer pairs specific to the proximal region of the gene promoter (Table I).

Statistical analysis

All data were analyzed with Prism (GraphPad Software) and statistical significance was determined using the two-tailed unpaired Student *t* test.

Results

SARM is required for optimal TLR-induced CCL5 expression in macrophages

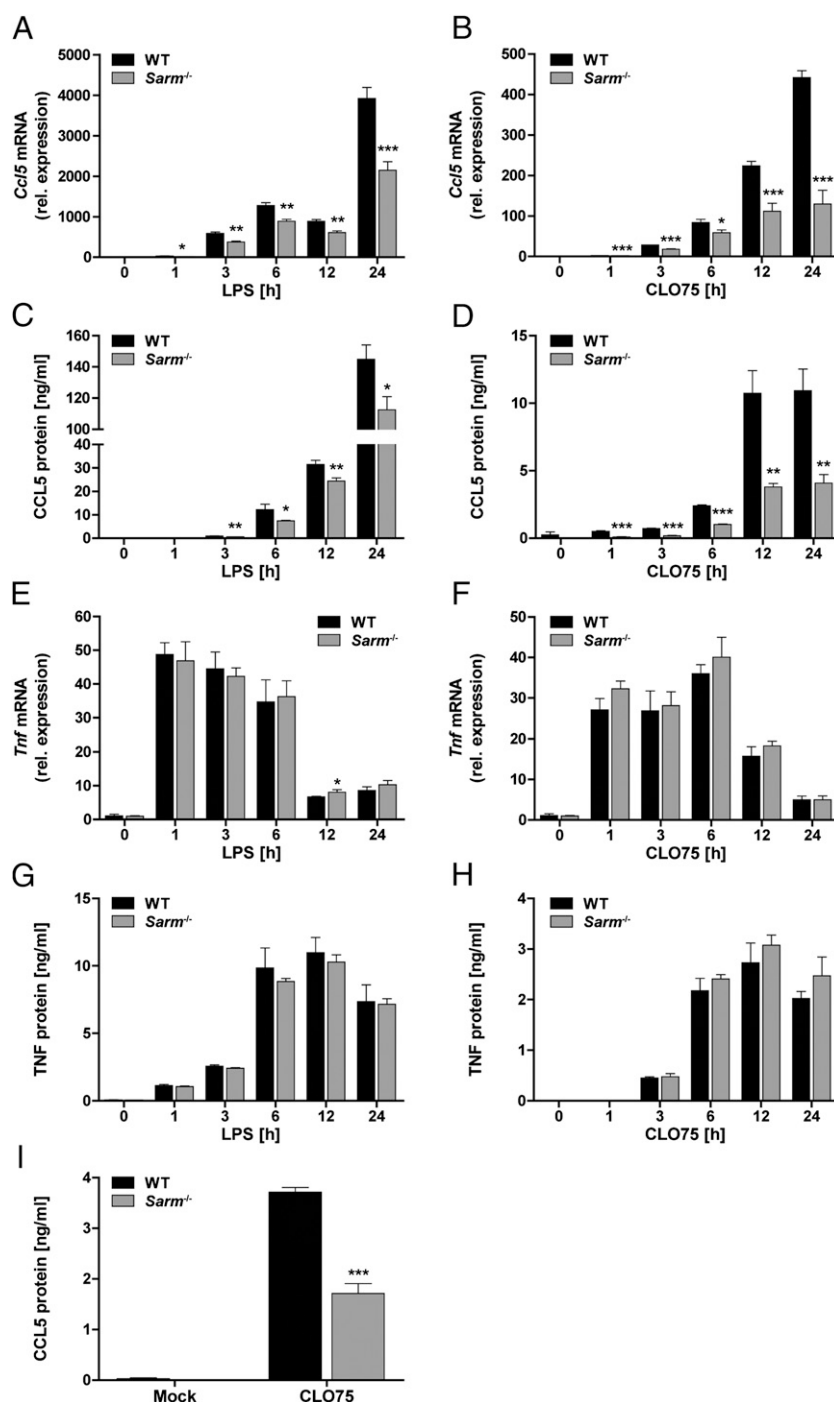
To investigate a potential function of SARM in murine macrophages, we generated BMDMs from WT and *Sarm*^{−/−} mice. The genotype of the cells was confirmed by PCR analysis of the genomic *Sarm* locus (Supplemental Fig. 1A). As previously reported, SARM protein expression was difficult to assess in peripheral cells with currently available Abs (data not shown) (8, 16). However, analysis of *Sarm* mRNA revealed clear expression of SARM in WT BMDMs (Supplemental Fig. 1B). Two mouse SARM isoforms have been reported, of 724 and 764 aa in length (here termed SARM_724 and SARM_764, respectively), with the latter being a splice variant with an extended region between the second sterile α motif and the TIR domain. Further PCR analysis determined that BMDMs predominantly expressed SARM_724, whereas SARM_764 was barely detectable (Supplemental Fig. 1C), which was in agreement with the expression profile of SARM in murine T cells (22).

To determine whether SARM had a role in TLR-mediated cytokine induction in macrophages, TLR4- and TLR7-dependent responses were examined, because TLR4 signaling is partly TRIF-dependent whereas TLR7 is solely MyD88-dependent. BMDMs were treated with the TLR4 agonist LPS or the TLR7 agonist CL075 and cytokine mRNA induction was measured over 24 h. Strikingly, *Ccl5* mRNA induction was significantly impaired in SARM-deficient BMDMs compared with WT cells, both for LPS and CL075 (Fig. 1A, 1B), as was CCL5 protein production (Fig. 1C, 1D). At the same time, SARM deficiency did not affect the induction of TNF mRNA or protein in response to TLR stimulation (Fig. 1E–H), nor did it affect TLR-stimulated induction of *Il1b*, *Ccl2*, or *Cxcl10* mRNA (Supplemental Fig. 2). Importantly, the requirement for SARM was not restricted to bone

marrow-derived macrophages, because CL075-induced CCL5 production was also significantly impaired in SARM-deficient peritoneal macrophages compared with WT cells (Fig. 1I).

To confirm the role of SARM in CCL5 induction, we reconstituted *Sarm*^{−/−} BMDMs with either SARM_724 or SARM_764 using retroviral transduction. *Sarm*^{−/−} BMDMs reconstituted with SARM_724 completely restored TLR7-induced CCL5 production and also augmented CCL5 to almost WT levels in response to TLR4 (Fig. 2A, 2B). SARM_764-expressing cells also regained some ability to produce CCL5 in response to TLR stimulation (Fig. 2A, 2B), but to a lesser extent than SARM_724-reconstituted cells, which correlated with the lower expression of the SARM_764 protein compared with SARM_724 (Fig. 2C). Next, we overexpressed both isoforms of SARM in WT BMDMs, which

FIGURE 1. SARM is required for optimal TLR-induced CCL5 expression, but is dispensable for TNF. Primary WT and *Sarm*^{−/−} BMDMs were stimulated with 100 ng/ml LPS (A, C, E, G) or 5 μg/ml CL075 (B, D, F, H) for the indicated times, or medium as control. *Ccl5* (A, B) and *Tnf* (E, F) mRNA were assayed by quantitative RT-PCR, normalized to the housekeeping gene *β-actin*, and are presented relative to the untreated WT control. Supernatants were assayed for CCL5 (C, D) or TNF (G, H) protein by ELISA. (I) Peritoneal macrophages were generated from WT and *Sarm*^{−/−} mice and stimulated with 10 μg/ml CL075 for 24 h. Supernatants were assayed for CCL5 by ELISA. The data are means ± SD of triplicate samples and are representative of at least three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with WT (Student *t* test).



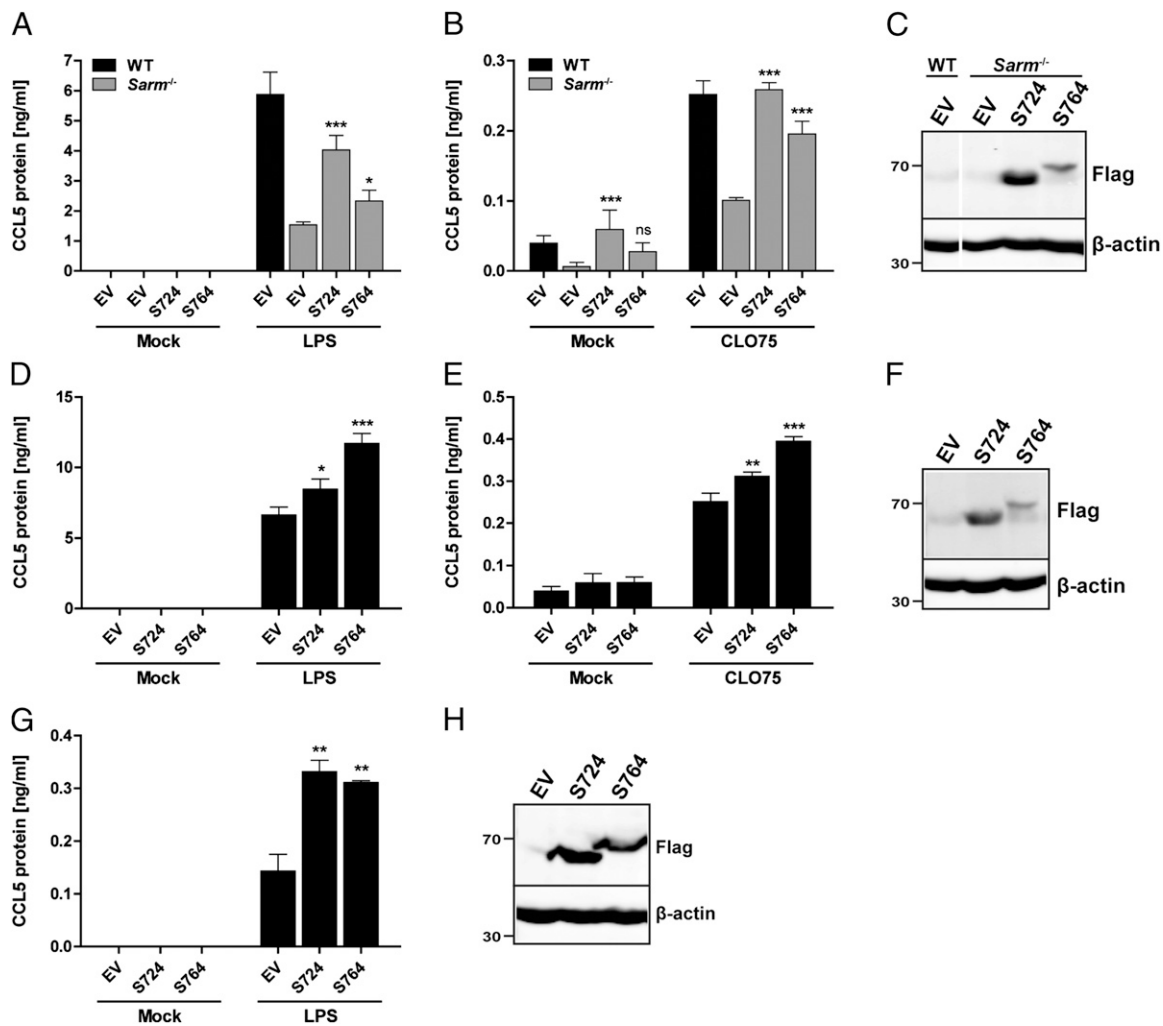


FIGURE 2. Retroviral expression of SARM rescues CCL5 production in *Sarm*^{-/-} BMDMs and augments CCL5 expression in WT cells. Immortalized WT and *Sarm*^{-/-} BMDMs (**A–C**), immortalized WT BMDMs (**D–F**), or NIH3T3 cells (**G, H**) were stably transfected with SARM_724-Flag (S724), SARM_764-Flag (S764), or pMSCV empty vector (EV) control using a retroviral system. Cells were stimulated for 24 h with 1 ng/ml LPS (**A**), 100 ng/ml LPS (**D, G**), 5 μ g/ml CLO75 (**B, E**), or medium as control (mock), and supernatants were assayed for CCL5 protein by ELISA. The data are means \pm SD of triplicate samples and are representative of at least three independent experiments. (**C, F, and H**) Cell lysates were subjected to SDS-PAGE and immunoblotted for SARM using a specific Ab to Flag, or β -actin as a loading control. The molecular mass markers are indicated to the left of the gel (in kDa). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with *Sarm*2/2 EV (**A, B**) or WT EV (**D, E, G**) (Student *t* test).

significantly elevated TLR4- and TLR7-induced CCL5 levels compared with the empty vector control (Fig. 2D–F). In a similar manner, we examined TLR4-induced CCL5 protein in NIH3T3 cells stably overexpressing SARM. Consistent with the results in BMDMs, this different murine cell type also showed significantly enhanced TLR4-induced CCL5 expression when either SARM isoform was ectopically expressed (Fig. 2G, 2H). Taken together, these data clearly show a role for SARM in TLR-induced CCL5 expression in peripheral mouse cells.

SARM is dispensable for the activation of transcription factors implicated in CCL5 expression

To gain insights into the mechanism of SARM-mediated CCL5 regulation, we first defined the signaling pathways that upregulated CCL5 expression in BMDMs. TLR stimulation leads to activation of MAPKs and several transcription factors, including NF- κ B and IRF3, which collectively induce the expression of primary response genes (PRGs). PRGs themselves mediate further responses, and some are involved in the induction of secondary response genes (SRGs). A particular example of this is TLR-induced type I IFNs, which subsequently signal via the IFN- α/β receptor (IFNAR) to

upregulate numerous IFN-stimulated genes (ISGs) (23). To determine whether TLR-induced CCL5 was a PRG and/or a SRG in BMDMs, we pretreated WT BMDMs with the protein synthesis inhibitor cycloheximide (CHX) before eliciting CCL5 production through TLR4 stimulation. As expected, the ISG *Ifit2*, which is known to be upregulated through type I IFNs secreted in response to TLR4 stimulation (24), showed reduced mRNA expression in CHX-treated cells (Supplemental Fig. 3B). In contrast, the induction of *Ccl5* mRNA was not impaired in the presence of CHX (Supplemental Fig. 3A), suggesting the direct transcriptional induction of *Ccl5* as a PRG after TLR4 stimulation. Furthermore, blocking type I IFN-mediated responses with an IFNAR blocking Ab did not affect CCL5 induction by TLR4 (Supplemental Fig. 3C), whereas this did inhibit induction of the ISGs *Ifit2* and *Irf7* (25), as it inhibited type I IFN signaling (Supplemental Fig. 3D–F). Thus, concordant with published data (26), *Ccl5* is a PRG in TLR-stimulated BMDMs.

Consequently, SARM was predicted to directly regulate the TLR signaling pathway to promote CCL5 expression, and we therefore tested whether SARM was required for the activation of transcription factors required for CCL5 induction. To define the

transcription factors relevant for TLR4-mediated CCL5 induction in BMDMs, we assessed their binding to the *Ccl5* promoter using ChIP analysis. Among the transcription factors previously implicated in CCL5 induction in different cell types by different stimuli (27–30), we observed a transient recruitment of the NF- κ B p65 subunit, IRF3 and IRF1, but not IRF8, to the *Ccl5* promoter in BMDMs in response to LPS (Fig. 3A). Recruitment of p65 and IRF3 to the promoter peaked after 1 h LPS stimulation, whereas IRF1 promoter occupancy was maximal at 3 h after LPS (Fig. 3A). Because IRF1 is induced as an SRG in response to TLR stimulation, via type I IFN induction (31), it was unlikely to be essential for TLR4-induced CCL5 because *Ccl5* mRNA induction was type I IFN-independent (Supplemental Fig. 3C). Thus, we examined whether the activation of NF- κ B or IRF3 was impaired in the absence of SARM. The activation of NF- κ B was first assessed indirectly by monitoring the LPS-induced degradation of the cytosolic inhibitor of NF- κ B, I κ B, which was found to be unaffected by the absence of SARM (Fig. 3B). Furthermore, confocal microscopy showed that LPS-induced translocation of p65 to the nucleus was also normal in cells lacking SARM (Fig. 3C). This was also the case for IRF3 translocation to the nucleus (Fig. 3C). We also demonstrated no role for SARM in the upregulation of IRF1 protein in response to type I IFN (Fig. 3D), whereas IRF8,

which was not recruited to the *Ccl5* promoter in response to LPS stimulation, was not induced by TLR4 or IFNAR signaling (Fig. 3D and data not shown).

Given that neither NF- κ B nor IRF3 activation by TLR4 was found to be SARM-dependent, and in light of the role of worm SARM (TIR-1) upstream of a p38 MAPK pathway in *C. elegans* (6), we additionally assessed LPS-induced activation of the three main MAPKs, p38, JNK, and ERK, which might contribute to *Ccl5* promoter activity (28). However, we observed a similar degree of MAPK phosphorylation in *Sarm*^{-/-} BMDMs compared with the corresponding WT (Supplemental Fig. 4), suggesting that mammalian SARM does not regulate MAPK pathways in BMDMs. Taken together, the data demonstrated that SARM was not required for the activation of the main transcription factors implicated in TLR4-stimulated CCL5 expression, nor was it involved in activating the MAPKs p38, JNK, and ERK.

SARM regulates the recruitment of transcription factors and Pol II to the Ccl5 promoter

The finding that SARM was not involved in the activation of NF- κ B and IRF3 in BMDMs was consistent with the lack of requirement for SARM for the induction of other NF- κ B- and IRF3-dependent TLR-induced genes (Fig. 1E–H, Supplemental Fig. 2) and sug-

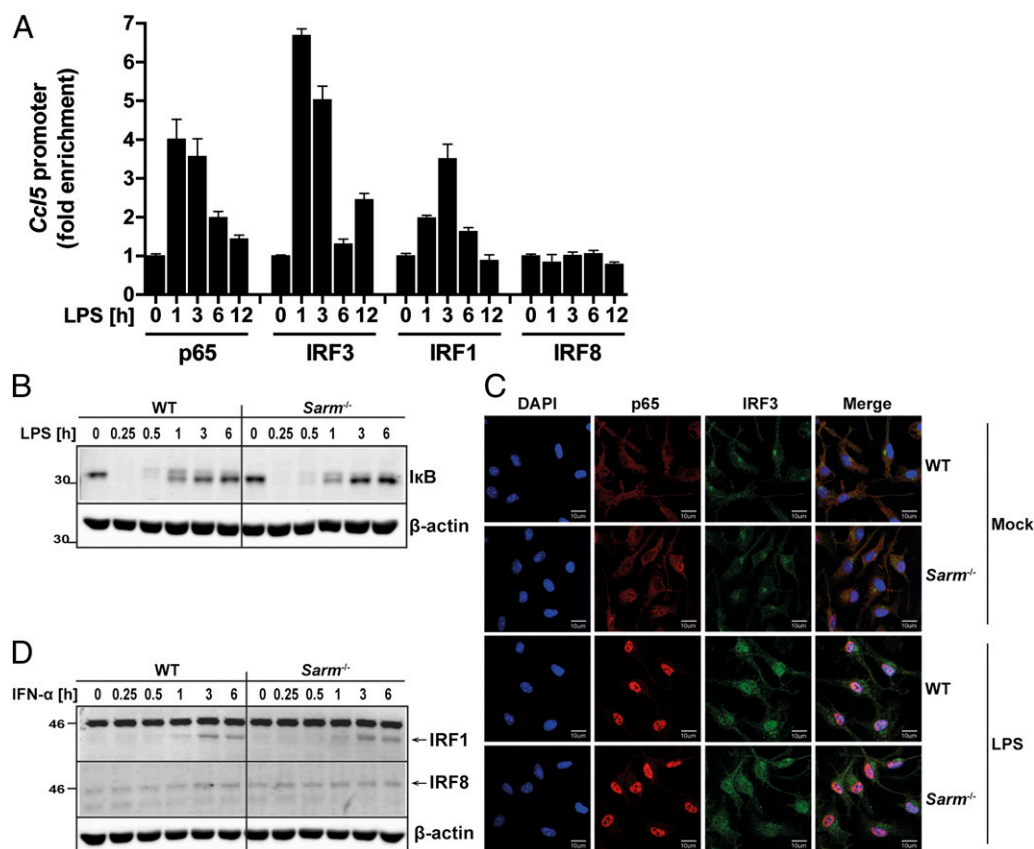


FIGURE 3. SARM is dispensable for the activation of transcription factors that are recruited to the *Ccl5* promoter. **(A)** Immortalized WT BMDMs were stimulated with 100 ng/ml LPS for the indicated times or medium as control. Sheared chromatin lysates were subjected to ChIP with Abs specific to NF- κ B p65, IRF3, IRF1, and IRF8, or an isotype-control Ab (IgG; not shown). Precipitated DNA and input DNA were analyzed by quantitative RT-PCR using primer pairs specific for the *Ccl5* promoter. Results are normalized to input and presented as fold enrichment of each transcription factor at the *Ccl5* promoter relative to the untreated control. The data are means \pm SD of PCR technical triplicates and are representative of at least two independent experiments. **(B and D)** Primary WT and *Sarm*^{-/-} BMDMs were stimulated with 100 ng/ml LPS **(B)** or 1000 U/ml IFN- α **(D)** for the indicated times, or medium as control. Cell lysates were subjected to SDS-PAGE and immunoblotted with specific Abs to I κ B **(B)** or IRF1 and IRF8 **(D)**; arrow indicates the specific band. β -actin served as loading control. The molecular mass markers are shown to the left of the gel (in kDa). **(C)** Primary WT and *Sarm*^{-/-} BMDMs grown on glass coverslips were stimulated for 1 h with 100 ng/ml LPS or medium as control (mock), then fixed and stained with Abs specific to NF- κ B p65 (red) or IRF3 (green). Nuclei were visualized using the DNA-intercalating dye DAPI (blue). Scale bars, 10 μ m. The data **(B)–(D)** are representative of at least three independent experiments.

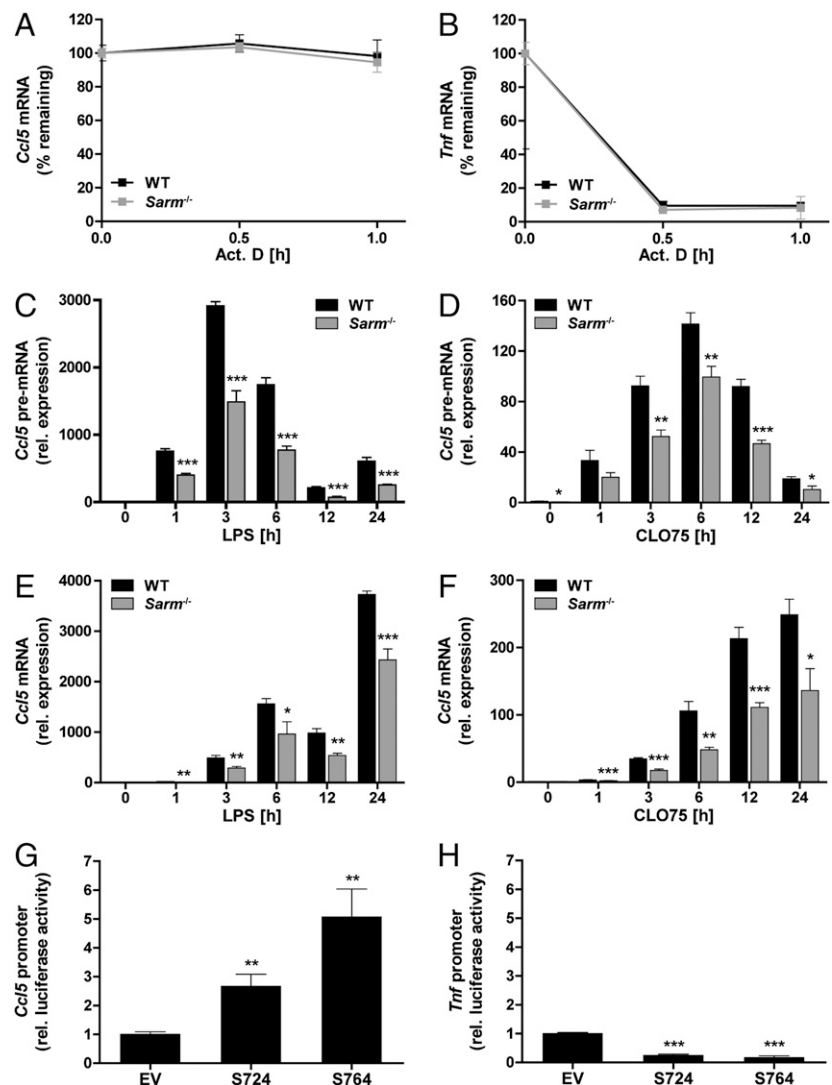
gested a more direct gene-specific role for SARM in CCL5 regulation. We therefore next examined a potential role for SARM in posttranscriptional regulation of CCL5 expression. In contrast to the induction of *Tnf* mRNA, which is rapid and transient, *Ccl5* mRNA induction is more delayed and gradually increases over a longer time course (32) (Fig. 1A, 1B, 1E, 1F). The different mRNA induction kinetics are mainly due to *Tnf* mRNA being unstable due to AU-rich elements in the 3' untranslated region, whereas *Ccl5* mRNA lacked those elements and was found to be particularly stable (32). To test whether SARM had any role in maintaining *Ccl5* mRNA stability after immune stimulation, the stability of LPS-induced *Ccl5* mRNA was monitored in WT and *Sarm*^{-/-} BMDMs after transcription was blocked with actinomycin D. As expected, *Tnf* mRNA showed rapid decay after actinomycin D treatment, whereas no decay was observed for *Ccl5* mRNA over 60 min (Fig. 4A, 4B). Importantly, the absence of SARM did not provoke any instability of *Ccl5* mRNA, indicating that SARM did not exert its CCL5 regulatory function at the level of mRNA stability.

A recent study demonstrated that the delayed expression of genes, such as *Ccl5*, resulted from a prolonged splicing time rather than a lag in transcriptional initiation (33). Thus, we asked whether there would be a defect in regulation of *Ccl5* RNA splicing in the absence of SARM. If so, *Ccl5* precursor mRNA (pre-mRNA) would be expected to accumulate in *Sarm*^{-/-} BMDMs in comparison with WT cells. Thus, we designed specific primer pairs

to distinguish unspliced *Ccl5* RNA (intron-containing) from spliced transcripts and performed a time course to monitor the expression kinetics of both forms following TLR4 and TLR7 activation. We observed peak expression of *Ccl5* pre-mRNA 3 h after LPS and 6 h after CLO75 stimulation, which gradually decreased thereafter (Fig. 4C, 4D). The decrease in unspliced transcripts coincided with a steady increase of spliced *Ccl5* mRNA that continued up to 24 h after stimulation (Fig. 4E, 4F). However, *Ccl5* pre-mRNA did not accumulate in SARM-deficient cells, but rather similar to mature mRNA, it was significantly reduced in the absence of SARM compared with WT cells (Fig. 4C, 4D). This excluded a role for SARM in RNA splicing and suggested SARM regulated CCL5 at or prior to transcriptional induction of the *Ccl5* promoter.

Thus, we next examined the potential of SARM ectopic expression to activate the *Ccl5* promoter directly using a luciferase reporter gene under the control of the *Ccl5* promoter in mouse NIH3T3 cells. The expression of either isoform of mouse SARM significantly triggered *Ccl5* promoter induction while having no positive effect on *Tnf* promoter induction (Fig. 4G, 4H), supporting a role for SARM in stimulating the transcriptional initiation of the *Ccl5* promoter. To further explore a role for SARM in inducing the endogenous *Ccl5* promoter, we performed ChIP analysis in WT and *Sarm*^{-/-} BMDMs for the recruitment of Pol II. This was a key experiment, as, in the case of CCL5, stimulus-

FIGURE 4. SARM regulates CCL5 expression at the promoter and not posttranscriptionally. (A and B) Primary WT and *Sarm*^{-/-} BMDMs were prestimulated for 3 h with 100 ng/ml LPS and then treated with 5 μ g/ml actinomycin D (Act. D) for the indicated times or DMSO as control. *Ccl5* (A) and *Tnf* (B) mRNA were assayed by quantitative RT-PCR, normalized to the housekeeping gene β -actin, and are presented as percentage remaining compared with each WT or *Sarm*^{-/-} control. (C–F) Primary WT and *Sarm*^{-/-} BMDMs were stimulated with 100 ng/ml LPS (C, E) or 5 μ g/ml CLO75 (D, F) for the indicated times, or medium as control. *Ccl5* pre-mRNA (C, D) or mature mRNA (E, F) were assayed by quantitative RT-PCR, normalized to the housekeeping gene β -actin, and are presented relative to the untreated WT control. (G and H) NIH3T3 cells were transfected for 48 h with 150 ng SARM₇₂₄-Flag (S724), SARM₇₆₄-Flag (S764), or pEF-BOS empty vector (EV) control, along with the pGL3-*Ccl5* promoter luciferase reporter gene (G) or pGL3-*Tnf* promoter luciferase reporter gene (H), and the control pGL3-*Renilla* luciferase gene. Then luciferase activity was measured, normalized to *Renilla*, and is presented relative to EV control. All data are means \pm SD of triplicate samples and are representative of at least two independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with WT (C–F) or EV (G, H) (Student t test).



induced Pol II recruitment to the promoter is a hallmark of transcriptional induction (17). The analysis revealed that SARM-deficient cells were significantly impaired in their ability to recruit Pol II to the *Ccl5* promoter in response to TLR4 and TLR7 signaling (Fig. 5A, 5B). To determine the specificity of this observation, we also examined Pol II occupancy at the *Tnf* promoter. In contrast to CCL5, for TNF Pol II is known to be already present at the *Tnf* promoter in the absence of cell stimulation (17), and this was also evident in our ChIP study, where Pol II was readily detectable at the *Tnf* promoter in unstimulated cells and did not significantly increase with TLR4 or TLR7 stimulation over 3 h (Fig. 5C, 5D). In contrast to the *Ccl5* promoter, Pol II abundance at the *Tnf* promoter was comparable between WT and SARM-deficient BMDMs (Fig. 5C, 5D). This was consistent with the fact that TNF induction is independent of SARM (Fig. 1E, 1F).

Recruitment of Pol II to the *Ccl5* promoter is known to be tightly controlled and highly dependent on transcription factor binding and chromatin remodeling (34, 35). Thus, although transcription factor activation was found to be unaffected by SARM ablation (Fig. 3), we performed ChIP analysis to investigate whether SARM would be required for transcription factor binding to the *Ccl5* promoter. Intriguingly, both TLR4- and TLR7-mediated recruitment of transcription factors to the *Ccl5* promoter was significantly impaired in *Sarm*^{-/-} BMDMs compared with the corresponding WT cells (Fig. 5E–I). Hence, optimal recruitment of p65, IRF3, and IRF1 to the *Ccl5* promoter in response to TLR4 stimulation, and of p65 and IRF1 to the promoter in response to TLR7, all depended on SARM (Fig. 5E–I). IRF3 recruitment was not examined for TLR7, as IRF3 is generally not activated by this pathway (1). In contrast to the case for the *Ccl5* promoter, p65 recruitment to the *Tnf* promoter after LPS or CL075 stimulation was normal in the absence of SARM (Fig. 5J, 5K). Overall, the ChIP data showed that SARM regulated *Ccl5* transcription by mediating the proper assembly of transcription factors at the *Ccl5* promoter and subsequently recruitment of Pol II, and thus the formation of the transcriptional machinery on the *Ccl5* promoter.

SARM is also required for optimal CCL5 induction in response to non-TLR pathways

In light of the promoter regulatory role of SARM for CCL5 induction, we wondered whether SARM had an intrinsic stimulus-independent role in CCL5 induction and therefore examined CCL5 expression elicited by non-TLR cytosolic PRRs. Transfection of the synthetic dsRNA poly(I:C) was used to stimulate the cytosolic RNA sensor melanoma differentiation-associated gene 5 (3), whereas the VACV 70-bp dsDNA oligonucleotide (dsVACV 70mer) was transfected into cells to stimulate STING-dependent DNA sensing pathways involving sensors such as the IFN-inducible protein 16 homolog p204 and cyclic GMP-AMP synthase (20, 36). CCL5 and TNF induction in response to the transfected nucleic acids was then compared in *Sarm*^{-/-} and WT BMDMs. Interestingly, this revealed that SARM was required for optimal CCL5 mRNA and protein expression downstream of cytosolic receptor stimulation (Fig. 6A–D), whereas it was dispensable for TNF induction (Fig. 6E–H), concordant with the data obtained for TLR-induced CCL5 and TNF.

Cumulatively, the data reveal a new role for SARM in induction of CCL5 expression in macrophages by both TLR and non-TLR pathways, and that SARM is required for the proper assembly of transcription factors and for subsequent RNA Pol II recruitment at the *Ccl5* promoter.

Discussion

It has become increasingly apparent recently that SARM might execute quite different roles compared with the other TIR adaptors,

MyD88, MAL, TRIF, and TRAM, which initiate downstream signaling from TLRs. Several groups have demonstrated that mouse SARM was predominantly expressed in neurons of the brain, and that it had a role in regulating neuronal morphology, stress-induced neuronal cell death, and axon degeneration (8–12). The role of SARM in the nervous system was concordant with its evolutionary counterpart TIR-1 in *C. elegans* (13, 14). However, there are fewer data on SARM functions in the periphery.

In this study, we have identified a crucial role for SARM in macrophages in the regulation of CCL5 expression by PRRs. This regulatory function of SARM was found to be quite selective for CCL5, as other cytokines and chemokines tested, namely TNF, IL-1 β , CCL2, and CXCL10, were not affected by SARM ablation. A previous study by Kim et al. (8) showed that TLR-induced cytokine responses were normal in *Sarm*^{-/-} BMDMs; however, that study only examined TNF and CCL2 and not CCL5, and so is consistent with our data in the present study. SARM function had been suggested to be likely CNS-restricted owing to its abundant expression in the brain compared with other tissues and immune cells (8), but apart from the present study, another recent paper showed a role for SARM in peripheral cells. That report demonstrated that SARM-mediated T cell death in immune-activated and expanded T cells to restore homeostasis after infection (22).

Roles for SARM in the regulation of cytokine and chemokine induction in the CNS have also emerged. Thus, infecting *Sarm*^{-/-} mice with neurotropic WNV indicated a requirement of SARM for virus-induced TNF production in the brainstem (16). Furthermore, Hou et al. (37) demonstrated that SARM-deficient mice showed a significant defect in upregulating cytokines in the CNS in response to vesicular stomatitis virus (VSV) infection. However, the range of cytokines dependent on SARM seems to be much broader in the CNS compared with our data for peripheral macrophages in the present study and includes IL-6, TNF, CCL2, CCL5, and type I IFNs. A broader requirement for SARM for cytokine gene induction in the CNS might result from the higher SARM expression in CNS-resident cells.

Of note, therefore, SARM has been found to regulate CCL5 both in the CNS and in peripheral macrophages, which could have particular implications in disease pathogenesis. For example, CCL5 and its receptor CCR5 were shown to be particularly important for leukocyte recruitment to the CNS, and subsequent control of WNV infection in mice (38, 39), whereas humans with a homozygous CCR5 mutation (CCR5 Δ 32) have an increased risk for symptomatic WNV infection (40). Strikingly, *Sarm*^{-/-} mice were shown to be more susceptible to WNV (16). Clearly SARM is required for immune cell infiltration into the brain, because after either WNV (16) or VSV infection (37) there were reduced macrophage numbers in the brain of mice lacking SARM compared with normal mice, whereas macrophage/monocyte infiltration of lesioned nerves was suppressed in *Sarm*^{-/-} mice (10).

Whether SARM-dependent CCL5 production contributes to virus- and insult-stimulated cellular infiltration needs to be studied in detail.

In this study, we focused on understanding mechanistically how SARM regulated CCL5 expression in BMDMs. A key observation was that SARM function was not restricted to TLR pathways, as would have been expected for a cytosolic TIR domain-containing protein, and given the role of human SARM in regulating TRIF signaling (7). Instead, SARM also regulated CCL5 induction downstream of cytosolic retinoic acid-inducible gene I-like receptors and DNA sensors, which signal via the non-TIR domain-containing adaptor proteins mitochondrial antiviral signaling and STING, respectively (1). However, the TLR-independent function of SARM in the present study is actually consistent with

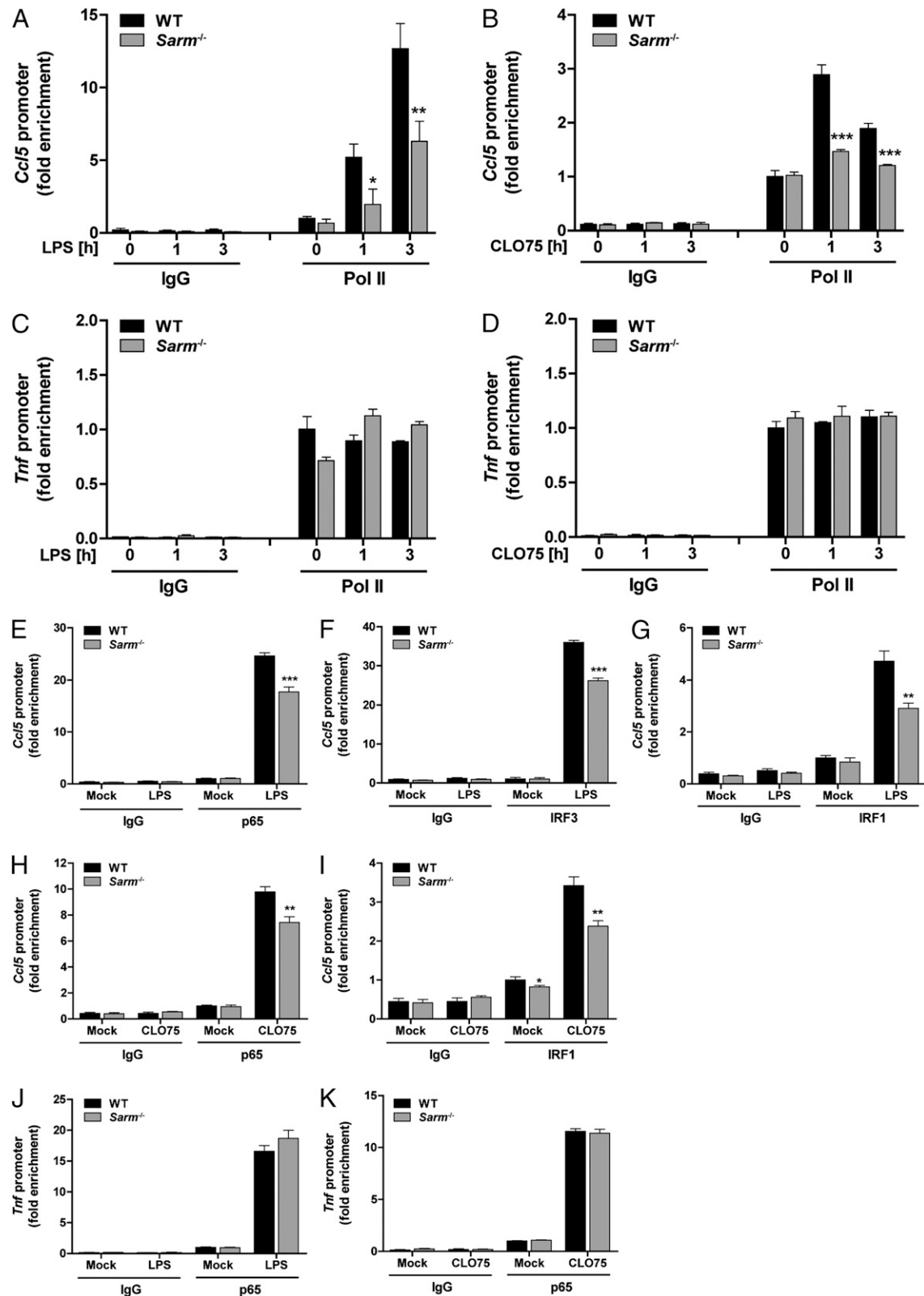


FIGURE 5. SARM is required for optimal Pol II recruitment and the assembly of transcription factors at the *Ccl5* promoter. (**A–D**) Primary WT and *Sarm*^{-/-} BMDMs were stimulated with 100 ng/ml LPS (**A, C**) or 5 μ g/ml CLO75 (**B, D**) for the indicated times, or medium as control. Sheared chromatin lysates were subjected to ChIP with an Ab specific to RNA Pol II or an isotype-control Ab (IgG). Precipitated DNA and input DNA were analyzed by quantitative RT-PCR using primer pairs specific for the *Ccl5* or *Tnf* promoter. Results are normalized to input and are presented as fold enrichment of Pol II at the *Ccl5* promoter (**A, B**) or *Tnf* promoter (**C, D**) relative to the untreated WT control. (**E–K**) Primary WT and *Sarm*^{-/-} BMDMs were stimulated for 1 h with 100ng/ml LPS (**E–G, J**), 5 μ g/ml CLO75 (**H, I, K**), or medium as control (mock). Sheared chromatin lysates were subjected to ChIP with Abs specific to NF- κ B p65 (**E, H, J, K**), IRF3 (**F**), IRF1 (**G, I**), or IgG control. Samples were analyzed as in (**A**)–(**D**) and are presented as fold enrichment of each specific transcription factor at the *Ccl5* promoter (**E–I**) or *Tnf* promoter (**J, K**) relative to mock-treated WT cells. All data are means \pm SD of PCR technical triplicates and are representative of at least three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with WT (Student t test).

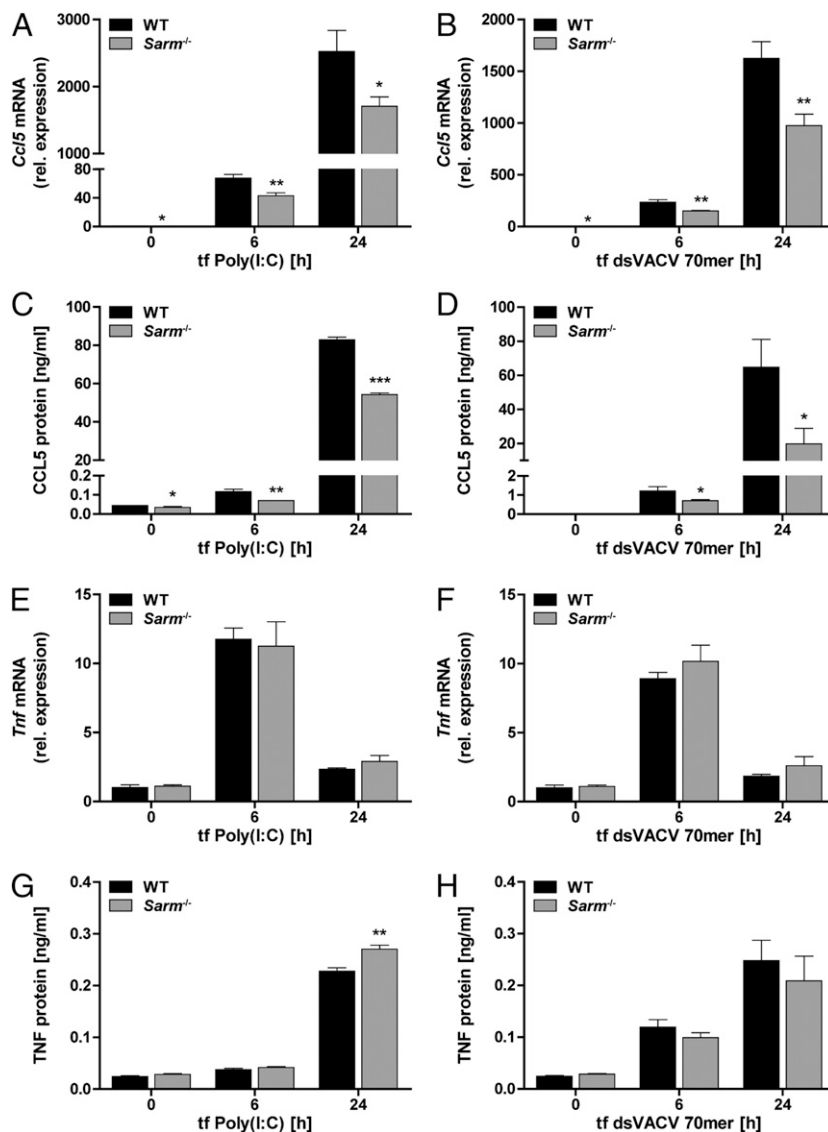


FIGURE 6. SARM is also required for optimal CCL5 expression of non-TLR pathways. Primary WT and *Sarm*^{-/-} BMDMs were transfected with 2 μ g/ml poly(I:C) (A, C, E, G) or 1 μ g/ml dsVACV 70mer (B, D, F, H) for the indicated times, or medium as control. *Ccl5* (A, B) and *Tnf* (E, F) mRNA were assayed by quantitative RT-PCR, normalized to the housekeeping gene β -actin, and are presented relative to the untreated WT control. Supernatants were assayed for CCL5 (C, D) or TNF (G, H) protein by ELISA. The data are means \pm SD of triplicate samples and are representative of at least three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with WT (Student t test).

the fact that its ortholog TIR-1 in *C. elegans* triggered immune responses independent of the sole *C. elegans* TLR ortholog, TOL-1 (15). Of interest, TLR-independent roles for other mammalian TIR adaptors have recently emerged in that MyD88 and TRIF apparently function as signaling adaptors downstream of DExD/H-box helicase proteins implicated in cytosolic RNA and DNA sensing (41, 42).

To define how SARM was regulating CCL5 induction, we first confirmed the mechanism by which PRRs induced CCL5 in BMDMs. This showed that PRR-induced CCL5 was a PRG up-regulated directly in response to TLR stimulation independently of any secondary-induced factor or of type I IFN signaling. Consequently, SARM was predicted to modulate the PRR pathway directly, but the main transcription factors implicated in CCL5 induction were activated normally in the absence of SARM, namely NF- κ B, IRF3, and IRF1. Also, the activation of the MAPKs, p38, JNK, and ERK, was normal in *Sarm*^{-/-} BMDMs. Apart from transcriptional regulation, *Ccl5* mRNA expression was recently demonstrated to be controlled by regulated RNA splicing (33). However, we could clearly exclude a role for SARM in the posttranscriptional events of *Ccl5* RNA splicing, as well as mRNA stability. Instead, SARM was found to enhance *Ccl5* promoter activity, indicating its regulatory function operated at the transcriptional level. This was confirmed for the endogenous pro-

motor, where SARM was required for optimal Pol II recruitment to the *Ccl5* promoter, but was dispensable for Pol II binding to the *Tnf* promoter. Notably, the accumulation of Pol II at the *Ccl5* promoter is regarded as the rate-limiting step in *Ccl5* transcription and depends on prior transcription factor binding and chromatin remodeling to open the otherwise tightly closed promoter (17, 34, 35). A detailed ChIP analysis revealed that recruitment of NF- κ B p65, IRF3, and IRF1 to the *Ccl5* promoter was significantly impaired in the absence of SARM. Reduced transcription factor recruitment to the promoter likely explains the impaired Pol II recruitment to *Ccl5* in the absence of SARM, because IRF3 in particular is implicated in stimulating chromatin remodeling at the *Ccl5* promoter (35).

Thus, our study in macrophages provides compelling evidence that SARM promotes CCL5 expression via controlling the proper recruitment of transcription factors, and subsequently Pol II, to the *Ccl5* promoter. Future experiments will seek to elucidate the exact mechanism used by SARM to achieve this. Given the TLR-independent nature of this SARM function, other protein motifs in SARM apart from the TIR domain might be involved in mediating *Ccl5* promoter induction. For example, sterile α motif domains, which are known to mediate diverse protein-protein interactions (43), may recruit a transcriptional regulator to the *Ccl5* promoter. Also, SARM may recruit a DExD/H-box protein to

the *Ccl5* promoter, because such helicases can interact with TIR adaptors (41, 42) and are also known to regulate gene promoters (44). Furthermore, it will be interesting to define whether SARM acts in close proximity to the *Ccl5* promoter, or whether it functions in a more upstream role. In support of the latter model is the fact that SARM is reported to be expressed in the cytosol and mostly located at the mitochondria (8, 45). However, we and others (46) have also detected SARM in the nucleus, although we have no evidence to date of PRR-stimulated relocalization of SARM (data not shown). Apart from elucidating the exact mechanism of *Ccl5* transcriptional regulation by SARM in macrophages, it will also be of interest to determine whether SARM-dependent cytokine induction in the CNS (37) is also due to regulation of recruitment of transcription factors and Pol II to selected gene promoters.

Overall, this study has identified a novel function of mouse SARM in selectively promoting PRR-induced CCL5 expression in macrophages. Accordingly, the data contribute to establishing SARM as a positive regulator of cytokine responses, concordant with previous murine *in vivo* studies. Importantly, the present study highlights the TLR-independent role of another mammalian TIR adaptor protein.

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Disclosures

The authors have no financial conflicts of interest.

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