Mal Mediates TLR-Induced Activation of CREB and Expression of IL-10

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TLRs initiate immune responses by direct detection of molecular motifs that distinguish invading microbes from host cells. Five intracellular adaptor proteins, each containing a Toll/IL-1R (TIR) domain, are used by TLRs and play key roles in dictating gene expression patterns that are tailored to the invader. Such gene expression is mediated by transcription factors, and although TIR adaptor-induced activation of NF-κB and the IFN regulatory factors have been intensively studied, there is a dearth of information on the role of TIR adaptors in regulating CREB. In this paper, we describe a role for the TIR adaptor Mal in enhancing activation of CREB. Mal-deficient murine bone marrow-derived macrophages show a loss in responsiveness to TLR2 and TLR4 ligands with respect to activation of CREB. Mal-deficient cells also fail to express the CREB-responsive genes IL-10 and cyclooxygenase 2 in response to Pam_2Cys-Ser-(Lys)4 and LPS. We reveal that Mal-mediated activation of CREB is dependent on Pellino3 and TNFR-associated factor 6, because CREB activation is greatly diminished in Pellino3 knockdown cells and TNFR-associated factor 6-deficient cells. We also demonstrate the importance of p38 MAPK in this pathway with the p38 inhibitor SB203580 abolishing activation of CREB in murine macrophages. MAPK-activated protein kinase 2 (MK2), a substrate for p38 MAPK, is the likely downstream mediator of p38 MAPK in this pathway, because Mal is shown to activate MK2 and inhibition of MK2 decreases TLR4-induced activation of CREB. Overall, these studies demonstrate a new role for Mal as a key upstream regulator of CREB and as a contributor to the expression of both pro- and anti-inflammatory genes. ***The Journal of Immunology*****, 2011, 186: 4925–4935.**

The mammalian innate immune system has evolved an array of pattern-recognition receptors (PRRs) that detect a host of pathogen-associated molecular patterns or microbial motif signatures that are absent from host cells (1). PRRs are the first line of defense in immunity and regulate the inflammatory response according to the type of threat they encounter. TLRs have emerged as an important family of transmembrane PRRs and directly interact with infectious agents via extracellular regions containing numerous leucine-rich repeats (2, 3). TLRs also possess a highly conserved intracellular Toll/IL-1R (TIR) domain, so named because it shares high homology to the intracellular domain of the IL-1R family (4, 5). The TIR domain is crucial to TLR signaling as demonstrated by a mutation in the TIR domain of TLR4 resulting in loss of responsiveness to LPS (6). The TIR domains of TLRs allow for their engagement of TIR domain-containing adaptor proteins that trigger downstream signaling cascades culminating in complex patterns of gene expression profiles (7). TLRs tailor quite specific responses to different invading microorganisms and recruit several TIR adaptor proteins to initiate downstream signaling. With the exception of TLR3, all TLRs use MyD88 to activate NF-κB, and this defines a MyD88-dependent pathway (8). MyD88 associates with and activates members of the IL-1R–associated kinase (IRAK) family that in turn recruit TRAF6, an E3 ubiquitin ligase that can promote strong autoubiquitination. IRAK1 is also ubiquitinated in this complex by members of the Pellino family of E3 ubiquitin ligases (9–11). The polyubiquitinated forms of IRAK1 and TRAF6 recruit the mitogen-activated protein 3 kinase TGF-β–associated protein kinase 1 (TAK1) and associated proteins TAK1-binding proteins 1–3 (12). TAK1 becomes phosphorylated, and this promotes downstream activation of the IκB-kinases and subsequent phosphorylation and degradation of IκBs that normally sequester NF-κB in the cytosol (13). Interestingly, TAK1 can also stimulate activation of the p38 and JNK MAPK pathways (14).

Although most TLRs can directly engage the above MyD88-dependent pathway, TLR2 and TLR4 require the bridging adaptor MyD88 adaptor-like (Mal) (15, 16) to associate with MyD88 (17, 18) and to trigger its downstream pathway to activate NF-κB. In contrast, TLR3 does not use MyD88 or Mal but instead directly recruits the TIR adaptor Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) to trigger downstream signaling both to NF-κB and IFN regulatory factor (IRF) transcription factors (19–21). TLR4 can also engage this MyD88-independent, TRIF-dependent pathway but requires the bridging adaptor TRIF-related adaptor molecule (TRAM) to associate with TRIF (22). In addition to promoting activation of NF-κB, the TRIF-dependent

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; ChIP, chromatin immunoprecipitation; COX2, cyclooxygenase 2; HA, hemagglutinin; HEK, human embryonic kidney; IRAK, IL-1R–associated kinase; IRF, IFN regulatory factor; Mal, MyD88 adaptor-like; MEF, murine embryonic fibroblast; MK, MAPK kinase; MSK, mitogen- and stress-activated protein kinase; Pam_2Cys, Pam_2Cys-Ser-(Lys)4; PKA, protein kinase A; PRR, pattern-recognition receptor; Rp-8, Rp-8-CPT-cAMP; shRNA, short hairpin RNA; siRNA, small interfering RNA; TAK1, TGF-β–associated protein kinase 1; TIR, Toll/IL-1R; TRAF6, TNFR-associated factor 6; TRAM, Toll/IL-1R domain-containing adaptor-inducing IFN-β–related adaptor molecule; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β; WT, wild-type.

pathway is also a key activator of IRF transcription factors, and the relative roles of the various TIR adaptors in driving activation of NF-κB and IRF transcription factors has been investigated intensively (23). However, TLRs can also trigger activation of other transcription factors, and one that is receiving increasing attention is CREB.

CREB represents the key transcription factor that mediates regulation of gene expression in response to the second messenger cAMP (24). Stimuli that elevate the latter can activate cAMP-dependent protein kinase A (PKA) that in turn will phosphorylate CREB at Ser-133 and thus increase the transactivation potential of CREB. Various TLRs can also trigger Ser-133 phosphorylation of CREB, and in association with agents that elevate cAMP, this can lead to increased CREB-mediated transcription (25, 26). CREB-responsive genes include proinflammatory ones such as TNF (27, 28) and cyclooxygenase 2 (COX2) (29) and anti-inflammatory genes such as IL-10 (30). Although some studies have identified p38 MAPK and its substrate kinases mitogen- and stress-activated protein kinases (MSKs) (31, 32) and MAPK-activated protein kinases (MKs) (9, 33, 34) as immediately upstream of CREB, there is currently no detail on the role of TLR proximal molecules such as the TIR adaptors in regulation of the CREB pathway. To this end, we sought to assess the importance of each of the TIR adaptors in mediating TLR-induced activation of CREB. We demonstrate a key role for Mal in mediating TLR2 and TLR4 activation of CREB and induction of CREB-responsive genes. We also dissect the downstream effectors of Mal that mediate activation of CREB and reveal a signaling axis involving Pellino3, TRAF6, p38 MAPK, and MK2. Thus, to our knowledge, this study represents the first detailed delineation of the signaling cascade that is used by TLRs in regulation of CREB.

Materials and Methods

Plasmids and reagents

Y86A Mal-hemagglutinin (HA) and Y187A Mal-HA were as described previously (35). Mal E190A was created using Pfu Turbo (Stratagene, La Jolla, CA) and the following primers: 5′-AGCTGCCTACCCACCTGAGCTCCGATTCATGTACT-3′ and 5′-AGTACATGAATCGGAGCTCAGGTGGGTAGGCAGCT-3′. MyD88-Flag was provided by Dr. M. Muzio (Mario Negri Institute, Milan, Italy). TRIF-Flag and TRAM-Flag were gifts from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). TRAF6 was from Tularik (San Francisco, CA). TK *Renilla*-luciferase reporter construct (phRL-TK) was from Promega (Madison, WI). CREB-luciferase was from BD Biosciences (San Jose, CA). MK2-DsRed expression plasmid was as described previously (9). MSCV-IRES-GFP and MSCV encoding HA-tagged mMal were provided by Dr. S. Miggin (National University of Ireland Maynooth). cAMP, H89, and PGE_2 were from Sigma-Aldrich (St. Louis, MO). MK2 inhibitor, SB203580, and SP600125 were from Calbiochem. Rp-8-CPT-cAMPS (Rp-8) was from BioLog (Life Science Institute, Bremen, Germany). Pam_2Cys-Ser-(Lys)4 (Pam_2Cys) was from InvivoGen (San Diego, CA), and LPS *Escherichia coli* serotype EH100 was from Alexis.

Anti–phospho-CREB, anti-CREB, anti–phospho-p38, and anti-p38 Abs were from Cell Signaling Technology (Danvers, MA). Anti–β-actin Ab was supplied by Sigma-Aldrich. The anti-COX2 Ab was from Thermo Fischer Scientific (Fremont, CA). Anti-TRAF6 Ab was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-HA Ab was from Covance (Princeton, NJ).

Cell culture

The human embryonic kidney (HEK)293 cells engineered to stably express TLR4 receptor, wild-type (WT) murine embryonic fibroblasts (MEFs), and TRAF6-deficient MEFs (gifts from Prof. A. Bowie, Trinity College Dublin, Dublin, Ireland) were maintained in DMEM, which was supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. G418 (500 μg/ml) was used to select for the stably transfected TLR4 cell line. WT bone marrow-derived macrophages (BMDMs), Mal-deficient, MyD88-deficient, TRIF-deficient, and TRAM-deficient BMDMs (gifts from Dr. K. Fitzgerald, University of Massachusetts Medical School) were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) FBS, and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Cells were maintained in a 37°C humidified atmosphere with 5% CO_2.

Lentiviral production and transduction

HEK293 T cells were seeded (2×10^5 cells/ml; 3 ml) in 6-well plates and grown for 24 h to ~80% confluency. The cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with the packaging plasmid pCMV-dR 8.91 (900 ng), envelope plasmid VSV-G (100 ng), sh-pLKO.1 vector (1 μg), and Pellino3 short hairpin RNA (shRNA) or control shRNA (Sigma-Aldrich): Pellino3 shRNA 5′-GCACTTGCTGATAGCCACTAT-3′. The control shRNA is a nontargeting shRNA vector that encodes for shRNA that does not match to any known human or mouse gene. The conditioned medium was changed 24 h post-transfection and replaced with fresh growth medium containing 30% (v/v) FBS. The cells were then incubated for an additional 24 h. The lentivirus-containing medium was harvested and stored at −20°C. Fresh growth medium containing 30% (v/v) FBS was again added to cells and incubated for another 24 h. The virus-containing medium was again harvested and stored for use. U373 cells were seeded (2×10^5 cells/ml; 3 ml) in 6-well plates and grown for 24 h. The growth medium was then removed and replaced with fresh medium containing polybrene (8 μg/ml) and 600 μl lentivirus-containing medium from above. The plates were incubated at 37°C for 24 h, after which the medium was removed and replaced with fresh growth medium containing puromycin (5 μg/ml) to select for cells showing integration of shRNA constructs. Prior to experiments, cells were selected for another 2–3 wk in the presence of puromycin to continue selection of cells showing stable integration of shRNA constructs.

Retroviral production and transduction

HEK293 T cells were seeded (2×10^5 cells/ml; 3ml) in 6-well plates and grown for 24 h to ~80% confluency. The cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with the packaging vector ψ (1 μg) and MSCV plasmid (1 μg) or MSCV-mMal (1 μg). The cells were then incubated for an additional 24 h. The retrovirus-containing medium was harvested and stored at −20°C. Fresh growth medium containing 30% (v/v) FBS was added to cells and incubated for another 24 h. The virus-containing medium was again harvested and stored for use. WT MEFs and TRAF6-deficient MEFs were seeded (2×10^5 cells/ml; 6 ml) in 25-cm^2 flasks and grown for 4 h. The growth medium was then removed and replaced with fresh medium containing polybrene (8 μg/ml) and 1 ml retrovirus-containing medium from above. The flasks were incubated at 37°C for 48 h prior to experiments.

Quantitative real-time PCR

U373 cells stably transfected with control or Pellino3 shRNA were seeded (2×10^5 cells/ml; 3 ml) into 6-well plates and grown for 24 h. Cells were washed in PBS, and RNA was extracted using Tri-Reagent (Sigma-Aldrich). After DNase I digestion, cDNA was generated from normalized RNA using avian myeloblastosis virus reverse transcriptase (Promega). Samples were assayed by quantitative real-time PCR for levels of Pellino3 cDNA using Brilliant SYBR Green QPCR Master mix (Stratagene). PCR was conducted with the CFB-322001G Opticon thermal cycler (Bio-Rad). Reactions were performed using prevalidated primers (Eurofins MWG Operon): forward Pellino3 5′-TGGCCTGATGGATGGACTGA-3′, reverse Pellino3 5′-CAGCACGTTGGACTCGTTTTC-3′, forward HRPT 5′-GGTGAAAAGGACCCCACGAA-3′, and reverse HPRT 5′-GGCGATGTCAATAGGACTCCAGAT-3′.

Transfection and luciferase reporter systems

HEK293 cells stably expressing TLR4 were seeded (1.5×10^5 cells/ml; 200 μl) in 96-well plates and grown for 24 h. Cells were then transfected using Lipofectamine 2000 transfection reagent, with NF-κB firefly luciferase reporter construct (80 ng) or CREB-regulated firefly luciferase (80 ng), constitutively expressed TK *Renilla*-luciferase reporter construct (phRL-TK) (40 ng) (Promega Biosciences) and varying amounts of expression constructs. Total DNA was kept constant (220 ng/well) using the appropriate empty vector. The activation of the p38 MAPK pathway was assessed by performing similar transfections using the *trans*-activator plasmid pFA-CHOP (1 ng; Stratagene) with firefly luciferase reporter plasmid pFR-Luc (60 ng), phRL-TK (20 ng), and varying amounts of the expression constructs. Cell extracts were generated 24 h post-transfection using Reporter Lysis Buffer (Promega Biosciences), and extracts were assayed for firefly luciferase and *Renilla*-luciferase activity using the Luciferase Assay system (Promega Biosciences) and coelenterazine (0.1 μg/

ml Insight Biotechnology), respectively. Luminescence was monitored with the Glomax microplate luminometer (Promega).

Western blot analysis

BMDMs, MEFs (5×10^5 cells/ml), or HEK293-TLR4 cells (2×10^5 cells/ml) were seeded in 6-well plates and grown for 24 h. In some experiments, cells were transfected, using Lipofectamine, with small interfering RNA (siRNA) targeting Pellino3 (sense sequence: 5′-GCACAGCAUCUCGUAUACATT-3′) or with control siRNA targeting lamin (20 nM) (Ambion). Cells were stimulated with various ligands for different time points as indicated in the figure legends. Cells were washed in 1 ml ice-cold PBS and lysed in 100 μl Nonidet P-40 lysis buffer (50 mM Tris-HCl [pH 7.5] containing 150 mM NaCl, 0.5% [w/v] igepal, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, and protease inhibitor mixture [leupeptin (25 μg/ml), aprotinin (25 μg/ml), benzamidine (1 mM), and trypsin inhibitor (10 μg/ml)]). The cell lysates were centrifuged at 12,000 × *g* for 10 min, supernatants were collected, and samples containing equal protein concentrations were generated using 4× sample loading buffer (0.125 M Tris-HCl [pH 6.8] containing 20% [w/v] glycerol, 4% [w/v] SDS, 1.4 M 2-ME, and 0.0025% [w/v] bromophenol blue). Samples were then resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed for immunoreactivity using anti–phospho-CREB, phospho-p38, phospho-p65, COX2, CREB, p38, α-tubulin–, or β-actin–specific Abs. Immunoreactive bands were detected using the Odyssey Infrared Imaging System from LI-COR Biosciences, according to the instructions of the manufacturer.

ELISA-based detection of IL-10

BMDMs were seeded (3×10^5 cells/ml; 200 μl) in 96-well plates and grown for 24 h. Cells were subsequently stimulated with LPS (100 ng/ml) for 24 h and/or pretreated with various inhibitors as indicated. Conditioned medium was collected from each well and measured for levels of IL-10 by sandwich ELISA (using mouse IL-10 DuoSet kits [R&D Systems]).

EMSA

BMDMs were grown in 6-well plates for 24 h. Cells were then stimulated with LPS or cAMP for various times. Nuclear extracts were generated as described previously (36). Nuclear protein (10 μg) was incubated with LI-COR IRDye 700-labeled oligonucleotide containing the CREB binding site 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ (underlined nucleotides constitute the binding site) according to the manufacturer's instructions. For supershift analysis, CREB polyclonal Ab (1 μg) or nonimmune IgG was added to the extracts and chilled for 1 h on ice prior to incubation with labeled oligonucleotide. All incubations were subjected to electrophoresis on a 4% native polyacrylamide gel for 2 h at 110 V and subsequently analyzed, and images were captured using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Chromatin immunoprecipitation assay

BMDMs cells were grown to confluency in 90-mm dishes and stimulated with LPS (100 ng/ml) for 30 min and 4 h. Chromatin immunoprecipitation (ChIP) assays were performed as previously described with some modifications (37). Cells were cross-linked with 1% formaldehyde for 10 min at 37°C. Isolated nuclei were subjected to four 12-s sonication pulses from a Bandelin Sonoplus HD2200 at 50% of the total power. This procedure yielded chromatin fragments of 200–1000 bp. Chromatin preparation aliquots were incubated overnight at 4°C with anti–phospho-CREB Ab (R&D Systems) or rabbit IgG. The immunoprecipitate was then incubated with protein A-agarose beads (Upstate Biotechnology, Waltham, MA) and the Ab/protein/DNA/beads complex was washed and collected for subsequent reverse cross-linking by overnight incubation at 65°C. DNA was then extracted using the QIAquick purification kit (Qiagen), according to the manufacturer's instructions. Standard PCR was conducted with specific primers designed for both IL-10 and COX2 promoter as follows: IL-10, 5′-CAGAAGTTCATTCCGACCAGT-3′ and 5′-CCTTCCTGGCAAAGGTTTTT-3′; and COX2, 5′-TTGGGGAAAGCCTAAGCGGAA-3′ and 5′-CAGAGTCCTGACTGACTCCT-3′. PCR products were then analyzed by 2% agarose gel electrophoresis.

Confocal microscopy

HEK293 TLR4 cells were seeded (2×10^5 cells/ml) in 4-well chamber slides (Lab-Tek; Nunc) and grown for 24 h to ~80% confluency. Cells were transfected using Lipofectamine 2000 with Mal-CFP and/or MK2-dsRed (400 ng). Total DNA was kept constant (800 ng/well) using the appropriate empty vector. Medium was removed, and the cells were gently washed three times in chilled PBS (500 μl). Cells were then fixed by addition of 4% (v/v) paraformaldehyde (500 μl) for 20 min. Cells were washed three times with PBS. An aliquot (500 μl) of DAPI (1.5 μg/ml) in water was added to each well for 1 min. Cells were mounted with Vectashield hard-set mounting medium (Molecular Probes). Confocal images were captured using the ×63 objective lens on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets. Acquired images were analyzed using the LSM 5 browser imaging software.

Statistical analysis

All data are expressed relative to untreated cells and are the means ± SEM of triplicate determinations from three independent experiments. For comparison between two groups, one-way ANOVA with the Neuman-Keuls post hoc test was used. A p value <0.05 was considered significant.

Results

Overexpression of Mal enhances CREB activation

The roles of TIR adaptors in triggering activation of NF-κB and IRF transcription factors have been well characterized, but their involvement in regulating CREB has not been addressed. We sought to address this deficiency in our understanding of TLR signaling pathways and our initial studies investigated the ability of each of the TIR adaptors to activate CREB. Thus, HEK293 cells were transfected with varying amounts of expression constructs encoding MyD88, Mal, TRIF, or TRAM and were assessed for their potential to induce expression of a cotransfected CREB-regulated reporter (luciferase) gene. The expression of the adaptors was confirmed by immunoblotting, but all four adaptors failed to show a significant induction of CREB-regulated luciferase (Fig. 1*A*). Given that previous studies have shown that TLR ligands such as LPS can only increase CREB-mediated transcription in conjunction with stimuli that elevate cAMP and activate PKA (25, 26), we examined the potential of each of the adaptors to induce the expression of the CREB reporter gene in the presence of coexpressed PKA (Fig. 1*B*). Although PKA alone was sufficient to activate CREB activation, this was greatly enhanced when PKA was coexpressed with Mal. Although TRAM also synergized with PKA in activating CREB, its efficacy was only half of that displayed by Mal. In contrast, MyD88 and TRIF failed to show any statistically significant synergy with PKA. The augmentation of PKA-induced activation of CREB by Mal was further characterized by demonstrating the dose dependent nature of this response (Fig. 1*C*).

Mal mediates TLR2- and TLR4-induced activation of CREB

The above overexpression studies showed that Mal is capable of enhancing the activation of CREB, and this prompted an investigation into the physiological role of Mal as a mediator of TLR-induced activation of CREB. Thus, WT, MyD88-deficient, Mal-deficient, TRIF-deficient, and TRAM-deficient BMDMs were treated with LPS (100 ng/ml) and cAMP (100 μM) for 30 min, and activation of CREB was initially determined by assaying CREB binding to DNA by EMSA (Fig. 2*A*). LPS, alone or in combination with cAMP, induced the binding of CREB to DNA in WT cells. The presence of CREB as part of a complex with DNA was confirmed by an upward shift in the mobility of the complex in the presence of an anti-CREB Ab. In Mal-deficient BMDMs, LPS failed to induce the formation of this CREB–DNA complex consistent with a role for Mal in mediating LPS-induced activation of CREB. In contrast, LPS stimulated CREB binding to DNA in BMDMs lacking MyD88, TRIF, or TRAM, indicating that LPS-induced activation of CREB can occur in a manner independent of these adaptors. Given that a prerequisite for activation of CREB is the phosphorylation of Ser-133, we also characterized LPS-induced phosphorylation of Ser-133 in the various BMDM cell

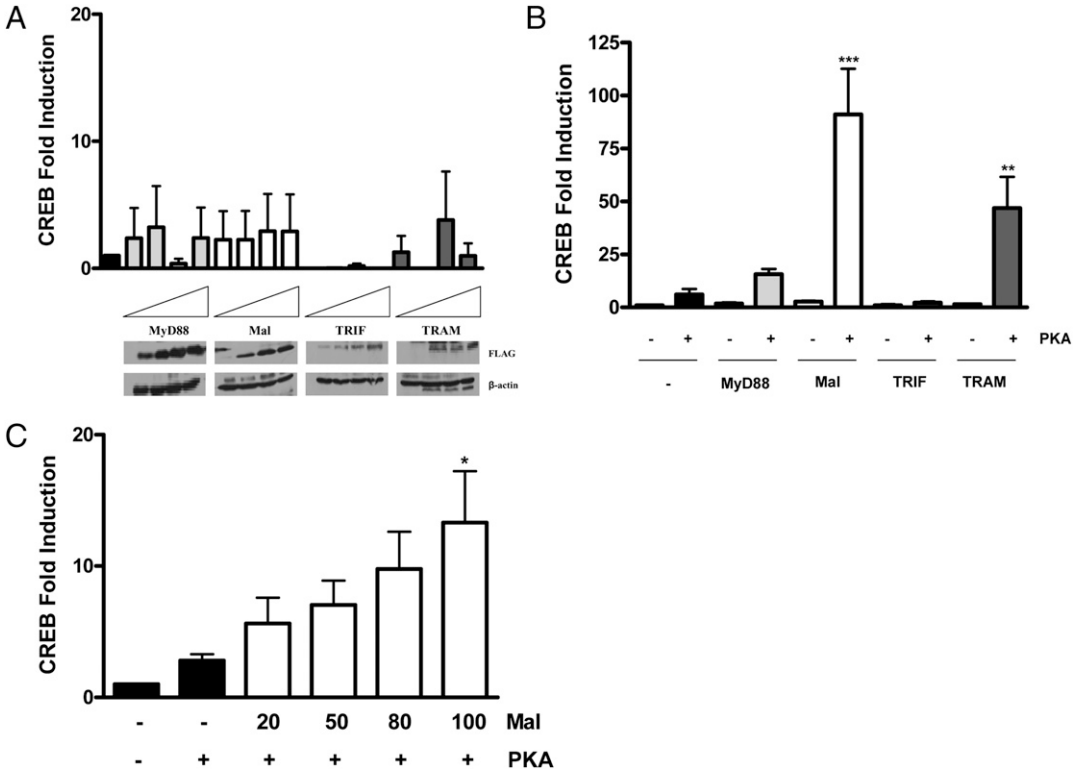

FIGURE 1. Overexpression of Mal enhances CREB activation. HEK293 cells stably transfected with TLR4 were cotransfected with plasmids encoding CREB-regulated firefly luciferase (80 ng), constitutively expressed TK Renilla luciferase (40 ng) and MyD88, Mal, TRIF, and TRAM (0, 20, 50, 80, and 100 ng) (*A*), MyD88, Mal, TRIF, and TRAM (50 ng) in the presence or absence of PKA (0.1 ng) (*B*), and Mal (0–100 ng) with or without PKA (0.1 ng) (*C*). Empty vector pcDNA3.1 was used to normalize the amount of total DNA transfected. Transfected cells were harvested 24 h post-transfection. Cell lysates were assayed for firefly luciferase activity and normalized for transfection efficiency using *Renilla* luciferase activity. Data are presented as the mean ± SEM of three independent experiments. Results were subjected to one-way ANOVA statistical analysis with Neuman-Keuls post hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$); cells transfected with PKA versus cells cotransfected with PKA and adaptors.

lines to further confirm the varying roles of the adaptors in the CREB pathway. The adaptor-deficient BMDM cell lines were stimulated with LPS (100 ng/ml) over various times, and the phosphorylation status of CREB was examined by immunoblotting using a phospho–Ser-133 specific Ab (Fig. 2*B*). In WT cells, LPS-induced phosphorylation of CREB was detectable after 5 min and was maintained for up to 1 h. The phospho–Ser-133 Ab detects two bands, with the slower mobility one representing phospho-CREB and the faster one corresponding to phosphorylated ATF-1, a close relative of CREB. In contrast to WT cells, LPS fails to induce phosphorylation of CREB in Mal-deficient BMDMs, whereas LPS-induced phosphorylation of CREB was still evident in MyD88-, TRIF-, and TRAM-deficient cells (Fig. 2*B*). We also assessed the role of Mal in cosignaling of LPS and a physiological stimulus that elevates levels of cAMP. For the latter purpose, we used the PG PGE_2 because it is induced by LPS, and thus, the simultaneous presence of LPS and PGE_2 is a likely scenario in an inflammatory setting. LPS and PGE_2 showed strong synergy in inducing phosphorylation of CREB, and this is lost in Mal-deficient cells (Fig. 2*C*). These findings are all consistent with a key role for Mal in mediating LPS-induced activation of CREB.

Because Mal is also required for TLR2 signaling, immortalized WT and Mal-deficient BMDMs were also treated with the TLR ligand Pam_2Cys, and lysates were probed for levels of phospho-CREB (Fig. 2*D*). Phosphorylated CREB is detectable after 5 min of Pam_2Cys stimulation and increases further at 15 min before declining at 1 h. BMDMs lacking Mal protein fail to show any Pam_2Cys-induced phosphorylation of CREB, indicating a key role for Mal in TLR2-mediated activation of CREB.

Mal mediates TLR4-induced expression of CREB-responsive genes

The above studies suggest that Mal is a key mediator of CREB activation, and thus, we next examined the role of Mal in regulating expression of CREB-responsive genes. We focused on *il-10* and *cox2* as genes that have been well characterized in terms of being positively regulated by CREB (29, 30). ChIP was used to characterize the in vivo binding of CREB to the promoters of these genes. Stimulation of WT BMDMs with LPS promoted the in vivo binding of CREB to the *il-10* promoter as evidenced by anti-CREB immunoprecipitation of increased levels of the DNA fragment of the *il-10* promoter that contains the CREB-binding motif (Fig. 3*A*). In contrast, LPS fails to induce CREB binding to the *il-10* promoter in Mal-deficient BMDMs. The functional consequence of the Mal-triggered binding of CREB to the *il-10* promoter was explored by measuring the expression levels of IL-10 in response to LPS stimulation in WT and Mal-deficient BMDMs (Fig. 3*B*). WT BMDMs showed a 3.5-fold increase in IL-10 production upon LPS stimulation, and this was modestly enhanced when cells were cotreated with a cell-permeable analog of cAMP. Mal-deficient macrophages showed a substantial impairment in IL-10 production in response to LPS in the absence and presence of cAMP. These findings show that Mal plays a key role in mediating TLR-4–induced binding of CREB to the *il-10* promoter and expression of IL-10.

We also explored the role of Mal in regulating LPS-induced expression of the CREB-responsive gene *cox2*. As with the *il-10* promoter, we initially confirmed the LPS-induced binding of CREB to the *cox2* promoter in WT BMDMs by ChIP analysis

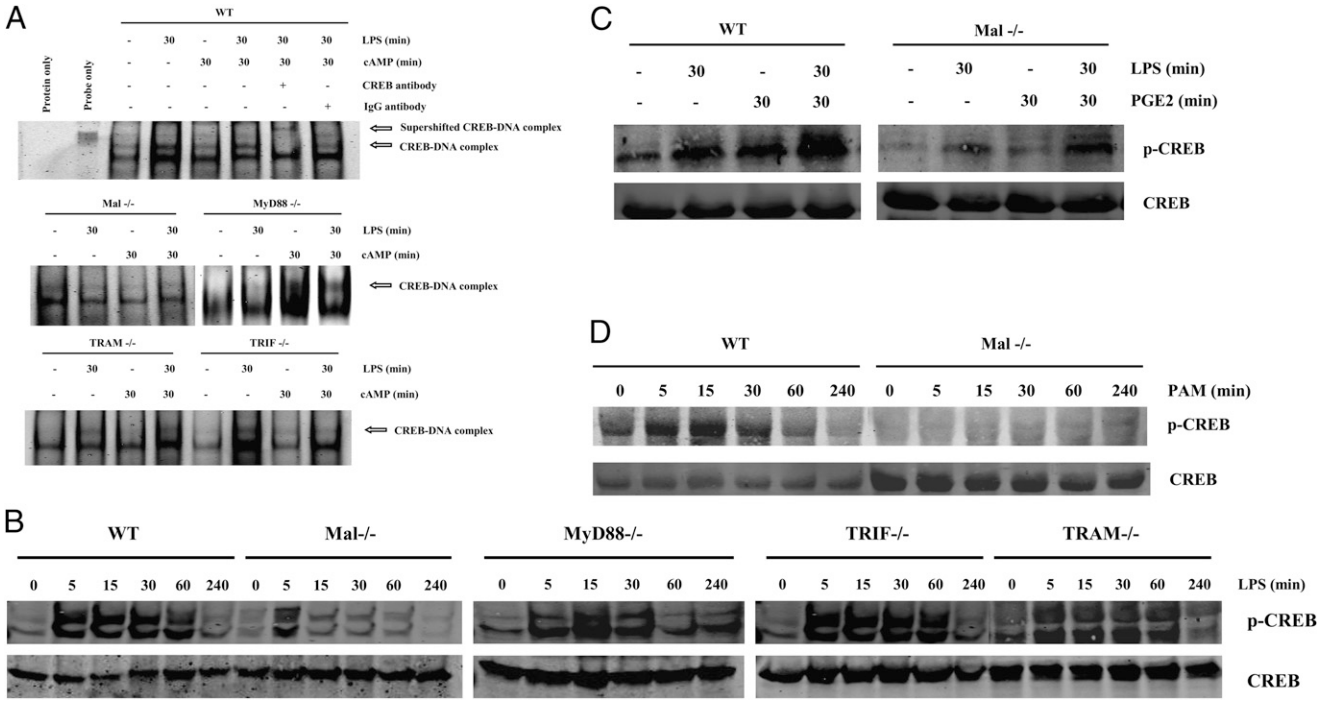

FIGURE 2. Mal mediates TLR2- and TLR4-induced activation of CREB. *A*, Immortalized BMDMs from WT, MyD88-deficient, Mal-deficient, TRIF-deficient, and TRAM-deficient mice were stimulated with LPS (100 ng/ml) and/or cAMP (100 μM) for 30 min. Nuclear extracts (10 μg protein) were generated and assayed for binding to an oligonucleotide containing a consensus CREB-binding motif by EMSA. Nuclear extracts from WT cells stimulated for 30 min with LPS were also preincubated with anti-CREB or nonimmune (IgG) Ab before assaying CREB-DNA–binding activity. *B*, Immortalized BMDMs from WT, MyD88-deficient, Mal-deficient, TRIF-deficient, and TRAM-deficient mice were treated for the indicated times with LPS (100 ng/ml). *C*, WT and Mal-deficient BMDMs were treated with/without LPS (100 ng/ml) and/or PGE_2 (100 ng/ml) for 30 min. *D*, WT and Mal-deficient BMDMs were treated for the indicated times with Pam_2Cys (100 ng/ml). Cell lysates were subjected to SDS-PAGE and analyzed by Western immunoblotting using anti–phospho-CREB and anti-CREB Abs. Data are representative of three independent experiments.

(Fig. 3*C*). This TLR4-induced binding of CREB to the *cox2* promoter was not apparent in Mal-deficient BMDMs. The functional importance of this Mal-induced binding of CREB to the *cox2* promoter was emphasized by the LPS-induced expression of COX2 protein in WT cells being considerably diminished in Mal-deficient cells (Fig. 3*D*). The above findings emphasize the importance of Mal in promoting the expression of CREB-responsive genes.

Pellino3 and TRAF6 are downstream of LPS/Mal in CREB activation pathway

Having confirmed a key role for Mal in mediating activation of CREB and promoting induction of CREB-responsive genes, we next aimed to delineate the downstream signaling molecules used by Mal in effecting activation of CREB. We have previously reported that the E3 ubiquitin ligase Pellino3 strongly activates CREB (9), and given that this activity of Pellino3 is dependent on its association with the Mal-interacting protein IRAK1, we probed the potential role of Pellino3 as a downstream mediator of Mal in its activation of CREB. The ability of Pellino3 to activate CREB was initially confirmed, and there was some further modest enhancement of this activation with a cell-permeable analog of cAMP (Fig. 4*A*). In contrast, a mutant form of Pellino3 that contains point mutations in its RING-like domain (Pellino3S C374A/C377A) proved incapable of activating CREB, demonstrating that the activation of CREB by Pellino3 is dependent on the catalytic E3 ubiquitin ligase activity of the latter. To determine whether Pellino3 is a mediator of TLR-induced activation of CREB, we initially used one of our previously described models in which we suppress endogenous expression of Pellino3 using Pellino3-specific siRNA (9), and we now show siRNA-mediated knockdown of Pellino3 causes a dramatic decrease in LPS-induced phosphorylation of CREB in HEK293-TLR4 cells (Fig. 4*B*). To complement this approach and to directly assess the role of Mal in CREB activation, U373 cell lines were infected with lentivirus containing shRNA constructs that specifically target Pellino3. Cells showing stable integration of the constructs were puromycin selected over a number of weeks, and quantitative real-time PCR was used to confirm knockdown of Pellino3 in these cells (Fig. 4*C*). These cells showed a strong reduction in the ability of Mal to induce expression of a CREB-regulated reporter gene relative to cells that were infected with lentivirus-containing control shRNA (Fig. 4*D*). Pellino3 knockdown cells also displayed greatly reduced responsiveness to LPS and Pam_2Cys in terms of activation of CREB (Fig. 4*E*), consistent with major loss in phosphorylation of CREB (Fig. 4*B*), and taken together, these findings strongly support the proposal that Pellino3 is a mediator of Mal-induced activation of CREB in TLR pathways.

We have previously shown that TRAF6 can act downstream of Pellino3, and thus, we next assessed the role of TRAF6 in mediating activation of CREB. First, overexpression of TRAF6 was sufficient to cause low activation of basal CREB and strong enhancement of cAMP-induced activation of CREB (Fig. 4*F*). Furthermore, whereas LPS stimulated strong phosphorylation of CREB in WT MEFs, this effect was totally absent in TRAF6-deficient MEFs (Fig. 4*G*), demonstrating that TRAF6 is indispensable for TLR4-induced activation of CREB. To directly assess the importance of TRAF6 as a mediator of Mal-induced activation of CREB, WT and TRAF6-deficient MEFs were transduced with retrovirus encoding or lacking murine Mal. Transduction of WT MEFs with retrovirus encoding Mal led to a slight increase in basal phosphorylation of CREB and enhanced

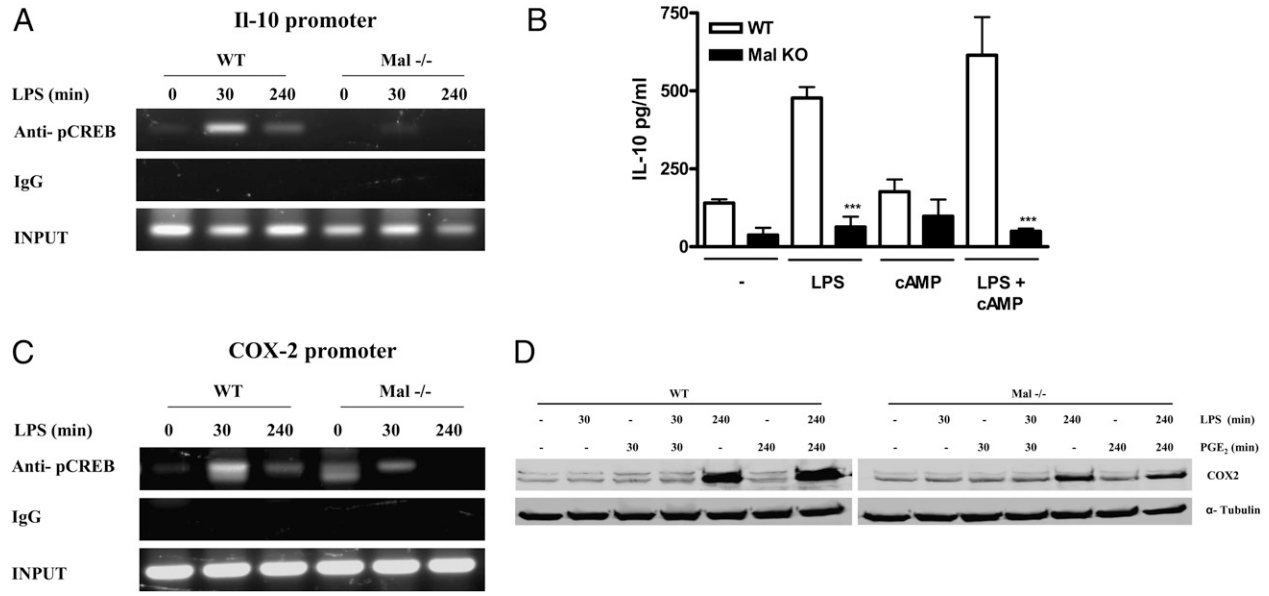

FIGURE 3. Mal mediates TLR4-induced expression of CREB-responsive genes. *A* and *C*, WT and Mal-deficient BMDMs were stimulated with LPS (100 ng/ml) for indicated times. Cells were then fixed in formaldehyde, and sonicated nuclear lysates were immunoprecipitated with an anti–phospho-CREB or rabbit IgG. Input DNA and immunoprecipitated chromatin were analyzed by 37 cycles of PCR with primers specific for promoters of genes encoding IL-10 (*A*) and COX2 (*C*). Results are representative of two independent experiments. *B*, BMDMs from WT and Mal-deficient mice were seeded into 96-well plates and stimulated with LPS (100 ng/ml) and/or cAMP (100 μM) for 24 h. Supernatants were analyzed for IL-10 production using sandwich ELISA. Data are presented as the mean ± SEM of three independent experiments. Results were subjected to one-way ANOVA statistical analysis with Neuman-Keuls post hoc test (*** $p < 0.001$); WT cells treated with ligand(s) versus Mal-deficient cells treated with ligand(s). *D*, WT and Mal-deficient BMDMs were treated with/without LPS (100 ng/ml) and/or PGE_2 (100 ng/ml) for 30 min and 4 h. Cell lysates were subjected to SDS-PAGE and analyzed by Western immunoblotting using anti-COX2 and anti–α-tubulin Abs. Data are representative of three independent experiments.

LPS-induced phosphorylation of CREB (Fig. 4*H*). In contrast, Mal failed to affect basal or LPS-induced phosphorylation of CREB in TRAF6-deficient cells, indicating a key role for TRAF6 as a mediator of Mal-induced activation of CREB. Mal contains a TRAF6-binding motif/activation site (38), and we next explored the effect of mutating this site (E190A) on the ability of Mal to activate CREB. However, the Mal E190A mutant mirrored the ability of WT Mal to activate CREB (Fig. 4*I*). To demonstrate specificity of action for Mal in this assay, mutation of tyrosine residue 88 or 187, critical residues for Mal signaling (35), abolishes the ability of Mal to activate CREB. It is interesting that the E190 mutant is capable of interacting with TRAF6, no longer activates NF-κB (38), but can still activate CREB. This suggests that although Mal can interact with TRAF6 and activate both NF-κB and CREB, the structural features of Mal that are required for these pathways are not identical.

Mal activation of CREB is p38 MAPK dependent

We have previously demonstrated that TRAF6 and p38 MAPK act as downstream mediators of Pellino3-induced activation of CREB (9), and in an effort to further map the signaling cascade used by Mal in activation of CREB, we characterized the role of p38 MAPK in this pathway. We initially probed the role of Mal in mediating LPS-induced activation of p38 MAPK in our BMDM experimental system. The activation of p38 MAPK was assayed by measuring the dual phosphorylaton of the threonine and tyrosine residues in its activation motif. LPS induced strong phosphorylation of p38 MAPK pathway in a time-dependent manner in WT BMDMs, but this was greatly diminished in Mal-deficient cells, demonstrating a key role for Mal in activation of the p38 MAPK pathway (Fig. 5*A*, densitometric analysis shown in the right panel). The role of p38 MAPK as a mediator in activating CREB in response to LPS was next examined. The p38 MAPK inhibitor SB203580 ablated the ability of LPS to induce phosphorylation of CREB, whereas the JNK inhibitor SP600125 was without effect (Fig. 5*B*), demonstrating a specific role for p38 MAPK in activation of CREB. This was further corroborated by a study showing that SB203580 blocked LPS induction of the CREB-responsive protein IL-10, whereas again, SP600125 was ineffective (Fig. 5*C*). Given that the findings above had also implicated a role for TRAF6 in mediating Mal-induced activation of CREB, we also sought to confirm that TRAF6 is an upstream regulator of p38 MAPK in this pathway. Indeed, this is demonstrated by LPS inducing strong time-dependent phosphorylation of p38 MAPK in WT MEFs, whereas this is completely absent in TRAF6-deficient cells (Fig. 5*D*).

Mal-induced activation of CREB is MK2 dependent

We finally aimed to define the downstream effector kinase used by p38 MAPK in mediating Mal-induced activation of CREB. Given that PKA is the classical CREB kinase, we initially assessed the effects of the PKA inhibitor H89 on activation of CREB. Pretreatment of BMDMs with H89 attenuated the LPS induced phosphorylation of CREB (Fig. 6*A*) and also reduced the expression of IL-10 in response to LPS (Fig. 6*B*), suggesting a role for PKA. However, we were mindful that H89 has also been reported to act as an inhibitor of MSK2, a known upstream kinase of CREB, and thus, we also used Rp-8-CPT-cAMPs (Rp-8), a more selective inhibitor of PKA (39). However, Rp-8 failed to affect the ability of LPS to induce phosphorylation of CREB (Fig. 6*C*) or expression of IL-10 (Fig. 6*D*). As a positive control for the inhibitory activity of Rp-8, the latter was shown to cause a dose-dependent inhibition of cAMP-induced activation of CREB (Fig. 6*E*). These findings suggest that the inhibitory effects described above for H89 are not attributable to targeting of PKA but instead more likely represents an inhibitory effect on MSKs.

We also noted that the inhibitory effects of H89 on LPS-induced activation of CREB and induction of IL-10 were incomplete,

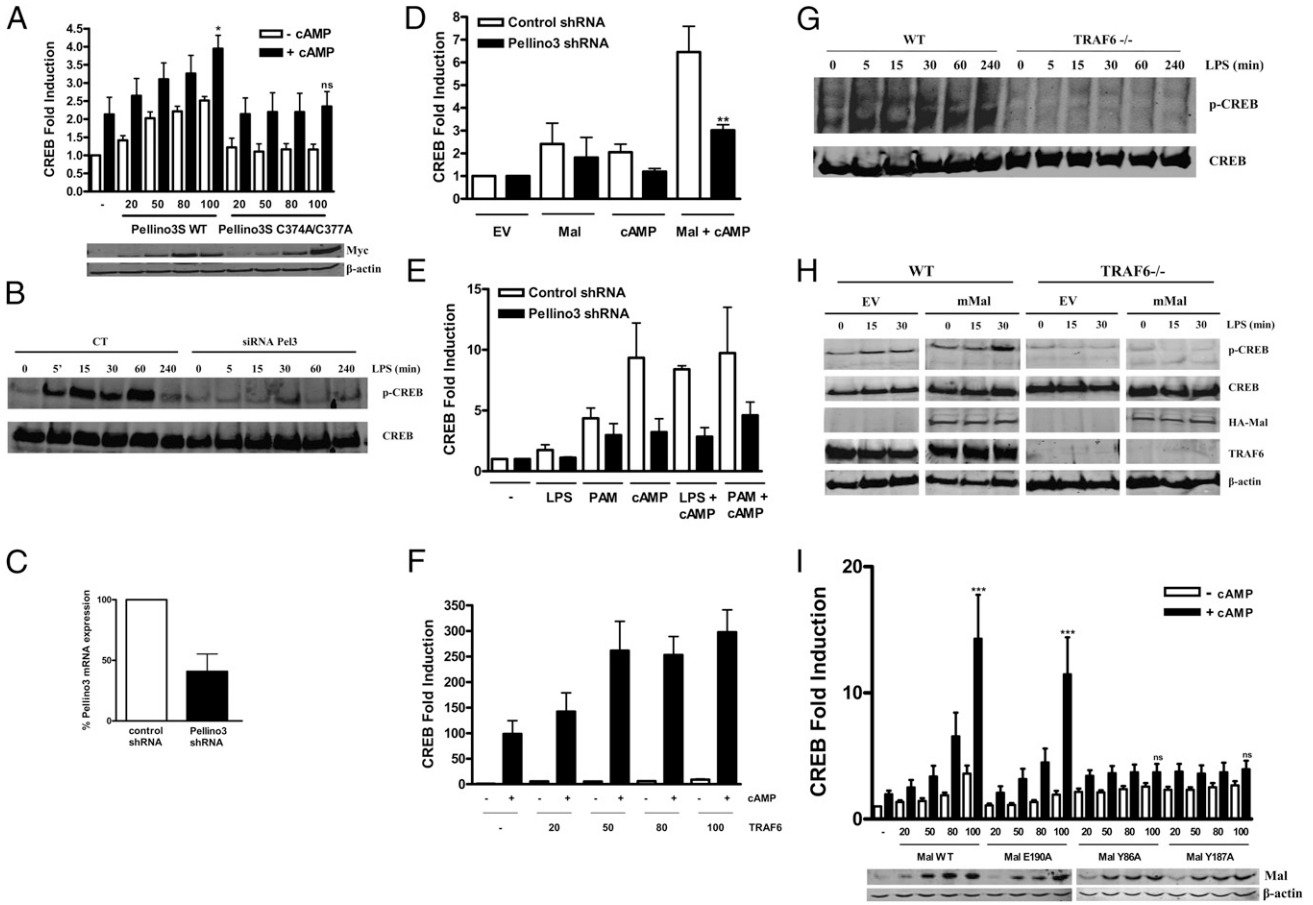

FIGURE 4. Pellino3 and TRAF6 are downstream effectors of LPS/Mal induced activation of CREB. *A*, HEK293 TLR4 cells were cotransfected with CREB-regulated firefly luciferase (80 ng), TK Renilla luciferase (40 ng), and a construct encoding Pellino3S or Pellino3S C374A/C377A (0–100 ng). Cell lysates were subsequently assessed for normalized firefly luciferase activity. *B*, HEK293 TLR4 cells were transfected with Pellino3-specific siRNA or control Lamin siRNA (CT) (20 μM) and incubated at 37˚C for 48 h. Cells were treated with LPS (100 ng/ml) for indicated times, and cell lysates were generated and subjected to Western immunoblotting using anti–phospho-CREB and anti-CREB Abs. *C*, U373 cells were stably transduced with control or Pellino3-specific shRNA. RNA was extracted, and cDNA was generated and assayed for Pellino3 expression using quantitative PCR. Data represent mean ± SEM of two independent experiments. *D* and *E*, U373 cells, stably transduced with control or Pellino3-specific shRNA, were cotransfected with CREB-regulated firefly luciferase (80 ng) or TK Renilla luciferase (40 ng) in the absence or presence of Mal (50 ng). Cells were treated, as indicated, with cAMP (100 μM), LPS (100 ng/ml), or Pam_2Cys (100 ng/ml) for 6 h, after which lysates were assayed for normalized firefly luciferase activity. *F* and *I*, HEK293 TLR4 cells were cotransfected with TK Renilla luciferase (40 ng), CREB-regulated firefly luciferase (80 ng), and TRAF6 (0–100 ng) (*F*) or Mal, MalE190A, MalY86A, or MalY187A (0–100 ng) (*I*) with and without cAMP treatment. Cell lysates were subsequently assessed for normalized firefly luciferase activity. *G*, MEFs from WT and TRAF6-deficient mice were seeded in 6-well plates (5×10^5 cells/ml) and grown for 24 h or were first transduced with a MSCV murine retroviral plasmid or MSCV encoding mMal-HA for 48 h before seeding in 6-well plates (*H*). Cells were then stimulated with LPS (100 ng/ml) for the indicated time periods. Cell lysates were generated and subjected to Western blotting using anti–phospho-CREB, anti-CREB, anti-HA, anti-TRAF6, and anti–β-actin Abs. Results are indicative of three independent experiments. All luciferase data represent mean ± SEM of three independent experiments (except *E*, which is the mean ± SEM of two independent experiments). Results were subjected to one-way ANOVA statistical analysis with Neuman-Keuls post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, nonsignificant); cells treated with cAMP versus cells treated with cAMP and transfected with Pellino3S or Pellino3S C374A/C377A (*A*); cells stably expressing control shRNA versus cells stably expressing Pellino3 shRNA both transfected with Mal and treated with cAMP (*D*); and cells treated with cAMP versus cells treated with cAMP and transfected with Mal, MalE190A, MalY86A, or MalY187A (*I*).

suggesting a contributory role for other kinases in addition to MSKs. MKs have also been described as CREB kinases (40), and indeed, we have previously shown that Pellino3 can activate MK2 in a p38 MAPK-dependent manner (9). The availability of a specific MK2 inhibitor facilitated an assessment of the potential role of MK2 in a mediator in the present pathways. Indeed, the MK2 inhibitor showed some inhibitory effects on LPS-induced phosphorylation of CREB (Fig. 6*F*) and expression of IL-10 (Fig. 6*G*), suggesting a role for MK2 in this CREB pathway. However, it was necessary to show that Mal was capable of stimulating activation of MK2. The latter is a known substrate of p38 MAPK and in unstimulated cells is found primarily in the nucleus because of a functional nuclear localization signal and masking of its nuclear export signal (41, 42). However, active p38 MAPK can phosphorylate MK2, which exposes the nuclear export signal leading to the shuttling of the p38/MK2/MK3 complex to the cytoplasm. Thus, as an index of MK2 activation, we sought to probe whether Mal could promote nuclear-cytoplasmic shuttling of MK2. HEK293 cells were transfected with MK2-DsRed (previously described in Ref. 9) in the absence or presence of Mal-CFP, and the subcellular localization of the proteins was examined using confocal microscopy (Fig. 6*H–J*). The expression of Mal-CFP (shown as green to differentiate from DAPI) manifested as very distinct staining at the plasma membrane in the absence (Fig. 6*H*) or presence of MK2 (Fig. 6*J*). However, when transfected alone, MK2-DsRed was found predominantly in the nuclei of cells (Fig.

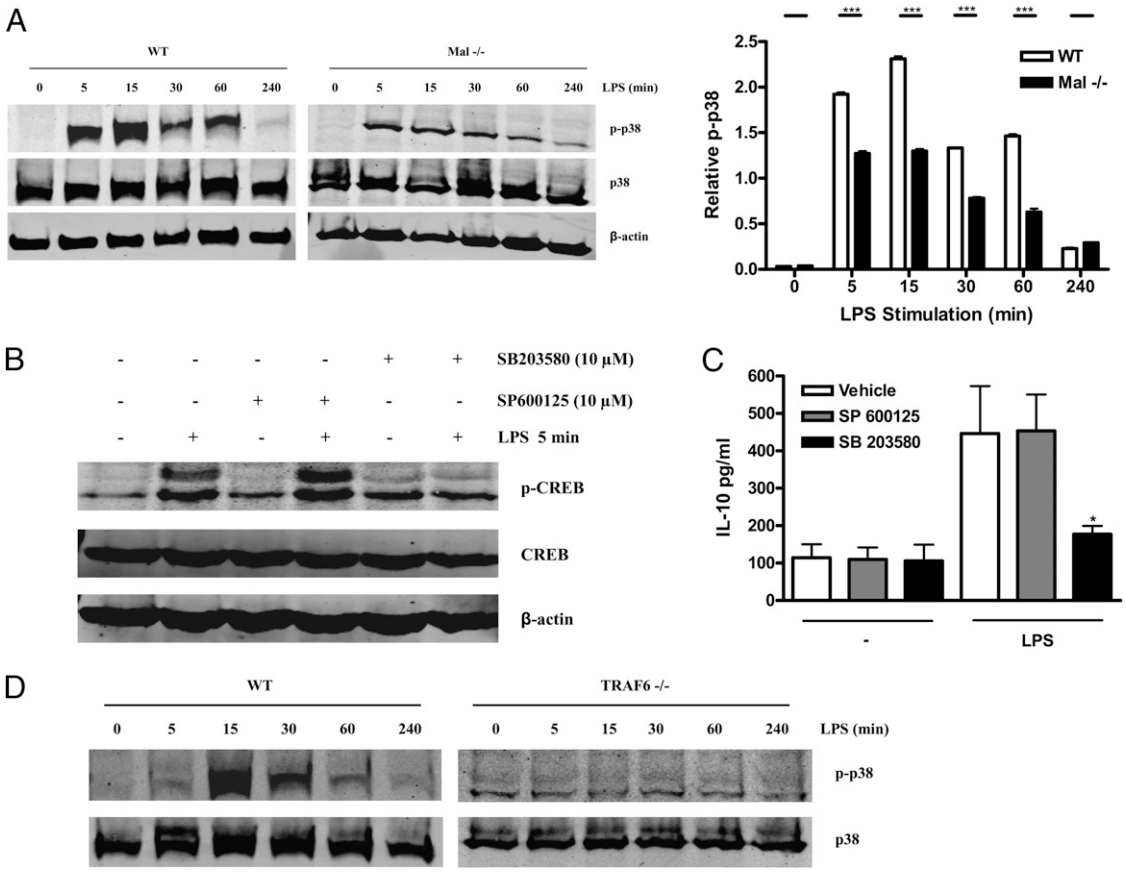

FIGURE 5. LPS/Mal induced activation of CREB is p38 MAPK dependent. *A*, Immortalized BMDMs from WT and Mal-deficient mice were treated with or without LPS (100 ng/ml) for the indicated time periods. Cell lysates were collected and subsequently subjected to Western immunoblotting using anti–phospho-p38, anti-p38, and β-actin Abs. Densitometric analysis (*right panel*) was used to calculate intensity of phospho-p38 staining (normalized to total p38), and the data are represented relative to unstimulated cells. *B* and *C*, Immortalized BMDMs from WT mice were pretreated for 30 min with SB203580 (10 μM) or SP600125 (10 μM) and then stimulated with LPS (100 ng) for indicated times or 24 h (*C*). *B*, Cell lysates were collected and subjected to Western immunoblotting analysis using anti–phospho-CREB, anti-CREB, and anti–β-actin Abs. *C*, Conditioned media from cells were assayed for IL-10 levels by sandwich ELISA. Data are presented as the mean ± SEM of three independent experiments and subjected to one-way ANOVA statistical analysis with Neuman-Keuls post hoc test (*$p < 0.05$, LPS-treated cells versus LPS-treated cells pretreated with SB203580). *D*, MEFs from WT or TRAF6-deficient mice were treated with or without LPS (100 ng/ml) for the indicated time periods. Cell lysates were collected and subsequently subjected to Western immunoblotting using anti–phospho-p38, anti-p38, and β-actin Abs. Data are representative of three experiments.

6*I*), whereas it redistributed to the cytoplasm in those cells showing coexpression of Mal-CFP (Fig. 6*J*). This demonstrates that Mal is capable of promoting downstream activation of MK2 and, in conjunction with the above MK2 inhibitor studies, suggests that MK2 is a likely mediator in Mal-induced activation of CREB.

Discussion

TIR adaptor proteins play key roles in defining the intracellular signaling pathways and gene expression profiles that are triggered by distinct TLRs. One of the challenges to our complete understanding of TLR biology is a lack of appreciation of how varying gene expression patterns can be generated in response to different pathogens and TLRs, and yet, the intracellular signaling pathways tend to be shared extensively by the various TLRs. In addition, all of the signaling pathways that are used by TLRs are ultimately triggered by TIR adaptor molecules, and thus, it is vitally important to define all of their downstream signaling cascades. To date, most research effort has focused on the ability of the adaptors to promote activation of two major families of transcription factors namely NF-κB and IRFs. However, other transcription factors are undoubtedly critical to TLR function, and the current study focused on CREB and the role of TIR adaptors, especially Mal, in regulating its activation.

The initial findings in this study highlighted the predominant role of Mal in promoting activation of CREB. Although the overexpression studies suggest that TRAM may also be capable of activating CREB, this may not be relevant in a physiological setting because LPS can still induce activation of CREB in cells lacking TRAM. In the case of Mal, it strongly enhances activation of CREB, and it is essential for LPS-induced activation of CREB because cells that lack Mal are unresponsive to LPS in terms of CREB activation. Interestingly, Mal alone is a very weak activator of CREB and requires a stimulus that can increase levels of cAMP and/or activate PKA. This is hardly surprising because it is already known that LPS can only induce activation of CREB and increase its transactivation potential in the presence of a cAMP stimulus (25, 26). In a physiological setting, there are multiple G protein-coupled receptors that can enhance levels of cAMP and potentially integrate with TLR signaling, and some of the lead candidates that may fill this role in an inflammatory setting are PGs. The latter are synthesized in response to inflammatory stimuli such as LPS, and this process is generally facilitated by the induction of the enzyme COX2, a key component in the biosynthetic pathway leading to PG production. Indeed, in the current study, we show a critical role for Mal in mediating LPS-induced expression of COX2. Thus, the Mal-induced CREB pathway described in the current study may

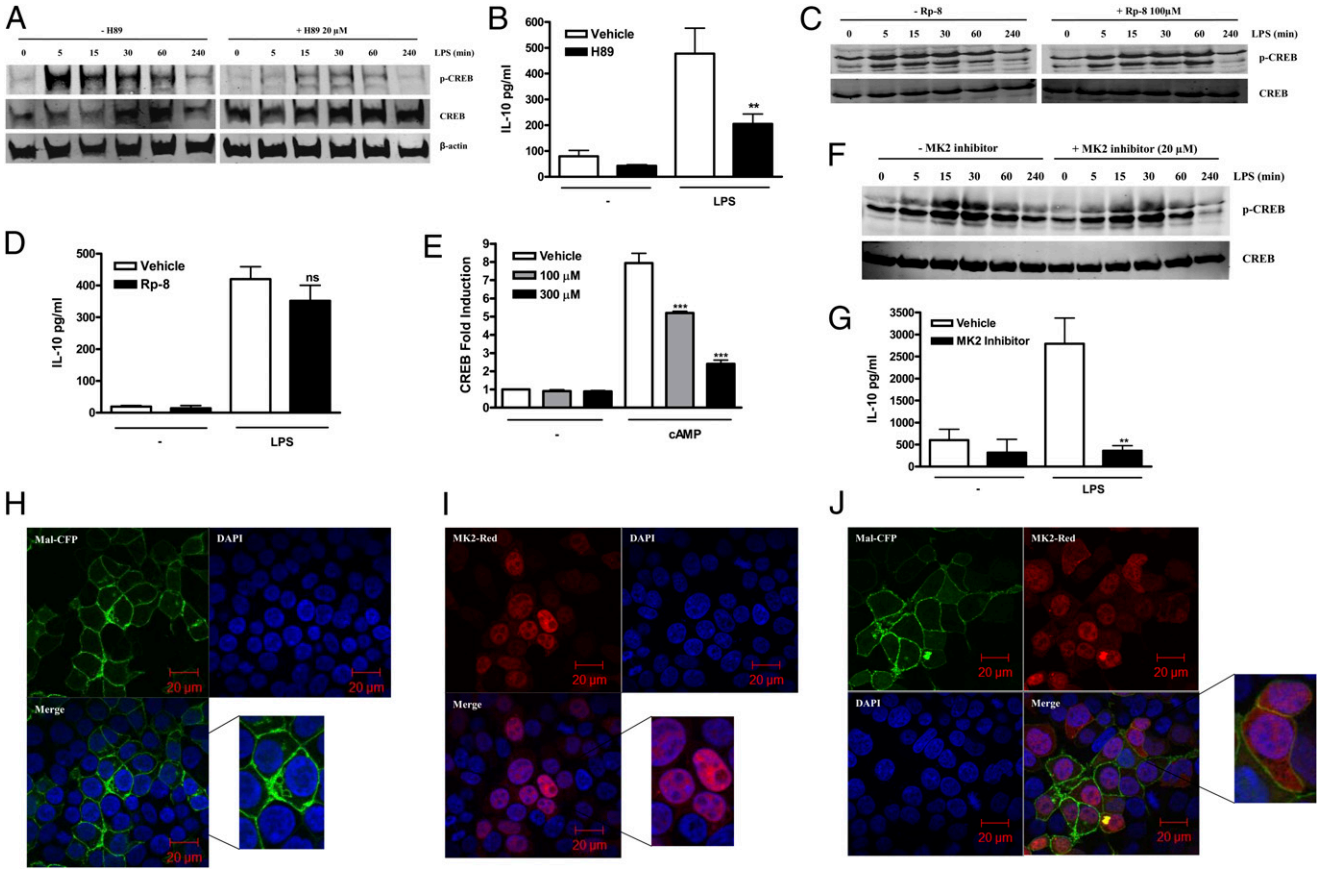

FIGURE 6. Mal-induced activation of CREB is MK2 dependent. *A–D*, *F*, and *G*, Immortalized BMDMs from WT mice were pretreated with H89 (20 μM) (*A*, *B*) for 30 min Rp-8-CPT-cAMPS (100 μM) (*C*, *D*) for 30 min, or MK2 inhibitor (20 μM) (*F*, *G*) for 1 h and then stimulated with LPS (100 ng/ml) for the indicated time periods or 24 h (*B*, *D*, *G*). *A*, *C*, and *F*, Cell lysates were collected and subjected to Western immunoblotting using anti–phospho-CREB, anti-CREB, and anti–β-actin Abs. *B*, *D*, and *G*, Conditioned media from cells were assayed for IL-10 levels by sandwich ELISA. *E*, HEK293 TLR4 cells were transfected with CREB-regulated firefly luciferase (80 ng) and TK Renilla luciferase (40 ng). Transfected cells were left overnight and then pretreated with/without Rp-8-CPT-cAMPS (100 or 300 μM) for 30 min and then stimulated with cAMP (100 μM) for 6 h. Cell lysates were assayed for firefly luciferase activity and normalized for transfection efficiency using *Renilla* luciferase activity. *H–J*, HEK293 cells were transfected with Mal-CFP (*H*), MK 2-DsRed (*I*), or Mal-CFP and MK2-DsRed (*J*). Confocal images were captured using the ×63 objective (oil immersion) on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets and analyzed using the LSM 5 browser imaging software. DAPI staining of the nuclei is also included. Scale bars, 20 μm. All data are representative of three independent experiments. Luciferase and ELISA data are presented as the mean ± SEM of three independent experiments and were subjected to one-way ANOVA statistical analysis with Neuman-Keuls post hoc test (** $p < 0.01$, *** $p < 0.001$, ns, nonsignificant); LPS-treated cells versus LPS-treated cells pretreated with H89 (*B*), Rp-8 (*D*), or MK2 inhibitor (*G*); cAMP-treated cells versus cAMP-treated cells pretreated with 100 or 300 μM Rp-8 (*E*).

facilitate a positive feedback system in dysregulated inflammatory responses where Mal can activate CREB that will induce COX2, and this will lead to PG production. The latter, including molecules such as PGE_2, can then feed back on the cells, promoting increased levels of cAMP and activation of PKA that will then synergize with the Mal pathway and promote further activation of CREB and induction of CREB-responsive genes. Indeed, the current study demonstrates the synergy of LPS and PGE_2 in relation to activation of CREB, and this is lost in Mal-deficient cells.

It is interesting to note that although LPS and Mal are incapable of promoting CREB-mediated transcription in the absence of a cAMP stimulus, they can induce Ser-133 phosphorylation of CREB without a costimulus. This is consistent with the notion that Ser-133 phosphorylation of CREB is insufficient to promote its activation, and it may require additional modifications and/or associations with ancillary proteins (24), and it will be of interest to further explore how Mal may be able to facilitate these modifications and/or associations.

To our knowledge, the present study also highlights for the first time the key role of Mal in regulating the expression of anti-inflammatory genes. To date, most studies on Mal have focused on its role in the induction of proinflammatory genes and more recently on its negative regulatory effects on IFN production (43). We now report that Mal is also a crucial mediator in the induction of the anti-inflammatory protein IL-10 via its ability to trigger activation of CREB. This raises the intriguing notion that Mal may be a crucial modulator in controlling the magnitude of the inflammatory response, and indeed, this is supported by studies showing that some polymorphic forms of Mal can bestow protection to infectious diseases and the autoimmune disease systemic lupus erythematosus (44–46), and it will be of interest to explore the regulation of the CREB pathway by these forms of Mal. The dualist role of Mal in mediating the induction of pro- and anti-inflammatory genes also warrants further investigation into the signaling pathways that are used by Mal in promoting these opposing responses. Although NF-κB is obviously central to its induction of proinflammatory genes, this study offers the CREB pathway as a crucial mediator of its potential to induce anti-inflammatory proteins such as IL-10. Findings from this study also highlight the intriguing notion that the structural requirements

of Mal for activating the CREB pathways may differ from those essential for the NF-κB pathway. Thus, the Mal E190A mutant mimics its WT counterpart in being capable of strongly activating CREB, whereas it no longer activates NF-κB and indeed can act as a dominant negative for the latter. This suggests that it may be possible to target Mal to differentially regulate its pro- and anti-inflammatory effects, and thus, Mal may act as a valuable target to modulate the inflammatory response.

The studies described presently propose a signaling cascade involving Pellino3, TRAF6, p38 MAPK, and MK2 that mediates the Mal-induced activation of CREB. CREB has been reported to act as a substrate for a plethora of kinases (24). Although PKA is the key kinase used in response to elevation of cAMP, in the context of an inflammatory stimulus, the MSKs and MKs are the lead candidates as the immediate upstream CREB kinases. In the current study, we exclude a role for PKA, and our data are consistent with contributory roles for both of the latter classes of kinases. Given that other TIR adaptors such as MyD88 can also trigger activation of these downstream components, it is somewhat surprising that Mal at this stage appears to be unique among the TIR adaptors as a mediator on the CREB pathway. However, this may reflect a situation where Mal may stimulate an additional process that is not promoted by the other adaptors. One can speculate that such a process may facilitate some additional modification of CREB that is required for its activation. Indeed, in addition to phosphorylation, CREB is subject to a variety of post-translational modifications including acetylation, ubiquitination, as well as sumoylation (24), and it will be interesting to assess whether Mal can regulate such processes. Alternatively, Mal may uniquely stimulate a process that facilitates the interaction of CREB with proteins that are essential for its activity. These could include co-activator proteins such as CREB-binding protein (24). Irrespective of the exact mechanism, the current study emphasizes a new role for Mal as a key mediator in TLR-induced activation of CREB. It also suggests that Mal may be an important signaling molecule for dictating the balance of proinflammatory versus anti-inflammatory gene expression and warrants further investigation into how Mal may be targeted to control dysregulated inflammatory responses.

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Disclosures

The authors have no financial conflicts of interest.

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