The NLR Adaptor ASC/PYCARD Regulates DUSP10, Mitogen-activated Protein Kinase (MAPK), and Chemokine Induction Independent of the Inflammasome*1

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ASC/PYCARD is a common adaptor for a diverse set of inflammasomes that activate caspase-1, most prominently the NLR-based inflammasome. Mounting evidence indicates that ASC and these NLRs also elicit non-overlapping functions, but the molecular basis for this difference is unclear. To address this, we performed microarray and network analysis of ASC shRNA knockdown cells. In pathogen-infected cells, an ASC-dependent interaction is centered on the mitogen-activated protein kinase (MAPK) ERK and on multiple chemokines. ASC did not affect the expression of MAPK but affected its phosphorylation by pathogens and Toll-like receptor agonists via suppression of the dual-specificity phosphatase, DUSP10/MKP5. Chemokine induction, DUSP function, and MAPK phosphorylation were independent of caspase-1 and IL-1β. MAPK activation by pathogen was abrogated in Asc−/− but not Nlrp3−/−, Nlrc4−/−, or Casp1−/− macrophages. These results demonstrate a function for ASC that is distinct from the inflammasome in modulating MAPK activity and chemokine expression and further identify DUSP10 as a novel ASC target.

The secretion of pro-inflammatory cytokines and chemokines by macrophages in response to pathogens is an important innate immune event orchestrated by a complex signaling network. Pathogenic signaling leads to the formation of an intracellular protein complex termed the inflammasome. A conventional inflammasome is composed of caspase-1, which promotes cleavage and maturation of the inflammatory cytokines IL-1β and IL-18, ASC4 (apoptotic speck protein, also known as TMS1 or pycard), and a nucleotide-binding domain leucine-rich repeat/NBD-LRR protein (NLR) (1). ASC serves as a bridge between caspase-1 via CARD-CARD interactions, and NLRs via pyrin/pyrin interactions (2, 3). Mutations in several NLRs are associated with inflammatory diseases, underscoring their importance in innate immunity (4, 5). More recently, AIM2 and RIG-1 have also been identified in separate inflammasome complexes that rely on the ASC adaptor (6–10).

Association of specific NLRs within the inflammasome may be dictated by the type and dose of infectious microorganism, whereas ASC is thought to assume a broader role as an NLR adaptor. For example, ASC and Nlr4 each have defined roles for caspase-1 activation and cell death by Salmonella typhimurium (11, 12), whereas ASC and caspase-1 are required for T-cell activation and protective immunity against flu challenge (13), however each of these processes are independent of Nlrp3. Moreover, both Francisella tularensis-mediated IL-1β processing and sensitivity to lethal doses of lipopolysaccharide (LPS) are ASC-dependent, but Nlr4- and Nlrp3-independent (11, 12). The pyrin and HIN domain-containing protein, AIM2, recognizes cytosolic DNA within an NLR-free ASC inflammasome, further supporting a mechanism for ASC independent of NLRs (6–9). Thus, ASC may play a role as a common downstream factor for different sets of NLRs and may also function within an inflammasome complex exclusive of NLR family proteins.

Additional evidence suggests that ASC function extends beyond the NLR/caspase-1 inflammasome. ASC was identified by its unique ability to condense into cytosolic speck structures and induce apoptosis in tumor cell lines (14). It also is silenced in certain cancers (15). Moreover, a caspase-independent type of necrosis induced by high dose Shigella (≥50 multiplicity of infection) (16), Neisseria gonorrhoeae (17), or Porphyromonas gingivalis (Pg) (18) is ASC-dependent. Asc−/− mice exhibit increased susceptibility to Mycobacterium tuberculosis without reduction in IL-1β, implying additional ASC function that is distinct from cytokine cleavage (19). Two recent studies of antigen-induced murine arthritis show dependence on Asc but caspase-1, Nlrp3, and Nlrc4 independence (20, 21). A requirement for Asc, but not Nlrp3 or Caspase-1, was also recently demonstrated for antigen-specific humoral immunity after vaccination with MF59-adjuvanted influenza (22). ASC has been proposed to regulate cytokine transcription through activation of NF-κB (23, 24). AP1, STAT3, ISGF3, and NF-AT have also been identified as transcriptional ASC targets in a reconstituted cell system with exogenously expressed ASC and...
a chimeric CARD12/NOD2 protein (25). Given the complexity of inflammatory signaling, it is likely that additional signaling pathways contribute to inflammasome-independent ASC function in macrophages.

One crucial feature of pathogenic signaling is the activation of the mitogen-activated protein (MAP) kinase kinase kinases (MAP3Ks), a family of signaling proteins that regulate a variety of physiological processes, including proliferation, cell death, stress response, and differentiation. The MAP3Ks act as nodes in the Toll-like receptor (TLR) signaling cascade for both the NF-κB and MAPK pathways (26, 27). Transcription factors downstream of these signaling pathways then collaborate to regulate the expression of immune and inflammatory mediators. By contrast, the role of components of the inflammasome complex in MAP3K signaling has not been directly explored. MAPK is at the center of many innate immune responses, thus the link between ASC and MAPK is an important topic to explore.

Pg is a Gram-negative oral pathogen associated with chronic adult periodontal disease. Pg surface components, including LPS, fimbriae, and hemagglutinin B, induce host inflammatory responses that result in breakdown of periodontal ligaments and destruction of the local alveolar bone (28–30). Although periodontal disease is localized to the tissues surrounding the tooth, Pg infection predisposes people to more serious systemic conditions such as cardiovascular disease and delivery of preterm infants (29, 31, 32). Recently, we showed that, during Pg infection, ASC exhibits inflammasome-independent functions, including TNF-α and NF-κB activation (23). We therefore elected to use this pathogen to reveal new ASC functions that might be separable from that of the inflammasome. Results described herein provide a novel microbial pathogen-induced mechanism for ASC in activating chemokine expression through MAPK activation independent of the conventional caspase-1 inflammasome.

**EXPERIMENTAL PROCEDURES**

**Generation of Cell Lines, Isolation of Mouse Macrophages, and Cell Culture**—THP1 monocytic cells (ATCC) were cultured in RPMI, 10% FCS. shASC#1, shASC#2, and shASC#1mut THP1 cell lines were generated using retroviral vectors and have been characterized previously (18, 23). DUSP10-expressing THP1 cells were generated using the full-length open reading frame cloned into lentiviral vector pLex-JRed (catalogue #OHS4493-98905681; Open Biosystems). A control empty vector, pLex-EV, was generated by digesting pLex-DUSP10 with XhoI to remove the DUSP10 cDNA and religating the empty vector. DUSP10 shRNA plasmid pHKO-shDUSP10 was obtained from the TRC collection (Open Biosystems). Lentivirus was packaged in 293T cells using vectors pMD2.G and psPAX2 (Addgene plasmids 12259 and 12260) as described previously (33). Cells were selected with 1 μg/ml puromycin for 2 weeks, and JRed expression was confirmed by using FACS. Asc<sup>−/−</sup> and Nlrp4<sup>−/−</sup> mice were obtained from Dr. Vishva Dixit at Genentech (South San Francisco, CA); Nlrp3<sup>−/−</sup> mice were obtained from Millennium Inc.; Casp1<sup>−/−</sup> mice were obtained from Dr. Richard Flavell, Yale University (12); and MyD88<sup>−/−</sup> mice were obtained from Dr. Shizuo Akira. All mice were backcrossed for a minimum of nine generations to C57BL/6 mice. Bone marrow-derived macrophages were harvested and cultured in DMEM, 10% FCS, macrophage-colony stimulating factor without replating for 6–7 days. Cells were maintained at low density and serum-starved 16 h prior to infection. Where indicated, cells were stimulated with 10 multiplicity of infection Pg, 0.5 multiplicity of infection *Escherichia coli*, or pharmacological agents detailed in supplemental Table S1.

**Bacterial Culture**—Pg strain A7436 was cultured anaerobically, and *E. coli* strain DH5α aerobically until late exponential phase (optical density of 0.8–1.2 at 660 nm). Aliquots were stored in media containing 20% glycerol at −80 °C and used within 3–4 months of preparation. Replating of frozen cultures confirmed the accuracy of bacterial counts to within 2–3 cfu.

**Microarray Analysis**—RNA was isolated using Qiagen RNeasy columns following 2-h infection with Pg. Two-color microarray analysis was performed at the Duke Microarray facility using 34,000 spot custom chips based on the version 3 Human oligonucleotide set (Operon). All samples were compared with a universal control created by pooling RNA over a time course of LPS treatment. Gene lists were generated from the averages from three independent experiments using GeneSpring 7.0. To identify Pg-regulated genes the following criteria were used: minimum raw signal of 50 in the universal control and 100 in at least one condition; and ±5-fold difference between expression in control versus Pg-infected cells. The criteria for ASC-dependent genes was as follows: minimum raw signal of 60 in the universal control and 100 in at least one condition; ±3-fold difference between expression in Pg-infected THP1 versus shASC#1 cells and shASC#1mut versus shASC#1 cells; and ≤50% difference between Pg-infected THP1 and shASC#1mut cells. Genes were further analyzed in GeneSpring 11 for statistical validation using modernized normalization methods, as presented in supplemental Tables S3 and S4 (*lanes* 5–7). Genes that passed the filtering schemes were uploaded into the Ingenuity Pathways analysis application, which overlaid them onto a global molecular network developed from information within the Ingenuity Pathways Knowledge Base. Each network was algorithmically generated based on the connectivity of genes. *p* values, which indicate the likelihood that the same number of genes taken from a random set would appear in the network, were calculated using Fischer’s exact test.

**Real-time PCR and PCR-based Expression Profiling**—Real-time PCR was performed as described (23) using the primers listed in supplemental Table S2. Mouse RNA was quantified using TaqMan<sup>®</sup> Assays on Demand (Applied Biosystems). Values represent averages ± S.D. of triplicates for RNA isolated on different days unless otherwise stated. All values were standardized to 18 S rRNA expression. PCR-based expression profiling was performed according to manufacturer recommendations using equal amounts of RNA from six mice with the Mouse Inflammatory Cytokines and Receptors array (SA Biosciences). Values were standardized to an average of five housekeeping genes.

**Assessment of RNA Stability and de Novo mRNA Synthesis**—To assess RNA stability, cells were treated for 2 h with LPS,
washed to remove the LPS, and treated with 50 \( \mu \text{M} \) 5,6-di-chloro-1-\( \beta \)-D-ribofuranosylbenzimidazole (DRB, Sigma) for 30 min. RNA was quantified by real-time PCR over a time course as described above. To assess de novo transcriptional, RNA was isolated from nuclear extracts using the PARIS kit (Ambion) and assessed by real-time PCR using primers that target nascent transcripts. Efficient separation of nuclei was confirmed by the absence of GAPDH on Western blots.

**Antibody Array**—Cell culture supernatants were collected 24 h following exposure to \( \text{Pg} \) and applied to RayBio\textsuperscript® Human Cytokine Antibody Array 5 glass slides according to manufacturer’s protocols. The signal strength for each cytokine was normalized to the average signal strength of spiked, internal controls. Data for each treatment group were assessed as change compared with uninfected samples.

**ELISA Analysis**—Supernatants were assessed 18–24 h following stimulation using human ELISA sets for TNF and IL-1\( \beta \) (BD Biosciences), DuoSets for CCL3 and CCL20 (R&D Systems), or the QuantiKine\textsuperscript® ELISA kit for human IGF-1 (R&D Systems). Samples were assayed within linear range.

**Western and Immunoprecipitation/Immunoblot Analyses**—Cells were washed in 1× PBS and lysed for 20 min in ice-cold 1× lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X) supplemented with Complete EDTA-free Protease Inhibitor and PhosSTOP Phosphatase Inhibitor mixture (Roche Applied Science). Lysates were centrifuged for 10 min and supernatants boiled for 5 min in 1/3 volume 3× SDS Sample Buffer (187.5 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 150 mM DTT, 0.03% bromphenol blue). Immunoblots were processed using Abs sc-7383 for p-ERK, equal volumes of sc-93 (ERK1) and sc-154 (ERK2) for total ERK, sc-474 for JNK, sc-1615 for actin (Santa Cruz Biotechnology), #4668 for p-JNK (Cell Signaling Technology), and MAB374 for GAPDH (Millipore). For caspase-1 immunoprecipitation/Western analyses, cells were plated at 10\(^{6}\)/ml and lysed following infection by addition of 0.1% Nonidet P-40 and 1× Complete EDTA-free Protease Inhibitors (Roche Applied Science). 1 ml of supernatant was recovered and incubated overnight with 25 \( \mu \)l of anti-caspase-1 (sc-515, Santa Cruz Biotechnology) and 30 \( \mu \)l of protein A/G UltraLink resin (Thermo Scientific). Immunoprecipitates were washed and then boiled in 3× SDS Sample Buffer. Immunoblots were performed using antibody IMG-5028 (Imgenex).

**RESULTS**

**Identification of \( \text{Pg} \) Interactomes by Microarray and Bioinformatics**—We selected \( \text{Pg} \) for this study because a previous study suggested that it activates ASC-mediated functions that are distinct from IL-1\( \beta \) processing and inflammasome activation (23). To identify biological pathways activated by \( \text{Pg} \), RNA from untreated THP1 cells and cells treated with 10 multiplicity of infection \( \text{Pg} \) for 2 h was assessed using human genome microarray chips comprising over 35,000 oligonucleotide probes and representing \( \sim 25,000 \) unique genes (Operon Biotechnologies, Inc). Using GeneSpring 7.0 microarray analysis software, a list was compiled of \( \sim 150 \) genes that \( \text{Pg} \) modulates by \( \geq 5\)-fold (supplemental Table S3). Additional statistical analysis using GeneSpring 11.0 verified the inclusion of the majority of the genes in this table (columns 5–7). Unbiased software analysis was performed to identify interactomes of genes that encode physically or functionally interacting proteins (Ingenuity Systems). Interactomes are networks of genes that are interconnected based on information derived from the literature, a textbook, or canonical knowledge. Three primary networks of \( \text{Pg} \)-modulated genes were identified. The first interactome encompasses the inflammatory cytokine TNF-\( \alpha \). TNFA RNA is induced 134–160-fold by \( \text{Pg} \) infection (Fig. 1A). Other genes in the TNF-\( \alpha \) interactome were modulated from 5- to 319-fold by \( \text{Pg} \). The extremely low \( p \) value of this interactome (\( p < 10^{-46} \)) suggests very high likelihood of functionality. Consistently, TNF-\( \alpha \) pathways are known to contribute to \( \text{Pg} \)-associated pathogenesis during periodontitis (34, 35). The second interactome (Fig. 1B; \( p < 10^{-46} \)) encompasses IL1B, which itself is activated \( >200\)-fold. Both interactomes show high induction for multiple chemokines. The third interactome (\( p < 10^{-41} \)) is unique in that its central molecule, NF-\( \kappa \)B, does not appear to be significantly regulated at the transcriptional level but was identified based on the genes that it is known to modulate (Fig. 1C). This is consistent with the role of the NF-\( \kappa \)B members as early signaling transcriptional regulators (36). Induction of the transcript for the CARD-containing serine/threonine kinase RIPK2 by 15- to 17-fold (Fig. 1A) could potentially contribute to the enhanced NF-\( \kappa \)B pathway activity by \( \text{Pg} \) (37, 38). The identification of each of these \( \text{Pg} \) interactomes is consistent with known biological effects of \( \text{Pg} \) in the activation of cellular signaling pathways toward inflammation.

**Identification of an ASC-dependent \( \text{Pg} \) Interactome**—ASC is an adaptor molecule important in the induction of apoptosis and inflammatory response (2, 11, 15). Our previous studies demonstrate that, in addition to regulating IL-1\( \beta \) processing through caspase-1 activation, ASC regulates the transcription of a panel of cytokines, including IL-6, IL-8, IL-10, and TNF-\( \alpha \) (23). Because induction of these cytokines is thought to be NLR- and caspase-1-independent, their regulation by ASC implies ASC-mediated functions that are distinct from the inflammasome. For example, several reports have shown that TNF-\( \alpha \) expression is not altered by the deletion of Nlrp3 (12, 39). Thus we reasoned that \( \text{Pg} \) represents a good system to reveal differences between ASC-associated inflammasome-dependent and inflammasome-independent signaling.

To identify additional genes and pathways regulated by ASC, two different ASC knockdowns were utilized with reduced ASC expression. The lentiviral shRNA construct shASC\#1 caused \( \sim 90\% \) knockdown and shASC\#2 \( \sim 70\% \) knockdown when compared with controls, including non-transfected THP1 cells, and cells bearing an empty vector (EV) or a mutated target site (shASC\#1mut) (23) (Fig. 1D). The shRNAs reduced IL-1\( \beta \) cytokine induction, verifying that the knockdowns reduce ASC function as previously observed (Fig. 1E). Microarray analysis was performed to identify genes that were differentially regulated by ASC. Approximately 80 genes were modulated by \( \geq 3\)-fold in shASC\#1 cells as compared with control cells (supplemental Table S4). This set of ASC-dependent genes was evaluated by Ingenuity Pathways Analysis, and an ASC-dependent interactome was identified (Fig. 1F, \( p < 10^{-32} \)). As confirmation of the technology, ASC was reduced 8.8- to 12.6-fold in
shASC cells. Several chemokines, including CCL3, CCL3L1, and CCL4, were reduced in the absence of ASC, while CXCL10 was increased ~4-fold. NF-κB was also identified as an ASC-dependent regulator of these genes, consistent with our previous findings linking ASC-dependent gene expression to NF-κB activation (23). Because the expression and activity of NF-κB
and several chemokines within this interactome are also highly regulated by Pg (Fig. 1, A–C), the role of ASC may extend to many of the same pathways that Pg induces. All of the genes within the ASC-dependent interactome could be interconnected to the signaling molecule MAPK1/ERK2, albeit expression of the latter, like NF-κB, was not affected by ASC. Because MAPKs are primarily regulated post-transcriptionally, the central position of MAPK1 within this interactome provides novel insight into potential post-transcriptional mechanisms of ASC function.

Assessment of ASC-dependent Cytokine and Chemokine Expression—To verify ASC-dependent chemokine expression, RNA levels in ASC knockdown THP1 cells were measured following a time course of infection with Pg. Consistent with the microarray results, TNFA was induced by Pg in control cells, with a peak at 2–4 h post-infection, and this induction was reduced in the ASC-knockdown cells (Fig. 2A).

CCL3, CCL4, and CXCL3 each showed a similar pattern of ASC-dependent RNA expression (Fig. 2, B–D). These findings support the identification of the ASC interactome and show a role for ASC in Pg-dependent induction of chemokines.

Regulation of mRNA expression can occur either at the level of transcription or RNA stability. To distinguish these possibilities, RNA stability was examined following LPS treatment using DRB to block de novo transcription (Fig. 2E). The RNA decay profiles were similar in shASC#1mut and shASC#1 cells, following a time course of infection with Pg. Consistent with the microarray results, TNFA was induced by Pg in control cells, with a peak at 2–4 h post-infection, and this induction was reduced in the ASC-knockdown cells (Fig. 2A). CCL3, CCL4, and CXCL3 each showed a similar pattern of ASC-dependent RNA expression (Fig. 2, B–D). These findings support the identification of the ASC interactome and show a role for ASC in Pg-dependent induction of chemokines.

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indicating that regulation of RNA stability is not a major determinant for ASC-dependent differences in chemokine expression. Conversely, assessment of de novo mRNA synthesis using nuclear extracts and primers that target unspliced message indicates that regulation is primarily transcriptional (Fig. 2F).

To further verify a role for ASC in chemokine induction by *Pg* we performed protein array analysis using antibody chips comprising 79 cytokines, chemokines, and growth factors (RayBiotech, Inc.). Supernatants were assayed from shASC#1mut and shASC#1 cells before and following 24-h infection with *Pg*. Six proteins were differentially induced by *Pg* in shASC#1mut versus shASC#1 cells (Fig. 3A). This includes IL-1β, which is a known target for ASC regulation via cleavage by caspase-1 within the inflammasome complex (2). TNF-α was also identified, further confirming our real-time results. The chemokines from Fig. 2 either were not detected at high levels (for CCL4) or were not included on this array (for CCL3 and CXCL3). However, the chemokines, GRO/GRO-α, CCL13, and CCL20, were identified, further supporting a broad role for ASC in regulating chemokine expression. The latter proteins are not thought to be regulated by caspase-1, suggesting a potential role for ASC in caspase-1-independent regulation of chemokine expression.

To further verify these findings, supernatants were collected from control and ASC knockdown lines 24 h following *Pg* infection and assessed by ELISA. Levels of secreted TNF-α, CCL3, and CCL20 were reduced in shASC#1 cells, with somewhat less reduction in shASC#2 cells suggesting dose-dependent regulation (Fig. 3, B–D). IGF-1 expression was not ASC-dependent, demonstrating specificity of this effect (Fig. 3E). These findings further support ASC-dependent chemokine expression suggested by the interactome described in Fig. 1F.

**MAPK Activation Is ASC-dependent in THP1 Cells**—MAPKs are activated by phosphorylation by upstream kinases (26, 27). To provide support for the central position of MAPK1/ERK2 within the ASC interactome (Fig. 1F), we assessed phosphorylation levels of ERK in ASC-knockdown cells following *Pg* infection. Consistent with the microarray results, total expression of ERK1 and ERK2 was not significantly different in control shASC#1mut versus shASC#1 knockdown cells and was not regulated by *Pg* infection. Levels of phospho-ERK (p-ERK) induction peaked at ~60–90 min for both cell lines but were quantitatively reduced in ASC-knockdown cells over a 120-min time course of infection (Fig. 4A). These findings are consistent with a role for ASC in ERK activation.

To determine whether effects of ERK activation by ASC have broad significance during microbial infection, control and ASC-knockdown cells were treated for 60 min with *E. coli* and a variety of agents known to activate cells through different TLRs (Fig. 4B). For each of these treatments, levels of p-ERK were reduced in the absence of ASC. These findings indicate that, in addition to *Pg*, ASC mediates ERK activation through *E. coli* and a variety of TLR agonists.

ERK is one of three related MAPK pathways activated in stimulated cells. To test whether the JNK and p38 pathways also display reduced activation in ASC-knockdown cells, phos-
phoblots were repeated. Levels of p-p38 in THP1 cells were too low to be measured accurately (data not shown). However, similar to p-ERK, levels of p-JNK activation by \( \text{Pg} \) were reduced in ASC-knockdown cells, indicating that the role of ASC in MAPK activation extends to other MAPK pathways (Fig. 4C).

To determine whether reduction in ERK and JNK activation might explain the reduced chemokine levels in ASC knockdown THP1 cells, ELISA assays were repeated in the presence of specific inhibitors of ERK and JNK pathways (Fig. 4D). Levels of CCL3 and CCL20 following \( \text{Pg} \) infection were reduced by both inhibitors, either alone or in combination, whereas the carrier, DMSO, did not affect expression. This effect was specific, because levels of IGF-1 were unchanged (Fig. 4E). These results confirm the importance of the ERK and JNK pathways in chemokine induction by \( \text{Pg} \).

**MAPK Activation and Chemokine Induction by \( \text{Pg} \) in Primary Mouse Macrophages Are ASC-dependent**—To confirm our findings in a primary cell system, macrophages were harvested from wild-type C57BL/6 mice and matched \( \text{Asc}^{-/-} \) mice and infected with \( \text{Pg} \) over a time course. Levels of p-ERK activation were nearly eliminated in macrophages from \( \text{Asc}^{-/-} \) mice, whereas levels of p-JNK and p-p38 were modestly reduced (Fig. 5, A–C). These results confirm the role for ASC in \( \text{Pg} \)-induced MAPK phosphorylation in a primary cell system.

To determine whether reduced MAPK activity in mouse macrophages correlates with reduced chemokine activation, RNA was pooled from six WT and \( \text{Asc}^{-/-} \) mice following \( \text{Pg} \) infection and assessed by PCR-based expression profiling. Several chemokines were identified (Fig. 5D). This list of candidate genes includes Ccl3, which was decreased, and Cxcl10, which was increased both in the RNA from pooled Asc-deficient mouse macrophages and THP1 shASC#1 cells (Fig. 1F). Other chemokines identified in the two-model system differed, most likely explained by differences between species and the transformed or non-transformed state of the cells. Nonetheless, the regulation of these chemokines would be consistent with our overall finding that ASC modulates chemokine expression.

To verify Asc-dependent expression of chemokines in mouse macrophages, TaqMan® PCR was performed before and following 2-h \( \text{Pg} \) infection, and -fold induction was calculated. Consistent with the above results, Asc was required for high level inducibility of Ccl3, Ccl19, and Ccl25, whereas Cxcl10 was increased in the absence of Asc (Fig. 5E). Ccl3 expression was dependent upon ERK, JNK, and p38 (Fig. 5F), verifying an essential role for MAPKs in chemokine expression in primary mouse macrophages.

**The Dual-specificity Phosphatase DUSP10/MKP5 Negatively Regulates MAPK Phosphorylation and Chemokine Activation by \( \text{Pg} \)**—The modulation of multiple MAPKs by ASC suggests that ASC might lie upstream of a common regulator of MAPKs. Examination of ASC-dependent genes identified by microarray analysis (supplemental Table S4) revealed that \( \text{DUSP10/MKP5} \) is \( \sim3.5 \)-fold higher in shASC-containing cells. The dual-specificity phosphatases (DUSPs) are known to negatively regulate the activity of multiple MAPKs through dephosphorylation (40, 41). To verify the effect of ASC on \( \text{DUSP10} \) expression, real-
time PCR analysis was performed. DUSP10 levels were increased in both ASC-knockdown lines relative to the controls (Fig. 6A, top panel). As a control, levels of transcript for MAPK1/ERK2 were ASC-independent (Fig. 6A, bottom panel). Increased DUSP10 in ASC-knockdown cells was observed over a time course of infection with Pg in THP1 cells (Fig. 6B) and mouse macrophages (Fig. 6C). Because DUSP10 is known to correlate negatively with the activity of MAPKs (42), these findings are consistent with the reduced post-transcriptional activation of MAPKs in ASC-knockdown cells following Pg stimulation and provide a potential mechanism for diminished MAPK phosphorylation.

To directly assess the role of DUSP10 in MAPK phosphorylation, DUSP10 was exogenously expressed in THP1 cells (Fig. 6D). Cells were infected with Pg and MAPK phosphorylation levels were measured by immunoblotting. ERK phosphorylation was significantly reduced and JNK phosphorylation nearly eliminated in pLex-DUSP10 cells as compared with non-transfected THP1 cells and the empty vector control, pLex-EV (Fig. 6E). These results verify that DUSP10 can negatively regulate MAPK phosphorylation following Pg infection. To determine whether increased DUSP10 expression can regulate chemokine induction, supernatants from pLex-EV control cells and pLex-DUSP10 cells were collected following infection with Pg and assessed by ELISA. Levels of secreted CCL3 and CCL20 were ablated in pLex-DUSP10 expression as determined by TaqMan Assays. Expression levels were normalized to 100 in control cells. Data represent averages ± S.D. for three independent experiments.

FIGURE 5. MAPK activation and chemokine induction in primary mouse macrophages is Asc-dependent. A–C, Western blot of p-ERK, p-JNK, and p-p38 in primary mouse macrophages from WT C57BL/6 and Asc−/− mice following a time course of infection with Pg. Blotting for the Asc protein is shown as a verification of the knockout, and GAPDH is shown as a loading control. Representative of at least three independent experiments. D, chemokines modulated in Asc−/− mice as assessed by pathway-focused gene expression profiling of pooled RNA from six mice. Values represent -fold expression for Pg-infected Asc−/− versus WT macrophages. Negative values represent a decrease in Asc−/− macrophages, and positive values represent an increase. A complete list of genes is provided in supplemental Table S5. E, induction of chemokines following 2-h Pg infection as assessed by TaqMan PCR. Data represent averages ± S.D. for four independent experiments; *, p < 0.05; **, p < 0.01. F, effects of MAPK inhibitors on Ccl3 expression as determined by TaqMan Assays. Expression levels were normalized to 100 in control cells. Data represent averages ± S.D. for three independent experiments.
phosphorylation was no longer dependent on ASC (Fig. 6H). These results suggest that DUSP10 expression is required for ASC-mediated ERK phosphorylation.

To determine how *DUSP10* expression is regulated, THP1 cells were treated with a panel of inhibitors for specific MAPK pathways. Inhibition of ERK caused increased *DUSP10* expression.
sion with the greatest increase achieved using a combination of ERK and JNK inhibitors (Fig. 6, top panel). The converse results were observed for TNFA as expected (bottom panel). Inhibition of p38 did not affect DUSP10 nor TNFA expression. Because the p38 inhibitor SB203580 also can potently inhibit RIPK2, these results could suggest that RIPK2 is not involved in the short term regulation of DUSP10 in THP1 cells (43). For primary mouse macrophages, all three inhibitors affected Dusp10 expression, although the greatest enhancement of expression was observed following inhibition of either JNK or ERK and p38/RIPK2 in combination (Fig. 6f). These findings suggest that a negative feedback loop occurs in which DUSP10 regulates MAPK activation, and MAPKs in turn regulate DUSP10 expression. The complement of the specific MAPKs involved appears to vary for THP1 cells versus mouse macrophages. However, a similar pattern of reciprocal regulation occurs in each system.

**Induction of Chemokines and ERK phosphorylation by Pg is IL-1β-, caspase-1-, and NLRP3-independent.** A, real-time PCR of chemokine RNA in THP1 cells treated with Pg and/or the IL-1 receptor antagonist, Kineret®. Data are normalized to an average of 100 in Pg-treated THP1 cells and represent averages ± S.D. for three independent experiments. N.D., not detectable. B, ELISA of IL-1β, CCL3, and CCL20 in supernatant from THP1 cells treated with Pg, Kineret®, and/or the caspase-1 inhibitor YVAD-cmk. C and D, Western analysis of p-ERK in THP1 cells following a time course of infection with Pg, Kineret® (C) or YVAD-cmk (D) was added as indicated. GAPDH is shown as a loading control. Representative of three independent experiments. E, caspase-1 activation in shASC#1 and pLex-DUSP10 cells following Pg infection. Immunoprecipitation/immunoblotting was performed in uninfected cells (lanes 1–4) and following 2.5-h Pg infection (lanes 5–8). An immunoblot for actin in cell lysates is shown as a loading control. Representative of three independent experiments. F–I, Western blot of p-ERK following a time course of infection with Pg in primary mouse macrophages. Supernatant from Pg-infected WT or ASC−/− mouse macrophages was applied to WT and ASC−/− mouse macrophages 5 min prior to infection.
following *Pg* infection (Fig. 7E). Activation of caspase-1 was ablated in shASC#1 cells as expected (*lane 6 versus lane 5*). However, expression of DUSP10 did not reduce caspase-1 activation following *Pg* infection (*lane 8 versus lane 7*). These findings suggest that the regulation of MAPK signaling by DUSP10 constitutes a distinct ASC-dependent activity that is independent of caspase-1 and the conventional inflammasome. Further studies will be necessary to determine whether the inflammasome independence extends to other genes and pathways within the ASC-dependent inflammasome identified in Fig. 1F.

Macrophages from wild-type B6 and gene deletion mice were tested to confirm inflammasome independence of MAPK activation in a primary cell system. Following *Pg* infection, phosphorylation of p-ERK was dramatically reduced in the positive control, macrophages from *MyD88*−/− mice. This is expected due to the prominent role of TLR/IL-1R in this pathway (26, 27) (Fig. 7F). However, an examination of macrophages from *Casp1*−/−, *Nlrp3*−/−, and *Nlrc4*−/− mice show that these genes do not affect ERK activation. The persistence of ERK activation in the *Nlrp3*−/− and *Nlrc4*−/− macrophages shows independence from Nlrp3 and Nlrc4 inflammasome function. The use of *Casp1*−/− mice most clearly indicates that p-ERK activation is independent of the caspase-1 inflammasome. These findings further argue for a role as ASC-dependent MAPK activation that is exclusive of the caspase-1 inflammasome.

**DISCUSSION**

Using a combination of gene-profiling analysis and bioinformatics we identified several pathways activated by *Pg*. The finding that *Pg* activates NF-κB- and TNF-related signaling is consistent with other microarray studies of *Pg* infection (28, 34). Our further assessment of ASC-modulated genes revealed several chemokines that are ASC-dependent. These findings are novel, and led us to the identification of MAPK1/ERK2 as a potential ASC-regulated protein within a complex interactome of chemokines, signaling molecules, and transcriptional regulators. MAPK regulation was not transcriptional, but rather at the level of phosphorylation. This work demonstrates the power of combining microarray technology with software-based network analysis to study cell-signaling pathways. In this instance the relevant proteins are regulated post-transcriptionally and would therefore be missed by microarray analysis alone.

Western analysis confirmed the role of MAPKs in ASC-mediated signaling in THP1 cells and *Asc*−/− mice following *Pg* infection. ASC also was important in the activation of ERK in response to *E. coli* and to agonists of TLR2, -4, and -5. The contribution of MAPKs to the activation of chemokines by *Pg* correlates with these results and provides one potential functional outcome for reduced MAPK activation in ASC-deficient cells. The chemokines identified and the dependence on specific MAPKs in each model system had partial overlap. Differences in the two-model system might be explained by human *versus* mouse differences, the transformed *versus* non-transformed state of the cell, or perhaps by differences in the stage of differentiation; however, the fundamental finding of ASC-dependent MAPK activation and chemokine induction was conserved.

Our results identify DUSP10 as a key regulator in ASC-dependent MAPK activation. The DUSPs function by reversing the tyrosine and serine/threonine phosphorylation of the MAPKs that occurs upon activation (41). *DUSP10* expression was enhanced in the absence of ASC. Furthermore, exogenous expression of DUSP10 reduced MAPK phosphorylation and chemokine induction, and conversely, reduction of *DUSP10* by RNA interference reversed the ASC-dependent MAPK phosphorylation. Interestingly, *DUSP10* expression also was regulated reciprocally by the MAPKs in a classic negative feedback loop. The reciprocal regulation of DUSP10 and MAPKs could explain the profound effect on the expression of chemokines within the ASC-dependent interactome.

It is noteworthy that a connection between ASC and the transcription factor AP1 has recently been established using a reconstituted cell system that is engineered to respond to the bacterial cell wall component muramyl dipeptide (25). AP1 is among a large number of transcriptional regulators that are downstream targets of MAPKs (26). The regulation of AP1 described in the former study appears to be at the level of transcription, whereas our results reveal a post-transcriptional regulatory mechanism. Further studies will be necessary to define effects of ASC-dependent DUSP repression and MAPK activation on AP1 activity. ASC-dependent post-transcriptional activation of AP1 could provide a complementary mode of controlling AP1 activity. Given the broad role of MAPKs in a number of biological pathways, it is likely that ASC-dependent MAPK activation controls the activity of multiple additional transcription factors and pathways.

Initial studies of *Asc*−/− mouse macrophages failed to show a difference in MAPK activation (11). Differences between these findings and ours could reflect the use of different stimuli to activate p-ERK, or different methods of macrophage isolation or culture. MAPKs can be readily activated by a wide array of stimuli. In our protocol, macrophages were plated for at least 6 days without disruption, because MAPKs can be easily induced by changes in adherance. There are multiple types of proliferative stimuli and pathways that can lead to MAPK activation (41), and for this reason we have serum-starved the primary cell macrophages to reduce background stimulation from serum components. Serum starvation is one major difference between our study and earlier studies and is typically required to see ERK activation.

Classically, ASC acts within an inflammasome complex that also contains caspase-1 and one of several different NLR proteins (1) or the HIN domain protein AIM2 for cytosolic DNA viruses (6–9). In contrast to IL-1β processing, activation of MAPK and chemokines via an ASC-dependent pathway did not require caspase-1. Furthermore, exogenous DUSP10 expression reduced MAPK phosphorylation and chemokine activation without affecting caspase-1 processing. ERK phosphorylation was not reduced in *Casp1*−/−, *Nlrp3*−/−, or *Nlrc4*−/− mice. These findings suggest that the MAPK pathway of chemokine activation is caspase-1- and NLR-independent, providing a novel inflammasome-independent function for ASC. ASC also is required for caspase-independent activation of necrosis (16–18) and for antigen-induced arthritis (20, 21). Previously we showed that NF-κB activation by *Pg* is caspase-
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1-independent (23). The present study provides an additional caspase-independent mechanism of ASC that could explain ASC functionality in the absence of the inflammasome.

In summary, ASC has important roles in both inflammasome-dependent and inflammasome-independent signaling cascades. We have revealed a novel mechanism for ASC in the activation of MAPKs that is regulated through DUSP10 suppression, and a seminal result of this activation is the alteration of chemokines necessary for host response to microbial pathogens.

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