# Supporting Information

## Grönwall et al. 10.1073/pnas.1211868109

### SI Materials and Methods

**Antibody Assays.** Standard sandwich ELISA were performed with precoats of goat anti-IgM, anti-IgG, phosphorylcholine (PC)–albumin, malondialdehyde (MDA)–albumin, and 4HNE–albumin for control Ag with detection with anti-IgM or anti-IgG as described previously (1). For studies of the inhibitory effect of sera, concentrations were assessed before and after adsorption based on $OD_{280}$ and IgG and IgM content by ELISA.

**Detection Antibodies.** Immunoblots used antibodies to the following: MAPK phosphatase-1 (MKP-1) (catalog 07–535; Upstate Biotechnology), P-p38 (catalog 9211; Cell Signaling), P-ERK (catalog 4377; Cell Signaling), p38 (catalog 9212; Cell Signaling), ERK (catalog 4695; Cell Signaling), P-JNK/SAPK (catalog 4688; Cell Signaling), JNK/SAPK (catalog 9258; Cell Signaling), P-Elk-1 (catalog 9181; Cell Signaling), P-STAT1 (catalog 9171; Cell Signaling), or STAT1 (catalog 9172; Cell Signaling), detected with anti-rabbit HRP (catalog 7078; Cell Signaling). Anti–pan-actin provided a loading control (catalog 4968; Cell Signaling).

Intracellular flow cytometry assays used mouse anti–P-p38-Alexa 488 (catalog 4551; Cell Signaling) or rabbit anti–MKP-1 (catalog sc1102; Santa Cruz) antibodies or isotype controls, then after washing, detection utilized anti-rabbit IgG-FITC (catalog sc-2012; Santa Cruz). Costaining was performed with anti–MHCII-PE/Cy5 (catalog 15–5321-82; eBioscience).

The following antibodies were used in the immunofluorescence studies: rabbit anti–MKP-1 (catalog sc1102; Santa Cruz), mouse anti–P-p38-Alexa488 (catalog 4551; Cell Signaling), rabbit anti–P-JNK (catalog 4668; Cell Signaling), or rabbit anti–P-ERK1/2 (catalog 9101; Cell Signaling) and counterstained with fluorochrome-conjugated anti-rabbit IgG (Santa Cruz). The anti-chromatin IgG stimulation studies used rabbit anti–P-p38 mAb (catalog 4511; Cell Signaling) for detection by immunofluorescence microscopy.

**Bone Marrow-Derived Dendritic Cells.** As previously described (1) bone marrow (BM) cells from C57BL/6 femurs/tibias were washed and cultured in RPMI 1640 containing 10% (vol/vol) FBS, 1% (vol/vol) penicillin–streptomycin–glutamine, GM-CSF (10 ng/mL), and IL-4 (4 ng/mL) in petri dishes at $5 \times 10^6$ cells/mL and an equal volume of media added on day 3 (2). On day 5, dentritic cells (DCs) were selected in the presence of Fc block with magnetic anti-CD11c beads using LS magnetic columns (Miltenyi Biotec) to >96% $CD11c^+$ purity. For studies of activated DCs, cells were then cultured in STEMSPAN SF Expansion (StemCell Technologies) serum-free media supplemented with C1q (80 μg/mL), unless otherwise indicated. For antigen-specific inhibition, aliquots of sera were preincubated for 1 h at 37 °C with PC-BSA, MDA-BSA, or 4HNE-BSA at 40 μg/mL (Biosearch) as described (1, 3).

For stimulation studies, DCs were further cultured for 24–48 h without/with agonists for Toll-like receptor 3 (TLR3), poly(I:C) at 3.3 μg/mL; TLR4, LPS at 0.1 μg/mL; TLR7, imiquimod (InvivoGen) at 1 μg/mL; or TLR9, phosphorothioate CpG oligo 1018 at 0.5 μg/mL, as described (3). Replicate cultures included serial concentrations of T15-IgM or IgM isotype control, or as indicated. Cultures with anti-apoptotic cell (AC) IgM blockade or isotype control also included Fc block. Soluble factors in culture supernatants were evaluated as described (3) or for activated TGF-β as previously described (4). To assess DC maturation, cells were costained with PE–anti-mouse CD80 (clone 16–10A1) and for intracellular Alexa Fluor 647 anti-MHCII, as per manufacturer's protocol (eBioscience). Quantitative transcript analysis was performed by TaqMan (Applied Biosystems), using manufacturer's directions, as described (3).

**Antichromatin IgG Stimulation.** The antichromatin IgG hybridoma (PL2-3) was provided by Monestier (5) and IgG was purified from cell supernatants by protein A (Repligen) affinity chromatography following manufacturer's recommendations. C57BL/6 BM cells were cultured in complete media supplemented with GM-CSF (6.67 ng/mL) and IL-4 (0.4 ng/mL) for 3 d. On the fourth day, an equal amount of media supplemented as above was added. On day 6, BM cultures were purified with anti-CD11c magnetic beads (Miltenyi). Stimulations were performed as previously described (6) and anti-AC NAb or isotype control IgM pretreated BM-DCs were stimulated with chromatin IgG (8 μg/mL) for 30 min.

**Immunofluorescence Studies.** After DC in vitro stimulation for 30 min, cells were incubated for 15 min at room temperature (RT) on poly-L-lysine–coated coverslips, washed with PBS, and fixed for 30 min with 3.7% (vol/vol) formaldehyde. Cells were washed with PBS twice and then permeabilized with 0.5% (vol/vol) Triton X-100 in PBS for 3 min at RT. After treatment with Fc block, staining used either appropriate isotype control or rabbit anti–MKP-1, anti–P-p38-, rabbit anti–P-JNK, or rabbit anti–P-ERK1/2 for 30 min RT, washed and counterstained with fluorochrome-conjugated anti-rabbit IgG (Santa Cruz) and Hoechst dye (catalog H21492; Invitrogen), Images were captured with a 60× oil immersion lens (Nikon Eclipse TE200) using a Deltavision Deconvolution Microscope (Nikon inverted), Deconvolution occurred after image acquisition using Deltavision SoftWorx 4.0.0., which was also used for immunofluorescence density determinations.

For cells stimulated with antichromatin IgG, cells were added to poly-L-lysine–treated coverslips as above, followed by fixation with 4% (vol/vol) paraformaldehyde for 10 min at RT, blocking with PBS 5% goat serum, 0.3% (vol/vol) Triton X, and incubation with rabbit anti–P-p38 mAb or rabbit anti-MKP1 overnight at 4 °C in PBS 1% (wt/vol) BSA, 0.3% (vol/vol) Triton X following manufacturer's protocol. Cells were then washed and incubated with goat anti-rabbit IgG-FITC (Santa Cruz) in PBS 1% (wt/vol) BSA, 0.3% (vol/vol) Triton X, followed by addition of Hoechst 34580 (Invitrogen) in PBS. Coverslips mounted in Prolong Gold Antifade (Invitrogen) were visualized at RT on an Applied Precision Personal DC live-cell imaging system, using the 60× lens and the CoolSnap HQ2 CCD camera operated by Softworx software. Acquired images were further analyzed by Image J.

All studies were repeated 3–12 times.


1. Chen Y, Park YB, Patel E, Silverman GJ (2009) IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol* 182(10):6031–6043.
2. Inaba K, et al. (1998) Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J Exp Med* 188(11): 2163–2173.
3. Chen Y, et al. (2009) Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol* 183(2):1346–1359.
4. Abe M, et al. (1994) An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 216(2):276–284.

5. Monestier M, Novick KE (1996) Specificities and genetic characteristics of nucleosome-reactive antibodies from autoimmune mice. *Mol Immunol* 33(1): 89–99.

6. Vas J, Grönwall C, Marshak-Rothstein A, Silverman GJ (2012) Natural antibody to apoptotic cell membranes inhibits the proinflammatory properties of lupus autoantibody immune complexes. *Arthritis Rheum* 64(10):3388–3398.

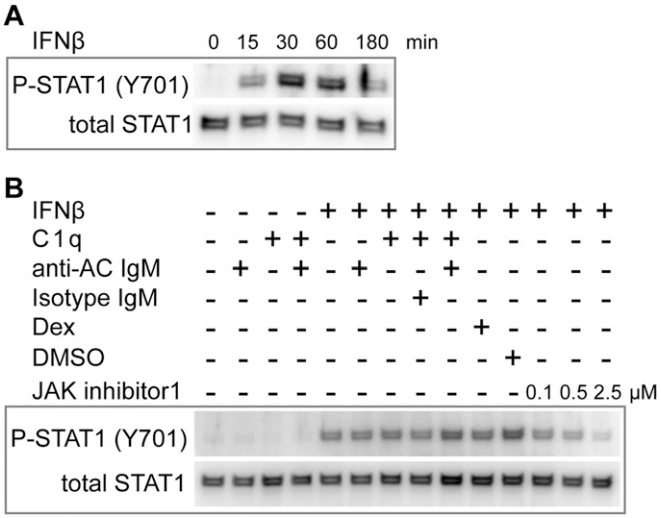


**Fig. S1.** Anti-AC IgM does not inhibit IFN type I-mediated phosphorylation of STAT1. BM-DCs cultured in serum-free media and stimulated with IFNβ (300 IU/mL; PBL InterferonSource) and Janus-activated linase 1 (JAK1)/tyrosine kinase 2 (TYK2) activity was assessed by immunoblot for phosphorylated STAT1. (*A*) Kinetic studies showed a peak of activation at 30 min after addition of IFNβ. (*B*) BM-DC cultures were incubated with anti-AC IgM (20 μg/mL), isotype control IgM (20 μg/mL), C1q (80 μg/mL), dexamethasone (1 μM), the pan JAK inhibitor JAK inhibitor 1 (0.1, 0.5, or 2.5 μM; Calbiochem), or vehicle (DMSO) 30 min before stimulation with IFNβ for 30 min at indicated conditions.

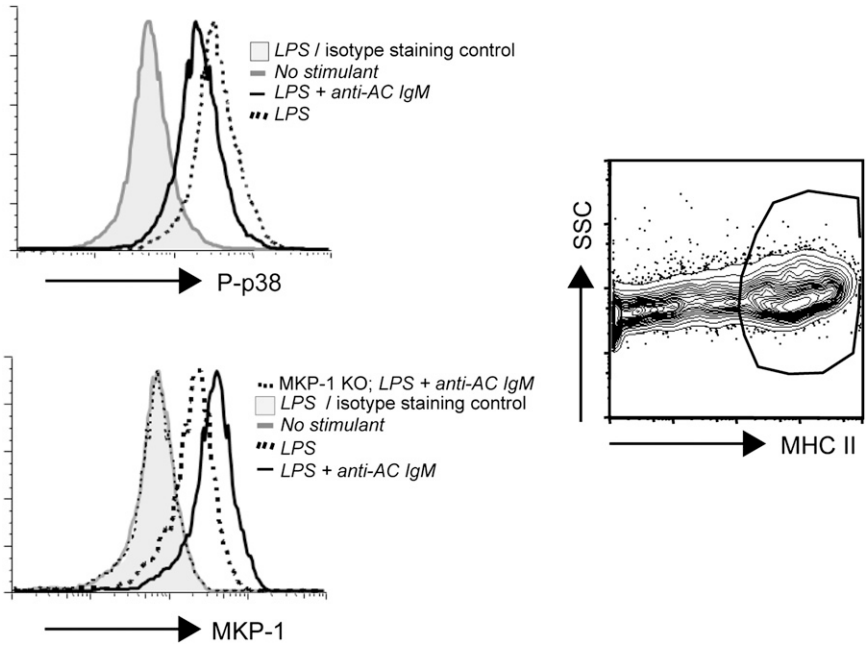


**Fig. S2.** Intracellular flow cytometry for P-p38 and MKP-1 in BM-DCs. Intracytoplasmic P-p38 or MKP-1 expression in BM-DCs after anti-AC IgM and LPS. As previously described MHC II high DCs were most responsive to LPS stimulation (6). Flow cytometry plot of MHCII-high DC gate (*Right*). Overlaid histograms of P-p38 (*Upper Left*) or MKP-1 expression (*Lower Left*) in the gated DC subset. The specificity of the anti–MKP-1 detection antibody was confirmed by lack of reactivity of DCs from MKP-1 KO mice (*Lower Left*). DCs were stimulated for 30 min in replicate serum-free cultures with conditions and detection reagents as indicated. C1q (80 μg/mL) was included in all cultures with IgM, and DCs were from wild-type mice unless indicated.

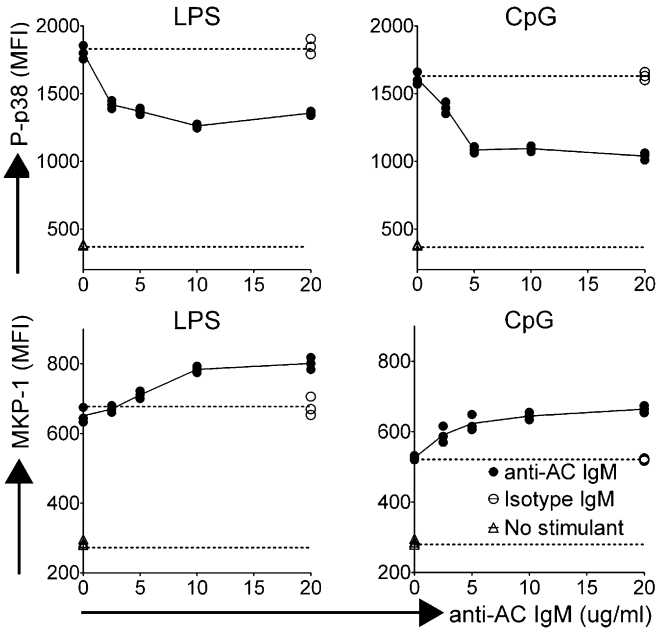


**Fig. S3.** Anti-AC IgM displays concentration-dependent modulation of TLR-induced P-p38 MAPK inhibition and MKP-1 induction. Intracellular flow cytometry for P-p38 and MKP-1 in BM-DCs. Intracellular P-p38 or MKP-1 expression in BM-DCs after anti-AC IgM and TLR ligand. Values for each of the triplicate cultures at 30 min are depicted. Higher concentrations of IgM anti-AC to TLR-stimulated DCs exhibited a direct dose–response relationship with induction of higher MKP-1 levels, and with inversely less p38 activation. These effects reach a plateau above an anti-AC IgM of ~10 μg/mL.

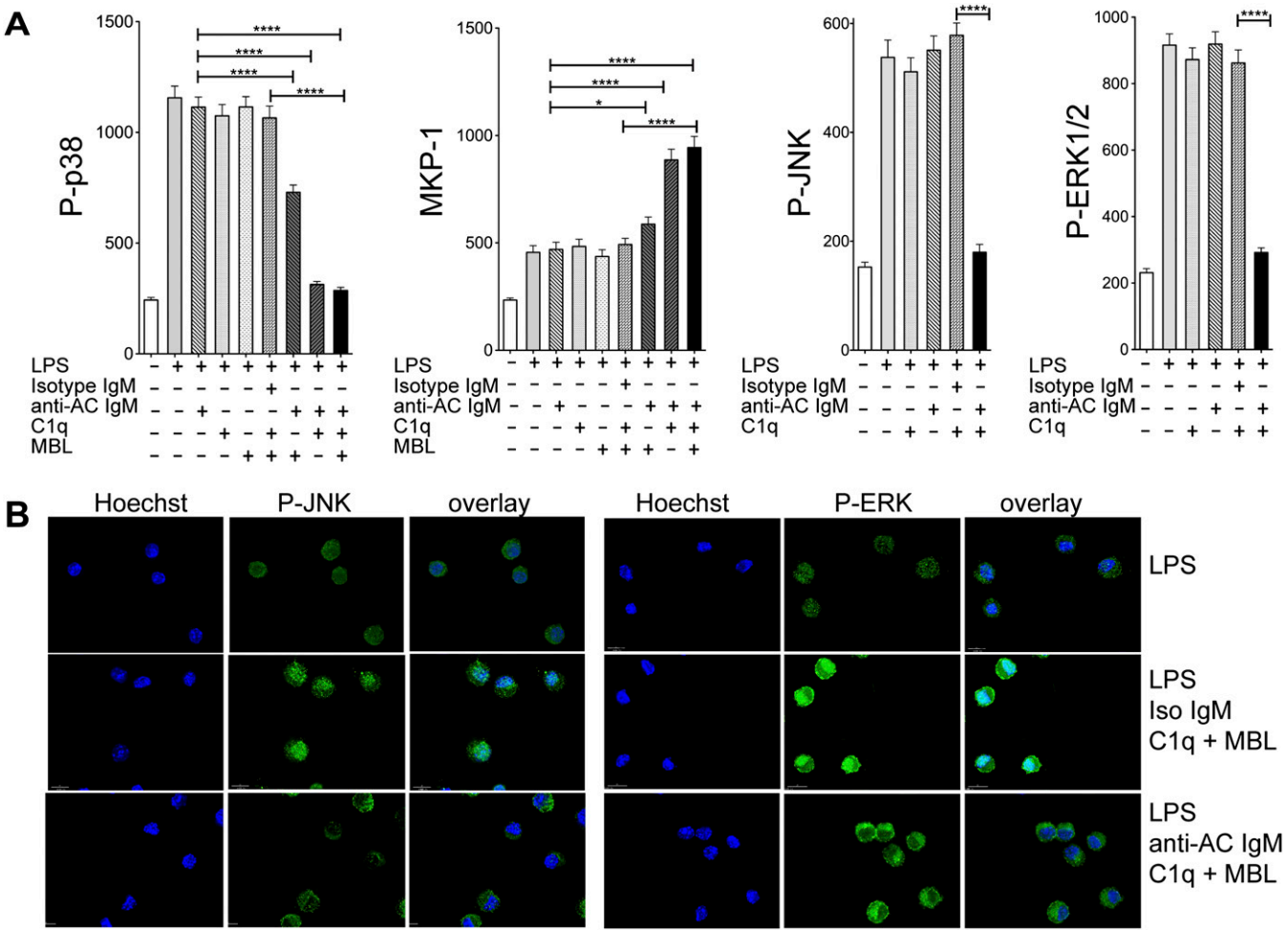


**Fig. S4.** Anti-AC IgM requires C1q and/or MBL for induction of nuclear MKP-1 and inhibition of nuclear localized P-p38, P-JNK, and P-ERK1/2 in LPS-stimulated DCs. (*A*) Immunofluorescence intensity (measured with SoftWorx 4.0.0) levels of P-p38 and MKP-1 in the nuclei of DCs from studies shown in Fig. 3. Values are means ± SD for 30 DCs. (*B*) Intracellular immunofluorescence studies for P-JNK and P-ERK were performed on LPS-stimulated DCs cultured in serum-free media for 30 min under the indicated conditions. Staining used rabbit anti–P-JNK (catalog 4668; Cell Signaling) or rabbit anti–P-ERK1/2 (Thr202-Tyr204) (catalog 9101; Cell Signaling), washed and counterstained with anti-rabbit IgG-FITC (Santa Cruz) and Hoechst dye. Microscope and software used were the same as in Fig. 3. Error bars represent SD. $*P < 0.05$, $****P < 0.0001$.

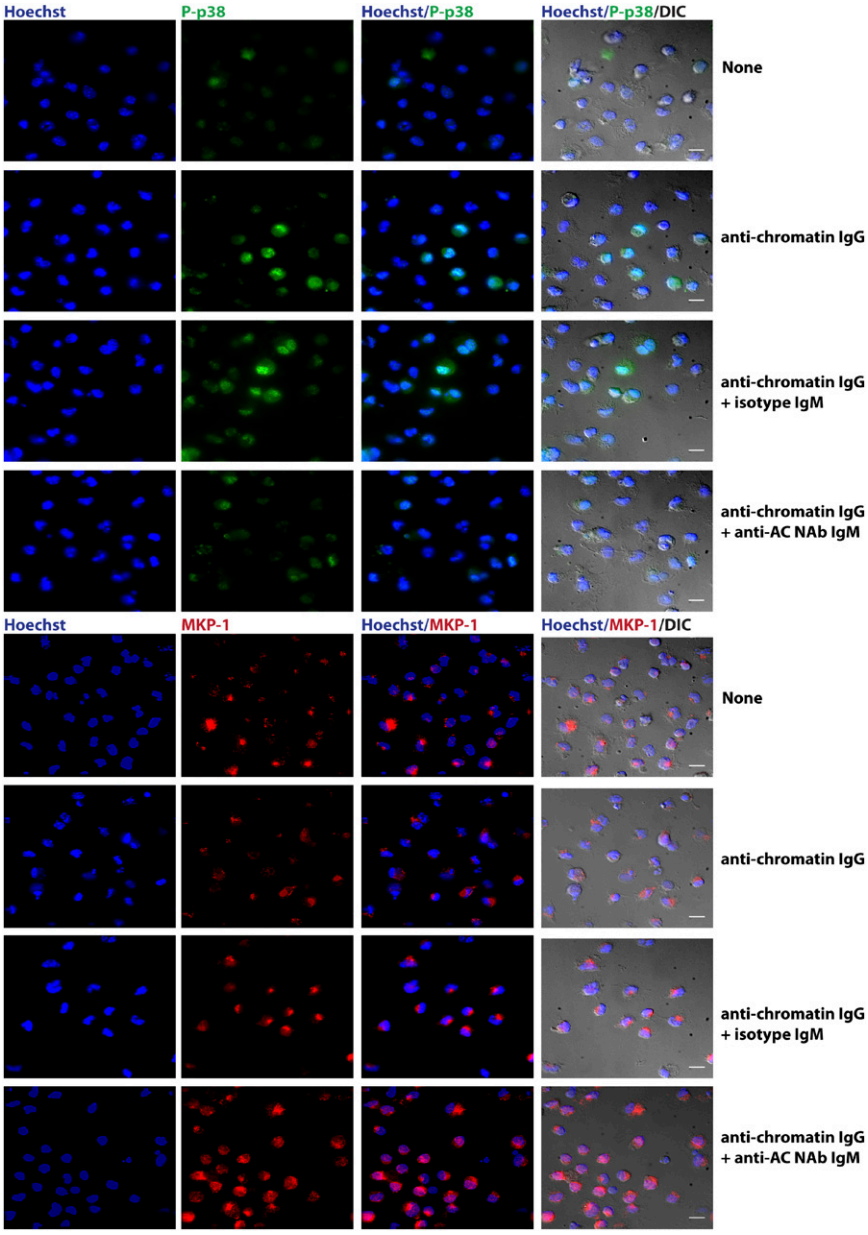


**Fig. S5.** IgM anti-AC reduces nuclear P-p38 in antichromatin IgG-stimulated cells. BM-DCs cultured in complete media and 30 μg/mL C1q. Isotype or anti-PC NAb IgM was added before stimulation with antichromatin IgG (8 μg/mL) for 30 min. DCs attached to poly-L-lysine–coated coverslips were stained for P-p38 FITC or rabbit anti–MKP-1 counterstained with anti-rabbit IgG-FITC (green) and Hoechst 34580 (blue) to identify the nucleus and imaged by fluorescent microscopy (*SI Materials and Methods*). DIC images were overlaid with FITC (P-38 or MKP-1) and Hoechst 34580 using Photoshop software. (Scale bar, 10 μM.) Data are representative of three independent experiments.

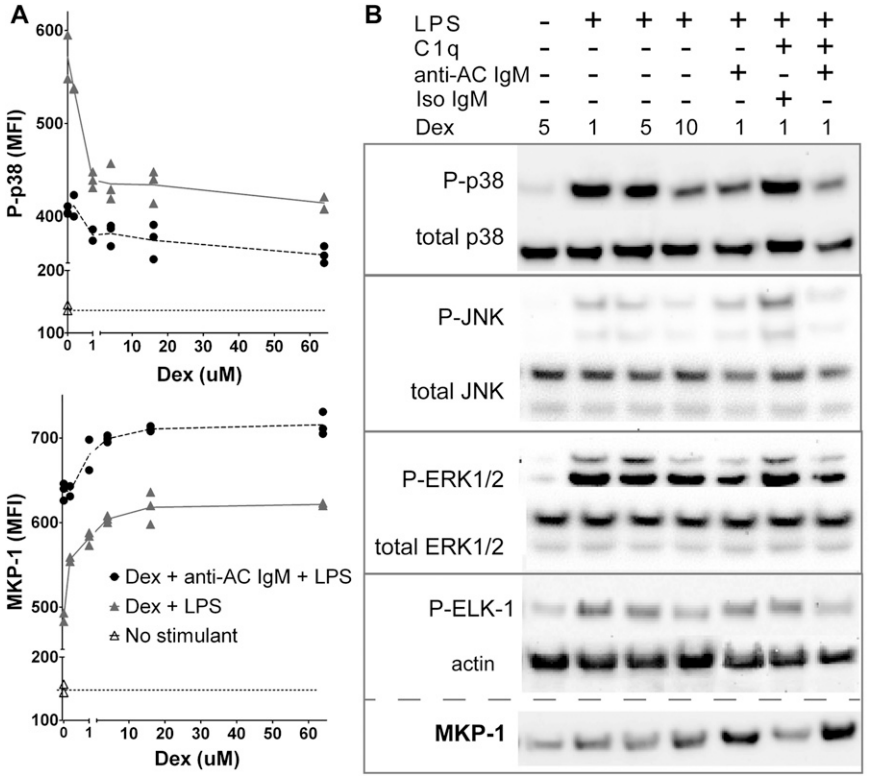


**Fig. S6.** Dexamethasone enhances anti-AC IgM-mediated induction of MKP-1 and inhibition of P-MAPK levels. Costimulation with a TLR4 agonist and dexamethasone together induced significantly earlier and higher peak MKP-1 levels than after stimulation with the TLR agonist alone. Addition of even modest doses of dexamethasone (i.e., 1 μM) to DCs cultured with anti-AC IgM and C1q caused significant further increases in MKP-1 expression along with inhibition of the activation of the primary MAPKs. Notably, coincubation of LPS with dexamethasone at concentrations up to 60 μM never attained the effects found with anti-AC IgM costimulation on either MKP-1 or P-p38 expression. (*A*) Flow cytometry studies demonstrate that dexamethasone induces an additive dose-dependent induction of MKP-1 and inhibition of P-p38. Values for replicate DC cultures in serum-free media are depicted ($n = 3$). C1q was added to all wells. (*B*) Immunoblot studies of DCs confirm the additive effects of dexamethasone on MAP kinase signaling. Methods were the same as in Fig. 1*A*. Data are representative of three independent sets of experiments.

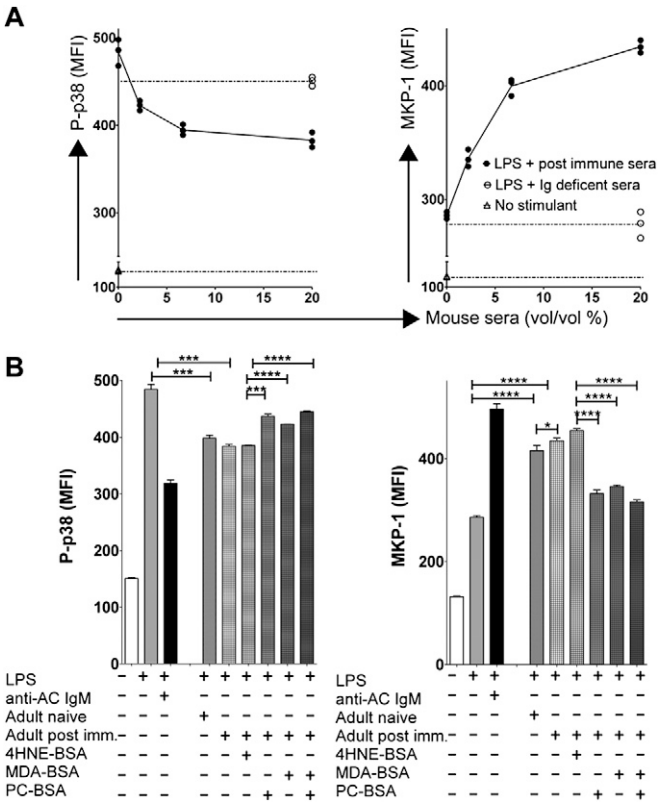


**Fig. S7.** Natural IgM antibodies with PC and MDA specificities in sera induce MKP-1 and inhibit P-p38 in LPS-stimulated BM-DCs. (*A*) Sera from adult mice inhibit P-p38 and induce MKP-1 in LPS-stimulated DCs. Coculture of LPS-stimulated DCs in serum-free media with C1q (80 μg/mL) for 30 min without or with the addition of titrated percentages of sera (%vol/vol) from 14-wk-old adult C57BL/6 immunized with AC or Ig-deficient mice. (*B*) Coculture of LPS-stimulated DCs in serum-free media with C1q (80 μg/mL) for 30 min without or with the addition of sera from 14-wk-old adult naive C57BL/6 mice, mice immunized with AC, or Ig-deficient mice at indicated conditions. PC and MDA specificities in adult sera promote induction of MKP-1 and inhibition of P-p38. Replicates included serum (20% vol/vol) after incubation for 1 h at 4 °C with PC-BSA, MDA-BSA, or 4HNE-BSA at 50 μg/mL, T15-IgM or isotype control (20 μg/mL), or C1q (80 μg/mL). Intracellular flow cytometric mean fluorescence intensity (MFI) values are depicted for individual replicate cultures ($n = 3$). Error bars indicate SD. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

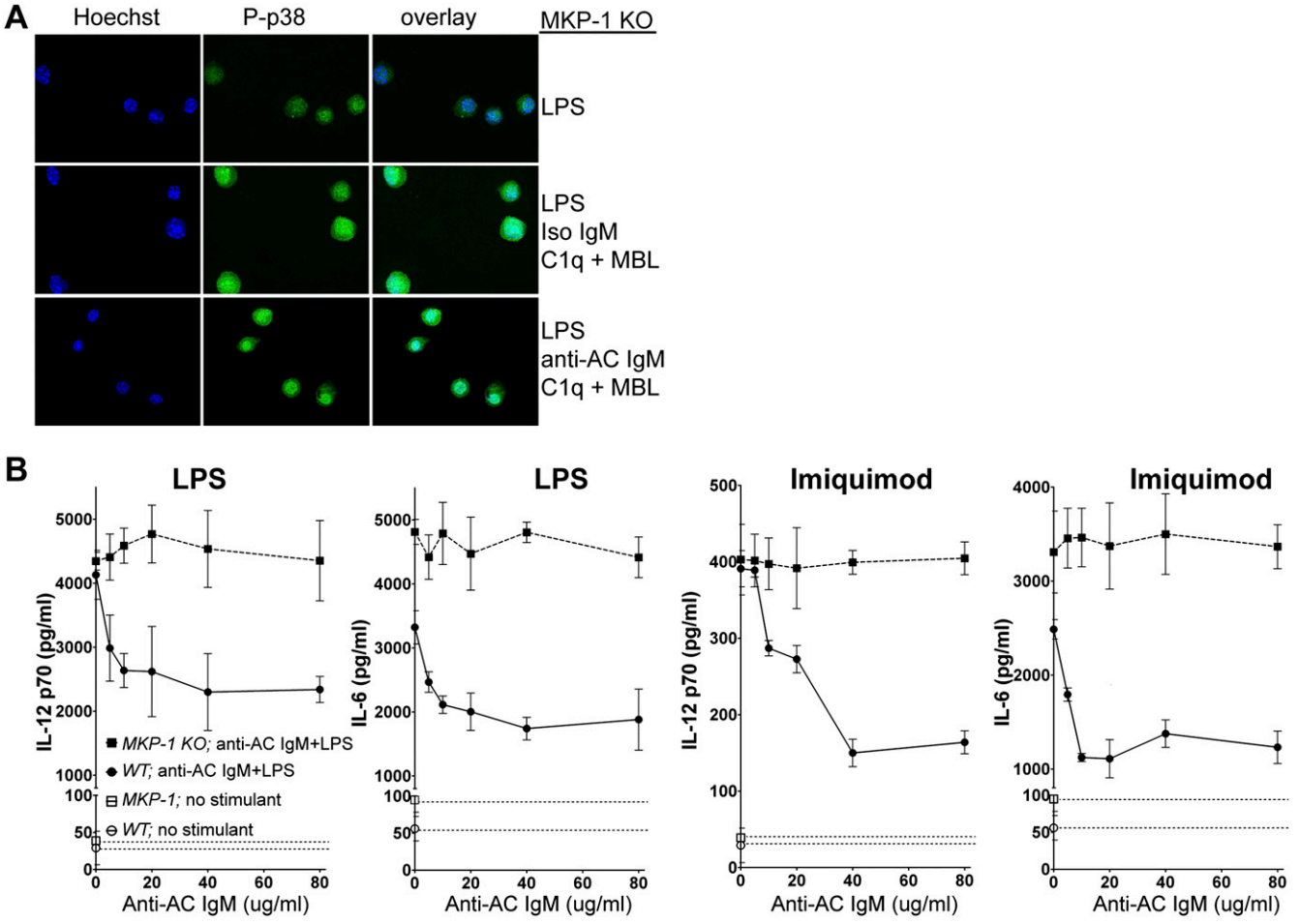


**Fig. S8.** MKP-1 is required for anti-AC IgM-mediated inhibition of TLR signaling and inflammatory cytokine production. (*A*) Immunofluorescence of MKP-1–deficient DCs, cultured for 30 min in serum-free media, under indicated conditions. Studies were performed as shown in Fig. 3. Anti-AC IgM and C1q of LPS-stimulated MKP-1–deficient DCs inhibited neither the induction of high levels nor the nuclear localization of P-p38 induced by LPS, compared with the responses of wild-type DCs (Fig. 3). (*B*) Inflammatory cytokine production after 48 h in culture with different levels of IgM anti-AC under indicated conditions ($n = 3$ for each condition). These studies demonstrated that, in MKP-1–deficient DCs, even high concentrations of IgM anti-AC (i.e., 60 μg/mL) did not inhibit production of IL-6 or IL-12p70. Error bars represent SEM.