SUPPORTING INFORMATION

p38γ and p38δ kinases regulate TLR4-induced cytokine production by controlling ERK1/2 pathway activation

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SI Materials and Methods

Antibodies, reagents and mice. Anti-p38β antibody was purchased from Zymed (Cambridge Bioscience, UK). Anti-p38γ and -p38δ antibodies for immunoprecipitation and immunoblotting were raised and purified as described (1). Antibodies for total and phosphorylated forms of ERK1/2, MKK1 and NFκB1 p105, as well as antibodies to total JNK1/2, total IκBα, TNFα and phosphorylated p38MAPK were from Cell Signaling Technology. Anti-TPL-2, -β-actin and -p38α were from Santa Cruz, anti-phosphorylated JNK1/2 from Biosource, and anti-β-tubulin from Zymed (Cambridge Bioscience, UK). All antibodies used in flow cytometry analysis (anti-Gr1 (RB6-8C5), -CD11b (M1/70), -F4/80 (BM8)) were from BD PharMingen). PD184352 was made by custom synthesis (2).

Escherichia coli lipopolysaccharide (LPS) and D-galactosamine (D-gal) were purchased from Sigma. Protesome inhibitor I (PSI) was from Calbiochem (Nottingham, UK).

All DNA constructs and proteins used in this study are described elsewhere (4-6).

Mice lacking p38γ, p38δ and p38γ/δ have been described (6). All strains were backcrossed onto the C57BL/6 strain for at least nine generations. Mice were housed in specific pathogen-free conditions in accordance with European Union regulations; work was approved by the CNB/CSIC ethical review.

Bone marrow-derived dendritic cells (BMDC) cultured. BMDC were cultured following a modified protocol (3). In brief, total bone marrow cells were grown on bacteria-grade plastic dishes, in RPMI with 10% FBS and 20 ng/ml GM-CSF (Peprotech; Rocky Hill, NJ) at an initial density of 0.5 x 10^6 cells/ml. Every 3 days, non-adherent cells were removed and replated in the same conditions. After 9 days, the cultures were enriched in dendritic cells by magnetic purification using anti-CD11c-biotin antibody (BD PharMingen; San Diego, CA) and avidin-MACS (Miltenyi; Bergisch Gladbach, Germany). Final purity of the cultures was >95% based on FACS analysis using an anti-CD11c-APC antibody (eBioscience, Hatfield, UK). These dendritic cell cultures were seeded at a constant density of 0.25 x 10^6 cells/ml in non-tissue-culture treated p24 plates in RPMI/10% FBS.

Immunoprecipitation and immunoblotting. BMDM extracts (15 mg) were incubated with 2 μg anti-p38γ or -p38δ antibody coupled to protein G-Sepharose. After incubation (2 h, 4°C), captured proteins were centrifuged (13,000 x g), supernatants discarded and the beads washed twice in buffer A (see Methods) containing 0.5 M NaCl, then twice in buffer A alone. For immunoblotting, protein samples were resolved in SDS-PAGE and transferred to nitrocellulose membranes, which were blocked (30 min) in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 0.05% (v/v) Tween (TBST buffer) containing 10% (w/v) non-fat dry milk, then incubated in TBST buffer with 10% (w/v) non-fat dry milk and 0.5-1 μg/ml antibody (2 h, room temperature or overnight, 4°C). Protein was detected using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Cytokine quantification and Flow cytometry. For BMDC cytokine measurements, we used OptEIA Set ELISA kits for TNFα, IL1-1β, IL-6, IL-10 and IL-12p70 (BD Biosciences). IFNβ levels were detected using a Verikine IFN-Beta ELISA kit (PBL

Interferon Source). Cytokine concentrations in BMDM supernatants and mouse serum samples were measured using the Luminex-based MilliPlex Mouse cytokine/chemokine immunoassay and the Luminex-based Bio-Plex Mouse Grp I Cytokine 23-Plex Panel (Bio-Rad). Antibodies used were PE-conjugated, and cells were stained at saturating concentrations (4°C). Cells were analyzed on an EPICS XL with System II software (Beckman Coulter).

Gene expression analysis. cDNA for real-time quantitative PCR (qPCR) was generated from 1 μg total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 10 μl final reaction volume. Real-time qPCR reactions were performed in triplicate using 3 μl/well of two serial dilutions (1/50, 1/500) of each cDNA, 0.3 μM of each primer, and 1x Fluocycle SYBR Green Mix for real-time qPCR (Genycell-EuroClone) in a volume of 8 μl in MicroAmp Optical 384-well plates (Applied Biosystems). PCR reactions were carried out in an ABI PRISM 7900HT (Applied Biosystems) and SDS v2.2 software was used to analyze results by the Comparative Ct Method (ΔΔCt). X-fold change in mRNA expression was quantified relative to unstimulated wild-type cells, and 18S or GAPDH RNA was used as loading control.

Primer sequences used for quantitative real-time PCR. *p38α*, forward 5'-AACCAGACAGTGGATATTTGGTC and reverse 5'- TGAGCTTCAACTGATCA-ATATGGT; *p38β*, forward 5'-GTCCTGAAGTTCTGGCAAAGA and reverse 5'-CACTGATGAGGTCCTTCTGG; *p38γ*, forward 5'-ACCTGATGAGTCTCTGGACGA and reverse 5'-CCAGATCAGTGCCCATGAAT; *p38δ*, forward 5'-GGACCCTGAGG-AGGAGACA and reverse 5'-GTTTGAGATCTCTTTGTAGATGTGTTG; *TPL-2*, forward 5'-CAACTTCCTGAGAACATTGCTG and reverse 5'-CGCTGTCTCCTGA-GCACTT; *IL-6*, forward 5'-GCTACCAAACTGGATATAATCAGG and reverse 5'-

CAGGTAGCTATGGTACTCCAGAA; *IL10*, forward 5'-AGGCGCTGTCATCGATT-TCTC and reverse 5'-TGGCCTTGTAGACACCTTGGTC; *IL12(p35)*, forward 5'-AAGAACGAGAGTTGCCTGGCT and reverse 5'-TTGATGGCCTGGAACTCTGTC; *IL12(p40)*, forward 5'-GGAAGCACGGCAGCAGAATA and reverse 5'-AACTTGA-GGGAGAAGTAGGAATGG; *IL1β*, forward 5'-TTGACGGACCCCAAAAGAT and reverse 5'-GAAGCTGGATGCTCTCATCTG; *TNFα*, forward 5'-CTGTAGCCCAC-GTCGTAGC and reverse 5'-TTGAGATCCATGCCGTTG; *IFNβ*, forward 5'-GGAA-AAGCAAGAGGAAAGATTGAC and reverse 5'-CCACCATCCAGGCGTAGC; *ABIN-2*, forward 5'-GACGCACTTCTGGATCAGGT and reverse 5'-CGCTCCGTAA-GTCTTTCAACTT; *18S*, forward 5'-CGCGGTTCTATTTTGTTGGT and reverse 5'-AGTCGGCATCGTTTATGGTC; *GAPDH*, forward 5'-GCCTTCCGTGTTCCTACCC and reverse 5'-TGCCTGCTTCACCACCTTC.

Statistical analysis. Data were expressed as mean ± SD. Statistical analysis was performed with Student's t test for two samples. Kaplan-Meier analysis was used for survival analyses. Values of $p < 0.05$ were considered to be significant.

SI References

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2. Kuma Y, et al. (2005) BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *J Biol Chem* 280, 19472-19479.

3. Inaba K, *et al.* (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176(6):1693-1702.
4. Cerezo-Guisado MI, *et al.* (2011) Evidence of p38gamma and p38delta involvement in cell transformation processes. *Carcinogenesis* 32(7):1093-1099.
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6. Sabio G, *et al.* (2005) p38gamma regulates the localisation of SAP97 in the cytoskeleton by modulating its interaction with GKAP. *Embo J* 24(6):1134-1145.

SI Figure Legends

Figure S1. Expression of p38MAPK isoforms in BMDM. (**A**) Cell extracts from WT, p38$\gamma^{-/-}$, p38$\delta^{-/-}$ and p38$\gamma/\delta^{-/-}$ BMDM were examined by immunoblot using anti-p38α and -p38β antibodies. Duplicate lanes are shown. (**B**) Endogenous p38γ or p38δ were immunoprecipitated from 15 mg of WT BMDM extracts and p38 protein in the pellet was immunoblotted with anti-p38γ or -p38δ antibodies. Total tubulin protein was used as a loading control. Representative blots are shown from three independent experiments. The p38α isoform was most abundant, followed by p38β, p38δ, and finally p38γ, which was expressed at very low levels (see also Table S1). Moreover, the level of p38β was approximately two-fold higher in p38$\gamma^{-/-}$ and p38$\gamma/\delta^{-/-}$ macrophages than in WT or p38$\delta^{-/-}$ cells (see also Table S1), indicating that p38β expression might be negatively regulated by p38γ. In BMDC, p38δ was the most abundant isoform after p38α.

Figure S2. Lack of p38γ and p38δ do not affect *in vitro* BMDM or BMDC development. (**A**) BMDM or (**B**) BMDC from WT and p38γ/δ$^{-/-}$ mice were stained with anti-F4/80 or -CD11c antibody, respectively, and analyzed by flow cytometry. Results are representative plots ($n \geq 3$). White histograms show isotype control.

Figure S3. BMDM cytokine production from p38γ and p38δ-deficient mice in response to LPS. **(A)** BMDM from WT (black bars), p38γ$^{-/-}$ (dark grey) or p38δ$^{-/-}$ (light grey) mice were exposed to LPS (100 ng/ml) for the indicated times and culture supernatants harvested for luminex cytokine analysis of TNFα, IL-1β, IL-10, IL-6 and IL-12(p70). Values show mean ± SD for one representative experiment of three performed in duplicate. **(B)** Quantitative PCR of TNFα, IL-1β, IL-10, IL-6, IL-12(p35) and IL-12(p40) mRNA in total RNA from WT (black bars), p38γ$^{-/-}$ (dark grey) or p38δ$^{-/-}$ (light grey) BMDM stimulated with LPS (100 ng/ml) for the indicated times. Results were normalized to 18S RNA expression and x-fold induction was calculated relative to WT expression at 0 h. Data show mean ± SD from one representative experiment of two in triplicate, with similar results. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ relative to WT BMDM exposed to LPS, in each time.

Figure S4. p38γ/δ deficiency in BMDC affects cytokine production in response to LPS. (**A**) BMDC from WT (black bars) or p38γ/δ$^{-/-}$ mice (white) were exposed to LPS (100 ng/ml) for the indicated times and culture supernatants harvested for ELISA of TNFα, IL-1β, IL-10, IL-6 and IL-12(p70). Data are mean ± SD of one representative experiment of three performed. (**B**) Quantitative PCR of TNFα, IL-1β, IL-10, IL-6, IL-12(p35) and IL-12(p40) mRNA in total RNA from WT (black bars) or p38γ/δ$^{-/-}$ (white)

BMDC stimulated with LPS (100 ng/ml) as indicated. Results were normalized to 18S RNA expression and x-fold induction was calculated relative to WT expression at 0 h. Data show mean ± SD of one representative experiment of two with similar results. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ relative to WT BMDM exposed to LPS, in each time.

Figure S5. Role of IL-10 in LPS-induced cytokine production. (**A**, **B**) BMDM from WT (black bars) or p38γ/δ$^{-/-}$ mice (white) were exposed to LPS (100 ng/ml) alone or in the presence of neutralizing antibody to IL-10 (Anti-IL10) or an isotype control antibody (IgG) (both at 1 μg/ml, 12 h); culture supernatants were harvested for luminex cytokine analysis of (**A**) IL-10 and (**B**) TNFα. Data are mean ± SD of one representative experiment of two with similar results. Real-time qPCR analysis of (**A**) *IL-10* and (**B**) *Tnfα* mRNA in total RNA from WT (black bars) or p38γ/δ$^{-/-}$ (white) BMDM stimulated as before for the indicated times. Results show mean ± SD of triplicate wells normalized to GAPDH mRNA. (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (***) $p \leq 0.001$ relative to WT BMDM in each experimental condition.

Figure S6. Deletion of p38γ and p38δ blocks LPS activation of ERK1/2 in BMDC. BMDC from WT or p38γ/δ$^{-/-}$ mice were unstimulated or stimulated with 100 ng/ml LPS for the indicated times and cell lysates immunoblotted with anti-P-ERK1/2, -ERK1/2, -IκBα, -P-p38α, -P-JNK1/2, anti-JNK1/2, anti-TPL2, anti-ABIN2 and -β-actin (loading control). Representative blots are shown from two independent experiments.

Figure S7. Deletion of p38γ and p38δ decreases TPL-2 protein levels in BMDM. BMDM from WT, p38γ$^{-/-}$, p38δ$^{-/-}$ or p38γ/δ$^{-/-}$ mice were stimulated with 100 ng/ml LPS

for the times indicated. Following cell lysis and SDS-PAGE, immunoblotting was carried with antibodies to total TPL-2 protein and tubulin (loading control). TPL-2 activation after LPS treatment is followed by degradation of its "long" form by the proteasome.

Figure S8. ERK1/2 pathway regulates cytokine production. WT BMDM were preincubated with PD184352 (2 μM) or DMSO (vehicle) and then cells were stimulated with 100 ng/ml LPS for (**A**) 6 h, and TNFα, IL-1β, IL-12(p70), IL-10 and IL-6 levels were measured in a Luminex-based assay; or (**B**) for 1 or 6 h and total RNA was isolated. TNFα, IL-1β, IL-12(p35), IL-10 and IL-6 mRNA expression analyzed by qPCR. Results are normalized for 18S RNA quantities. Data show mean ± SEM of three independent stimulations.

Figure S9. Bone marrow-derived macrophages from TPL-2-deficient mice show altered cytokine production in response to LPS. BMDM from WT (black bars) or TPL-$2^{-/-}$ (white) mice were exposed to LPS (10 ng/ml) for the indicated times and total RNA was isolated. TNFα, IL-1β, IL-10, IL-6, IL-12(p35) and IL-12(p40) mRNA expression was analyzed by qPCR. Target gene mRNA levels were normalized against *Hprt* mRNA levels. Each data point represents the average ± SEM for three independent macrophage pools. Experiments are representative of three independent experiments with consistent results. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ relative to WT BMDM exposed to LPS, in each time.

Figure S10. Effect of p38γ and p38δ deletion on cytokine production in response to LPS/D-Gal challenge. Mice received injections of LPS (50 mg/kg) and D-Gal (1 g/kg). Serum from WT (black bars), p38$\gamma^{-/-}$ (dark grey), p38$\delta^{-/-}$ (light grey) or p38$\gamma/\delta^{-/-}$ (white)

mice was collected 2 h after LPS and D-Gal challenge and cytokines measured in a Multiplex cytokine assay. Data show mean ± SD (n = 4-6 mice/group). (*) $p \leq 0.05$ relative to WT mouse serum.

Table S1. Comparative expression pattern of p38MAPKs mRNA in bone marrow derived macrophages (BMDM) and derived dendritic cells (BMDC)[a]

		p38 family member mRNA[b]			
		α	β	γ	δ
BMDM	Wild type	1.000 ± 0.104	0.100 ± 0.005	0.003 ± 0.0005	0.045 ± 0.0071
	p38γ -/-	1.077 ± 0.092	0.204 ± 0.0005	0	0.045 ± 0.0041
	p38δ -/-	0.661 ± 0.229	0.067 ± 0.033	0.0012 ± 0.0001	0
	p38γ/δ -/-	0.930 ± 0.106	0.173 ± 0.000	0	0
BMDC	Wild type	1.0000 ± 0.17203	0.0002 ± 0.00004	0.0021 ± 0.00030	0.0520 ± 0.02021

a. RT-PCR analysis was performed using p38MAPK isoforms specific primers as described in Methods.

b. Values represent the mean ± SD of determinations using RNA from three to four independent cell cultures. Values are relative to p38α mRNA in each cell type as reference (fold value = 1).

Figure S1 (Risco et al)

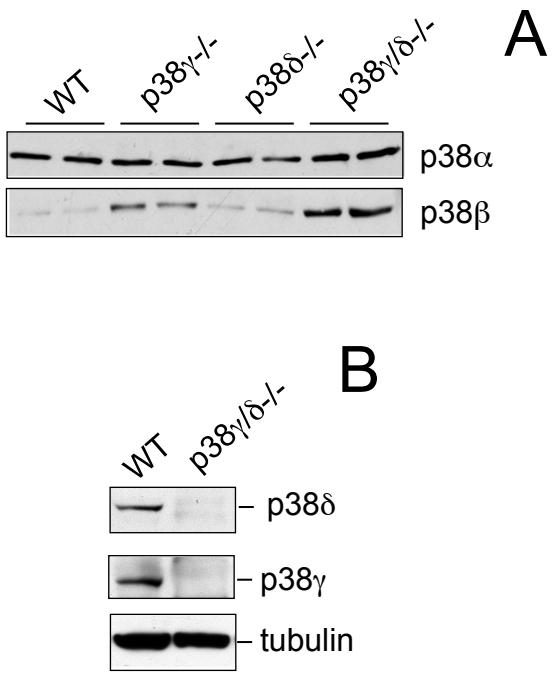


Figure S2 (Risco et al)

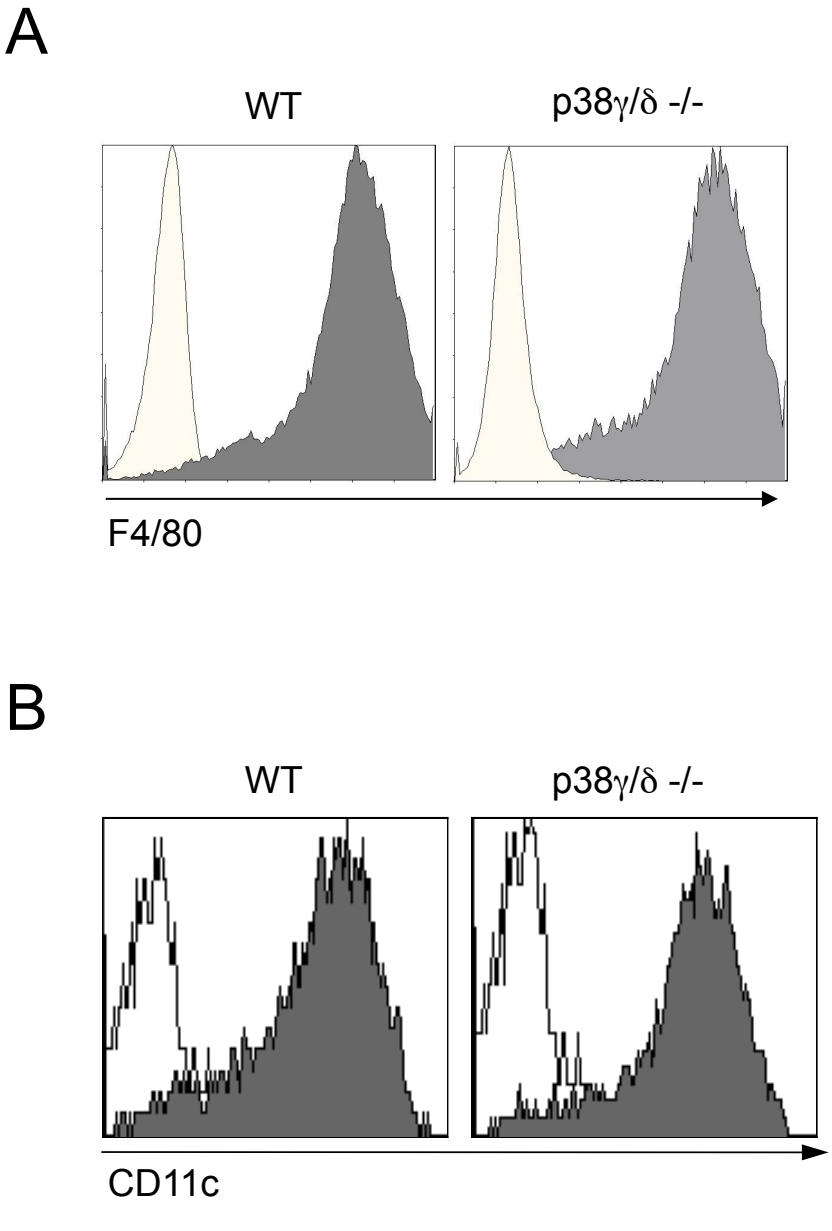
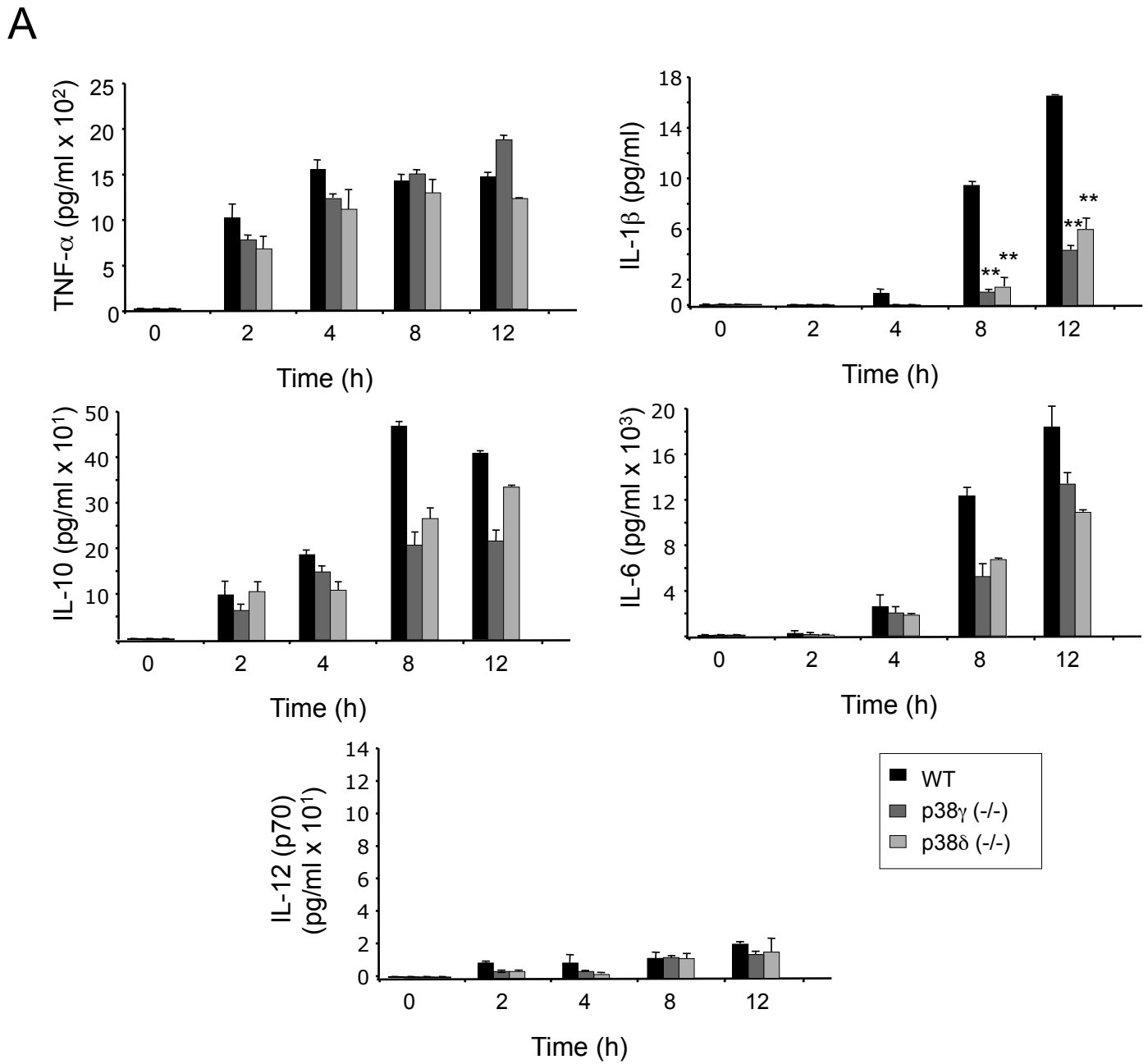


Figure S3 (Risco et al)



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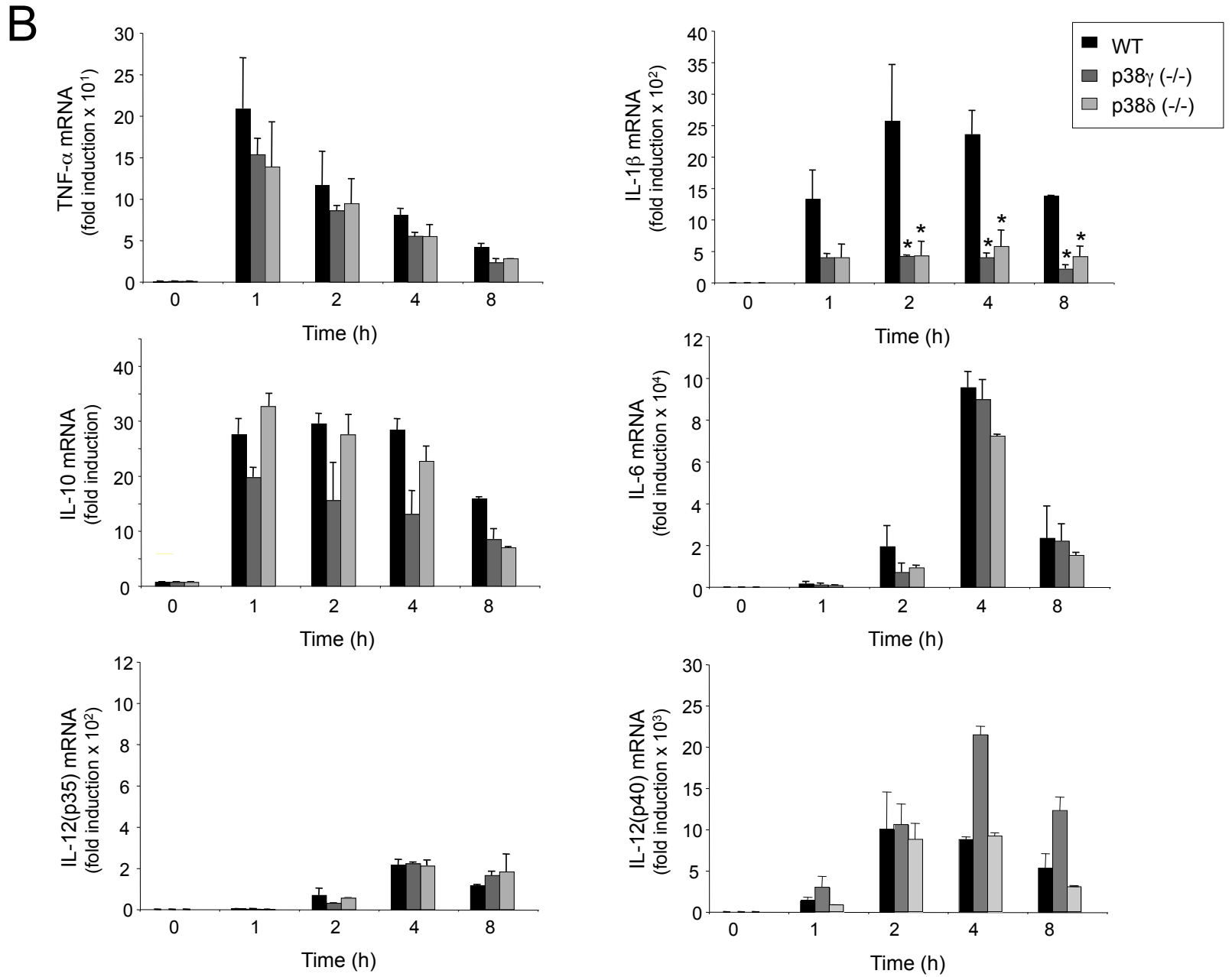

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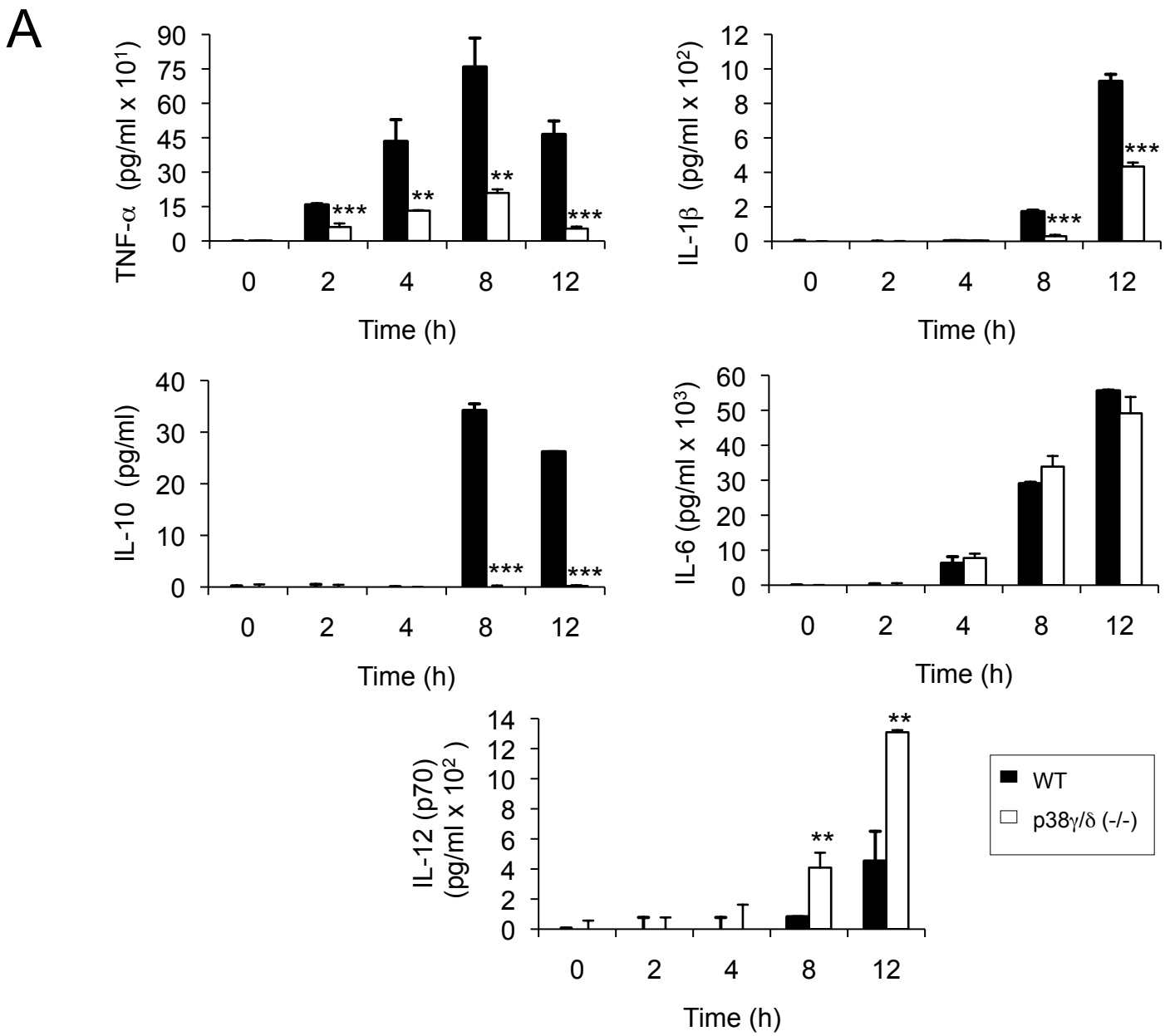

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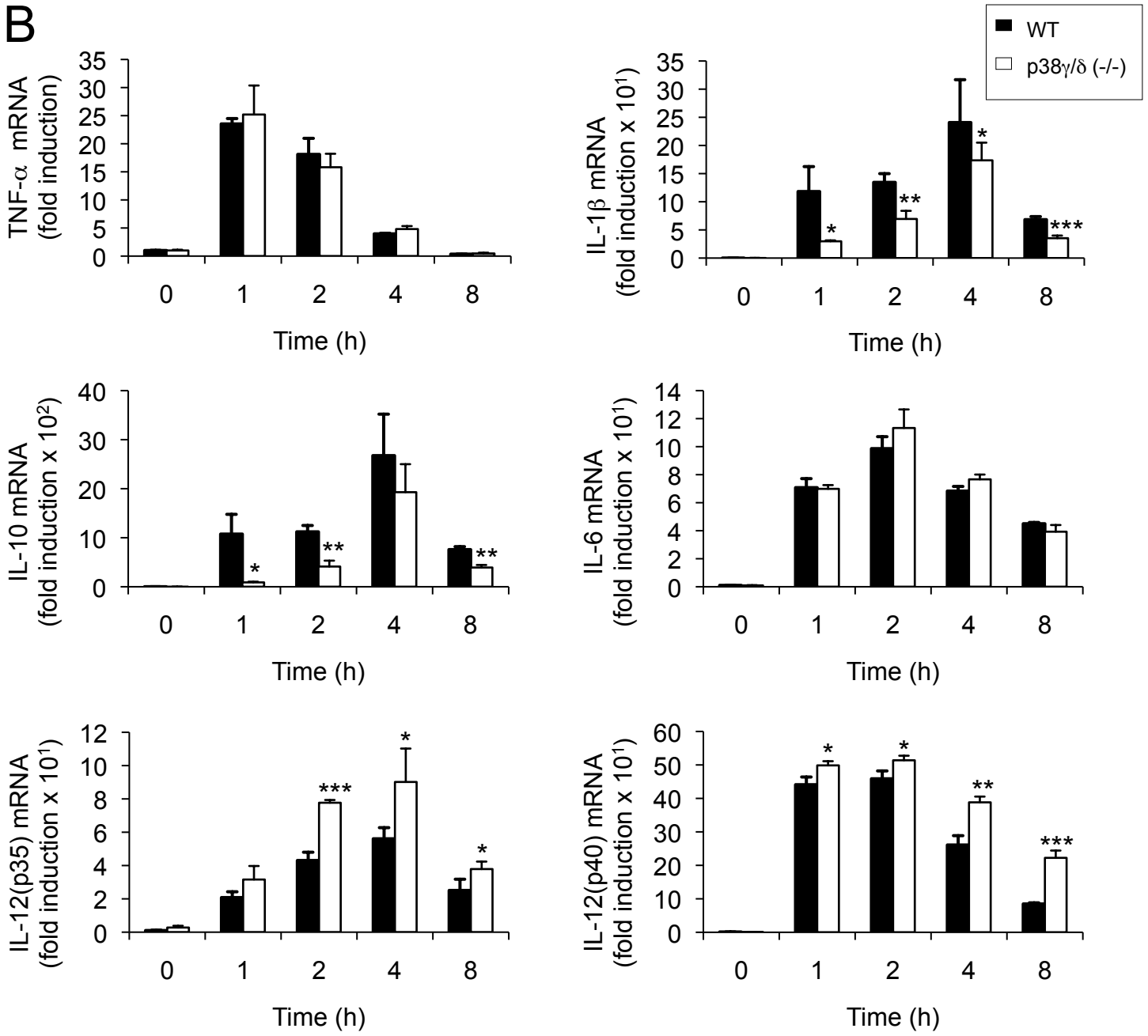
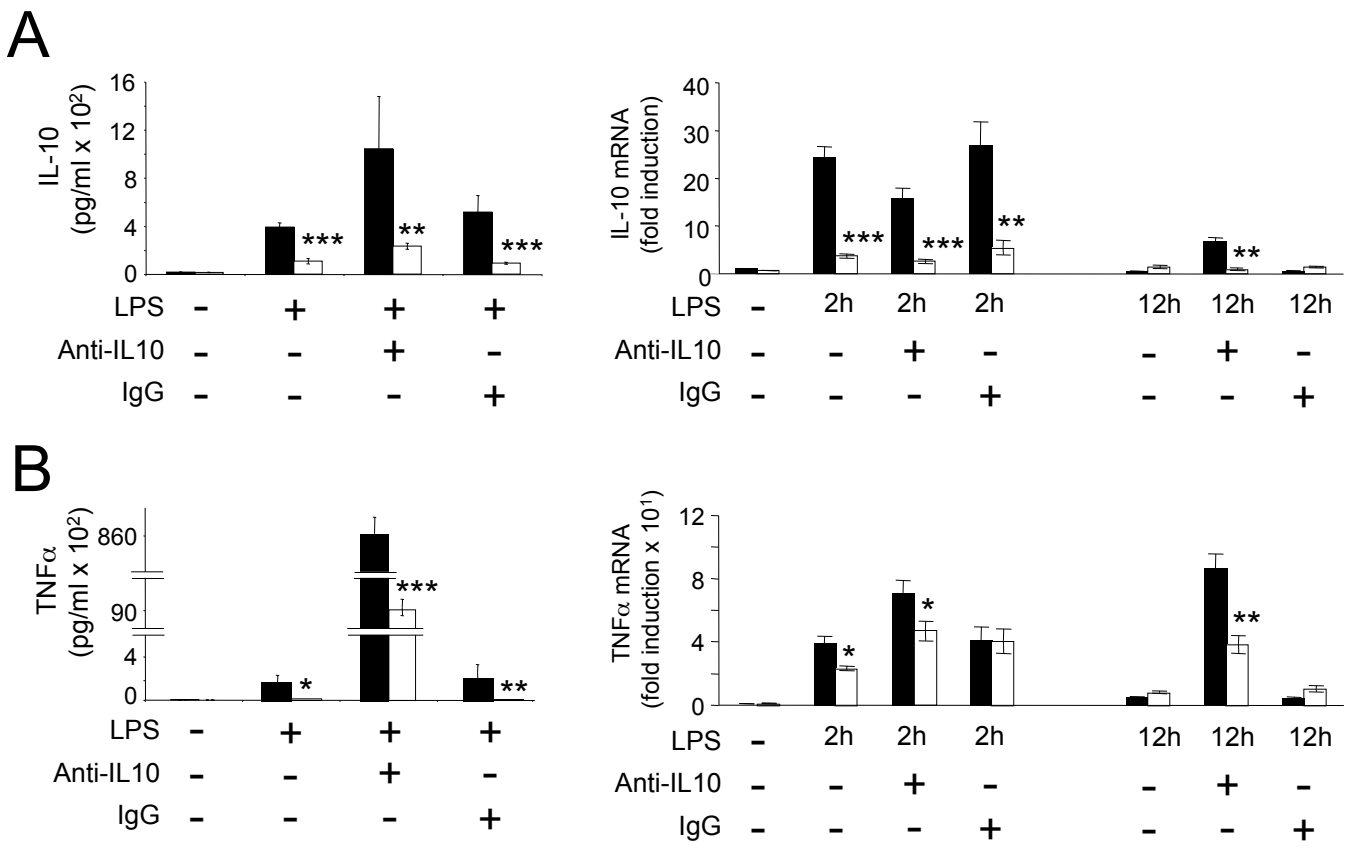


Figure S5 (Risco et al)



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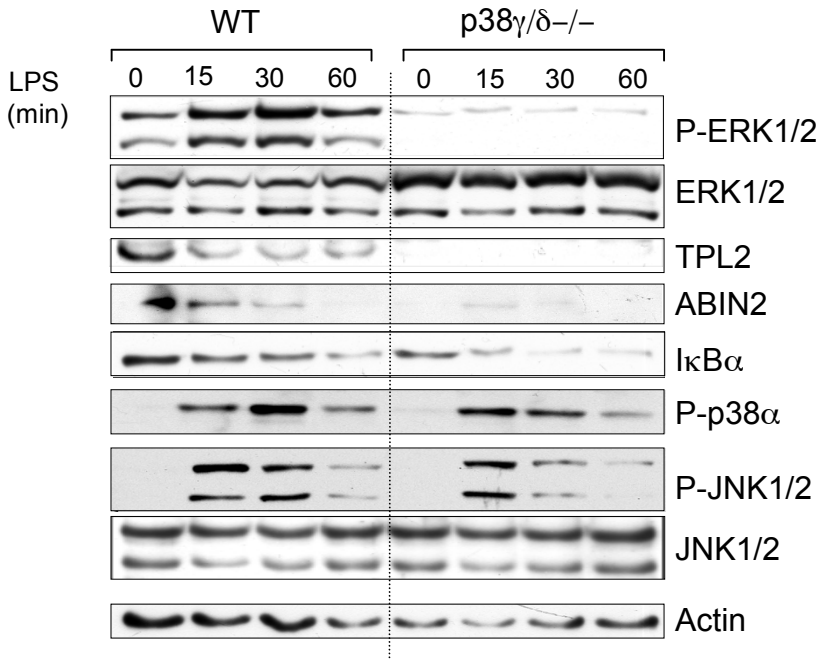

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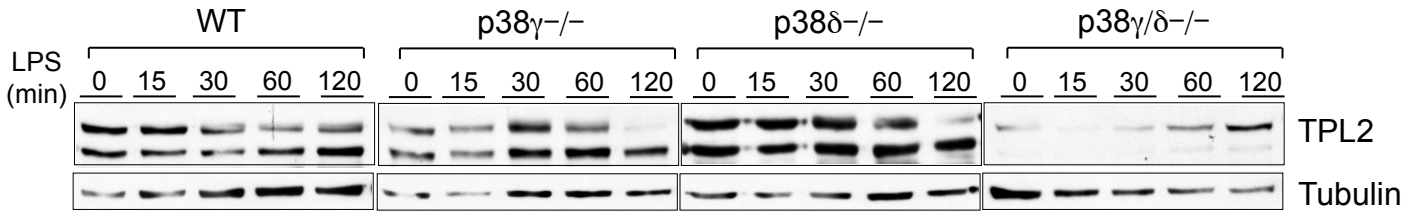

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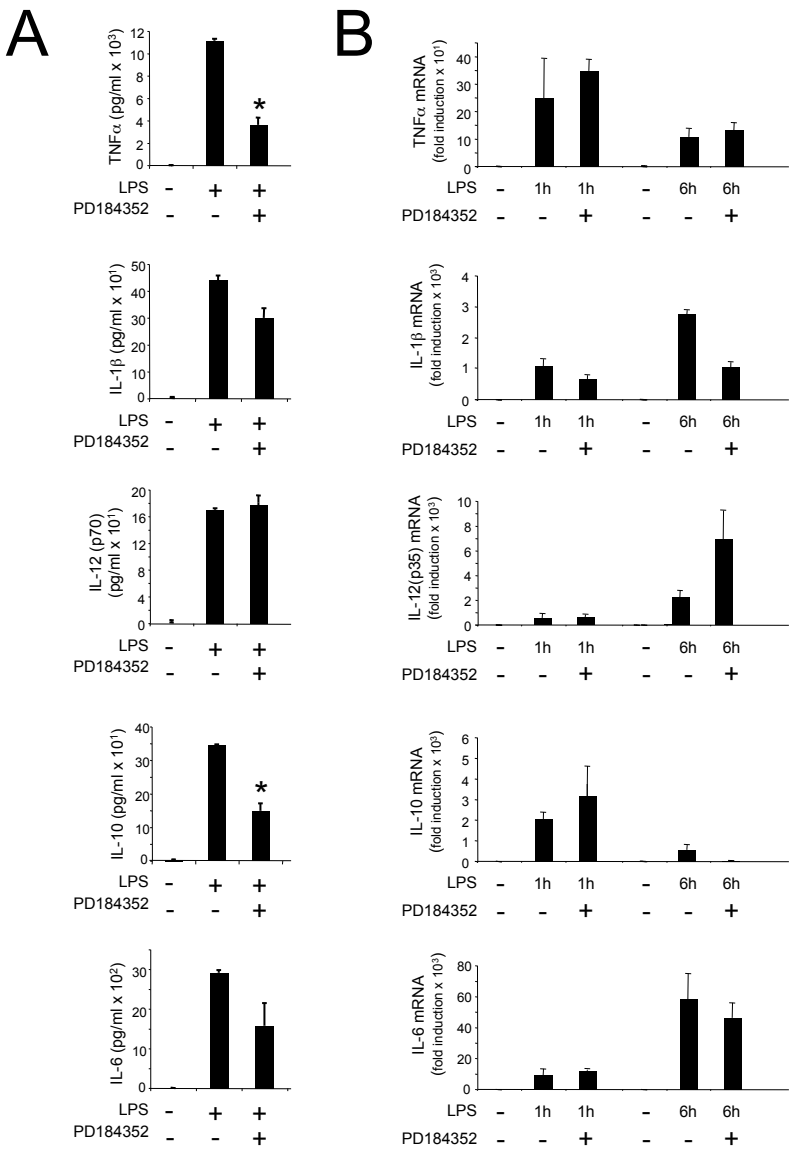

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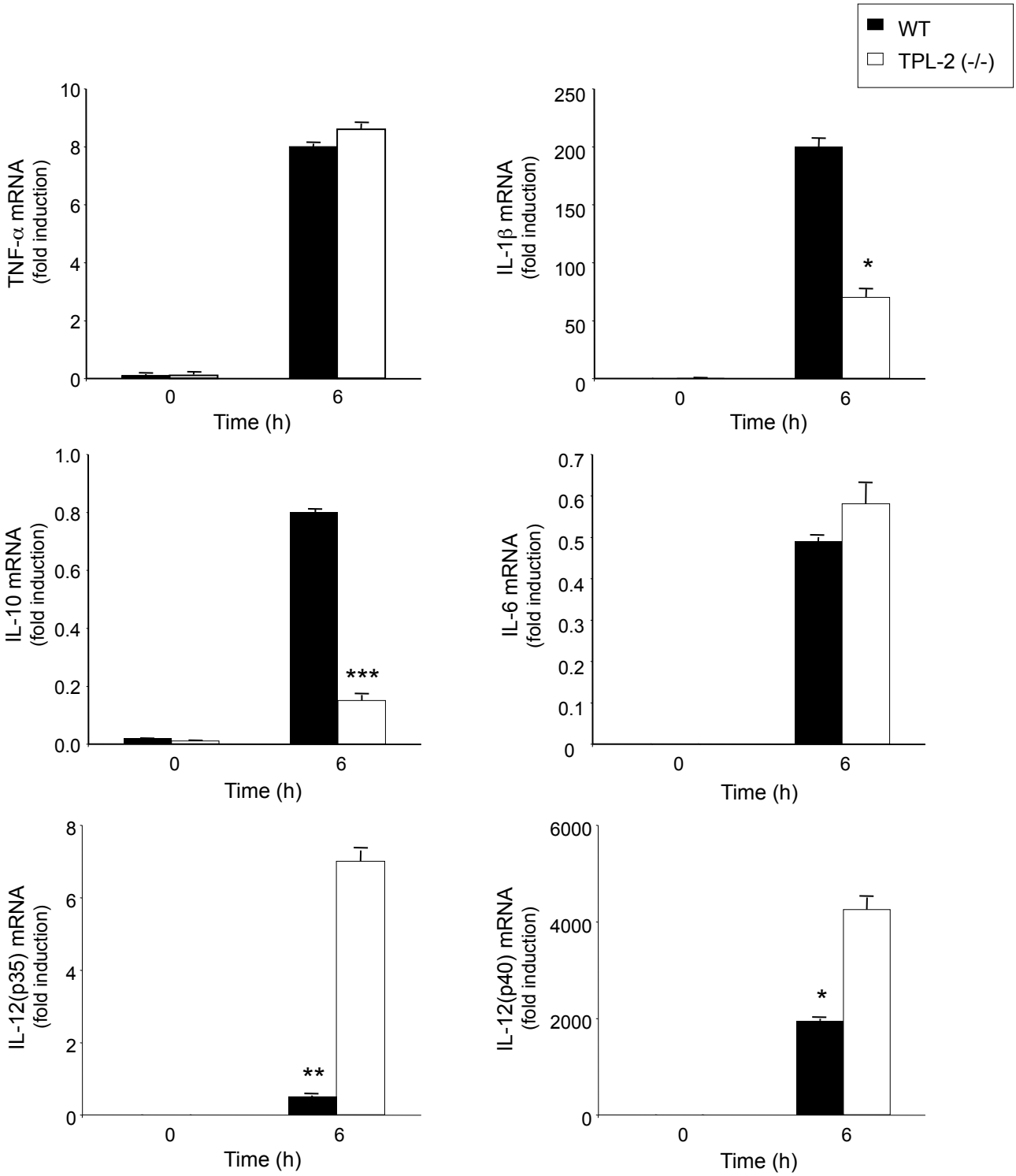

Figure S10 (Risco et al)