Supplemental Information

Myeloid Differentiation Primary Response Protein 88 Couples Reverse Cholesterol Transport to Inflammation

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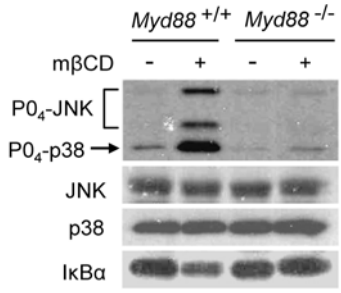
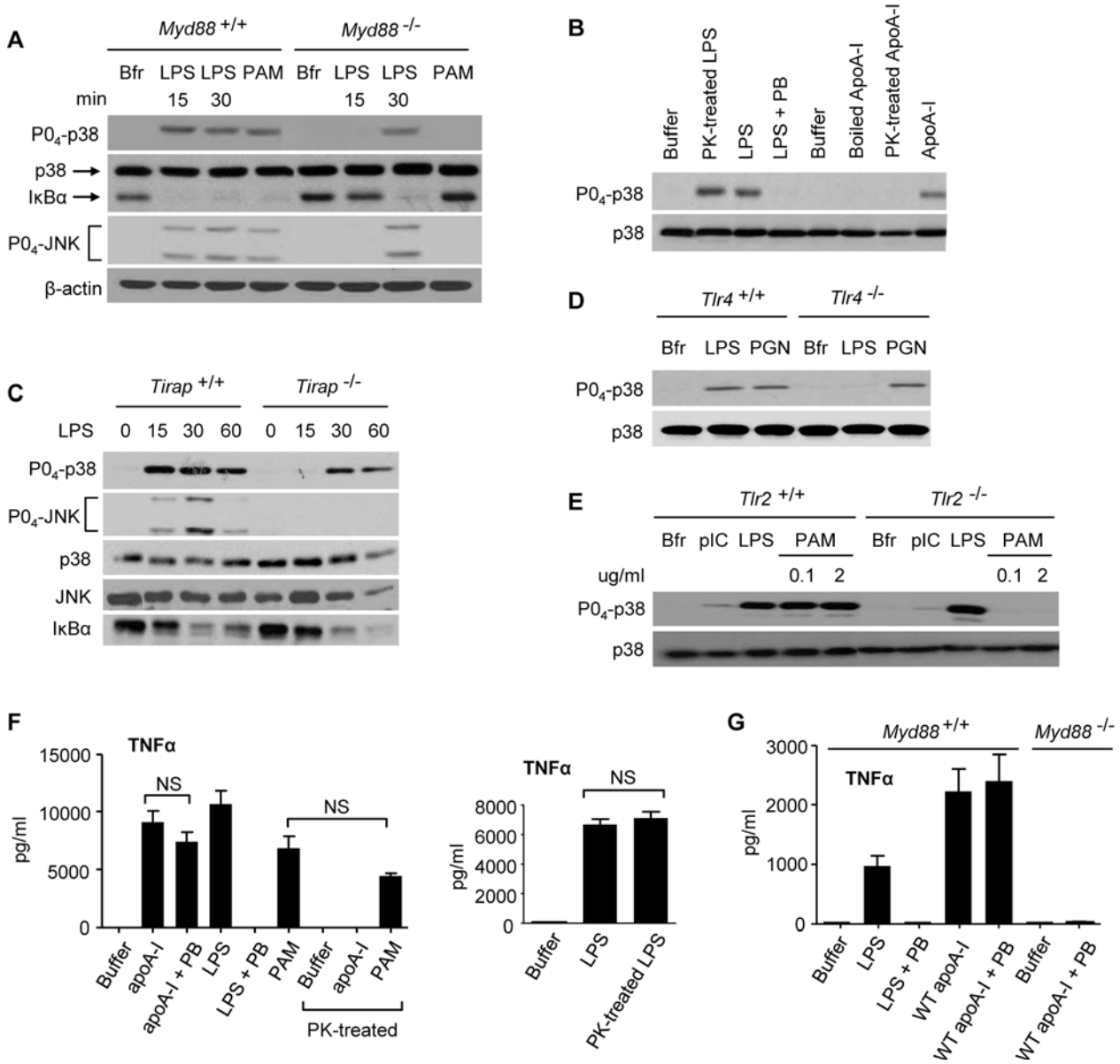
Figure S1. Cyclodextrin stimulates MyD88-dependent signals in the macrophage.



Myd88$^{+/+}$ and *Myd88*$^{-/-}$ PEMs were treated with methyl-β-cyclodextrin (mβCD) (10 mM, 15 min, 37°C) in the presence of polymyxin B, and lysates were then immunoblotted for the indicated targets.

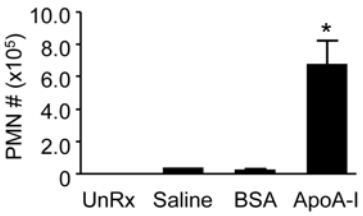
Figure S2. Controls for apoA-I exposure and for TLR- and TLR adaptor-deficient macrophages.



(A) *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ PEMs were treated with buffer (Bfr), 1 μg/ml Pam3CSK4 (PAM; 30 min), or 100 ng/ml LPS as shown. Lysates were immunoblotted for the indicated targets. (B) C57BL/6 PEMs were treated with LPS (100 ng/ml) or apoA-I (10

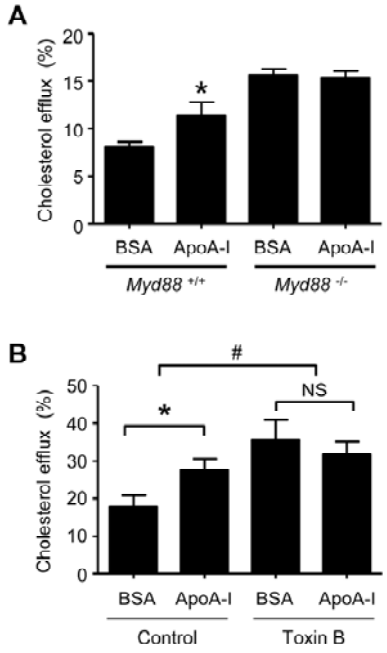
μg/ml) which had been left untreated, pretreated with proteinase K (PK-treated) as in Fig. 1D, or boiled (15 min), as shown. Polymyxin B (PB, 25 μg/ml; 30 min pretreatment) was used in all conditions except for 'LPS' and 'PK-treated LPS'. (C) *Tirap*$^{+/+}$ or *Tirap*$^{-/-}$ PEMs were treated with LPS (10 ng/ml) as shown, and lysates immunoblotted. (D) *Tlr4*$^{+/+}$ or *Tlr4*$^{-/-}$ PEMs were treated with LPS (100 ng/ml; 30 min), or peptidoglycan (PGN; 10 μg/ml; 30 min) and lysates immunoblotted as shown. (E) *Tlr2*$^{+/+}$ or *Tlr2*$^{-/-}$ PEMs were treated with polyI:C (pIC; 50 μg/ml; 1h), LPS (100 ng/ml; 30 min), or Pam3CSK4 (PAM, concentrations shown; 30 min). (F) ApoA-I (30 μg/ml), PAM3CSK4 (PAM, 1 μg/ml), or LPS (100 ng/ml) were pretreated with PK as in Fig. 1D, wild type PEMs exposed as shown, and TNFα quantified in media supernatants by ELISA. Data on left are results from 3 independent experiments, and on right are results from a representative experiment of three, each with triplicate samples. NS, nonsignificant. (G) *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ PEMs were exposed (3 hrs) to buffer, LPS with or without PB, and wt recombinant apoA-I (425 nM) with or without PB as shown, and TNFα was quantified in media supernatants by ELISA. Data shown represents 3 independent experiments.

Figure S3. ApoA-I injection induces neutrophilic peritonitis *in vivo.*



C57BL/6 mice were injected i.p. with endotoxin-free apoA-I (50 μg), delipidated BSA (50 μg), PBS, or left untreated (UnRx). Neutrophils (PMN) were counted in peritoneal lavage fluid 6h later. Data are representative of two experiments, both with n=4 receiving each treatment. *, $P<0.01$ compared to BSA. All values are means $\pm$ s.e.m.

Figure S4. Supplementary cholesterol efflux studies.



(A) Cholesterol efflux to BSA and apoA-I from *Myd88*$^{+/+}$ or *Myd88* $^{-/-}$ PEMs was calculated as in Fig. 6, however cholesterol mass was measured and used for efflux calculations in place of ^{3}H-cholesterol signal. *, P=0.03 compared to BSA (one-tailed t test). (B) C57BL/6 PEMs pretreated with *C. difficile* toxin B (500 ng/ml, 4h) were assayed for cholesterol efflux (^{3}H-cholesterol measurement) to BSA or apoA-I (10μg/ml). *, P=0.04; #, P<0.001. Data are results from 3 independent experiments. All values are means $\pm$ s.e.m.

Table S1. (A) *Myd88*$^{+/+}$ ('C57') or *Myd88* $^{-/-}$ ('MyD88 KO') PEMs were pretreated with polymyxin B (25 μg/ml, 30 min) prior to stimulation with buffer (control, 'C') or ApoA-I ('AI', 10 μg/ml, 2h). RNA was extracted from all four conditions from three independent experiments, and analyzed by microarray. The table shows ratios of gene expression for apoA-I to buffer in both genotypes with p-values (ANOVA) for the apoA-I expression change, and for the inter-genotype ratio comparison. As previously reported (Bjorkbacka et al., 2004a), the term 'MyD88-dependent' was assigned to genes whose apoA-I-induced expression change was significantly reduced (ANOVA of expression ratio) in the absence of MyD88; 'MyD88-modulated' genes were significantly dependent upon MyD88, but showed increased induction (or repression) in the absence of MyD88; 'MyD88-independent' genes had no statistically significant ($p>0.01$) requirement for MyD88 in their apoA-I induction. (B) Select inflammation-related genes significantly regulated by apoA-I are shown, as is the dependence of this regulation upon MyD88.

Supplemental Experimental Procedures

Cholesterol Mass Analysis

100-200 μl of medium or cell lysate (including 5α-cholestane as internal standard) were mixed with 1 ml of 100% ethanol and 100 μl of 50% KOH, incubated at 65 °C for 45-60 min, and then allowed to cool to room temperature. Then, 2.0 ml of hexane and 1.0 ml of water were added and the sample was vortexed vigorously and centrifuged at 2000 rpm for 5 min. The upper phase (hexane) was removed, placed in a clean tube, dried down, brought up in 200 μl of hexane and analyzed by gas-liquid chromatography (GLC) (Furbee et al., 2002). Values were normalized to GLC analysis volume.

ApoA-I expression

Wild type apoA-I was expressed in *E. coli* and purified as previously reported (Lagerstedt et al., 2007; Oda et al., 2003). Recombinant protein was confirmed by SDS-PAGE, quantified (BCA assay, Pierce), and subjected to LPS removal (Proteospin, Norgen Biotek), after which it was used for cell exposure at 10 μg/ml (~0.06 EU [6 pg]/ml LPS by Limulus assay) or 20 μg/ml in the presence and absence of polymyxin B (25 μg/ml). The recombinant apoA-I has been extensively characterized as structurally and functionally comparable to the native protein (Lagerstedt et al., 2007; Oda et al., 2003).

Microarray Analysis

Total RNA from 3 independent experiments (4 conditions/experiment: *Myd88*$^{+/+}$/buffer, *Myd88*$^{+/+}$/apoA-I (2 hrs; 10 μg/ml), *Myd88*$^{-/-}$/buffer, *Myd88*$^{-/-}$/apoA-I (2 hrs)) was extracted from macrophages using the RNeasy Mini Kit (Qiagen) and subjected to DNase treatment using Qiagen's DNase set. Gene expression analysis was conducted using Agilent Whole Mouse Genome (014868) 4x44 multiplex format oligo arrays (Agilent Technologies) following the Agilent 1-color microarray-based gene expression analysis protocol. Starting with 500 ng of total RNA, Cy3 labeled cRNA was produced according to manufacturer's protocol. For each sample, 1.65 μg of Cy3 labeled cRNAs were fragmented and hybridized for 17 hours in a rotating hybridization oven. Slides were washed and then scanned with an Agilent Scanner. Data was obtained using the Agilent Feature Extraction Software (v9.5), using the 1-color defaults for all parameters. The Agilent Feature Extraction Software performed error modeling, adjusting for additive and

multiplicative noise. The resulting data were processed using the Rosetta Resolver® system (version 7.1)(Rosetta Biosoftware, Kirkland, WA). The microarray data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO series accession number GSE13772.

References

Furbee, J.W., Jr., Sawyer, J.K., and Parks, J.S. (2002). Lecithin:cholesterol acyltransferase deficiency increases atherosclerosis in the low density lipoprotein receptor and apolipoprotein E knockout mice. The Journal of biological chemistry 277, 3511-3519.

Lagerstedt, J.O., Budamagunta, M.S., Oda, M.N., and Voss, J.C. (2007). Electron paramagnetic resonance spectroscopy of site-directed spin labels reveals the structural heterogeneity in the N-terminal domain of apoA-I in solution. The Journal of biological chemistry 282, 9143-9149.

Oda, M.N., Forte, T.M., Ryan, R.O., and Voss, J.C. (2003). The C-terminal domain of apolipoprotein A-I contains a lipid-sensitive conformational trigger. Nat Struct Biol 10, 455-460.