SUPPLEMENTARY APPENDIX

Activated factor X signaling via protease-activated receptor 2 suppresses pro-inflammatory cytokine production from lipopolysaccharide-stimulated myeloid cells

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Supplementary Methods

Materials

Plasma-purified human FVIIa, FIXa, FXa, FXa_{DEGR}, FXa_{DESGLA}, bovine FXa, FVa and APC were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Synthetic peptides mimicking FX sequence $L^{83}FTRKL^{88}G$ and a scrambled version of the same FX sequence (KFTGRLL) were synthesized on request by Genscript Inc. (Piscataway, NJ, USA). TLR agonists (Pam3CSK4, Heat-killed *Listeria monocytogenes* (HKLM), bacterial flagellin, FSL-1) and wortmannin were purchased from Invivogen (Toulouse, France). Endotoxin-free receptor-associated protein (RAP) was purchased from Innovative Research, Inc. (Novi, MI, USA). Histopaque 1077 was purchased from Sigma-Aldrich Life Sciences (Cambridge, UK). Anti-ApoER2 monoclonal antibodies and mouse IgG_1 isotype control antibody were obtained from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Boston, MA, USA).

Isolation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from buffy coat whole blood component obtained from a healthy donor pool provided by the Irish Blood Transfusion Service. PBMCs were isolated by centrifugation at 2000 rpm in Ficoll-Hypaque density gradient using the Boyum method[32] and maintained in RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies, Paisley, UK). Cell viability before each assay was assessed by Trypan Blue staining. Cells were seeded in 96-well microtitre plates at a density of 2 $x10^6$ cells/ml and incubated at 37ºC and 5% CO_2 for 3 hours (after which any non-adherent cells were removed) then cultured for a further 24 hours.

Mice

$PAR2^{-/-}$ mice, originally from Jackson Laboratories, were on a BALB/c background and maintained in house. Animals were kept in individually ventilated and filtered cages under positive pressure (Teniplast, Northants, UK) and Specific Pathogen-Free (SPF) conditions. Mice were fed an irradiated diet and housed on irradiated bedding. Food and water were supplied *ad libitum*. All animal experiments were performed in compliance with Irish Department of Health and Children regulations and were approved by the Trinity College Dublin BioResources ethical review board.

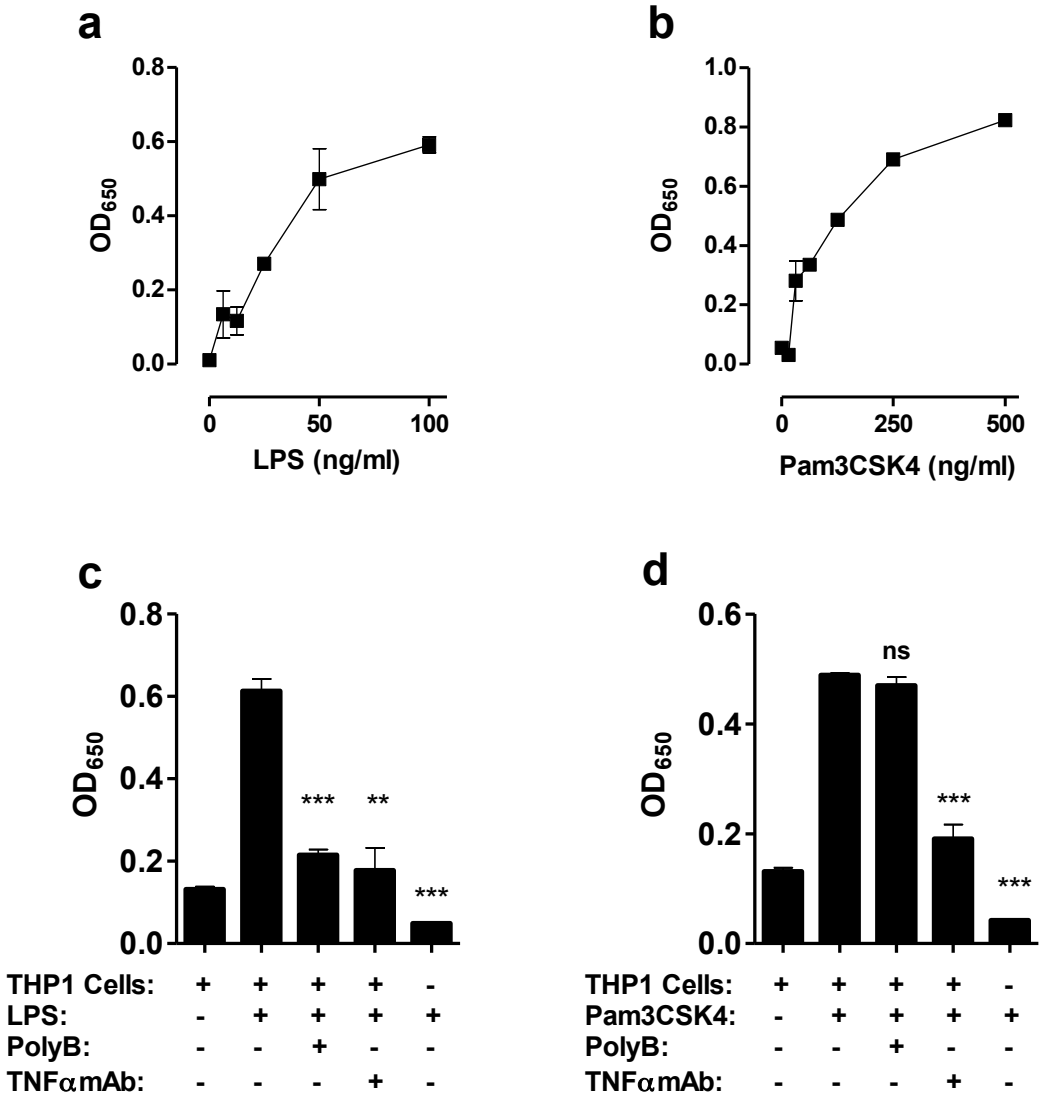
Isolation and culture of murine bone marrow-derived macrophages

Bone marrow-derived macrophages were prepared from mice by standard techniques. [33] Briefly, bone marrow cells obtained from the femurs and tibia of PAR2$^{-/-}$ and BALB/c wild type mice. Bone marrow cells were plated in 6-well plates and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin and 10 ng/ml GM-CSF (R&D Systems, Minneapolis, MN, USA) for 7 days. Purity of macrophages was analysed using flow cytometry with data collection on a CyAn (Beckman Coulter). Data were analyzed using FlowJo software (Tree Star). Cultured cells were stained with BD Biosciences mAb; F4/80-APC (BM8) and eBiosciences mAb; CD11b-PerCP (M1/70). Flow buffers used contained 2mM EDTA to exclude doublets. Using appropriate isotype-controls, quadrants were drawn and data were plotted on logarithmic scale density-plots. Cultured cells expressed both CD11b and F4/80 markers for macrophages.

Statistical analysis

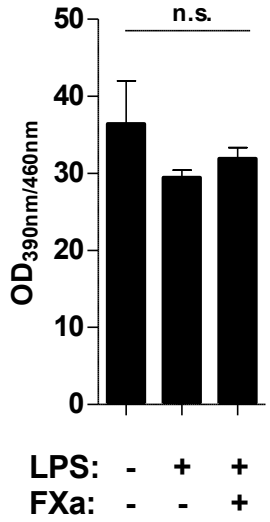
All experiments were performed in triplicate as a minimum and plotted as the mean ± standard deviation (S.D.). Unpaired Student t-tests were used to determine statistical significance, and p values < 0.05 were deemed significant.

Supplementary Figure 1:



Supplementary Figure 1: Exposure of THP-1 cells to LPS and Pam3CSK4 results in expression of TNFα that can be measured using HEK Blue™ TNF-α/IL-1β reporter cells. THP-1 cells were treated with *(a and c)* LPS (6.25-100ng/ml) *(b and d)* Pam3CSK4 (31.3-500ng/ml) for 4 hours. TNFα secretion was determined by incubating treated THP-1 cell supernatant with HEK Blue™ TNF-α/IL-1β reporter cells, resulting in a dose-dependent increase in secreted ALP expression. THP-1 cells were treated with the LPS inhibitor polymixin (PolyB), prior to exposure to (*c*) LPS (200ng/ml; 4hrs) or (*d*) Pam3CSK4 (500ng/ml; 4hrs). PolyB completely prevented LPS-induced TNFα secretion but had no effect on Pam3CSK4-induced TNFα secretion. The presence of an anti-TNFα monoclonal antibody prevented induction of secreted ALP expression by both LPS/Pam3CSK4 treated THP-1 supernatant, confirming ALP activity was a consequence of TNFα in the supernatant.

Supplementary Figure 2:



Supplementary Figure 2: The effect of FXa on LPS-induced TNFα secretion does not occur as a consequence of altered THP-1 cell viability. To verify that the observed effect of FXa on LPS-induced cytokine secretion is not a consequence of altered monocyte viability, THP1 cell viability after FXa treatment was assessed using the CellTiter-Flour Viability assay. This assay uses constitutive protease activity within live cells, lost upon degradation of membrane integrity during apoptosis, as a marker of cell viability. Cells were treated with a flourogenic, cell-permeable, peptide substrate cleaved only in viable cells to generate a fluorescent signal that is proportional to the number of non-apoptotic cells [Niles, A.L. *et al*. Anal Biochem. 2007 Jul 15;366(2):197-206]. THP-1 cells were incubated with PBS or FXa (20nM) for 3 hours prior to stimulation with LPS (500ng/mL) for 4 hours. Cell viability was assessed using the CellTiter-Flour viability assay. Viability of cells treated with LPS alone or in combination with FXa did not differ significantly from that of untreated cells.