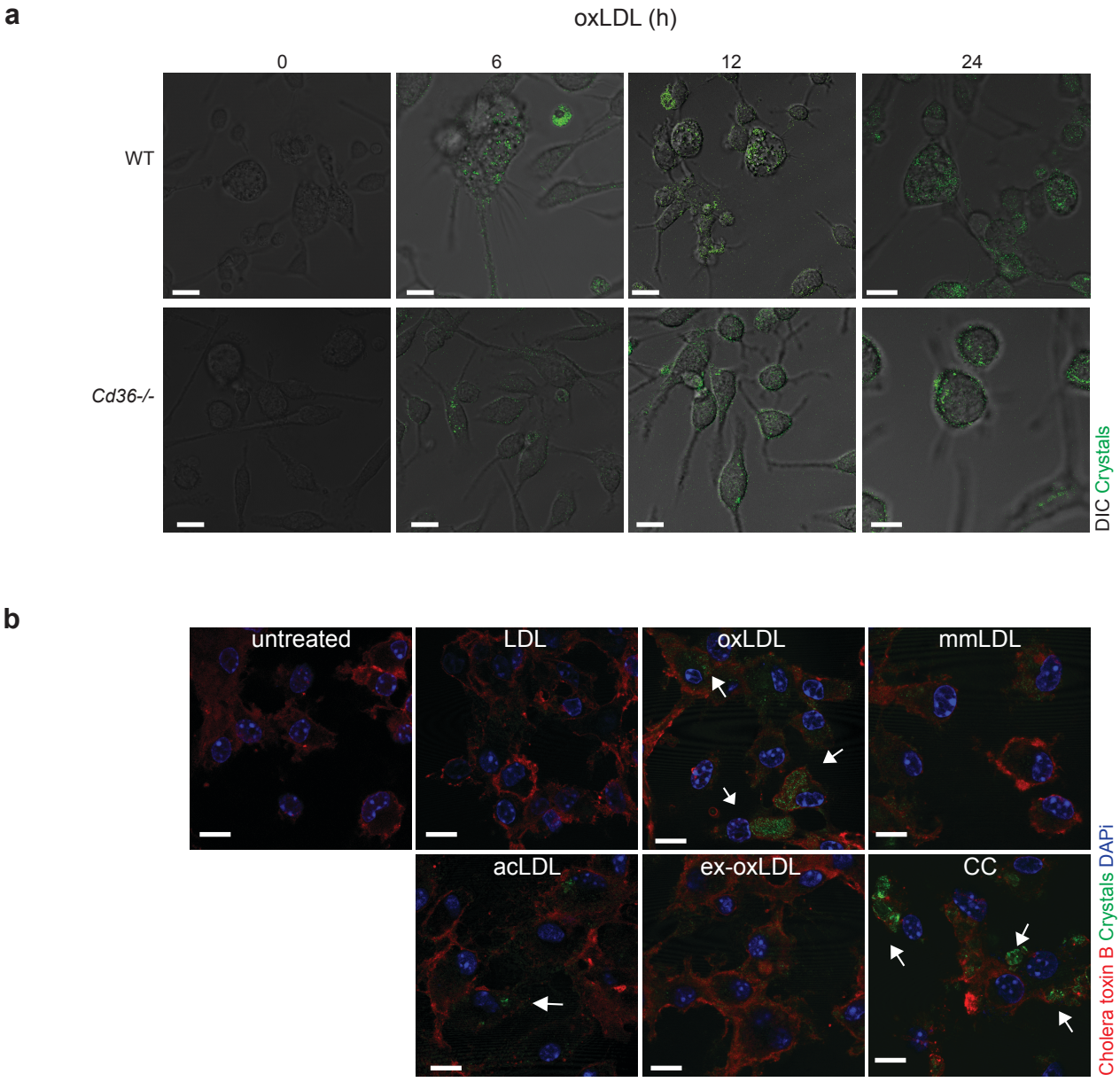
CD36 coordinates NLRP3 inflammasome activation by facilitating the intracellular nucleation from soluble to particulate ligands in sterile inflammation

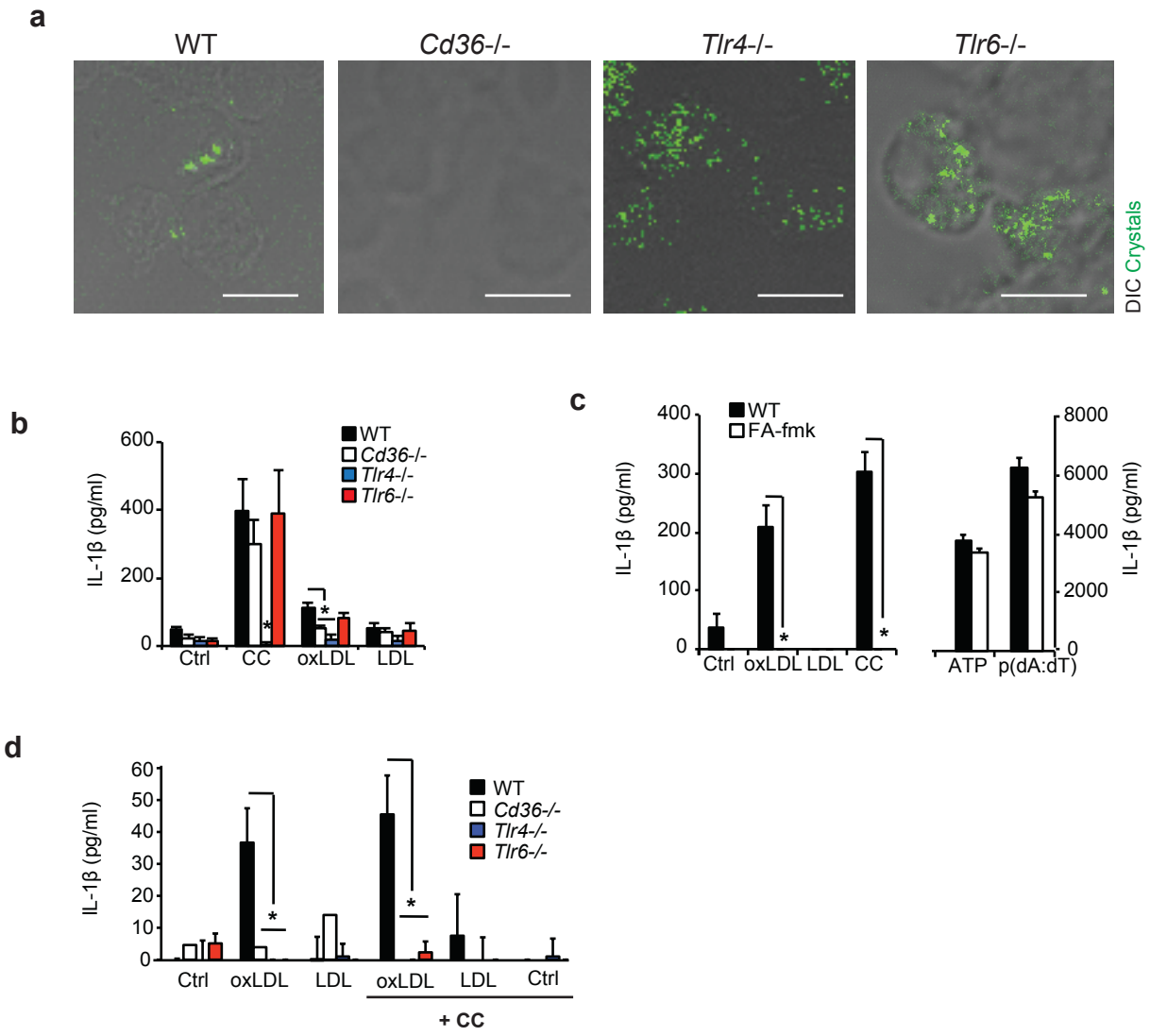
Frederick J. Sheedy[1], Alena Grebe[2], Katey J. Rayner[1], Parisa Kalantari[3], Bhama Ramkhelawon[1], Susan B. Carpenter[3], Christine E. Becker[4], Hasini N. Ediriweera[1], Adam E. Mullick[5], Douglas T. Golenbock[3], Lynda M. Stuart[4], Eicke Latz[2,3,6], Katherine A. Fitzgerald[3] and Kathryn J. Moore[1,7]

[1]Department of Medicine, Marc and Ruti Bell Program for Vascular Biology and Disease, The Leon H. Charney Division of Cardiology, New York University School of Medicine, New York, NY, USA, [2]Institute of Innate Immunity, University of Bonn, Bonn, Germany, [3]Department of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA, USA, [4]Laboratory of Developmental Immunology, Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA, [5]Isis Pharmaceuticals, Carlsbad, CA, USA, [6]German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany.

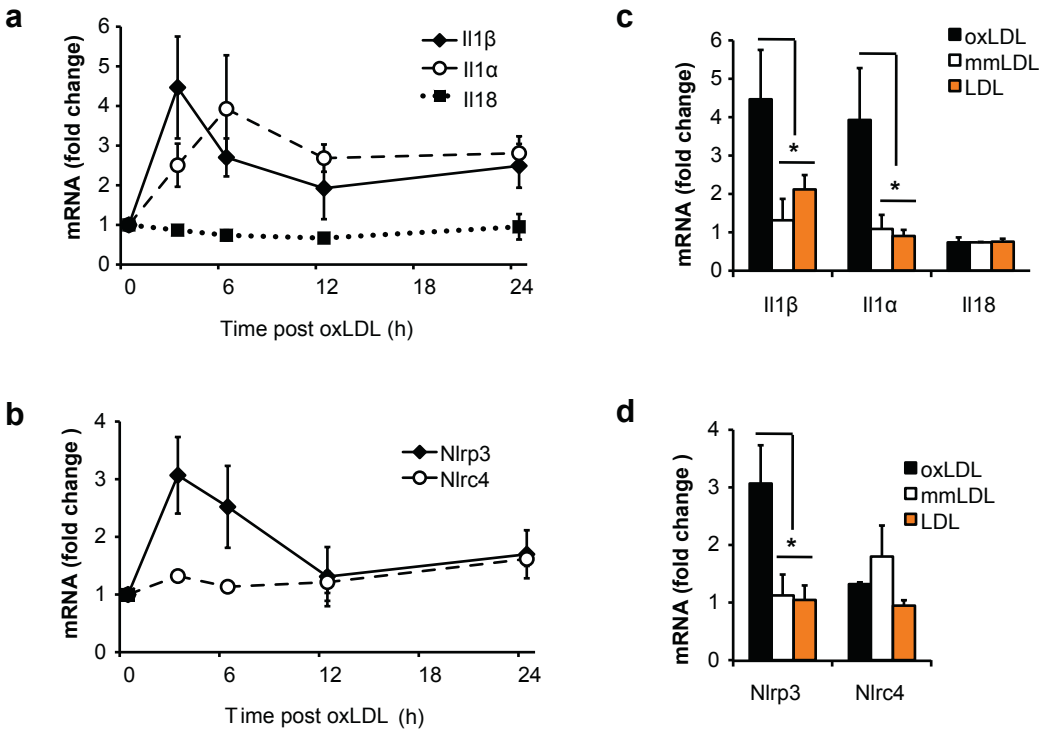
[7]Corresponding Author: Dr. Kathryn J Moore, Telephone: +1-212-2639498, E-mail: Kathryn.Moore@nyumc.edu



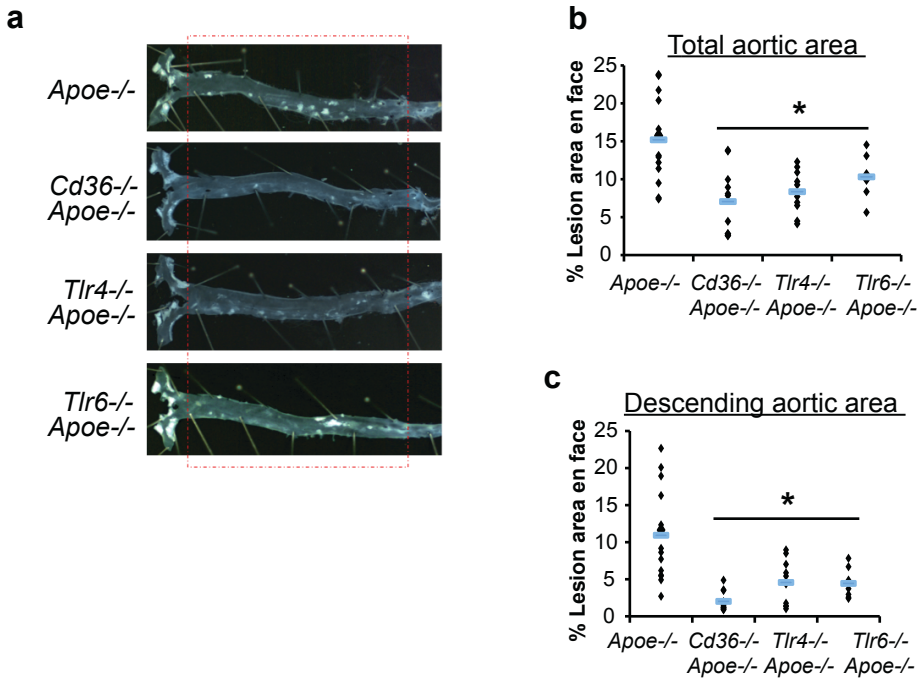
Supplementary Figure 1. Crystal formation induced by modified LDL. **a)** Time course analysis of the appearance of crystals in oxLDL-loaded macrophages of the indicated genotype. **b)** Confocal reflection analysis of crystals in macrophages loaded with the indicated species of modified LDL (all at 50 µg/mL, 24h) following staining with DAPi (to stain nuclei) and Cholera toxin B (to stain plasma membranes). Images are representative of 2-3 independent experiments. Scale bar = 10 µm.



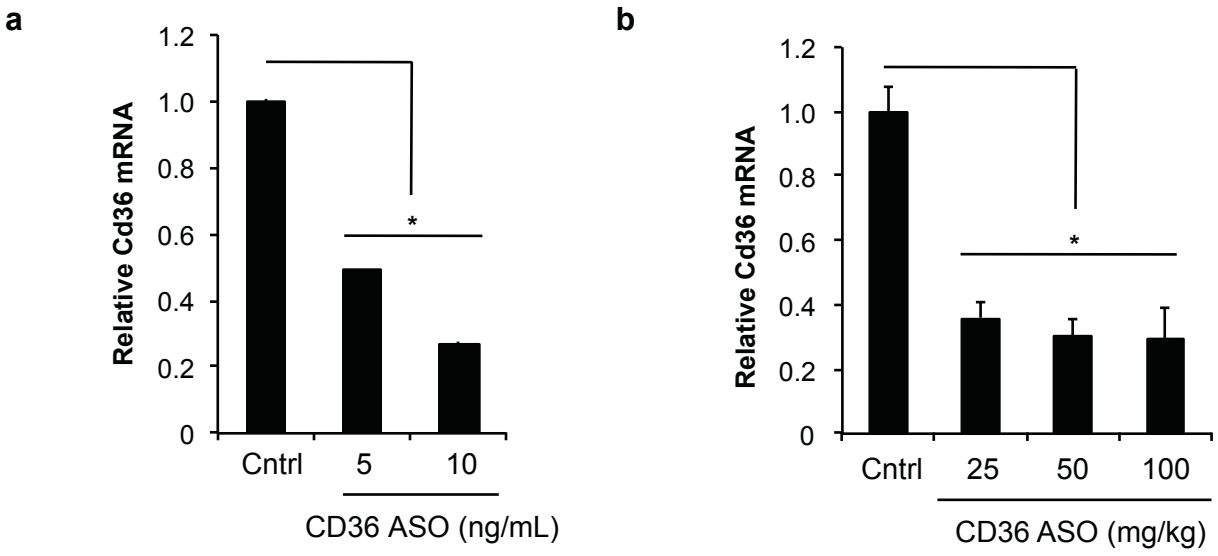
Supplementary Figure 2. Role of TLR4/TLR6 heterodimer in cholesterol crystal mediated inflammasome activation and priming by oxLDL **a)** Confocal reflection microscopy of macrophages from the indicated genotype follwing incubation with oxLDL (50 µg/mL, 24 h). **b)** IL-1β ELISA of supernatants from LPS-primed BMDM of the indicated genotype incubated with the indicated inflammasome activators as follows; cholesterol crystals (CC – 1 mg/mL, 24 h), oxLDL (50 µg/mL, 24 h) and unmodified LDL (LDL - 50 µg/mL, 24 h). TLR4 null macrophages are resistant to priming with LPS and therefore produce no IL-1β and serve as a background control in the assay. **c)** IL-1β ELISA of supernatants from LPS-primed BMDM pre-treated with FA-fmk peptide (20 µM, 1 h) prior to incubation with the indicated inflammasome activator as before or alongside ATP (5 mM, 1 h) or transfection with poly(dA:dT). **d)** IL-1β ELISA of supernatants from peritoneal macrophages of the indicated genotype primed as follows; oxLDL (50 µg/mL, 6 h), unmodified LDL (LDL - 50 µg/mL, 6 h) or PBS (Ctrl), followed by incubation with cholesterol crystals (+CC, 1 mg/mL, 12 h) to activate NLRP3. Data in b-d are mean ± s.d. of triplicate samples within a single experiment and all panels are representative of three independent experiments. Scale bar = 10 µm. *P<0.05.



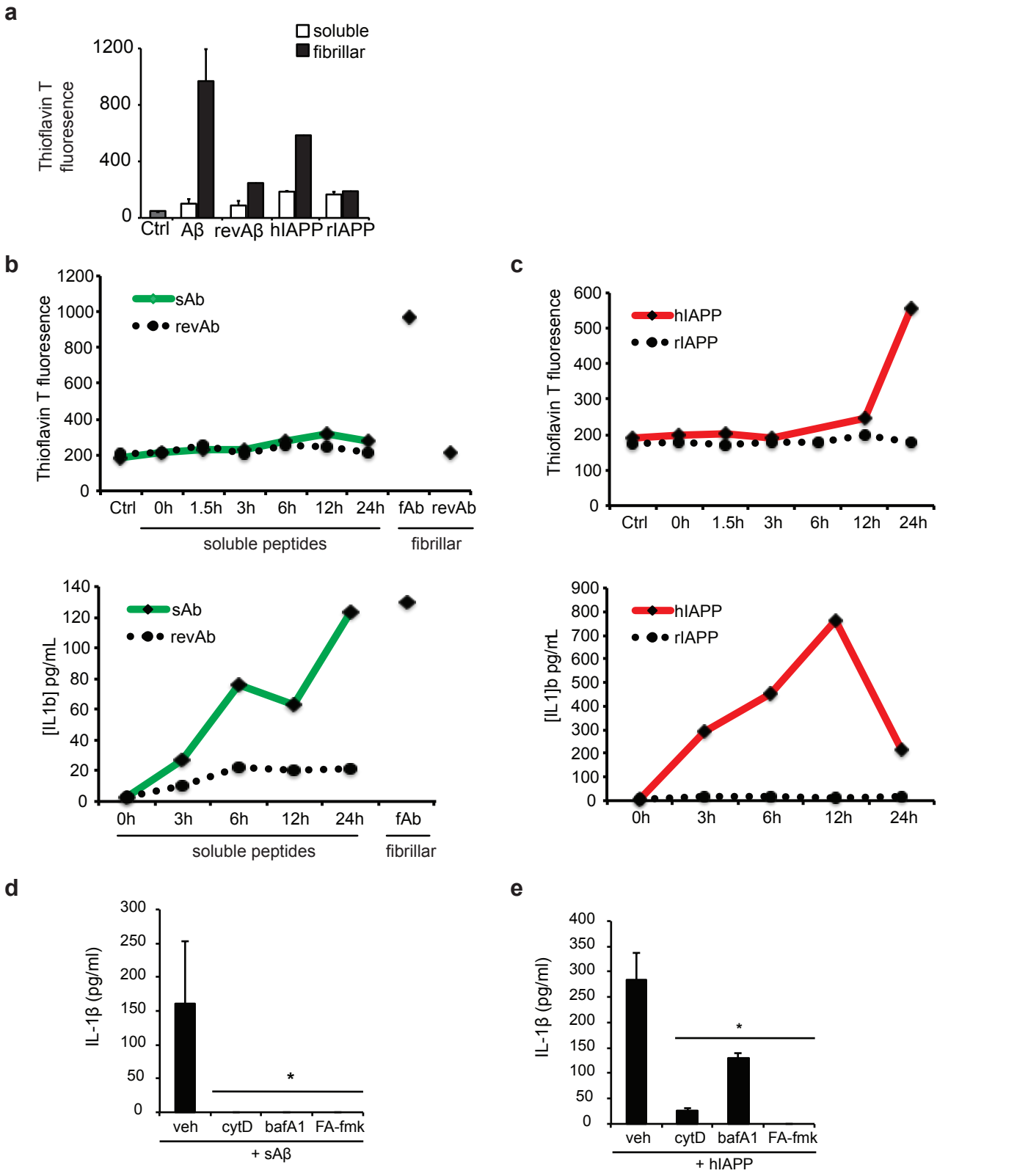
Supplementary Figure 3. Induction of inflammasome components by oxLDL **a & b)** Relative mRNA expression of the indicated gene in peritoneal macrophages treated with oxLDL (50 μg/mL) for the indicated times, measured by qRT-PCR. **b & d)** mRNA expression of the indicated gene in peritoneal macrophages following treatment with the indicated LDL species (oxLDL, minimally modified LDL or unmodified LDL at 50 μg/mL for 3 h (Il1β, Nlrp3, Il18, Nlrc4) or 6 h (Il1α) measured by qRT-PCR. Data are mean ± s.e.m. of three independent experiments. *P<0.05.



Supplementary Figure 4. Aortic atherosclerosis is reduced in atherogenic mice deficient in CD36, TLR4 or TLR6. Mice of the indicated genotype (*Apoe*$^{-/-}$, *Cd36*$^{-/-}$*Apoe*$^{-/-}$, *Tlr4*$^{-/-}$*Apoe*$^{-/-}$, *Tlr6*$^{-/-}$*Apoe*$^{-/-}$) were fed a western diet for 12 weeks and aortic atherosclerosis was assessed. **(a)** Representative images of aortae from each genotype **(b-c)** Region specific lesion area in the aorta en face measured as a % of total aortic area (b) or as a % of the abdomino-thoracic (descending) aortic area (c). Data is presented for n=15 mice (*Apoe–/–*), n=10 mice/group (*Cd36–/–Apoe–/–* and *Tlr4–/–Apoe–/–*) and n=7 mice (*Tlr6–/–Apoe–/–*). Horizontal bars indicate the mean and symbols indicate individual mice (b-c). *P<0.05.



Supplementary Figure 5. Knockdown of CD36 mRNA by CD36-specific ASO. **a)** Relative CD36 mRNA expression in immortalized macrophages (iMø) transiently transfected with the indicated concentration of CD36-specific antisense oligonucleotide (ASO) or a control non-targeting oligonucleotide (Ctrl at 10 ng/mL) using Lipofectamine RNAiMax, as measured by qRT-PCR. **b)** Relative CD36 mRNA expression in resident peritoneal macrophages derived from mice treated with CD36-specific ASO at the indicated concentrations (sub-cutaneous injection, 1x weekly for 3 weeks) or control non-targeting oligonucleotide, as measured by qRT-PCR. Data are mean ± s.d. of triplicate samples within a single experiment and are representative of 2-3 experiments. *P<0.05.



Supplementary Figure 6. Soluble amyloidogenic peptides activate the inflammasome via a phagolysosomal pathway. **(a)** Thioflavin T fluorescence of the indicated peptide preparations (soluble or made fibrillar *in-vitro*) to determine fibrillar content. Abbrevations - β-amyloid peptide (Aβ), reverse β-amyloid control (revAβ), human IAPP (hIAPP), rat IAPP (rIAPP). **(b-c)** Monitoring of thioflavin T fluorescence of the indicated soluble peptides at 37 ºC in DMEM media (top) measured in parallel with IL-1β secretion from LPS primed BMDM treated with (b) sAβ or revAβ (10 μM), or (c) hIAPP or rIAPP (10 μM) over the same time course (bottom). **(d-e)** IL-1β ELISA of supernatants from LPS-primed BMDM pre-treated with cytochalasin D (cytD, 1 μM), bafilomycin A1 (bafA1, 500 nM) or FA-fmk peptide (20 μM) prior to incubation with sAβ (10 μM, 24 h) (d) or hIAPP (10 μM, 6 h) (e). Data are mean ± s.d. and representative of 3 independent experiments. *P<0.05.

Supplementary Table 1: Serum levels of total cholesterol in mice after 12 weeks western diet.

Total Cholesterol [mg/dL]

(mean ± s.e.m.)

$Apoe^{-/-}$ **(n = 21)**	**$Cd36^{-/-}Apoe^{-/-}$** **(n = 14)**	**$Tlr4^{-/-}Apoe^{-/-}$** **(n = 15)**	**$Tlr6^{-/-}Apoe^{-/-}$** **(n = 7)**	**C57BL6** **(n = 14)**
786.08 ± 259.24	859.31 ± 235.86	867.79 ± 331.21	1099.14 ±121.95	97.72 ± 15.88

Supplemental References (from Methods section):

53. Boltz-Nitulescu G, Wiltschke C, Holzinger C, Fellinger A, Scheiner O, Gessl A, et al. Differentiation of rat bone marrow cells into macrophages under the influence of mouse L929 cell supernatant. Journal of leukocyte biology 1987, 41(1): 83-91.

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