CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation

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Particulate ligands, including cholesterol crystals and amyloid fibrils, induce production of interleukin 1β (IL-1β) dependent on the cytoplasmic sensor NLRP3 in atherosclerosis, Alzheimer’s disease and diabetes. Soluble endogenous ligands, including oxidized low-density lipoprotein (LDL), amyloid-β and amyloid peptides, accumulate in such diseases. Here we identify an endocytic pathway mediated by the pattern-recognition receptor CD36 that coordinated the intracellular conversion of those soluble ligands into crystals or fibrils, which resulted in lysosomal disruption and activation of the NLRP3 inflammasome. Consequently, macrophages that lacked CD36 failed to elicit IL-1β production in response to those ligands, and targeting CD36 in atherosclerotic mice resulted in lower serum concentrations of IL-1β and accumulation of cholesterol crystals in plaques. Collectively, our findings highlight the importance of CD36 in the accrual and nucleation of NLRP3 ligands from within the macrophage and position CD36 as a central regulator of inflammasome activation in sterile inflammation.

Interleukin 1β (IL-1β) has been linked to the pathogenesis of several sterile inflammatory diseases, including atherosclerosis, Alzheimer’s disease and type 2 diabetes. In each of those conditions, the accumulation of particulate materials at sites of inflammation (cholesterol crystals in subendothelial spaces of atherosclerotic plaques1, fibrillar amyloid-β in senile plaques in Alzheimer’s disease2 or amyloid-containing amylin–islet amyloid polypeptide (IAPP) on pancreatic beta cells in type 2 diabetes3) is a hallmark of disease. Studies have identified such particulate endogenous ligands as activators of inflammasomes formed by the cytoplasmic sensor NLRP3 (refs. 4–7). The NLRP3 inflammasome complex regulates activation of caspase-1, which catalyzes the cleavage of the precursor cytokines pro-IL-1β and pro-IL-18 into their mature, active forms8 and promotes the secretion of the now biologically active cytokines, as well as of IL-109. However, the mechanisms by which such structurally distinct molecules activate the NLRP3 inflammasome remain elusive.

Full activation of the NLRP3 inflammasome in macrophages requires the following two steps: priming (signal 1) and activation of inflammasome complex assembly (signal 2). Priming of macrophages increases expression of both pro-IL-1β and NLRP3 (refs. 10–12) and can be achieved in vitro through ligation of the Toll-like receptors (TLRs). Thus, experimental studies typically use bacterial products to prime the NLRP3 inflammasome, as the nature of the immunostimulatory priming signals in atherosclerosis, Alzheimer’s disease and type 2 diabetes are not well described. Signal 2 seems to be triggered by perturbation of cytoplasmic homeostasis due to ‘lysosomal destabilization’13. This process, triggered by certain aggregated and insoluble materials, is characterized by the cytotoxic release of lysosomal contents13 (such as cathepsins) and/or reactive oxygen species14, which results in assembly of the NLRP3 inflammasome and activation of caspase-1. Whether signal 1 and signal 2 are unrelated in vivo or whether common molecular events underlie these apparently distinct processes remains undefined.

The mechanisms that control the generation of NLRP3 activators in atherosclerosis, Alzheimer’s disease and type 2 diabetes are not well understood. For example, the deposition of cholesterol-rich lipoproteins in the vessel wall is a key pathogenic step in atherosclerosis15, yet the origin of cholesterol crystals that accumulate in plaques1 is poorly understood. Studies of combined confocal-reflection microscopy have shown that crystals are present in early plaques, both in extracellular spaces and in macrophages14,16. In the brain of patients with Alzheimer’s disease and the pancreas of patients with diabetes,
amyloid plaques arise from the aggregation of pre fibrillar amyloidogenic peptides such as the amyloid-β peptide of amino acids 1–42 (refs. 17–19) or IAPP20–22, which oligomerize and give rise to higher order structures, including fibrils. Extracellular deposits of IAPP-amyloid in the pancreas of patients with type 2 diabetes are associated with the loss of insulin-producing beta cells, and amyloid is also detected in pancreatic macrophages that secrete IL-1β23,24. Similarly, peptides of fibrillar amyloid-β accumulate in extracellular plaques in the brain of patients with Alzheimer’s disease, and increasing evidence indicates that soluble forms of amyloid-β are also damaging25,26. The present paradigm suggests that the immunostimulatory aggregates of such NLRP3 agonists form in the extracellular space and that their engulfment by macrophages triggers a process of dysfunctional phagocytosis or lysosomal damage that activates the NLRP3 inflammasome and the release of cytokines of the IL-1 family.

CD36 is an archetypal pattern-recognition receptor that binds polyanionic ligands of both pathogen and self origin27 and has been linked to the pathogenesis of atherosclerosis and Alzheimer’s disease through its recognition of modified endogenous ligands, including oxidized low-density lipoprotein (oxLDL)28, fibrillar amyloid-β29 and soluble amyloid-β30. CD36 acts together with a TLR heterodimer of TLR4-CD14 to recognize oxLDL and fibrillar amyloid-β and to induce the inflammatory response to these endogenous ligands31. Mice that lack CD36 (Cd36−/−) mice are protected from the toxic effects of fibrillar amyloid-β32, atherosclerosis33,34, insulin resistance and obesity35–37. Such observations prompted us to investigate the role of CD36 in the priming and activation of the inflammasome in atherosclerosis, Alzheimer’s disease and type 2 diabetes.

RESULTS

Uptake of oxLDL by CD36 generates NLRP3-activating crystals

Oxidized LDL is a key inflammatory component of atherosclerotic plaques that contains oxidation-specific epitopes that share molecular identity with phosphorylcholine moieties present on Streptococcus pneumoniae38. Recognition of that danger-associated molecular pattern by CD36 leads to uptake of oxLDL by recruited monocytes and the formation of cholesterol-laden macrophage foams cells that establish

Figure 1 CD36-mediated uptake of oxLDL generates intracellular crystals and activates the NLRP3 inflammasome. (a) Enzyme-linked immunosorbent assay (ELISA) of IL-1β in supernatants of LPS-primed wild-type (WT) and Cd36−/− bone marrow–derived macrophages (BMDMs) incubated for 24 h with PBS vehicle (control (Ctrl)), various concentrations of oxLDL (horizontal axis) or unmodified LDL (LDL), or with cholesterol crystals (CC; 1 mg/ml), ATP (5 μM; 1 h) or poly(dA:dT) (p(A:T); 100 ng/ml; 6 h). (b) Confocal reflection microscopy of wild-type and Cd36−/− peritoneal macrophages treated for 24 h with 0, 25 or 50 μg/ml of oxLDL. DIC, differential interference contrast. (c) Confocal reflection microscopy of peritoneal macrophages treated for 24 h with oxLDL (50 μg/ml) and then for 1 h with methyl-β-cyclodextrin (MβCD; 10 μM) or vehicle (Veh). (d) Confocal reflection microscopy of BMDMs pretreated for 1 h with cyclohexatin (CytD; 1 μM) or vehicle control and then treated for 24 h with oxLDL (50 μg/ml). (e) ELISA of IL-1β in supernatants of LPS-primed wild-type BMDMs pretreated with cyclohexatin D or vehicle (key) and then treated for 24 h with PBS (Ctrl) or oxLDL (50 μg/ml) or with cholesterol crystals or ATP (as in a). (f) Confocal reflection microscopy of wild-type and Nlrp3−/− BMDMs treated for 24 h with oxLDL (50 μg/ml). (g) ELISA of IL-1β in supernatants of LPS-primed wild-type and Nlrp3−/− BMDMs treated as in a. (h) ELISA of IL-1β in supernatants of LPS-primed wild-type BMDMs pretreated for 1 h with ZVAD-fmk (20 μM) or YVAD-fmk (20 μM) and then treated with PBS, oxLDL, unmodified LDL or cholesterol crystals or with ATP or poly(dA:dT). ND, not detected. Scale bars (b–d,f), 10 μm. *P < 0.05 (Student’s t-test). Data are from one experiment representative of three independent experiments (mean and s.d. of triplicates in a,e,g,h).
the atherosclerotic lesion. Notably, oxLDL induced the release of IL-1β from lipopolysaccharide (LPS)-primed wild-type macrophages but not from their Cd36−/− counterparts, but unmodified LDL did not (Fig. 1a, left). CD36 deficiency did not affect the induction of IL-1β by other activators of NLRP3, including cholesterol crystals generated in vitro or ATP or the synthetic double-stranded DNA poly(dA:dT), which activates the AIM2 inflammasome (Fig. 1a, right). To understand how CD36-mediated uptake of oxLDL activated the NLRP3 inflammasome, we used combined confocal-reflection microscopy to monitor crystalline material in macrophages. We observed a dose- and time-dependent increase in the appearance of crystals in macrophages treated with oxLDL but not in those treated with native LDL (Fig. 1b and Supplementary Fig. 1), and this was much lower in Cd36−/− macrophages treated similarly (Fig. 1b and Supplementary Fig. 1a). Acetylated LDL and minimally oxidized LDL, which bind SR-A and CD14 (but not CD36), also induced intracellular formation of crystals, albeit to a lesser extent than did oxLDL (Supplementary Fig. 1b). Treatment with methyl-β-cyclodextrin, to deplete cells of cholesterol, reversed the appearance of reflective material in oxLDL-treated macrophages (Fig. 1c), which confirmed that this crystalline material was cholesterol. Although CD36 can act as a coreceptor with TLR4-TLR6, oxLDL-induced crystal formation was unimpeded in TLR4-deficient (Tlr4−/−) or TLR6-deficient (Tlr6−/−) macrophages, and oxLDL-induced secretion of IL-1β from LPS-primed Tlr6−/− macrophages was similar to that of their wild-type counterparts (Supplementary Fig. 2a,b). However, blocking CD36-mediated endocytosis of oxLDL with cytochalasin D inhibited both the formation of intracellular crystals and secretion of IL-1β by LPS-primed macrophages (Fig. 1d,e), whereas the induction of IL-1β secretion by ATP was unaffected (Fig. 1e). Notably, despite the similar accumulation of crystals in oxLDL-treated wild-type and NLRP3-deficient (Nlrp3−/−) macrophages (Fig. 1f), secretion of IL-1β was abrogated in Nlrp3−/− macrophages (Fig. 1g), which confirmed the dependence of this response on the NLRP3 inflammasome. Nlrp3−/− macrophages treated with other activators of NLRP3, including synthetic cholesterol crystals or ATP, also had less secretion of IL-1β (Fig. 1g). Finally, oxLDL-induced secretion of IL-1β was dependent on caspase-1, as this response was lower in LPS-primed macrophages treated with either a broad caspase-1 inhibitor peptide (ZVAD-fmk) or a specific caspase-1 inhibitor peptide (YVAD-fmk) than in control cells treated with vehicle (Fig. 1h). This also inhibited IL-1β secretion induced by synthetic cholesterol crystals, ATP and AIM2 (Fig. 1h). These data established CD36-regulated formation of cholesterol crystals as a key driver of inflammasome activation upstream of NLRP3.

### Lysosomes control crystal formation and NLRP3 activation

Unlike the receptor for LDL, CD36 is not downregulated by its ligands, which leads to unabated uptake of oxLDL and its cholesterol cargo. To examine the subcellular localization and fate of oxLDL-induced crystals, we used lysosomal tracers combined with confocal-reflection fluorescence microscopy. After treatment of wild-type macrophages with oxLDL, crystals appeared within 8 h in acidic lysosomal compartments that stained with the lysosomotropic dye LysoTracker (Fig. 2a). Labeling of lysosomes by incubation of macrophages with fluorescent dextran demonstrated that crystal-containing lysosomes began to destabilize over 24 h, as shown by the translocation of

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**Figure 2** CD36-mediated formation of cholesterol crystals and activation of NLRP3 occurs via a lysosomal pathway. (a) Combined confocal reflection microscopy of wild-type and Cd36−/− BMDMs incubated for 8 h with oxLDL (50 µg/ml) and LysoTracker Red. (b) Combined confocal reflection microscopy of wild-type and Cd36−/− BMDMs incubated for 24 h with PBS (Ctrl), oxLDL (50 µg/ml), unmodified LDL (50 µg/ml) or cholesterol crystals (1 mg/ml) plus fluorescent dextran red. (c) LysoTracker Red fluorescence in wild-type and Cd36−/− macrophages, and oxLDL-induced secretion of IL-1β (pg/ml) from lipopolysaccharide (LPS)-primed wild-type macrophages (Cd36−/− BMDMs treated as in b, assessed by flow cytometry. Numbers above bracketed lines indicate percent LysoTracker-negative cells. (d) ELISA of IL-1β in supernatants of LPS-primed BMDMs pretreated with vehicle (Veh) or 500 nM bafilomycin A1 (BafA1) and then incubated PBS (Ctrl) or oxLDL or with cholesterol crystals, ATP or poly(dA:dT). (e,f) Confocal reflection microscopy of BMDMs pretreated with vehicle or with 500 nM BafA1 (e) or 20 nM LatB (f) and then loaded for 24 h with oxLDL (50 µg/ml). (g) ELISA of IL-1β in supernatants of LPS-primed BMDMs pretreated with vehicle or 20 nM LatB and then incubated as in d. Scale bars (a,b,e,f), 10 µm. *P < 0.05 (Student’s t-test). Data are from one experiment representative of three independent experiments (mean and s.d. of triplicates in d,g).
Figure 3 CD36 regulates priming of the NLRP3 inflammasome by oxLDL via TLR4-TLR6. (a) ELISA of IL-1β in supernatants of wild-type, Cd36−/−, Tlr4−/− or Tlr6−/− peritoneal macrophages primed for 18 h with PBS (Ctrl), oxLDL or unmodified LDL (50 µg/ml) alone (left half) or then treated for 1 h with 5 µM ATP (+ ATP). (b) Confocal microscopy of immortalized macrophages expressing ASC-CP, left untreated (UT) or treated with oxLDL alone, LPS and ATP, or oxLDL and ATP (above images); plasma membranes are stained with Alexa Fluor 594–coupled cholera toxin subunit B, and nuclei are stained with the DNA-intercalating dye DAPI. Scale bars, 10 µm. (c) ASC oligomerization in cells primed with various concentrations of oxLDL (horizontal axis, left) or LPS (right) and then left untreated (No ATP) or treated with ATP, presented as speck formation and s.d. of triplicates in cholera toxin subunit B, and nuclei are stained with the DNA-intercalating dye DAPI. Scale bars, 10 µm. (d-f) Quantitative RT-PCR analysis of mRNA in oxLDL-treated wild-type, Cd36−/−, Tlr4−/− or Tlr6−/− peritoneal macrophages (d) or in wild-type peritoneal macrophages pretreated with vehicle or 10 µM BAY 11–7083 (BAY; e) or 1 µM diphenylene iodonium (DPI; f), then treated with oxLDL; results are presented relative to those of untreated cells. (g) Quantitative RT-PCR analysis of mRNA in in vivo–formed foam-cell macrophages from wild-type, Apoe−/− or Cd36−/−/Apoe−/− mice fed a Western diet; results are presented relative to those of macrophages from chow-fed wild-type mice. (h,i) Production of IL-1β (h) or IL-1α (i) by in vivo–formed wild-type, Apoe−/− or Cd36−/−/Apoe−/− foam cells left untreated (no ATP) or treated for 1 h ex vivo with 5 µM ATP. *P < 0.05 (analysis of variance). Data are from one experiment representative of three independent experiments (a,b,h,i; mean and s.d. of triplicates in a,b,h,i or are from three independent experiments (c-f,g; mean and s.e.m.).

dextran into the cytosol (Fig. 2b). Cd36−/− macrophages treated similarly had a punctate perinuclear staining pattern characteristic of intact lysosomes (Fig. 2a,b), consistent with oxLDL–induced crystals’ being the source of the lysosomal damage. In contrast, treatment with pre-formed cholesterol crystals induced the release of lysosomal dextran to a similar extent in wild-type and Cd36−/− macrophages (Fig. 2b). Unmodified LDL did not disrupt macrophage lysosomal integrity (Fig. 2b). We confirmed those findings with a fluorescent LysoTracker dye (Fig. 2c), but did not affect IL-1β secretion by macrophages treated with oxLDL alone induced modest secretion of IL-1β from unprimed macrophages, a response dependent on CD36 (Fig. 3a). As signal 1 often proceeds through TLRs, we investigated whether cooperative signaling via CD36 with TLR4–TLR6 provided the oxLDL priming signal. Treatment of macrophages with oxLDL alone induced modest secretion of IL-1β that was not enhanced in the presence of the NLRP3 activator ATP (Fig. 3a); this confirmed that oxLDL was able to provide both signal 1 and signal 2 for NLRP3 activation. Notably, IL-1β production was impaired in Cd36−/−, Tlr4−/− or Tlr6−/− macrophages (Fig. 3a), which indicated that the heterotrimeric (Cd36–TLR4–TLR6) signaling complex was required for the priming of NLRP3 by oxLDL. We obtained similar results when we primed macrophages with oxLDL but used cholesterol crystals as the NLRP3 activator (Supplementary Fig. 2d). Because we detected such modest amounts of IL-1β when oxLDL was the priming agent, we assessed the oligomerization of ASC (a component

**CD36 and TLR4-TLR6 together prime the inflammasome**

The dual requirement for priming and activating signals for full activation of NLRP3 acts as a safeguard against the accidental or uncontrolled release of IL-1β. During the course of our studies, we noted that oxLDL had the unusual ability to induce the secretion of mature IL-1β from unprimed macrophages, a response dependent on CD36 (Fig. 3a). As signal 1 often proceeds through TLRs, we investigated whether cooperative signaling via CD36 with TLR4–TLR6 provided the oxLDL priming signal. Treatment of macrophages with oxLDL alone induced modest secretion of IL-1β that was not enhanced in the presence of the NLRP3 activator ATP (Fig. 3a); this confirmed that oxLDL was able to provide both signal 1 and signal 2 for NLRP3 activation. Notably, IL-1β production was impaired in Cd36−/−, Tlr4−/− or Tlr6−/− macrophages (Fig. 3a), which indicated that the heterotrimeric (Cd36–TLR4–TLR6) signaling complex was required for the priming of NLRP3 by oxLDL. We obtained similar results when we primed macrophages with oxLDL but used cholesterol crystals as the NLRP3 activator (Supplementary Fig. 2d). Because we detected such modest amounts of IL-1β when oxLDL was the priming agent, we assessed the oligomerization of ASC (a component...
of the NLRP3 inflammasome) labeled with cyan fluorescent protein (ASC-CFP) to further investigate the role of oxLDL in NLRP3 priming. ASC-CFP was polydispersed throughout the cytoplasm in unstimulated macrophages; however, supramolecular assembly of ASC occurred after signal 1 and signal 2, as noted in cells primed with LPS and subsequently treated with ATP (Fig. 3b, bottom left). Treatment with oxLDL alone induced the formation of ASC complexes (Fig. 3b), consistent with the idea that oxLDL not only primed but also activated NLRP3. Treatment with ATP after priming with oxLDL also induced the formation of speck-like complexes, albeit at a lower frequency than that in LPS-primed cells (Fig. 3b,c).

**Figure 4** Inflammasome activity is impaired in atherosclerosis-susceptible mice deficient in CD36 and its signaling partners TLR4 and TLR6. (a) Lesion area in the aorta en face in Aposer−/−, Cd36−/− Aposer−/−, Tlr4−/− Aposer−/− and Tlr6−/− Aposer−/− mice fed a Western diet for 12 weeks, relative to total aortic area. (b) Protein array analysis (IL-1β and IL-1α) and ELISA (IL-1β) of cytokines in serum from mice as in a and their wild-type (Aposer+/+) C57BL/6 counterparts. (c) Quantitative RT-PCR analysis of mRNA in the aorta of mice as in b; results are presented relative to those of chow-fed wild-type mice. (d,e) Plaque crystal content in serial sections throughout the aortic root of mice as in a, assessed by combined confocal reflection microscopy (e) followed by quantification (d). Scale bars (e), 200 μm. (f) Activity of caspase-1 in plaques in serial sections (n = 8 per mouse) throughout the aortic root of mice as in a, assessed as FAM-YVAD-fmk fluorescence with a FLICA probe (left) or immunofluorescence staining with antibody to IL-1β (right), presented relative to total plaque area. (g) IL-1β in serum from Aposer−/− mice before (Chow) or after (WD) being fed a Western diet for 4 weeks, along with treatment with a nontargeting ASO (NT) or CD36-specific ASO (CD36). Each symbol represents an individual mouse (a,d: n = 15 (Aposer−/−), 10 (Cd36−/− Aposer−/−), 10 (Tlr4−/− Aposer−/−) or 7 (Tlr6−/− Aposer−/−); small horizontal lines indicate the mean. *P < 0.05 (analysis of variance). Data are representative of three experiments (a), one experiment (b,c,g) or two experiments (d–f); mean and s.e.m. of four (b,c), six (f) or five (g) mice per group in b,c,f,g). The endogenous NLRP3-priming signals in atherosclerosis remain unclear. We postulated that recognition by CD36 of oxidized forms of LDL that accumulate during hypercholesterolemia can prime macrophages in vivo. To test this, we used an in vivo model of foam-cell formation by isolating peritoneal macrophages from atherosclerosis-susceptible mice deficient in apolipoprotein E (Aposer−/− mice) and Cd36−/− Aposer−/− mice fed an atherogenic, high-fat, high-cholesterol Western diet for 12 weeks. Macrophages isolated from those Aposer−/− mice had a greater abundance of Nlrp3, Nlrp3, Il1b and Nlrp3 mRNA than did macrophages from normalolipidemic wild-type mice (Fig. 3g), which indicated that these cells were primed in vivo for NLRP3 activity. That enhancement of NLRP3-priming signals was specific, as we detected no difference between the cells in their abundance of Il18 mRNA or Nlr4c mRNA. In contrast, the abundance of Il1a, Il1b and Nlrp3 mRNA in macrophages from Cd36−/− Aposer−/− mice fed a Western diet was similar to that of macrophages from wild-type mice fed regular chow. Furthermore, for macrophages obtained from Aposer−/− mice fed a Western diet, treatment ex vivo with the NLRP3 activator ATP (signal 2) induced secretion of IL-1β, whereas we detected no enhanced secretion of IL-1β by macrophages from Cds6−/− Aposer−/− mice treated similarly (Fig. 3h). Moreover, secretion of IL-1α, a function dependent on the inflammasome, was similarly enhanced in macrophages obtained from Aposer−/− mice fed a Western diet and treated with ATP, but not in their Cds6−/− Aposer−/− counterparts (Fig. 3i). These data demonstrated a requirement for CD36 in the priming of NLRP3 in macrophages by atherogenic stimuli in vivo.

**Targeting CD36 diminishes inflammasome activation in vivo** To further investigate the role of CD36 and its signaling partners TLR4-TLR6 in priming and activation of the inflammasome in vivo, we generated Aposer−/− mice deficient in CD36, TLR4 or TLR6 and fed them a Western diet for 12 weeks. Despite similar concentrations of total cholesterol in all groups (Supplementary Table 1), Cds6−/− Aposer−/−, Tlr4−/− Aposer−/− and Tlr6−/− Aposer−/− mice had significantly
CD36 regulates amyloid formation and NLRP3 activation

Like atherosclerosis, Alzheimer’s disease is characterized by a protracted inflammatory response driven by cells of the monocyte lineage. Fibrillar amyloid-β activates the NLRP3 inflammasome and secretion of IL-1β in LPS-primed macrophages and microglia, and activation of NLRP3 has been shown to contribute to Alzheimer’s disease. CD36 binds to soluble (prefibrillar) amyloid-β, which prompted us to investigate whether uptake of that soluble ligand by CD36 would also promote the intracellular generation of an NLRP3 activator. We treated macrophages with soluble amyloid-β, which we confirmed was nonfibrillar (Supplementary Fig. 6a), and stained the cells with thioflavin-S, a dye that becomes fluorescent after it binds amyloid oligomers and fibrils. Confocal microscopy showed a time-dependent increase in the appearance of intracellular thioflavin-S fluorescence in wild-type macrophages treated with soluble amyloid-β but not in their CD36−/− counterparts (Supplementary Fig. 5a). Macrophages incubated with a nonamyloidogenic ‘reverse’ amyloid-β peptide (with the amyloid-β amino acid sequence in reverse order) showed no evidence of thioflavin-S-reactivity for up to 24 h, whereas thioflavin-S-reactive amyloid was apparent in wild-type macrophages as early as 6 h after the addition of soluble amyloid-β, and it localized to lysosomes (Fig. 5a,b). We detected no extracellular or cell-associated fibrils by confocal microscopy (Fig. 5a,b), and soluble amyloid-β incubated for up to 24 h at 37 °C in cell culture medium showed no evidence of reactivity with thioflavin-T (an analog of thioflavin-S for in vitro assays; Supplementary Fig. 6b). Over that time frame (3–24 h), soluble amyloid-β induced the progressive release of IL-1β when incubated with LPS-primed wild-type macrophages but not when incubated with their Nlrp3−/− or caspase-1-deficient (Casp1−/−) counterparts (Fig. 5c and Supplementary Fig. 6b, bottom), which indicated that uptake of this soluble ligand resulted in activation of NLRP3. Of note, soluble amyloid-β was as effective an NLRP3 activator as was fibrillar amyloid-β, and pretreating macrophages with Congo red to interfere with the formation of β-sheets blocked the secretion of IL-1β induced by soluble amyloid-β (Fig. 5d). Finally, the secretion of IL-1β induced by soluble amyloid-β was abrogated in CD36−/− macrophages (Fig. 5e), which indicated a critical role for this receptor-mediated uptake pathway in the generation of intracellular NLRP3-activating amyloid.

Studies have identified activation of the NLRP3 inflammasome by another amyloidogenic peptide, IAPP, as a mechanism for the enhanced IL-1β in type 2 diabetes. Notably, IAPPs delivered to macrophages in a soluble form are the ‘preferential’ activators of NLRP3 (ref. 6). We postulated that CD36-mediated uptake of IAPP may promote intracellular amyloid formation and NLRP3 activation,
as observed with soluble amyloid-β. Binding of fluorescence-labeled human IAPP or amyloid-β to Chinese hamster ovary cells was greater in cells stably overexpressing CD36 than in control cells expressing empty vector (Fig. 6a). Furthermore, wild-type macrophages incubated with soluble human IAPP showed a time-dependent increase in intracellular thioflavin-S fluorescence, but their CD36−/− counterparts did not (Fig. 6b). We observed no such increase in thioflavin-S staining in macrophages treated for up to 24 h with rat IAPP, which is nonamyloidogenic because of species-specific differences in amino acids (Fig. 6b), or when we incubated soluble IAPP at 37 °C in cell culture medium alone (Supplementary Fig. 6c). The intracellular accumulation of thioflavin-S-reactive amyloid altered lysosomal stability, as wild-type macrophages treated with fluorescent dextran and incubated for 24 h with human IAPP or soluble amyloid-β showed a loss of lysosomal integrity and leakage of dextran into the cytoplasm (Fig. 6c). Soluble IAPP induced the progressive release of IL-1β, from as early as 3 h, when incubated with LPS-primed wild-type macrophages but not when incubated with their Nlrp3−/− or Casp1−/− counterparts (Fig. 6d and Supplementary Fig. 6c). Notably, the lysosomal damage induced by those amyloidogenic peptides, and subsequent IL-1β secretion, were abrogated in CD36−/− macrophages (Fig. 6e,c). Furthermore, pretreatment of macrophages with Congo red blocked the accumulation of thioflavin-reactive IAPP and secretion of IL-1β (Fig. 6f), which indicated that the intracellular transformation of soluble IAPP resulted in activation of NLRP3. Moreover, and consistent with the results we obtained with oxLDL-induced cholesterol crystals, phagolysosomal activity was required for the activation of NLRP3 by human IAPP and soluble amyloid-β, as pretreatment with cytochalasin D to block endocytosis, with baflomycin A1 to block lysosomal acidification or with FA-fmk to inhibit the serine-protease family of cathepsins blunted IL-1β production (Supplementary Fig. 6d,e). These data supported the proposal of a common pathway through which CD36-mediated uptake of the soluble precursors of NLRP3 ligands facilitated the intracellular generation of pathogenic activators of NLRP3.

**DISCUSSION**

Our work here has identified CD36 as a master regulator of inflammasome activation in atherosclerosis, Alzheimer’s disease and type 2 diabetes that facilitated the accrual and intralysosomal conversion of soluble endogenous ligands (oxLDL, soluble amyloid-β and IAPP) into insoluble crystals or amyloid oligomers and fibrils and, consequently, the activation of NLRP3 that led to IL-1β secretion. Lysosomal dysfunction has a critical role in both the generation of crystalline and amyloid ligands and the transmission of the damaging effects of those ligands sensed by NLRP3. Our results
suggest a new model of NLRP3 activation by particulate ligands formed in the macrophage and place CD36 upstream of a common mechanism of NLRP3-dependent IL-1β secretion in several sterile inflammatory diseases.

In each of the diseases focused on in this study, the accumulation of particulate materials at sites of inflammation is a pathological hallmark. Unlike previously proposed models of NLRP3 activation in these diseases in which 'frustrated phagocytosis' of extracellular particulate materials triggers the inflammasome4,5, our findings suggested that the earliest activation of NLRP3 by cholesterol crystals or amyloid-β–IAPP fibrils resulted from recognition and endocytosis of the soluble precursors of particulate ligands (such as oxLDL, soluble amyloid-β and soluble IAPP) by CD36 and the subsequent intracellular nucleation of insoluble NLRP3 activators. Our data fit with published observations of intracellular formation of amyloid fibrils in vitro23–26,42,43, and an increasing literature that suggests that soluble amyloid-β and soluble IAPP are the damaging species in Alzheimer’s disease and type 2 diabetes, respectively. Macrophages incubated with soluble amyloid-β and soluble IAPP may contain higher order oligomeric forms of these peptides, as well as fibrillar forms of amyloid in the lysosome that may activate NLRP3. Although our results do not exclude the possibility that extracellular crystals or amyloid fibrils that accumulate during disease also activate the NLRP3 inflammasome through the ‘frustrated phagocytosis’ process, here we have expanded on that paradigm by suggesting that such ligands may be secondary triggers of NLRP3 that arise later in disease because of their active export from macrophages or the release of crystals or fibrils from dying cells. Macrophage death may itself be triggered by activation of the inflammasome through the process of pyroptosis44, which is linked to the release of proatherogenic IL-1β45–47, or as a result of lysosomal dysfunction due to the prolonged accumulation of insoluble crystals or fibrils.

In atherosclerosis, CD36 has a dual role in initiating both priming (signal 1) and activation (signal 2) of the NLRP3 inflammasome. Although oxLDL has been known to trigger IL-1β production in foam cells48–50, many of those studies were done before the discovery of the NLRP3 inflammasome. Our work has identified the molecular mechanism by which oxLDL induces macrophage secretion of IL-1β by showing that binding of oxLDL by CD36 controlled engagement of the two pathways necessary for full activation of the inflammasome: signal 1 (via the TLRs) and signal 2 (via the NLRs). Although published studies of inflammasome activity in host defense and inflammatory disease have suggested that signal 1 and signal 2 are derived from the engagement of distinct signaling pathways by separate danger-associated molecular patterns, our data have suggested that a single such pattern (oxLDL) provided both signals in atherosclerosis by engaging CD36. Although the amount of priming signal induced by oxLDL via CD36–TLR4–TLR6 was modest compared with that induced by bacterial stimuli, it was consistent with the idea of atherosclerosis arising from low-grade inflammation over a protracted period of time, and our in vivo data confirmed the importance of this receptor complex in both the priming of NLRP3 and the formation of atherosclerotic plaques. Notably, CD36 deficiency in ApoE−/− mice also resulted in much less accumulation of cholesterol crystals in plaques and serum concentrations of cytokines of the IL-1 family (IL-1β, IL-1α and IL-18), which indicated that the CD36-dependent pathway for intracellular crystal generation identified in vitro was also active in vivo. The endocytosis of ligands by CD36, which lead to the nucleation of NLRP3 activators, can therefore be thought of as ‘signal 0’ in models of inflammasome activation by sterile inflammatory stimuli, which provides the common cellular event required for priming and activation of NLRP3 in metabolic diseases.

The incidence of atherosclerosis and type 2 diabetes continues to increase, and the development of new therapeutics for metabolic disease is crucial. Appreciation of the critical role of the NLRP3 inflammasome in both atherosclerosis and type 2 diabetes indicates the promise of this pathway for therapeutic targeting. Although many studies have described regulators of inflammasome activity at the level of complex assembly and caspase-1 activation51, the identification of CD36 as a key upstream regulator of NLRP3 activation by endogenous ‘danger ligands’ in atherosclerosis, Alzheimer’s disease and type 2 diabetes provides a new target for therapeutic intervention that does not compromise the inflammasome activity required for host defense against pathogens. To our knowledge, this is the first such upstream regulator of NLRP3 described in sterile inflammation, with CD36 fulfilling a role analogous to that of the receptor P2X7 in ATP-mediated NLRP3 activation52. Targeting CD36 for amelioration of NLRP3 activation may be particularly useful for atherosclerosis, in which CD36 is essential for priming and activation of the inflammasome in macrophage foam cells, as well as the accumulation of cholesterol crystals in plaques. In support of that, we found that targeting CD36 with ASOs resulted in lower serum concentrations of IL-1β in atherosclerotic mice, which suggests that such strategies may hold promise for diminishing inflammasome activation in human disease.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

F.S. designed, did and analyzed experiments; A.G. analyzed cholesterol crystals in atherosclerotic plaques; K.I.R. and H.N.E. assisted with mouse atherosclerosis experiments. P.K., B.R., S.B.C. and C.E.B. assisted with microscopy experiments; A.E.M. provided CD36-specific ASOs and contributed to experimental design; D.T.G., L.M.S., E.L. and K.A.F. contributed to study design, data analysis, and manuscript preparation; and K.J.M. conceived of the study, designed and analyzed experiments and, along with F.S., prepared the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice and cell culture. Cd36−/−, Tlr4−/−, Tlr6−/−, Nlrp3−/− and Casp1−/− mice have been described5,31. C57BL/6J mice were from Jackson Laboratories. For isolation of peritoneal macrophages, mice were injected intraperitoneally with 1 ml 4% thioglycollate medium solution (Gibco) and the peritoneal cells elicited were then washed twice of the peritoneal cavity with 5 ml PBS 4 d after injection. For separation of macrophages, red blood cells were lysed and the resulting cells were seeded at a density of 2 × 10^6 cells per ml in DMEM (Gibco) containing 10% FBS. Macrophages were allowed to adhere overnight and then medium was changed to DMEM containing 2% FBS. BMDMs were isolated by flushing the bone marrow from femur and tibia of hind legs from mice with DMEM containing 10% FBS supplemented with antibiotics as described above. Cells were collected, centrifuged and lysed as described above and the resulting cells were seeded at a density of 1 × 10^6 cells per ml in DMEM containing 10% FBS and supplemented with conditioned medium from L929 mouse fibroblasts (15%) as described53. Cells were allowed to differentiate until day 5, after which they were collected with 10 mM EDTA and were plated at a density of 1 × 10^6 cells per ml in DMEM containing 2% FBS and 7.5% conditioned medium from L929 mouse fibroblasts. Immortalized macrophages stably transfected with a construct for the overexpression of CFP-tagged mouse ASC have been described31. Chinese hamster ovary cell lines stably transfected with plasmids overexpressing Cd36 or empty vector control have been described30 and were maintained in RPMI medium containing 10% FBS supplemented with antibiotics.

Mice and atherosclerosis studies. Apoe−/− and Cd36−/−Apoe−/− mice have been described31. Tlr4−/−Apoe−/− and Tlr6−/−Apoe−/− mice were generated by intercrossing of Apoe−/− mice and Tlr4−/− or Tlr6−/− mice on the C57BL/6J background31. Doubly deficient offspring were viable and born at the expected Mendelian ratios. All mice were housed in a pathogen-free environment in autoclaved filter-top cages with autoclaved water and kept on a 12-hour light-dark cycle. All experiments were approved by the New York University School of Medicine Institutional Animal Care and Use Committee. Beginning at 5–6 weeks of age, mice were fed a modified Western diet (containing 3 g cholesterol per kg chow; DYET#100244; Dyets) for 12 weeks. Then, mice were anesthetized with isoflurane and exsanguinated by cardiac puncture. Hearts were perfused with PBS and the arterial system was perfusion-fixed with 10% formalin for lesion analysis or with RNAlater solution (Ambion) for analysis of RNA. The heart and upper section of the aorta were removed, cleaned of peripheral fat under a dissecting microscope and dissected directly under and parallel to the aortic leaflets. The upper section was embedded in optimum cutting temperature compound and was snap-frozen immediately. For quantification of lesions in the aorta en face, the aorta was dissected so that it was free of all branching vessels, then was incised ventrally from the aortic root to the bifurcation of the iliac arteries and stored in PBS. Images of aortas were digitally captured and lesion area selected with IP Lab Spectrum software. Lesion area was calculated relative to the total area of the whole aorta or defined regions of the aorta, including the aortic arch, thoracic, abdominal and iliac areas (descending). Hearts embedded in optimum cutting temperature compound were sectioned under a dissecting microscope and dissected directly under and parallel to the aortic root. The aortic arch, thoracic, abdominal, and ileal areas (descending) were distributed in aliquots and frozen immediately as described30. The nonamyloidogenic control reverse peptide was made soluble by similar treatment of plasmids effects that could affect inflammasome activation.

Reagents. Human LDL (BT-903) and acetylated LDL (BT-906) were from Biomedical Technologies. For preparation of oxLDL, LDL was diluted in PBS to a concentration of 250 µg/ml and was dialyzed overnight at 4 °C in 3 liters of PBS. Moderately oxidized LDL was prepared by dialysis for 6 h against 5 µM CuSO4, which was terminated by the addition of EDTA (200 mM) and butylated hydroxytoluene (500 mM). To obtain minimally modified LDL and extensively oxidized LDL, dialysis against 5 µM CuSO4 was terminated after 2 h or 24 h, respectively. Cholesterol (Sigma-Aldrich) was crystalized as described27. ATP and poly(dA:dT) were from Sigma-Aldrich. The poly(dA:dT) was delivered to the cytoplasm by transfection with Lipofectamine 2000 (Invitrogen). Amyloid-β and IAPP were from California Peptides. For the generation of soluble amyloid-β, human amyloid-β peptide (amino acids 1–42) was resuspended at a concentration of 5 mg/ml in dimethyl sulfoxide, distributed in aliquots and frozen immediately as described28. The nonamyloidogenic control reverse peptide was made soluble by similar treatment of a human amyloid-β peptide of amino acids 1–42 in reverse order (42–1). This method of preparation did not generate fibrillar amyloid-β, as assessed by thioflavin-T fluorescence and electron microscopy. Soluble human IAPP was resuspended in endotoxin-free water at a concentration of 1 mg/ml as described26. Nonamyloidogenic control rat IAPP was prepared similarly. All reagents were sterile and endotoxin free, as confirmed by Limulus amebocyte lysate analysis with a PYROGENT kit (Lonza). Ultrapure LPS (Alexis) was used for 6 h at a concentration of 200 ng/ml for priming. In some experiments, after 6 h of priming, the medium was changed to DMEM containing 2% FBS and cells were pretreated with the following inhibitors for 1 h before stimulation with NLRP3 activators: cytochalasin-D (Sigma-Aldrich), baflomycin A1 (Sigma-Aldrich), Congo red (Sigma-Aldrich) or Laisist (a gift from F. Maxfield). The broad spectrum caspase inhibitor ZVAD-fmk (benzoylcarbonyl–Val-Ala–Asp–fluoromethylketone), the caspase-1-specific inhibitor z-YVAD-fmk (benzoylcarbonyl–Val–Va–Asp–fluoromethylketone) and z-FA-fmk (benzoylcarbonyl–Phe–Ala–fluoromethylketone), an irreversible inhibitor of cysteine proteases (such as cathepsins B, L and S) that do not require an aspartic acid at position 1, were from Santa Cruz Biotechnology. In some experiments, cells were pre-treated with BAY 11-7082 (Calbiochem) or diphencyclidinium chloride (Sigma-Aldrich) 1 h before priming.

Molecular biology. RNA was isolated from cells and tissues with TRIzol reagent according to the manufacturer's protocol (Invitrogen, now known as Ambion). RNA (1 µg) was reverse-transcribed to generate cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). Gene expression was measured by real-time quantitative RT-PCR with SYBR Green for quantification (KAPA Molecular biology). As Ambion). RNA (1 µg) was reverse-transcribed to generate cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). Gene expression was measured by real-time quantitative RT-PCR with SYBR Green for quantification (KAPA Molecular biology). As Ambion). RNA (1 µg) was reverse-transcribed to generate cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). Gene expression was measured by real-time quantitative RT-PCR with SYBR Green for quantification (KAPA Molecular biology). As Ambion). RNA (1 µg) was reverse-transcribed to generate cDNA with the iScript cDNA Synthesis Kit (Bio-Rad).
Silencing of CD36 in vitro was assessed by transfection of immortalized macrophages with 10 ng ASO per well in 12-well plates with Lipofectamine RNAiMax (Invitrogen).

Serum array and ELISA. Mouse serum was used for multiplex analysis of cytokines with Quantibody Mouse Inflammation Array 1 (QAM-INF-1) according to the manufacturer’s instructions (RayBiotech). The Mouse IL-18 ELISA Kit (7625; MBL) was used for ELISA of IL-18 in serum. Specific DuoSet ELISA kits (DY401 (IL-1β) and DY400 (IL-1α); R&D Systems) were used ELISA of supernatants.

Microscopy. For analysis of speck formation in macrophages transfected to express ASC-CFP, cells were seeded in eight-well Lab-Tek chamber slides (Thermo Scientific) and, after the experiments, were fixed in 4% paraformaldehyde (IC Fixation Buffer, 1x concentrate; Invitrogen), washed with PBS and stained with Alexa Fluor 594–coupled cholera toxin subunit B (2 μg/ml; Invitrogen) and counterstained with DAPI (4′,6-diamidino-2-phenylindole; 0.5 μg/ml; Sigma) before being mounted with Fluorescent Mounting Medium (Dako). Cells were visualized by confocal microscopy (Zeiss 600 System). Images obtained at a magnification of ×63 were merged with ImageJ software. For quantification of speck formation, images were obtained by epifluorescence microscopy at a magnification of ×20. For analysis of crystal formation alongside lysosomal markers, live-cell imaging was done. For this, cells were seeded in 35-mm Petri dishes on 0.16-mm coverglasses and were allowed to adhere overnight. Cells were then pretreated for 1 h with Alexa Fluor 647–coupled dextran (10 kDa; Sigma) before being mounted with Fluorescent Mounting Medium (Thermo Scientific) and, after the experiment, were fixed in 4% paraformaldehyde, washed with PBS, stained with Alexa Fluor 594–coupled cholera toxin subunit B (2 μg/ml; Invitrogen) and counterstained with DAPI (for the preservation of crystalline material, nuclei were stained with Hoechst 33342 (Immunochemistry) and combined confocal reflection microscopy was done with a Leica TCS SP5 II AOBS confocal laser-scanning microscope as described13. Plaque crystal content was quantified with Velocity Quantitation (PerkinElmer) and is presented relative to total plaque area.

Flow cytometry. For evaluation of lysosomal damage, cells were incubated with LysoTracker Red DND-99 (1 μM) for 1 h before stimulation with inflammasome activators. Lysosomal damage was defined as loss of LysoTracker fluorescence, as assessed by flow cytometry. For evaluation of the binding of soluble peptides, human IAPP peptide with a fluorescent dye conjugate on the amino terminus and Hilyte-Fluor 488–conjugated human amyloid-β peptide of amino acids 1–42 (AnaSpe) were made soluble as described above. Binding to Chinese hamster ovary cells was assessed after 2 h of incubation by flow cytometry to measure associated fluorescence as described54. Extracellular fluorescence was quenched with 0.2% trypan blue shortly before analysis, and binding was calculated as the difference between total cell-associated fluorescence and intracellular fluorescence. An Accuri C6 Flow Cytometer (BD Biosciences) was used for all flow cytometry.

Statistical analysis. Statistical significance was assessed by one-way analysis of variance for multiple comparisons or an unpaired, two-tailed Student's t-test for single comparison. P values of less than 0.05 were considered significant.

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