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Coordinate Stimulation of Macrophages by Microparticles and TLR Ligands Induces Foam Cell Formation

Peter A. Keyel,* Olga A. Tkacheva, † Adriana T. Larregina,* † and Russell D. Salter*  

Aberrant activation of macrophages in arterial walls by oxidized lipoproteins can lead to atherosclerosis. Oxidized lipoproteins convert macrophages to foam cells through lipid uptake and TLR signaling. To investigate the relative contributions of lipid uptake and TLR signaling in foam cell formation, we established an in vitro assay using liposomes of defined lipid compositions. We found that TLRs signaling through Toll/IL-1R domain-containing adapter inducing IFN-β promoted foam cell formation by inducing both NF-κB signaling and type I IFN production, whereas TLRs that do not induce IFN, like TLR2, did not enhance foam cell formation. Addition of IFN-α to TLR2 activator promoted robust foam cell formation. TLR signaling further required peroxisome proliferator-activated receptor α, as inhibition of peroxisome proliferator-activated receptor α blocked foam cell formation. We then investigated the ability of endogenous microparticles (MP) to contribute to foam cell formation. We found that lipid-containing MP promoted foam cell formation, which was enhanced by TLR stimulation or IFN-α. These MP also stimulated foam cell formation in a human skin model. However, these MP suppressed TNF-α production and T cell activation, showing that foam cell formation can occur by immunosuppressive MP. Taken together, the data reveal novel signaling requirements for foam cell formation and suggest that uptake of distinct types of MP in the context of activation of multiple distinct TLR can induce foam cell formation.  

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Macrophages are one of the first-line responders of the innate immune system. As such, they help coordinate the immune response to pathogens and other forms of danger. Correct signaling is vital to a protective immune response, and aberrant activation can lead to a number of diseases, including atherosclerosis (1). Indeed, a prominent characteristic of atherosclerosis, the formation of fatty streaks, is caused by conversion of macrophages into foam cells. Foam cell formation is characterized by an accumulation of lipids, predominantly cholesterol esters (1, 2). Oxidized low-density lipoprotein (oxLDL) is the archetypal source of cholesterol and inducer of foam cell formation (1). However, other lipids can also contribute to foam cell formation. For example, treatment of macrophages with LPS and LDL can lead to foam cell formation, even without oxidation of LDL (3).

LPS signals through TLR4. TLRs are sensors of pathogen-associated molecular patterns, including molecules that range from bacterial and viral nucleic acids to bacterial and fungal cell wall components. Typically, TLRs signal through adaptors, the best understood being Toll/IL-1R domain-containing adapter inducing IFN-β (Trif) and MyD88, which propagate the immune response through activation of NF-κB, AP-1, and/or type I IFN production (4). TLR signaling in the context of pathogen infection can lead to atherosclerosis. For example, activation of TLR2 by Chlamydia leads to enhanced atherosclerosis (5). OxLDL itself can signal through a CD36/TLR4/TLR6 heterotrimer to induce foam cell formation (6), though it has also been reported that oxLDL impairs LPS-induced NF-κB signaling (7, 8). One way in which TLR4 controls foam cell formation is through induction of cellular programs that alter the metabolic state of a macrophage (9). These changes are designed to trap and eliminate internalized pathogens, but they also result in decreased cholesterol efflux, which enhances foam cell formation (10). Thus, TLR4 can influence foam cell formation, though the signaling requirements remain unclear.

OxLDL and LDL are not the only lipid sources that can promote foam cell formation. Exosomes derived from T cells have also been implicated in foam cell formation (11). Unlike oxLDL, these exosomes are internalized by the phosphatidylinerine receptor on macrophages (11). Microparticles (MP) derived from endothelial cells and platelets have also been implicated in a number of cardiovascular diseases, including hypertension, coronary and peripheral artery diseases, and atherosclerosis (12). Indeed, MP are increased in patients with cardiovascular disease (12) and can be predictive of disease (13). MP also lead to neovascularization, progression, and eventual destabilization of atherosclerotic plaques (14). MP can promote vascular inflammation and expression of inflammatory cytokines, partially through delivery of IL-1β to endothelial cells (15). They can also lead to macrophage apoptosis (16). Many of these functions have been attributed to the protein profile of the MP (12). However, what role the lipids present in the MP play is less well understood. Similarly, the role of MP in the initial stage of foam cell formation is poorly understood.

We hypothesized that foam cell formation requires both activation through a TLR pathway and any source of abundant exogenous lipid. OxLDL fulfills both of these requirements and can drive foam cell formation independently of other ligands. In the current study, we further investigated additional TLR ligands and...
biologically relevant lipid sources, specifically nonoxidized MP, that might also promote foam cell formation.

**Materials and Methods**

**Reagents**

Unless otherwise noted, reagents were from Sigma-Aldrich (St. Louis, MO). Phosphatidylycholine, phosphatidyleserine, and phosphatidylethanolamine were from Avanti Polar Lipids (Alabaster, AL). Ultrapure LPS, Pan3CSK4, polyinosinic-polycytidylic acid (polycyclc), and type B CpG-ODN were purchased from Invivogen (San Diego, CA). Anti-CD69 conjugated to FITC and anti-CD80 conjugated to allophycocyanin Abs were from BD Biosciences (San Jose, CA). Streptolysin O (SLO) was purified from *Escherichia coli* as previously described (17). LDL and oxLDL were from Biomedical Technologies (Stoughton, MA).

**Cell culture**

Bone marrow-derived macrophages (BMDM) were isolated and cultured as previously described (18). Bone marrow from B6, TLR4−/−, MyD88−/−, and Tnf−/− mice were generous gifts from Lisa Borghesi and Timothy Billiar. HeLa, D2, T3A/3Ha, and B16 cells were cultured in DMEM supplemented with 10% FCS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. 3T3 cells were also cultured in this medium, though it was fortified with 1 mM sodium pyruvate and 1X nonessential amino acids. To rule out MP contamination from FCS, TA3/3Ha cells were also cultured in AIM V media (Invitrogen, Carlsbad, CA). T27A cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. B3Z cells were also cultured in this media, along with 500 μg/ml G418. Human macrophages were differentiated from plastic-adhered PBMCs obtained anonymously from the Central Blood Bank (Pittsburgh, PA) in DMEM supplemented with 10% FCS, 2 mM t-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10 ng/ml GM-CSF for 5 d.

**Liposome preparation**

Liposomes were prepared as previously described (19). Individual lipids in chloroform or ethanol (cholsterol) were mixed at a molar ratio of 45:45:10:0 or 22:5.2:5:10:50 phosphatidylycholine/phosphatidylethanolamine/phosphatidyleserine/cholestrol liposomes lacking cholesterol (chool) and liposomes containing 50% cholesterol (50chol), respectively) in glass tubes and dried under nitrogen. The lipids were resuspended in 15 mM HEPES (pH 7.4), 50 mM sorbitol, and 1 mM magnesium acetate at a concentration of 4 mg/ml and incubated at 37°C for 1 h. Liposomes were formed through four freeze-thaw cycles and stored −80°C. No oxidation of the liposomes was detected by thiobarbituric acid reactive substances assay nor was LPS detected by *Limulus* amebocyte lysate assay.

**MP preparation**

Spontaneously released vesicles (SRV) were prepared by collecting the supernatant of cells cultured for 2 to 3 d at 37°C and centrifuging first at 300 × g and then at 107,000 × g using a Sorvall Surespin 630/36 rotor (Thermo Scientific). The pellet was resuspended in RPMI 1640 and used for assays. For ectosome (MV) production, 50–100 million target cells were harvested, centrifuged at 300 × g and resuspended in RPMI 1640. SLO was added at a sublytic dose (300–1500 U/ml, depending on cell type) and the cells incubated at 37°C for 15 min. The cells were pelleted at 300 × g and the MV isolated from the supernatant via centrifugation at 107,000 × g using a Beckman SW60 Ti rotor (Beckman Coulter). The pellet was resuspended in RPMI 1640. Protein content was determined by Bradford assay and cholesterol content colorimetrically according to manufacturer instructions (Cayman Chemicals, Ann Arbor, MI). Electron microscopy (EM) analysis was performed by adsorbing MP onto EM grids for 10 min at room temperature and staining for 30 s with 1% uranyl acetate. Grids were examined on a JEOL 1011 transmission EM (Jeol).

**Foam cell assay**

A total of 10^5 BMDM were incubated in IMDM supplemented with 10% FCS, 1X t-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin for 2 d in the presence or absence of 112 μg/ml liposomes, 25 μg/ml MV or SRV, 1 μg/ml Pan3CSK4, 10 ng/ml LPS, 10 μg/ml polyC, 1.68 μM CpG, and 100 U/ml IFN-α (PBL InterferonSource, Piscataway, NJ) and fixed in 2% p-formaldehyde for 15 min. Cells were washed in 60% isopropanol, stained with 0.3% Oil Red O in 60% isopropanol, washed once each in 60% isopropanol and PBS, stained for 1 min with Harris hematoxylin, washed in PBS, and mounted in gelvatol. Images were acquired on an Olympus Provis using a 40X objective (Olympus). All macrophages containing one or more Oil Red O-positive lipid droplets were counted as foam cells, consistent with previous methods (20). Foam cell formation was also assessed by measuring the intensity of Oil Red O staining using Meta-morph ( Molecular Devices). Images were separated into red/green/blue images and the green image subtracted from the red to provide the red Oil Red O-specific image. An image mask was prepared through dilation and erosion, and the integrated intensity above background was measured. This intensity was normalized to the number of cells in the field. The per-cell intensity was expressed as a percentage of the maximal intensity in the experimental set, which enabled comparison across sets. Filtration of the media through a 0.1-μm filter to remove serum MP did not alter the extent foam cell formation (data not shown).

**Cholesterol assay**

A total of 5 × 10^5 BMDM were incubated in IMDM supplemented with 10% FCS, 1X t-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin for 2 d in the presence or absence of 112 μg/ml liposomes, 10 ng/ml LPS, or 10 μg/ml polyC, fixed in 2% p-formaldehyde for 15 min, and washed in PBS and 60% isopropanol. Cholesterol was extracted with 250 μl isopropanol for 30 min at room temperature and cholesteryl esters measured using a fluorimetric cholesterol assay (Cayman Chemicals) according to the manufacturer’s instructions. Protein was extracted using 0.1 M NaOH and measured by Bradford assay.

**Skin assays**

Samples of normal human skin were obtained from healthy donors undergoing abdominal plastic surgery and distributed by the tissue bank, a division of the Department of Pathology of the University of Pittsburgh Medical Center. Human skin samples obtained following deidentification by honest brokers are classified as human nonsubjects by our institutional review board. Skin explants composed of epidermis and dermis were obtained by a dermatone as previously published (21) and injected with oxLDL, 50chol, or MV. Following 3 d, skin samples were paraffin embedded, sectioned, stained with H&E, and analyzed by light microscopy using an Axiosstar plus microscope (Zeiss).

**Activation status of BMDM**

A total of 5 × 10^5 BMDM were incubated with lipids and/or TLR ligands for 18 h at 37°C, harvested with Cellstripper (Cellgro, Manassas, VA), stained with Abs for 30 min on ice, and analyzed by FACS using an LSR II (BD Biosciences). Supernatants were saved for measurement of TNF-α by ELISA (BioLegend, San Diego, CA).

**B3Z assay**

T cell activation was measured as previously described (22). Briefly, 10^4 B3Z cells were cultured at 37°C overnight in 96-well plates in the presence or absence of 0.1–100 ng/ml OVA peptide (SIINFEKL), 5000 B16 cells, 112 μg/ml liposomes, or 25 μg/ml MV. B3Z cells were washed, incubated for 4 h at 37°C in 150 μM chlorphenol red-b-D-galactopyranoside (CPRG) and 0.1% Triton X-100, and A575 read on a PowerWave XS plate reader (BioTek, Winouski, VT).

**Statistics**

Statistics were determined with two-way ANOVA followed by Bonferroni posttesting using Prism 3.0 (GraphPad, La Jolla, CA).

**Results**

Multiple types of lipid MP induce foam cell formation

To dissect the relationship among TLR signaling, lipids, and foam cell formation, we compared the ability of oxLDL, LDL, and liposomes with defined compositions to promote foam cell formation in BMDM with or without LPS by Oil Red O staining. Although LPS alone did not promote any foam cell formation, oxLDL robustly did so regardless of LPS, consistent both with its own ability to activate TLR4 and ability to antagonize LPS-induced signaling (6–8) (Fig. 1A, 1B). As previously reported (3), LDL promoted foam cell formation in the presence of LPS (Fig. 1C). We tested the ability of 0chol or 50chol to promote foam cell formation. We found that in the absence of cholesterol, liposomes only stimulated foam cell formation in the presence of LPS (Fig.
Consistent with the ability of cholesterol to drive foam cell formation (1), 50chol promoted foam cell formation without LPS, though LPS further enhanced this effect (Fig. 1E). Thus, LPS can enhance or even drive foam cell formation in the presence of exogenous lipids.

TLR4 independent foam cell formation

Because LPS enhanced foam cell formation, we asked if the spontaneous formation of foam cells in the presence of cholesterol-containing liposomes was due to TLR4 activation. We determined that the liposome preparations were endotoxin-free by Limulus amebocyte lysate assay and then tested whether they stimulated foam cell formation in TLR4−/− macrophages singly or in combination with either LPS or the TLR3 ligand polyI:C. Both TLR ligands promoted robust foam cell formation in B6 BMDM (Fig. 1F). We found that TLR4−/− BMDM were able to form foam cells following liposome treatment (Fig. 1G) when coexposed to polyI:C, but not to LPS (Fig. 1G). Quantitation of the Oil Red O staining in these macrophages showed that an increase in the amount of cholesterol in the liposomes increased the severity of foam cell formation (Supplemental Fig. 1A, 1B). Oil Red O staining recapitulated cholesteryl ester levels, as LPS and polyI:C both induced an increase in cholesteryl esters (Supplemental Fig. 1C). We next asked if 0chol or 50chol could induce foam cell formation in primary human macrophages. The human macrophages had a higher degree of spontaneous foam cell formation in culture (Supplemental Fig. 1D), which has been observed in other systems (23). The 50chol significantly increased this basal level of foam cell formation (Supplemental Fig. 1D) in the presence or absence of LPS. Taken all together, these data show that TLR3, in addition to TLR4, can promote foam cell formation.

Synergism between TLR and type I IFN signaling for foam cell formation

To determine if multiple TLRs could promote foam cell formation, we treated macrophages with either 0chol or 50chol and ligands for
TLR2, -3, and -4. Although we found that stimulation through TLR3 and TLR4 increased the incidence of foam cell formation with either 0chol and 50chol as a lipid source (Fig. 2A), TLR2 did not provide significant augmentation when ligated with Pam3CSK4. Because TLR2 activation by Pam3CSK4 is mediated at the cell surface, we considered whether TLRs that function in endosomes might selectively enhance foam cell formation. Traditionally, TLRs are divided into surface TLRs, which predominantly signal at the surface, and endosomal TLRs, which predominantly signal following internalization. The surface TLRs signal predominantly through MyD88, leading only to NF-κB activation. The endosomal TLRs signal through Trif (TLR3 and TLR4) or MyD88 (TLR4, TLR9), leading to both NF-κB and IFN regulatory factor (IRF) 3 (Trif) or IRF7 (MyD88) activation (4). IRF signaling leads to type I IFN production (4). To test whether type I IFN production is responsible for the observed difference in the ability of surface and endosomal TLRs to enhance foam cell formation, we added IFN-α in combination with liposomes to BMDM. IFN-α promoted foam cell formation only in 50chol, but not 0chol (Fig. 2B). Because the other primary signaling outcome of TLR signaling is NF-κB activation, we asked if treatment with both IFN-α and Pam3CSK4, neither of which independently promote 0chol liposome-mediated foam cell formation, would enhance foam cell formation. We found that the combination of Pam3CSK4 and IFN-α together with either 0chol or 50chol drove robust foam cell formation, measured either by total number of foam cells or intensity of Oil Red O staining (Fig. 2). This was comparable to the foam cell formation provided by LPS or polyIC and liposomes (Fig. 2). Thus, type I IFN enhances foam cell formation.

**Trif is required for polyIC- and LPS-dependent foam cell formation**

To further probe the signaling role TLRs play in foam cell formation, we asked which TLR adaptors were responsible for foam cell formation. We treated BMDM from wild-type, Trif−/−, or MyD88−/− mice with no lipid, 0chol, or 50chol and various TLR ligands. Ablation of Trif reduced the spontaneous foam cell formation that occurs following polyIC treatment (Fig. 3A). Foam cell formation due to 0chol in the presence of polyLC was Trif dependent (Fig. 3B). Foam cell formation following treatment with 50chol was Trif dependent, as was the enhancement of foam cell formation by either LPS or polyIC (Fig. 3C). No significant difference was observed between wild-type and knockout mice when treated with Pam3CSK4 plus IFN-α (Fig. 3B, 3C), indicating TLR adaptor deficiencies could be overcome. Thus, we believe that both type I IFN and NF-κB signaling, which are provided by TLR ligation, may be needed for liposome-mediated foam cell formation.

**Activation of peroxisome proliferator-activated receptor α, but not peroxisome proliferator-activated receptor γ, enhances foam cell formation**

TLR ligation has previously been implicated in atherogenic signaling factors. For example, in a transfected cell system, IRF3 can act as a switch between LXR activation and foam cell formation in an IFN-independent manner (24). Given that LXR activation can also lead to peroxisome proliferator-activated receptor (PPAR) γ activation, which is antiatherogenic (25), we wanted to determine what role PPAR played in MP-induced foam cell formation. When we treated macrophages with agonists or antagonists to PPARγ in the presence of TLR ligands and liposomes, we found no effect on the extent of foam cell formation (data not shown). Similarly, the LXR agonist GW3965 had no effect on foam cell formation in our system (data not shown). However, we found that PPARα agonists and antagonists had a profound effect on foam cell formation. Fenofibrate, a PPARα agonist, promoted foam cell formation in macrophages treated with either 0chol or 50chol (Fig. 4A). Conversely, GW6471, a PPARα antagonist, blocked foam cell formation by 50chol (Fig. 4A). Although GW6471 did not significantly change the number of foam cells formed by treatment with LPS and 0chol, it did reduce the overall Oil Red O staining (Fig. 4). When BMDM were treated with LPS and liposomes, fenofibrate further augmented foam cell formation by 0chol (Fig. 4B). Conversely, GW6471 impaired foam cell formation by the combination of either 0chol or 50chol and LPS (Fig. 4B). Thus, PPARα activation plays a decisive role in foam cell formation.

**Foam cell formation by endogenous lipid-bearing vesicles**

To further characterize the requirements for lipid sources that could promote foam cell formation and substantiate the biological relevance of these findings, we next addressed whether endogenous MP could promote foam cell formation. T cell-derived exosomes promote foam cell formation (11). MP similarly play a role in atherogenesis (26). We examined the ability of two types of MP to promote foam cell formation and whether the TLR pathway operatively with liposomes was used. One source of MP was SRV by TA3/Ha adenocarcinoma or B16 melanoma cells. EM examination of SRV derived from TA3/Ha or B16 cells indicated that they were
80–100 nm in size, consistent with exosomes (Supplemental Fig. 2A, 2B). A second source of MP was microvesicles shed by cells in response to damage by toxins (MV), also known as ectosomes (17, 27–29). Ectosomes are shed as part of the cellular resistance mechanism to pore-forming toxins (17, 30) and may be taken up by macrophages responding to the bacterial infection. MV prepared from four different cell lines were larger and more heterogeneous than SRV (Supplemental Fig. 2C–F), as previously described (30). As expected from membranes containing large numbers of cholesterol-binding toxin pores, MV were significantly enriched in cholesterol relative to their parent cell line (Supplemental Fig. 2G). We compared the ability of SRV and MV to promote foam cell formation with oxLDL. Both SRV and MV stimulated varying degrees of foam cell formation (Fig. 5A, Supplemental Fig. 3). We found that cholesterol content of the MV correlated with robustness of foam cell formation, reminiscent of 0chol and 50chol (Fig. 5A, Supplemental Fig. 2G). Thus, two types of MP, SRV and MV, both promote foam cell formation, and this is correlated with the amount of cholesterol present in the shed vesicle.

FIGURE 3. Trif is required for foam cell formation. BMDM from B6, MyD88−/−, or Trif−/− mice were treated and quantitated as described in Fig. 1 with no lipid source (A) or either 0chol (B) or 50chol (C). An average of 1300 cells from nine independent experiments were counted for B6 and Trif−/−; and an average of 1000 cells from six independent experiments were counted for MyD88−/−. *p < 0.05, **p < 0.001 from B6. KO, Knockout.

FIGURE 4. PPARα is needed for foam cell formation. BMDM were treated as described in Fig. 1, except that PPARα agonist fenofibrate or PPARα antagonist GW6471 were included where indicated. The number of foam cells (A) or the intensity of Oil Red O staining (B) was quantitated. An average of 500 cells from five independent experiments was counted for each condition. *p < 0.05, **p < 0.01, ***p < 0.001 compared with no inhibitors.
cholesterol showed enhanced foam cell formation when treated with LPS (Fig. 5A). Treatment with either polyI:C or both Pam3CSK4 and IFN-α also enhanced SRV and MV-induced foam cell formation (data not shown). Treatment with PPAR agonists and antagonists similarly promoted or reduced expression as observed with the liposomes (Fig. 5B). Finally, given that macrophages can signal through p38 in response to ATP-derived MP (31), we tested whether p38 played a role in foam cell formation. We found that chemical inhibition of p38 with SB203580 had no effect upon foam cell formation (Fig. 5A). Thus, MV show similar TLR and PPAR dependence for foam cell formation as liposomes.

**MP promote foam cell formation in tissue**

We next asked whether MP were capable of inducing foam cell formation in a tissue model. We used total human skin because it was a readily available source of healthy tissue for which the histology is well understood and manipulations easily performed (21). Vessel triads present in the skin contain endogenous macrophages, removing the variable of cell migration into the tissue. Importantly, injection of PBS intradermally led only to limited edema 3 d later and no disruption of vessel triads (Fig. 6A). Injection of oxLDL, however, led to lipid accumulation in macrophages and a classic foam cell phenotype (Fig. 6B, thick arrowheads). Similarly, injection of MV derived from B16 or 3T3 cells led to foam cell formation, as did injection of SRV and 50chol (Fig. 6C–F, thick arrowheads). The melanin present within B16 MP allowed us to confirm that these MP were internalized by the foam cells (Fig. 6C, 6E, thin arrowheads). To confirm that the foam cells were macrophages, frozen sections of oxLDL-treated tissue were cut and dual stained with the macrophage marker anti-CD68 and endothelial marker anti-CD31 (Fig. 6G, 6H). Although CD31+ cells did not show any foam cell formation, CD68+ cells in the vessel triad did become foamy, as seen by dilution of the CD68 signal and lipid inclusions present in the cell (compare intensity of CD68 staining in Fig. 6G and 6H). Thus, we conclude that MP can promote foam cell formation in tissue.

**FIGURE 5.** MP promote foam cell formation. Microvesicles were generated by treating the indicated cell lines with sublytic doses of SLO for 15 min at 37°C (MV) or collecting SRV from cultures and centrifuging the supernatants at 107,000 g. The pellet was resuspended and added to BMDM in the presence or absence of 10 ng/ml LPS as cells treated as in Fig. 1. Approximate cholesterol levels in MV were 2.5 µg/ml (3T3), 1.2 µg/ml (D2), and 0.6 µg/ml (T27A). In addition to MP, BMDM received either LPS (A) or LPS plus PPARα agonist and antagonist (B). For each condition, an average of 700 cells from three to nine independent experiments (A) or 350 cells from three experiments (B) were counted. *p < 0.05, **p < 0.01, ***p < 0.001 compared with no LPS (A) or no inhibitor (B).

**FIGURE 6.** MP promote foam cell formation in a human skin model. MP from the indicated sources or 50chol were injected intradermally into skin explants, cultured for 3 d at 37°C fixed in formaldehyde, embedded in paraffin, sectioned and stained with H&E (A–F), or stained with anti-CD68 (red) and anti-CD31 (brown) to identify macrophages and endothelial cells, respectively (G, H). Images illustrate clearly the presence of macrophages with clear and vacuolated cytoplasm characteristic of foam cells, shown by thick arrowheads. Thin arrowheads indicate MP derived from B16 cells, which contain melanin. Images from one representative experiment of two performed are shown. Scale bar, 20 µm.
Endogenously derived MP only weakly activate macrophages in the absence of TLR ligands

One difference between foam cell formation due to MP and that due to oxLDL is the need of MP for an exogenous TLR ligand. To determine whether there are other differences in macrophage phenotype, we tested the ability of MP or oxLDL to activate macrophages after 18 h. We found that LPS induced surface CD69 expression on a large percentage of cells and CD80 to a lesser extent (Fig. 7). Similarly, oxLDL induced primarily CD69, but also CD80 (Fig. 7). In contrast, MV induced CD80 expression and minimal CD69 induction (Fig. 7). Both 0chol and 50chol did not induce either CD69 or CD80 surface expression (data not shown). Thus, we observe distinct effects of oxLDL and MP on macrophages, even though both sources can drive macrophages to become foam cells.

SRV and MV suppress T cell function

Because we observed limited activation of BMDM treated with MV or SRV, we measured the ability of BMDM to produce TNF-α following TLR stimulation. When BMDM were treated with either LPS or TLR9 ligand CpG, they produced large amounts of TNF-α (Fig. 8A). In the presence of MV or SRV, the TNF-α production was attenuated (Fig. 8A). SRV were not as able to impair TNF-α production as robustly as MV (Fig. 8A).

To confirm that MP were acting in an immunosuppressive manner, we tested their ability to block Ag stimulation of the B3Z T cell hybridoma. B3Z cells secrete β-galactosidase in response to stimulation through H2-K<sup>d</sup> with the OVA peptide SIINFE(H/K)L (OVA peptide) (22). We incubated B3Z cells with OVA peptide, B16 cells (which express H2-K<sup>d</sup>), and B16 or 3T3-derived MV or SRV and measured β-galactosidase activity. We found that both MV and SRV blocked activation following treatment with 0.1 nM OVA peptide, though this inhibition could be overcome with a 100-fold increase in OVA peptide (Fig. 8B). B3Z cells can also present OVA peptide to one another, although the efficiency is greatly reduced (Fig. 8C). MV and SRV also blocked this activation of B3Z cells (Fig. 8C). These data indicate that MV and SRV are immunosuppressive and yet can promote foam cell formation.


**Discussion**

In this study, we have dissected the novel signaling pathways leading to foam cell formation. We have characterized a pathway for foam cell formation wherein three signals are required: exogenous lipid, NF-κB signaling, and IFN-α. These signals lead to PPARα activation and subsequent foam cell formation. We offer support for the hypothesis that in the presence of TLR ligands, MP, such as ectosomes or SRV, promote foam cell formation, even though these MP are themselves weakly activating or inhibitory.

These data shed light on the link between TLR activation and foam cell formation. We find in this study that both NF-κB and type I IFN production are required for optimal foam cell formation. OxLDL activates both arms of this pathway through its interaction with TLR4. TLR ligands that do not accomplish both of these functions, such as TLR2, are predicted to require another source of type I IFN for optimal foam cell formation. In *Chlamydia* infection, TLR2 is critically required for foam cell formation (5). Stimulation with zymosan, a potent TLR2 ligand, however, did not result in foam cell formation (32). The model presented in this study resolves this seeming paradox, as *Chlamydia*-induced foam cell formation uses both MyD88-dependent signaling and Trif-dependent IRF3 signaling (33), whereas zymosan only signals through MyD88. A previous study observed foam cell formation from Pam3CSK4 alone (5). However, this study used peritoneal macrophages elicited with peptone, which could be contaminated with LPS. It is also possible elicitation provides one of the signals necessary for foam cell formation. Likewise, spontaneous foam cell formation in cell lines (23) or primary human macrophages is necessary for foam cell formation. Likewise, spontaneous foam cell formation in cell lines (23) or primary human macrophages is likely due to cell-type differences. Mice are less susceptible to atherosclerosis than humans, so for in vitro studies, murine macrophages may be a more suitable model system. In the BMDM used in this study, we find that additional stimulation is needed beyond TLR2 ligation, and an exogenous lipid source is necessary. We provide one possible mechanistic link between infection and atherosclerosis.

In addition to *Chlamydia*, other pathogens can induce foam cell formation. There is a link established between periodontal disease and atherosclerosis (34–36). Periodontopathic bacteria have been isolated from atherosclerotic plaques (37). Similar to *Chlamydia* infection, TLR2 and TLR4 signaling occurs in the endothelium in response to infection with these bacteria (38). Foam cell formation has also been observed in the context of tuberculosis granuloma formation (10). Based on the results in this study, we predict that blocking either type I IFN production or NF-κB signaling will reduce the ability of these bacteria to accelerate atherosclerosis.

These data also demonstrate that multiple sources of lipid can drive foam cell formation. We show that in addition to cholesterol carried on LDL or oxLDL particles, chemically defined liposomes can promote foam cell formation. This suggests that circulating MP could promote foam cell formation if NF-κB activation and type I IFNs are also present. We show that two types of MP, SRV and ectosomes, have the capacity to induce foam cell formation, including in vivo in a human skin model. There is a growing appreciation for the number of cell types in the body that emit SRV and the biological role these MP play. The concentration of MP circulating in the serum of acute myeloid leukemia patients reaches 75 μg/ml (39). In this study, we found 25 μg/ml of MV sufficient to promote robust foam cell formation. If circulating MP contained a similar amount of cholesterol to the MV described in this study, the amount of cholesterol would be 3-fold less than the 50chol. Hence, we believe 50chol serve as a simple model system for studying MP-induced foam cell formation at physiologically relevant concentrations. We find that a wide variety of MP can induce foam cell formation and therefore could be directly relevant to atherosclerosis.

Our results expand work performed with platelet and endothelial cell MP to include SRV derived from a range of cell lines and ectosomes. Ectosomes are shed in response to cellular attack by pore-forming toxins (17, 40). Because bacteria produce many pore-forming toxins, we suggest that these ectosomes could provide the source of lipid for foam cell formation in the context of bacterial infections. In the absence of pore-forming toxin, clearance of the bacteria through complement can induce ectosomes (40). Finally, certain strains of oral pathogens themselves secrete MP, which may incorporate TLR ligands (41, 42). Depending on the bacteria and immune response involved, all three components needed for foam cell formation may be present during infection. This model provides a mechanistic link between the invading bacteria and foam cell formation, which can lead to atherosclerosis.

Typically foam cells have an inflammatory phenotype, and oxLDL induces inflammatory cytokines (6). However, the MP and liposomes described in this study did not induce inflammation on their own. Indeed, the MP blocked TNF-α secretion and T cell activation, consistent with an immunosuppressive phenotype. An immunosuppressive phenotype is consistent with the phenotype of MP. SRV released by tumor cells are capable of diminishing cellular responses to IL-2 (43), induce apoptosis in T cells (44, 45), and reduce NK cell lytic capacity (46, 47). Furthermore, they can also express Ags that sequester anti-tumor Abs, protecting the tumor cell from Ab-dependent cell-mediated cytotoxicity (48). Likewise, MV shed by platelets can be immunosuppressive due to phosphatidylycerine and protein expression (49). Importantly, platelet-derived MV inhibit induction of TNF-α in macrophages (50), similar to what we observe. Platelet-derived MV are not provoked by toxin attack, but instead are spontaneously shed after 5 d of platelet storage (50). Despite the absence of toxin, these MV are immunosuppressive (50). This suggests that SLO itself does not affect the overall immunosuppressive nature of plasma membrane-derived vesicles. Given that platelet-derived MP account for 70–90% of circulating MP in blood (51–53), tolerance to these MP may be more important for survival than mounting an inflammatory response to toxin-induced MV. Although these MP impair inflammatory cytokine responses following TLR ligation, they also induce foam cell formation under these circumstances. This widens the range of potentially atherogenic stimuli and reveals novel mechanisms for foam cell formation.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Figure Legends

Supplemental Figure S1. Foam Cell Formation in Macrophages. (A, B) The intensity of Oil Red O staining from B6 (A) or TLR4<sup>-/-</sup> (B) mice in Fig. 1 was quantitated. (C) B6 BMDM were treated as described in Fig 1 with the indicated lipids and TLR ligands for 2 days, and the level of cholesteryl esters (normalized to ng cholesterol/µg protein) measured in the cells. Stars indicate conditions not determined. (D) Primary human macrophages were treated with the indicated lipids and TLR ligands, and the intensity of Oil Red O staining measured. * p<0.05, ** p<0.01, *** p<0.001.

Supplemental Figure S2. Characterization of microparticles. (A-F) Microparticles derived from culture supernatants (A, B) or SLO-treated cultures (B-F) were negatively stained with 1% uranyl acetate and examined by EM. (G) Microparticles or the remaining cell pellets were analyzed for protein and cholesterol content. ** p<0.01, *** p<0.001 Scale bar = 100 nm

Supplemental Figure S3. Foam cell formation induced by microparticles. BMDM were treated, fixed and stained as in Fig 1, using the indicated lipid sources in the presence or absence of LPS. Approximate cholesterol levels in MV were 2.5 µg/mL (3T3) and 5.5 µg/mL (B16). Panels showing no lipid or oxLDL are identical to those displayed in Fig 1. Scale bar = 10 µm.
Supplemental Figure S2

A  SRV TA3  B  SRV B16  C  MV 3T3
D  MV T27A  E  MV D2  F  MV B16

G

relative cholesterol (ng chol/μg prot)

3T3  B16  D2  T27A

Cell Line

microvesicle  cell

***  **  **  *
Supplemental Figure S3