Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyreactive IgM producing B1a lymphocytes

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Aims
To investigate whether activation of atheroprotective peritoneal B1a cells by apoptotic cells or phosphatidylserine liposomes (PSLs) can enhance their protective actions during atherosclerosis development.

Methods and results
Male apolipoprotein E-knockout (ApoE−/−) mice were treated with apoptotic cells or PSLs at the beginning of 8-week high-fat diet. Intraperitoneally administered apoptotic cells attenuated atherosclerosis in hypercholesterolemic ApoE−/− mice by 53% and macrophage accumulation by 52%, effects mimicked by administering PSLs and abolished by B1a cell depletion by splenectomy. These effects were associated with reduced lesion CD4+ and CD8+ T cells, mRNAs of MCP-1, VCAM-1, TNF-α, IL-1β, IL-12, and IL-18 while anti-inflammatory TGF-β mRNA levels doubled. Apoptotic cells or PSLs increased B1a lymphocytes including TIM-1 + B1a cells in vivo and in vitro while other lymphocyte populations were unaffected. Total plasma IgM, anti-leucocyte, anti-CD3, anti-CD4, and anti-oxLDL IgM were elevated. IgM in atherosclerotic lesions was also elevated and this was associated with reduced lesion MDA-LDL (oxLDL), apoptotic cells and necrotic core size. These effects of activating B1a cells could be attributed to B1a-derived polyreactive IgM deposited in lesions that reduce inflammatory cytokines by lowering lesion ox-LDL via anti-oxLDL IgM, T-cells via anti-leucocyte, anti-CD3, and anti-CD4 IgM, apoptotic cells and necrotic core size via IgM binding to apoptotic cells and enhancing phagocytosis, which also elevates anti-inflammatory cytokines.

Conclusion
Targeting B1a cell activation by PSLs may be a potentially potent therapeutic strategy to attenuate atherosclerosis and reduce the incidence of atherosclerosis-dependent myocardial infarction and stroke.

Keywords
B1a cells • IgM • Apoptotic cells • Phosphatidylserine liposomes • Atherosclerosis

1. Introduction
Despite lipid-lowering statins, heart attack and stroke are atherosclerosis-related cardiovascular diseases remaining the leading cause of mortality worldwide. Atherosclerosis is a chronic inflammatory disease of medium and large arteries characterized by accumulation of lipids and immune cells that modulate atherosclerotic lesion development and progression. Atherosclerosis becomes clinically significant upon severe lumen encroachment or thrombotic occlusion following lesion rupture. B cells have been identified within atherosclerotic lesions and associated adventitia in humans and in mice. We and others reported that conventional B2 cells promote atherosclerosis. We also provided evidence that peritoneal B1a cells are atheroprotective by producing natural IgM that is required for protection against atherosclerosis. We have reviewed the opposing roles of conventional B2 cells and peritoneal B1a cells in atherosclerosis development.

Administration of apoptotic cells protects mice from autoimmune inflammation by inducing regulatory B cells that secrete IL-10, through direct interaction of apoptotic cells with B cells. DNA complexes expressed on the surface of apoptotic cells and their interaction with toll-like receptor 9 (TLR9) expressed by B cells via leucocyte, anti-CD3, and anti-CD4 IgM, apoptotic cells and necrotic core size via IgM binding to apoptotic cells and enhancing phagocytosis, which also elevates anti-inflammatory cytokines.

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Use of Laboratory Committee (AEC no: E/1277/2012/B) and conform to the Guide for the Care and use of laboratory animals were approved by institute animal ethics committee. We investigated the effects of apoptotic cell administration on atherosclerosis development and its dependency on phosphatidyserine. We demonstrate that administration of apoptotic cells significantly ameliorates atherosclerosis development. Phosphatidylserine liposomes (PSLs) mimicked these effects and were associated with activation of B1a cells and production of polyreactive IgM antibodies targeting leukocytes, CD3 and CD4+T cells, oxidized LDL (oxLDL), and apoptotic cells. PSL treatment ameliorates atherosclerosis development and changes the lesion proinflammatory cytokine milieu to anti-inflammatory.

2. Methods

2.1 Animals and experimental protocol

Six-week-old male ApoE-KO mice commenced on an HFD were treated every alternate week for 8 weeks with i.p. injection with 30 × 10^6 irradiated apoptotic thymocytes controlled by PBS injection or with 0.5 mg/mouse PSL controlled by 0.5 mg/mouse phosphocholine liposomes (PCL) injection. Mice were euthanized at 14 weeks of age and effects on atherosclerosis, lymphocytes, and immune cells assessed.

All experimental procedures complied with national guideline for the care and use of laboratory animals were approved by institute animal ethics committee (AEC no: E/1277/2012/B) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 2011).

An expanded Materials and methods section is available in Supplementary material online.

2.2 Statistical analysis

Results are expressed as means ± SEM. Comparisons between groups were carried out using Student t-test or Mann–Whitney U test, depending on whether the data were normally distributed, as assessed using the Kolmogorov–Smirnov test. For multiple comparisons, results were analysed using one-way ANOVA (after confirming normality of distribution) followed by Bonferroni post-test. A value of P < 0.05 was considered statistically significant.

3. Results

3.1 Apoptotic cells attenuate development of atherosclerotic lesions

Treatment of hyperlipidaemic ApoE−/− mice intraperitoneally with irradiated apoptotic thymocytes every 2 weeks markedly attenuated development of atherosclerotic lesions. On average, lesion areas were reduced by 53% in mice treated with apoptotic cells (P < 0.05; Figure 1A), whilst Oil Red O stained areas were reduced by 61% (P < 0.05; Figure 1B); viable thymocytes did not affect lesion or lipid stained areas (P > 0.05; Figure 1A and B). Macrophage accumulation was also reduced, by 52% (P < 0.05; Figure 1C) following administration of apoptotic cells but unaffected by viable thymocytes. Apoptotic cell treatment also reduced CD4+ and CD8+ T-cell numbers in atherosclerotic lesions by 56 and 63%, respectively (P < 0.05; Figure 1D and E). Expression of inflammatory and adhesion molecules was also reduced. Expression of MCP-1 and VCAM-1 were reduced by 54 and 56%, respectively (P < 0.05; Figure 1F), expression of proinflammatory cytokines TNF-α, IL-1β, IL-12, and IL-18 were reduced by between 47 and 80% (all P < 0.05; Figure 1G) and expression of anti-inflammatory cytokine TGF-β mRNA levels doubled (P < 0.05; Figure 1H). Body weight and plasma cholesterol levels were unaffected by administering apoptotic cells (P > 0.05; Supplementary material online, Figure S1A and B).

3.2 Phosphatidylserine liposomes mimic the effects of apoptotic cells on atherosclerotic lesions

Multiple mechanisms can account for the reduced lesion size and inflammation mediated by apoptotic cells. Apoptotic cells can induce tolerogenic immunosuppressive B1a cells by activating TLR9 via extracellular DNA bound to apoptotic cells. Suppressive B cells can be activated by apoptotic cells binding phosphatidylserine receptors expressed by B1a cells or via other phagocytic immune cells. To determine whether the effects are mediated by phosphatidylserine receptors expressed by B1a cells, we next assessed the effects of intraperitoneal PSL treatment on development of atherosclerosis; PSLs are free of extracellular bound DNA. Chronic treatment with PSL attenuated atherosclerosis by 42% (P < 0.05; Figure 2A and B); PC liposomes were without affect (P > 0.05; Figure 2A and B). Macrophage accumulation was also reduced in PSL-treated mice, by 47% and CD4+ and CD8+ T-cell numbers were reduced by 64 and 52%, respectively (all P < 0.05; Figure 2C–E). Like apoptotic cells, PSL also reduced expression of VCAM-1 and MCP-1 by 76 and 58%, respectively (both P < 0.05; Figure 2F) whilst anti-inflammatory cytokines TGF-β mRNA and IL-10 nearly doubled (P < 0.05; Figure 2G) and proinflammatory cytokines IFN-γ, IL-17, and IL-18 were all reduced by between 60 and 83%, respectively (all P < 0.05; Figure 2H). PSL as well as AC treatment resulted in a significant (~30%) increase in plasma IL-5 levels (P < 0.05; Supplementary material online, Figure S1C). Body weight and plasma cholesterol levels were unaffected by PSL treatment (P > 0.05; Supplementary material online, Figure 1A and B).

3.3 Phosphatidylserine liposomes and apoptotic cells expand B1a cells during development of atherosclerosis

Apoptotic cells protect mice from inflammation by expanding regulatory B cells. To determine whether apoptotic cells and PSL liposomes exert similar effects on B1a cells during atherosclerosis development, we assessed peritoneal and spleen B1a cell numbers after treating hyperlipidaemic ApoE−/− mice with apoptotic cells or PSL liposomes; B1a cells were defined as CD5−/CD19+ CD14low/CD16− CD1d−/IgM− that are CD43low (Supplementary material online, Figure S2A and B). Following treatment with apoptotic cells peritoneal B1a cell numbers increased by 76% (P < 0.05; Figure 3A) whilst the increase in spleen cells was not statistically significant (P > 0.05; Figure 3A). Treatment with PSL liposomes also increased peritoneal B1a cells by 50% (P < 0.05; Figure 3B) and spleen cells by 52% (P < 0.05; Figure 3B). Both treatments increased TIM-1 + B1a cells in the peritoneal cavity and spleen (P < 0.05; Figure 3C–E). Other immune cell types were unaffected by the treatments including follicular, marginal zone, transitional (T1 and T2) B cells as well as CD4+, CD8+, NK, and NKT cells (all P > 0.05; Supplementary
To determine whether this increase in B1a cell numbers was due to direct interaction of PS liposomes with B1a cells, we compared the effects of phosphatidylserine and phosphatidylcholine liposomes on B1a cell proliferation in vitro. Compared with phosphatidylcholine liposomes, PSLs significantly stimulated B1a-cell proliferation ($P < 0.05$; Figure 3F) and their IgM production (data not shown).

### 3.4 PSLs and apoptotic cells increase polyreactive IgM levels and reduce local inflammation

Since PS liposomes stimulated the expansion of B1a cells, we next examined whether apoptotic cells and PS liposomes increased natural IgM

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**Figure 1** ACs transfer attenuated atherosclerosis development, proatherogenic leucocytes accumulation and local inflammation in atherosclerotic lesions. Aortic sinus lesions were stained by Oil-red O for lipid content, showing (A) a reduction in total atherosclerotic lesion area and (B) lipid accumulation in ACs transferred group. Representative images showed total intimal lesion areas and ORO-stained lipid accumulation. Aortic sinus lesions were stained by anti-CD68, CD4, and CD8 Ab for macrophage, CD4$^+$ T cells and CD8$^+$ T cells accumulation in the lesions, respectively. The graphs showing (C) reduction in macrophage accumulation in ACs transferred group, (D) reduction in CD4$^+$ T cells accumulation ACs transferred group and (E) reduction in CD8$^+$ T cells accumulation in ACs transferred group. Representative microimages showed CD68$^+$ macrophage, CD4$^+$ T cells, and CD8$^+$ T cells accumulation. Real-time PCR analysis of arterial mRNA showed (F) reduction in mRNA expression of MCP-1 and VCAM-1 and (G) a decrease in expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IL-18 in mice that transferred ACs compared with PBS. (H) Real-time PCR analysis of arterial mRNA also showed reduction in mRNA expression of anti-inflammatory cytokine TGF-β and IL10. Data represent mean ± SEM, representative results of two experiments (AC: $n = 6$, PBS: $n = 6$ and Thy: $n = 6$) $^*P < 0.05$ compared with both controls, one-way ANOVA with Bonferroni post-test or $^*P < 0.05$ compared with PBS control, unpaired $t$-test. Scale bar presents 100 μm.
antibody secretion during atherosclerosis development. Treatment with either PS liposomes or apoptotic cells increased plasma IgM levels by 67 and 115%, respectively (both $P < 0.05$; Figure 4A). Anti-oxLDL and anti-leucocyte IgM antibodies were also significantly increased by both treatments, as were anti-CD3 and anti-CD4 IgM antibodies (all $P < 0.05$; Figure 4B–E). IgM accumulation in atherosclerotic lesions also increased following treatment with PS liposomes or apoptotic cells, by 46 and 42%, respectively ($P < 0.05$; Figure 4F and G).

These increases in IgM were associated with reductions in accumulated lesion oxidized LDL (MDA-LDL), by 42 and 28% after PS liposomes and apoptotic cell treatments (both $P < 0.05$; Figure 4H and I). Since IgM facilitates the removal of apoptotic cells,$^{10,21}$ we also compared the effects of the two treatments on lesion apoptotic cell numbers and necrotic core size. Lesion apoptotic cell numbers and necrotic core size were reduced by 52 and 20%, respectively, after treatment with apoptotic cells whilst following treatment with PS liposomes, apoptotic...
cells were reduced by 57% and necrotic cores by 34% (all $P < 0.05$; Figure 5A–D).

Body weights and plasma cholesterol levels were also unaffected ($P > 0.05$; Figure 6D and E).

4. Discussion

Our findings indicate that targeting peritoneal B1a cells with apoptotic cells is highly effective in attenuating atherosclerosis development, an effect mimicked by PS liposomes. B1a-cell activation with either apoptotic cells or PS liposomes induces B1a-cell expansion together with an increased secretion of polyreactive IgM antibodies, accounting for much of the immunosuppression by apoptotic cells and PS liposomes on atherosclerosis.

Apoptotic cells can mediate immunosuppressive effects via multiple ligands on their cell surface. Annexin A1, a cytosolic protein that translocates to the surface of early stage apoptotic cells acts as an inhibitory effector molecule that prevents induction of inflammatory dendritic cells and facilitates development of tolerogenic dendritic cells that mediate immunosuppression. DNA and PS are also abundant on the surface of apoptotic cells. DNA complexes on the surface of apoptotic cells interact with toll-like receptor 9 (TLR9). Such interactions of DNA with TLR9 expressed by B1a B cells results in their differentiation to IgM producing plasma cells as well as induction of tolerogenic IL-10 secreting B cells, which suppress experimental autoimmune encephalitis.
whilst PS can interact with PS receptors encoded by TIM to regulate both innate and adaptive immunity. Our findings that partial deletion of B1a B cells by splenectomy prevented the attenuation of atherosclerosis by apoptotic cells and PS liposomes indicate an important role for spleen dependent B1a B cells in regulating atherosclerosis. Earlier studies have suggested at least two populations of peritoneal B1a B cells, CD5+ B220DULL and CD5+ B220+. Hox11−/− spleenless mice only possess CD5 + B220 + B1a B cells in the peritoneal cavity whilst C57Bl6 mice possess two populations, CD5 + B220DULL (major population) and CD5 + B220+ (minor population), indicating that only the CD5 + B220DULL population is dependent on the presence of a spleen. Whilst we used a different gating strategy to detect peritoneal B1a B cells, it is very likely that both CD5+ populations (CD5 + B220DULL and CD5 + B220+) are included in the CD5 + CD19 + IgM+ peritoneal population of ApoE−/− mice. Presumably the latter CD5 + B220+ population whose survival is not spleen dependent is

Figure 4  Treatment with PSL and ACs increased plasma and lesions IgM. ELISA showed that PSL and ACs treatment increased (A) plasma total IgM and (B) MDA-oxLDL IgM antibodies. ELISA also showed that PSL and ACs treatment increased (C) plasma anti-leucocyte IgM, (D) anti-CD3 IgM and (E) anti-CD4 IgM antibodies. Immunohistochemical analysis of aortic sinus atherosclerotic lesions showed that increased total IgM deposits in (F) ACs transferred group and (G) PSL treated group compared with their own control. Representative microimages showed IgM deposits in the lesions. Immunostaining of aortic sinus lesions showed reduction of oxLDL accumulation in the lesions in (H) ACs and (I) PSL treated groups. Representative microimages showed oxLDL accumulation in the lesions. Data represent mean ± SEM; PSL: n = 11, PCL: n = 10, AC: n = 6, PBS: n = 6; *: P < 0.05 compared with PBS or PCL control, unpaired t-test. Scale bar presents 100 μm.
not responsive to either apoptotic cells or liposomes. Whilst our study indicates that apoptotic cells are anti-inflammatory and attenuate atherosclerosis, an earlier report indicated apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. The difference between our study and the previous study may be due to different methods of inducing cell apoptosis; only previously stressed apoptotic cells appear to be proinflammatory.27 Our study indicating that apoptotic cells can be anti-inflammatory during development of atherosclerosis are in accord with other studies demonstrating that their administration can induce tolerance in other inflammatory disorders, protect against autoimmune inflammation, and suppress inflammatory arthritis.

Our finding that PS liposomes mimic the suppressive effects of apoptotic cells on atherosclerosis indicates a major role for PS in attenuating atherosclerosis. TIM-1 is highly expressed on regulatory B cells and we demonstrate high TIM-1 expression on B1a cells, suggesting a role for their activation by PS in attenuating atherosclerosis. Deletion of B1a cells by splenectomy prevented the attenuation of atherosclerosis by both apoptotic cells and PS liposomes. Direct B1a cell activation by PS in vitro stimulated expansion of B1a cells including the TIM-1+ B1a cell population together with increased secretion of polyreactive IgM. The findings suggest that PS interacts with TIM-1 to initiate B1a cell expansion and secretion of polyreactive IgM antibodies. TIM-1 may act as a membrane signalling receptor. TIM-1 on B cells interacts with the kinase Fyn resulting in TIM-1 phosphorylation which is increased when TIM-1 is activated. Fyn promotes B cell proliferation mediated by T-independent antigens. Whilst TIM-1 expressed by B1a cells is likely responsible for the suppressive effects of PS on atherosclerosis, we cannot exclude a role for other PS receptors expressed by B1a cells, e.g. term-like transcript 2 (TLT2) receptors or a role for

Figure 5 Mice treated with PSL and ACs show reduced necrotic core and apoptotic cells in atherosclerotic lesions. H&E stained aortic sinus lesions showed reduced necrotic cores of atherosclerotic lesions, identified as acellular areas in mice that received (A) ACs and (B) PSL. Apoptotic cells as TUNEL-positive cells in atherosclerotic lesions in (C) ACs and (D) PSL treated groups were reduced compared to their respective controls. Representative microimages showed necrotic core and apoptotic cells in atherosclerotic lesions. Data represent mean ± SEM; PSL: n = 11, PCL: n = 10, AC: n = 6, PBS: n = 6; *: P < 0.05 compared to PBS or PCL control, unpaired t-test. Scale bar presents 100 μm.
macrophages in also activating peritoneal B1a B cells. The small (~30%) increase in plasma IL-5 levels after apoptotic cell and PS liposome treatment suggests some involvement of indirect stimulation of B1a B cells by macrophages. B1aB cells can be activated to produce IgM by IL-5, a cytokine secreted by macrophages. Apoptotic cell engulfment by macrophages activates liver X receptor (LXR) signalling and LXR activation in macrophages induces IL-5 expression.

Antibody production by B1a cells appears critically dependent on their location within body cavities, including the peritoneal cavity. Their activation in the peritoneal cavity by either apoptotic cells or PS

Figure 6 PSL and ACs treatment failed to protect against atherosclerosis development in absence of B1a cells. Splenectomized ApoE −/− mice were received PSL and ACs as test and PBS as controls while fed an HFD for 8 weeks. Oil-red O stained for lipid content, showed no difference in (A) total atherosclerotic lesion area and (B) lipid accumulation in the splenectomized mice. Representative microimages showed total intimal lesion areas and ORO-stained lipid accumulation. FACS analysis showed (C) peritoneal B1a cells and non-B1a lymphocytes across the experimental groups after 8 weeks HFD and macrophages, monocytes, DC and neutrophils after 4 weeks HFD in splenectomized mice compared with sham operated mice. No difference in (D) body weight and (E) lipid profile was observed across the groups. Data represent mean ± SEM, (SO; n = 6, SX-PBS; n = 7, SX-PSL; n = 6, SX-AC; n = 6) *P < 0.05 compared with SX groups, one-way ANOVA with Bonferroni post-test or unpaired t-test. Scale bar presents 100 μm.
Liposomes markedly increases secretion of polyreactive IgM antibodies, including anti-leucocyte, anti-CD3, and anti-CD4 IgM antibodies. IgM antibodies were elevated in both plasma and within atherosclerotic lesions of treated atherosclerotic mouse. Natural anti-leucocyte as well as anti-CD3 and anti-CD4 IgM antibodies inhibit both T-cell activation and chemotaxis. Our findings of reduced numbers of CD4+ and CD8+ T cells within atherosclerotic lesions of mice treated with apoptotic cells or PS liposomes is consistent with such inhibitory effects of IgM antibodies on T-cell activation and migration. Also, the reduction in oxidized LDL (MDA-LDL) accumulation in lesions indicates an important role for MDA-oxLDL IgM antibodies in removing oxLDL from atherosclerotic lesions. OxLDL enhances pro-inflammatory responses of macrophages including their secretion of TNF-α, IL-1β, and MCP-1 in addition to promoting macrophage apoptosis. Our findings of reductions in lesion proinflammatory cytokines are consistent with the IgM-mediated reduction in oxLDL accumulation. In addition to IgM antibodies affecting T-cell and macrophages proinflammatory responses, IgM also recognizes and promotes phagocytosis of apoptotic cells as well as apoptotic microparticles, attenuating accumulation of post-apoptotic necrotic cells in developing lesions. Our finding of significant reductions in lesion necrotic core size is consistent with such an effect of IgM within atherosclerotic lesions, together with increased expression of anti-inflammatory cytokines IL-10 and TGF-β in lesions of treated mice. Phagocytosis of apoptotic cells by macrophages markedly increases expression of both IL-10 and TGF-β. Thus PS activated B1a cell derived IgM markedly also alters the cytokine milieu within developing atherosclerotic lesions from one that is predominantly proinflammatory to anti-inflammatory.

Our findings indicate that B1a cells can be specifically activated by PS to attenuate atherosclerosis. Administration of PS in liposomes activates and expands peritoneal B1a cells augmenting secretion of polyreactive IgM which in turn profoundly dampens inflammation in atherosclerotic lesions. Polyreactive IgM secreted by PS stimulated B1a cells include T-cell targeting IgM antibodies, anti-oxLDL IgM antibodies that attenuate inflammation, and IgM antibodies interacting with apoptotic cells that promotes their clearance, reducing necrotic core size and increasing anti-inflammatory cytokine expression. Given these atheroprotective effects of PS from stimulated B1a cells, B1a cell activation by PS may be a useful therapeutic strategy to reduce the morbidity and mortality of atherosclerosis associated with myocardial infarction and stroke.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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