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

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1. Introduction

Despite lipid-lowering statins, heart attack and stroke are atherosclerosis-related cardiovascular diseases remaining the leading cause of mortality worldwide.1 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.2 B cells have been identified within atherosclerotic lesions and associated adventitia in humans3 and in mice.4 We5,6 and others7,8 reported that conventional B2 cells promote atherosclerosis. We also provided evidence that peritoneal B1a cells are atheroprotective5,9 by producing natural IgM that is required for protection against atherosclerosis.10 We have reviewed the opposing roles of conventional B2 cells and peritoneal B1a cells in atherosclerosis development.11

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.12 DNA complexes expressed on the surface of apoptotic cells and their interaction with toll-like receptor 9 (TLR9) expressed by B cells at least in part accounts for the anti-inflammatory actions of B cells.13 Phosphatidylserine (PS), normally sequestered on the inner leaflet of the plasma membrane, is exposed on the outer leaflet in apoptotic cells.14 As B1a cells express...
TIM-1 and TIM-4, receptors for PS, is it also likely that B1a cell activation by apoptotic cells involves TIM receptors. TIM receptors exhibit both phagocytic and costimulatory properties and antibody ligation of TIM-1 induces tolerogenic IL-4 and IL-10 producing B1a cells which promote long-term graft survival.

Given that apoptotic cells induce tolerance, suppress development of type I diabetes, augment bone marrow engraftment, and suppress inflammatory arthritis, the latter via an IgM-dependent mechanism, we investigated the effects of apoptotic cell administration on atherosclerosis development and its dependency on phosphatidylserine. 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 CD4T cells, oxidized LDL (oxLDL), and apoptotic cells. PSL treatment attenuates 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 guidelines for the care and use of laboratory animals were approved by institute animal ethics committee (AEC no: E/1277/2012/8) 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 8). 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 2I). Bone weight and plasma cholesterol levels were unaffected by PSL treatment (P > 0.05; Supplementary material online, Figure 2A 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 PSLsomes 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 PSLsomes; B1a B cells were defined as CD5+ CD19+ CD14low 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 PSLsomes also increased peritoneal B1a cells by 56% (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...
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, 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...
3.5 Splenectomy abolished the anti-atherosclerotic effects of PS liposomes and apoptotic cells

To confirm the dependency of anti-atherosclerotic effects of PS liposomes and apoptotic cells on peritoneal B1a cells we splenectomized ApoE−/− mice before treating with PS liposomes or apoptotic cells; as splenectomy specifically deletes B1a cells from the peritoneal cavity,9 splenectomized ApoE−/− mice fed a high-fat diet for 8 weeks exhibited larger lesions than sham-operated mice (P < 0.05; Figure 6A and B) and treating with either PS liposomes or apoptotic cells lesions size was unaffected and identical to mice that received vehicle, measured as total intimal or Oil Red O stained lesion areas (P > 0.05; Figure 6A and B). Peritoneal B1a cells were on average reduced by nearly 64% in splenectomized mice (P < 0.05; Figure 6C) affecting CD5+CD19+CD1dlowIgM+B1a cells (Supplementary material online, Figure S2C). Similar findings of B1a cells in blood and lymph nodes were observed (all P < 0.05; Supplementary material online, Figure S5A and B), whilst splenectomy did not affect lymphocyte populations, dendritic cells, macrophages, and monocytes in PC (all P > 0.05; Figure 6C), blood and lymph nodes (Supplementary material online, Figure S5A and B).

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.22 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 cells23 as well as induction of tolerogenic IL-10 secreting B cells, which suppress experimental autoimmune encephalitis.13

Figure 3 Liposomes containing phosphatidylserine and ACs treatment increased B1a cells expansion in peritoneal cavity and spleen. FACS analysis of B1a cells in peritoneal cavity and spleen of ApoE−/− mice at the end point of 8 week high-fat diet showed that (A) ACs were able to proliferate and expand B1a cells in spleen and peritoneal cavity and (B) PSL treatment also showed proliferation and expansion of B1a cells in spleen and peritoneal cavity. (C) B1a cells expressing TIM-1 were increased in spleen of ACs transferred group and in (D,E) spleen and peritoneal cavity of PSL treated mice without affecting the percentage of TIM1+B1a cells in peritoneal cavity and spleen. In vitro stimulation of peritoneal B1a cells by PSL revealed (F) a dose—response increase in B1a cell proliferation as assessed by 3H-thymidine incorporation. 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.
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 depended is...
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. 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 mice. Natural anti-leucocyte as well as anti-CD3 and anti-CD4 IgM antibodies inhibit both T-cell activation and chemotaxis. 35 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, 36 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, 37 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 38 and TGF-β. 39 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 includes 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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