Lysophospholipids induce innate immune transdifferentiation of endothelial cells, resulting in prolonged endothelial activation

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**Abstract**

Innate immune cells express danger-associated molecular pattern (DAMP) receptors, T-cell co-stimulation/co-inhibition receptors, and the major histocompatibility complex II (MHC-II). We have recently proposed that endothelial cells can serve as innate immune cells, but the molecular mechanisms involved still await discovery. Here, we investigated whether human aortic endothelial cells (HAECs) could be transdifferentiated into innate immune cells by exposing them to hyperlipidemia-upregulated DAMP molecules, \textit{i.e.} lysophospholipids. Performing RNA-Seq analysis of lysophospholipid-treated HAECs, we found that lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI) regulate largely distinct gene programs, as revealed by principal component analysis. Metabolically, LPC up-regulated genes that are involved in cholesterol biosynthesis, presumably through sterol regulatory element–binding protein 2 (SREBP2). By contrast, LPI up-regulated gene transcripts critical for the metabolism of glucose, lipids, and amino acids. Of note, we found that LPC and LPI both induce adhesion molecules, cytokines, and chemokines in HAECs, which are all classic markers of endothelial cell activation. Moreover, LPC and LPI shared the ability to transdifferentiate HAECs into innate immune cells, including induction of potent DAMP receptors, such as CD36 molecule, T-cell co-stimulation/co-inhibition receptors, and MHC-II proteins. The induction of these innate-immunity signatures by lysophospholipids correlated with their ability to induce up-regulation of cytosolic calcium and mitochondrial reactive oxygen species. In conclusion, lysophospholipids such as LPC and LPI induce innate immune cell transdifferentiation in HAECs. The concept of prolonged endothelial activation, discovered here, is relevant for designing new strategies for managing cardiovascular diseases.

**Introduction**

Cardiovascular disease (CVD) is a leading cause of death in well-developed countries. As a chronic autoimmune inflammatory disease, atherosclerosis is fueled by both the innate and adaptive immune responses, which mediate the initiation, progression, and ultimate thrombotic complications\textsuperscript{(1)}. We and others have reported that hyperlipidemia, together with other CVD risk factors, such as hyperglycemia, chronic kidney disease, obesity, and hyperhomocysteinemia (HHcy), promote atherosclerosis development via several mechanisms. These mechanisms include endothelial cell (EC) activation and injury\textsuperscript{(2-6)}; monocyte recruitment and differentiation\textsuperscript{(7,8)};
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decreased function of regulatory T cells (Tregs)(9-11) and transdifferentiation of Tregs into antigen-presenting cell (APC) like Tregs(12); impaired vascular repair ability of bone marrow-derived progenitor cells(13-15); increased migration and proliferation of vascular smooth muscle cells(16,17), and high fat-induced adipocyte hypertrophy and metabolic healthy obesity(18). However, the underlying mechanisms of how hyperlipidemia promotes prolonged EC activation response during atherosclerosis development remain poorly defined.

Lysophospholipids are a group of bioactive, proinflammatory lipid molecules, which include lysophosphatidylecholine (LPC), lysophosphatidylinositol (LPI), and others. As our newly proposed conditional danger-associated molecular patterns (DAMPs)(19,20), lysophospholipids contribute to aortic EC activation(4,6,21) and development of atherosclerosis(22). It has been suggested that LPC-induced EC activation is an initiation step of atherogenesis(4). During early hyperlipidemia, LPCs are induced and activate ECs to produce adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), which mediate the adhesion and migration of leukocyte into the aortic artery intima(23). However, whether other members of the lysophospholipids, such as LPI, could activate aortic ECs in a manner differentially from LPC; and additional lysophospholipid-regulated gene targets in EC remain unknown.

As we reported, during acute inflammation elicited by pathogen infection, ECs are transiently activated and express adhesion molecules, cytokines, and chemokines, which mediate leukocyte recruitment into the aorta(24). In addition, we proposed a new concept of physiological EC activation for facilitation of patrolling immune surveillance cell trans-EC migration(21). During chronic metabolic inflammation, however, constant stimulation by cardiovascular stressors causes prolonged pathogenic EC activation, which is responsible for the prolonged pathogenic monocyte recruitment into the aorta, contributing to the development of CVD. We have recently reported that the caspase-1 and NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome play a critical role in this process, as deficiencies in caspase-1/inflammasomes decrease EC activation, aortic monocyte recruitment, and early atherosclerosis induced hyperlipidemia(2) and hyperhomocysteinemia (HHcy)(25). These findings strengthen our recently proposed new concept that ECs are innate immune cells(26). More specifically, we proposed that in response to inflammatory stimuli and vascular stressors during CVD development, ECs could be transdifferentiated into antigen presenting cells by expressing MHC II molecules and T cell co-stimulation/co-inhibition molecules, which present endothelial cell antigens to activate T cells through MHC II (signal 1) and T cell co-stimulation (signal 2). ECs could also upregulate the expressions of DAMP receptors, proinflammatory cytokines and chemokines, which can modulate the activities of immune cells (signal 3). Nevertheless, it remains poorly defined whether conditional DAMPs and chronic CVD stressors, such as lysophospholipids, could induce prolonged/sustained EC activation in a manner different from acute pathogen-induced EC activation.

ECs from different tissues are heterogeneous with respect to their protein and surface marker expressions(26). The heterogeneity of ECs contributes to their diversity in function at different vascular sites. More specifically, ECs are highly flexible and may be induced to stem-cell like properties via endothelial-mesenchymal transition (EndMT)(27). During EndMT, a mesenchymal phenotype is acquired by mature- and progenitor-ECs, which can give rise to other cell types. EndMT was found to be common in atherosclerotic lesions and to be associated with plaque instability. Mechanistically, transforming growth factor-β (TGF-β) and Notch pathways have been shown to be important in regulating endothelial plasticity(26). However, it remains unknown whether lysophospholipids are involved in the process of EndMT.

In response to bacterial and viral infections, innate immune cells, such as macrophages, undergo extensive metabolic reprogramming. These metabolic changes not only provide energy and biosynthesis power, but also directly regulate macrophage effector functions. In lipopolysaccharide (LPS)-stimulated macrophages,
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the increased mitochondrial oxidation of succinate drives mitochondrial reactive oxygen species (mtROS) production. As a result, LPS-stimulated macrophages shift their mitochondrial metabolism from ATP production towards mtROS production that promotes a pro-inflammatory state(28). In human aortic ECs, we reported that lysophospholipid-induced mtROS are crucial in mediating adhesion molecule gene expression, as well as site-specific histone 3 lysine 14 acetylation(6), a long-term epigenetic reprogramming of EC activation(4). Nevertheless, the roles of lysophospholipids in the specific transcriptional regulation of endothelial metabolic pathways remains to be elucidated.

Thus, despite significant progress including our new concept of ECs as innate immune cells(26), several important knowledge gaps exist: First, the global transcriptomic targets of lysophospholipids, especially LPI, in ECs is unknown; second, whether lysophospholipids could induce “professional” innate immune cell signatures in ECs is unclear; third, whether lysophospholipids are involved in the process of EndMT remains unknown; and fourth, the role of lysophospholipids in EC metabolic reprogramming into prolonged and sustained activation status is uncharacterized. In this study, we hypothesized that lysophospholipids, as conditional DAMPs, could induce innate immune transdifferentiation of ECs, which might contribute to sustained EC activation and vascular pathology. Using RNA-sequencing (Seq), we determined the transcriptomic effects of LPI and LPC treatment in human aortic ECs (HAECs). We found that LPC and LPI engage with distinct cellular metabolic pathways in HAECs. More importantly, LPI, similar to LPC, not only induces the transcriptional upregulation of transient EC activation marker genes including cytokines, chemokines and EC adhesion molecules, but also contributes to the transcriptional induction of additional DAMP receptors, such as CD36 and caspase-1, T cell co-stimulation and co-inhibition receptors, and major histocompatibility complex class II (MHC-II) molecules. Thus, a new system of innate immune transdifferentiation of EC, induced by lysophospholipids, is uncovered. Our in-depth analysis of the roles of lysophospholipids in EC activation as well as our new concept of characterizing prolonged and sustained EC activation provide novel therapeutic potential for vascular inflammation and other CVDs.

Results

LPI induces both acute and sustained endothelial inflammation — To examine the global gene expression changes in ECs after LPI stimulation, we performed RNA-Seq analysis in HAECs treated with either vehicle control or LPI (10 µM) for 18 hours. The results showed that LPI regulated gene expression extensively in HAECs, with more upregulated genes (437 genes) than downregulated genes (215 genes), indicating that LPI acts mostly by promoting gene transcription in HAECs (Figures 1A and 1B). Next, we performed Ingenuity Pathway Analysis (IPA) on LPI-upregulated genes and found that the “cellular infiltration by leukocytes” pathway was the most significantly enriched pathway (Supplemental Figure 1A), suggesting that LPI promoted EC activation, which is an initiation step for leukocyte infiltration into the aorta and early atherogenesis. Further examination of the LPI-upregulated genes in this pathway reveals that LPI not only promoted the upregulation of cytokines (IL1A, IL1B, IL15, and IL33), chemokines (CCL2, CX3CL1, CXCL3, CXCL5, and CXCL8), and adhesion molecule (ICAM1, and SELE) gene expression changes, but also increased the gene expression changes related to the DAMPs (CASP1, TLR3, and CD36) (Figure 1C). Furthermore, IPA predicted TLR3 as one the top upstream regulator of LPI-induced genes (Supplemental Figure 1B), and as many as 22 LPI-upregulated genes might be induced indirectly through the upregulation of TLR3 (Supplemental Figure 1C). IPA also predicted ERK1/2, which leads to AP-1 transcription factor activation, as top upstream regulator of LPI-promoted transcriptome. Moreover, gene set enrichment analysis (GSEA) confirms that LPI treatment is associated with the gene signatures of “positive regulation of cytokine secretion”, “positive regulation of leukocyte chemotaxis”, “cell adhesion molecules”, “innate immune response” (Figures 1D to 1G). These results indicate that LPI not only provokes “classic” acute endothelial cell activation process, as judged by increased expressions of adhesion molecules, cytokine and chemokines, but also activates a previously uncharacterized “innate immune” EC activation process. We classified this as the first
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As innate immune cells, endothelial cells (ECs) are characterized by engaging with amplification of the gene expression of additional DAMP receptors, allowing ECs to continuously receive the stimulation from DAMPs in blood circulation and tissues and sustain the EC activation (20, 26, 30). Of note, the gene signature of “adaptive immune response (functioning as antigen presenting cells to activate T cell responses)” was also positively correlated with the LPI treatment group (Figure 1I), indicating that LPI also promotes “adaptive immune” EC activation, which we classified as the second key features of ECs’ antigen presenting function as innate immune cells. Examination of gene expression fold changes further consolidates with these findings, with several scavenger receptors (CD36, STAB2, OLR1, and CD5), DAMPs (CASP1 and TLR3), T cell co-stimulation and co-inhibition receptors (TNFSF15, TNFRSF4, and CD274), and major histocompatibility complex (MHC) class II molecules (HLA-DRB1 and CD74) also highly upregulated in HAECs after LPI treatment (Figure 1I). Taken together, these results indicate that LPI induces both classic EC activation process for acute vascular inflammation and unique “innate immune” and “adaptive immune” EC activations for prolonged inflammatory process.

LPI extensively reprograms endothelial cell metabolism — During innate immune cell activation induced by proinflammatory stimuli such as lipopolysaccharide (LPS), macrophages undergo extensively cellular metabolic reprogramming process, which is crucial for both meeting its synthetic and energetic needs and inducing inflammatory response (31, 32). We hypothesized that LPI treatment also drives metabolic gene expression changes during innate immune reprogramming of ECs. We found that besides the induction of adhesion molecules, cytokines, and chemokines, LPI treatment also strongly induced the transcripts of several metabolic genes, which were related to the metabolism of glucose, amino acid, lipid, and mitochondrial genes (Figure 2).

These molecules included master mitochondria transcription factors, mitochondrial transcription factor A (TFAM); mitochondrial reactive oxygen species scavenger, superoxide dismutase 2, (SOD2); rate-limiting glycolysis enzymes (phosphofructokinase, muscle, PFKM and lactate dehydrogenase A, LDHA) that drive the first and final step of glycolysis; enzymes that carry the conversion between glutamine and glutamate (glutaminase, GLS and glutamate-ammonia ligase GLUL); critical cholesterol and fatty acid metabolism enzymes (carnitine palmitoyltransferase 1A, CPT1A and insulin induced gene 1, INSIG1). These results indicate that LPI globally reprograms EC metabolisms, which are presumably required for the innate reprogramming of EC activation, similar to that reported for macrophages (31, 32).

Lysophosphatidylcholine (LPC) induces both acute activation and prolonged innate reprogramming in HAECs. — Next, we hypothesized that other lysophospholipids could also induce innate reprogramming of EC for sustained inflammatory response. We performed RNA-Seq analysis in HAECs treated with either vehicle control or LPC (10 µM) for 18 hours, the same experimental conditions as we performed for LPI treatment of HAECs. Similarly, we found that LPC induced the enrichment of gene transcription signatures of “cell adhesion molecules”, “chemokine receptors bind chemokines”, and “cytokine-cytokine receptor interaction” (Figures 3A to 3C). More importantly, the gene modulation patterns of LPC also positively correlated with the gene signatures of innate “NOD-like receptor signaling pathway” and adaptive “co-stimulation by the CD28 family” (Figures 3D and 3E). Further examination of selected genes from these signatures revealed that LPC similarly promoted the gene expression of cytokine (IL1B), chemokine (CCL2 and CXCL8), adhesion molecules (ICAM1 and SELE), DAMPs (CD36, NOD2, and NAIP), costimulatory/coinhibitory molecules (TNFSF15, TNFRSF9, CD70, and CD274), and MHC class II molecules (CD74, HLA-DRB1, and HLA-DMA) (Figure 3F). These results expanded on our previous findings and suggested that innate reprogramming of EC could be a common shared feature of DAMPs lysophospholipids.

LPC positively regulates genes downstream of master regulator of lipid metabolism, sterol regulatory element-binding protein 2 (SREBP2) — To examine whether LPC could drive metabolism changes during EC activation, we performed IPA pathway enrichment profiling on LPC-regulated genes. Strikingly, IPA results showed that “steroid metabolism” is the most significantly enriched pathway in LPC-treated HAECs (Figure 4A).
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GSEA of “steroid biosynthesis” also showed that LPC treatment group strongly correlates with the gene signature of “steroid biosynthesis” (Figure 4B). Furthermore, IPA predicted that Sterol Regulatory Element Binding Transcription Factor 2 (SREBF2), which encodes the protein SREBP2, to be the upstream transcription factor of LPC-regulated genes in HAECs (Figure 4C). Other protein members related to the regulation of SREBP2 (SCAP and INSIG1), as well as its other family member SREBP1, were also predicted to be the upstream regulators. SREBP2 is a master transcription factor and a proinflammatory M1 macrophage polarization marker(33), important for the regulation of cholesterol metabolism. In addition, we found that as many as 20 SREBP2-regulated genes are significantly changed by LPC in HAECs, including 9 out of 13 critical cholesterol biosynthesis enzymes (Figure 4D). These results indicate that LPC drives EC activation and inflammation via inducing unique cholesterol metabolism pathways when comparing with LPI, potentially mediated by SREBP2.

LPI and LPC similarly activate mitochondrial reactive oxygen species (ROS), cytosolic calcium and acute EC activation marker genes, but regulates largely distinct gene programs in human aortic endothelial cells — Since LPI and LPC have similar structures(34), we hypothesized that LPC could activate similar cellular signaling pathways with LPI. Using flow cytometry method with specific mtROS and cellular calcium probes, we found that both LPC and LPI acute treatment (1 hour) strongly upregulated both mitochondrial ROS production and cytosolic calcium concentrations (Figures 5A and 5B). The induction of cytosolic calcium correlated with the induction of calcium signaling-dependent genes (Figure 5C), suggesting that cytosolic calcium influx-mitochondrial ROS signaling and endoplasmic reticulum-mitochondrial tethering pathways(35) might partially mediate lysophospholipid-induced inflammatory gene expression, as we reported(4). Although LPI and LPC both induced cytosolic calcium influx-mitochondrial ROS signaling pathways, they regulate largely distinct gene expression changes in HAECs, as determined by principal component analysis (PCA)(http://setosa.io/ev/principal-component-analysis/). Although PC2 axis, which explains 20.7% of the variance among the data, separated the control group with LPI-, and LPC- groups. The LPI and LPC treatment groups were in the opposite directions from the control group in the PC1 axis, which showed the largest variations and explained 48.3% of the variances among the groups (Figure 5D). Differential gene regulations by LPC and LPI was also evident from the differential gene expression heat map, as there was a small overlap between LPC-induced and LPI-induced genes (purple group, Figures 5E and 5F). Furthermore, IPA enrichment profiling of the genes commonly induced by LPI and LPC revealed that “attraction of leukocytes” was the most enriched gene signature, suggesting that LPC and LPI could commonly induce acute EC activation, presumably through induction of mitochondrial ROS-cytosolic calcium influx signaling pathways (Figures 5G and 5H). Moreover, by analyzing published microarray from LPS-induced endothelial cell activation (GSE5883), we found that although LPS acutely induced cytokine IL1B, adhesion molecule ICAM1, scavenger receptor CD36, T cell costimulatory molecule TNFSF15, and MHC class II molecule CD74, it could only upregulate ICAM1 expression after 24 hours. By contrast, both LPC and LPI could sustain the upregulation of these genes in HAECs (Figure 5I). These results indicate that during acute inflammation, LPS could transiently induce EC activation by upregulation of adhesion molecules, such as ICAM-1. In comparison, lysophospholipids could induce prolonged endothelial activation and transform ECs into innate immune cells by upregulating additional DAMP receptors, costimulatory molecules and MHC class II molecules (Figure 6).

LPI and LPC commonly induce proinflammatory cytokines and chemokines that are downstream of NF-kB transcription factor — To consolidate the conclusion of innate immune transdifferentiation of HAECs by lysophospholipids, we analyzed the expression profile of a comprehensive list of inflammation-modulating cytokines (Supplemental Table 1). Although LPI and LPC commonly induced the gene expression of a few proinflammatory cytokines including IL1A, IL1B, and TNFSF15, they also differentially regulate other cytokine gene expression. LPC highly induced the expression of CD70 and IL32, while LPI highly upregulated CSF2, IL16, and LTB. We also examined the gene expression of all the chemokine family members after LPC/LPI
stimulation. We found that both LPC and LPI commonly upregulated a few alpha chemokines (CXC) and delta chemokine (CXC3C) family members, including CXCL3, CXCL6, CXCL8, CXCL11, and CX3CL1, whereas they largely regulate distinct beta chemokines (CC), (Supplemental Table 2). Next, we hypothesized that LPI and LPC could activate certain transcription factor pathways, leading to the upregulation of cytokine and chemokine gene expressions. To examine this, we analyzed the predicted transcription factors for LPI (Supplemental Figure 2A) and LPC (Supplemental Figure 2B) using IPA. IPA listed interferon regulatory factor 1 (IRF1), nuclear factor NF-kappa-B p65 subunit (RELA), signal transducer and activator of transcription 3 (STAT3), and NF-kB inhibitor epsilon (NFKBIE) as top upstream transcription factors for LPI-regulated transcriptomes and listed sterol regulatory element-binding protein 2 (SREBF2), SREBF1, nicotinamide adenine dinucleotide (NAD)-dependent deacetylase silent information regulator 2 (sirtuin 2, SIRT2), and tumor protein p53 (TP53) as top upstream transcription factors for LPC-regulated transcriptomes in HAECs. Strikingly, there is an overlap of nearly half (9 out of 20) of the predicted transcription factors for both LPI and LPC, many of which are related to the NF-kB transcription factor pathway (Supplemental Figure 2C). Further examination of the genes downstream of RELA revealed that LPI and LPC commonly regulated genes downstream of RELA transcription factor, such as inflammatory cytokines IL1A and IL1B, chemokines such as CCL2 and CXCL8, and adhesion molecules ICAM1 and SELE (Supplemental Figure 2C). These results indicated that NF-kB might be involved in the upregulation of EC activation marker genes induced by LPC and LPI.

**LPC and LPI differentially regulate T cell co-stimulation and co-inhibition receptors as well as mesenchymal regulators/markers in EndMT**—To further consolidate immune transdifferentiation of HAECs by lysophospholipids, we examined the gene expression changes of a comprehensive list of T cell co-stimulation and co-inhibition receptors after LPC and LPI stimulation in HAECs(36). We found that LPC upregulated CD28, tumor necrosis factor receptor superfamily member 9 (TNFRSF9, CD137), and TNFRSF18 (Supplemental Table 3). While LPI also upregulated CD28 gene expression, LPI differentially upregulated B and T lymphocyte attenuator (BTLA, CD272), TNFRSF4, hepatitis A virus cellular receptor 1 (HAVCR1), leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), and CD274. The induction of different T cell co-stimulation and co-inhibition receptors suggested that LPC and LPI stimulation might lead to distinct immune transdifferentiation programs of HAECs. Finally, we examined the hypothesis that lysophospholipids might be involved in the process of EndMT process. To test this hypothesis, we examined the gene expression changes of previously characterized EndMT regulators and mesenchymal markers in HAECs(37). The results showed that LPI upregulated gene expression of Caldesmon 1 (CALD1), Snail superfamily of C2H2-type zinc finger transcription factor 2 (SNAI2), WNT gene family secreted signaling protein 11 (WNT11), disintegrin and metalloproteinase domain-containing protein 12 (ADAM12), basic smooth muscle protein Calponin 1 (CNN1), matrix metalloproteinase 9 (MMP9), and S100 calcium binding protein A4 (S100A4); while LPI induced Nidogen-2 (NID2) and Notch receptor family 3 (NOTCH3) (Supplemental Table 4 and Supplemental Table 5). Thus, LPC and LPI might be involved in the process of EndMT, but they regulated distinct regulators/markers in this process.

**Discussion**

Anti-inflammatory strategies are promising therapeutic approaches for prevention and treatment of atherosclerotic CVD(38). The majority of lysophospholipids serve as conditional DAMPs(19,20); and they were considered to play a causative role in atherogenesis. For this reason, inhibitors of the key enzymes responsible for the generation of lysophospholipids were developed as potential anti-inflammatory therapies against atherosclerosis(38). Despite the beneficial effects of these inhibitors in atherosclerotic animal models(39,40) and human phase 2 clinical trials(41,42), human phase 3 clinical trials failed to show their therapeutic efficacy(43-45). One possibility that may explain these failures is that lysophospholipids might also be important under normal physiological conditions; and blocking its
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physiological effects could be detrimental to the host. To consolidate this argument, we recently reported that LPC-induced mtROS, uncoupled from ATP synthesis, determine EC activation for both physiological recruitment of patrolling cells and pathological recruitment of inflammatory cells(4,5,46). Thus, targeting the pathogenic downstream effects of LPC, instead of blocking the production of LPC itself, may lead to better therapeutics. In our current study, we found that different lysophospholipid species including LPC and LPI regulated largely distinct gene signatures in HAECs. In terms of cellular metabolism regulation, while LPC was involved in cholesterol biosynthesis potentially mediated by SREBP2, LPI broadly regulated critical enzymes involved in cellular glucose, lipids, and amino acid metabolism. Nevertheless, LPC and LPI could both induce EC activation marker genes such as adhesion molecules, cytokines, and chemokines. More importantly, they could both induce innate immune reprogramming/transdifferentiation of ECs into innate immune cells by upregulating additional DAMP receptors such as CD36 to receive additional DAMPs for prolonged/sustained activation; and MHC-II molecules such as HLA-DRB1 to present endothelial cell-specific, and non-EC specific self-antigen epitopes to activate T cells(47,48). Similar to the findings in this paper on cell transdifferentiation, we also reported recently that in response to environmental stimulations including DAMPs, hypoxia and master gene mutations, cell identification could be plastic as we recently reported in several papers: first, transcription factor GATA binding protein 3 (GATA3), histone deacetylase 6 (HDAC6) and B-cell lymphoma 6 (BCL6) regulate forkhead box P3 (FOXP3)+Treg plasticity, and determine Treg conversion into either novel antigen-presenting cell (APC)-like regulatory T cell (Treg), or type 1 T helper cell (Th1)-Treg(47), which further consolidated our recently proposed new working model that pathological conditions re-shape physiological Tregs into pathological Tregs(49); second, thrombus leukocytes exhibit more endothelial cell-specific angiogenic markers than peripheral blood leukocytes do in acute coronary syndrome patients, suggesting a possibility of transdifferentiation of leukocytes into angiogenic endothelial cells(50); and third, in patients with lymphomas, Tregs can function in four different formats(51). Collectively, our data presented here also indicated that targeting the innate immune systems in ECs, such as by targeting CD36, could become novel anti-inflammatory therapies against hyperlipidemia-induced endothelial cell activation and atherosclerotic CVD.

When there is ample oxygen in the environment, cancer cells utilize mainly glycolysis rather than mitochondrial oxidative phosphorylation, an effect termed “Warburg effect”(52). The Warburg effect is beneficial for tumor cells as it supports macromolecule biosynthesis. Quiescent ECs also display the Warburg effect, but very little is known about the roles of cellular metabolisms during hyperlipidemia stimuli-induced EC activation(53). Although there was a report indicating that LPC shares the same receptor, G protein-coupled receptor 55 (GPR55), with LPI(54), our detailed analyses of the differential EC transcriptomes induced by LPC and LPI emphasize strongly that LPI and LPC may use different receptors-initiated signaling pathways, which correlated with others’ report that LPI species 20:4 is the most potent GPR55 agonist(55), emphasizing the high specificity of LPI on GPR55. By using transcriptomic profiling, our study here demonstrated for the first time that in ECs, LPC-induced global reprogramming of cellular metabolism by inducing specific isoforms of metabolic gene transcripts, such as LDHA. Notably, a large portion of these metabolic enzymes are regulated at transcript levels, suggesting that LPI uses transcription and alternative splicing mechanisms to reprogram cellular metabolism. Further studies are needed to elucidate the specific molecules that mediate LPI-regulated metabolic pathways.

Lipid biosynthesis is regulated by SREBPs and liver X receptor (LXR) transcription factors. SREBPs are master transcription factors that control the expression of a range of enzymes required for cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis. Cholesterol and its derivatives are established to be an upstream regulator of SREBPs by directly binds to the protein and prevents its translocation to the nucleus(56). Recent studies suggested that maturation of nuclear, transcriptionally active SREBP protein is also controlled by phospholipids(57). In addition,
proatherogenic oscillatory flow as DAMP, activates SREBP2 and induces NLRP3 inflammasome in ECs(58). However, to our knowledge, our results for the first time indicate that SREBP might be regulated by conditional DAMPs lysophospholipids. Upregulation of SREBP2-regulated genes has been found to be a proinflammatory M1 macrophage polarization marker(33), suggesting that proinflammatory immunometabolism remodeling can be the same in different cell types(59,60). Future studies are needed to determine whether LPC could directly binds to and regulates SREBP, as cholesterol does. Remarkably, LPI does not regulate SREBP-dependent genes, indicating the specificity of LPC in regulating cellular lipid homeostasis.

LPC and LPI both induce comparable levels of mitochondrial ROS and cytosolic calcium influx, which correlate with their common induction of EC adhesion molecules, cytokines, chemokines, DAMP receptors and adaptive immune molecules such as T cell co-stimulation/co-inhibition receptors and MHC class II molecules. These results indicate that calcium influx/mitochondrial ROS pathway might mediate lysophospholipid-induced EC activation. By contrast, LPC and LPI differentially regulate metabolic pathways in ECs, suggesting that calcium influx/mitochondrial ROS are not responsible for these physiological responses. Thus, as we reported with mitochondrial ROS inhibitor MitoTempol(4), targeting mitochondrial ROS signaling-histone 3 lysine 14 acetylation pathway(6) might be superior than targeting lysophospholipids themselves as therapies, since it does not interfere with normal physiological metabolism of ECs but specifically targets the innate immune response in ECs.

Taken together, we proposed a new system of characterizing prolonged and sustained EC activation and innate immune transdifferentiation (Figure 6) based on the differentiating criteria (1) induction of DAMP receptors and (2) induction of T cell co-stimulation/co-inhibition receptors and MHC-II molecules. During acute inflammation induced by pathogens such as bacteria and virus, ECs are transiently activated by upregulation of adhesion molecules and secretion of cytokines and chemokines. When ECs are equipped to sense, and are activated by chronic metabolic stressors/DAMPs, such as lysophospholipids, additional DAMP receptors, T cell co-stimulation/co-inhibition receptors, and MHC-II molecules are also upregulated, besides the induction of classic EC activation marker genes. By doing this, ECs are transdifferentiated into innate immune cells, which can keep receiving stimulations from additional DAMPs and mediate long-term and consistent pathogenic leukocyte recruitment to the aorta. Moreover, activated ECs with upregulated T cell co-stimulation/co-inhibition receptors, and MHC class II can activate/modulate encountered T cells and enhancing T cell participation of vascular inflammation and atherogenesis. Our new working model of EC activation provides novel insight into how ECs are differentially activated and innate immune transdifferentiated during CVD development. Our findings are significant for future design of novel anti-inflammatory/immunosuppressive therapies against CVD.

**Experimental procedures**

*Chemicals and Antibodies* — All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. LPC (16:0) and LPI (16:0) were purchased from Avanti Polar Lipids, Inc (#855675P; Alabaster, Alabama). For mtROS measurement, MitoSOX Red Mitochondrial Superoxide Indicator (#M36008; Life technologies, Carlsbad, CA) was used. Fluo-4 (Life technologies) was purchased for the measurement of cytosolic calcium.

*Human Samples* — All the experiment procedures were performed in accordance with protocols approved by the Institutional Review Board at Temple University, which confirmed to the National Institutes of Health Guidelines. All studies involving human subjects abide by the Declaration of Helsinki principles.

*Cell Culture* — Human aortic endothelial cells (HAECs) (Lonza, CC2535; Walkersville, MD) were cultured in medium M199 (HyClone laboratories, Logan, UT) supplemented with 15% fetal bovine serum (FBS; HyClone); endothelial cell growth supplement (ECGS, 50µg /mL); BD Biosciences, San Jose, CA); heparin (50µg/mL); and 1% penicillin, streptomycin, and amphotericin
Lysophospholipids induce sustained endothelial activation (PSA; Invitrogen, Carlsbad, CA). HAECs were grown on 0.2% gelatin-coated flasks, plates, or dishes and experiments were performed at passage 9.

**Fluorescence Activated Cell Sorting (FACS)** — For mtROS and cytosolic calcium measurement: After staining with MitoSOX and Fluo-4, HAECs were incubated at 37°C for 10min and washed with PBS twice afterwards. After LPC and LPI treatment, cells were washed once with ice-cold PBS and Trypsin-EDTA was added to detach cells. Trypsinization was terminated by adding FACS buffer (2% FBS in PBS) and cells were collected by centrifugation. After re-suspension in 0.2ml FACS buffer, samples were subjected to flow cytometry analysis, where fluorescence emissions were measured by FACSCalibur machine (BD).

**RNA-Seq of LPC** — RNA-Seq of LPC was performed by BGI (Shenzhen, China). Total RNAs were extracted from samples, then mRNA and non-coding RNAs were enriched by removing rRNA from the total RNA with kit. By using the fragmentation buffer, the mRNAs were fragmented into short fragments (about 200–500 nucleotides (nt)), then the first-strand cDNA was synthesized by random hexamer-primer using the fragments as templates, and dTTP was substituted by dUTP during the synthesis of the second strand. Short fragments were purified and resolved with elution buffer for end reparation and single nucleotide A (adenine) addition. After that, the short fragments were connected with adapters, then the second strand was degraded finally using UNG (Uracil-N-Glycosylase) [2]. After agarose gel electrophoresis, the suitable fragments were selected for the PCR amplification as templates. During the quality control steps, Agilent 2100 Bioanalyzer (https://www.genomics.agilent.com/en/Bioanalyzer-System/2100-Bioanalyzer-Instruments/?cid=AG-PT-106) and ABI StepOnePlus Real-Time PCR System (https://www.thermofisher.com/order/catalog/product/4376600) were used in quantification and qualification of the sample library. At last, the library was sequenced using Illumina HiSeq4000 using PE100 strategy. Primary sequencing data that produced by Illumina Hiseq4000 called as raw reads, were filtered into clean reads by remove adaptor contained and low quality reads by BGI (Shenzhen, China) in-house software. Reference annotation based assembly method was used to reconstruct the transcripts by Tophat (v2.0.10) + Cufflinks (v2.1.1), while background noise was reduced by using FPKM (Fragments Per Kilobase Million) and coverage threshold. Data were deposited as E-MTAB-6604 at ArrayExpress.

**RNA-Seq of LPI** — RNA-Seq of LPI was performed by Novogene (Beijing). Briefly, mRNA from HAECs was purified from total RNA using poly-T oligo-attached magnetic beads. The mRNA was first fragmented randomly by addition of fragmentation buffer. Then first strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Double-stranded cDNA was purified using AMPure XP beads (Beckman Coulter, Beverly, USA). Remaining overhangs of the purified double-stranded cDNA were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter). Finally, the final library was gotten by PCR amplification and purification of PCR products by AMPure XP beads. After library construction, diluting library to 1.5ng/ul with the preliminary quantitative result by Qubit2.0 and detecting the insert size by Agilent 2100. Q-PCR was used to accurately quantify the library effective concentration (> 2nM), in order to ensure the library quality. Libraries were fed into HiSeq machines (https://www.illumina.com/Documents/systems/hiseq/datasheet_hiseq_systems.pdf) after pooling according to activity and expected data volume. Data were deposited as E-MTAB-6604 at ArrayExpress.

**Sequencing data analysis** — Data analysis was carried out using the statistical computing environment, R, the Bioconductor suite of packages for R, and RStudio (https://www.rstudio.com/) (29). Raw data were background subtracted, variance stabilized, and normalized by robust spline
Lysophospholipids induce sustained endothelial activation normalization. Differentially expressed genes were identified by linear modeling and Bayesian statistics using the Limma package. Probes sets that were differentially regulated (≥1.4 fold, P value < 0.05; after controlling for multiple testing using the Benjamini-Hochberg method were used for hierarchical clustering and heatmap generation in R. Clusters of co-regulated genes were identified by Pearson correlation using the hclust function of the stats package in R. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/), and Gene Set Enrichment Analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp). Briefly, IPA was a web-based software application that goes beyond pathway analysis by identifying key upstream regulators to explain expression patterns and predicting downstream effects on biological and disease processes. GSEA is a computational method that determines whether a priori defined set of genes shows statistically significant, concordant differences between two biological states. GSEA does not focus on only significantly/highly changed genes, but examines all the genes that belongs to a certain biological process instead.

Statistical analysis — Data were expressed as the mean ± standard deviation (SD) throughout the manuscript. For comparisons between two groups, two-tailed Student t test was used for evaluation of statistical significance or, when the data were not normally distributed, a nonparametric Mann-Whitney U test was used. For comparisons across multiple groups, one-way ANOVA with Bonferroni post-test adjustment was used or, when the data were not normally distributed, the data were analyzed using one-way ANOVA with the Kruskal-Wallis test, followed by pairwise comparison using the Dunn test. For linear regression tests, simple linear regression analyses were performed using GraphPad Prism (https://www.graphpad.com/scientific-software/prism/) to determine coefficient of determination and p value. Data shown were representative of two to three independent experiments. NS, not significant; *, p<0.05; **, p<0.01; *** p<0.001.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

X.L., L.W., and X.F.Y designed research; X.L. and L.W. performed research; P.F., Y.S., X.J., and H.W. provided reagents and critically read the manuscript; X.L. and X.F.Y analyzed data; and X.L., and X.F.Y wrote the paper.
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Figure Legends

Fig. 1. RNA-Seq analysis reveals that LPI induces both transient and sustained endothelial cell activation. Human aortic endothelial cells (EC) were treated with either vehicle control or lysophosphatidylinositol (LPI) (10µM) for 18 hours and RNA-Seq experiments were performed. n=3 in each group. A. Volcano plot showing log(Fold change, FC) and −log10 (P value) of control vs LPI treatment. Red genes indicate significantly changed genes by more than 1.4-folds by LPI. B. Heat map of genes that are significantly changed by more than 1.4 folds by LPI in ECs. C. The LPC-upregulated genes from its top regulated pathway “cellular infiltration by leukocyte” were shown. Genes that are related to the innate immunity are boxed. D to H. Gene set enrichment analysis (GSEA) of the gene signatures that are significantly enriched in the LPI-treated EC group. I. Representative gene expression changes in different categories corresponding to the GSEA plots.

Fig. 2. LPI reprograms endothelial cell metabolism extensively besides upregulating adhesion molecules and cytokines/chemokines in human aortic endothelial cells. Human aortic endothelial cells (EC) were treated with either vehicle control or lysophosphatidylcholine (LPC) (10µM) for 18 hours and RNA-Seq experiments were performed. The transcript level in the unit of transcripts per million (TPM) of the genes related to different categories, including EC adhesion molecules, cytokines/chemokines, and metabolic regulators were shown.

Fig. 3. RNA-Seq analysis reveals that LPC induces both acute and sustained endothelial cell activation. Human aortic endothelial cells (ECs) were treated with either vehicle control or lysophosphatidylcholine (LPC) (10µM) for 18 hours and RNA-Seq experiments were performed. n=3 in each group. A to E. Gene set enrichment analysis (GSEA) of the gene signatures that are significantly enriched in the LPC-treated EC group. F. Representative gene expression changes in different categories corresponding to the GSEA plots.

Fig. 4. LPC positively regulates genes downstream of master regulator of lipid metabolism, sterol regulatory element-binding protein 2 (SREBP2). Human aortic endothelial cells (HAECs) were challenged with LPC (10 µM) for 18 hours and RNA-Seq with Ingenuity Pathway Analysis (IPA) was performed. A. Top regulated diseases or functions annotation of the genes that are significantly changed by LPC in HAECs. B. Gene set enrichment analysis from the top LPC-regulated pathway “steroid biosynthesis” was shown. C. Top upstream regulator analysis of the genes that are changed by LPC in HAECs predicted by the IPA. D. The 20 SREBP2-regulated genes that are significantly changed by LPC in HAECs. The red genes are induced by LPC and the blue genes are decreased by LPC.

Fig. 5. LPI and LPC similarly activate mitochondrial reactive oxygen species (ROS), cytosolic calcium and acute EC activation marker genes, but regulate largely distinct gene programs in human aortic endothelial cells. A&B. Human aortic endothelial cells (HAECs) were treated with either LPC (10µM) or LPI (10µM) for 1 hour. Flow cytometry analysis with mitochondrial ROS (panel A) and cytosolic calcium (panel B) probes was performed afterwards. C. gene set enrichment analysis (GSEA) of the LPI-upregulated genes in the “calcium-mediated signaling” collection. D. principal component analysis showing the global transcription profile relationship among control, LPI, and LPC. E. heatmap showing the similarity and difference from LPC-regulated and LPI-regulated genes. F. Venn diagram showing the number of LPC&LPI co-upregulated genes. G. Top enriched pathways of the LPI&LPC co-upregulated genes (58 genes from panel F), as determined by Ingenuity Pathway Analysis. H. The LPC&LPI co-upregulated genes from their top regulated pathway “attraction of leukocytes” (in panel G) were shown. I. Comparison of Lipopolysaccharide (LPS) and lysophospholipids-induced endothelial activation. Red-
colored numbers indicate gene expression fold changes that are more than 1.4 folds. For all panels, data are expressed as mean ± SD. **, p<0.01, ***, p<0.001,

**Fig. 6. A new working model.** Left: During acute inflammation, Danger signal from pathogen or virus infection induces transient endothelial cell activation, as characterized by two features, including upregulated adhesion molecule expression and increased secretion of cytokines and chemokines. Right: In the process of chronic metabolic inflammation during cardiovascular disease development, constant stimulation from endogenous metabolic danger-associated molecular patterns (DAMPs), such as lysophospholipids (during hyperlipidemia), glucose (during hyperglycemia), and homocysteine (during hyperhomocysteinemia), transformed endothelial cells into innate immune cells and induced prolonged endothelial cell activation. The innate reprogramming of endothelial cells is characterized not only by upregulation of cytokine/chemokines and adhesion molecule gene expression, but also by upregulation of additional DAMP receptors, such as Caspase-1 and CD36, as well as upregulation of costimulatory molecules and histocompatibility complex (MHC) class II molecules.
Figure 1

| Gene     | Category              | Fold change |
|----------|-----------------------|-------------|
| IL1B     | Cytokine              | 2.21        |
| CXCL6    | Chemokine             | 2.54        |
| CXCL8    | Chemokine             | 1.86        |
| SELE     | Adhesion              | 3.66        |
| ICAM1    | Adhesion              | 1.42        |
| CD36     | Scavenger receptor    | 2.98        |
| STAB2    | Scavenger receptor    | 12.1        |
| OLR1     | Scavenger receptor    | 3.24        |
| CD5      | Scavenger receptor    | 3.10        |
| CASP1    | Nod-like receptor     | 1.42        |
| TLR3     | Toll-like receptor    | 1.40        |
| TNFSF15  | Adaptive              | 2.64        |
| TNFRSF4  | Adaptive              | 1.72        |
| CD274 (PDL1) | Adaptive            | 1.43        |
| HLA-DRB1 | MHCII                 | 4.11        |
| CD74     | MHCII                 | 1.71        |
### Table

| Gene          | Category | Fold change |
|---------------|----------|-------------|
| IL1B          | Cytokine | 1.85        |
| CCL2          | Chemokine| 1.78        |
| CXCL8         | Chemokine| 1.43        |
| SELE          | Adhesion | 2.87        |
| ICAM1         | Adhesion | 1.45        |
| CD36          | Innate   | +∞          |
| NOD2          | Innate   | 1.92        |
| NAIP          | Innate   | 1.63        |
| TNFSF15       | Adaptive | 1.79        |
| TNFRSF9       | Adaptive | 4.38        |
| CD70          | Adaptive | 2.92        |
| CD274 (PDL1)  | Adaptive | 1.39        |
| HLA-DRB1      | MHCII    | 4.16        |
| HLA-DMA       | MHCII    | +∞          |

### Figure 3

- **A**: Enrichment plot for KEGG_CELL_ADHESION_Molecules_CAMS
  - LPC (positively correlated)
  - Control (negatively correlated)
  - P = 0.018

- **B**: Enrichment plot for REACTOME_CHEMOKINE_RECEPTORS BIND CHEMOKINE
  - LPC (positively correlated)
  - Control (negatively correlated)
  - P = 0.000

- **C**: Enrichment plot for KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTIONS
  - LPC (positively correlated)
  - Control (negatively correlated)
  - P = 0.000

- **D**: Enrichment plot for KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY
  - LPC (positively correlated)
  - Control (negatively correlated)
  - P = 0.006

- **E**: Enrichment plot for REACTOME_COSTIMULATION_BY_THE_CD28_FAMILY
  - LPC (positively correlated)
  - Control (negatively correlated)
  - P = 0.043

- **F**: Genes and their categories with fold changes:
  - IL1B: Cytokine, 1.85
  - CCL2: Chemokine, 1.78
  - CXCL8: Chemokine, 1.43
  - SELE: Adhesion, 2.87
  - ICAM1: Adhesion, 1.45
  - CD36: Innate, +∞
  - NOD2: Innate, 1.92
  - NAIP: Innate, 1.63
  - TNFSF15: Adaptive, 1.79
  - TNFRSF9: Adaptive, 4.38
  - CD70: Adaptive, 2.92
  - CD274 (PDL1): Adaptive, 1.39
  - CD74: MHCII, 4.16
  - HLA-DRB1: MHCII, +∞
### A

| Diseases or Functions Annotation          | p-Value   | z-score | # Molecules |
|------------------------------------------|-----------|---------|-------------|
| steroid metabolism                       | 1.71E-10  | 2.694   | 19          |
| metabolism of terpenoid                  | 5.39E-10  | 2.694   | 20          |
| tumorigenesis of tissue                  | 1.22E-09  | 0.392   | 170         |
| metabolism of cholesterol                | 1.26E-09  |         | 12          |
| neoplasia of epithelial tissue           | 1.41E-09  | 0.392   | 168         |
| metabolism of membrane lipid derivative  | 3.00E-09  | 0.862   | 22          |
| malignant solid tumor                    | 3.27E-09  | 1.353   | 189         |
| epithelial cancer                        | 3.55E-09  | -0.109  | 166         |

### C

| Upstream Regulator      | Molecule Type         | Predicted State | z-score   | p-value  |
|-------------------------|-----------------------|-----------------|-----------|----------|
| SREBF2                  | transcription regulator| Activated       | 3.739     | 6.32E-25 |
| SCAP                    | other                 | Activated       | 3.837     | 8.39E-19 |
| SREBF1                  | transcription regulator| Activated       | 3.241     | 9.53E-15 |
| INSIG1                  | other                 | Inhibited       | -2.235    | 3.15E-14 |
| POR                     | enzyme                | Inhibited       | -3.835    | 1.32E-11 |
| PPARA                   | ligand-dependent nuclear receptor | Inhibited   | -1.589 | 4.23E-11 |
| TGFBI                   | growth factor         | Inhibited       | -2.715    | 9.28E-10 |
| CYP51A1                 | enzyme                | Inhibited       | -2.449    | 9.37E-10 |

### B

![Figure 4](http://www.jbc.org/Downloaded from)
## Diseases or Functions Annotation

| Annotation                        | p-Value       |
|-----------------------------------|---------------|
| Attraction of leukocytes          | 1.62E-11      |
| Adhesion of monocytes             | 4.46E-11      |
| Attraction of phagocytes          | 9.23E-11      |
| Quantity of myeloid cells         | 1.12E-10      |
| Activation of neutrophils         | 1.44E-10      |
| Adhesion of immune cells          | 1.98E-10      |
| Chemotaxis of phagocytes          | 3.29E-10      |
| Recruitment of lymphocytes        | 5.52E-10      |
| Adhesion of vascular endothelial cells | 6.37E-10  |
| Mobilization of neutrophils       | 6.83E-10      |

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### Table: Gene expression fold changes

| GEO ID    | Treatment | Cell type                             | Time | Gene expression fold changes |
|-----------|-----------|---------------------------------------|------|------------------------------|
| GSE5883   | LPS       | Human lung microvascular endothelial cells | 4 h  | IL1B  | 9.22 | 13.6 | 0.81 | 4.6  | 1.85 |
|           |           |                                       | 8 h  | ICAM1 | 6.60 | 17.9 | 0.55 | 6.19 | 0.66 |
|           |           |                                       | 24 h | CD36  | 1.27 | 3.8  | 0.57 | 1.04 | 1.15 |
| This study | LPI       | Human aortic endothelial cells         | 18 h | TNFSF15 | 2.21 | 1.42 | 2.98 | 2.64 | 1.71 |
| This study | LPC       | Human aortic endothelial cells         | 18 h | CD74  | 1.85 | 1.45 | +∞   | 1.79 | 4.16 |
**Acute inflammation**

- Pathogen/viral/danger signal
- Upregulated adhesion molecules (ICAM-1/VCAM-1/E-selectin)
- Increased secretion of cytokines/chemokines

**Chronic metabolic inflammation**

- Constant hyperlipidemia/hyperglycemia/hyperhomocysteinemia (DAMPs)
- Upregulated adhesion molecules (ICAM-1/VCAM-1/E-selectin)
- Increased secretion of cytokines/chemokines
- Regulated additional DAMP receptors
- Upregulated costimulatory molecules and MHC class II

**Feasibility 1**
Upregulated adhesion molecules

**Feasibility 2**
Increased secretion of cytokines/chemokines

**Feature 3**
Regulated additional DAMP receptors

**Feature 4**
Upregulated costimulatory molecules and MHC class II

**Figure 6**

- Prolonged endothelial activation and “transformation” into innate immune cells
Lysophospholipids induce innate immune transdifferentiation of endothelial cells, resulting in prolonged endothelial activation
Xinyuan Li, Luqiao Wang, Pu Fang, Yu Sun, Xiaohua Jiang, Hong Wang and Xiao-Feng Yang

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