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Lysophospholipid Receptors, as Novel Conditional Danger Receptors and Homeostatic Receptors Modulate Inflammation - Novel Paradigm and Therapeutic Potential

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Abstract

There are limitations in the current classification of danger associated molecular patterns (DAMP) receptors. To overcome these limitations, we propose a new paradigm by using endogenous metabolites lysophospholipids (LPLs) as a prototype. By utilizing a data mining method we pioneered, we made the following findings: 1) Endogenous metabolites such as LPLs at basal level have physiological functions; 2) under sterile inflammation, expression of some LPLs are elevated. These LPLs act as conditional DAMPs or anti-inflammatory homeostasis-associated molecular pattern molecules (HAMPs) for regulating the progression of inflammation or inhibition of inflammation, respectively; 3) receptors for conditional DAMPs and HAMPs are differentially expressed in human and mouse tissues; and 4) complex signaling mechanism exists between pro-inflammatory mediators and classical DAMPs that regulate the expression of conditional DAMPs and HAMPs. This novel insight will facilitate identification of novel conditional DAMPs and HAMPs, thus promote development of new therapeutic targets to treat inflammatory disorders.

Keywords

Lysophospholipid receptor; Rheumatoid arthritis; Atherosclerosis; Danger-associated molecular pattern molecules receptor; Homeostasis-associated molecular pattern molecules receptor

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1. Introduction

Pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) generated during a tissue injury act as sensors and activate the immune system to respond to infection or injury [1]. One of the key cellular receptors that recognize the “threat” signals initiated by PAMPs and DAMPs are Toll-like receptors (TLRs). These receptors are mainly located on the plasma membrane and activate inflammatory genes to counteract tissue injury and mediate repair. Moreover, TLRs work in synergy with cytosolic sensing receptor families like NLRs [NOD (nucleotide binding and oligomerization domain)-like receptors] to recognize DAMPs, particularly in inflammation privileged tissues where inflammasome component genes that activate pyroptosis are not constitutively expressed [2,3]. In addition to TLRs and NLRs, four additional DAMP receptors including transmembrane C-type lectin receptors, retinoid acid inducible gene I (RIG-I), AIM2 (absent in melanoma 2), receptor for advanced glycation end products (RAGE, also a receptor for high mobility group box 1(HMGB1)) have been characterized [4]. Herein, we refer the above mentioned 6 categories of DAMP receptors as classical DAMP receptors.

Dr. Matzinger’s proposal of danger model has significantly improved our understanding on the role of endogenous metabolites in the pathogenesis of inflammatory and immunological diseases [5]. Based on the current understanding, we can identify following principles that classify DAMPs and their receptors: (1) endogenous metabolites released under cellular stress could serve as the DAMPs; (2) DAMPs has to bind to classical DAMP receptors to initiate inflammation; (3) classical DAMP receptors initiates pro-inflammatory transcription factor-mediated signaling pathways [6]; and (4) DAMP receptor genes are evolutionally conserved from mice to humans. However, the current classification and paradigm of DAMP receptors raise the following concerns: (1) Do all the endogenous metabolites that can act as DAMPs initiate inflammation via binding only to the classical DAMP receptors? (2) Do all the pro-inflammatory endogenous metabolites have high affinity and signal-generating interactions to classical DAMP receptors to facilitate the downstream signaling cascades? Is it feasible in the biochemical point of view? (3) Does the endogenous metabolites that regulate normal cellular functions at physiological concentrations initiate inflammatory response under elevated conditions? (4) The current DAMP receptor model emphasizes only the danger signals generated from endogenous metabolic processes. It fails to recognize the roles of potential endogenous metabolites in anti-inflammatory responses, inflammation resolution and maintenance of homeostasis; and (5) the current DAMP receptor model emphasizes only the danger signals released from damaged/dying cells [7] but fails to identify the roles of pathological elevated endogenous metabolites released from activated viable cells.

The significance of addressing these limitations and shifting the paradigm to form a new model is that it will encourage investigators [8] to recognize novel conditional DAMP receptors and anti-inflammatory and homeostatic signals derived from endogenous metabolites. Recent advances in immunology has clearly demonstrated the well-published “two arms model”. This model states that in addition to the pro-inflammatory immunoeffector and T cell co-stimulatory mechanisms, there are several immunotolerance and anti-inflammatory mechanisms mediated by the immune system. These anti-

inflammatory mechanisms include T cell co-inhibition/co-suppression pathways [9], T cell anergy [10], regulatory T cells [11], and secretion of anti-inflammatory/ immunosuppressive cytokines such as transforming growth factor- β (TGF- β), interleukin-10 (IL-10), IL-35, and IL-37 as we and others reported [12–15], etc.

In order to shift the current paradigm on DAMP receptors and address the limitations mentioned above, we utilized an extensive literature survey and database mining method, which we have pioneered and published previously [16]. For this study we used the endogenous metabolite lysophospholipids (LPLs) and its receptors as the prototype. LPLs are bioactive, lipid-derived metabolites that act through G-protein coupled receptors (GPCRs) to either serve as signaling molecules at physiological concentrations that control cellular homeostasis or as inflammatory regulators at elevated concentrations [17]. LPLs are generated by regiospecific phospholipases on substrates such as membrane phospholipids and sphingolipids.

Some of the LPLs that have been identified and studied so far are lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lipophosphoglycan (LPG), lysophosphatidylinositol (LPI), and lysophosphatidylserine (LysoPS) [17]. The role of LPLs in chronic inflammatory disorders such as coronary artery disease (CAD), hypertension [18,19], atherosclerosis and severe vascular diseases is well established [20,21]. Furthermore, LPLs contribute to pathophysiology of autoimmune disorders such as rheumatoid arthritis, and is involved in exacerbating the progression of the disease [22,23].

Our comprehensive literature survey revealed that LPLs can either act as pro- or anti-inflammatory agents that follow the logic of aforementioned “two arms” model in immunity. For an example, LysoPS [24] and LPE [25] were identified as endogenous metabolites that have anti-inflammatory and homeostatic functions, while elevated LPA, LPI and LPC levels were shown to induce inflammation. Therefore, we propose a conceptually innovative paradigm that identifies endogenous metabolites such as LPLs that act as pro-inflammatory mediators at elevated concentrations but mediate normal cellular functions at physiological concentrations as ***conditional DAMPs***. Moreover, we designate the receptors that exert the effects of conditional DAMPs as ***conditional DAMP receptors*** and suggest that activation of inflammation by conditional DAMPs may not necessarily always involve or “converge to” TLRs, NLRs and other classical DAMP receptors. Herein, we further advance the current paradigm by suggesting that endogenous metabolites such as LysoPS and LPE that maintain homeostasis at physiological levels, but act as anti-inflammatory mediators to inhibit inflammation and promote inflammation resolution as ***homeostasis-associated molecular patterns (HAMPs)***. Furthermore, we propose that these HAMPs bind to their receptors (***HAMP receptors***) to initiate anti-inflammatory/homeostatic signaling and promote inflammation resolution. Moreover, we suggest that LPL-GPCRs can be classified as novel conditional DAMP receptors for the following reasons: (1) LPLs, which are endogenous pro-inflammatory lipid metabolites, are elevated during cellular stress and bind to their LPL-GPCRs; (2) Elevated pro-inflammatory LPLs initiate signaling cascade to activate inflammatory genes via LPL-GPCRs.

In this study, we have used LPLs as a group of prototypic molecules to demonstrate the proof of principle and do not exclude the possibilities of other bio-lipids such as n-6 polyunsaturated fatty acids to function as DAMPs or HAMPs to regulate inflammation [26]. Our newly proposed HAMPs include lipid-based specialized pro-resolving mediators (SPMs) [27] and other anti-inflammatory endogenous metabolites. Our new classification of LPL-GPCRs as conditional DAMP receptors and our novel concept of anti-inflammatory endogenous metabolite receptors as HAMP receptors will lead to the future development of novel therapeutics for autoimmune diseases, inflammatory diseases and inflammatory cancers.

2. Methods

2.1 Tissue expression profile of genes encoding G protein-coupled LPLs receptors (LPL-GPCRs)

We used our previously reported experimental data mining strategy to analyze the expression profile of LPL-GPCRs (Part A, B in Figure 1) [28,2]. We analyzed LPL-GPCR gene expression in cardiovascular and other tissues by mining experimentally verified human and mouse mRNA expressions in the expressed sequence tag (EST) databases of the National Institutes of Health (NIH)/ National Center of Biotechnology Information (NCBI) UniGene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene>) (see the justification for this database mining approach in the Discussion).

Transcripts per million of genes of interest were normalized with that of housekeeping β -actin in each given tissue to calculate the arbitrary units of gene expression. A confidence interval of the expression variation of housekeeping genes was generated by calculating the mean plus two times that of the standard deviation of the arbitrary units of three randomly selected housekeeping genes (ARHGDIA, GADPH and PRS27A in human; Ldha, Nono and Rpl32 in mouse) normalized by β -actin in the given tissues (Figure 4). If the expression variation of a given gene in the tissues was larger than the upper limit of the confidence interval (the mean plus two times the standard deviation) of the housekeeping genes, the high expression levels of genes in the tissues were considered statistically significant. Gene transcripts lower than one per million were technically presented as no expression.

2.2 Expression profiles of LPL-GPCRs in human disease, mouse disease models and several TLR genes deficient mouse tissues, and expression profiles of TLRs in LPLs stimulated tissues

Gene expression profiles were collected from 15 microarray datasets in NIH-Geo database (Part C in Figure 1). Specific samples were chosen as disease or treatment groups and parallel control. The number of samples was always greater than 3 except for the pooled samples. By searching for the gene symbols from the microarray data, we selected the genes with significant changes ($p < 0.05$). The genes of which the expression changes were more than 1 fold were defined as the upregulated genes while genes with their expression changes less than 1 fold were defined as downregulated genes.

3. Results

3.1. LPLs bind to distinct LPL-GPCRs to exert its pro-/ anti-inflammatory effects

The biological effects of LPLs are exerted via GPCRs. By an extensive literature survey, we found 15 GPCRs that are recognized as LPL receptors and tabulated this data in Table 1 [29–34]. Of note, previously claimed LPL-GPCRs including GPR3, GPR4, GPR6, GPR12, GPR35, GPR45, GPR65 (TDAG8), GPR68 (ORG1), GPR87, GPR119, GPR132 (G2A) and PAFR are now not recognized as LPL receptors by the LPL community (unidentified leading LPL expert, personal communication).

Our comprehensive literature survey revealed that LPLs bind to distinct LPL-GPCRs. For example, so far only GPR55 is recognized as the receptor for LPI [35] while LPA, S1P, SPC (sphingosylphosphorylcholine) and LysoPS bind to more than one type of LPL-GPCRs. As shown in Table 1, LPA binds to six LPL-GPCRs, S1P and SPC bind to five LPL-GPCRs.

So far, GPR 34, which is the receptor for anti-inflammatory LysoPS [36,37] was identified to bind to the subunit $G_{i/o}$ explicitly. Similarly, the LPI receptor GPR55 specifically binds to G_q and $G_{12/13}$ subunits. Nevertheless, the other LPL-GPCRs which we have tabulated in Table 1 seem to bind to a variety of G protein subunits (the information of G protein subunits for GPR174 and P2RY10 was not available). This data suggested that certain LPLs such as LPI and LysoPS exerts distinct signaling mechanisms via GPR34 and GPR55, respectively, while some LPLs trigger broad spectrum of signaling by binding to variety of LPL-GPCRs. In order to have a comprehensive understanding about the signaling mechanisms of LPLs, we further examined the down-stream targets of currently identified LPL-GPCRs and illustrated our findings in Figure 2.

As shown in Figure 2, the LPLs act as signaling mediators by binding to seven-transmembrane domain GPCRs and either activate or inhibit downstream signaling pathways such as Rho-associated kinase (Rock), diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP3), mitogen-activated protein kinase (MAPK), Ca^{2+} , adenylate cyclase (AC), phosphoinositide-3-kinase (PI3K) [29,38,39] etc. The specificities of LPL signaling via LPL-GPCRs may depend on the number of available receptors, as well as differentiation status, co-signaling pathways, and pathophysiological status in the cells.

3.2. For the first time, we propose a new paradigm that LPLs are either pro-inflammatory conditional DAMPs or anti-inflammatory HAMPs for regulating the balance between inflammation and inhibition/ resolution of inflammation

The Table 1 and Figure 2 clearly depicts that; (1) pro-inflammatory LPLs specifically bind to distinct LPL-GPCRs; (2) binding of pro-inflammatory LPLs to LPL-GPCR induce downstream inflammatory signaling cascades such as PKC (Protein Kinase C) [40–42], MAPK [43,44], ROCK [45] and SRF (Serum Response Factor) [46]. Therefore, it is evident that endogenous metabolites such as LPLs exert its inflammatory responses via LPL-GPCRs, thus it can be justified to designate LPL-GPCRs as conditional DAMP receptors. Furthermore, anti-inflammatory LysoPS has the capability of activating the survival PI3K and AKT pathway suggesting its receptors can be classified as HAMP receptors. To further

consolidate this new hypothesis on conditional DAMPs and HAMPs, an extensive literature search was conducted.

Further supporting evidence for classifying LPLs as conditional DAMPs or HAMPs are presented in Table 2. As illustrated in Table 2A, endogenous LPLs were upregulated in the presence of metabolic and inflammatory disorders such as type 2 diabetes, diabetic retinopathy, acute coronary syndrome and autoimmune disorders. High serum levels of S1P, LPA and LPI was observed in patients with juvenile-onset systemic lupus erythematosus [47], proliferative diabetic retinopathy [48] and obese type 2 diabetes [49] respectively. Interestingly, acute coronary syndrome increased a bunch of pro-inflammatory LPLs (LPA, LPG, LPI and LPC) and anti-inflammatory LPLs (LysoPS and LPE). This indicates the presence of a complex signaling cascade that takes place to retain the balance of inflammation via conditional DAMPs and its resolution via HAMPs [21].

More supporting evidence for LPLs working as conditional DAMPs or HAMPs are presented Table 2B. Table 2B indicates that; (1) LPA, S1P, LPC, LPI and SPC induced the secretion of pro-inflammatory cytokines [50–56]; (2) in contrast, LysoPS and LPE suppressed the production of pro-inflammatory cytokines [57,25]; (3) additionally LPE also induced the secretion of anti-inflammatory cytokine IL-10; and (4) LPL mediated secretion of pro-inflammatory/ anti-inflammatory cytokines was dose dependent.

We have previously demonstrated that under low concentrations, LPLs induce physiological signaling without exerting pro/ anti-inflammatory effects at a threshold that is detectable [58]. The data provided in the Table 2B indicates that LPLs have distinct functions in a dose dependent manner indicating LPLs can indeed be classified as conditional DAMPs or HAMPs. We have presented our novel working model in Figure 3.

As illustrated in Figure 3A, pro-inflammatory LPLs such as LPA, S1P, LPC, LPI and SPC act as conditional DAMPs to induce systemic inflammatory responses. However, LysoPS and LPE act as HAMPs to suppress systemic inflammatory response and induce the resolution of inflammation. Thus, LPLs regulate the balance between initiation and resolution of inflammation via newly designated conditional DAMP receptors and HAMP receptors (Figure 3B). This new paradigm significantly improve our understanding of endogenous metabolites' biological functions at pathophysiological conditions and their ability to modulate inflammation, systemic homeostasis and immune tolerance.

3.3. Some conditional DAMPs such as LPA, S1P and LPC regulate physiological signaling ubiquitously while others may have tissue specific signaling functions

We hypothesized that conditional DAMPs fulfill differential physiological roles at basal level. We further postulated that physiological functions of conditional DAMPs are regulated by differential expression of conditional DAMP receptors and also by tightly controlling the receptor level in the tissues. To validate this hypothesis, a database mining method we pioneered (Part 2 in Figure 1) was used to examine experimentally-verified expression profiles of mRNA transcripts of LPL-GPCRs in NCBI-UniGene database. The expression of 15 LPL-GPCR genes in 23 human tissues and 21 mouse tissues that were available in the

NCBI-Unigene database was examined (Figure 4A). The analyzed data are summarized in Figures 4B and 4C.

The analysis implicated that the expression level of human LPL-GPCRs were relatively higher compared to mouse counterparts. Human brain, eye, intestine, lung and placenta expressed a variety of LPL-GPCRs, suggesting that LPLs mediate vital physiological functions in these tissues. Nevertheless, human adrenal gland, bone, bone marrow, salivary gland, thyroid, trachea and vascular tissues expressed least variety (less than eight) of LPL-GPCRs. Moreover, human heart and vascular tissues had less LPL-GPCRs compared to connective tissue and lymph nodes, suggesting that LPL-GPCRs signaling is stronger in connective tissue and lymph nodes than in the heart and vascular tissues. LPL-GPCRs were not expressed in mouse adrenal gland, muscle and thyroid.

Table 3 is a summary of figure 4 that shows the tissue distribution of LPL-GPCRs based on the ligand. Our data clearly demonstrates that GPCRs for LPA, S1P and SPC are widely expressed in most of the human and mouse tissues examined, suggesting that these metabolites may have more ubiquitous physiological roles. In contrast, LPI-GPCR expression is limited in humans and are only seen in intestine, muscle, liver and spleen, while it was not detected in the mouse tissues. Furthermore, GPCRs for anti-inflammatory LysoPS was expressed in 15 out of 23 human tissues and 10 out of 21 mouse tissues respectively, implicating that LysoPS is a potential HAMP as its presence may regulate homeostatic functions in tissues at basal level. Moreover, LysoPS, as a potential HAMP may play role in inflammation resolution if its levels are elevated due to cellular stress or pathological conditions.

3.4. The expressions of conditional DAMP receptors are significantly upregulated in rheumatoid arthritis, coronary artery disease, and other metabolic inflammatory diseases of human and mouse

Table 2A and 2B implicate that LPLs may regulate physiological signaling at basal levels. In addition, we hypothesized that the expressions of LPL-GPCRs, the newly designated conditional DAMP receptors are significantly upregulated in human and mouse inflammatory diseases. To examine this hypothesis, we extensively analyzed microarray experimental datasets deposited in NIH-Geo database by other laboratories. These depositors have utilized these microarray data for various other non-LPL studies. We analyzed the expression of LPL-GPCRs in five metabolic inflammatory diseases including coronary artery disease, metabolic syndrome, type 2 and type 1 diabetes as well as autoimmune rheumatoid arthritis and have tabulated the data in Table 4.

Interestingly, our analysis indicated that 4 LPL-GPCRs in synovial tissue of patients with rheumatoid disease, and 3 LPL-GPCRs in peripheral blood cells in patients with coronary artery disease were significantly upregulated with the scales ranging from 1.5 to 3.0. In addition, 5 LPL-GPCRs in peripheral blood mononuclear cells in patients with type 1 diabetes were upregulated in a low scale <1.5. This suggested that specific LPL-GPCR signaling contributes to the pathogenesis of these diseases. The downregulation of LPL-GPCRs was found in patients with rheumatoid arthritis, type 2 and type 1 diabetes but not in the patients with coronary artery disease. P2RY10 GPCR upregulation was common in both

rheumatoid arthritis and coronary artery disease; and the majority of upregulated LPL-GPCRs were somewhat disease-specific.

These results suggest that, (1) pro-inflammatory LPLs including SPC, LPA, and S1P may contribute to the pathogenesis and progression of rheumatoid arthritis, coronary artery disease and type 1 diabetes; (2) expression of GPCRs for anti-inflammatory/ homeostatic LysoPS was attenuated in type 1 and type 2 diabetes; and (3) however, LysoPS-GPCR is induced in rheumatoid arthritis and coronary artery disease, indicating LysoPS may act as a potential HAMP under these pathologies. This is similar to our recently reported responsive anti-inflammatory cytokine IL-35 [13,14]. Similarly, LysoPS-GPCR expression was elevated in the aortas of ApoE^{-/-} mice fed with high fat diet further validating the role of anti-inflammatory LysoPS as responsive HAMPs (Table 4B).

As shown in Table 4B, the expressions of GPCRs for pro-inflammatory LPLs and anti-inflammatory LysoPS were elevated in collagen-induced arthritis model in mice. Out of these receptors most significant upregulation was seen for LPA-GPCRs, which was also the case observed in diabetes (db/db mice). Taken together, our results suggest that differential expression of LPL-GPCRs promote the pathogenesis of human and mouse rheumatoid arthritis, coronary artery disease, and other metabolic inflammatory diseases.

3.5. Pro-inflammatory cytokine signaling regulates the expression of LPL receptors

In order to determine how inflammatory and metabolic diseases mediate the upregulation of conditional DAMPs, we hypothesized that pro-inflammatory cytokines play an important role in regulating the expression of LPL-GPCRs, which is the prototype used for this study. To test this hypothesis, we examined the LPL-GPCRs expression in several GEO microarray datasets with inflammatory cytokine stimulated endothelial cells and monocytes. It is well established that the pro-inflammatory cytokines that were examined in this study are involved in development and progression of rheumatoid arthritis [22], coronary heart disease [59] and other metabolic diseases [60], suggesting that our hypothesis is pathophysiologically relevant.

Table 5 implicate that tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) induces Lpar2 (LPA-GPCR) in mouse endothelial cells and LPAR6 in human endothelial cells, respectively. Further, interleukin-1 β (IL-1 β) induces the expressions of GPR55 (LPI-GPCR). Since the GPR55 expression is not found in human vascular tissue in unstimulated conditions as shown in Figure 2, the upregulation of GPR55 induced by IL-1 β in human endothelial cells is significant. Interestingly, IL-1 β increased the expression of S1PR1 (receptors for SPC and SP1) while decreasing the other receptors (S1PR5, S1PR3) where the functions are mediated by the same ligands in human endothelial cells. This indicates that cytokines can differentially modulate the functions of conditional DAMPs in response to inflammation. Additionally, the data shown in Table 5 implicate that TNF- α and IL-1 β can significantly upregulate the expression of GPR34, which is receptor for anti-inflammatory LysoPS. This indicates the presence of complex signaling mechanism where cytokines can directly or indirectly regulate the expression of conditional DAMPs and HAMPs in inflammatory diseases.

Of note, due to the limitations of microarray datasets in the current GEO database, the extent we could analyze the LPL-GPCRs in different tissues/ cells in response to different pro-inflammatory mediators were limited. Further, we also acknowledge that variety of factors other than pro-inflammatory cytokines may contribute to the disease mediated upregulation of LPL-GPCRs. However, our results demonstrated the proof of principle that pro-inflammatory cytokine signaling regulates the expression of conditional DAMPs and its receptors.

3.6. TLR signaling regulates the expression of LPL receptors

By utilizing GEO microarray data sets, we further studied the interrelation between classical DAMP receptors such as TLRs and LPL-GPCRs, which we have newly designated as conditional DAMP/ HAMP receptors. We hypothesized that in response to endogenous DAMPs, TLRs act as a set of prototypic DAMP receptors and play an important role in regulating the expressions of LPL-GPCRs. To test this hypothesis, we examined LPL-GPCR expression in several GEO microarray datasets in TLR gene deficient mouse tissues. This data is shown in Table 6. So far, four TLRs have been identified to bind to DAMPs such as HMGB1, RNA and DNA. These four receptors are TLR2 (HMGB1), TLR3 (RNAs), TLR4 (HMGB1) and TLR9 (HMGB1 and DNAs) [61].

Table 6 showed that the expressions of P2ry10 (LysoPS-GPCR) and Gpr55 (LPI-GPCR) are downregulated in TLR2 gene knock-out (KO) mouse in proximal jejunum. In addition, we found that the expressions of S1pr2 (S1P/ SPC-GPCR), P2ry10 (LysoPS-GPCR), and Lpar1 (LPA-GPCR) are downregulated in TLR4 KO mouse kidney. Moreover, the expression of S1pr2 (S1P/ SPC-GPCR) is decreased in TLR3 KO mouse liver.

This further validates that TLRs and pro-inflammatory mediators (Table 5) together with other classical DAMPs are involved in regulating the expression of conditional DAMPs and HAMPs to mediate the progression and resolution of inflammation in tissues.

Of note, the GEO datasets for TLR9 KO mice were not available for the analysis. Although the GEO datasets are limited, our results demonstrated the proof of principle that TLR signaling regulates the expressions of LPL receptors; and that classical DAMP receptor signaling regulates the expression of LPL receptors.

3.7. LPL receptor signaling regulates the expression of TLRs

We further studied whether newly designated conditional DAMPs regulate expression of classical DAMP receptors such as TLRs. Therefore, we hypothesized that in response to endogenous LPL metabolite danger signals, LPL-GPCRs regulate the expressions of TLR receptors. To test this hypothesis, we examined the TLR receptor expressions in several GEO microarray datasets that was conducted on LPA-stimulated human PBMCs and one S1P-stimulated mouse liver (Table 7).

Of note, ten human TLRs have been identified including TLR1-TLR10 whereas twelve mouse TLRs have been found including TLR1-TLR9, and TLR11-TLR13 [62]. Table 7 illustrates that the expression several TLRs/ Tlrs are modulated in the presence of pro-inflammatory LPLs in human and mouse tissues. This suggests that, when elevated,

endogenous metabolites such as LPLs can regulate the expression of TLRs and can modulate the inflammatory signals in response to classical DAMPs and other pro-inflammatory cytokines.

Again, although the GEO datasets are limited, our results demonstrated the proof of principle that LPLs regulate the expressions of TLR receptors. Of note, the reason for LPA to downregulate TLR7 and TLR3, and for S1P to downregulate Tlr5 may be due to signal complexity of these LPL receptors with stimulatory GPCRs and inhibitory GPCRs shown in Figure 3. Once again, our results suggest that: (1) the interplay between classical DAMP receptors and newly designated conditional DAMP receptors collaborate in endogenous danger signal-initiated sterile inflammation; (2) LPL-GPCR (as conditional DAMP receptors and HAMP receptors) signaling not only regulates various inflammatory cytokine signaling but also modulates other DAMP receptor-initiated signaling.

3. Discussion

Recently DAMPs and its six types of receptors had gained widespread attention as major players in regulating sterile inflammation. This was further validated by the recent reports from our lab, which demonstrated that NLR/ caspase-1 signaling pathway is responsible for inducing endothelial activation and subsequent atherogenesis during early hyperlipidemia [58] and hyperhomocysteinemia [63], for decreasing vascular endothelial growth factor receptor 2 (VEGFR2) mediated angiogenesis in ischemia [64], and for weakening of the progenitor cell mediated vessel repair [3,64].

However, despite the well-established roles of currently identified DAMPs and their receptors, there are limitations in the classification of DAMP receptors. To overcome the limitations we have elaborated above, we utilized a family of endogenous lipid metabolites (LPLs) and their receptors as a prototype. We performed a panoramic analysis on the tissue expression of all currently identified 15 LPL receptors in 23 human tissues and 21 mouse tissues. In addition, we also examined the expression changes of the 15 LPL receptors in five prototypic chronic sterile inflammations including autoimmune rheumatoid disease, coronary artery disease, metabolic syndrome, type 2 diabetes and type 1 diabetes. Based on our analysis we propose the following paradigm: (1) endogenous metabolites play a role in regulating normal cellular functions at basal level. However, the expression of these metabolites can be elevated in sterile inflammatory conditions and can induce inflammation in tissues. One potential mechanism that pro-inflammatory LPLs induce inflammation is by activating pro-inflammatory transcription factors (NF-kB, C/EBP6 and RUNX1) [6]. Thus, we named these endogenous metabolites as conditional DAMPs; (2) anti-inflammatory metabolites expression can get elevated in response to inflammation, and may play a role in inhibition or resolution of inflammation. We designated such metabolites as HAMPs; (3) conditional DAMPs and HAMPs exert their pathophysiological signaling via their receptors, and we designated these receptors as conditional DAMP receptors or HAMP receptors respectively; (4) conditional DAMPs and HAMPs are differentially expressed in tissues at normal physiological conditions (healthy state). Some conditional DAMPs are ubiquitously expressed while some may have distinct tissue specific functions; (5) under inflammatory and metabolic diseases, expression of conditional DAMP receptors and HAMP receptors are

modulated, which will be responsible for either progression or inhibition/ resolution of inflammation; (6) complex signaling mechanism exists between various pro-inflammatory mediators and classical DAMPs and their receptors, that regulate the expression of conditional DAMPs and HAMPs and their receptors. We have illustrated our working model in Figure 3.

As we have emphasized, LPLs and LPL-GPCRs were used as a prototype to formulate this novel paradigm and we acknowledge that there may be myriad of other endogenous metabolites that can behave similarly as LPLs, thus they can either act as conditional DAMPs or HAMPs. At present, various types of LPLs and LPL-GPCRs were identified to play a role in inflammatory disorders including cardiovascular diseases [65]. Most recently, our lab further validated that the pro-inflammatory LPC is induced at early stages of atherogenesis and it induces mitochondrial reactive oxygen species production in viable endothelial cells [66].

We previously reported that although LPLs at low levels cannot initiate pro-inflammatory/ anti-inflammatory cytokine secretions above the lowest threshold of current technique-detectable levels, still they can initiate a physiological signaling [58]. This finding is important since it emphasizes that LPLs and other pro-inflammatory metabolites are not pathologically generated only for the purpose to be the danger signals and HAMPs for regulating inflammation. Of note, recent reports attributed that LPA receptors may also initiate immune-suppressive signals [67–69] in addition to pro-inflammatory signals [70]. Similar conflicting results have been reported to pro-inflammatory mediators such as caspase-1 [71], anti-inflammatory estrogen [72] and pro-inflammatory cytokine interleukin-17 [73]. The contextual dependence of these biologicals systems that were used to study these inflammatory mediators may contribute to these dissimilarities observed [74]. Future work is needed to solve these discrepancies.

As we pointed out previously, our sets of experimental data on LPL receptor tissue expression are collected from cDNA cloning and DNA sequencing experiments deposited in NIH-UniGene database, rather than theoretical data derived from computer modeling. Therefore, it is our belief that this data require no further experimental verification. Further, these gene expression databases have been established based on precise DNA sequencing data, thus the data we obtained by NIH-UniGene database mining are more precise in providing the tissue expression profiles of genes than traditional hybridization- and primer annealing-based approaches like Northern blots and RT-PCRs [2].

In addition, the gene expression data we gathered by analyzing NIH-GEO databases have been generated by high throughput microarray experiments. Of note, the expression of LPL receptor genes among tissues and disease-related microarrays are significantly higher relative to housekeeping genes. Since a huge amount of “omics” experimental data are deposited in various NIH and other databases, it had become a well-accepted norm to analyze those large sets of experimental data to validate hypothesizes and formulate new paradigms without having additional experimental verification. Furthermore, the laboratories that have deposited the microarray data that we used for this study were conducted on other non-LPL-related projects. Therefore, to the best of our knowledge, the findings that we

reported here have not been published elsewhere. This well-accepted practice allows us to claim that our analysis of existing data are novel and unpublished research findings, as such, do not represent a traditional literature review.

Our proposal of the new paradigm and new concept of conditional DAMPs, DAMP receptors, HAMPs and their receptors would significantly improve our understanding on the roles of endogenous metabolites in regulating the pathogenesis of inflammations. This will also eventually lead to future development of novel therapeutics for rheumatoid diseases, metabolic inflammatory diseases, cardiovascular diseases, inflammatory cancers and other diseases.

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Abbreviations

PAMP	Pathogen associated molecular patterns
DAMP	Danger associated molecular patterns
TLR	Toll like receptors
NLR	NOD like receptors
NOD	Nucleotide binding and oligomerization domain
RIG-I	Retinoid acid inducible gene I
AIM2	Absent in melanoma 2
RAGE	Receptor for advanced glycation end product
HMGB1	High mobility group box 1
IL	Interleukin
TGF-β	Transforming growth factor- β
LPLs	Lysophospholipids
GPCRs	G-protein coupled receptors
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylentaolamine
LPG	Lysophosphoglycan
LPI	Lysophosphatidylinositol

LysoPS	Lysophosphatidylserine
CAD	Coronary artery disease
SPM	Specialized pro-resolving mediators
EST	Expressed sequence tags
NIH	National institute of health
NCBI	National center of biotechnology information
ROCK	Rho-associated kinase
DAG	Diacylglycerol
IP3	Inositol 1,4,5-triphosphate
MAPK	Mitogen activated protein kinase
AC	Adenylate cyclase
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
SRF	Serum response factor
SPC	Sphingosylphosphorylcholine
IFN-γ	Interferon- γ

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Figure 1. Flow chart of database mining strategy and three parts of data organization

A) We propose a new paradigm for the first time that LPLs are pro-inflammatory danger-associated molecular pattern molecules (DAMPs) or anti-inflammatory homeostasis-associated molecular pattern molecules (our newly proposed HAMPs). B) The strategy in generating tissue expression profile of LPL-GPCRs. C) Result analysis and presentation.

Figure 2. LPLs act as signaling mediators by binding to GPCRs

GPR34, the receptor of LysoPS, binds to G_i to trigger specific signaling, GPR55, the receptors of LPI, bind to G protein (G_q and $G_{12/13}$) to trigger signaling while the receptors of LPA, S1P and SPC are associated with more than one G protein subunits (G_q , $G_{12/13}$, G_i and G_s) for signaling.

Figure 3. Our new working model: LPL-GPCRs are a new type of conditional DAMPRs or HAMPRs (receptors for DAMPs or HAMPs)

A) Upregulated LPA, S1P, LPC, LPI and SPC act as new types of conditional DAMPs to induce systemic /local inflammatory responses while LysoPS and LPE act as newly proposed HAMPs to suppress systemic/local inflammatory responses and induce the resolution of inflammation. **B)** LPLs regulate the balance between the progression of inflammation and resolution of inflammation via newly designed conditional DAMPR signaling and HAMPR signaling. Our new report showed that LPC stimulates mitochondrial reactive oxygen species generation without compromising ATP production in human aortic endothelial cells [66].

Figure 4. LPL-PCRs genes are differently expressed in human and mouse tissues

A. Data presentation format (The data presented in X-, Y-axis, and tissue order of ARHGDI A and Ldha were applied to all the human and mouse genes examined respectively). As an example, the gene expression profiles of human housekeeping gene Rho GDP dissociation inhibitor (GDI) alpha (ARHGDI A) in the twenty-three tissues were presented, with the tissue names and position numbers shown on the X-axis. The gene expression data were normalized by the β -actin (Hs. 520640) expression data from the same tissue, which were presented on the left Y-axis. The expression ratios among tissues were

generated by normalizing the arbitrary units of the gene in the tissues with the median level of the arbitrary units of the gene in all the tissues which are presented on the right Y-axis. In order to define confidence intervals for statistically higher expression levels of given genes, we calculated the confidence intervals of tissue expression for three housekeeping genes [the mean $X + 2 \times$ standard deviations (SD) = 3.84] including ARHGDI1 (Hs. 159161), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs. 544577), and ribosomal protein S27a (RPS27A, Hs. 311640). The expression variations of given genes in tissues, when they were larger than 3.84-fold, were defined as the high expression levels with statistical significance (the right Y-axis). To define confidence intervals for statistically higher expression levels of given genes in 21 mouse tissues, we calculated the confidence intervals of tissue expression [the mean $X + 2 \times$ standard deviations (SD) = 2.86] for three mouse housekeeping genes including Lactate dehydrogenase A (Ldha, Mm. 29324), non-POU-domain-containing octamer binding protein (Nob1, Mm. 280069), and ribosomal protein L32 (Rpl32, Mm. 104368). The expression variations of given genes in tissues, when they were larger than 2.86-fold, were defined as the high expression levels with statistical significance (the right Y-axis). **B.** The expression profiles of LPL-GPCRs genes in human tissues (Left 2 columns, with LPL-GPCRs genes designated with capital letters). **C.** The expression profiles of LPL-GPCRs genes in mouse tissues (right 2 columns, with LPL-GPCRs genes designated with lowercase letters).

Table 1

Three lysophospholipids (LPLs) including LPI and PAF bind to one specific G protein-coupled LPLs receptor (LPL-GPCRs), respectively, while LPA, S1P, SPC and LysoPS bind to more than two LPL-GPCRs.

Receptors	Ligands	Gene Symbols of receptors	Alias of receptor	G-proteins
LPA ₁	LPA	LPAR1	EDG2, vzg-1, GRCP26	G _{i/o} G _q G _{12/13}
LPA ₂	LPA	LPAR2	EDG4	G _{i/o} G _q G _{12/13}
LPA ₃	LPA	LPAR3	EDG7	G _{i/o} G _q G _s
LPA ₄	LPA	LPAR4	P2Y9, GPR23	G _s G _{i/o} G _q G _{12/13}
LPA ₅	LPA	LPAR5	GPR92, GPR93	G _s G _{i/o} G _q G _{12/13}
LPA ₆	LPA	LPAR6	P2Y5	G _{12/13}
S1P ₁	S1P, SPC	S1PR1	EDG1, LPB1	G _{i/o}
S1P ₂	S1P, SPC	S1PR2	EDG5, GPCR13, LPB2	G _s G _{i/o} G _q G _{12/13}
S1P ₃	S1P, SPC	S1PR3	EDG3, LPB3	G _s G _{i/o} G _q G _{12/13}
S1P ₄	S1P, SPC	S1PR4	EDG6, LPC1	G _s G _{i/o} G _{12/13}
S1P ₅	S1P, SPC	S1PR5	EDG8, LPB4	G _{i/o} G _q G _{12/13}
GPR34	LysoPS	GPR34		G _{i/o}
GPR55	LPI	GPR55		G _q G _{12/13}
GPR174	LysoPS	GPR174	FKSG79, GPCR17, LYPSR3	
P2RY10	LysoPS	P2RY10	P2Y10	

Table 2A

The supporting evidence 1 for classifying LPLs as conditional DAMPs or HAMPs: LPLs are upregulated during the progression of some autoimmune, cardiovascular and metabolic diseases.

Disease	Tissue	Upregulated Lysophospholipid	Downregulated Lysophospholipid	PMID
Juvenile-onset systemic lupus erythematosus	Human serum	S1P	-	22648459
Acute Coronary Syndrome	Human plasma	LPA, LPE, LPG, LPI, LysoPS, LPC	-	25425621
Proliferative diabetic retinopathy	Human vitreous fluid	LPA	-	25496321
Obese type 2 diabetes	Human plasma	LPI	-	22179809

Table 2B

The supporting evidence 2 for classifying LPLs as conditional DAMPs or HAMPs: The bindings of LPLs to their receptors regulate inflammation cytokine generation as the inflammation readouts.

Treatment	Dose	Cell type/tissue	Induced cytokines/signaling	Suppressed cytokines/signaling	PMID
LPA	1μM	Human Synoviocytes	IL-8	-	18006645
	2.5μM		IL-6, IL-8	-	
	5μM		IL-6, IL-8	-	
SIP lyase knock-out	-	Mouse liver	TNF, IFN-γ, MCP-1, IL-6	-	20097939
SIP	0.5μM	Human CD4+ T cells	NF-κB ligand (RANKL)	-	22326262
SIP	0.01–0.5μM	MH7A synovial cells	NF-κB ligand (RANKL)	-	22326262
LPC	10μM	Mouse THP-1-derived macrophages	GM-CSF, TNF-α, MCP-1, IL-1β, IL-6, IL-8	-	21957201
LPI	-	hGPR55-HEK293 Cells	MAPK Signaling (ERK1/2 Phosphorylation)	-	22027819
LPI	1μM	hGPR55-HEK293 Cells	RhoA-dependent Ca^{2+} signaling and NFAT activation	-	18757503
SPC	10μM	Human keratinocytes	TNF-α, IL-6, ICAM-1	-	9886270
LysoPS	-	Mouse peritoneal macrophages	-	TNF-α, MMP-9, L-6, MCP-1	25445889
LysoPS	-	RAW 264.7 cells	-	TNF-α, MMP-9	25445889
LPE (2-ARA-LPE)	50μg/kg 150μg/kg	Mouse peritoneal lavage fluids of Zymosan A-Induced Peritonitis	- IL-10	TNF-α, IL-1β, IL-6 TNF-α, IL-1β, IL-6	21744277
LPE (2-DHA-LPE)	15μg/kg	Mouse peritoneal lavage fluids of Zymosan A-Induced Peritonitis	IL-10	IL-6	21744277

LPA, SIP and SPC have ubiquitous physiological signaling functions in human tissues whereas other LPLs, especially LPI, may have tissue-specific signaling functions.

Table 3A

tissue	LPA-GPCRs	SIP-GPCRs	SPC-GPCRs	LPI-GPCRs	LysoPS-GPCRs
intestine	+	+	+	+	+
blood	+	+	+	-	+
brain	+	+	+	-	+
kidney	+	+	+	-	-
connective tissue	+	+	+	-	+
lung	+	+	+	-	+
thymus	+	+	+	-	+
pancreas	+	+	+	-	+
eye	+	+	+	-	+
lymph node	+	+	+	-	+
placenta	+	+	+	-	+
muscle	+	+	+	+	+
liver	+	+	+	+	+
spleen	+	+	+	+	-
vascular	+	+	+	-	+
thyroid	-	+	+	-	-
bone	+	+	+	-	-
bone marrow	+	+	+	-	-
heart	+	+	+	-	-
skin	+	+	+	-	-
trachea	+	+	+	-	+
adrenal gland	+	+	+	-	+
salivary gland	-	+	+	-	-

Table 3B
LPA, SIP and SPC have ubiquitous physiological signaling functions in mouse tissues.

tissue	LPA-GPCRs	SIP-GPCRs	SPC-GPCRs	LPI-GPCRs	LysoPS-GPCRs
lymph node	+	+	+	-	+
skin	+	+	+	-	+
spleen	+	+	+	-	+
thymus	+	+	+	-	+
brain	+	+	+	-	+
bone	+	+	+	-	+
bone marrow	-	+	+	-	+
eye	+	+	+	-	-
heart	+	+	+	-	-
intestine	+	+	+	-	+
joint	+	+	+	-	-
lung	+	+	+	-	-
salivary gland	+	+	+	-	+
pancreas	+	+	+	-	+
kidney	+	+	+	-	-
blood	+	+	+	-	-
liver	+	+	+	-	-
connective tissue	+	+	+	-	-
muscle	-	-	-	-	-
adrenal gland	-	-	-	-	-
thyroid	-	-	-	-	-

Table 4A

The expression of certain LPL-GPCRs are significantly upregulated in rheumatoid arthritis, coronary artery disease, and other metabolic inflammatory diseases in human.

Disease	Tissue or cell type	Upregulated genes	Downregulated genes	GEO Dataset ID
Rheumatoid Arthritis	synovial tissue	P2RY10 ^{**} (LysoP) LPAR2 [*] (LPA) LPAR6 [*] (LPA) S1PR5 (S1P, SPC)	LPAR1 (LPA)	GSE55235
Coronary Artery Disease	peripheral blood	GPR34 [*] (LysoPS) GPR174 [*] (LysoPS) P2RY10 [*] (LysoPS)	-	GSE23561
Metabolic Syndrome	peripheral blood	-	-	GSE23561
Type 2 Diabetes	peripheral blood	-	P2RY10 (LysoPS)	GSE23561
Type 1 Diabetes	peripheral blood mononuclear cell	LPAR1 (LPA) LPAR2 (LPA) LPAR3 (LPA) S1PR3(S1P, SP) S1PR4(S1P, SP)	S1PR5 (S1P, SPC), GPR174 (LysoPS)	GSE55100

These microarray experiments were not designed for LPL-GPCRs studies; FC, fold change;

* 1.5 FC<3,

** 3 FC<10,

*** 10 FC<100,

**** FC 100

Table 4B

The expression of certain LPL-GPCRs are significantly upregulated in collagen induced arthritis, high fat diet mediated atherosclerosis, and diabetes in mouse.

Model	Tissue or cell type	Upregulated genes	Downregulated genes	GEO Dataset ID
CIA mice, without joint swelling	knee joint synovium	-	S1pr1 (S1P, SPC) [#]	GSE13071
CIA mice, with joint swelling	knee joint synovium	Lpar4** (LPA) P2ry10** (LysoPS) Lpar1* (LPA) [#] Lpar6* (LPA) S1pr2* (S1P, SPC) [#] S1pr3* (S1P, SPC) Gpr34* (LysoPS) [#] Gpr174* (LysoPS) [#]	-	GSE13071
APOE ^{-/-} mice, with 8/16/24 weeks high fat diet	aortic arch	P2ry10*** (LysoPS) Gpr34** (LysoPS) [#]	-	GSE18443
diabetic mice	glomerular endothelial cell	Lpar4* (LPA) Lpar6* (LPA) S1pr1 (S1P, SPC)	-	GSE21324

Table 5

Pro-inflammatory cytokine signaling regulates the expression of LPL receptors.

Treatment	Cell type	Upregulated gene	Downregulated gene	GEO Datasets ID
TNF- α vs Control	Mouse endothelial cells	Lpar2**	-	GSE4518
IL-1b vs Control	Human endothelial cells	GPR55**	S1PR5**	GSE19240
		S1PR1*	S1PR3*	
		-	LPAR2**	
IFN- γ vs Control	Human endothelial cells	LPAR6*	-	GSE3920
TNF- α vs Control (2 hours)	Human Peripheral Blood Mononuclear Cells (PBMCs)	GPR34***	-	GSE40838
IL-1b vs Control (6 hours)	Human Peripheral Blood Mononuclear Cells (PBMCs)	GPR34*	-	GSE40838

Table 6

DAMPs/HAMPs signaling interactions (1): TLR signaling regulates the expression of LPL receptors

Genotype	Tissue	Upregulated gene	Downregulated gene	GEO Dataset ID
TLR2 ^{-/-} mouse	Mouse proximal jejunum	-	P2ry10* Gpr55*	GSE56426
TLR4 ^{-/-} mouse	Mouse kidney		S1pr2* P2ry10* Lpar1*	GSE34351
TLR3 ^{-/-} mouse	Mouse liver	-	S1pr2*	GSE14719

Table 7

DAMPs/HAMPs signaling interactions (2): LPL receptor signaling regulates the expression of TLRs

Treatment	Tissue or cell type	Upregulation	Downregulation	GEO Dataset ID
LPA	Human peripheral blood mononuclear cells	TLR2* -	TLR7* TLR3*	GSE40889
S1P	Mouse liver	Tlr2** Tlr1* Tlr13* Tlr7* Tlr4* Tlr3*	Tlr5*	GSE18745